Benzodiazepine use attenuates cortical beta-amyloid and is not associated with progressive cognitive decline in non-demented elderly adults: A pilot study using F¹⁸-Florbetapir Positron Emission Tomography

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A complete listing of ADNI investigators can be found at:

http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

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Abstract

Objective: There are as yet inconclusive findings as to whether benzodiazepines (BZD) are related to cognitive deterioration in the elderly populations. Animal studies suggest that gamma-aminobutyric acid A receptor (GABA-A) agonists, such as BZD, may prevent A β -neurotoxicity and reduce beta-amyloid (A β). However, no studies have investigated the effects of BZD use on A β in humans.

Design: A cross-sectional and prospective study

Setting: Alzheimer's Disease Neuroimaging Initiatives (ADNI) sites in the United States and Canada

Participants: Non-demented elderly adults between 55 to 90 years of age

Measurements: Cortical A β levels were assessed by positron emission tomography radiotracer F¹⁸-Florbetapir. Changes in global cognitive function and verbal memory performance over 2 years were assessed using scores on Montreal Cognitive Assessment and five domains of Rey Auditory Verbal Learning Test, respectively.

Results: Previous BZD users (PRE+BZD)(n = 15) had lower cortical A β levels in frontal (F (1, 26) = 8.82, p = 0.006), cingulate (F (1, 26) = 8.58, p = 0.007), parietal (F (1, 26) = 7.31, p = 0.012) and temporal (F (1, 26) = 7.67, p = 0.010) regions compared to matched BZD non-users (n = 15), after controlling for history of psychiatric disorders and antidepressant use. There was also no difference in global cognitive function and changes in cortical A β over 2 years between continuous BZD users (CON+BZD)(n = 15) and the matched non-user group (n = 15).

Conclusion: Previous BZD use was associated with lower cortical A β levels in non-demented elderly controls. Future studies with larger samples are required to replicate our findings.

Keywords: Benzodiazepine, beta-amyloid, cognition, function, GABA-A, Alzheimer's disease

Background

Benzodiazepines (BZDs) are among the most commonly used psychotropics targeting anxiety, agitation, and insomnia in the elderly population. Neuropsychiatric symptoms are prevalent in non-demented elderly adults aged 65 years or older (1) with about 25% of elderly individuals without cognitive impairment, and 16% depressive symptoms (1).

BZDs act as gamma-amino butyric acid A (GABA-A) receptor agonists, which allosterically modulate GABA-A receptors by increasing their affinity for GABA (2). The adverse effects of BZDs on cognition are well documented and likely occur as a result of GABAergic agonism. BZD associated cognitive deficits include amnesic effects, namely anterograde and retrograde amnesia (3) and non-amnesic cognitive deficits (4).

However, studies investigating the effects of BZD use on progressive cognitive decline in adults in later life have yielded conflicting results. While there are several epidemiological studies showing an elevated incidence of cognitive decline (5) and risk of dementia in BZD users (6), there are findings suggesting that BZD use is not associated with cognitive impairment or an increased incidence of dementia. A study of community-dwelling women (n = 510) found that current BZD use did not increase the risk of cognitive decline (7). Another study showed that BZD use was related to dizziness and sleep disturbance, but not directly linked to cognitive dysfunction (8). A study with a large sample size (n = 5423) reported that there was no difference between BZD users and non-users in changes in cognition over time (9). In addition, a recent case controlled study also showed that BZD use was not associated with an increased risk of AD or vascular dementia (10).

In conjunction with the studies that show the absence of deleterious effects of BZD on cognition, there are several findings, which suggest that BZDs may have a neuroprotective role against dementia (11, 12). Excessive glutamatergic signaling is a common pathological pathway seen in patients with dementia (13). GABA-A receptor agonists, including certain types of BZDs, may have neuroprotective effects via the attenuation of glutamate-mediated neuronal excitability and beta-amyloid (A β) neurotoxicity (11, 14, 15). Furthermore, several animal studies showed that BZD administration reduced

cortical A β plaques deposition in mouse brains (16, 17). A recent study also highlighted that midazolam, a benzodiazepine, suppressed A β formation, suggesting its protective effect against A β aggregation (18).

To the best of our knowledge, we are not aware of any study that has investigated whether BZDs attenuate the cortical A β in humans. Using data from Alzheimer's Disease Neuroimaging Initiatives (ADNI), we aimed to see whether there is decreased level of cortical A β in elderly BZD users in comparison to matched BZD non-users. Additionally, we analyzed whether long-term BZD use was linked to progressive cognitive deterioration over 2 years of follow-up. We had three hypotheses: First, we hypothesized that BZDs would lower the levels of cortical A β based on the aforementioned literature in support of the possible neuroprotective effects of BZDs. Second, BZDs would not promote progressive cognitive deficits in non-demented elderly controls. Finally, we hypothesized that BZD use would strongly predict the level of cortical A β in a dose and duration dependent manner.

Materials and Methods

Participants and assessments

The database was downloaded from ADNI -1, ADNI-2, and ADNI Grand Opportunity databases on March 3rd, 2015 (19). Alzheimer's Dementia severity was assessed using the Alzheimer's Disease Assessment Scale (ADAS) 11 and 13 item versions (20), and the Clinical Dementia Rating Scale Sum of Boxes (CDR-SB)(21). Cognition was assessed using the Montreal Cognitive Assessment (MOCA) (22). Verbal memory performance was assessed using the Rey Auditory Verbal Learning Test (RAVLT) (23), including (i) RAVLT immediate recall score, which consists of correctly recalling 15 words across five trials (the maximum score is 60 with a higher score indicating better performance) (24); (ii) RAVLT learning score, which consists of the difference between the number of words correctly recalled after the fifth trial and the first trial (24); (iii) RAVLT forgetting score, which represents the difference between the numbers correctly recalled after the fifth trial and a 20 minute delay period (24); and (iv) RAVLT percentage of forgetting, which is calculated by following equation: (RAVLT forgetting score/Trial 5 score) x 100 (24). Neuropsychiatric Inventory (NPI) total score (25) was used to measure the

neuropsychiatric symptoms of participants. Overall functioning was assessed using the Functional Activities Questionnaire (FAQ) (26). History of psychiatric disorders of included participants was obtained from medical history database. Data for antidepressant usage (users vs. non-users) and BZDs were collected from concurrent medication log database from ADNI-1, ADNI-2, and ADNI-GO. Types of BZDs, which were included in this study, were GABAergic BZD derivatives, including alprazolam (n = 3), clonazepam (n = 1), diazepam (n = 1), lorazepam (n = 2), and temazepam (n = 1). GABAergic non-BZD derivatives included eszopiclone (n = 1) and zolpidem (n = 7). One participant was using eszopiclone along with zolpidem, and another participant was using alprazolam along with zolpidem.

Previous BZD users

Previous BZD users (PRE+BZD) were defined as those controls in ADNI database using any type of BZDs for at least for 1 year before the baseline clinical assessment. The duration of BZD use was calculated as follows: medication date ended minus date began from the concurrent medication log in the ADNI database. If participants were still on the medication at the time of this analysis, the duration of BZD was calculated as follows: day of current clinical assessment minus medication date began. Medication dose was standardized by converting the various BZD doses to diazepam dose equivalents. Subsequently, dose x duration was calculated by multiplying the duration of BZD use by the BZD diazepam dose equivalent. Next, PRE+BZD participants were matched to non-demented elderly adults who never took BZD (PRE-BZD) at baseline based on age, gender, apolipoprotein E4 (apoE4) genotype, race, ethnicity, marital status, education years, and MOCA scores.

Continuous BZD users

Continuous BZD users (CON+BZD) were defined as those controls who continued to use BZD from baseline to the follow-up endpoint. Similarly, CON+BZD participants were matched to non-demented elderly adults who never took BZD (CON-BZD) in their lifetime based on aforementioned demographic and clinical variables listed above.

Positron Emission Tomography Analysis

Data for F¹⁸-Florbetapir (AV-45) were available from ADNI-2 and ADNI-GO database. ADNI reported regions of interest (ROIs) included bilateral frontal, anterior/posterior cingulate, lateral parietal, and lateral temporal cortices. ROI-based AV-45 standardized uptake value ratios (SUVR) was calculated by dividing the AV-45 mean SUV from one of the ROIs, as reported by the ADNI (updated on 3 March 2015), by a composite reference region (average of whole cerebellum, brainstems/pons, and eroded subcortical white matter).

A voxel-based confirmatory analysis was carried out in PRE+BZD and PRE-BZD users using Analyze 6.0 (AnalyzeDirect, Overland Park, KS) software and Statistical Parametric Mapping 2 (SPM2, <http://www.fil.ion.ucl.ac.uk/spm/>) running on Matlab 6.5 (MathWorks, Inc). 4 PRE+BZD and 3 PRE-BZD participants were excluded from the voxel-based analysis due to image artifact or incomplete data. The same voxel-based analysis procedure used in the previous study (27) was carried out.

Voxel-based morphometry

All the T1 scans were analyzed using the Voxel-based morphometry (VBM)-8 toolbox (<http://www.neuro.uni-jena.de/vbm/>) to obtain total brain volume (TBV) using Statistical Parametric Mapping 8.0 (SPM8 - <http://www.fil.ion.ucl.ac.uk/spm/>) running on Matlab 6.5. TBV was used as one of the clinical variables in the later mentioned statistical analysis. TBV was equivalent to the sum of grey matter and white matter.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) Version 21.0 (IBM, New York, US). Pair t-tests and McNemar's tests were performed to compare baseline demographic and clinical profiles where appropriate.

An analysis of covariance (ANCOVA) was performed to investigate group differences for AV-45

SUVR, controlling for history of psychiatric disorders and antidepressant usage. Bonferroni correction for multiple comparisons was performed for the 4 a priori regions-of-interest (ROIs): frontal, parietal, cingulate, and temporal regions, setting the threshold for statistical significance at $p < 0.013$ ($p = 0.05/4$).

A stepwise regression analysis was performed with to see whether previous BZD use predicted the average AV-45 SUVR (Average SUVR of frontal, cingulate, precuneus, and parietal cortex with the cerebellum as a reference region) in a dose by duration dependent manner. Average AV-45 SUVR was selected as the dependent variable. For the first regression analysis, previous BZD use, age, gender, ApoE4 genotype, MOCA, antidepressant use, FAQ score, and history of psychiatric disorders were the independent variables. For the second analysis, previous BZD use was replaced with BZD dose x duration, while the other independent variables remained unchanged.

A mixed-effect model for repeated measurements (MMRM) analysis was performed to compare AV-45 from 4 different ROIs over 2 years. The MMRM model included frontal, cingulate, parietal, and temporal AV-45 SUVR as dependent variables, and the year and AV-45-by-year interaction as fixed effects. Another MMRM analysis was performed to investigate changes in cognition over 2 years between the CON-BZD and CON+BZD group. The MMRM model included scores on cognition (MOCA, ADAS-11, ADAS-13, RAVLT immediate recall, learning, forgetting, and percentage of forgetting) as dependent variables, and the year (baseline, year 1, and year 2) and scores on cognition x year interaction as fixed effects. Dose x duration and antidepressant use were included as covariates.

Statistical parametric map comparison using two-sample t-test was performed in SPM 12 using SUVR images of PRE+BZD and PRE-BZD participants with history of psychiatric disorders and antidepressant use as covariates. The analysis was performed using a threshold p value < 0.01 with a cluster threshold of at least 100 voxels (2 mm isotropic).

Results

Demographic and clinical profile

Fifteen non-demented elderly adults (i.e., controls in ADNI database) who were prescribed BZD for at least one year (PRE-BZD) were identified in the ADNI medical history database. BZD use ranged from 1 to 25 years, with a mean of 7.47 years. Fifteen PRE+BZD users were matched to 15 PRE-BZD users based on age, gender, education years, ethnicity, race, marital status and apoE4 genotype.

CON+BZD users were also selected based on the ADNI medical history database. Fifteen participants were identified who had been using BZD continuously for 2 years from baseline to the final clinical assessment. The mean duration of BZD use of CON+BZD users was 6.40 years. Fifteen CON+BZD users were matched to 15 CON-BZD users based on aforementioned variables listed above.

PRE-BZD vs. PRE+BZD users

Pair t-test and McNemar's test results highlighted that the PRE-BZD and PRE+BZD groups did not differ in age, gender, race, ethnicity, marital status, apoE4 genotype profile, education years, MOCA score, cognitive functions measured by scores on RAVLT and ADAS, CDR-SB, FAQ scores and TBV (**Supplementary Table 1**). However, PRE+BZD users had higher forgetfulness, and percentage of forgetting than matched PRE-BZD, but they did not reach statistical significance. There was no difference in history of psychiatric disorders between the PRE-BZD and PRE+BZD group.

CON-BZD vs. CON+BZD users

Pair t-test and McNemar's test results showed that the two groups did not differ in aforementioned clinical and demographic variables listed above (**Supplementary Table 2**).

The effects of BZD uses on cortical beta-amyloid

The ANCOVA analysis, controlling for the history of psychiatric disorders and antidepressant usage, revealed that PRE+BZD users had lower frontal, cingulate, parietal, and temporal AV-45 SUVR than the PRE-BZD group (**Figure 1**). All these a priori regions survived correction for multiple comparisons.

First regression analysis showed that previous BZD use and ApoE4 genotype were the predictors of average AV-45 SUVR ($B = -0.50$, $t = -3.27$, $df = 27$, $p < 0.01$; $B = 0.35$, $t = 2.27$, $df = 27$, $p = 0.03$, respectively). ApoE4 genotype accounted for 10.0% (adjusted R^2) of the total variance; both ApoE4 genotype and previous BZD use accounted for 32.3% (adjusted R^2) of the total variance. Second analysis showed that dose x duration did not predict the average AV-45 SUVR ($B = -0.13$, $t = -0.72$, $df = 28$, $p = 0.48$), while FAQ scores predicted it ($B = 0.38$, $t = 2.17$, $df = 28$, $p = 0.04$), accounting 11.3% (adjusted R^2) of the total variance.

The confirmatory voxel-based analysis showed that PRE-BZD group showed higher AV-45 SUVR in bilateral frontal, cingulate and parietal region in comparison to PRE+BZD users (**Figure 2**). No cortical region showed higher AV-45 SUVR in PRE+BZD users in comparison to the PRE-BZD group.

The effects of chronic BZD use on cognitive function

The mixed model analysis, controlling for dose x duration and antidepressant use, showed no difference in changes in MOCA, ADAS-13, RAVLT immediate recall and RAVLT percentage of forgetting between the CON+BZD and matched CON-BZD group (**Figure 3**). There was also no difference in changes in scores on ADAS-11 ($F(1, 27.46) = 0.18$, $p = 0.68$), RAVLT learning ($F(1, 26.65) = 0.87$, $p = 0.36$), and RAVLT forgetting ($F(1, 26.87) = 0.39$, $p = 0.54$) between the two groups.

The effects of chronic BZD uses on changes in cortical beta-amyloid

The mixed model analysis, controlling for dose x duration and antidepressant use, showed no difference in AV-45 SUVR changes in frontal, cingulate, parietal, and temporal regions between CON-BZD and CON+BZD users (**Figure 4**).

Discussion

To the best of our knowledge, this is the first PET study investigating the effect of BZDs on cortical A β levels in elderly non-demented adults. We observed that PRE+BZD users had lower cortical A β , in comparison to the matched PRE-BZD group, which was confirmed by the voxel-based analysis. In terms of progressive cognitive decline, there was no difference between CON-BZD and CON+BZD groups on measures of cognition. Together, these findings suggest that BZDs may not contribute to progressive cognitive deterioration, which may, in part, be attributable to the neuroprotective effects of lower cortical A β levels in elderly adults.

Abnormally excessive neuronal excitability is also a common feature in AD. Disturbance in calcium (Ca²⁺) homeostasis is highly implicated in neurodegenerative disorders (28). There is a bi-directional link between Ca²⁺ and A β . Altered Ca²⁺ signaling promotes A β formation (28), which in turn, induces Ca²⁺ influx and elevated intracellular Ca²⁺ levels, leading to increased neuronal excitability (29). Furthermore, neuronal excitability increases vulnerability to glutamate-mediated excitotoxicity and oxidative injury (30). A β decreases the reuptake of glutamate from synapses, leading to increased extracellular glutamate levels (31). This interaction between N-methyl-D-aspartate (NMDA) glutamate receptors and A β appears to be highly neurotoxic, through A β promotion of NMDA receptor excessive activation (32). In support of the theory of glutamate-mediated excitotoxicity in patients with AD, NMDA glutamate receptor antagonists, including memantine, are effective, albeit modestly, for patients with AD.

A principal function of BZDs is neuronal depression. BZDs are positive allosteric modulators of GABA-A receptors. After BZDs bind to the allosteric binding site, the chloride ion channel's response to GABA increases, facilitating neuronal membrane hyperpolarization (33). Anticonvulsants have similar effects as BZDs, by enhancing GABAergic signaling, are shown to attenuate neuronal injuries and A β -mediated neurotoxicity (34). Several animal studies have suggested that BZDs reduce A β levels, although the exact mechanism by which this occurs is currently unknown. Repetitive diazepam use significantly reduced A β levels in the cortex and hippocampus in an AD mouse model (17); furthermore, a recent *in-vitro* study showed that midazolam prevented A β formation (18). Another animal study showed that diazepam lowered the levels of A β deposition, but exacerbated impaired memory in transgenic mice (16).

Based on A β level lowering, BZDs may have neuroprotective effects by possibly preventing glutamate-mediated excitotoxicity and cortisol suppression (35).

In addition to enhancing the GABAergic signaling, BZDs bind to translocator proteins 18kDA (TSPO), formerly known as peripheral BZD receptors, which are not coupled to GABA-A receptors. There is evidence suggesting that ligands that modulate TSPO promote neurosteroid synthesis; this could elicit therapeutic effects by reducing the expression of pro-inflammatory genes and cytokines, and eliminate neuroinflammation, which is linked to A β (36). However, it is unclear whether BZD binding to TSPOs would be of benefit to patients with AD.

In the present study, confounders may have influenced our finding of decreased A β levels in PRE+BZD users. One confounder consisted of possible differences in severity of neuropsychiatric symptoms between the PRE+BZD and PRE-BZD group. PRE+BZD users were treated with medications that alleviate neuropsychiatric symptoms, such as anxiety and depression, which are known to elevate the risk of dementia. On the other hand, the PRE-BZD group may have had untreated neuropsychiatric symptoms. There is also well-documented evidence supporting the association between sleep disturbances and increased A β levels (37). Nonetheless, our results showed no difference in neuropsychiatric symptoms at baseline, as assessed by total NPI scores, after controlling for the temporal discrepancy between the clinical assessment and PET scan between PRE-BZD and PRE+BZD users. Furthermore, history of psychiatric disorders, such as anxiety disorders, may influence the level of A β . However, no difference in the number of individuals with a history of psychiatric disorders was found between groups. Thus, higher A β levels in the PRE-BZD group were likely not attributable to the differences in neuropsychiatric symptoms or history of psychiatric disorders. No difference in the NPI total scores between the PRE-BZD and PRE+BZD users, however, suggests that the former may not have sought treatment for their neuropsychiatric symptoms. Relatedly, another potential confounder was antidepressant use. There is evidence that antidepressants lower A β levels (38). Although it did not reach a statistical significance, our finding indicates that there was higher number of patients treated with antidepressants among PRE+BZD users (n= 7) in comparison to the PRE-BZD group (n = 2). To address

this, we controlled for antidepressant use in order to diminish the confounding effect on A β levels. Furthermore, results from our regression analyses revealed that antidepressant use did not predict A β levels. Another confounder is the possible differential effect of BZD derivatives and non-BZD derivatives on A β accumulation. A subgroup analysis using independent t-test between BZD derivative users (n = 7) and non-BZD derivative users (n = 7) revealed no difference in AV-45 SUVR in the frontal, cingulate, parietal and temporal regions between the two groups. Therefore, classes of BZDs did not seem to influence our findings.

Our results indicate that continuous BZD administration did not contribute to progressive cognitive decline in elderly adults, which is consistent with findings from other studies (7-9). In fact, a recent study showed a lower relative risk of developing AD in the long-term BZD users in comparison to non-users (10). Findings of increased risk of dementia in BZD takers may be attributable to possibilities that BZDs are prescribed to patients with prodromal symptoms of dementia prior to a diagnosis of the disorder (9). Our findings suggest that BZDs use may not be directly linked to progressive cognitive impairment, at least in non-demented elderly individuals. Furthermore, it is important to note that in the PRE-BZD group, there was a bimodal distribution of high amyloid (n = 9) and low amyloid (n = 6) individuals. In order to find an explanation for the difference in the level of A β , we compared their clinical and demographic variables. There was no difference in any of the variables between groups. This leads to the variability of level of A β among non-demented elderly individuals potentially due to other environmental risk factors, such as accumulated high level of stress (27).

There were several limitations to our study. First, the sample size was small. Contrary to literature suggesting a high proportion of BZD users in the elderly population, there was a limited number of BZD users based on the ADNI medication log database. Because of the small sample size, we were unable to explore the effect of BZD formulations with different half-lives on A β levels and cognition. Nonetheless, given that this is the first neuroimaging study to investigate the effect of BZD on beta-amyloid, further research is needed to use larger sample sizes to investigate the effects of BZDs on cortical A β . Second, the duration of follow-up for the longitudinal analysis was only 2 years. This is relatively short to observe

drastic changes in cognitive function and cortical A β levels, especially in non-demented elderly adults. Third, data regarding BZD usage was obtained from the primary caregiver or self-reported medication record log. Additionally, different neuropsychiatric conditions for which BZDs are prescribed may exert differential effects on AD pathology. Fourth, another limitation is that individuals who used or have been used different types of BZDs, from atypical BZD derivatives to BZD agonists derivatives, were included in the same BZD user group. Fifth, radiotracer AV-45 may bind non-specifically to white matter in addition to A β (39), potentially leading to a spillover effect from white matter to the reference region. Lastly, cortical A β accumulation is an early-occurring pathological change, whose rate of deposition slows down over the course of the disease in elderly individuals (40). As a result, we may not have observed drastic changes in cortical A β levels over the 2 years of follow-up in both the CON+BZD and CON-BZD groups.

In conclusion, we observed that BZD use was associated with lower cortical A β in non-demented elderly adults and that chronic BZD was not linked to progressive cognitive decline. As BZD use is linked to amnestic effects, long-term use of BZD is still discouraged and must be cautiously prescribed to elderly individuals. Future studies with larger samples of elderly individuals must be carried out to reproduce and confirm the findings of our study.

Declaration of Interest statement

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Appendix A

The ADNI was launched in 2003 by the National Institute on Aging (NIA), the National Institute of Biomedical Imaging and Bioengineering (NIBIB), the Food and Drug Administration (FDA), private

pharmaceutical companies and non-profit organizations, as a \$60 million, 5-year public private partnership. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early Alzheimer's disease (AD).

Determination of sensitive and specific markers of very early AD progression is intended to aid researchers and clinicians to develop new treatments and monitor their effectiveness, as well as lessen the time and cost of clinical trials.

The Principal Investigator of this initiative is Michael W. Weiner, MD, VA Medical Center and University of California – San Francisco. ADNI is the result of efforts of many coinvestigators from a broad range of academic institutions and private corporations, and subjects have been recruited from over 50 sites across the U.S. and Canada. The initial goal of ADNI was to recruit 800 subjects but ADNI has been followed by ADNI-GO and ADNI-2.

To date these three protocols have recruited over 1500 adults, ages 55 to 90, to participate in the research, consisting of cognitively normal older individuals, people with early or late MCI, and people with early AD. The follow up duration of each group is specified in the protocols for ADNI-1, ADNI-2 and ADNI-GO. Subjects originally recruited for ADNI-1 and ADNI-GO had the option to be followed in ADNI-2. For up-to-date information, see www.adni-info.org.

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Supplementary Table 1. Comparison of demographic and clinical variables between non-demented elderly adults who had been using benzodiazepines for at least one year (PRE+BZD) and matched non-demented elderly adults who had never used benzodiazepines (PRE-BZD). * indicates statistical significance $p < 0.05$.

Demographic variables	PRE+BZD		PRE-BZD		t	df	p-value
	(n = 15)		(n = 15)				
	mean	SD	mean	SD			
Age, years	75.36	8.35	76.27	4.18	0.69	14	0.50
Education years	16.73	2.52	15.53	2.53	-1.34	14	0.20
MOCA score	26.27	2.46	25.6	2.64	-0.62	14	0.55
CDRSB score	0.07	0.18	0.13	0.52	0.82	14	0.42
ADAS-11	4.53	3.16	4.27	1.49	-0.26	14	0.80
ADAS-13	7.33	5	7.2	2.78	-0.08	14	0.94
RAVLT immediate	47	13.88	48.6	6.8	0.38	14	0.71
RAVLT_learning	6.07	2.55	6.4	2.35	0.39	14	0.70
RAVLT forgetting	4.27	2.34	2.6	2	-1.77	14	0.10
RAVLT per.forgetting	41.14	27.7	22.35	20.15	-1.74	14	0.10
FAQ	0.13	0.52	0.33	0.72	0.82	14	0.42
TBV	1134.56	125.97	1135.34	96.79	0.03	13	0.98

Demographic variables	Number (frequency [%])	Number (frequency [%])	p-value (two-tailed)
CVD+	12 (80.00)	9 (60.00)	0.51
Smoking+	5 (33.33)	6 (40.00)	1.00
White	13 (86.67)	14 (93.33)	0.60
Hispanic/Latino	0 (0)	1 (6.67)	1.00
Married	12 (80.00)	13 (86.67)	0.50
Females	11(73.33)	11(73.33)	1.00
No ApoE4	9 (60.00)	9(60.00)	1.00
Psychiatric +	8 (53.33)	6(40.00)	0.73

There was no difference in all demographic variables listed above between the PRE+BZD and PRE-BZD groups, except for the RAVLT forgetting and RAVLT percentage of forgetting (pair t-test, two-tailed for the continuous demographic variables; McNemar's test, two-tailed for the dichotomous variables).

Abbreviations: ADAS-11 = Alzheimer's Disease Assessment Scale 11 items; ADAS-13 = Alzheimer's Disease Assessment Scale 13 items; ApoE4 = Apolipoprotein E4; CDR = the Clinical Dementia Rating; CVD+ = presence of history of cardiovascular disease; df = degree of freedom; FAQ = the Functional Activities Questionnaire score; MOCA = the Montreal Cognitive Assessment score; NPI = the Neuropsychiatric Inventory; PRE+BZD = non-demented elderly adults who have been using benzodiazepine at least for a year; PRE-BZD = non-demented elderly adults who have never used benzodiazepine; Psychiatric + = Individuals with a history of psychiatric disorders; RAVLT immediate = Rey Auditory Verbal Learning Test immediate recall score (sum of 5 trials); RAVLT forgetting = Rey Auditory Verbal Learning Test forgetting (trial 5- delayed); RAVLT learning = Rey Auditory Verbal Learning Test learning (trial 5- trial 1) ; RAVLT per_forgetting = Rey Auditory Verbal Learning Test percentage of forgetting ; Smoking+ = currently smoking

Supplementary Table 2. Comparison of demographic and clinical variables between non-demented elderly adults who had been continuously using benzodiazepines during the entire duration of this study (CON+BZD) and matched non-demented elderly adults who had never used benzodiazepines (CON-BZD). * indicates statistical significance $p < 0.05$.

Demographic variables	Con+BZD (n=15)		Con-BZD (n=15)		t	df	p-value
	mean	SD	mean	SD			
Age, years	75.1	9.3	75.7	4.33	0.38	14	0.71
Education years	16.4	2.67	15.27	2.46	-1.08	14	0.30
MOCA score	25.93	3.1	25.73	2.59	-0.18	14	0.86
CDRSB score	0.03	0.13	0	0	-1.00	14	0.33
ADAS-11	4.33	3.04	4.47	1.55	0.13	14	0.90
ADAS-13	7.13	4.73	7.2	2.62	0.04	14	0.97
RAVLT immediate	46.67	13.74	48.07	7.19	0.34	14	0.74
RAVLT_learning	5.53	2.29	6.13	2.2	0.78	14	0.45
RAVLT forgetting	4	2.33	2.87	1.92	-1.23	14	0.24
RAVLT per.forgetting	38.4	25.13	25.53	20.89	-1.24	14	0.24
FAQ	0.13	0.52	0.27	0.59	0.62	14	0.55
TBV	1121.93	100.82	1147.4	95.02	0.62	11	0.55

Demographic variables	Number (frequency [%])	Number (frequency [%])	p-value (two-tailed)
CVD+	10 (66.67)	9 (60.00)	1.00
Smoking+	6 (40.00)	8 (53.33)	0.69
White	14 (93.33)	15 (100.00)	1.00
Hispanic/Latino	1 (6.67)	0 (0)	1.00
Married	11 (73.33)	13 (86.67)	0.50
Females	11(73.33)	11(73.33)	1.00
No ApoE4	9 (60.00)	10 (66.67)	1.00
Psychiatric +	7 (46.67)	5 (33.33)	0.73

There was no difference in all the demographic variables listed above between the CON+BZD and CON-BZD groups (pair t-test, two-tailed for the continuous demographic variables; McNemar's test, two-tailed for the dichotomous variables).

Abbreviations: ADAS-11 = Alzheimer's Disease Assessment Scale 11 items; ADAS-13 = Alzheimer's Disease Assessment Scale 13 items; ApoE4 = Apolipoprotein E4; CDR = the Clinical Dementia Rating; CON+BZD = non-demented elderly adults who have been continuously using benzodiazepine for the entire duration of the study; CON-BZD = non-demented elderly adults who have never used benzodiazepine; CVD+ = presence of history of cardiovascular disease; df = degrees of freedom; FAQ = the Functional Activities Questionnaire score; MOCA = the Montreal Cognitive Assessment score; RAVLT immediate = Rey Auditory Verbal Learning Test immediate recall score (sum of 5 trials); RAVLT forgetting = Rey Auditory Verbal Learning Test forgetting score (trial 5- delayed); RAVLT learning = Rey Auditory Verbal Learning Test learning (trial 5- trial 1) ; RAVLT per_forgetting = Rey Auditory Verbal Learning Test percentage of forgetting ; Smoking+ = currently smoking

Figure 1. Scattergraphs showing F^{18} -Florbetapir standardized uptake value ratios (AV-45 SUVR) with a reference to a composite reference region between PRE+BZD users ($n = 15$) and the matched PRE-BZD group ($n = 15$) in the a) frontal region; b) cingulate region; c) parietal region; d) temporal region

PRE-BZD group showed higher frontal, cingulate, parietal and temporal AV-45 SUVR in comparison to PRE+BZD group. The analysis of covariance was carried out in SPSS (Version 21.0).

Figure 2. a) Brain image from the voxel-based analysis showing the difference in the AV-45 SUVR between PRE+BZD users ($n = 11$) and the matched PRE-BZD group ($n = 12$)($p = 0.01$, voxel extent of 100)

b) Coordinates showing cortical regions of higher beta-amyloid in PRE-BZD users (n = 12) in comparison to the PRE+BZD group (n = 11)

Coordinate mm								
x	y	z	Regions	Hemisphere	BA	Cluster size	T	P
0	-10	48	Medial frontal gyrus	Left	6	292	3.64	0.00087
0	-18	38	Cingulate gyrus	Left	24		2.97	0.0040
4	-24	42	Paracentral lobule	Right	31		2.90	0.0046
48	-42	52	Inferior parietal lobule	Right	40	114	3.60	0.00095
42	-42	44	Inferior parietal lobule	Right	40		3.11	0.0029
56	-30	48	Postcentral gyrus	Right	40		2.90	0.0046
30	56	20	Middle frontal gyrus	Right	10	157	3.49	0.0012
0	38	42	Medial frontal gyrus	Left	8	704	3.43	0.0014
4	42	22	Medial frontal gyrus	Right	9		3.33	0.0018
6	22	32	Cingulate gyrus	Right	32		3.33	0.0018
-30	6	56	Middle frontal gyrus	Left	6	104	3.40	0.0015

PRE-BZD showed higher AV-45 SUVR mainly in the frontal, cingulate and parietal regions. Statistical parametric map comparison was performed with a two-sample t-test with covariates as implemented in SPM12. The degrees of freedom = (1,19)

Figure 3. Graphs showing the changes in the a) MOCA score; b) ADAS-13 score; c) RAVLT immediate recall score; d) RAVLT percentage of forgetting, over the 2 year period between the CON+BZD (n = 15) and the matched CON-BZD group (n = 15).

No difference in changes in scores on MOCA, ADAS-13, RAVLT-immediate and RAVLT-% forgetting was found between the CON-BZD and CON+BZD group. Comparison was performed with a mixed-effect model for repeated measurements analysis implemented in SPSS (Version 21.0).

Abbreviations: ADAS-13 = Alzheimer's Disease Assessment Scale 13 items; CON+BZD = non-demented elderly adults who have been continuously using benzodiazepine for the entire duration of the study; CON-BZD = non-demented elderly adults who have never used benzodiazepine; MOCA = the Montreal Cognitive Assessment score; RAVLT immediate = Rey Auditory Verbal Learning Test immediate recall score (sum of 5 trials); RAVLT-% forgetting = Rey Auditory Verbal Learning Test percentage of forgetting

Figure 4. Graphs showing the changes in the a) frontal AV-45 SUVR; b) cingulate AV-45 SUVR; c) parietal SUVR; d) temporal SUVR, over the 2 year period between the CON+BZD (n = 15) and the matched CON-BZD group (n = 15).

No difference in changes in frontal, cingulate, parietal and temporal AV-45 SUVR was found between the CON-BZD and CON+BZD group. Comparison was performed with a mixed-effect model for repeated measurements analysis implemented in SPSS (Version 21.0).

Abbreviations: AV-45 = F^{18} -Florbetapir standardized uptake value ratios; CON+BZD = non-demented elderly adults who have been continuously using benzodiazepine for the entire duration of the study; CON-BZD = non-demented elderly adults who have never used benzodiazepine