2. Materials and methods

The CIRCS is an ongoing dynamic community cohort study involving 5 communities in Japan. Details of the CIRCS protocol have been described elsewhere [13]. In this study, we included only 1 community, Kyowa, where disabling dementia surveillance is being carried out, and serum CoQ10 values have been measured. A total of 65 cases of patients who were diagnosed between 1999 and 2004 as having disabling dementia, participated in annual health checkups (baseline) at least 5 years before receiving the dementia diagnosis (ie, between 1984 and 1994), were aged 40–69 years at baseline, and provided sera for storage at baseline were identified. The criteria of disabling dementia were the same as those of our previous study [14], which also includes the validation of the criteria and the details of the study protocol. As supplemental analysis, we further classified the dementia cases into cases with and without history of stroke on the basis of the systematic stroke registration system described elsewhere [14]. We excluded individuals aged 70 years or older at baseline, because serum CoQ10 would be likely affected by the baseline age, and residents aged 70 years and over were not invited systematically to the baseline examination. One hundred thirty randomly selected controls whose age (±3 years), sex, and follow-up time were matched at a ratio of 2:1 with the cases were also identified from the risk set. Venous blood was collected at baseline, and sera were prepared from the blood samples as soon as possible after the blood collection at the checkup sites. The serum samples were collected in 0.3 mL tubes and stored at −80 °C until measurement in 2005. Serum CoQ10 was measured using high-performance liquid chromatography (HPLC) at the Public Health Institute of Kochi Prefecture with modification of the reported methods [15]. Briefly, a 10 μL serum sample was pretreated with 380 μL of 2-propanol. After centrifugation at 7000 g for 5 min, 10 μL of supernatant was applied to the HPLC system (L-7000 series; Hitachi High-Technologies Corporation, Tokyo, Japan) using a SUPELCOSIL LC-8 HPLC Column (Sigma–Aldrich Japan, Tokyo, Japan), an RC-10 reduction column (Shiseido Company, Tokyo, Japan), and an electrochemical detector (SI-2; Shiseido Company). The oxidation potential for the electrochemical detector was 600 mV. The mobile phase consisted of 50 mmol/L sodium perchlorate in methanol/2-propanol (100/10, v/v) with a flow rate of 1 mL/min. Serum total cholesterol was measured at baseline using the Liebermann-Burchard direct method at the Osaka Medical Center for Cancer and Cardiovascular Disease, an international member of the US National Cholesterol Reference Method Laboratory Network (CRMLN) [16].

For statistical analyses, we conducted conditional logistic analyses using SAS 9.1.3. Service Pack 4 (SAS Institute, Cary, NC, USA) with adjustments for body mass index, smoking status, alcohol consumption, diastolic blood pressure, total cholesterol, diabetes mellitus, and use of medication for hypertension or hypercholesterolemia. Since CoQ10 and total cholesterol were strongly correlated (Spearman $r = 0.34$), we also examined the association of the CoQ10/total cholesterol ratio with risk of disabling dementia. All probability values for the statistical tests were 2-tailed, and probability values below 0.05 were considered significant. Informed consent was obtained from community leaders and, verbally, from individual participants according to the guidelines of the Council for International Organizations of Medical Science [17]. The study was approved by the institutional review boards of the Osaka Center for Cancer and Cardiovascular Disease Prevention and of the University of Tsukuba.

3. Results

As shown in Table 1, the baseline characteristics did not differ materially between the cases and the non-cases, although diastolic blood pressure levels tended to be higher, and prevalence of cholesterol-lowering medication use, lower for cases than for non-cases.

Table 1
Baseline characteristics of dementia cases and non-cases, CIRCS aged 40–69 years.

	Dementia cases	Non-cases	*P* for difference
Number	65	130	
Age, y	64.5	64.1	0.64
Male gender, %	34	34	1.00
Body mass index, kg/m^2	24.3	24.1	0.77
Current smokers, %	26	22	0.47
Current drinkers, %	28	28	1.00
Systolic blood pressure, mm Hg	137	136	0.43
Diastolic blood pressure, mm Hg	81	79	0.08
Antihypertensive medication, %	28	33	0.45
Diabetes mellitus, %	14	7	0.12
Serum total cholesterol, mg/dL	205	201	0.53
Cholesterol-lowering medication, %	3	10	0.09
Serum coenzyme Q10,[a] nmol/L	731	762	0.32
Coenzyme Q10/total cholesterol ratio,[a] 10^{-6}	138.5	151.4	0.15

[a] Median values and *P* values for differences tested by the Wilcoxon rank sum test.

Serum coenzyme Q10 was inversely associated with risk of incident dementia (Table 2). The multivariate odds ratios and 95% confidence intervals were 0.68 (0.26–1.78), 0.92 (0.33–2.56), and 0.23 (0.06–0.86), for individuals with the second, third, and highest quartiles of CoQ10, respectively, compared with the lowest quartile (*P* for trend = 0.05). A similar association was observed for the CoQ10/total cholesterol ratio. The respective odds ratios were 0.67 (0.25–1.78), 0.73 (0.28–1.92), and 0.21 (0.05–0.90) (*P* for trend = 0.04).

As supplemental analysis, we stratified dementia cases into those with history of stroke and those without it. Although the number of cases was limited, the associations were stronger for dementia without stroke for both serum CoQ10 and the CoQ10/total cholesterol ratio. The unadjusted odds ratios and 95% confidence intervals of dementia without stroke were 1.18 (0.37–3.75), 0.50 (0.15–1.70), and 0.46 (0.12–1.74) for individuals with the second, third, and highest quartiles of CoQ10 (*P* for trend = 0.13), and were 0.47 (0.15–1.48), 0.34 (0.11–1.09), and 0.24 (0.06–0.98) for respective quartiles of CoQ10/total cholesterol ratio (p for trend = 0.04). The corresponding odds ratios and 95% confidence intervals of dementia with stroke were 0.82 (0.26–2.57), 2.24 (0.63–7.96) and 0.35 (0.06–2.05) according to quartiles of CoQ10 (p for trend = 0.45), and were 1.68 (0.45–6.29), 2.05 (0.51–8.30) and 0.43 (0.06–2.97) according to quartiles of CoQ10/total cholesterol ratio (p for trend = 0.43).

4. Discussion

We found a strong inverse association between serum CoQ10 concentration and risk of disabling dementia in the Japanese population. To date, this is the first prospective study to examine the association between CoQ10 and incident dementia in a general setting.

Several animal studies have shown that CoQ10 may have a beneficial effect on dementia progression. In transgenic mice, dietary supplementation with CoQ10 reduced brain oxidative stress and deposition of amyloid plaque or amyloid-β and improved behavioral performance [11]. Rats with damaged hippocampi and cerebral cortices showed an adverse alteration in the markers of oxidative damage, but with supplementation with CoQ10, this alteration was reversed [8]. Thus, CoQ10 has been considered a

Table 2
Age and sex-matched and multivariate adjusted conditional odds ratios and 95% confidence intervals of incident dementia according to quartiles of serum coenzyme Q10 and coenzyme Q10/total cholesterol ratio.

	Men and women				
	Quartiles of serum coenzyme Q10 (nmol/L)				
	Q1 228–558	Q2 559–765	Q3 766–1015	Q4 1016–2353	Trend *P*
Median, nmol/L	459	635	850	1253	
Total disabling dementia					
Number of cases	18	20	18	9	
Number of non-cases	32	33	32	33	
Matched OR (95%CI)[a]	1.0	1.03 (0.47–2.29)	0.94 (0.41–2.14)	0.43 (0.16–1.19)	0.10
Multivariable OR (95%CI)[c]	1.0	0.68 (0.26–1.78)	0.92 (0.33–2.56)	0.23 (0.06–0.86)	0.05
	Quartiles of serum coenzyme Q10/total cholesterol ratio (10^{-6})				
	Q1 54.1–107.5	Q2 107.6–151.3	Q3 151.4–203.6	Q4 203.7–489.1	Trend *P*
Median, 10^{-6}	91.2	127.3	173.6	230.7	
Total disabling dementia					
Number of cases	21	19	17	8	
Number of non-cases	32	33	33	32	0.02
Matched OR (95%CI)[a]	1.0	0.80 (0.35–1.81)	0.72 (0.31–1.67)	0.28 (0.09–0.84)	
Multivariable OR (95%CI)[b]	1.0	0.67 (0.25–1.78)	0.73 (0.28–1.92)	0.21 (0.05–0.90)	0.04

[a] Matched with age (±3 years), sex and baseline-year.
[b] Multivariable model further includes body mass index, smoking status, alcohol consumption, diastolic blood pressure, diabetes mellitus and medication of hypertension and hypercholesterolemia.
[c] Further includes serum total cholesterol.

promising treatment for Alzheimer disease. However, a recent randomized control trial of patients with mild-to-moderate Alzheimer disease did not show that antioxidant treatment, including supplementation with high-dose CoQ10, improved the indices of markers of oxidative stress or of neurodegeneration in the cerebrospinal fluid [12]. Although this finding warrants confirmation by replication studies, when it is taken together with the findings of the present study, we assume that CoQ10 could have a more prominent impact on prevention, rather than on treatment, of dementia.

The limitations of this study include (1) the relatively small number of cases, although the associations were strong enough to be detected; (2) the diagnoses of disabling dementia conducted by attending physicians, although such diagnosis by attending physicians was previously validated [14]; and (3) the use of long-stored sera, although a previous study showed that the plasma CoQ10 value was unchanged after being deep-frozen and stored for 3 years [18]. When we excluded the serum samples collected before 1989 (23 cases and 46 matched controls excluded), the results were similar (not shown). Fourth limitation is that we did not classify dementia into Alzheimer type and vascular type. Instead, we have information on dementia with and without history of stroke. The associations seemed stronger for dementia without history of stroke, which needs to be confirmed by further studies. Last, we did not survey the dementia at baseline. However, we constructed the baseline at least 5 years prior to the beginning of dementia survey, so the possibility of reverse causation may be small.

The inverse association between serum CoQ10 levels and incident dementia did not directly assure that dietary intake of CoQ10 would prevent dementia. Furthermore, CoQ10 supplements were not generally available in Japan at the era of the baseline period (1984–1994). The impact of dietary or supplementary intake of CoQ10 was not tested in the present study, which must be examined by a randomized control trial in the future.

In conclusion, the serum level of CoQ10 was inversely associated with disabling dementia in this Japanese general population, which suggests that higher serum CoQ10 levels may have a beneficial effect on prevention of dementia.

Acknowledgments

The authors thank Kimiko Yokota, Yumi Shiina, Miyuki Hori, Tomoko Tanaka, and Kumi Otsuka, for technical assistance. We also thank Ms Flaminia Miyamasu, Medical English Communications Center, University of Tsukuba, for editorial assistance. This work was supported by Health and Labour Science Research Grants for Dementia (H21-Ninchisho-Wakate-007, 2009–2012, and H24-Ninchisho-Wakate-003, 2012–2015), Ministry of Health, Labour and Welfare, Japan. We have no potential conflicts of interest to declare. The full list of CIRCS investigators is presented in reference [13].

References

[1] Block G, Jensen CD, Norkus EP, Dalvi TB, Wong LG, McManus JF, Hudes ML. Usage patterns, health, and nutritional status of long-term multiple dietary supplement users: a cross-sectional study. Nutr. J. 2007;6:30.
[2] Shults CW, Oakes D, Kieburtz K, Beal MF, Haas R, Plumb S, Juncos JL, Nutt J, Shoulson I, Carter J, Kompoliti K, Perlmutter JS, Reich S, Stern M, Watts RL, Kurlan R, Molho E, Harrison M, Lew M. Effects of coenzyme Q10 in early parkinson disease: evidence of slowing of the functional decline. Arch. Neurol. 2002;59:1541–50.
[3] Feigin A, Kieburtz K, Como P, Hickey C, Claude K, Abwender D, Zimmerman C, Steinberg K, Shoulson I. Assessment of coenzyme Q10 tolerability in huntington's disease. Mov. Disord. 1996;11:321–3.
[4] Lodi R, Rajagopalan B, Bradley JL, Taylor DJ, Crilley JG, Hart PE, Blamire AM, Manners D, Styles P, Schapira AH, Cooper JM. Mitochondrial dysfunction in friedreich's ataxia: from pathogenesis to treatment perspectives. Free Radic. Res. 2002;36:461–6.
[5] Cooper JM, Schapira AH. Friedreich's ataxia: disease mechanisms, antioxidant and coenzyme Q10 therapy. Biofactors 2003;18:163–71.
[6] Rosenfeldt F, Hilton D, Pepe S, Krum H. Systematic review of effect of coenzyme Q_10 in physical exercise, hypertension and heart failure. Biofactors 2003;18:91–100.
[7] Burke B, Neuenschwander R, Olson R. Randomized, double-blind, placebo-controlled trial of coenzyme q10 in isolated systolic hypertension. South Med. J. 2001;94:1112–7.
[8] Ishrat T, Khan MB, Hoda MN, Yousuf S, Ahmad M, Ansari MA, Ahmad AS, Islam F. Coenzyme Q10 modulates cognitive impairment against intracerebroventricular injection of streptozotocin in rats. Behav. Brain Res. 2006;171:9–16.
[9] Yang X, Yang Y, Li G, Wang J, Yang ES. Coenzyme Q10 attenuates beta-amyloid pathology in the aged transgenic mice with alzheimer presenilin 1 mutation. J. Mol. Neurosci. 2008;34:165–71.

[10] Li G, Jack CR, Yang XF, Yang ES. Diet supplement CoQ10 delays brain atrophy in aged transgenic mice with mutations in the amyloid precursor protein: an in vivo volume mri study. Biofactors 2008;32:169–78.
[11] Dumont M, Kipiani K, Yu F, Wille E, Katz M, Calingasan NY, Gouras GK, Lin MT, Beal MF. Coenzyme Q10 decreases amyloid pathology and improves behavior in a transgenic mouse model of alzheimer's disease. J. Alzheimers Dis. 2011;27:211–23.
[12] Galasko DR, Peskind E, Clark CM, Quinn JF, Ringman JM, Jicha GA, Cotman C, Cottrell B, Montine TJ, Thomas RG, Aisen P. Antioxidants for alzheimer disease: a randomized clinical trial with cerebrospinal fluid biomarker measures. Arch. Neurol. 2012;69:836–41.
[13] Imano H, Iso H, Kiyama M, Yamagishi K, Ohira T, Sato S, Noda H, Maeda K, Okada T, Tanigawa T, Kitamura A. Non-fasting blood glucose and risk of incident coronary heart disease in middle-aged general population: the Circulatory Risk in Communities Study (CIRCS). Prev. Med. 2012;55:603–7.
[14] Ikeda A, Yamagishi K, Tanigawa T, Cui R, Yao M, Noda H, Umesawa M, Chei C, Yokota K, Shiina Y, Harada M, Murata K, Asada T, Shimamoto T, Iso H. Cigarette smoking and risk of disabling dementia in a japanese rural community: a nested case-control study. Cerebrovasc. Dis. 2008;25:324–31.
[15] Yamashita S, Yamamoto Y. Simultaneous detection of ubiquinol and ubiquinone in human plasma as a marker of oxidative stress. Anal. Biochem. 1997;250:66–73.
[16] Nakamura M, Sato S, Shimamoto T. Improvement in Japanese clinical laboratory measurements of total cholesterol and hdl-cholesterol by the us cholesterol reference method laboratory network. J. Atheroscler. Thromb. 2003;10:145–53.
[17] International guidelines for ethical review of epidemiological studies. Law Med. Health Care 1991;19:247–58.
[18] Kaikkonen J, Nyyssonen K, Salonen JT. Measurement and stability of plasma reduced, oxidized and total coenzyme Q10 in humans. Scand. J. Clin. Lab. Invest 1999;59:457–66.

Geriatrics Gerontology International — Medline Indexed

Geriatr Gerontol Int 2015

Prevention of cognitive and physical decline by enjoyable walking-habituation program based on brain-activating rehabilitation

Tatsuhiko Murai,[1] Tomoharu Yamaguchi,[1]† Yohko Maki,[1]†† Mikie Isahai,[2] Ayumi Kaiho Sato,[2] Tetsuya Yamagami,[3] Chiaki Ura,[4] Fumiko Miyamae,[4] Ryutaro Takahashi[4] and Haruyasu Yamaguchi[1]

[1]Gunma University Graduate School of Health Sciences, Maebashi, [2]Department of Longevity, Takasaki City Office, [3]School of Health Care, Takasaki University of Health and Welfare, Takasaki, and [4]Research on Social and Human Sciences, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

Aim: Evaluating effects of an enjoyable walking-habituation program.

Methods: We carried out a 12-week intervention, consisting of an enjoyable walking-habituation program based on five principles of brain-activating rehabilitation: pleasant atmosphere, interactive communication, social roles, praising each other and errorless support. The program, once a week for 90 min, was carried out in small groups. Participants were 71 community-dwelling people (72.2 ± 4.3) without dementia. Cognitive function was evaluated in five cognitive domains: memory, executive function, word fluency, visuospatial abilities and sustained attention. Additionally, quality of life, depressive state, functional capacity, range of activities, social network and subjective memory complaints were assessed using questionnaires. Motor function was also evaluated. Measurement was carried out before the observation period, after observation and after intervention.

Results: A total of 63 participants were included in the analysis. Daily steps, executive function, subjective memory complaints, functional capacity and 5-m maximum walking time significantly improved during the intervention period (after observation to after intervention) compared with the observation period (before the observation period to after observation). No significant differences were seen in other evaluations. At 6 months after the intervention, 52 of 63 participants (82.5%) continued to walk once a week or more, and all of them were confident about continuing to walk in the future. Furthermore, all participants were satisfied with our walking-habituation program and all replied that they felt delighted.

Conclusion: The intervention program, based on the five principles of brain-activating rehabilitation, resulted in improvement of some cognitive and physical functions, as well as a high walking-habituation rate at 6 months' follow up. **Geriatr Gerontol Int 2015; ••: ••–••.**

Keywords: brain-activating rehabilitation, dementia, prevention, quality of life, walking-habituation.

Introduction

In Japan, the number of elderly people with dementia is estimated at 4.5 million, which is approximately 15% of the elderly population. Increased prevalence of dementia is caused by longevity as a result of a decreased mortality rate by stroke, cancer and myocardial infarction. The Japanese Public Long-Term Care (LTC) Insurance Act was enacted in April 2000 to respond to the falling birthrate and the growing elderly population with the intention to build a sustainable and fair social security system.

People aged over 40 years pay premiums for long-term care insurance not covered by their public health insurance, and those who are qualified under this system can receive care services. The revision of the act in 2008 led to a greater emphasis on preventive LTC,

Accepted for publication 26 April 2015.

Correspondence: Mr Haruyasu Yamaguchi MD PhD, Gunma University Graduate School of Health Sciences, 3-39-15 Showama-machi, Maebashi, Gunma 371-8514, Japan. Email: yamaguti@gunma-u.ac.jp
†*Present address:* Gunma University Graduate school of Health Sciences.
††*Present address:* National Center for Geriatrics and Gerontology.

© 2015 Japan Geriatrics Society
doi: 10.1111/ggi.12541

and local municipalities are expected to play leading roles in building the platform and network for preventive activities.[1]

The preventive LTC program was initiated under the leadership of the Ministry of Health, Labor and Welfare in Japan, where disuse was regarded as one of the leading risks of LTC. In agreement with the concept of community-based rehabilitation, municipality-led preventive interventions have been encouraged.[2] However, municipalities have undertaken interventions without reliable evidence of prevention against mental decline. Evidence is required to underpin the policies. Herein, we evaluated the efficacy of a community-based program for prevention of mental decline as a national project in three areas in Japan: Tokyo, Ohbu and Takasaki.

Previous studies have reported that regular exercise is beneficial in lowering the risk of mental decline in elderly individuals.[3] In particular, aerobic exercise has been shown to be beneficial. Erickson *et al.* reported that aerobic exercise could bring about a positive change in elderly individuals not only in memory function, but also in reversing hippocampal volume loss, which was not observed in non-aerobic exercise.[4] Among aerobic exercises, the efficacy of walking has been specifically supported by evidence.[5] Furthermore, greater amounts of walking are associated with greater gray matter volume, as shown in a 9-year follow up of 299 elderly individuals.[6] Another merit of walking is its feasibility at a low cost; effective prevention strategies would also have public health implications by reducing economic and social burdens.

The effects of an intervention program in Takasaki City, the Takasaki Project, has already been reported in a randomized controlled trial (RCT).[7] We modified the walking-habituation program to maximize the efficacy of the walking habit by introducing the five principles of brain-activating rehabilitation: pleasant atmosphere, interactive communication, social roles, praising each other and errorless support. We have already shown that brain-activating rehabilitation improves cognitive function, and reduces behavioral and psychological symptoms of dementia in demented subjects.[8] The intervention program, used in the current study, requested the participants to have tasks during walking, and to praise each other for the products of their tasks after walking. This made walking more pleasurable. Here, we examined the effects of the modified program based on the five principles of brain-activating rehabilitation as a preventive care program in the community. In the current study, the control group of the RCT study participated: 3 months of observation as a control group of the RCT study, and then 3 months of intervention in the current study with a modified walking-habituation program.[7]

Methods

Participants

The intervention program was carried out for the prevention of cognitive decline as a service of the municipality of Takasaki City in 2010. Participants were recruited between April and July 2010 in four areas of Takasaki City. In the first step, 162 participants were screened by a questionnaire and medical interview (precise information in Maki *et al.*[7]). Inclusion criterion was age between 65 and 80 years. Exclusion criteria were demented subjects and subjects who could not walk. After the RCT study, 71 subjects, who were in the control group in the RCT study and completed a second evaluation at the end of the RCT study, joined the modified walking-habituation program for 3 months (12 weeks), and 67 completed a third evaluation and 63 attended 12 sessions eight times or more (Fig. 1). Four participants were absent from the third evaluation, and four participants joined the program less than eight times.

The ethics board of Gunma University School of Health Sciences approved all procedures (No. 21–26). We obtained written consent from all participants.

Intervention with walking-habituation program

An intervention of 90 min was carried out for 12 weeks in four classes (sites). Two instructors, who were

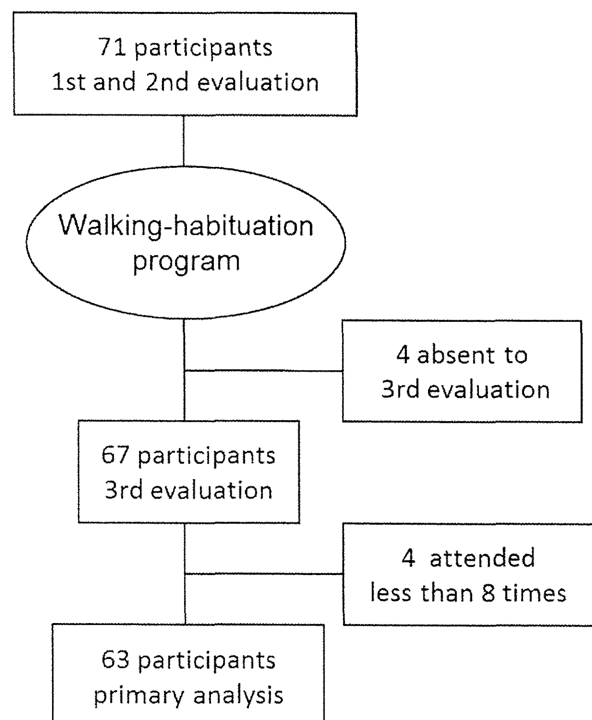

Figure 1 Flow of the study.

© 2015 Japan Geriatrics Society

pretrained through a Takasaki City office-sponsored seminar, carried out the program in each class. Approximately 15 participants were divided into three small groups (approximately 5 members) to discuss group-walking plans and tasks for facilitating communication between participants. In the present study, we made a staff manual and a guide book for participants of this project (downloadable from https://www.city.takasaki.gunma.jp/docs/2014012000147/).

The intervention program consisted of a group program in the class and a home program. The group program consisted of group activities: (i) report on home exercise (pleasant activity and self-walking) and praising each other; (ii) planning pleasant group walking; and (iii) original pleasant exercise (*Takasaki City song exercise with Hirameki*).

The aim of the home program was to develop a walking habit and to increase self-efficacy every day. To achieve these, the home program consisted of the following: (i) choice of pleasant activity or self-walking; (i) check of the daily number of steps; (iii) setting of goal of the number of steps; and (iv) setting of self-rewards.

Evaluation

The effects of the intervention were evaluated by cognitive tests, motor function and questionnaires. Evaluation was carried out three times: before the observation period (Ev1; first), after observation/before intervention (Ev2; second) and after intervention (Ev3; third). The parameters of measurement are shown in Table 2.

Cognitive tests

The major outcome variable was change in cognitive function. A five cognitive domains (5-Cog) test was used to measure the following: (i) learning and memory (category cued delayed recall test consisting of 32 words in 8 categories); (ii) executive function abilities (dual task test that requires divided attention and abstract reasoning test similar to the Wechsler Adult Intelligence Scale-III [WAIS-III]); (iii) language (a categorical word fluency test of "animals" completed in two minutes); and (iv) visuospatial abilities (a clock drawing test requiring drawing clock hands showing the time "ten after eleven").[9] The mean ± SD scores in normal aged particinants ($n = 800$, age between 65 and 80 years) were as follows: delayed recall test 12.0 ± 5.8; dual task test 20.1 ± 9.1; abstract reasoning test 10.8 ± 4.3; word fluency test 13.9 ± 6.0; and clock drawing test 6.7 ± 1.4. The 5-Cog test is intended to be carried out in a group setting. The test set is distributed with a 35-min long instructional DVD, so that the instructions are identical every time.

To evaluate sustained attention, we added the Digit-Symbol Substitution Test (DSST), a subset of the WAIS-III, and the Yamaguchi Kanji-Symbol Substitution Test (YKSST).[10] The YKSST was developed for Japanese elderly individuals as a variation of the DSST; Japanese characters, *kanji*, were used instead of numbers, as in the DSST, because the elderly in Japan are more familiar with *kanji*. The mean ± SD score of the YKSST was 46.9 ± 10.9 in normal aged participants ($n = 170$, age between 60 and 80 years). The Trail Making Test (TMT) was also carried out to evaluate executive function. Improvement was reflected by an increase in the 5-Cog, DSST and YKSST, whereas improvement was reflected by a decrease in the TMT.

Questionnaires on memory, quality of life, mood, functional capacity, range of activity and social network

Participants were required to complete self-assessment questionnaires. Memory complaints were assessed using the Questionnaire for Subjective Memory Complaint, which consisted of four questions: 1. Are there times when you are unable to remember what date it is even if you look at your calendar? 2. Are there times when you forget where you placed your wallet or keys? 3. Are there times when you read something you had scheduled in your calendar or diary and are unable to recall what you had planned? 4. Are there times when you are unable to remember what you heard 5 min ago? Each item was evaluated on a scale of 0–3, with 0 equal to never, and 3 equal to always.[11] Quality of Life (QOL) was measured using a questionnaire on satisfaction in daily life.[12] Depressive state was evaluated using the Geriatric Depression Scale (GDS) 15-item version.[13] Functional capacity for independent living was assessed by the Tokyo Metropolitan Institute of Gerontology Index of Competence (TMIG-IC).[14] The TMIG-IC was designed to measure higher-level functional capacities above physical self-maintenance in community-dwelling elderly individuals, consisting of 13 items comprised of three subscales: instrumental self-maintenance, intellectual activity and social role.

The range of activity was measured by the Life-Space Assessment, which assesses how far and how often a person moves, ranging from moving around the bedroom only, to traveling out of the person's town.[15] The social network size was assessed using the Japanese version of the abbreviated Lubben Social Network Scale (Lubben).[16] Improvement was reflected by an increase in QOL and the TMIG-IC, Life-Space Assessment and Lubben, and by a decrease in the GDS.

Assessment of motor function

We carried out four tests: grip force to assess muscle strength, balance time on one foot (60 s as cut-off time), the Timed Up & Go test and maximum walking speed

© 2015 Japan Geriatrics Society

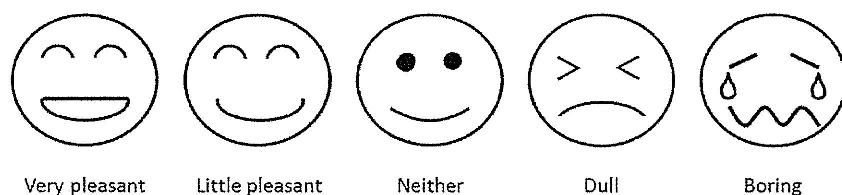

Figure 2 Original face scale.

over 5 m. Improvement was reflected by an increase in grip and balance, and by a decrease in the Timed Up & Go test and walking speed over 5 m.

In addition, average daily steps in a week were measured to evaluate the direct effect of the intervention program. All participants wore a pedometer (EX-500, Yamasa Tokei, Tokyo, Japan) to record the daily number of steps. The effect was assessed by comparison of the average number of steps in 7 days just before the observation, after the observation and after the intervention.

Assessment of pleasant feeling

The pleasant feeling in each session was self-checked using the original face scale (Fig. 2), and expressed as the mean of 11 (2nd to 12th) sessions.

Questionnaire 6 months later

At 6 months after the intervention, we surveyed the frequency of walking, confidence in continuing to walk and general impression of this program by questionnaire. All 63 participants of the analysis answered the questions.

Analysis of the data

The data of the 63 participants were analyzed using the Japanese version of SPSS for Windows version 19.0 (IBM Corporation, Armonk, NY, USA). Differences in longitudinal date between subgroups were tested by repeated measures analysis of variance (ANOVA) and Bonferroni correction post-hoc test. We regarded $P < 0.05$ as showing significance. Intention-to-treat analysis was also carried out; four participants who attended less than eight times and four participants who were absent at the third assessment were added in the intention-to-treat analysis ($n = 71$).

Results

The baseline data are shown in Table 1. The attendance rate of the program was 85.4% for the 71 participants. The analysis was carried out using data from 63 participants.

Table 1 Baseline characteristics

Participants	$n = 71$
Age years (mean ± SD)	72.2 ± 4.3
Sex (n)	
Male	20
Female	51
Education, years (mean ± SD)	11.9 ± 2.4
MMSE (mean ± SD)	27.8 ± 2.0

SD, standard deviation.

Direct effect of the program

The average daily steps in a week significantly increased during the intervention period (Ev2-Ev3) compared with the observation period (Ev1-Ev2) (F[2,110] = 41.925, $P < 0.001$; Ev1 4649 ± 2091, Ev2 5019 ± 2582, Ev3 7293 ± 2965 by repeated measures ANOVA). Post-hoc multiple comparisons with Bonferroni correction showed a significant increase during the intervention period (Ev2-Ev3; $P < 0.001$) alone (Table 2).

Cognitive tests

The Wechsler DSST (WDSST) significantly improved during the intervention period (Ev2-Ev3) compared with the observation period (Ev1-Ev2) (F[2,112] = 14.037, $P < 0.001$, Ev1 53.15 ± 13.07, Ev2 56.08 ± 15.02, Ev3 59.61 ± 16.94) with a significant increase during the intervention period (Ev2-Ev3; $P < 0.001$) alone (Table 2). Other cognitive tests, 5-Cog, YKSST, and TMT parts A and B, did not show a significant interaction between the intervention and observation periods.

In further analysis, we divided the participants into CDR 0 ($n = 48$) and 0.5 ($n = 15$) groups. The CDR 0 group (normal control) showed a significant improvement in the word fluency test of the 5-Cog test during the intervention period (F[2,94] = 4.685, $P < 0.05$, Ev1 16.19 ± 5.03, Ev2 15.73 ± 4.34, Ev3 17.25 ± 4.33) with a significant increase during the intervention period ($P < 0.01$) alone.

Questionnaires

Memory complaints (Questionnaire for Subjective Memory Complaint) significantly decreased during the

 © 2015 Japan Geriatrics Society

© 2015 Japan Geriatrics Society

Table 2 Results with repeated measures ANOVA in 63 participants

Assessment	Mean ± standard deviation			*F*-value	*P*-value	*P*-value of post-hoc		
	Ev1	Ev2	Ev3			Ev1 *vs* Ev2	Ev2 *vs* Ev3	Ev1 *vs* Ev3
5-Cog dual	19.1 ± 8.1	21.7 ± 7.3	22.6 ± 6.7	13.147	<0.001***	<0.01**	0.284	<0.001***
5-Cog memory	13.8 ± 5.1	16.5 ± 5.5	18.1 ± 5.8	68.496	<0.001***	<0.001***	<0.001***	<0.001***
5-Cog clock	6.8 ± 0.6	6.9 ± 0.5	6.9 ± 0.3	1.501	0.229	0.833	1.000	0.25
5-Cog fluency	15.8 ± 5.0	15.4 ± 4.2	16.4 ± 4.6	2.466	0.089	1.000	0.071	0.578
5-Cog reasoning	10.5 ± 3.2	10.8 ± 2.8	11.1 ± 2.7	2.741	0.076	0.638	0.634	0.124
WDSST	53.1 ± 13.1	56.1 ± 15.0	59.6 ± 16.9	14.037	<0.001***	0.077	<0.01**	<0.001***
YKSST	43.9 ± 10.0	45.6 ± 10.3	47.5 ± 11.3	14.508	<0.001***	<0.05*	<0.05*	<0.001***
TMT-A	42.7 ± 15.5	41.2 ± 16.2	42.1 ± 16.8	.374	0.689	1.000	1.000	1.000
TMT-B	143.4 ± 70.3	136.1 ± 65.0	124.0 ± 59.1	4.306	<0.05*	1.000	0.177	<0.01**
QSMC (memory)	6.2 ± 1.8	6.7 ± 2.0	6.1 ± 1.6	4.537	<0.05*	0.053	<0.05*	1.000
Lubben	18.0 ± 5.0	17.0 ± 5.4	17.1 ± 5.1	1.894	0.155	0.281	1.000	0.422
LSA	91.0 ± 19.6	96.3 ± 17.5	96.8 ± 18.9	4.341	<0.05*	0.092	1.000	<0.05*
TMIG-IC	12.1 ± 1.4	11.5 ± 1.6	12.1 ± 1.3	8.797	<0.001***	<0.01**	<0.001***	1.000
SDL (QOL)	21.0 ± 5.3	21.7 ± 6.2	20.7 ± 5.5	1.983	0.142	0.403	0.206	1.000
GDS (depression)	3.2 ± 2.7	3.2 ± 2.8	2.5 ± 2.6	4.636	<0.05*	1.000	0.061	<0.05*
Daily steps	4649 ± 2091	5019 ± 2582	7293 ± 2965	41.925	<0.001***	0.553	<0.001***	<0.001***
Grip	25.5 ± 6.5	27.8 ± 6.6	28.0 ± 6.0	15.425	<0.001***	<0.001***	1.000	<0.001***
Balance	37.0 ± 22.7	38.6 ± 21.6	49.4 ± 76.2	1.652	0.204	1.000	0.696	0.525
TUG	5.7 ± 1.0	5.4 ± 0.8	5.2 ± 0.7	27.239	<0.001***	<0.001***	<0.001***	<0.001***
5 m walking	2.7 ± 0.4	2.7 ± 0.4	2.4 ± 0.3	26.062	<0.001***	1.000	<0.001***	<0.001***

Results are expressed as mean ± standard deviation. *Significant at $P < 0.05$, **significant at $P < 0.01$, ***significant at $P < 0.001$. Daily steps, average daily steps in a week; Ev, evaluation; GDS, Geriatric Depression Scale; LSA, Life-Space Assessment; Lubben, Lubben Social Network Scale score; QSMC, Questionnaire for Subjective Memory Complaint; SDL, satisfaction in daily life; TMIG-IC, Tokyo Metropolitan Institute of Gerontology Index of Competence; TMT-A, Trail Making Test Part A; TMT-B, Trail Making Test Part B; TUG, Timed Up & Go Test; WDSST, Wechsler Digit Symbol Substitution Test; YKSST, Yamaguchi Kanji Symbol Substitution Test.

intervention period (Ev2-Ev3) compared with the observation period (Ev1-Ev2) ($F[2,124] = 4.537$, $P < 0.05$, Ev1 6.22 ± 1.78, Ev2 6.67 ± 2.04, Ev3 6.13 ± 1.61) with a significant increase during the intervention period (Ev2-Ev3; $P < 0.05$) alone (Table 2).

Functional capacity (TMIG-IC) showed a significant interaction ($F[2,124] = 8.797$, $P < 0.001$, Ev1 12.05 ± 1.39, Ev2 11.52 ± 1.56, Ev3 12.14 ± 1.31) with significant deterioration during the observation period (Ev1-Ev2, $P < 0.01$), and significant improvement during the intervention period (Ev2-Ev3, $P < 0.001$).

Depression (GDS) showed a significant interaction ($F[2,124] = 4.636$, $P < 0.05$, Ev1 3.21 ± 2.73, Ev2 3.19 ± 2.76, Ev3 2.49 ± 2.57) with marginal improvement during the intervention period (Ev2-Ev3, $P = 0.061$).

QOL (satisfaction in daily life) and range of activity (Life-Space Assessment) showed no significant interaction.

Assessment of motor function

By repeated measures ANOVA, the 5 m maximum walking time significantly decreased during the intervention period compared with the observation period ($F[2,122] = 26.062$, $P < 0.001$, Ev1 2.67 ± 0.42, Ev2 2.70 ± 0.43, Ev3 2.43 ± 0.34) with significant improvement during the intervention period ($P < 0.001$) alone (Table 2).

Significant interaction was not observed in tests of grip power, Timed Up & Go and balance (one-foot standing).

Intention-to-treat analysis

Improvement in average daily steps in a week, the TMIG-IC and 5 m maximum walking time was sustained after the intention-to-treat analysis: average daily steps in a week significantly increased during the intervention period (Ev2-Ev3) compared with the observation period (Ev1-Ev2) ($F[2,120] = 34.261$, $P < 0.001$; Ev1 4672 ± 2033, Ev2 5048 ± 2618, Ev3 7052 ± 3001) with a significant increase during the intervention period (Ev2-Ev3; $P < 0.001$) alone; the TMIG-IC showed a significant interaction ($F[2,140] = 9.989$, $P < 0.001$, Ev1 12.06 ± 1.36, Ev2 11.58 ± 1.53, Ev3 12.20 ± 1.26) with significant deterioration during the observation period (Ev1-Ev2, $P < 0.05$), and with significant improvement during the intervention period (Ev2-Ev3, $P < 0.001$); 5 m maximum walking time significantly decreased during the intervention period compared with the observation period ($F[2,138] = 28.573$, $P < 0.001$, Ev1 2.69 ± 0.44, Ev2 2.71 ± 0.44, Ev3 2.45 ± 0.37) with significant improvement during the intervention period (Ev2-Ev3, $P < 0.001$). In addition, the GDS significantly improved during the intervention period ($F[2,140] = 5.990$, $P < 0.01$, Ev1 3.37 ± 2.90, Ev2 3.35 ± 3.00, Ev3 2.63 ± 2.84). The WDSST and SMC did not significantly improve after the intention-to-treat analysis.

Face scale

The pleasant feeling in each session was self-checked using the face scale. A mean of 11 (2nd to 12th) sessions was 43% for "very pleasant," 49% for "a little pleasant," 8% for "neither,", 0% for "dull" and 0% for "boring."

Questionnaire at 6 months' follow up

In the questionnaire administered 6 months after the intervention, 52 of 63 participants (82.5%) continued to walk once a week or more, and all of them (52) were confident about continuing to walk in the future.

All participants (100%) were satisfied with the walking program.

Regarding the Takasaki *Hirameki* walking program, all of the participants replied that they felt delighted.

Discussion

The Takasaki *Hirameki* walking-habituation program was aimed at developing a walking habit to prevent cognitive and physical decline in community-dwelling elderly people. Accordingly, we showed significant improvement in the WDSST, a cognitive function test. The WDSST measures executive attention and processing speed.[17] The present results were consistent with a previous report showing that exercise training is associated with improvement in the WDSST.[18] Kuo showed that decline in the WDSST is associated with multiple domains of late-life disability, activities of daily living, instrumental activities of daily living, leisure, social activities and lower extremity mobility.[19]

In the present study, subjective memory complaints (Questionnaire for Subjective Memory Complaint) decreased significantly, meaning that participants felt forgetful less frequently after the intervention. The effect of aerobic exercise and physical activity has been reported to improve episodic memory in many studies.[4,20]

In the present study, depression (GDS) tended to improve, suggesting that physical activity reduces depression in older adults. Physical activity has effects on anxiety, social dysfunction and depression, as well as somatic symptoms.[21]

In addition, functional capacity (TMIG-IC) tended to improve after the intervention. Exercise was reported to improve walking performance, and to prevent the decline of ADL[22] and IADL.[23] Walking is well known as a good exercise for the lower extremities. Walking has

© 2015 Japan Geriatrics Society

been reported to improve balance, muscular strength of legs and cardiopulmonary function. A systematic review showed that exercise improves walking performance and reduces a decline in ADL.[24]

As a direct effect of the program, average daily steps in a week significantly increased by approximately 1.5-fold. Physical activity has positive effects on mental health,[25] cognitive function[7,26] and the prevention of dementia. Furthermore, we also showed significant improvement in 5 m walking time after the intervention. In a systematic review, van Iersel *et al.* showed that walking velocity and step length are related to the severity of dementia.[27]

In our walking-habituation program, each participant set their target steps in 3-week intervals to enhance motivation. In each session, participants reported their weekly steps, and praised each other, resulting in enhanced motivation. Motivation is important to continue walking (i.e. walking habit). There are various devices in this program for enhancing motivation. For example, two types of enjoyable walking were prepared: self-walking with activity and walking together with group members. Both included a pleasant atmosphere and praising each other.

The Takasaki *Hirameki* walking-habituation program is carried out in a small group, and is based on the five principles of brain-activating rehabilitation: pleasant atmosphere, communication, social roles, praising each other and supportive care. The aim of brain-activating rehabilitation is to enhance social relationships and motivation, and maximize the use of their remaining functions.[8,28] Activities with errorless learning enhance a pleasant atmosphere and dignity.[28] Brain-activating rehabilitation induces happy feelings and empathetic two-way communication between staff members and participants, as well as between participants. Midlife neuroticism was associated with increased risk of Alzheimer's disease dementia.[29] Taken together, social relationships, enhanced motivation, pleasant atmosphere, and happy feelings might contribute to cognitive and physical improvement.

In the present study, the five principles of brain-activating rehabilitation contributed to the high participation rate and the high habituation rate. It was shown that approximately 80% of the participants had developed the habit of walking once a week or more at 6 months after the intervention. Our walking-habituation program has some advantages in developing a walking habit, when compared with previous studies.[27,30] First, a pleasant atmosphere was developed. Second, our walking-habituation program included enjoyable activities (one's own interests and leisure activities). Third, each group member carried out self-walking every day. In addition, group members reported their daily steps and activities as a group. In the group work, members and facilitators praised each other to enhance motivation, resulting in increased self-efficacy and walking habituation.

Group work is associated with facilitators. If participants feel there is a pleasant and delightful atmosphere in the group work, they will be more likely to participate again. Motivation, self-efficacy and autonomy are closely related to walking habit.

In the present study, we showed improvement in some cognitive functions after a 3-month intervention program according to the Japanese LTC insurance service rule. This might result in limited efficacy. However, the most important aim of the intervention was obtaining walking habits to prevent cognitive decline. Further research is required to show the long-term effects of the program.

In conclusion, the *Takasaki Hirameki* walking-habituation program, once a week for 3 months, showed improvement in some cognitive functions, functional capacity and motor function, and contributed to developing a walking habit. The intervention program, based on the five principles of brain-activating rehabilitation, was enjoyable, and enhanced social communication and motivation of the participants, resulting in improvement of some cognitive and physical functions, as well as a high walking habituation rate at 6 months' follow up.

Acknowledgments

The authors thank Rumi Shinohara and Yuko Tsunoda (Gunma University, Maebashi, Japan) for technical assistance. We express our deep gratitude to all of the participants of Takasaki city.

Disclosure statement

No potential conflicts of interest were disclosed.

References

1 Takeda A, Tanaka N, Chiba T. Prospects of future measures for persons with dementia in Japan. *Psychogeriatrics* 2010; **10**: 95–101.
2 World Health Organization. Community-based rehabilitation. [Cited 11 Nov 2014.] Available from URL: http://www.who.int/disabilities/cbr/en/
3 Lautenschlager T, Cox KL, Flicker L *et al.* Effect of physical activity on cognitive function in older adults at risk for Alzheimer disease. *JAMA* 2008; **300**: 1027–1037.
4 Erickson KI, Voss MW, Prakash RS *et al.* Exercise training increases size of hippocampus and improves memory. *Proc Natl Acad Sci U S A* 2011; **108**: 3017–3022.
5 Weuve J, Kang JH, Manson JE, Breteler MM, Ware JH, Grodstein F. Physical activity, including walking, and cognitive function in older women. *JAMA* 2004; **292**: 1454–1461.

© 2015 Japan Geriatrics Society

6 Erickson KI, Leckie RL, Weinstein AM *et al.* Physical activity predicts gray matter volume in late adulthood: the Cardiovascular Health Study. *Neurology* 2010; **75**: 1415–1422.
7 Maki Y, Ura C, Yamaguchi T *et al.* Effects of intervention using a community-based walking program for prevention of mental decline: a randomized controlled trial. *J Am Geriatr Soc* 2012; **60**: 505–510.
8 Yamagami T, Takayama Y, Maki Y, Yamaguchi H. A randomized controlled trial of brain-activating rehabilitation for elderly participants with dementia in residential care homes. *Dement Geriatr Cogn Dis Extra* 2012; **2**: 372–380.
9 Miyamoto M, Kodama C, Kinoshita T *et al.* Dementia and mild cognitive impairment among non-responders to a community survey. *J Clin Neurosci* 2009; **16**: 270–276.
10 Yamaguchi T, Maki Y, Kaiho A *et al.* Yamaguchi kanji symbol substitution test as a scale of executive function in old people for preventing dementia in community. *Jpn J Geriatr Psychiatry* 2011; **22**: 587–594. (In Japanese.)
11 Sugiyama M, Miyamae F, Inagaki H *et al.* Development of a checklist for subjective cognitive decline in community-dwelling elderly (1): questionnaire for Subjective Memory Complaint. *Jpn J Gerontol* 2011; **33**: 300. (In Japanese.)
12 Hachisuka K, Tsutsumi Y, Kobayashi M, Iwata N. Factor structure of satisfaction in daily life of elderly residents in Kitakyushu. *J UOEH* 1999; **21**: 179–189.
13 Yesavage JA, Brink TL, Rose TL *et al.* Development and validation of a geriatric depression screening scale: a preliminary report. *J Psychiatr Res* 1982; **17**: 37–49.
14 Koyano W, Shibata H, Nakazato K, Haga H, Suyama Y. Measurement of competence: reliability and validity of the TMIG Index of Competence. *Arch Gerontol Geriatr* 1991; **13**: 103–116.
15 Peel C, Sawyer Baker P, Roth DL, Brown CJ, Brodner EV, Allman RM. Assessing mobility in older adults: the UAB Study of Aging Life-Space Assessment. *Phys Ther* 2005; **85**: 1008–1119.
16 Lubben JE. Assessing social networks among elderly populations. *Fam Community Health* 1988; **11**: 42–52.
17 Holtzer R, Wang C, Lipton R, Verghese J. The protective effects of executive functions and episodic memory on gait speed decline in aging defined in the context of cognitive reserve. *J Am Geriatr Soc* 2012; **60**: 2093–2098.
18 Laura DB, Laura LF, Karen FS. Effects of aerobic exercise on mild cognitive impairment. *Arch Neurol* 2010; **67**: 71–79.
19 Kuo HK, Leveille SG, Yu YH, Milberg WP. Cognitive function, habitual gait speed, and late-life disability in the National Health and Nutrition Examination Survey (NHANES) 1999–2002. *Gerontology* 2007; **53**: 102–110.
20 Sugano K, Yokogawa M, Yuki S *et al.* Effect of cognitive and aerobic training intervention on older adults with mild or no cognitive impairment: a derivative study of the nakajima project. *Dement Geriatr Cogn Dis Extra* 2012; **2**: 69–80.
21 Yoon JE, Lee SM, Lim HS, Kim TH, Jeon JK, Mun MH. The effects of cognitive activity combined with active extremity exercise on balance, walking activity, memory level and quality of life of an older adult sample with dementia. *J Phys Ther Sci* 2013; **25**: 1601–1604.
22 Littbrand H, Stenvall M, Rosendahl E. Applicability and effects of physical exercise on physical and cognitive functions and activities of daily living among people with dementia: a systematic review. *Am J Phys Med Rehabil* 2011; **90**: 495–518.
23 Rolland Y, Pillard F, Klapouszczak A *et al.* Exercise program for nursing home residents with Alzheimer's disease: a 1-year randomized, controlled trial. *J Am Geriatr Soc* 2007; **55**: 158–165.
24 Penninx BW, Messier SP, Rejeski WJ *et al.* Physical exercise and the prevention of disability in activities of daily living in older persons with osteoarthritis. *Arch Intern Med* 2001; **161**: 2309–2316.
25 Rosano C, Venkatraman VK, Guralnik J *et al.* Psychomotor speed and functional brain MRI 2 years after completing a physical activity treatment. *J Gerontol A Biol Sci Med Sci* 2010; **65**: 639–647.
26 Kamegaya T, Long-Term-Care Prevention Team of Maebashi City, Maki Y *et al.* Pleasant physical exercise program for prevention of cognitive decline in community-dwelling elderly with subjective memory complaints. *Geriatr Gerontol Int* 2012; **12**: 673–679.
27 Van Iersel MB, Hoefsloot W, Munneke M *et al.* Systematic review of quantitative clinical gait analysis in patients with dementia. *Z Gerontol Geriatr* 2004; **37**: 27–32.
28 Yamaguchi H, Maki Y, Yamagami T. Overview of non-pharmacological intervention for dementia and principles of brain-activating rehabilitation. *Psychogeriatrics* 2010; **10**: 206–213.
29 Johansson L, Guo X, Duberstein PR *et al.* Midlife personality and risk of Alzheimer disease and distress: a 38-year follow-up. *Neurology* 2014; **83**: 1538–1544.
30 Sofi F, Valecchi D, Bacci D *et al.* Physical activity and risk of cognitive decline: a meta-analysis of prospective studies. *J Intern Med* 2011; **269**: 107–117.

© 2015 Japan Geriatrics Society

Balakrishnan *et al. Acta Neuropathologica Communications* (2015) 3:41
DOI 10.1186/s40478-015-0217-z

ACTA NEUROPATHOLOGICA COMMUNICATIONS

RESEARCH Open Access

Impact of amyloid β aggregate maturation on antibody treatment in APP23 mice

Karthikeyan Balakrishnan[1†], Ajeet Rijal Upadhaya[1†], Julia Steinmetz[1†], Julia Reichwald[2], Dorothee Abramowski[2], Marcus Fändrich[3], Sathish Kumar[4], Haruyasu Yamaguchi[5], Jochen Walter[4], Matthias Staufenbiel[2,6] and Dietmar Rudolf Thal[1*]

Abstract

Introduction: The deposition of the amyloid β protein (Aβ) in the brain is a hallmark of Alzheimer's disease (AD). Removal of Aβ by Aβ-antibody treatment has been developed as a potential treatment strategy against AD. First clinical trials showed neither a stop nor a reduction of disease progression. Recently, we have shown that the formation of soluble and insoluble Aβ aggregates in the human brain follows a hierarchical sequence of three biochemical maturation stages (B-Aβ stages). To test the impact of the B-Aβ stage on Aβ immunotherapy, we treated transgenic mice expressing human amyloid precursor protein (APP) carrying the Swedish mutation (KM670/671NL; APP23) with the Aβ-antibody β1 or phosphate-buffered saline (PBS) beginning 1) at 3 months, before the onset of dendrite degeneration and plaque deposition, and 2) at 7 months, after the start of Aβ plaque deposition and dendrite degeneration.

Results: At 5 months of age, first Aβ aggregates in APP23 brain consisted of non-modified Aβ (representing B-Aβ stage 1) whereas mature Aβ-aggregates containing N-terminal truncated, pyroglutamate-modified $Aβ_{N3pE}$ and phosphorylated Aβ (representing B-Aβ stage 3) were found at 11 months of age in both β1- and PBS-treated animals. Protective effects on commissural neurons with highly ramified dendritic trees were observed only in 3-month-old β1-treated animals sacrificed at 5 months. When treatment started at 7 months of age, no differences in the numbers of healthy commissural neurons were observed between β1- and PBS-treated APP23 mice sacrificed with 11 months.

Conclusions: Aβ antibody treatment was capable of protecting neurons from dendritic degeneration as long as Aβ aggregation was absent or represented B-Aβ stage 1 but had no protective or curative effect in later stages with mature Aβ aggregates (B-Aβ stage 3). These data indicate that the maturation stage of Aβ aggregates has impact on potential treatment effects in APP23 mice.

Keywords: Amyloid, Immunization, Antibody, Protofibrils, Fibrils, Clearance

Introduction

The deposition of the amyloid β-protein (Aβ) in senile plaques is one of the hallmarks of Alzheimer's disease (AD) [1, 2]. Active and passive immunization against the Aβ peptide has been developed to treat AD [3, 4]. In amyloid precursor protein (APP) transgenic mice, peripheral administration of Aβ-antibodies lead to a reduced number of detectable plaques and prevented a further increase in the number of detectable Aβ plaques [3, 5]. Even the amount of fibrillar, Thioflavin S-positive plaques was reduced in anti-Aβ treated animals [6]. However, another group could not confirm Aβ reduction though an improvement of memory dysfunction was observed [7–9]. Active immunization in PDAPP mice demonstrated more effective rescue from cognitive impairment when treatment was started to prevent Aβ pathology compared to mice treated at a later point in life in a reversal trial [10]. First trials of active and passive Aβ immunization in AD patients, however, did not show a reduction of symptoms or prevention from further disease progression [11–16].

Given the discrepancy between the reduction of stained plaques and memory improvement in APP transgenic mice and the clinical disease progression in AD patients

* Correspondence: dietmar.thal@uni-ulm.de
†Equal contributors
[1]Institute of Pathology - Laboratory of Neuropathology, Center of Biomedical Research, University of Ulm, Helmholtzstrasse 8/1, D-89081 Ulm, Germany
Full list of author information is available at the end of the article

© 2015 Balakrishnan et al. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

the question arises whether Aβ immunization failed in AD patients because treatment was started too late after the onset of morphologically detectable neurodegeneration. Recently, we reported biochemical stages of Aβ aggregate maturation (B-Aβ stages) in the course of preclinical and symptomatic AD [17]. In addition to the most abundant forms of Aβ, i.e. $A\beta_{1-40}$ and $A\beta_{1-42}$, posttranslational modifications of Aβ have been identified in Aβ aggregates, including N-terminal truncations and pyroglutamate modifications at residues 3 or 11 ($A\beta_{N3pE}$ and $A\beta_{N11pE}$) and phosphorylation at Serine residue 8 (pAβ) [18–20]. The presence of $A\beta_{N3pE}$, thereby, indicates B-Aβ stage 2 and that of pAβ B-Aβ stage 3 [17]. It is not yet clear whether the B-Aβ stage has impact on the effect of Aβ-antibody treatment and whether maturation of Aβ aggregates explains the failure of treatment trials in AD patients.

To address these questions in a mouse model we chose APP23 mice, which overexpress human APP with the Swedish mutation (KM670/671NL) driven by a Thy1 promoter [21]. These mice produce soluble and insoluble Aβ aggregates, Aβ plaques and they show neuron loss in CA1, loss of asymmetric synapses in the frontocentral neocortex [22, 23] as well as more subtle degeneration of a subtype of layer III commissural neurons in the frontocentral neocortex, i.e. the degeneration of commissural neurons with a highly ramified dendritic tree. Degeneration of these highly ramified commissural neurons was seen as early as at 5 months of age in parallel with the onset of Aβ plaque deposition whereas no such changes were reported at 3 months of age in the absence of Aβ aggregates [24]. Degeneration of axons of commissural neurons represents the morphological correlative for corpus callosum atrophy, which is an early event in the pathogenesis of AD [25–27]. As such, these mice showing early degeneration of commissural neurons are ideally suited to study the effects of Aβ-antibody treatment on these Aβ-related neurodegenerative changes.

In this study, we treated APP23 mice with the Aβ-antibody β1 (Additional file 1: Table S1) beginning 1) at 3 months of age before the onset of plaque deposition and degeneration of commissural neurons and 2) at 7 months of age after plaque deposition and neurodegeneration started. The β1 antibody is directed against the N-terminus of Aβ and capable of preventing Aβ deposition in APP23 mice when it is transgenetically expressed [28]. The treated animals were sacrificed and the samples were analyzed 1) with 5 months - when first plaques and early dendrite degeneration can be identified in APP 23 mice - to detect protective effects and 2) with 11 months to clarify whether β1 antibody treatment allows recovery of altered highly ramified commissural neurons after a 4-month-treatment period. PBS-treated animals were used as controls.

Material and methods

Animals

APP23 mice were generated as described previously [21] and continuously back-crossed to C57BL/6. The murine Thy-1 cassette was used to drive neuron-specific expression of human APP751 with the Swedish double mutation 670/671 KM → NL. Heterozygous female APP23 mice of two age groups, 3 months ($n = 44$) and 7 months, ($n = 39$) were treated in this study and analyzed at 5 and 11 months of age, respectively. Animals were treated in agreement with German and Swiss laws on the use of laboratory animals.

Vaccination

Passive vaccination was performed in APP23 mice by weekly intraperitoneal (i.p.) injections of 500 μg of β1-anti-Aβ antibody [29] (Additional file 1: Table S1). As control group, APP23 mice received weekly i.p. injections of phosphate-buffered saline (PBS).

3-month-old animals were treated for 9 weeks, until 5 months of age, and were sacrificed 2–3 days after the last injection (β1: $n = 21$, PBS: $n = 23$). 7-month-old mice received injections for 12 weeks, until the age of 11 months, and were sacrificed for analysis 5–6 days after the last injection (β1: $n = 21$, PBS: $n = 18$). Animal experiments were carried out with permission of the Regierungspräsidium Tübingen/Germany (Permission: Animal Experiment No. 933) and the Animal Care and Use Committees of the Kanton Basel, Switzerland (Permission 1980).

Tissue preparation and DiI tracing

For DiI tracing and histopathological examinations, the brains of 5 and 11-months-old β1-treated and PBS-treated animals were used (5 months – β1: $n = 9$, PBS: $n = 10$; 11 months – β1: $n = 10$; PBS: $n = 10$). Mice were anesthetized and perfusion was performed transcardially with Tris-buffered saline (TBS) with heparin (pH 7.4) followed by the injection of 0.1 M PBS (pH 7.4) containing 2.6 % paraformaldehyde (PFA), 0.8 % iodoacetic acid, 0.8 % sodium periodate and 0.1 M D-L lysine. The brains were removed in total and post-fixed in 2.6 % phosphate-buffered PFA (pH 7.4) containing 0.8 % iodoacetic acid, 0.8 % sodium periodate and 0.1 M D-L lysine [30]. After 3 days, a single crystal (0.3 mm^3) of the carbocyanine dye DiI (Molecular Probes, Eugene, OR, USA) was implanted into the left frontocentral cortex, 1 mm rostrally from the central sulcus, 2 mm laterally from the middle line and 1 mm deep in the cortex as reported earlier [24]. This dye allows precise Golgi-like tracing of neurons in post-mortem fixed tissue in a quality similar to *in-vivo* tracing methods [24]. After incubation in 2.6 % phosphate-buffered PFA for at least 3 months at 37 °C, 100 μm thick coronal vibratome sections were cut. All sections of a given mouse brain were separately stored and continuously

numbered. Sections were temporarily mounted in TBS for microscopic analysis.

Microscopic and quantitative analysis

In layer III of the frontocentral cortex of the right hemisphere, contralateral to the implantation site of the tracer, the morphology of traced commissural neurons was examined. The traced neurons were assigned to different types according to their morphology [24] (Additional file 2: Table S2). Then the number of traced commissural neurons of each type in β1-treated APP23 mice was counted and compared with that in PBS-treated APP23 mice. For qualitative and quantitative analysis, 10 consecutive sections (100-μm thickness each) representing a tissue block of 1 mm thickness were studied for each mouse. Analysis started at the anterior commissure setting the caudal limit of the investigated tissue block. For each coronal section, the medial boundary of the region investigated was set as the vertical line at the cingulum that separated the cingulate cortex from secondary motor cortex (M2). The horizontal boundary was set as the horizontal line separating the primary somatosensory cortex (S1) from the insular cortex.

For the qualitative analysis, a laser scanning confocal microscope (Leica TCS NT, Leica, Bensheim, Germany) was used. Stacks of 2D images were superimposed digitally using the ImageJ image processing and analysis software (National Institutes of Health (NIH), Bethesda, MD, USA), and 3D data sets were generated for the visualization of neurons with their entire dendritic tree. For quantification, traced neurons in layer III were counted in the region of interest in 10 consecutive sections of the tissue block taken for qualitative and quantitative analysis using a fluorescence microscope (Leica DMLB, Leica, Germany). In so doing, we analyzed a cortex volume of 5–6 mm^3 in each mouse. Mean and median values of the number of traced neurons were calculated and compared between β1-treated and PBS-treated APP23 mice of a given age.

Immunohistochemistry

Morphological and immunohistochemical analysis were carried out on sections of the traced animals after obtaining the tracing results. Vibratome sections of the frontocentral cortex were immunostained with anti-$A\beta_{17-24}$, anti-$A\beta_{1-17}$, anti-$A\beta_{42}$, anti-$A\beta_{40}$, anti-$A\beta_{N3pE}$, anti-pAβ, anti-Aβ-β1, anti-APP, anti-mouse-IgG, anti-glial fibrillary acidic protein (GFAP) and the microglia marker iba-1 [18, 19, 29, 31, 32] (Additional file 1: Table S1). The primary antibodies were detected with the respective biotinylated anti-mouse and anti-rabbit IgG secondary antibodies and visualized with the ABC-complex (ABC-Kit, Vector Laboratories, Burlingame, CA, USA) and diaminobencidine-HCl (DAB) as chromogen. To avoid crossreactivity of intrinsic IgG with anti-mouse-IgG secondary antibodies, sections were preincubated with goat-anti-mouse-IgG [33]. Protofibrils and fibrils were detected with B10AP-antibody fragments coupled with alkaline phosphatase [34]. They were visualized with permanent red (DAKO, Glostrup, Denmark). For immunofluorescence, rabbit primary antibodies were detected with carbocyanine 3 (Cy3)-labeled anti-rabbit-IgG secondary antibodies whereas mouse IgG was detected with Cy2-labeled anti-mouse IgG antibodies without previous anti-mouse-IgG blocking to allow the detection of intrinsic IgGs. Amyloid material was identified in the double stained section by UV-light-induced amyloid autofluorescence, i.e. detection of unstained amyloid material by fluorescence microscopy (excitation filter: 360–370 nm; emission filter: > 420 nm) [35]. Respective positive and negative controls were performed.

Quantification of plaque loads for $A\beta_{42}$-, $A\beta_{40}$-, $A\beta_{N3pE}$-, pAβ-, β1- and B10AP-positive plaques

$A\beta_{42}$ load was determined as the percentage of the area in the frontocentral cortex covered by Aβ plaques detected with anti-$A\beta_{42}$ antibodies. Morphometry for $A\beta_{42}$ load determination was performed using ImageJ image processing and analysis software by interactive measurement of plaque areas in a region of interest as well as of the total area of interest. The plaque load was calculated as the percentage of the area of interest covered by amyloid plaques stained with the antibody (National Institutes of Health, Bethesda, USA) [23]. Accordingly, the $A\beta_{40}$, $A\beta_{N3pE}$, pAβ, $A\beta_{1-17}$, β1 and B10AP loads were determined as the percentage of the frontocentral cortex area covered by plaques positive for the respective antibodies.

Protein extraction from brain tissue and serum

For biochemistry, deeply anesthetized mice were sacrificed by decapitation. Blood was collected after decapitation. Serum was isolated after centrifugation at 3000 × g for 30 min at room temperature. Serum samples were immediately frozen at −80 °C until the performance of the experiments. The brains were taken, dissected, and unfixed left and right hemispheres as well as the brain stem and the cerebellum were kept separately at −80 °C for further analysis.

Protein extraction from frozen brain samples of 5- and 11-month-old APP23 treated with β1-antibodies (5 months: $n = 12$, 11 months: $n = 11$) or PBS (5 months: $n = 13$, 11 months: $n = 8$) was carried out for biochemical studies [17, 23]. Briefly, fresh frozen forebrain tissue samples (0.4 g) were homogenized in 2 ml of 0.32 M sucrose dissolved in Tris-buffered saline (TBS) containing a protease and phosphatase inhibitor-cocktail (Complete and PhosSTOP, Roche, Mannheim, Germany) with Micropestle

(Eppendorf, Hamburg, Germany) followed by sonication. The homogenate was centrifuged for 30 min at 14,000 × g at 4 °C. The supernatant (S1), containing both the soluble and dispersible fraction was kept for further ultracentrifugation. The pellet (P1) containing the membrane-associated and the insoluble, plaque-associated fraction was resuspended in 2 % SDS. Ultracentrifuging of the supernatant S1 at 175,000 × g was used to separate the soluble, i.e. the supernatant after ultracentrifuging (S2), from the dispersible fraction, i.e. the resulting pellet (P2). The pellet P2 with the dispersible fraction was resuspended in TBS.

The SDS-resuspended pellet P1 was centrifuged at 14,000 × g the supernatant (S3) was kept as membrane-associated SDS-soluble fraction. The pellet (P3) that remained was dissolved in 70 % formic acid and dried in a vacuum centrifuge (Vacufuge, Eppendorf, Hamburg, Germany) and reconstituted in 100 μl of 2X LDS (lithium dodecyl sulfate) sample buffer (Life Technologies, Carlsbad, CA, USA) followed by heating at 70 °C for 5 min. The resultant sample was considered as insoluble, plaque-associated fraction [36]. The total protein amounts of soluble, dispersible, and membrane-associated fractions were determined using BCA Protein Assay (Bio-Rad, Hercules, CA, USA).

Immunoprecipitation

For immunoprecipitation (IP), 200 μl of the native soluble and dispersible fractions from the brain lysates were incubated with 1 μl A11 antibodies against non-fibrillar oligomers, or 5 μl B10AP antibody fragments for precipitation of protofibrils and fibrils (Additional file 1: Table S1) or were kept without adding antibodies to identify already antibody-bound Aβ. 50 μl of protein G-coated Microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) were added to the mixture and incubated overnight at 4 °C. The mixture was then passed through the μColumns, which separate the microbeads by retaining them in the column, while the rest of the lysate flows through. After one mild washing step with 1X TBS at pH 7.4 the microbead-bound-proteins were eluted with 95 °C heated 1X LDS sample buffer (Life Technologies, Carlsbad, CA, USA).

SDS-PAGE and Western blot analysis

For SDS-PAGE, soluble (S2), dispersible (P2), membrane-associated (SDS-soluble; S3), insoluble, plaque-associated (formic acid soluble; P3) fractions and IP eluates (50 μg total protein) were electrophoretically resolved in a precast NuPAGE 4–12 % Bis-Tris gel system (Life Technologies, Carlsbad, CA, USA). Proteins were transferred onto Nitrocellulose membrane and membranes were boiled in 1X PBS for 5 min. The protein load was controlled either by Ponceau S staining (C4, 1/1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or by MemCode reversible protein stain kit (Pierce, Rockford, IL, USA) prior to immunoblotting.

Aβ was detected by western blotting with anti-$A\beta_{1-17}$, anti-pAβ and anti-$A\beta_{N3pE}$ antibodies (Additional file 1: Table S1). Blots were incubated with chemiluminescent ECL detection system (Supersignal Pico Western system, ThermoScientific-Pierce, Waltham, MA, USA) or Luminata Forte Western HRP substrate (Merck Millipore, Billerica, Massachusetts, USA) and acquired using either ECL Hyperfilm (GE Healthcare, Buckinghamshire, UK) or CCD imager Image Quant LAS 4000 (GE Healthcare, Buckinghamshire, UK). Since anti-$A\beta_{1-17}$ also stains APP we used the respective protein bands corresponding to ~110 kDa seen with this antibody as internal loading control to verify the protein content.

Because Aβ aggregates readily dissociate in the presence of SDS-containing buffers into monomers and small oligomers, such as dimers, trimers, or Aβ*56 [37, 38], we analyzed differences among the monomer bands that indicate changes in the protein levels of precipitated Aβ aggregates densitometrically using ImageJ software (NIH, Bethesda, MD, USA). This method allows a semi-quantitative assessment of Aβ [23]. Briefly, the X-ray films were scanned and image colors were inverted or the images acquired by CCD imager were exported as 8-bit grayscale TIFF files. The relative protein levels of the monomer bands were measured as integrated density values for each lane [39].

Biochemical stages of Aβ aggregation (B-Aβ stages) were determined on the basis of Aβ, $A\beta_{N3pE}$ and pAβ detection in the soluble, dispersible, membrane-associated, and plaque-associated fraction [17]: B-Aβ stage 1 = Aβ aggregates detected only with anti-$A\beta_{1-17}$; B-Aβ stage 2 = Aβ aggregates positive for anti-$A\beta_{1-17}$ and anti-$A\beta_{N3pE}$ but not for anti-pAβ; B-Aβ stage 3 = Aβ aggregates containing anti-$A\beta_{1-17}$-, anti-$A\beta_{N3pE}$- and anti-pAβ-positive material.

Stereology

Six β1-treated and six PBS-treated APP23 mice at the ages of 5 and 11 months, respectively, were chosen randomly for stereology. One brain section including the hippocampal formation already quantified for the number of DiI-traced neurons was selected by chance and stained with aldehyde fuchsin-Darrow red. Quantification of neurons was performed according to the principles of unbiased stereology [40]. The CA1 volume was measured in serial 100 μm thick sections of the entire mouse brain at 5 × magnification. Neurons were counted in three different, randomly chosen microscopic fields (40 × objective magnification) of an aldehyde fuchsin – Darrow red stained section of the frontocentral cortex and CA1, respectively. For optical dissection, stacks of 10 images in 2 μm focus distance were generated for each microscopic

field. Only those neurons having nuclei with dark and round nucleoli visible in the center of soma in one of the stack-images were considered for quantification using the ImageJ software (NIH, Bethesda, USA). The number of neurons in the frontocentral cortex and CA1 was calculated on the basis of the respective reference volumes and neuron densities as previously published [41].

Electron microscopy, immunoelectron microscopy and semiquantitative assessment of synapse densities

100 μm thick vibratome sections of the frontocentral cortex from six β1-treated and six PBS-treated APP23 mice, aged 5 and 11 months, respectively, were flat-embedded in Epon (Fluka, Germany). A part of the of the frontocentral cortex covering all six layers was cut and pasted on a second Epon block ultrathin sections were cut at 70 nm. Epon sections were block stained with uranyl acetate and lead citrate, and viewed with a Zeiss EM10 (Zeiss, Oberkochen, Germany), or a JEM-1400 (JEOL, Tokyo, JP). electron microscope. Digital pictures were taken.

Synaptic densities in the frontocentral cortex were measured in 20 randomly taken pictures of the layers II-VI at 4600-times magnification. The numbers of the symmetric and asymmetric synapses were counted and the length of the synapses was determined with the ImageJ software (NIH, Bethesda, USA). The synaptic density was determined separately for symmetric and asymmetric synapses according to DeFelipe et al. [42] (synaptic density = number of synapse-profiles in a given area / length of synaptic profiles). These semi-quantitative data were used to compare the synaptic densities between the different mouse lines. Asymmetric and symmetric synapses were distinguished according to published criteria [43, 44].

Statistical analysis

SPSS 21.0 (SPSS, Chicago, IL, USA) software was used to calculate statistical tests. Non-parametric tests were used to compare β1-treated and PBS-treated APP23 mice. p-values were corrected for multiple testing using the Bonferroni-method. Parametric data were analyzed by ANOVA with subsequent Games-Howell post-hoc test to correct for multiple testing or by using the Welch test. The results of the statistical analysis are summarized in Additional file 3: Table S3.

Results

Neurodegeneration in β1-treated and PBS-treated APP23 mice

Between 3 and 5 months of age degeneration of dendrites of DiI-traced highly ramified commissural layer III neurons in the frontocentral cortex was observed in β1-treated as well as in PBS-treated animals (Fig. 1). However, β1-treated mice showed slightly higher numbers of type I commissural neurons than PBS-treated animals (Mann-Whitney U-test: $p < 0.05$). The numbers of type II and III commissural neurons did not differ between β1- and PBS-treated animals (Fig. 1 and Additional file 3: Table S3). Treatment with the β1-antibody between 7 and 11 months of age had no obvious effect on the numbers of type I, II and III layer III commissural neurons traced in the frontocentral neocortex in comparison to PBS-treated controls (Fig. 1 and Additional file 3: Table S3a).

Significant differences in the synapse densities of asymmetric and symmetric synapses in the frontocentral cortex as well as differences in the numbers of CA1 neurons were not observed between β1-treated and PBS-treated animals in both age groups (Additional file 3: Table S3b, c and Additional file 4: Figure S1).

Aβ plaques in β1-treated and PBS-treated APP23 mice

Aβ plaques detectable with antibodies directed against $A\beta_{42}$, $A\beta_{40}$, $A\beta_{17-24}$, $A\beta_{1-17}$, $A\beta_{N3pE}$, and pAβ were found in both, β1-treated and PBS-treated APP23 mice (Figs. 2 and 3), without quantitative differences at 5 and 11 months of age, respectively (Additional file 3: Table S3d). At 5 months of age only single plaques in some of the mice were observed whereas in 11-month-old APP23 mice moderate numbers of plaques were seen. Further analysis of 11-month-old animals using the β1 antibody (used for passive immunization) showed that treated animals exhibited fewer plaques stained with β1 indicating epitope masking (Fig. 3 and Additional file 3: Table S3d). B10AP-positive plaques were also reduced in β1-treated 11-month-old animals (Fig. 3) whereas 5-month-old mice did not exhibit B10AP-positive plaques regardless of β1 treatment (Additional file 3: Table S3d). APP-positive dystrophic neurites were seen in APP-type neuritic plaques (i.e. amyloid plaques associated with APP-positive dystrophic neurites) of β1-treated and PBS-treated APP23 mice (Additional file 5: Figure S2). Mouse IgG was not observed in plaques of 5-month-old animals whereas amyloid plaques in 11-month-old β1- and PBS-treated mice exhibited IgG in similar amounts (Additional file 6: Figure S3 and Additional file 7: Figure S4). Plaque-associated astrocytes and microglial cells were seen in both β1- and PBS-treated APP23 mice at 11 months of age whereas no glial reaction was evident in 5-month-old animals (Additional file 6: Figure S3 and Additional file 7: Figure S4).

Brain Aβ, $A\beta_{N3pE}$, and pAβ in β1-treated and PBS-treated APP23 mice

Western blot analysis of forebrain homogenates revealed no obvious differences in the amounts of soluble, dispersible, and membrane-associated Aβ detectable with

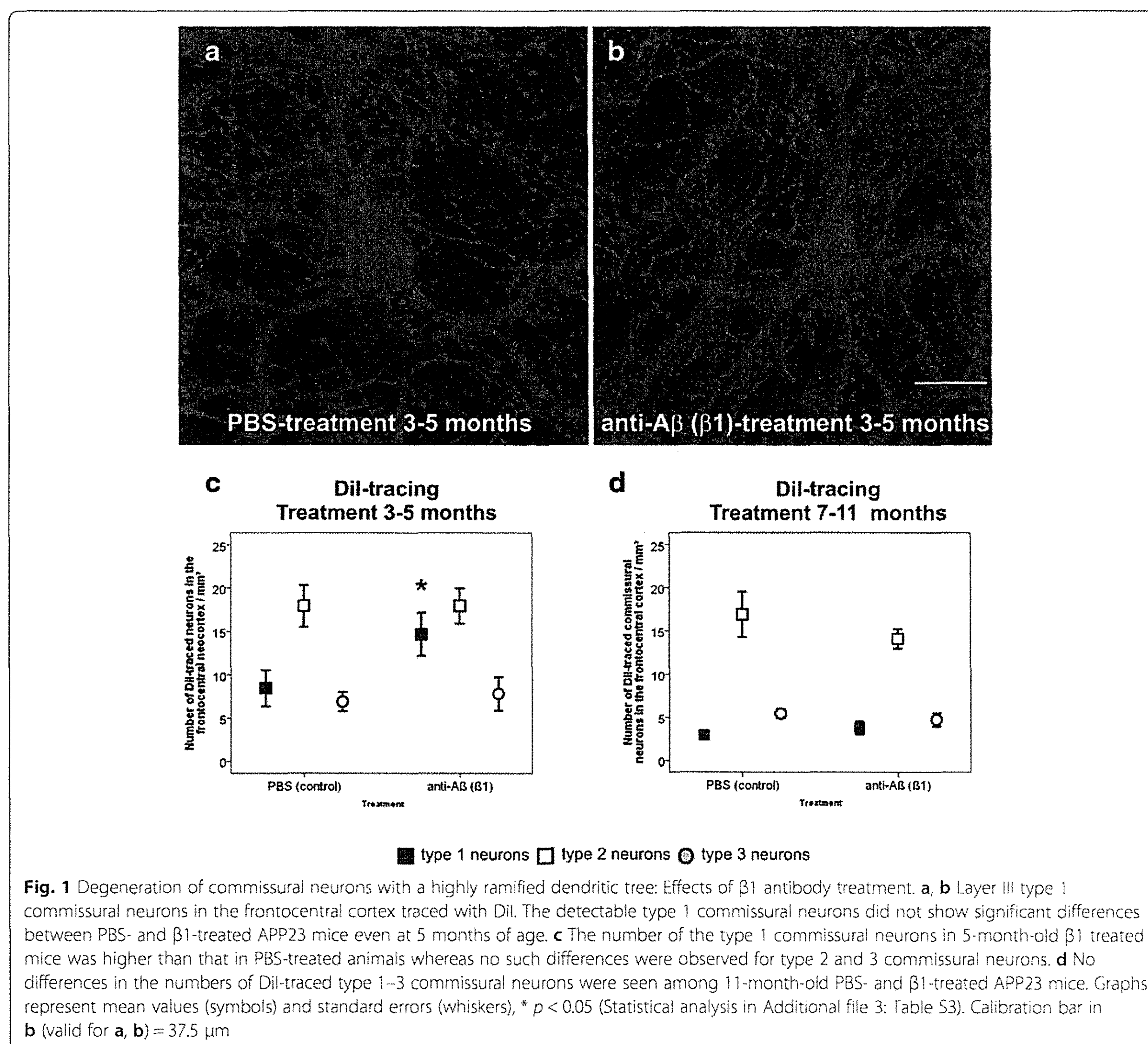

Fig. 1 Degeneration of commissural neurons with a highly ramified dendritic tree: Effects of β1 antibody treatment. **a**, **b** Layer III type 1 commissural neurons in the frontocentral cortex traced with DiI. The detectable type 1 commissural neurons did not show significant differences between PBS- and β1-treated APP23 mice even at 5 months of age. **c** The number of the type 1 commissural neurons in 5-month-old β1 treated mice was higher than that in PBS-treated animals whereas no such differences were observed for type 2 and 3 commissural neurons. **d** No differences in the numbers of DiI-traced type 1–3 commissural neurons were seen among 11-month-old PBS- and β1-treated APP23 mice. Graphs represent mean values (symbols) and standard errors (whiskers), * $p < 0.05$ (Statistical analysis in Additional file 3: Table S3). Calibration bar in **b** (valid for **a**, **b**) = 37.5 μm

anti-Aβ_{1-17} in β1-treated and PBS-treated animals of both age groups (at 5 and 11 months of age). β1-treated 5-month-old APP23 mice exhibited higher amounts of anti-Aβ_{1-17} detected plaque-associated Aβ than PBS-treated animals (Fig. 4, Additional file 3: Table S3e and Additional file 8: Figure S5). In 11-month-old mice, Aβ in all four fractions was elevated in comparison to 5-month-old APP23 mice. Differences between the treatment groups were not obvious at this age (Fig. 4, Additional file 3: Table S3e and Additional file 8: Figure S5). Aβ_{N3pE} and pAβ were not observed in brain homogenates of 5-month-old animals but in mice of 11 months of age. No differences in the pattern of Aβ_{N3pE} and pAβ aggregation between β1- and PBS-treated animals were seen (Fig. 4, Additional file 3: Table S3e and Additional file 8: Figure S5). In accordance with a previously published hierarchical pattern of Aβ aggregate maturation during the course of AD, the presence of Aβ in the absence of biochemically detectable Aβ_{N3pE} and pAβ in 5-month-old APP23 mice corresponded to B-Aβ stage 1 whereas Aβ aggregates in 11-month-old mice consisting of normal Aβ, Aβ_{N3pE}, and pAβ were referred to as B-Aβ stage 3 [17].

In 5-month-old mice, immunoprecipitation with the oligomer-specific A11 antibody and with protofibril- and fibril-specific B10AP antibody fragments and subsequent western blot analysis with anti-Aβ_{1-17} showed more Aβ oligomers, protofibrils and fibrils in the β1-treated group compared to the PBS-treated group selectively in the soluble fraction (Fig. 5, Additional file 3: Table S3 and Additional file 9: Figure S6). Such a difference was not found in 11-month-old animals. In the dispersible fractions

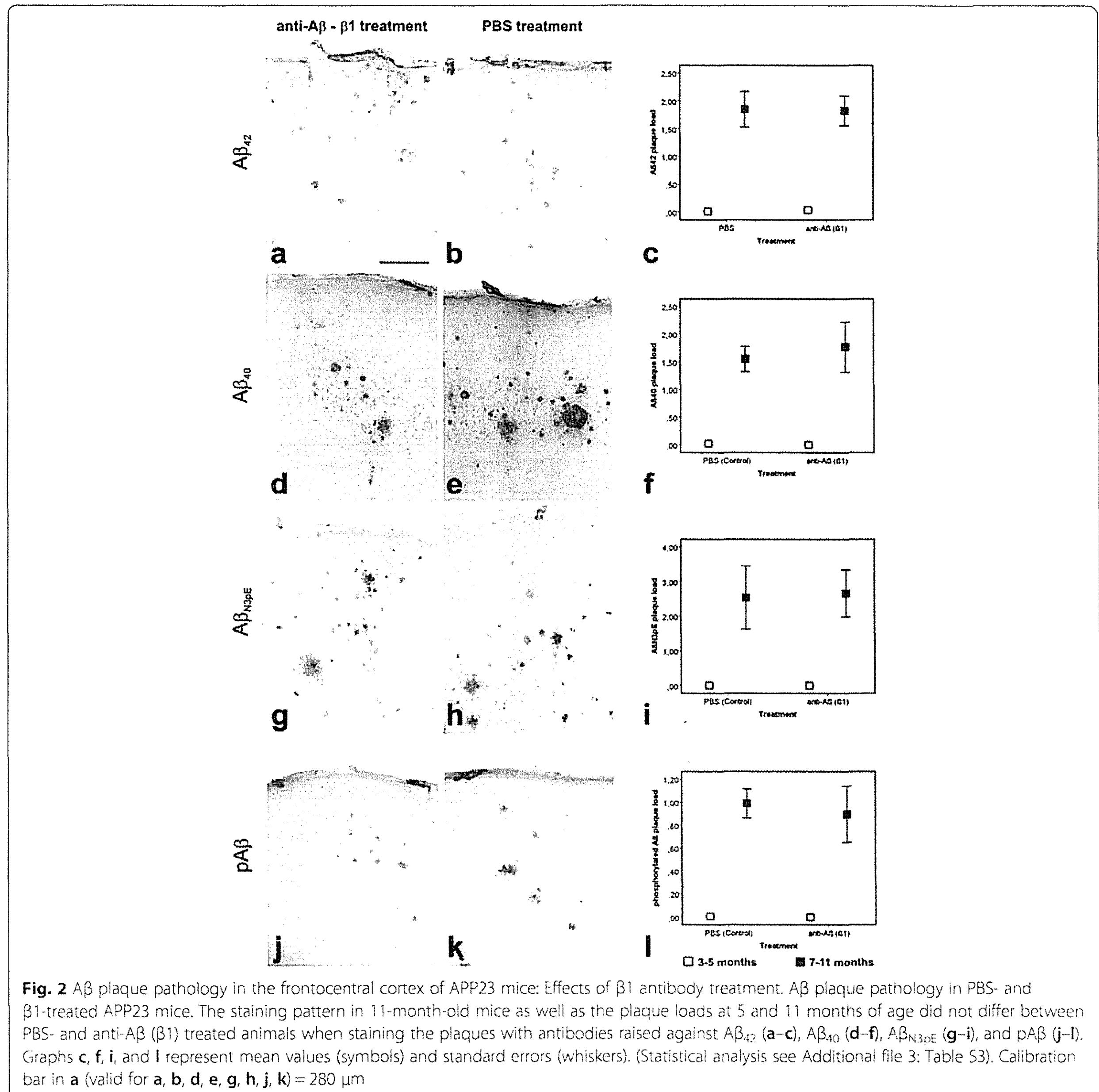

Fig. 2 Aβ plaque pathology in the frontocentral cortex of APP23 mice: Effects of β1 antibody treatment. Aβ plaque pathology in PBS- and β1-treated APP23 mice. The staining pattern in 11-month-old mice as well as the plaque loads at 5 and 11 months of age did not differ between PBS- and anti-Aβ (β1) treated animals when staining the plaques with antibodies raised against $Aβ_{42}$ (**a**–**c**), $Aβ_{40}$ (**d**–**f**), $Aβ_{N3pE}$ (**g**–**i**), and pAβ (**j**–**l**). Graphs **c**, **f**, **i**, and **l** represent mean values (symbols) and standard errors (whiskers). (Statistical analysis see Additional file 3: Table S3). Calibration bar in **a** (valid for **a**, **b**, **d**, **e**, **g**, **h**, **j**, **k**) = 280 μm

there were no differences in the amounts of Aβ oligomers, protofibrils and fibrils between the β1-treated and PBS-treated animals of both age groups (Fig. 5, Additional file 3: Table S3f and Additional file 9: Figure S6).

In 5-month-old APP23 mice, irrespective of the treatment, no $Aβ_{N3pE}$ and no pAβ was detected, in the soluble and dispersible fraction of samples immunoprecipitated with A11 or B10AP. Similarly, in 11-month-old mice, $Aβ_{N3pE}$ and pAβ were not detected in A11 and B10AP immunoprecipitated oligomers, protofibrils and fibrils from the soluble fraction. $Aβ_{N3pE}$ and pAβ were found in the dispersible fraction in Aβ protofibrils and fibrils precipitated with B10AP at 11 months of age but without significant differences among β1- and PBS-treated animals (Fig. 5, Additional file 3: Table S3f and Additional file 9: Figure S6). There was no detectable $Aβ_{N3pE}$ in both groups of 11-month-old mice in the dispersible fractions immunoprecipitated with non-fibrillar Aβ oligomer-specific A11 antibodies but pAβ was observed in β1- and PBS-treated mice (Fig. 5, Additional file 3: Table S3f and Additional file 9: Figure S6).

Immunoprecipitation of antibody-bound Aβ by incubation of the brain samples with protein G magnetic beads without previous coupling to primary antibodies

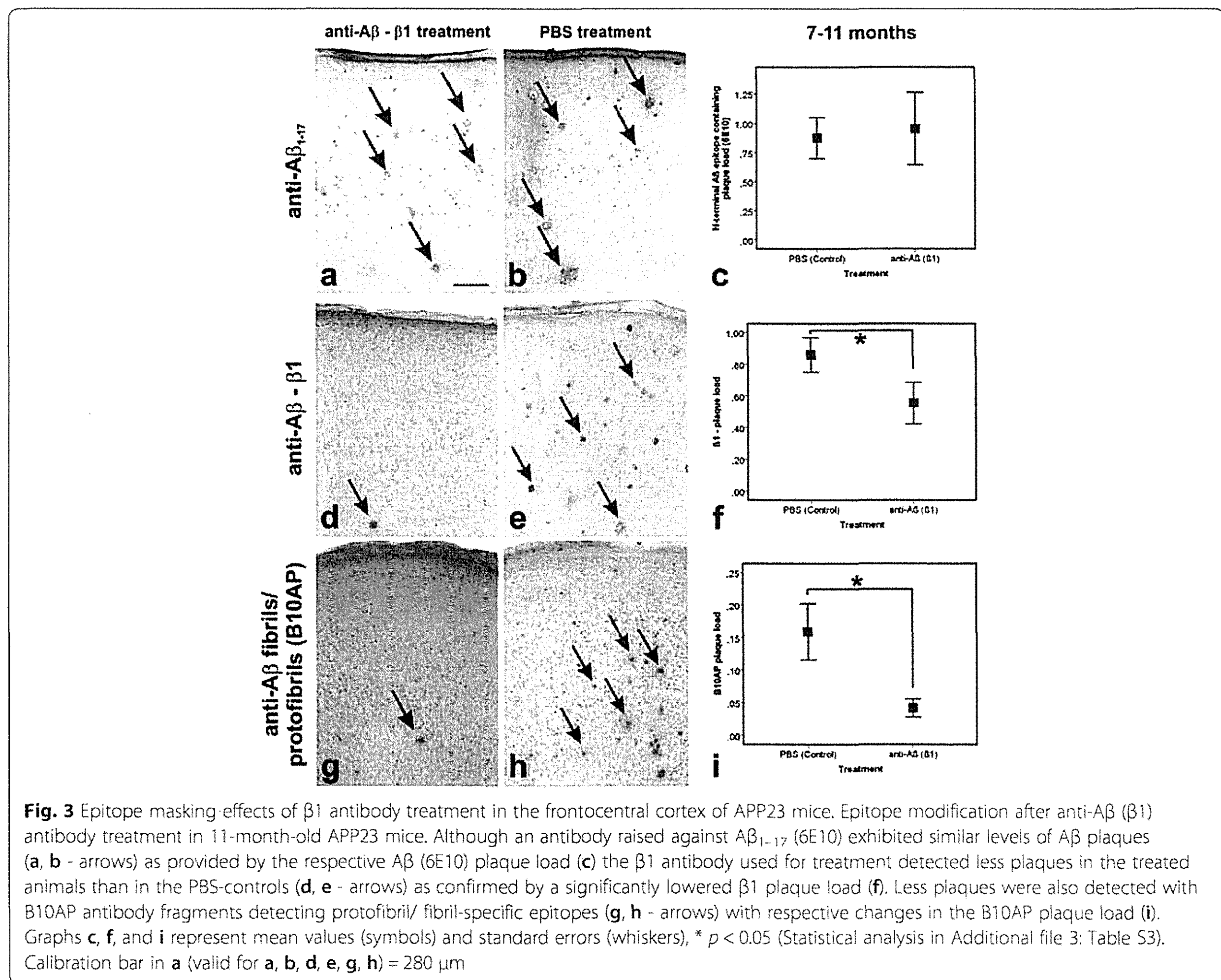

Fig. 3 Epitope masking effects of β1 antibody treatment in the frontocentral cortex of APP23 mice. Epitope modification after anti-Aβ (β1) antibody treatment in 11-month-old APP23 mice. Although an antibody raised against $A\beta_{1-17}$ (6E10) exhibited similar levels of Aβ plaques (**a**, **b** - arrows) as provided by the respective Aβ (6E10) plaque load (**c**) the β1 antibody used for treatment detected less plaques in the treated animals than in the PBS-controls (**d**, **e** - arrows) as confirmed by a significantly lowered β1 plaque load (**f**). Less plaques were also detected with B10AP antibody fragments detecting protofibril/ fibril-specific epitopes (**g**, **h** - arrows) with respective changes in the B10AP plaque load (**i**). Graphs **c**, **f**, and **i** represent mean values (symbols) and standard errors (whiskers), * $p < 0.05$ (Statistical analysis in Additional file 3: Table S3). Calibration bar in **a** (valid for **a**, **b**, **d**, **e**, **g**, **h**) = 280 μm

showed IgG-bound soluble Aβ in 5-month-old β1-treated mice detected with anti-$A\beta_{1-17}$ antibodies, but not in PBS-treated animals (Fig. 6, Additional file 3: Table S3 and Additional file 10: Figure S7). Such a difference was not observed in 11-month-old mice. No soluble IgG-bound $A\beta_{N3pE}$ and pAβ was seen in 5- and 11-month-old APP23 mice (Fig. 6). In the dispersible fractions, immunoprecipitation with protein-G coated magnetic beads without primary antibody exposure and subsequent western blotting with anti-$A\beta_{1-17}$ antibodies, showed detectable amounts of IgG-bound Aβ in both age and treatment groups, respectively, without significant differences among treatment (Fig. 6, Additional file 3: Table S3g and Additional file 10: Figure S7). $A\beta_{N3pE}$ was not seen in antibody-bound aggregates (Fig. 6, Additional file 3: Table S3g and Additional file 10: Figure S7). In 5-month-old APP23 mice no dispersible pAβ was observed in antibody-bound aggregates but 11-month-old animals exhibited dispersible pAβ in the precipitates without significant differences between the treatment groups (Fig. 6, Additional file 3: Table S3g and Additional file 10: Figure S7).

Detection of Aβ in the blood serum of β1-treated and PBS-treated APP23 mice

Western blot analysis did not reveal detectable amounts of Aβ in the serum of 5- and 11-month-old β1- and PBS-treated APP23 mice (Fig. 7 and Additional file 11: Figure S8). Only after immunoprecipitation of antibody-bound Aβ from blood serum using protein-G coated magnetic beads without previous primary antibody coupling showed anti-$A\beta_{1-17}$ detectable Aβ in β1-treated 11-month-old APP23 mice but not in PBS-treated animals (Fig. 7, Additional file 3: Table S3 and Additional file 11: Figure S8). This effect was not observed at 5 months of age. Antibody-bound $A\beta_{N3pE}$ and pAβ were not found in the blood serum of APP23 mice of both ages regardless of β1-treatment (Fig. 7 and Additional file 11: Figure S8).

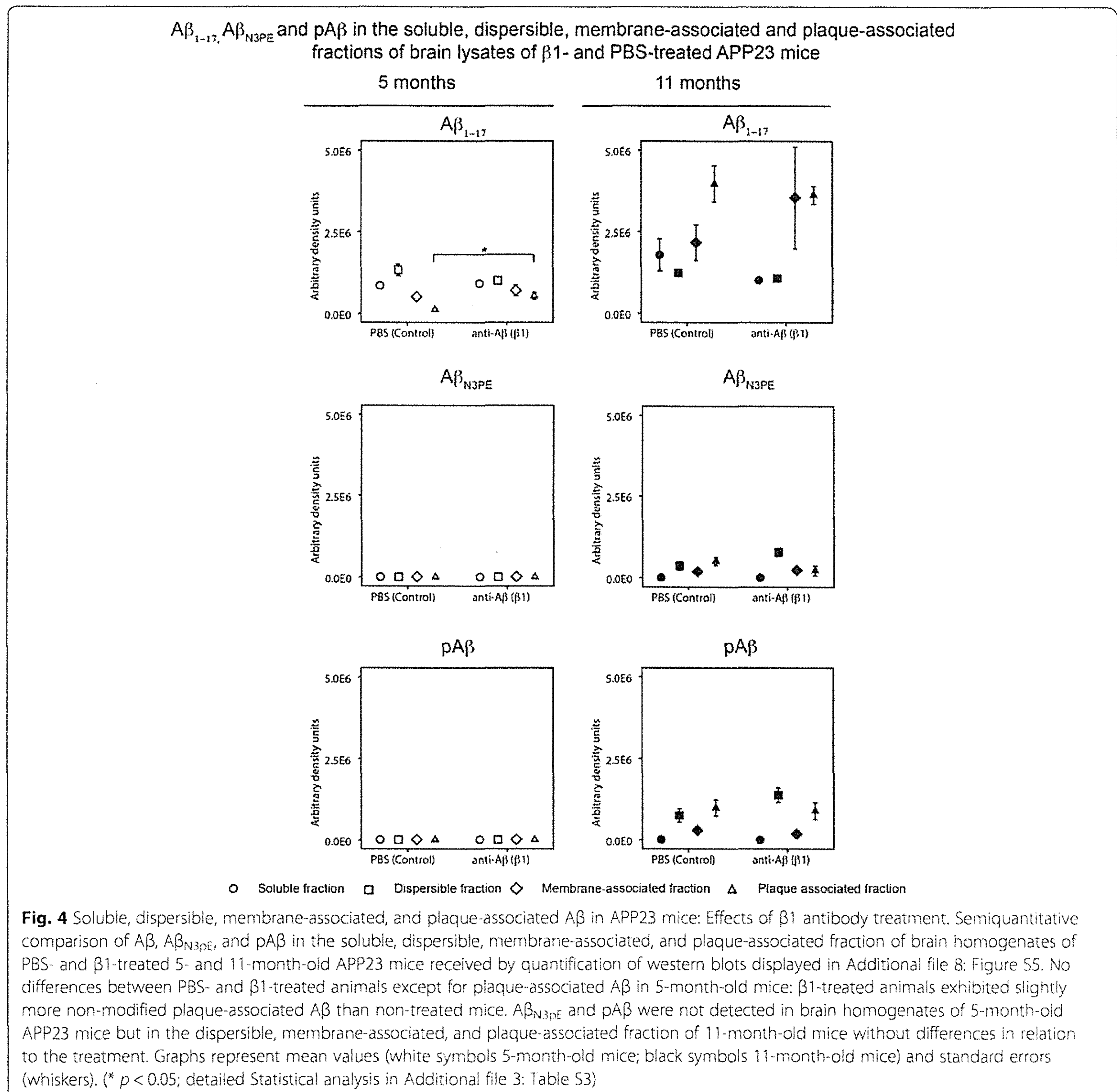

Fig. 4 Soluble, dispersible, membrane-associated, and plaque-associated Aβ in APP23 mice: Effects of β1 antibody treatment. Semiquantitative comparison of Aβ, $A\beta_{N3pE}$, and pAβ in the soluble, dispersible, membrane-associated, and plaque-associated fraction of brain homogenates of PBS- and β1-treated 5- and 11-month-old APP23 mice received by quantification of western blots displayed in Additional file 8: Figure S5. No differences between PBS- and β1-treated animals except for plaque-associated Aβ in 5-month-old mice: β1-treated animals exhibited slightly more non-modified plaque-associated Aβ than non-treated mice. $A\beta_{N3pE}$ and pAβ were not detected in brain homogenates of 5-month-old APP23 mice but in the dispersible, membrane-associated, and plaque-associated fraction of 11-month-old mice without differences in relation to the treatment. Graphs represent mean values (white symbols 5-month-old mice; black symbols 11-month-old mice) and standard errors (whiskers). (* $p < 0.05$; detailed Statistical analysis in Additional file 3: Table S3)

Discussion

Our study on β1-immunized APP23 mice in comparison to non-immunized mice revealed five major findings (Fig. 8): 1. A qualitative change of Aβ aggregate composition was observed between 5- and 11-month-old APP23 mice corresponding with the biochemical maturation of Aβ aggregates as also seen in human AD brain. 2. Passive immunization with β1 antibodies starting at 3 months of age prior to the onset of Aβ deposition and dendritic degeneration until 5 months of age provided a protective effect against dendritic degeneration. Such an effect was not seen when immunization started later at 7 months of age when plaques and dendritic degeneration were already present. 3. Soluble antibody-bound oligomeric, protofibrillar and fibrillar Aβ-containing aggregates were found in β1-treated 5-month-old APP23 mice but not in PBS-treated mice. In older animals soluble antibody-bound Aβ was observed in similar amounts in both β1- and PBS-treated APP23 mice. 4. Aβ immunization in animals with preexisting Aβ pathology showed epitope masking effects in Aβ plaques but no biochemically detectable differences of Aβ in the brain between β1- and PBS-treated mice. 5. An increase of antibody-bound non-modified Aβ in the blood serum, but not of antibody-bound $A\beta_{N3pE}$ and pAβ was detected in β1-treated APP23 mice.