

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; (ii) 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 \pm SD scores in normal aged participants ($n = 800$, 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 \pm 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

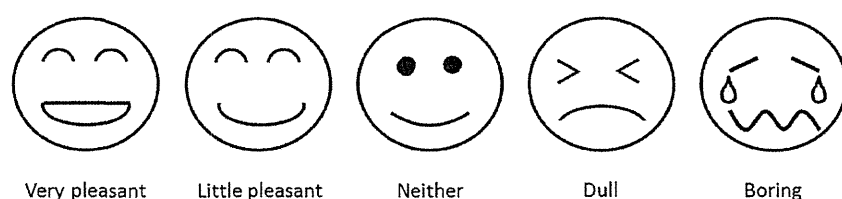


Figure 2 Original face scale.

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 data 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 \pm SD)	72.2 \pm 4.3
Sex (n)	
Male	20
Female	51
Education, years (mean \pm SD)	11.9 \pm 2.4
MMSE (mean \pm SD)	27.8 \pm 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 \pm 2091, Ev2 5019 \pm 2582, Ev3 7293 \pm 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 \pm 13.07, Ev2 56.08 \pm 15.02, Ev3 59.61 \pm 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 \pm 5.03, Ev2 15.73 \pm 4.34, Ev3 17.25 \pm 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

Table 2 Results with repeated measures ANOVA in 63 participants

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

Results are expressed as mean \pm 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 ADL²² and IADL.²³ Walking is well known as a good exercise for the lower extremities. Walking has

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.

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Disclosure statement

No potential conflicts of interest were disclosed.

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RESEARCH

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

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Abstract

Introduction: The deposition of the amyloid β protein ($A\beta$) in the brain is a hallmark of Alzheimer's disease (AD). Removal of $A\beta$ by $A\beta$ -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 $A\beta$ aggregates in the human brain follows a hierarchical sequence of three biochemical maturation stages (B- $A\beta$ stages). To test the impact of the B- $A\beta$ stage on $A\beta$ immunotherapy, we treated transgenic mice expressing human amyloid precursor protein (APP) carrying the Swedish mutation (KM670/671NL; APP23) with the $A\beta$ -antibody β 1 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 $A\beta$ plaque deposition and dendrite degeneration.

Results: At 5 months of age, first $A\beta$ aggregates in APP23 brain consisted of non-modified $A\beta$ (representing B- $A\beta$ stage 1) whereas mature $A\beta$ -aggregates containing N-terminal truncated, pyroglutamate-modified $A\beta_{N3pE}$ and phosphorylated $A\beta$ (representing B- $A\beta$ stage 3) were found at 11 months of age in both β 1- and PBS-treated animals. Protective effects on commissural neurons with highly ramified dendritic trees were observed only in 3-month-old β 1-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 β 1- and PBS-treated APP23 mice sacrificed with 11 months.

Conclusions: $A\beta$ antibody treatment was capable of protecting neurons from dendritic degeneration as long as $A\beta$ aggregation was absent or represented B- $A\beta$ stage 1 but had no protective or curative effect in later stages with mature $A\beta$ aggregates (B- $A\beta$ stage 3). These data indicate that the maturation stage of $A\beta$ 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 ($A\beta$) in senile plaques is one of the hallmarks of Alzheimer's disease (AD) [1, 2]. Active and passive immunization against the $A\beta$ peptide has been developed to treat AD [3, 4]. In amyloid precursor protein (APP) transgenic mice, peripheral administration of $A\beta$ -antibodies lead to a reduced number of detectable plaques and prevented a further increase in the number of detectable $A\beta$ plaques [3, 5]. Even the

amount of fibrillar, Thioflavin S-positive plaques was reduced in anti- $A\beta$ treated animals [6]. However, another group could not confirm $A\beta$ 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 $A\beta$ pathology compared to mice treated at a later point in life in a reversal trial [10]. First trials of active and passive $A\beta$ 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

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the question arises whether A β immunization failed in AD patients because treatment was started too late after the onset of morphologically detectable neurodegeneration. Recently, we reported biochemical stages of A β aggregate maturation (B-A β stages) in the course of pre-clinical and symptomatic AD [17]. In addition to the most abundant forms of A β , i.e. A β_{1-40} and A β_{1-42} , posttranslational modifications of A β have been identified in A β aggregates, including N-terminal truncations and pyroglutamate modifications at residues 3 or 11 (A β_{N3pE} and A β_{N11pE}) and phosphorylation at Serine residue 8 (pA β) [18–20]. The presence of A β_{N3pE} , thereby, indicates B-A β stage 2 and that of pA β B-A β stage 3 [17]. It is not yet clear whether the B-A β stage has impact on the effect of A β -antibody treatment and whether maturation of A β 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 A β aggregates, A β plaques and they show neuron loss in CA1, loss of asymmetric synapses in the fronto-central 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 A β plaque deposition whereas no such changes were reported at 3 months of age in the absence of A β aggregates [24]. Degeneration of axons of commissural neurons represents the morphological correlate 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 A β -antibody treatment on these A β -related neurodegenerative changes.

In this study, we treated APP23 mice with the A β -antibody β 1 (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 β 1 antibody is directed against the N-terminus of A β and capable of preventing A β 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 β 1 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 \rightarrow 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 μ g of β 1-anti-A β 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 (β 1: $n = 21$, 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 (β 1: $n = 21$, 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 β 1-treated and PBS-treated animals were used (5 months - β 1: $n = 9$, PBS: $n = 10$; 11 months - β 1: $n = 10$, 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 mm³) 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 μ m 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 β 1-treated APP23 mice was counted and compared with that in PBS-treated APP23 mice. For qualitative and quantitative analysis, 10 consecutive sections (100- μ m 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 mm³ in each mouse. Mean and median values of the number of traced neurons were calculated and compared between β 1-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 β _{17–24}, anti-A β _{1–17}, anti-A β ₄₂, anti-A β ₄₀, anti-A β _{N3pE}, anti-pA β , anti-A β - β 1, 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 diaminobenzidine-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 β ₄₂, A β ₄₀, A β _{N3pE}, pA β , β 1- and B10AP-positive plaques

A β ₄₂ load was determined as the percentage of the area in the frontocentral cortex covered by A β plaques detected with anti-A β ₄₂ antibodies. Morphometry for A β 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 β ₄₀, A β _{N3pE}, pA β , A β _{1–17}, β 1 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 \times 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 β 1-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 $14,000 \times g$ 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 $175,000 \times g$ 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 $14,000 \times g$ 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 A β . 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 μ Columns, 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 pre-cast 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.

A β was detected by western blotting with anti-A β_{1-17} , anti-pA β and anti-A β_{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 Lumi-nata 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 β_{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 A β aggregates readily dissociate in the presence of SDS-containing buffers into monomers and small oligomers, such as dimers, trimers, or A β^{*56} [37, 38], we analyzed differences among the monomer bands that indicate changes in the protein levels of precipitated A β aggregates densitometrically using ImageJ software (NIH, Bethesda, MD, USA). This method allows a semi-quantitative assessment of A β [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 A β aggregation (B-A β stages) were determined on the basis of A β , A β_{N3pE} and pA β detection in the soluble, dispersible, membrane-associated, and plaque-associated fraction [17]: B-A β stage 1 = A β aggregates detected only with anti-A β_{1-17} ; B-A β stage 2 = A β aggregates positive for anti-A β_{1-17} and anti-A β_{N3pE} but not for anti-pA β ; B-A β stage 3 = A β aggregates containing anti-A β_{1-17} , anti-A β_{N3pE} and anti-pA β -positive material.

Stereology

Six $\beta 1$ -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 $5 \times$ magnification. Neurons were counted in three different, randomly chosen microscopic fields ($40 \times$ 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 μ m thick vibratome sections of the frontocentral cortex from six β 1-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 semiquantitative 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 β 1-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 β 1-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 β 1-treated as well as in PBS-treated animals (Fig. 1).

However, β 1-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 β 1- and PBS-treated animals (Fig. 1 and Additional file 3: Table S3). Treatment with the β 1-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).

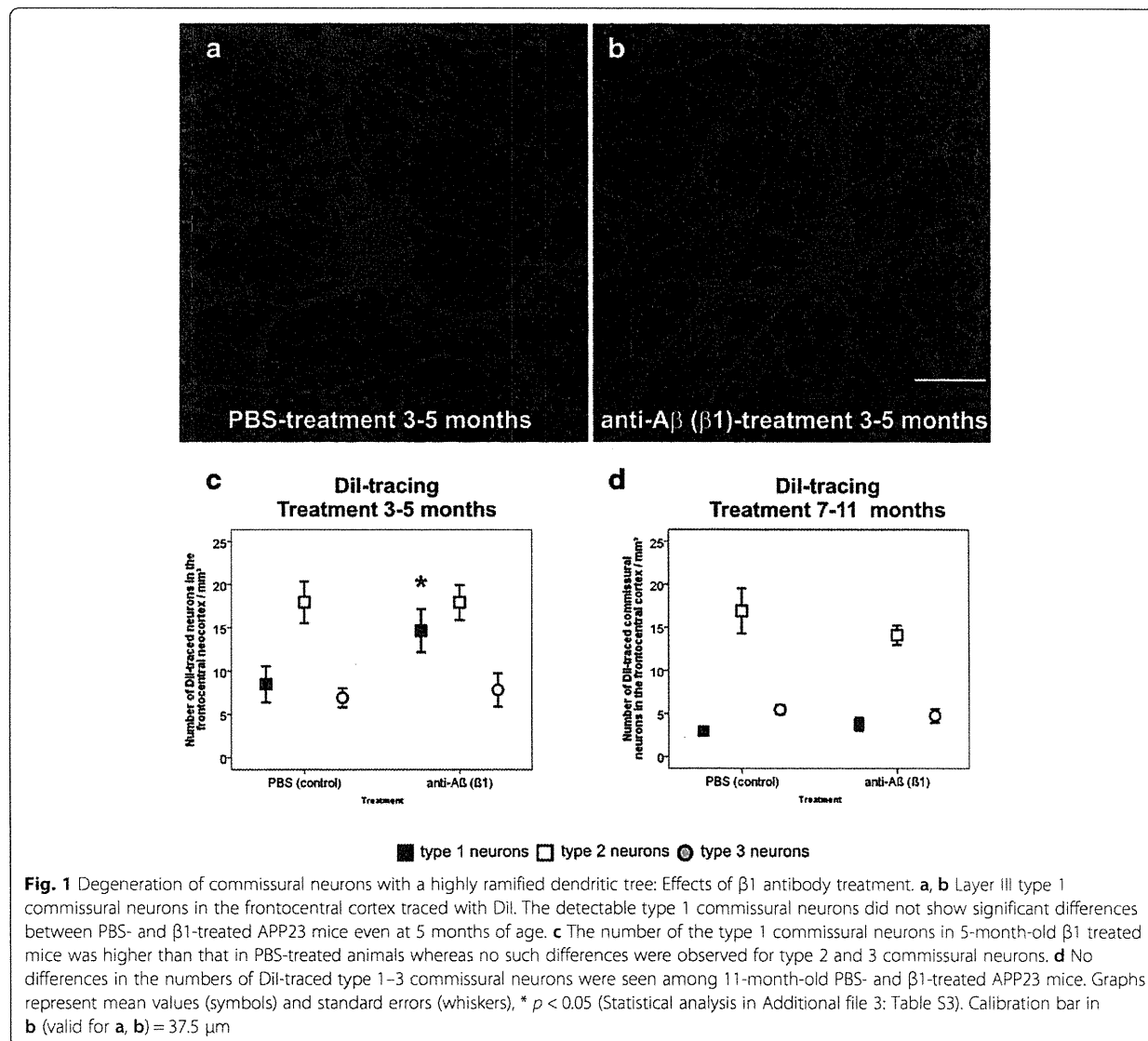
Significant differences in the synapse densities of asymmetric and symmetric synapses in the frontocentral cortex as well as differences in the numbers of CA1 neurons were not observed between β 1-treated and PBS-treated animals in both age groups (Additional file 3: Table S3b, c and Additional file 4: Figure S1).

A β plaques in β 1-treated and PBS-treated APP23 mice

A β plaques detectable with antibodies directed against A β ₄₂, A β ₄₀, A β ₁₇₋₂₄, A β ₁₋₁₇, A β _{N3pE}, and pA β were found in both, β 1-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 β 1 antibody (used for passive immunization) showed that treated animals exhibited fewer plaques stained with β 1 indicating epitope masking (Fig. 3 and Additional file 3: Table S3d). B10AP-positive plaques were also reduced in β 1-treated 11-month-old animals (Fig. 3) whereas 5-month-old mice did not exhibit B10AP-positive plaques regardless of β 1 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 β 1-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 β 1- 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 β 1- 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 A β , A β _{N3pE} and pA β in β 1-treated and PBS-treated APP23 mice

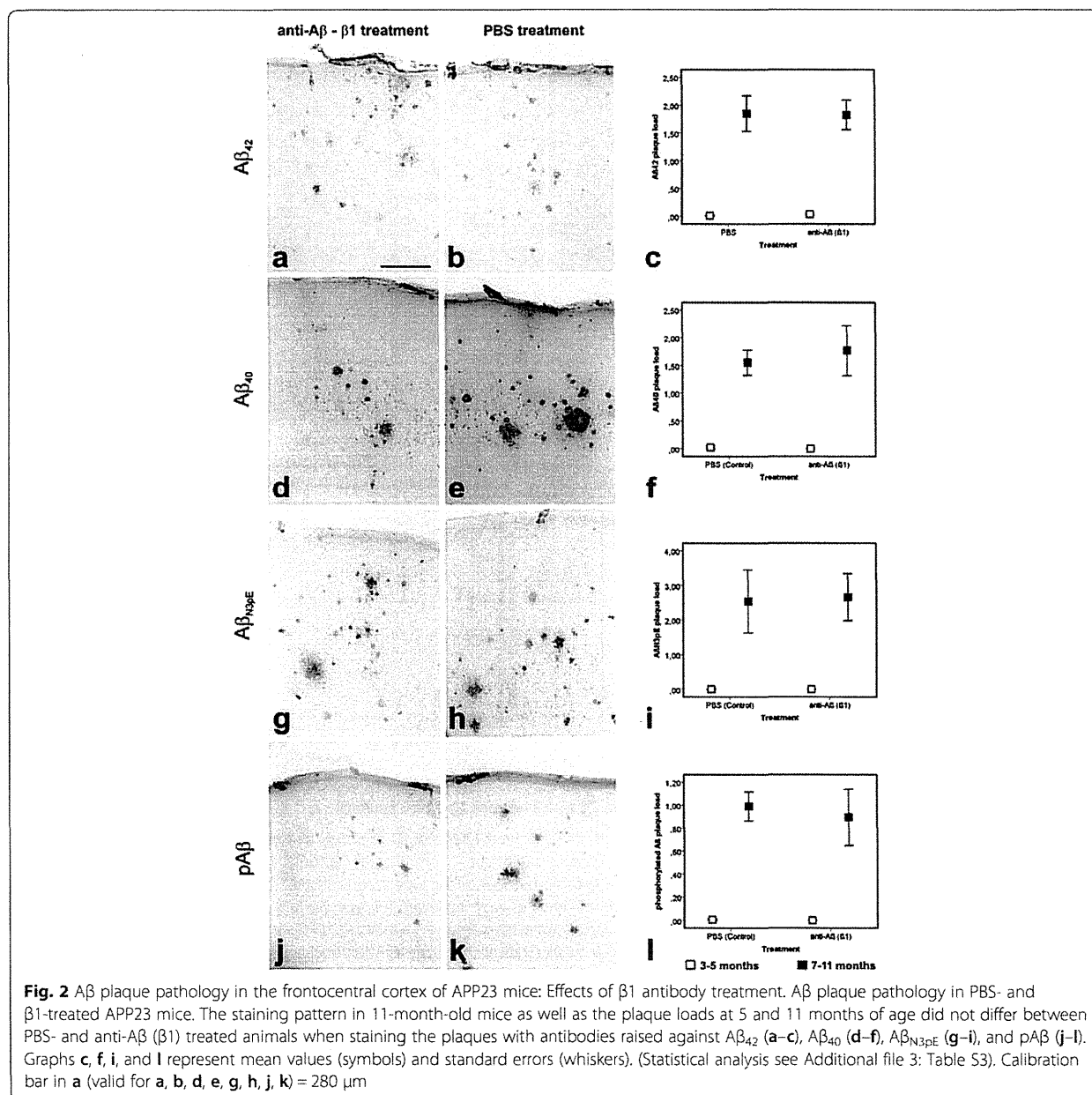
Western blot analysis of forebrain homogenates revealed no obvious differences in the amounts of soluble, dispersible, and membrane-associated A β detectable with



anti-A β_{1-17} in β 1-treated and PBS-treated animals of both age groups (at 5 and 11 months of age). β 1-treated 5-month-old APP23 mice exhibited higher amounts of anti-A β_{1-17} detected plaque-associated A β than PBS-treated animals (Fig. 4, Additional file 3: Table S3e and Additional file 8: Figure S5). In 11-month-old mice, A β 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). A β_{N3pE} and pA β 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 A β_{N3pE} and pA β aggregation between β 1- 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 A β aggregate maturation during the course of AD, the presence of A β in the absence of biochemically detectable A β_{N3pE} and pA β in 5-month-old APP23 mice corresponded to B-A β stage 1 whereas A β aggregates in 11-month-old mice consisting of normal A β , A β_{N3pE} , and pA β were referred to as B-A β 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-A β_{1-17} showed more A β oligomers, protofibrils and fibrils in the β 1-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

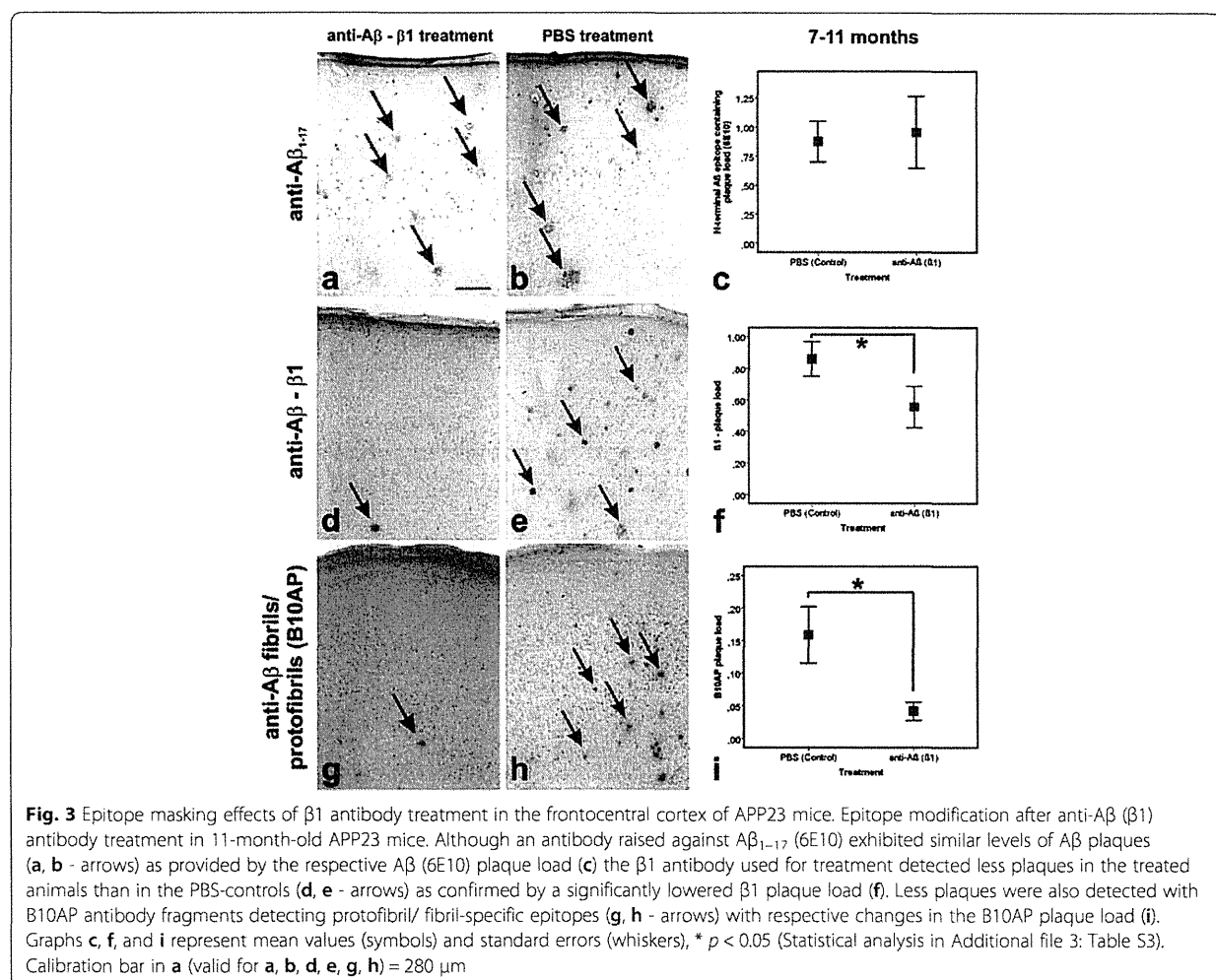


there were no differences in the amounts of A β oligomers, protofibrils and fibrils between the β 1-treated and PBS-treated animals of both age groups (Fig. 5, Additional file 3: Table S3f and Additional file 9: Figure S6).

In 5-month-old APP23 mice, irrespective of the treatment, no A β _{N3pE} and no pA β was detected, in the soluble and dispersible fraction of samples immunoprecipitated with A11 or B10AP. Similarly, in 11-month-old mice, A β _{N3pE} and pA β were not detected in A11 and B10AP immunoprecipitated oligomers, protofibrils and fibrils from the soluble fraction. A β _{N3pE} and pA β were found in the dispersible fraction in A β protofibrils and fibrils

precipitated with B10AP at 11 months of age but without significant differences among β 1- and PBS-treated animals (Fig. 5, Additional file 3: Table S3f and Additional file 9: Figure S6). There was no detectable A β _{N3pE} in both groups of 11-month-old mice in the dispersible fractions immunoprecipitated with non-fibrillar A β oligomer-specific A11 antibodies but pA β was observed in β 1- and PBS-treated mice (Fig. 5, Additional file 3: Table S3f and Additional file 9: Figure S6).

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



showed IgG-bound soluble A β in 5-month-old $\beta 1$ -treated mice detected with anti-A β_{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 β_{N3pE} and pA β 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 β_{1-17} antibodies, showed detectable amounts of IgG-bound A β 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 β_{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 pA β was observed in antibody-bound aggregates but 11-month-old animals exhibited dispersible pA β 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 A β in the blood serum of $\beta 1$ -treated and PBS-treated APP23 mice

Western blot analysis did not reveal detectable amounts of A β in the serum of 5- and 11-month-old $\beta 1$ - and PBS-treated APP23 mice (Fig. 7 and Additional file 11: Figure S8). Only after immunoprecipitation of antibody-bound A β from blood serum using protein-G coated magnetic beads without previous primary antibody coupling showed anti-A β_{1-17} detectable A β in $\beta 1$ -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 β_{N3pE} and pA β were not found in the blood serum of APP23 mice of both ages regardless of $\beta 1$ -treatment (Fig. 7 and Additional file 11: Figure S8).

$A\beta_{1-17}$, $A\beta_{N3PE}$ and $pA\beta$ in the soluble, dispersible, membrane-associated and plaque-associated fractions of brain lysates of $\beta 1$ - and PBS-treated APP23 mice

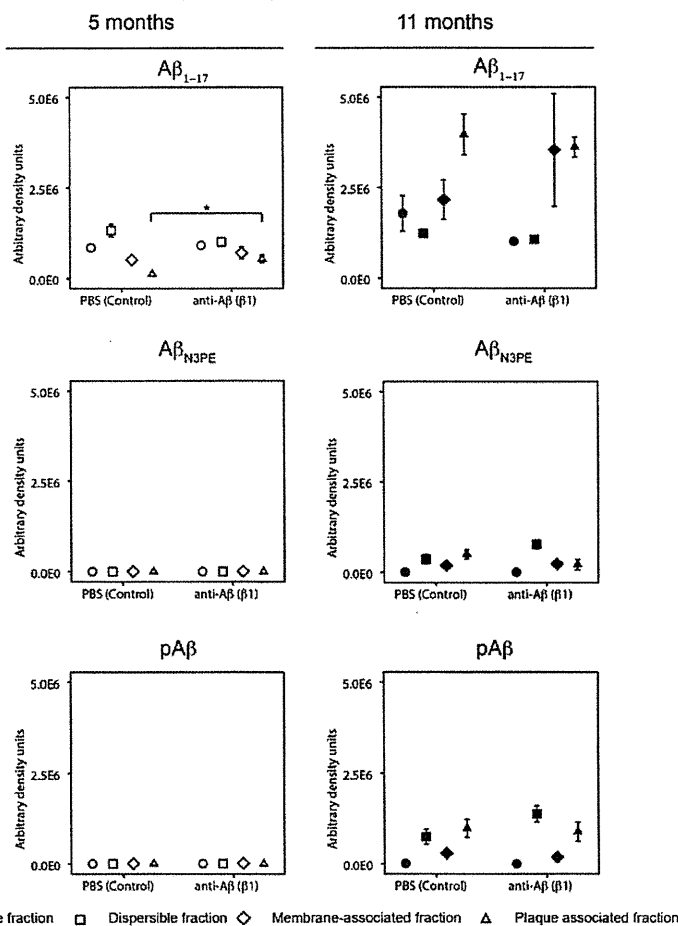
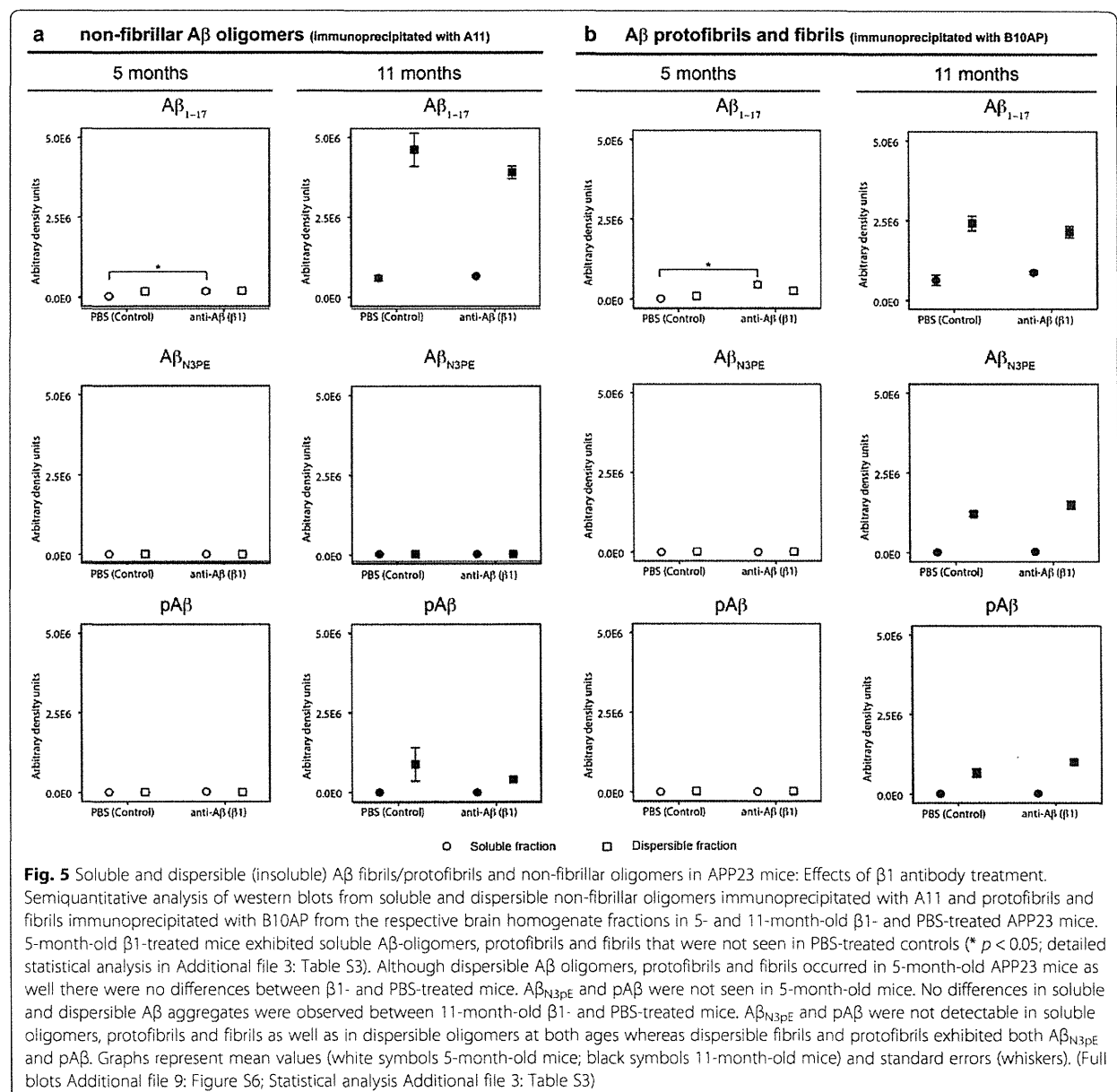


Fig. 4 Soluble, dispersible, membrane-associated, and plaque-associated $A\beta$ in APP23 mice: Effects of $\beta 1$ antibody treatment. Semiquantitative comparison of $A\beta$, $A\beta_{N3PE}$, and $pA\beta$ in the soluble, dispersible, membrane-associated, and plaque-associated fraction of brain homogenates of PBS- and $\beta 1$ -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 $\beta 1$ -treated animals except for plaque-associated $A\beta$ in 5-month-old mice: $\beta 1$ -treated animals exhibited slightly more non-modified plaque-associated $A\beta$ than non-treated mice. $A\beta_{N3PE}$ and $pA\beta$ 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 $\beta 1$ -immunized APP23 mice in comparison to non-immunized mice revealed five major findings (Fig. 8): 1. A qualitative change of $A\beta$ aggregate composition was observed between 5- and 11-month-old APP23 mice corresponding with the biochemical maturation of $A\beta$ aggregates as also seen in human AD brain. 2. Passive immunization with $\beta 1$ antibodies starting at 3 months of age prior to the onset of $A\beta$ 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 $A\beta$ -containing aggregates were found in $\beta 1$ -treated 5-month-old APP23 mice but not in PBS-treated mice. In older animals soluble antibody-bound $A\beta$ was observed in similar amounts in both $\beta 1$ - and PBS-treated APP23 mice. 4. $A\beta$ immunization in animals with preexisting $A\beta$ pathology showed epitope masking effects in $A\beta$ plaques but no biochemically detectable differences of $A\beta$ in the brain between $\beta 1$ - and PBS-treated mice. 5. An increase of antibody-bound non-modified $A\beta$ in the blood serum, but not of antibody-bound $A\beta_{N3PE}$ and $pA\beta$ was detected in $\beta 1$ -treated APP23 mice.



Thus, it is tempting to speculate that A β -antibody treatment with the β 1-antibody is most effective in early stages of A β aggregate maturation whereas late stage aggregates containing A β _{N3PE} and pA β may preclude sufficient clearance. In the following paragraphs, we discuss the hypothesis that biochemical maturation of A β aggregates modifies its response to antibody treatment in detail.

Protective effects of β 1-antibody treatment in APP23 mice without preexisting pathology (Fig. 8)

Our study showed that β 1 A β antibody treatment protected APP23 mice from severe dendrite degeneration in

type 1 commissural neurons when treatment was started at 3 months before A β and dendrite pathology develop in this mouse model [24]. Such an effect was not observed when treatment was started later, i.e. after the onset of A β plaque pathology and type 1 commissural neuron degeneration with 7 months of age. This finding is in line with a previous report showing the absence of beneficial effects after γ -secretase treatment in mice with preexisting plaques [45]. The fact that only tracing of commissural neurons revealed differences between β 1- and PBS-treated APP23 mice but neither CA1 neuron numbers nor synapse densities in the frontocentral cortex as further markers for neurodegeneration appear to

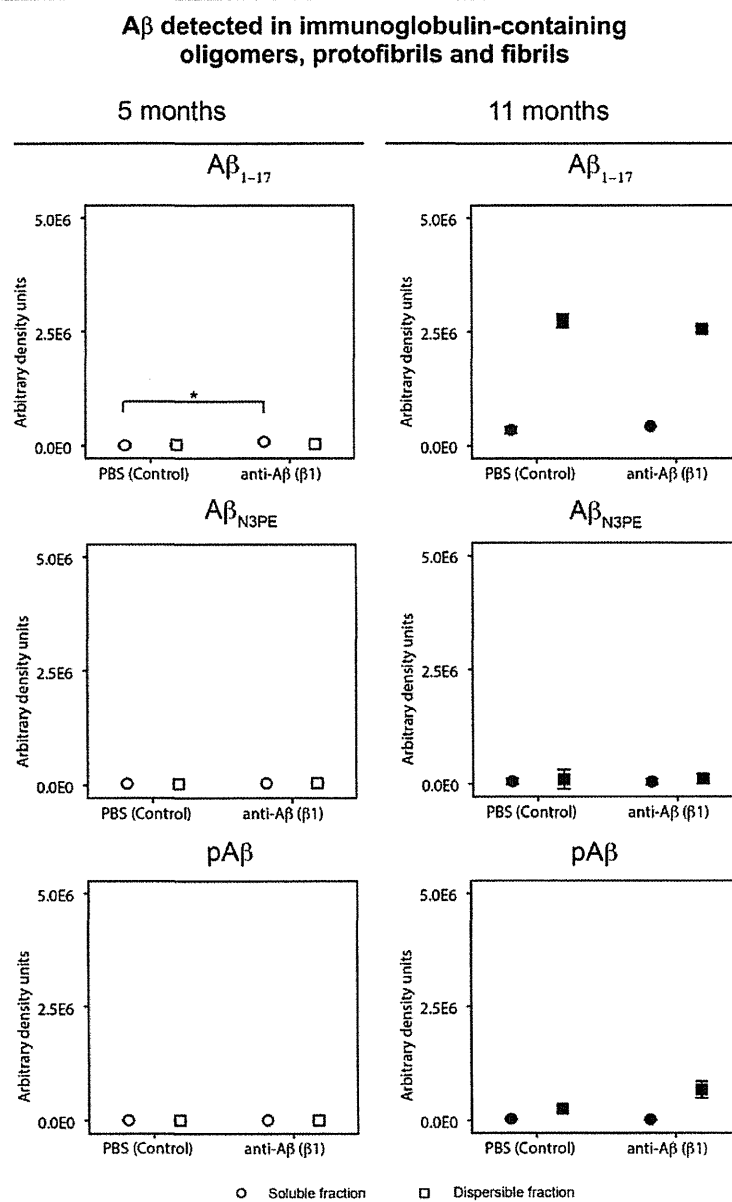
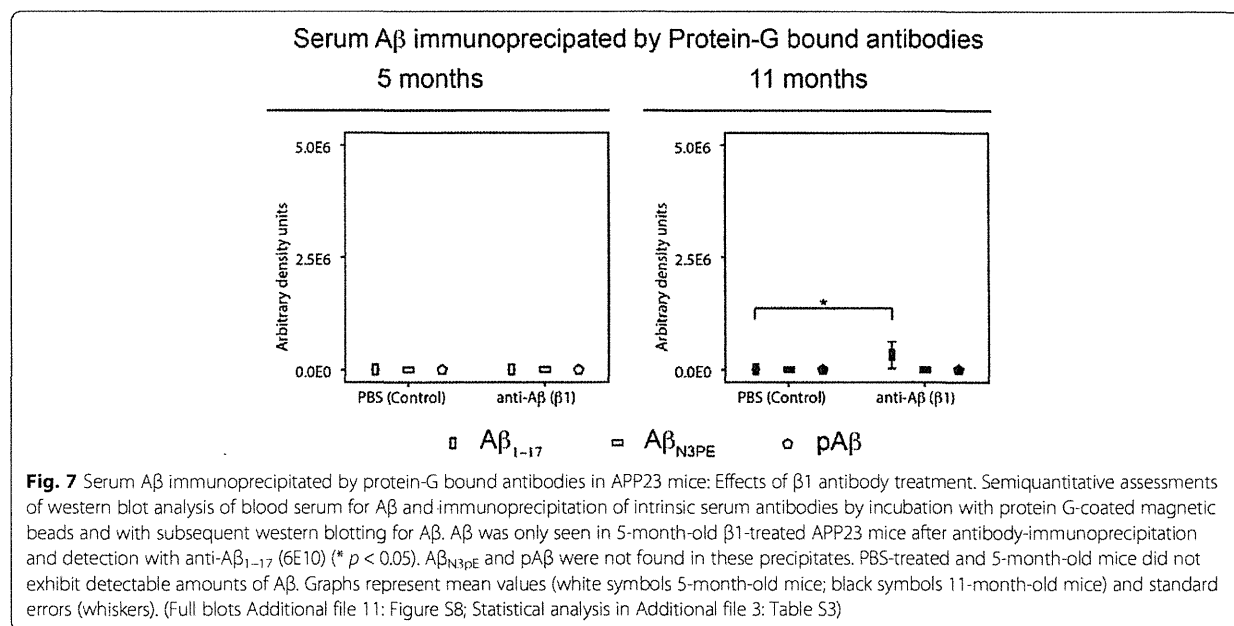


Fig. 6 Immunoglobulin-bound A β detected in oligomers, protofibrils and fibrils in APP23 mice: Effects of β 1 antibody treatment. Semiquantitative analysis of western blots after immunoprecipitation of immunoglobulin (antibody)-bound A β by precipitation of antibodies with protein G-coated magnetic beads. Subsequent western blot analysis with anti-A β_{1-17} revealed antibody-bound A β in the dispersible fraction of both β 1- and PBS-treated mice at both ages. In 5-month-old β 1-treated APP23 mice antibody-bound A β was found in the soluble fraction whereas no antibody-bound A β was precipitated in PBS-treated animals (* $p < 0.05$). At 11-months of age both, β 1 and PBS-treated animals exhibited antibody bound soluble A β in similar amounts. Antibody-bound A β_{N3PE} was not observed whereas 11-month-old (but not 5-month-old) APP23 mice showed similar amounts of dispersible antibody-bound pA β . No soluble antibody-bound pA β was seen. Graphs represent mean values (white symbols 5-month-old mice; black symbols 11-month-old mice) and standard errors (whiskers). (Full blots Additional file 10: Figure S7; Statistical analysis in Additional file 3: Table S3)

be related to the APP23 mouse model. Different types of neurons differ with respect to their vulnerability to A β -related neurodegeneration in APP23 mice [24] and the type 1 commissural neurons are among the most vulnerable neurons in the frontocentral neocortex.

Accordingly, degeneration of these neurons is a very sensitive indicator for A β -induced neurodegeneration in the APP23 mouse model that allows the detection of differences in A β -related neurodegeneration before other readouts such as synapse densities and neuron numbers



become positive. It is important to note that the APP23 mouse is, in contrast to other APP-transgenic mice, the only APP-transgenic mouse that shows neuron loss, synapse loss, and dendritic degeneration [22, 24, 41]. Other mouse models showing such signs of neurodegeneration do not only express APP as the APP23 mouse but also presenilin 1 or τ that can also be responsible for neurodegeneration [46, 47]. As such our study in APP23 mice shows that morphological alterations of dendrites presumably caused by A β aggregates were subject of protection by immunotherapy but were not repaired once the neurons were fallen victim to degeneration.

Our finding of soluble antibody-bound A β -containing oligomers, protofibrils and fibrils as detected by immunoprecipitation in 5-month-old β 1-treated APP23 mice that were not seen in PBS-treated animals indicates that β 1-antibody treatment increases soluble A β aggregates. The antibody may stabilize existing soluble aggregates similar to blood A β [48, 49]. An enhancement of A β -aggregation cannot be excluded. The lack of degeneration suggests that such soluble A β -IgG aggregates are less pathogenic and may become subject of clearance. No antibody-bound A β aggregates were found in the blood of 5-month-old APP23 mice. The increase of antibody-bound A β in the blood of 11-month-old β 1-treated APP23 mice but not in PBS-treated animals most likely reflects stabilization of plasma A β as previously described [48, 49]. No modified forms of A β were detected in the soluble fraction of 5- and 11-month old mice and in the blood due to their respective low concentrations or due to inefficient β 1-antibody binding. β 1-treatment did not cause a shift in soluble and

insoluble A β aggregates in 11-month-old APP23 mice exhibiting A β_{N3pE} and pA β . A possible explanation for the lack of a treatment effect on A β aggregates in 11-month-old APP23 mice may be the property of A β_{N3pE} and pA β to stabilize A β aggregates *in-vitro* [18, 50] and, in so doing, to preclude sufficient antibody treatment effects as shown here.

Since the APP23 mouse is a mouse model that showed morphological signs of neurodegeneration with neuron loss in CA1 [22], reduction of asymmetric synapse densities [23], and dendritic degeneration in commissural neurons [24] this mouse model appears to be useful to study morphological signs of A β -related neurotoxicity to better understand the lack of improvement in anti-A β treated patients [11, 14, 16]. In this context, our results suggest that treatment attempts in already demented individuals exhibiting mature A β aggregates in the B-A β -stage 3 indicated by A β_{N3pE} and pA β as its components [17] may not be successful because 1. irreversible degenerative changes already took place and 2. mature A β aggregates in B-A β stage 3 prevail [17], which were not removed by β 1-antibody treatment in APP23 mice as described here. Accordingly, treatment strategies aimed at protecting from A β maturation and early A β -related neurodegenerative effects appear to be more attractive than those targeting non-modified forms of A β including oligomeric, protofibrillar and fibrillar aggregates.

Moreover, in human symptomatic AD cases in addition to A β plaque pathology, there are already high amounts of neurofibrillary tangles as well as severe neuron and synapse loss. As such, the amount of brain damage at this stage of the disease is significant. Given that most degenerating

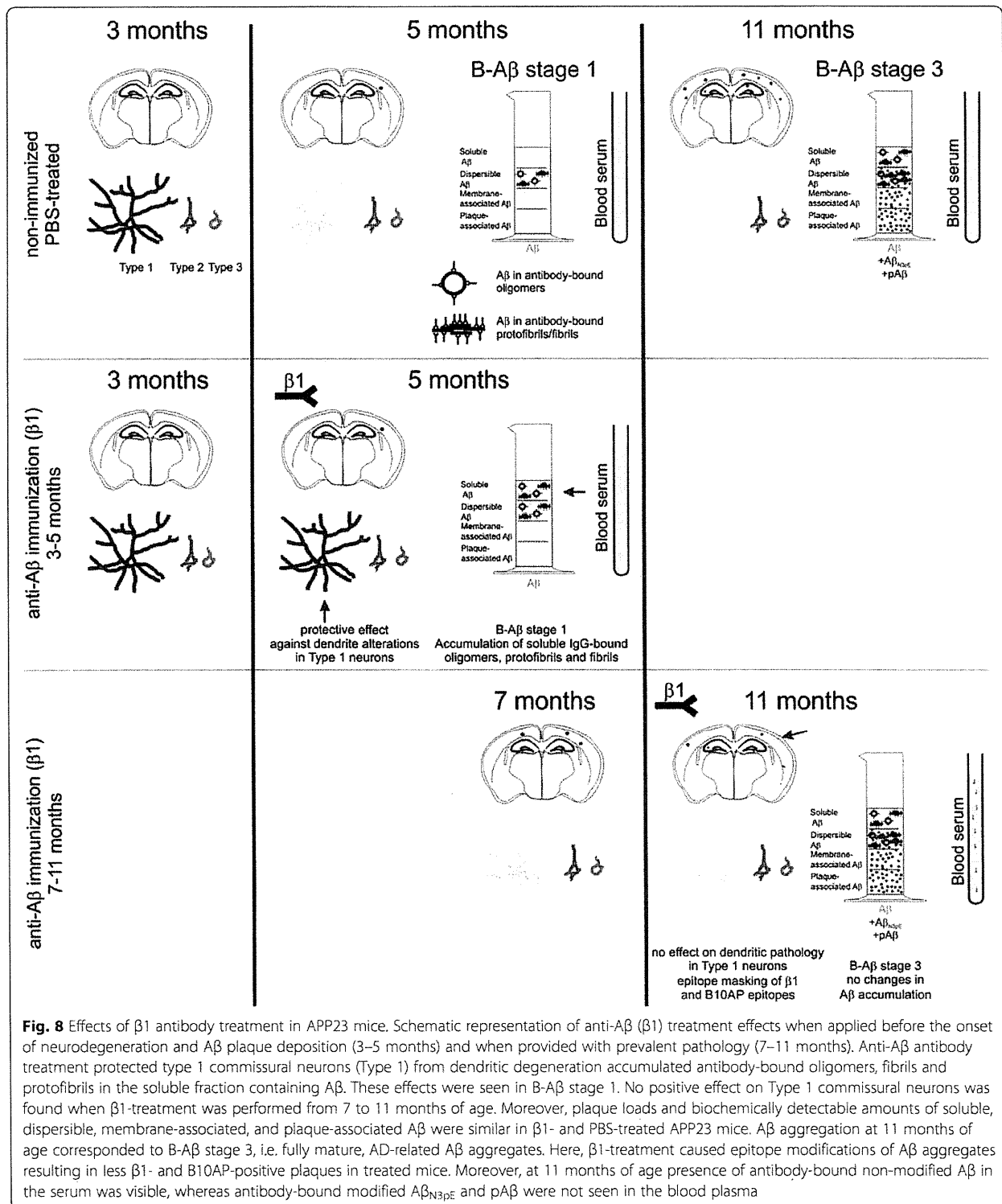


Fig. 8 Effects of β 1 antibody treatment in APP23 mice. Schematic representation of anti-A β (β 1) treatment effects when applied before the onset of neurodegeneration and A β plaque deposition (3–5 months) and when provided with prevalent pathology (7–11 months). Anti-A β antibody treatment protected type 1 commissural neurons (Type 1) from dendritic degeneration accumulated antibody-bound oligomers, fibrils and protofibrils in the soluble fraction containing A β . These effects were seen in B-A β stage 1. No positive effect on Type 1 commissural neurons was found when β 1-treatment was performed from 7 to 11 months of age. Moreover, plaque loads and biochemically detectable amounts of soluble, dispersible, membrane-associated, and plaque-associated A β were similar in β 1- and PBS-treated APP23 mice. A β aggregation at 11 months of age corresponded to B-A β stage 3, i.e. fully mature, AD-related A β aggregates. Here, β 1-treatment caused epitope modifications of A β aggregates resulting in less β 1- and B10AP-positive plaques in treated mice. Moreover, at 11 months of age presence of antibody-bound non-modified A β in the serum was visible, whereas antibody-bound modified A β _{N3pE} and pA β were not seen in the blood plasma

neurons are post-mitotic cells and will not be replaced [51] and that presence of A β plaques and neurofibrillary tangles induces gliosis [52–56] compensation or reconstruction of these lesions is very unlikely. Therefore, it is tempting to

speculate that early B-A β stage preclinical AD cases are best suited to protect from or slow down progression of the disease as shown in 3-months-old β 1-treated APP23 mice sacrificed at 5 months of age. Findings in APP23- β 1-

double transgenic mice clearly demonstrate that an efficient protective effect of the β 1-antibody is possible in the initial stage of A β aggregation [28].

Effects of β 1-antibody treatment on A β aggregates in animals with preexisting pathology

β 1-antibody treatment of APP23 mice between 7 and 11 months neither led to any improvement of neurodegenerative lesions nor to changes in the levels of soluble or the insoluble types of A β aggregates or in A β plaque-loads (Fig. 8). Modified forms of A β occurred in PBS- and β 1-treated APP23 mice in similar amounts (Fig. 8). Interestingly, detection of A β plaques in β 1-treated mice was attenuated for staining with the β 1-antibody and the B10AP antibody fragments recognizing protofibril/ fibril-related epitopes. In contrast, immunoprecipitation of soluble and dispersible fractions with A11 and B10AP revealed similar levels of soluble and dispersible A β oligomers, protofibrils and fibrils. It is tempting to speculate that β 1 antibody bound to plaques to a certain extent blocked binding of further β 1 and B10AP antibodies. This finding is in agreement with the binding of intravenously injected β 1 antibody to preexisting plaques in APP23 mice [49].

However, immunoprecipitation of antibody-bound peptides by incubating brain homogenates with protein G-coated beads revealed no significant differences between 11-month-old immunized and non-immunized animals although antibody-bound A β was immunoprecipitated in the serum of β 1-treated animals in contrast to PBS-treated ones. The unexpected finding of IgG in plaques and bound to A β aggregates in PBS-treated and β 1-treated animals in similar amounts may be explained by naturally occurring autoantibodies that interact with amyloid plaques in APP23 mice as previously demonstrated in AD patients [57, 58]. Further evidence in favor of this interpretation is provided by our finding that IgG was detected immunohistochemically in plaques of PBS- and β 1-treated animals in similar amounts and pattern.

Aspects of A β maturation for A β -immunotherapy

The major finding of this study is that the maturation stage of A β aggregates has impact on protective effects of A β immunotherapy related to A β toxicity. If this effect of the maturation stage on A β -immunotherapy seen in the APP23 mouse would also apply for human AD this would mean that immunotherapy should be started in early pre-clinical stages of the disease exhibiting very few plaques and B-A β stage 1. The non-favorable outcomes of recently completed trials on passive immunization against A β with bapineuzumab or solanezumab [13, 14] could be explained as a logical consequence of the selection of patients with early symptomatic AD, i.e. patients that very likely already had B-A β stage 3 [17] indicative for advanced stage A β pathology when they started the trial. Accordingly, it still

seems reasonable that A β -targeting immunotherapy trials which include non-diseased individuals in a preclinical phase of AD instead of symptomatic AD patients with late stage pathology, may show a protective effect as shown here in APP23 mice. However, in our mouse model we only studied the effects of A β but not that of τ -pathology, which prevails in human AD brain as well [59, 60] and appears capable of spreading into further brain regions even without being triggered by A β [61].

Conclusions

Our results confirm previous studies that the protective potential of passive A β immunization is much greater than its disease modifying potential and demonstrate for the first time that the maturation stage of the biochemical A β aggregate composition, i.e. the B-A β stage, has impact on A β -antibody treatment. As such, our results point to an important role of the B-A β stage for design of treatment strategies targeting A β .

Additional files

Additional file 1: Table S1. List of antibodies.

Additional file 2: Table S2. Types of commissural neurons traced with Dil [24].

Additional file 3: Table S3. Statistical analysis.

Additional file 4: Figure S1. Neuronal and synaptic pathology: Effects of β 1 antibody treatment. No differences between PBS- and β 1-treated APP23 mice at 5 and 11 months of age in the densities of asymmetric (a) and symmetric synapses in the frontocentral cortex (b) as well as in the numbers of CA1 neurons in the hippocampus (c). Graphs represent mean values (symbols) and standard errors (whiskers). (Statistical analysis in Additional file 3: Table S3).

Additional file 5: Figure S2. Neuritic plaques: Effects of β 1 antibody treatment. APP-type neuritic plaques (arrows) are detectable in both anti-A β (β 1)- (a) and PBS-treated 11-month-old APP23 mice (b). Calibration bar in b (valid for a, b) = 27.5 μ m.

Additional file 6: Figure S3. IgG accumulation in plaques and cerebral amyloid angiopathy and microglial activation: Effects of β 1 antibody treatment. Detection of microglial cells and mouse IgG in 5- and 11-month-old β 1- and PBS-treated APP23 mice. Mouse IgG comprises intrinsic mouse IgG and the mouse-IgG antibody β 1 used for treatment. Amyloid plaques in 11-month-old mice were detected by A β autofluorescence [35] whereas no autofluorescent plaques were found in 5 month old animals. a-h: In 5-month-old APP23 mice no IgG and no amyloid material was observed in both β 1 and PBS-treated mice. Iba-1 immunostaining showed comparable microglia pattern in both groups. i-t: Mouse IgG occurred in all plaques (white and lucent arrows) and cerebral amyloid angiopathy (arrowheads) affected vessels identified by amyloid autofluorescence in 11-month-old APP23 mice. Plaque-associated microglial activation was observed in β 1- and PBS-treated mice in this age group (white arrows). Calibration bar in a (valid for a-t) = 60 μ m.

Additional file 7: Figure S4. IgG accumulation in plaques and cerebral amyloid angiopathy and astroglial activation: Effects of β 1 antibody treatment. Detection of astrocytes and mouse IgG in 5- and 11-month-old β 1- and PBS-treated APP23 mice. Mouse IgG comprises intrinsic mouse IgG and the mouse-IgG antibody β 1 used for treatment. Amyloid plaques in 11-month-old mice were detected by A β autofluorescence [35] whereas no autofluorescent plaques were found in 5 month old animals. a-h: In 5-month-old APP23 mice no IgG and no amyloid material was observed in both β 1 and PBS-treated mice. GFAP immunostaining showed comparable