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Amyloid precursor protein knockdown by siRNA impairs spontaneous alternation in adult mice

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Abstract

The cleavage-product of amyloid precursor protein (APP) constitutes the core component of plaques found in the brains of Alzheimer's disease (AD) patients. APP is ubiquitously expressed and its precise physiological functions remain unclear. This protein has been proposed to regulate synaptic function and processes underlying learning and memory. While APP knockout mice show behavioral impairments, these may occur due to early changes during development and/or due to abolition of APP function in adult. To investigate the acute effects of APP knockdown without involving developmental processes, APP expression was reduced using RNA interference in adult mouse brain. Small interfering RNAs (siRNAs) that down-regulated mouse APP protein levels (APP-siRNA) were identified using an APP plasmid-siRNA co-transfection assay in mouse NIH/3T3 fibroblast cells. Infusion of APP-siRNAs into the ventricular system for 2 weeks also down-regulated APP mRNA in mouse brain. Highest knockdown of APP mRNA levels was found in the CA2-CA3 regions of the hippocampus. Mice treated with the most active APP-siRNA showed a significant reduction in spontaneous alternation rate in the Y-maze, without effects on forelimb grip strength or locomotor activity. These data suggest that acute knockdown of APP in adult mouse brain impairs hippocampus-dependent spatial working memory.

Keywords: amyloid precursor protein, RNA interference, hippocampus, spontaneous alternation, working memory.
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Alzheimer's Disease (AD) is characterized by progressive deterioration of cognitive function associated with neuronal loss and the presence of senile plaques, neurofibrillary tangles and synaptic defects (Selkoe 2001). Altered proteolytic processing of amyloid precursor protein (APP) has been suggested to play a key role in the pathogenesis of AD, through the generation of amyloidogenic Abeta (Aβ) peptides found in AD plaques (Tanzi and Bertram 2005). The study of Aβ and its implications for AD has been the major focus of neuropathological research. However, the precise functions of its precursor APP in the non-pathological state still remain elusive. Functions proposed for APP range from cell adhesion and neurite outgrowth to cognitive roles in learning and memory (Turner *et al.* 2003; Reinhard *et al.* 2005), but are still lacking substantial *in vivo* evidence.

Amyloid precursor protein knockout mice (KO) are viable and fertile and show a subtle phenotype including reduced body weight and locomotor activity (Muller *et al.* 1994; Zheng *et al.* 1995). These mice also display age-related deficits in cognitive tests and long-term potentiation, suggesting a role for APP in synaptic function within the hippocampus (Dawson *et al.* 1999; Fitzjohn *et al.* 2000). Since APP-null mice lack the gene from ontogeny to adulthood, development may be altered leading to changes in adult life. These limitations associated with the study of APP-KO mice may complicate the interpretation of cognitive

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Abbreviations used: AD, Alzheimer's disease; APLP, Amyloid Precursor Like Protein; APPC, amyloid precursor protein C; GFP, green fluorescent protein; PBS, phosphate-buffered saline; TBST, Tris Buffered Saline with Tween20.

phenotypes that are observed. Besides genetic deletion of APP in KO animals, the feasibility of acute knockdown of APP gene expression through the recent technologies of antisense and small interfering RNAs (siRNAs) has opened new avenues into the investigation of APP function *in vivo* in various model organisms (reviewed by Senechal *et al.* 2006).

RNA interference (RNAi) is a naturally occurring post-transcriptional gene-silencing mechanism that attenuates the expression of the targeted gene in a sequence specific manner (Fire *et al.* 1998; Dykxhoorn *et al.* 2003). The feasibility of APP knockdown *in vivo* using a siRNA-based technology has been demonstrated previously using siRNAs directly in the eye (Herard *et al.* 2006). Furthermore, the direct intracerebroventricular (i.c.v.) infusion of siRNA molecules in mice reduces target gene expression in the brain and produces behavioral effects (Thakker *et al.* 2004, 2005, 2006).

In the present study, we aimed to reduce acutely the APP expression in the adult mouse brain using siRNAs to investigate the physiological roles of APP thus avoiding developmental effects. Mouse APP-targeting siRNAs (mAPP-siRNAs) and mis-match controls were designed, and then characterized *in vitro*. In a subsequent *in vivo* study, these siRNAs were infused into the brains of adult mice and the behavioral effects were examined using tests relevant to the study of APP function including the hippocampus-dependent spontaneous alternation test. The most potent mAPP-siRNA reduced the APP expression in the hippocampal CA2–CA3 subregion and caused a reduction in spontaneous alternation indicating a possible role for APP in spatial working memory.

Materials and methods

Molecular biology (cDNA and siRNA)

The plasmids used for the expression of green fluorescent protein (GFP, in the pLL3.7 backbone) and mouse APP695 (in the pRK5 backbone) were under the control of the cytomegalovirus promoter. The siRNAs specifically targeting the mouse APP transcript were designed using the BIOPRED algorithm as previously described (Huesken *et al.* 2005). The sequences of the mAPP-siRNAs and control siRNAs are shown in Table 1. For cell transfection studies, the siRNA targeting GFP (Caplen *et al.* 2001) was used as a positive control. A scrambled control siRNA (Qiagen, Hombrechtikon, Switzerland) was used as negative control. The siRNAs were chemically synthesized as previously described (Dorn *et al.* 2004). The siRNAs used are 21-mer double-stranded oligonucleotides with a 19-bp oligoribonucleotide region and dinucleotide overhangs located on the 3′ end of each strand consisting of deoxynucleotide residues linked by means of a phosphodiester group. Prior to use, the sense and antisense strands of each siRNA were annealed in an isotonic RNAi buffer (100 mmol/L potassium acetate/30 mmol/L Hepes-KOH/2 mmol/L magnesium acetate/36 mmol/L NaCl, pH 7.4) as described previously (Dorn *et al.* 2004). After annealing, the final siRNA concentration was 66.7 μg/μL.

Table 1 Sequences of the siRNAs used in the mouse APP silencing study

siRNA name	Gene targeted	Sequence
Si1	mAPP	UAAGGAAUCACGAUGUGGGTdG
		TTAUUCCUUAGUGCUACACCC
Si2	mAPP	UAAUUCUCGAGGGCCAGGCdGdG
		TTAUUAAGAGCUCCCGGUCCG
Si3	mAPP	UUUACCACAGAACAUGGCGdAT
		TTAAAUGGUGUCUUGUACCGC
Si4	mAPP	UGUUUGUCAGCCCAGAACCTdG
		TTACAAACAGUCGGGUCUUGG
Si5	mAPP	UUGUGAUCUGCAGUUCAGGdGT
		TTAACACUAGACGUCAAGUCC
Si6	mAPP	AUGGGUCUCACAAACAUCCdAT
		TTUACCCAGAGUGUUUGUAGG
Si7	mAPP	UUGGCCAAGACAUCGUCGGdAdG
		TTAACCGGUUCUGUAGCAGCC
Si8	mAPP	UGCAUUUGCUCAAAGAACUTdG
		TTACGUAAACGAGUUUCUUGA
Si9	mAPP	UACUGCAAGAUGCCCUCCUTdG
		TTAUGACGUUCUACGGGAGGA
Si10	mAPP	UUGGCUUUCUGGAAAUGGGdCdA
		TTAACCGAAAGACCUUUACCC
Si11	mAPP	UUGAGUUUACCACAGAACATdG
		TTAACUCAAAUGGUGUCUUGU
siGFP	GFP	AUGAACUUCAGGGUCAGCUTdG
		TCUACUUGAAGUCCCAGUCGA
Control siRNAs		
MM8	mAPP	UGCAGUUGCGCAACGAACUTdG
	mis-match	TTACGUCAACGCGUUGCUUGA
MM7	mAPP	UUGGACAAGCCAGCGUCGGdAdG
	mis-match	TTAACCUGUUCGGUCGCAGCC
siYFP	YFP	UUGAAGUUCACCUUGAUGCdCdG
		TTAACUUCAAGUGGAACUACG
siCON	Non-targeting	ACGUGACACGUUCGGAGAATT
		TTUGCACUGUGCAAGCCUCUU

The sequences of the antisense strand (upper sequence, 5′–3′ orientation) and of the complementary sense strand (lower sequence, 3′–5′ orientation) are indicated for each siRNA. Two-deoxyribonucleotide overhangs (dA, dG, dC or T) are placed at the 3′ end of each siRNA strand. The abbreviation mAPP stands for mouse APP. Underlined bases indicate mis-match bases harbored by the control siRNAs as compared to the match siRNAs.

Cell culture and transfections

Mouse embryonic fibroblasts NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4500 mg/L glucose, 10% Bovine Calf Serum and 1% penicillin/streptomycin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded 24 h before transfection to be 70–80% confluent on the day of transfection. On the day of transfection, cell culture medium was replaced with OptiMEM® medium (Invitrogen, Basel, Switzerland). Per well, 0.2 μg of siRNA was combined with 0.4 μg of plasmid DNA and transfected using 5 μL of TransMes-

senger reagent according to the manufacturer's instructions (Qiagen). A final siRNA concentration of 25 nmol/L was used in each well of a twelve-well plate unless otherwise indicated. Gene silencing was monitored 48 h after transfection. Cells were washed with ice-cold phosphate-buffered saline (PBS) and collected by scraping. After centrifugation (5 min at 1000 *g*) cell pellets were resuspended in 60 μL of PT × E buffer (PBS with 1% Triton X-100 and 0.1 mmol/L EDTA) containing protease inhibitors (Complete™ Mini Protease Inhibitor Cocktail tablets, Roche Diagnostics, Rotkreuz, Switzerland). Following 30 min of incubation at 4°C to allow lysis, cell lysates were sonicated for 10 s and analyzed for gene silencing using western blotting.

SDS-PAGE and western blotting

Cell or brain lysates were mixed 1 : 1 (vol/vol) with 2×SDS-loading buffer (Tris-HCl 100 mmol/L, dithiothreitol 200 mmol/L, sodium dodecyl sulfate (SDS) 4%, Bromophenol blue 0.2%, Glycerol 20%) supplemented with 2% -mercaptoethanol and the proteins were denatured for 10 min at 90°C. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using 10% or 4–20% gradient PAGEr® Tris-Glycine polyacrylamide Gels (Cambrex Bio Science, Rockland, ME, USA). The proteins were then blotted on a polyvinylidene difluoride Immobilon-P Transfer Membrane (Millipore, Zug, Switzerland) using semi-dry blotting system. All blocking and antibody incubation steps were carried out in Tris Buffered Saline with Tween20 (TBST) buffer (25 mmol/L Tris, 150 mmol/L NaCl, 2.5 mmol/L KCl, and 0.05% Tween 20, adjusted to pH 7.5 by the addition of HCl) containing 5% milk and 1% bovine serum albumin. The blots were blocked for 1 h at 20°C, incubated with the primary antibody overnight at 4°C and after washing in TBST, the blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (dilution 1 : 100 000) for 1 h at 20°C. Immunoblots were then washed in TBST and incubated either with the Supersignal® West Femto Maximum Sensitivity Substrate (Pierce and Kalivas 1997) or the enhanced chemiluminescence ECL detection reagent (Amersham Bioscience, Otelfingen, Switzerland). Blots were developed on chemiluminescence films (Hyperfilm™ High Performance, Amersham Bioscience). The BIO-RAD pre-stained standards (Reinach, Switzerland) were used as molecular weight markers. Protein concentration was determined using the Bradford protein assay (BIO-RAD) with BSA (bovine serum albumin) as standard, following manufacturer's instructions. The primary antibodies and the dilutions used were as follows: anti-GFP mouse monoclonal 1 : 2000 (Sigma-Aldrich, Buchs, Switzerland), anti-APP N-terminal 22C11 mouse monoclonal 1 : 2000 (Chemicon, Lucerne, Switzerland), anti-APP C-terminal C8 rabbit polyclonal antibody raised against residues 676–695 of APP 1 : 10 000 (APPC), and anti-α tubulin mouse monoclonal 1 : 4000 which was used as a loading control (Sigma).

Immunocytochemistry

Cells were grown and transfected on coverslips in 12-well plates and all immunocytochemistry steps were performed at 20°C. Forty-eight hours after transfection, cells were washed in PBS and fixed for 20 min in freshly prepared 4% (w/v) paraformaldehyde in PBS pH 7.4. After washing in PBS, cells were incubated for 5 min in PBS containing 1% Triton X-100 and next washed. All subsequent washing steps were carried out using PBS 0.1% Triton X-100 followed by PBS. Blocking incubation steps were performed in PBS buffer containing 0.1% Triton X-100 and 10% normal goat serum, whereas antibody incubation steps were performed in PBS buffer containing 0.1% Triton X-100 and 1% normal goat serum. Cells were blocked for 1 h, incubated for 90 min with primary antibody (anti-APP C8 antibody 1 : 2000) and after washing, the cells were incubated for a further 1 h with the appropriate secondary antibody (Alexa Fluor® goat anti-rabbit antibody 1/1000). Cells were then washed and mounted inverted onto glass slides using Vectashield® HardSet Mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). For the detection of GFP, coverslips were fixed and mounted and the direct fluorescence was observed under the microscope. Cells were observed using an epi-fluorescence Axioskop 2 plus microscope (Carl Zeiss AG, Feldbach, Switzerland) equipped with an AxioCam HRc high-resolution digital camera (Carl Zeiss AG), and driven by the AxioVision 3.0 software (Carl Zeiss AG) for image acquisition and processing.

In situ hybridization

Fifteen days after surgery mice were anesthetized and decapitated. Brains were then rapidly removed and frozen by immersion in 2-methylbutane at −35°C. Sagittal brain sections of 12 μm were cut on a cryostat (Microm HM560; Microm International, Volketswil, Switzerland), thaw mounted onto poly-L-lysine coated slides (Electron Microscopy Sciences), and kept at −80°C until use. The following 40-mer oligonucleotide probe was used: 5′- TGC GTG GGC CAC CGA GTG CTC CGT GTG CGA GTG GAA GAT C- 3′ complementary to the 5′ untranslated sequence of mouse APP mRNA (Andra *et al.* 1996). *In situ* hybridization experiments were performed as described (Hannon *et al.* 2002). In brief, the probe was labeled at its 3′ end using terminal deoxynucleotidyltransferase (Roche) and [α ^{33}P]dATP (110 TBq/mmol, Hartmann Analytic, Braunschweig, Germany). Frozen brain sections were fixed for 20 min in freshly prepared 4% paraformaldehyde in PBS and rinsed in PBS. After dehydratation in graded ethanol solutions, sections were air-dried. For hybridization, slides were incubated in a humid chamber overnight at 37°C with the ^{33}P labeled APP-specific probe (2×10^5 cpm per slide). Sections were subsequently washed at 55°C and again dehydrated in a graded sequence of ethanol concentrations. The hybridized slides were then exposed to β-max films (Kodak Biomax MR films; Kodak, Renens, Switzerland) for 4–10 days prior to development. For semiquantitative analysis, the optical density was determined using a computerized image analysis system. The expression levels of APP were determined in two sections per brain at the level of the dorsal hippocampus. Cresyl violet staining facilitated the localization of the brain regions analyzed. Specificity of *in situ* hybridization signals was confirmed by competition experiments using excess matching or unrelated non-labeled oligonucleotide probes and by including brain slices derived from APP knockout mice in our *in situ* studies (*data not shown*).

Histology

Brains from wildtype C57BL/6, APP23 transgenic (Boncristiano *et al.* 2002) and APP-null mice (Lorenzo *et al.* 2000) aged 5 months were perfused for 5 min with PBS followed by 15 min perfusion with paraformaldehyde 4% in PBS and post-fixation for 1 week in PFA 4%. Brains were cut 40 μm thick using a Leica VT1000 M vibratome. Coronal sectioning was performed in the

hippocampal region. Slices were collected in 24-well plates containing anti-freeze medium (50 mg Na Methiolate; 392.5 mg NaH_2PO_4; 1362 g Na_2HPO_4; 75 mL Ethylene glycol; and 75 mL Glycerol in a total volume of 250 mL H_20) with three slices per well (160 μm anterio-posterior distance between each slice). Slices were washed 2 × 5 min with PBS and then incubated in PBS-H_2O_2 1% for 10 min to inactivate endogenous residual peroxidase activity. Sections were then permeabilized in PBS-Triton 2% for 10 min then quickly washed in PBS Triton 0.1%, and finally incubated in acidic citrate buffer (0.01 mol/L pH 6) or basic Tris buffer (0.1 mol/L pH 9). Microwave treatment (Sherriff *et al.* 1994a,b; Shi *et al.* 1991) was performed 1 × 5 min plus 1 × 2 min or for 3 × 3 min after which sections were allowed to cool to 20°C. Sections were washed 3 × 10 min in PBS Triton 0.1% and sections were then incubated overnight with primary antibodies using a range of dilutions 6E10 (1 : 1000), 22C11 (1 : 500–1 : 250) and C8 (1 : 1000–1 : 500). The details of the APP primary antibodies used are as follows: 6E10 mouse monoclonal antibody (Signet, antigen epitope spans the first amino acids of human Abeta peptide); 22C11 mouse monoclonal antibody (Chemicon MAB348, antigen epitope is found in the N-terminus of APP, amino acids 66–81); and C8 rabbit polyclonal antibody (last 19 C-term residues of APP). After washing for 3 × 10 min in PBS-Triton 0.1%, the sections were incubated with either donkey anti-mouse or anti-rabbit secondary antibodies (Chemicon) in PBS Triton 0.1% at a final concentration of 1 : 1000. Sections were washed 2 × 10 min in PBS 0.1% Triton, 1 × 10 min in PBS and then the Avidin-Biotin Complex was applied for 1 h at 20°C according to manufacturer's instructions (Avidin Biotin-HSP Complex, Vector Laboratories Inc.). Slices were washed 3 × 10 min in PBS and covered in 200 μL of DAB solution for 30 min at 20°C. Sections were washed 1 × 10 min in PBS, switched to distilled H_2O and mounted on microscopic slides. After dry heating for 1 h sections were Xylol dehydrated (by incubation for 2 × 2 min) and finally mounted using Roti-Histokitt II (Carl Roth, Karlsruhe, Germany).

Animal handling

For the siRNA studies, male C57BL/6JIco mice (10–12 weeks old, Iffa Crédo) were used weighing 24.7 ± 1.3 g at the beginning of the experiments. For the scopolamine studies, male CD-1 mice (20–25 g, Charles River) were used. Mice were housed individually in a temperature controlled room under artificial illumination with a 12 h light/dark cycle (lights on from 06 h00 to 18 h00). Food and water were available *ad libitum*. Animals were allowed to acclimatize to their housing conditions for a minimal period of 1 week before surgery. For the siRNA study, mice were habituated by regular handling, weighed on a daily basis and housed in the experimental room at least 1 day before behavioral tests. Animal handling and experimentation were performed during the light cycle, according to methods approved by the cantonal Veterinary Authority of the city of Basel, Switzerland.

siRNA infusion into mouse brain

Surgery was performed as described previously (Thakker *et al.* 2004). Briefly, mice were injected with Temgesic (0.05 mg/kg of body weight, Essex Chemie AG, Luzern, Switzerland) and anesthetized by intraperitoneal injection of a ketamine–xylazine mixture (100 mg/kg - and 10 mg/kg of body weight, in saline) before surgery. The infusion cannula (PlasticsOne, cut 3 mm below pedestal) was implanted into the dorsal third ventricle; anteroposterior: −0.5 mm, mediolateral: 0 mm, dorsoventral: −3 mm relative to Bregma; according to Paxinos and Franklin's mouse brain atlas (Paxinos and Franklin 2001). Control mice were infused with vehicle (RNAi buffer). The mini-osmotic pumps were implanted subcutaneously and connected to the cannula via a flexible tubular (sterile vinyl catheter tubing, Durect Corp., Cupertino, CA, USA). Pumps were pre-filled in order to infuse 6 μL of siRNA or vehicle per day. The corresponding dose of siRNA delivered was 0.4 mg/day. The dose and duration of infusion were chosen as optimal and well tolerated, based on previous studies (Thakker *et al.* 2004, 2005). The siRNAs were continuously infused into the brain over a 2-week period using this cannula/mini-osmotic pump assembly system (Alzet, model 1002; Durect Corp.).

Locomotor activity

Behavioral studies were performed on days 11 and 12 after surgery. This period was chosen based on a previous studies using similar siRNA infusion protocols (Thakker *et al.* 2004, 2005). Each animal was tested in a blind random manner. Locomotor activity was measured on day 11 after the start of siRNA infusion. The mice were placed singly in one of eight empty transparent activity cages (37 × 22 × 15 cm) under red light and the total number of beam interruptions (three beams per cage) was recorded every 5 min for a total duration of 3 h (from 9 h 30 min to 12 h 30 min).

Y-maze test

Mice were tested for spontaneous alternation and exploratory behavior on day 12 post-surgery in the Y-maze test. The experimental apparatus consisted of a slightly transparent black Y-maze made of Plexiglas. Its arms consisted of three compartments (10 cm×10 cm) connected with 8 cm long and 6 cm wide passages. The 10 cm high Y-maze was covered with a cage grid during the test, and a filter paper placed under the maze was changed after each mouse. Each animal received one trial. The mouse was placed into one compartment (always the same) and was then allowed free exploration of the maze for 5 min. The sequence of arm entries was manually recorded, an arm entry being determined as the four paws within that arm (four-paw criterion). A spontaneous alternation is defined as the entry into all three arms on consecutive choices. The percentage spontaneous alternation was calculated as the ratio of actual to maximum number of alternations. The maximum number of possible alternations is defined as the total number of arm entries minus 2. For example, if an animal made the following sequence of arm choices (3,2,1,3,3,2,1), the maximal number of possible alternations would be 5 (7 minus 2) and the percentage alternation would be 60% (three actual alternations out of 5 possible).

Grip strength

Grip strength tests were performed on day 12 after the Y-maze test. A grip strength meter (Model GS3, Bioseb, Chaville, France) was used to measure the forelimb grip strength of mice. Animals were held by the tail and allowed to place their forepaws on the grid of the pull bar connected to the force sensor. Mice were slowly pulled away until they released the grid, and the maximal grip force measured during the pulling session was recorded. Untrained mice were tested three times without rest following this protocol. The

maximal strength reached by each animal (in g) was recorded and normalized by the body weight (g/g). Mice were also tested in the loaded grid test for their ability to carry small grids of increasing weights (10, 20, 30 and 40 g). Mice were allowed to grip the grid with their four paws and were subsequently lifted by the tail. Mice were considered to succeed in this test when they were able to carry the grid for 10 s.

Statistical analysis

All results were expressed as mean ± SEM (standard error of the mean). Statistical calculations were performed with the software Sigmastat (Systat Softwares Inc., Erkrath, Germany). For multi-group comparisons, data were analyzed for statistical significance using one-way analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis ANOVA on ranks, when appropriate. If there were significant values, *post hoc* Bonferroni's or Dunnett's multiple comparison tests were subsequently used. For pairwise group comparisons, statistical significance was determined using Student's *t*-test or the non-parametric Mann–Whitney rank sum test when appropriate. The criterion for statistical significance was $p < 0.05$ in all evaluations.

Results

mAPP-siRNAs cause knockdown of amyloid precursor protein levels in transfected cells

In order to identify APP-targeting siRNAs, eleven different siRNA duplexes targeting mouse APP were designed together with controls (Table 1). Knockdown efficacy of the siRNAs was tested using a plasmid-siRNA co-transfection assay. Briefly, the different siRNAs were co-transfected with an APP-expressing construct in NIH-3T3 cells and the resulting APP expression levels were measured. The assay was first validated with green fluorescent protein (GFP) by co-transfecting a GFP-expression plasmid with an *in vitro* validated siRNA against GFP, or with a non-targeting siRNA as a negative control. Resulting GFP expression levels were then measured by western blot and immunocytochemistry (Fig. 1a). As expected, efficient knockdown of GFP was found using the GFP-siRNA, but not a negative control siRNA. From the eleven APP-siRNAs tested in the same assay, two different active siRNAs were selected, namely si7 and si8, that reproducibly induced knockdown of APP protein expression as compared to negative control siRNAs (Fig. 1b).

Control mis-match siRNAs show no effect on amyloid precursor protein levels in transfected cells

Inactive control siRNAs corresponding to the active mAPP-siRNAs si7 and si8 were subsequently generated. Three mis-matches were introduced at positions 5, 10 and 13 of si7 to generate MM7, and at positions 5, 10 and 14 of si8 to generate MM8 (Table 1). In contrast to the active APP-siRNAs (si7 and si8), the corresponding mis-match siRNAs (MM7 and MM8) showed negligible knockdown of APP protein levels

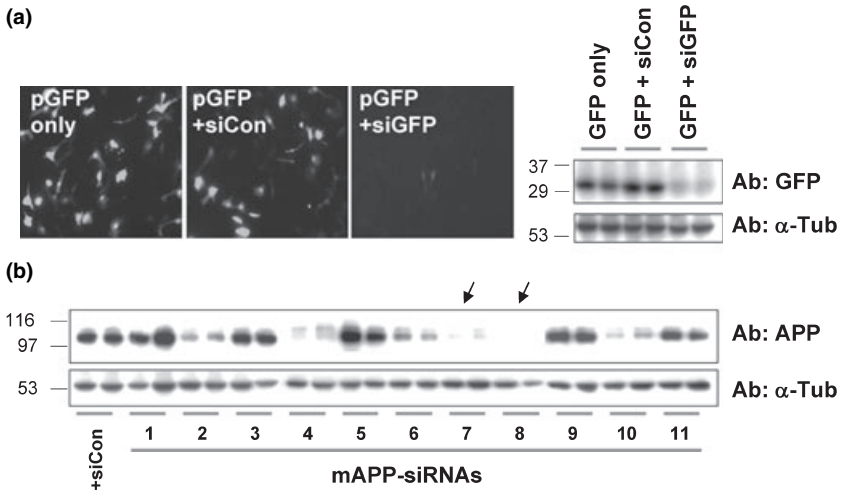

Fig. 1 Identification of active siRNAs targeting mouse APP. (a) Validation of the plasmid-siRNA co-transfection assay. GFP overexpressing plasmid was transfected alone (pGFP only), together with a negative control siRNA (si-Con), or with a previously validated GFP-siRNA si-GFP (Caplen *et al.* 2001), in NIH-3T3 cells at 25 nmol/L. Forty-eight hours later, cells were either directly observed under the microscope for GFP-fluorescence (left panel, same number of cells observed in each case as determined by DAPI staining), or harvested and analyzed by western blot for GFP expression-levels using the anti-GFP antibody (right panel, experiment performed in duplicate for each condition). The blot was additionally probed for α-tubulin (α-Tub), used as a loading control. (b) Evaluation of siRNAs targeting mouse APP. APP-siRNAs (numbered from 1 to 11) were transfected together with a mouse APP695 mammalian expression plasmid in NIH-3T3 cells at 25 nmol/L. Forty-eight hours later cells were harvested and APP expression levels were monitored by western blot using the 22C11 N-terminal antibody (experiment performed in duplicate for each condition). As a negative control an unrelated siRNA was also used (si-Con). The blot was additionally probed for α-tubulin (α-Tub) as a loading control. Western blot results are representative of two independent experiments. Black arrows indicate the most active APP-siRNAs identified in this assay (si7 and si8).

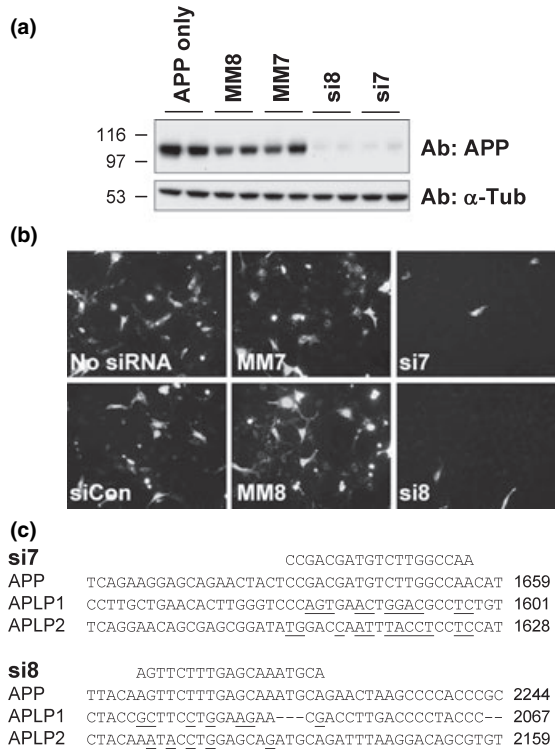

Fig. 2 Characterization of control mis-match mAPP-siRNAs. (a) Validation of control mis-match siRNAs. Mouse APP overexpressing plasmid was transfected in NIH-3T3 cells alone (APP only) or in combination with active siRNAs targeting mouse APP (si8 and si7), or in combination with corresponding mis-match controls (MM7 and MM8). Cell lysates were subsequently probed for APP by western blot using the 22C11 antibody. Each transfection was performed in duplicate. α-tubulin (α-Tub) was used as loading control. The western blot shown is representative of three independent experiments. Each condition was tested in duplicate. (b) Immunostaining was used to monitor APP-expression levels in transfected NIH-3T3 cells. APPC anti C-terminal APP antibody was used to detect APP, and secondary detection was performed with an Alexa488 fluorescent secondary antibody. Representative pictures are shown. Presence of equal number of cells on the observed field was verified by DAPI staining of cell nuclei. Results confirm that match siRNAs against APP (si7 and si8), but not inactive mis-match versions (MM7 and MM8) or control unrelated siRNA (siCon), resulted in knockdown of APP expression. (c) Overlap of si7 and si8 with mouse APP, APLP1 and APLP2. Sequence mis-matches are underlined. There were 11 and 12 mis-matches between APLP1 and APLP2 with si7 respectively and 7 and 5 mis-matches between APLP1 and APLP2 with si8 respectively. Accession numbers were as follows: BC070409 for APP, BC052396 for APLP2 and BC021877 for APLP1.

in the co-transfection assay (Fig 2a). In immunocytochemistry studies, a reduction in APP expression levels was found at the cellular level in the presence of the active mAPP-siRNAs (si7 and si8), but not in the presence of mis-match siRNAs (MM7 and MM8) (Fig. 2b). The inability of MM7 and MM8 to reduce APP levels showed sequence-specificity of APP-knockdown by si7 and si8. Further sequence specificity of si7 and si8 was examined with APP family members by alignment of sequences against Amyloid Precursor Like Protein (APLP)1 and APLP2. A poor overlap occurred (5–12 mis-matches) between si7 or si8 when aligned against APLP1 and APLP2, indicating a negligible likelihood that these genes are silenced by si7 or si8 (Fig. 2c).

APP-knockdown by si7 and si8 is concentration-dependent

To further determine the specificity and concentration-dependent efficacy of the mAPP-siRNAs, decreasing concentrations of siRNAs were tested in the co-transfection assay, and resulting APP expression levels were measured by western blot (Fig. 3a) and quantified by densitometric analysis (Fig. 3b). At a concentration of 25 nmol/L, si8 caused approximately 90% reduction in APP expression levels whereas si7 induced approximately 70% reduction. Both siRNAs were active at concentrations as low as 0.25 nmol/L (60% reduction for si8, and 40% for si7). Results showed reproducibly more efficacious APP knockdown activity of si8 compared to si7 at all concentrations tested. Large batch quantities of si8, si7 and corresponding mis-match controls were next synthesized for *in vivo* siRNA studies.

Infusion of active mAPP-siRNAs *in vivo* reduces APP mRNA levels in the hippocampus

Next, the *in vitro*-validated siRNAs were continuously infused *in vivo* for 2 weeks into the third ventricle of adult wild type mice using a previously described minipump-infusion method (Thakker *et al.* 2004). To investigate whether the active siRNAs reduced APP mRNA levels in the mouse brain, *in situ* hybridization was performed on brain sections from siRNA- and vehicle-infused mice using an oligonucleotide probe specific for mouse APP (Fig. 4a). Vehicle-control brains showed high levels of APP mRNA throughout the pyramidal cell layers of CA1–CA3, the granule cell layers of the dentate gyrus (DG), and layers of the cerebral cortex, as reported previously in untreated wild type mice (Sola *et al.* 1993). The active APP-siRNA si8 significantly reduced APP mRNA levels specifically in the CA2 to CA3 region of the hippocampus (30% knockdown compared to the vehicle-treated group, $p < 0.05$, two-tailed Dunnett's test following significant ANOVA) (Fig. 4b). The less active siRNA si7 showed a trend-reduction in APP mRNA levels in the CA2 to CA3 region (13% knockdown, $p = 0.091$) however these effects were not found to be statistically significant despite using two separate sections per brain and 5–6 individual brains. No significant reductions in APP mRNA levels were found when analyzing the entire cortex (average OD of the whole cortex of the saggital sections) or in the CA1 region of the hippocampus with any of the siRNA tested. Furthermore, the non-active mis-match controls MM7 and MM8 showed no effects on APP mRNA levels compared to vehicle in all brain regions analyzed (Fig. 4b).

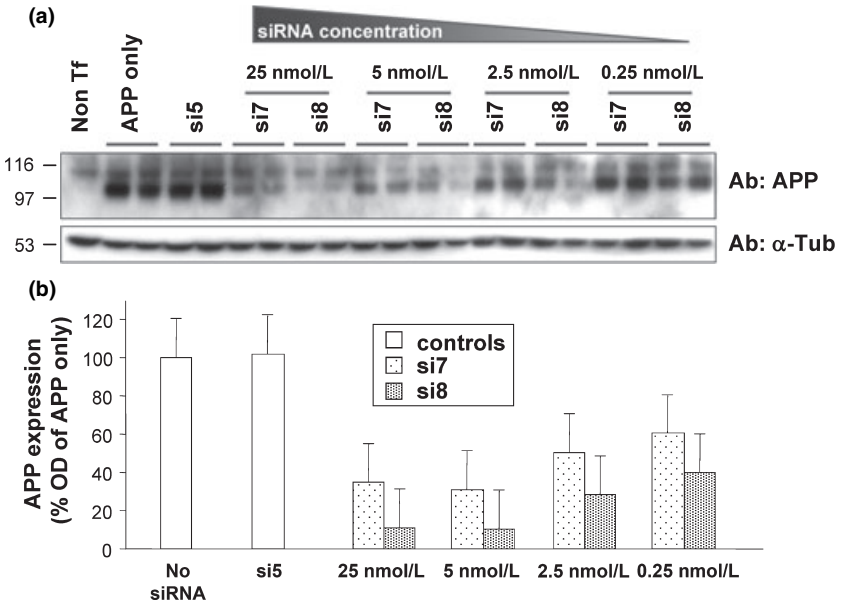

Fig. 3 Rank order of potency for active mAPP-siRNAs. (a) Active siRNAs targeting APP (si7 and si8) to be used *in vivo* were first tested in a concentration-response assay. APP-overexpressing plasmid was transfected alone (APP only lane) or with an inactive APP-siRNA (si5) or decreasing concentrations of the active siRNAs si7 and si8 (25, 5, 2.5 and 0.25 nmol/L) in NIH-3T3 cells. APP expression levels were then analyzed by western blot using the 22C11 antibody. Each condition was tested in duplicate. Lysate of non-transfected NIH-3T3 cells was also probed for APP in the same western blot. α-tubulin (α-Tub) was used as a loading control. (b) Densitometric analysis of APP expression levels. APP expression levels were quantified from bands of the western blot, normalized using the corresponding tubulin levels, and expressed as percentage of the APP levels obtained without siRNA transfection ('no siRNA' condition). Percentages are expressed as mean ± SD.

Infusion of active APP-siRNAs *in vivo* reduces amyloid precursor protein levels in the hippocampus

For efficient immunodetection of their target antigen, some antibodies require a preliminary antigen retrieval step using unmasking treatments like microwave heating in a defined buffer or protease digestion (see Sherriff *et al.* 1994a,b). We aimed to measure the knockdown of APP at the protein level using immunohistochemistry, however were unable to do so most likely due to technical reasons involving antigen retrieval of APP. In agreement with previous studies reporting weak immunoreactivity for APP under normal conditions (Sherriff *et al.* 1994a,b) and the use of APP as a marker of axonal damage (Cuthill *et al.* 2006), we found little or no specific APP immunohistological signal in wild type mice compared to APP-null mice despite testing a range of antigen-retrieval methods and harsh unmasking protocols that often lead to poor tissue preparations (Fig. 5a). Since we were able to detect APP staining in APP23 transgenic animals (Boncristiano *et al.* 2002) the lack of APP staining found in wild type mice is likely to be due to differences in expression levels (APP23 transgenic mice express sevenfold higher amounts of APP; Boncristiano *et al.* 2002), the different antibodies used and/or due to the differential masking of APP antibody epitopes (APP23 transgenic mice express human APP Swedish mutant). Thus to measure whether the most active APP-siRNA si8 was able to reduce APP protein levels in the mouse brain, western blot analysis was instead performed on hippocampal punctures from siRNA- and vehicle-infused mouse brains (Fig. 5b). Results show a reduction in APP expression with si8 compared to vehicle and mis-match control. APP protein levels were further quantified by densitometric analysis of bands (Fig. 5c). The active APP-siRNA si8 showed a trend towards reduced APP protein levels by 31% compared to vehicle (Fig. 5c).

Active mAPP-siRNAs show no effects on grip strength or locomotor activity

Before *in situ* analysis, behavioral tests were carried out from day 11 after implantation of the minipump/cannula assembly: locomotor activity (day 11), grip strength (day 12), and Y-maze (day 12). These tests were chosen based on previous observations made using APP KO mice, APP-antisense-treated animals, and APP antibody-infused animals (see Senechal *et al.* 2006).

No significant differences in weight were observed between active and inactive APP siRNA- and vehicle-treated animals (*data not shown*). Mice were examined for grip strength deficits using two different tests: forelimb grip strength measured by a digital grip strength meter and general grip strength evaluated by the ability to carry small grids of

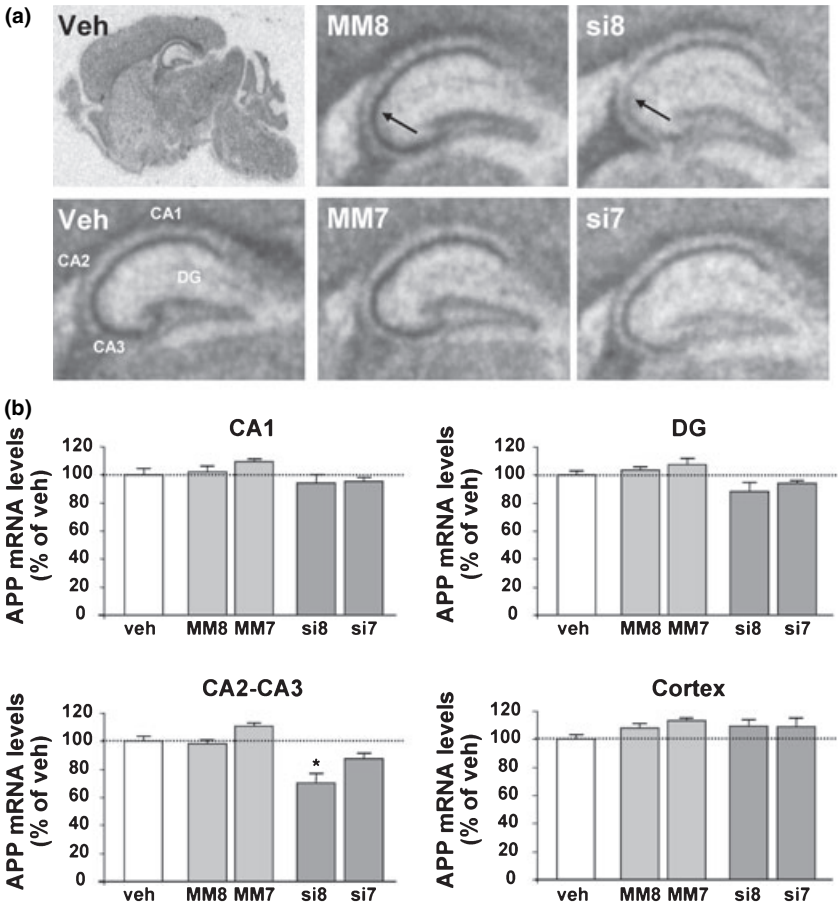

Fig. 4 APP-siRNAs reduce APP mRNA levels *in vivo* in the hippocampus. (a) *In situ* hybridization analysis of APP expression levels in the brain following acute APP-siRNA infusion. Mice were infused for 2 weeks with synthetic siRNAs targeting mouse APP (si7 $n = 5$ and si8 $n = 6$), or corresponding control inactive mis-match siRNAs (MM7 $n = 5$ and MM8 $n = 6$), or vehicle (veh $n = 5$). Brains were sagittally sectioned (1.08 mm lateral to the sagittal suture) and analyzed by *in situ* hybridization for quantification of APP mRNA levels. The resulting film autoradiograms show APP knockdown in the hippocampal region (indicated by black arrows). (b) Quantification of the densities in different brain regions. APP mRNA expression levels in the dentate gyrus (DG), in the CA1 and CA2 to CA3 fields of the hippocampus, and in the cerebral cortex are presented in the bar chart, expressed as a percentage of optical densities obtained in the vehicle-treated group. * $p < 0.05$ (two-tailed Dunnett's test following significant ANOVA, comparison vs. control vehicle group). All quantitative data are expressed as mean ± SEM.

increasing weight (loaded grid test, *data not shown*). In both tests APP-silenced mice did not exhibit reduced forelimb grip strength as compared to vehicle- or inactive siRNA-infused mice, indicating that neuromuscular function was not measurably impaired (Fig. 6a). The locomotor activity was measured as the total number of beam interruptions in successive 5 min intervals for a total of 3 h. APP-knockdown showed no effects on spontaneous locomotor activity behavior (Fig. 6b).

APP-knockdown impairs spontaneous alternation behavior

Spontaneous alternation in the Y-maze test is regarded as a measure of spatial working memory. This behavioral test was first validated by confirming the well documented effects of scopolamine (Sarter *et al.* 1988), a muscarinic antagonist that impairs memory in numerous cognitive paradigms (Fornari *et al.* 2000). Wild type mice ($n = 10$ per group) received i.p. administration of scopolamine hydrobromide (1 mg/kg) or vehicle (sterile saline), and were tested 30 min later in the Y-maze. As previously reported (Anisman 1975), scopolamine-treated animals alternated significantly less (55 ± 2%, $p < 0.01$, Mann-Whitney) than vehicle-treated controls (70 ± 5%, Fig. 7a). Furthermore, scopolamine-treated mice showed an increased number of arm entries (48 ± 3, $p < 0.001$, Mann-Whitney) as compared to vehicle control mice (31 ± 2).

Spontaneous alternation behavior of mAPP-siRNA infused mice was next tested in the Y-maze (Fig. 7b). Control mis-match siRNA-treatment showed no significant effects on alternation rates (MM8 65 ± 3, MM7 64 ± 4%), as compared to vehicle-treatment (veh 68 ± 1%). These alternation rates are consistent with previously reported spontaneous alternation rates in C57BL/6 mice (Sarter *et al.* 1988; Gerlai 2001). In contrast, mice infused with the most potent APP-siRNA si8 exhibited a significant reduction in their alternation rates (55 ± 6%, $p < 0.05$, two-tailed Dunnett's test following significant ANOVA). Infusion of the less potent si7 had no effect on alternation rates (63 ± 5%), consistent with its limited ability to knockdown APP mRNA level in the brain (see Fig. 4). APP siRNA-treated mice exhibited no significant change in the number of arm entries as compared to vehicle- and control siRNA-treated mice indicating similar motivation states and total activity in all animals (Fig. 7b).

Discussion

The physiological functions of APP and its contribution to AD pathogenesis still remain unclear. In order to further

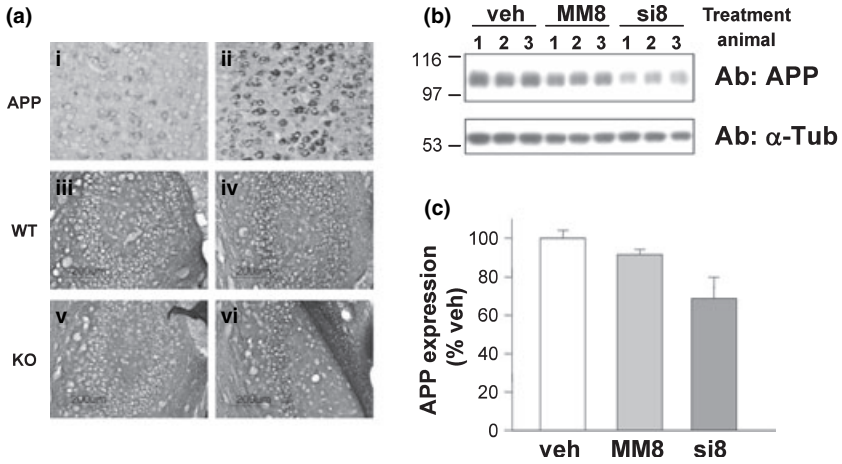

Fig. 5 APP-siRNA reduce APP protein levels *in vivo* in the hippocampus. (a) Different antibodies and microwave heating pre-treatment were tested both in an acidic citrate buffer (0.01 mol/L pH6) and a basic Tris buffer (0.1 mol/L pH 9). APP23 transgenic brain sections were used as positive control, where strong staining of neurons was detected. Brain sections from APP-null mice were used as negative controls and as expected no specific signal was detected. Microwave heating pre-treatment gave better immunostaining of APP23 transgenic mouse brain sections. APP23 transgenic mouse brain sections were immunostained with 6E10 antibody after no unmasking (i) and acidic unmasking (ii). Wildtype mouse brain sections were pre-treated with basic (*not shown*) or citrate buffer and immunostained with 22C11 (iii) and APPC (iv) antibodies. APP-null mouse brain sections were pre-treated with basic (*not shown*) or citrate buffer and immunostained with 22C11 (v) and APPC (vi) antibodies. While APP staining was seen in APP transgenic mouse brain sections, no selective APP immunostaining was detected in sections from wildtype animals when compared to APP null mice. (b). APP protein expression in the hippocampus of APP-siRNA infused mice. Mice were infused for 2 weeks with synthetic siRNAs targeting mouse APP (si8), or its corresponding control inactive mismatch siRNA (MM8), or vehicle (veh). Mechanical extraction of a hippocampal region was performed using a circular-shaped puncture tool of 2 mm diameter from a ~1 mm thick saggital section. After extraction the hippocampal punctures were homogenized and analyzed by western blot using the APPC antibody. The α-tubulin (α-Tub) antibody was used as normalization marker. Hippocampi from 3 different animals are shown for each treatment. (c) Quantification of the band densities obtained in the western blot. Densitometric analysis of the blot was performed to quantify APP protein levels. APP expression levels were normalized using the corresponding tubulin levels, and were expressed as percentage of APP-expression levels obtained in the vehicle group, as mean ± SEM (veh $n = 4$, MM8 $n = 3$, si8 $n = 5$).

investigate the role of APP *in vivo* and to overcome the potential compensation effects associated with gene KO technologies, in the present study a siRNA-based approach was used to acutely reduce APP expression in the adult mouse brain. C57BL/6 mice were chosen for the APP siRNA-behavioral studies since this mouse strain is considered to be a well accepted mouse model for investigating hippocampus-dependent learning and memory (Schimanski and Nguyen 2004). Eleven *in silico*-designed mAPP-siRNAs were tested for APP knockdown efficacy using a plasmid/siRNA co-transfection assay. The mAPP-siRNA induced different levels of APP-silencing, confirming that the identification of biologically effective siRNAs is still an empirical process, as previously reported (Dykxhoorn *et al.* 2003). The exact rules governing siRNA silencing efficiencies are not yet fully understood and additional factors beyond sequence information such as the secondary structure of the target mRNA may also influence siRNA-efficiency (Miyagishi and Taira 2005).

From the initial screen, two active siRNAs (si7 and si8) and their corresponding inactive mis-match controls were identified and characterized, where si8 was found to be more potent than si7 *in vitro*. In agreement, a 2 week infusion of these siRNAs resulted in higher *in vivo* silencing-efficacy by si8 compared to si7. The lack of significant behavioral effects of si7 compared to si8 are consistent with its lower APP-knockdown efficacy. Behavioral analysis of mice infused with the most active siRNA, si8 revealed no deficits in grip strength and locomotor activity. Importantly, in the Y-maze test, si8 infused mice showed a significant reduction in spontaneous alternation, suggesting a role for APP in spatial working memory.

The mAPP-siRNAs used in this study almost certainly did not cross-silence APLP2 (or APLP1), an APP family member thought to share redundant functions with APP, given that the number of base-mis-matches of si7 and si8 with the APLP2 mRNA sequence were 12 and 5 base-mis-matches, respectively. Furthermore, the compensatory regulation of APP family members has been studied in young APP-KO mice. The analysis of expression levels of APP homologs in young APP-KO mice (by qRT-PCR, Northern blot and *in situ*) did not reveal significant differences (Zheng *et al.* 1995) and no changes in the expression levels of APP family members was observed in single and double KO mice at P0 (Heber *et al.* 2000). The effects observed in our study may therefore be likely accounted for uniquely by the down-regulation of APP.

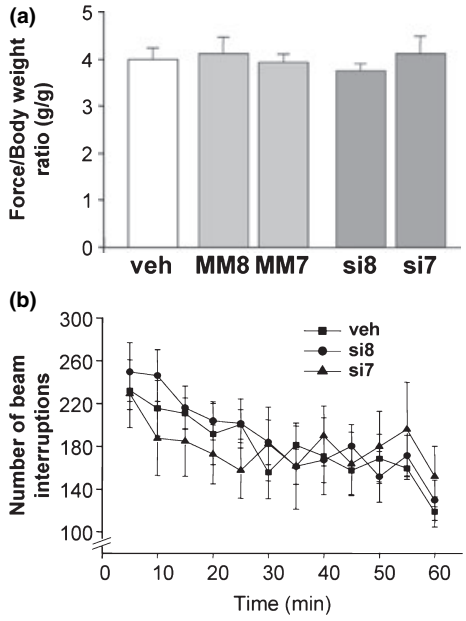

Fig. 6 APP-siRNA-treated mice show no deficits in forelimb grip strength and locomotor activity. (a) APP-silencing does not alter neuromuscular performance. Mice were infused with vehicle (Veh $n = 5$), mis-match control siRNAs (MM8 $n = 6$ and MM7 $n = 5$), or active APP-siRNAs (si8 $n = 6$ and si7 $n = 5$) and tested 12 days later for grip strength. Values are expressed as means ± SEM. Kruskal–Wallis one-way analysis of variance showed no statistical differences between experimental groups. (b) APP-silencing does not alter locomotor activity. On day 11 post-surgery, mice were placed in empty activity cages and the number of infrared beam interruptions were recorded every 5 min for a total period of 3 h. Results are shown for the first 60 min of locomotor activity. Each data point represents the total number of beam interruptions in the preceding 5 min. Values are expressed as means ± SEM. No significant difference was observed with active mAPP-siRNAs as compared to vehicle controls.

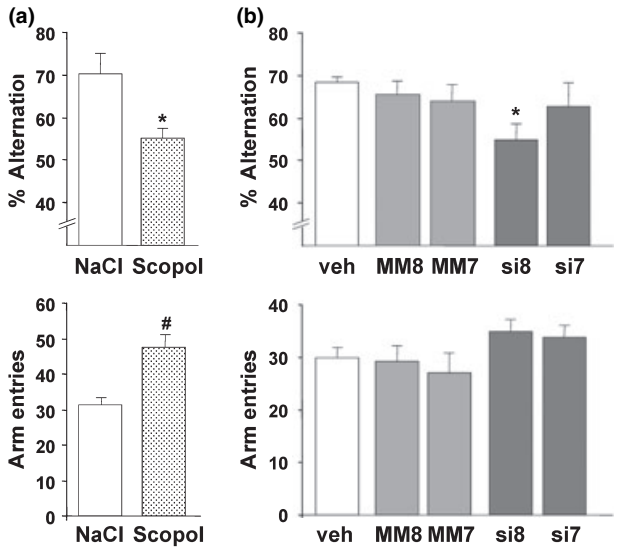

Fig. 7 *In vivo* application of APP-siRNA attenuates spontaneous alternation. (a) Effect of scopolamine on the performance of male CD1 mice in the Y-maze task. Percent spontaneous alternation and total number of arm entries were recorded over a 5 min Y-maze trial. Mice were injected i.p. with vehicle (NaCl group, $n = 10$) or 1 mg/kg scopolamine (scopol group, $n = 10$) and were tested 30 min later in the Y-maze. Scopolamine-treated animals displayed reduced alternation rates and increased number of arm entries. Values are means ± SEM, statistical comparisons are scopolamine versus NaCl treatment, $^{*}p < 0.01$ Mann Whitney, $^{\#}p < 0.001$ Mann-Whitney. (b) Effects of 2 weeks i.c.v. APP-siRNAs infusion on performance of wild type mice in the Y-maze task. The percent spontaneous alternation and total number of arm entries were recorded over a 5-min Y-maze trial. Values are expressed as means ± SEM. Mice were infused with vehicle (Veh $n = 12$), mis-match control siRNAs (MM8 $n = 6$ and MM7 $n = 5$), or active APP-siRNAs (si8 $n = 6$ and si7 $n = 5$). The highly active si8 reduces the rate of spontaneous alternation $^{*}p < 0.05$ (two-tailed Dunnett's test following significant ANOVA, comparison vs. vehicle control group). No significant difference was found in the number of arm entries.

Earlier studies have shown siRNA-induced knockdown to be widespread with greatest effect found in the hippocampal areas close to the site of infusion (Thakker *et al.* 2004, 2005). Consistent with high expression levels in the hippocampus, APP has been suggested to play an important role within this structure, making the siRNA-infusion method relevant for the study of hippocampal APP function. Using the same procedures as this present study, the level of siRNA-induced protein knockdown previously reported has ranged from approximately 30% for the GFP protein in GFP-transgenic mice as quantified by fluorescence microscopy (Thakker *et al.* 2004) to approximately 60% for the endogenous serotonin transporter (SERT) as measured by the density of serotonin transporter-binding sites (Thakker *et al.* 2005). Using a direct injection of siRNA into the rat eye in combination with transfection reagent, a 40–70% reduction in newly synthesized APP in retinal terminals has been reported, with no detectable knockdown of total APP mRNA and protein levels on whole retina (Herard *et al.* 2006). The range of protein knockdown observed across these studies may be accounted by differences in the protocols used for siRNA delivery and knockdown quantification methods and/or differences in the protein expression, turnover and distribution.

In our study, the most potent mAPP-siRNA, si8, caused approximately 30% reduction in APP expression at the mRNA level in the CA2-CA3 region of the hippocampus which translated to a similar degree of knockdown at the protein level. Although we have been unable to determine the level of APP knockdown induced by si7 or si8 using histology due to technical reasons, the comparable reduction in mRNA and protein levels seen with *in situs* and western blotting, respectively is in agreement with the short protein half life of APP; roughly 1–4 h in neurons (Allinquant *et al.* 1995; Lyckman *et al.* 1998). In our study, a higher siRNA-

induced knockdown of APP in the CA2-CA3 region compared to, for example the DG, may be explained by a more efficient uptake of RNAi and/or an enhanced silencing machinery in neurons of the CA2-CA3 region. This idea has been suggested in a recent report where RNAi-mediated silencing produced varying degrees of protein knockdown dependent on the cell-type used (Seibler *et al.* 2005).

In contrast to APP KO mice (Muller *et al.* 1994; Zheng *et al.* 1995), we observed no significant deficits in grip strength as well as no changes in body weight in our APP-silenced mice. The deficits in grip strength reported in KO animals may be due to a key role for APP during development and/or its function in the periphery, for example at the neuromuscular junction. In contrast to full KO mice, heterozygous APP KO mice do not show altered locomotor activity behavior compared to wild type mice in the open-field (Muller *et al.* 1994). This is consistent with our study where a partial knockdown of APP (30% down-regulation) in the mouse brain does not result in significant changes in locomotor activity. Also in agreement with our observations, antibody-mediated blockade of APP in rats does not result in activity changes in open field studies (Doyle *et al.* 1990). Finally, the overall limited behavioral effects of si7 compared to si8 are also consistent with a lower APP-knockdown efficacy, as shown *in vitro* by the concentration-response experiments and *in vivo* by the much lower down-regulation levels reached in the hippocampus compared to si8.

Amyloid precursor protein has been shown to play an important role in memory (for reviews see Dodart *et al.* 2000; Turner *et al.* 2003), as evidenced by the amnesia resulting from APP-antisense and APP-antibody blockade studies using avoidance learning paradigms in rats (Doyle *et al.* 1990; Huber *et al.* 1993) and chicks (Mileusnic *et al.* 2000). In addition, cognitive deficits (Dawson *et al.* 1999) as well as impairment in hippocampal long term potentiation observed in APP KO mice (Dawson *et al.* 1999; Fitzjohn *et al.* 2000) support the notion that APP is involved in memory processes. Furthermore, a direct role for secreted forms of APP in memory enhancing-effects has been demonstrated using various learning tasks involving short-term and long-term memory (Meziane *et al.* 1998). To further investigate the contribution of APP to memory processes, we tested the effects of acute APP knockdown on spontaneous alternation behavior. Importantly, APP-silencing resulted in significantly reduced alternation rates in the Y-maze spontaneous alternation test, suggesting that APP is involved in short term spatial working memory. Since we observed no change in total activity and number of arm entries indicating similar motivation states and found no changes in locomotor activity we believe the changes in spontaneous alternation to be dependent on the hippocampus rather than regulation of centers in the brain that control motor function or motivation. According to the working memory paradigms used by Lee and Kesner (Lee and Kesner 2003), the CA3 and DG regions appear to be required for short term spatial working memory, whereas the CA1 is required for longer term working memory (>5-min delay trials). In our study the CA3 was one of the most affected regions in terms of APP silencing, which is consistent with the reduced spontaneous alternation rates we observed and a putative role for APP in spatial working memory.

Several lines of evidence support an important role for APP, its cleaved products and family members in synaptic maintenance and function. For example, APP-APLP2 double KO mice exhibit defective neuromuscular synapses (Wang *et al.* 2005) and acutely decreasing APP and APLP2 levels at the presynaptic terminal alters synaptic function *in vivo* (Herard *et al.* 2006). The RERMS pentapeptide located within the N-terminal portion of APP has also been identified as a memory-promoting domain *in vivo* (Roch *et al.* 1994). This domain may directly interact with cell surface receptors leading to signal transduction events involved in memory formation. Interaction of APP with extracellular matrix components may also lead to the stabilization of synapses and contribute to synaptic plasticity mechanisms necessary for memory. Further studies using memory paradigms such as the radial arm-maze and the Morris water-maze will additionally allow us to strengthen evidence for the role of APP in spatial memory. In the present study, we postulate that synaptic dysfunction in the hippocampus as a result of silencing APP and/or APP derivatives (such as secreted APP) may account for the deficits observed in the spontaneous alternation test generally considered as a measure of spatial working memory (Sarter *et al.* 1988; Gerlai 2001; Dudchenko 2004).

To our knowledge, this is the first study reporting acute silencing of APP in the adult mouse brain using a siRNA-based approach. Our results demonstrate that moderate knockdown of APP in the adult mouse brain results in significant deficits in spontaneous alternation behavior, as shown in the Y-maze test. We propose that siRNA-based gene silencing constitutes an alternative to using APP-KO mice which may harbor compensatory mechanisms (Senechal *et al.* 2006). Investigating the behavior of APP KO mice in spontaneous alternation tests would determine whether any developmental mechanisms mask the normal contribution of APP. The general use of RNAi is now helping in understanding the function of molecular targets of neurodegenerative diseases and may contribute to the development of new therapeutic strategies.

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