Imaging functional brain connectivity : pharmacological modulation, aging and Alzheimer's disease

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Chapter 6

Imaging cholinergic and serotonergic neurotransmitter networks in Alzheimer's disease in vivo

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ABSTRACT

Disruption of cholinergic and serotonergic neurotransmitter systems is associated with cognitive, emotional and behavioural symptoms of Alzheimer´s disease (AD). To investigate the responsiveness of these systems in AD we measured the effects of a single-dose of the selective serotonin reuptake inhibitor citalopram and acetylcholinesterase inhibitor galantamine in 12 patients with AD and 12 age-matched controls on functional brain connectivity with resting state functional magnetic resonance imaging.

In this randomized, double blind, placebo-controlled crossover study, functional magnetic resonance images were repeatedly obtained before and after dosing, resulting in a dataset of 432 scans. Connectivity maps of ten functional networks were extracted using a dual regression method and drug vs. placebo effects were compared between groups with a multivariate analysis with cerebrospinal fluid, white matter, baseline and heart rate measurements as confound regressors (at $p < 0.05$, corrected).

A galantamine induced difference between groups was observed for the cerebellar network. Connectivity within the cerebellar network and between this network and the thalamus decreased after galantamine vs. placebo in AD patients, but not in elderly controls. For citalopram, voxelwise network connectivity did not show significant group x treatment interaction effects. However, we found default mode network connectivity with the precuneus and posterior cingulate cortex to be increased in AD patients, which could not be detected within the control group. Further, in contrast to the AD patients, elderly subjects showed a consistent reduction in mean connectivity with all networks after administration of citalopram.

Since AD has previously been characterized by reduced connectivity between the default mode network and the precuneus and posterior cingulate cortex, the effects of citalopram on the default mode network suggest a restoring potential of selective serotonin reuptake inhibitors in AD. The results of this study also confirm a change in cerebellar connections in AD, which is possibly related to cholinergic decline.

INTRODUCTION

In Alzheimer's disease (AD), destruction of neural tissue leads to loss of cholinergic nuclei in the basal forebrain and depleted cholinergic innervation towards the cerebral cortex, thalamus and hippocampus [229, 329, 335]. Acetylcholinesterase inhibitors (AChEIs) prevent the breakdown of acetylcholine and are often used as drug treatment to improve the cognitive symptoms of AD [189, 336]. In addition, reduced 5-hydroxytryptamine (5-HT; serotonin) activity plays a role in the cognitive deterioration [337, 338], as well as in behavioural and mood changes that frequently accompany AD [45, 51]. The cholinergic and serotonergic systems act in concert with each other with regard to functions like learning and memory [339-341], further suggesting the involvement of both systems in AD.

Single-dose administration of compounds that inhibit or excite synaptic activity can alter brain connectivity during rest, reflecting the responsiveness of neurotransmitter networks and related functions [77, 79, 311]. This pharmacological 'challenge' technique seems especially relevant for measuring deviant functional processes in AD, which is conceived as a disorder of large-scale network disconnections [18, 19]. Cholinergic network responses that have been studied so far substantiate the assumption that acetylcholine is involved in memory, learning and visual perception [189, 234]. A cholinergic challenge caused increased connectivity in healthy young subjects with regions that are implicated in visual processing, memory and attention [275]. Effects of AChEIs on connectivity in AD patients have only been examined after long-term cholinergic treatment, and show enhanced connectivity of the default mode network (DMN) and the interrelated hippocampus [89-94]. Despite the likelihood of disrupted serotonin transmission, serotonergic modulation of brain connectivity has not yet been studied in AD. Acute or short-term treatment with selective serotonin reuptake inhibitors (SSRIs) elicits reduced connectivity of the DMN and several other cortical and subcortical areas in healthy subjects [82, 83, 85-87, 199] and patients with a major depressive disorder [84].

In this randomized, placebo-controlled, crossover study, we used resting state functional MRI (RS-fMRI) to visualize cholinergic and serotonergic neurotransmitter networks in AD patients and age-matched controls. We hypothesized that single-dose AChEI and SSRI administration changes the functional integrity of neural networks differently in AD patients compared to controls, and that the altered connections would mostly apply to regions that are susceptible for AD related connectivity change such as the hippocampus, thalamus, precuneus and cingulate cortex [20, 25]. The outcomes of this study will provide fundamental knowledge on biochemical pathology in dementia, which might eventually benefit drug development and efficacy in neurodegenerative diseases.

MATERIALS AND METHODS

Included subjects

We included 12 patients with mild AD and 12 gender- and age-matched controls. The clinical diagnosis of probable AD was established according to the revised criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [15], including clinical and neuropsychological assessment. All AD patients participating in this study were recently diagnosed and had mild to moderate cognitive deficits with a Mini Mental State Examination (MMSE) score of at least 18 [274]. Furthermore, they were assessed by a physician (i.e. neurologist, geriatrician) as mentally capable of understanding the implications of study participation. The elderly subjects who served as controls had an MMSE score between 28 and 30 (see Table 6.1 for demographics).

Table 6.1. Demographics of mild AD patients and controls

	AD patients	Controls
n	12	12
Age (Mean ± SD)	74.0 ± 5.2	73.1 ± 5.2
Age range	65-81	64-79
Male/female	6/6	6/6
MMSE (Mean ± SD)	22.3 ± 2.5	29.3 ± 0.9
MMSE range	19-28	28-30
BMI (kg/m^2) range	22-30	22-31

All subjects underwent a thorough medical screening at the Centre for Human Drug Research (CHDR) to investigate whether they met the inclusion and exclusion criteria. They had a normal history of physical health and were able to refrain from using nicotine and caffeine during study days. Exclusion criteria included positive drug or alcohol screen on study days, regular excessive consumption of alcohol (>4 units/day), caffeine (>6 units/day) or cigarettes (>5 cigarettes/day), use of concomitant medication 2 weeks prior to study participation and involvement in an investigational drug trial 3 months prior to administration. The study was approved by the medical ethics committee of the Leiden University Medical Center (LUMC). Written informed consent was obtained from each subject prior to study participation.

Experimental design

This was a single centre, randomized, double blind, placebo-controlled crossover study with citalopram 30 mg and galantamine 8 mg [see 275]. Citalopram has an average time point of

maximum concentration (T_{max}) of 2-4 h, with a half-life ($T_{½}$) of 36 h. For galantamine, T_{max} = 1-2 h and $T_{½}$ = 7-8 h. To correct for the different pharmacokinetic (PK) profiles, citalopram 20 mg was administered at T = 0 h, followed by a second dose of 10 mg at T = 1 h (if the first dose was tolerated). Galantamine was given as a single 8 mg dose at T = 2 h. Blinding was maintained by concomitant administration of double-dummy placebo's at all three time points. All subjects received an unblinded dose of granisetron 2 mg at T = -0.5 h, to prevent the most common drug-induced adverse effects of nausea and vomiting.

Six RS-fMRI scans were acquired during study days, two at baseline and four after administering citalopram, galantamine or placebo (at T = 2.5, 3.5, 4.5 and 6 h) (Figure 6.1). Each scan was followed by performance of computerized cognitive tasks (taken twice at baseline) on the NeuroCart® test battery, for quantifying pharmacological effects on the CNS [167, 204, 205]. By including multiple measurements during the T_{max} interval, this repeated measures profile increases the statistical power of the analysis and allows for identification of time related effects, associated with changing serum concentrations. Nine blood samples were taken during the course of the day to define the PK profile of citalopram, citalopram's active metabolite desmethylcitalopram, galantamine and concentrations of cortisol and prolactin [182, 206]. Washout period between study days was at least 7 days.

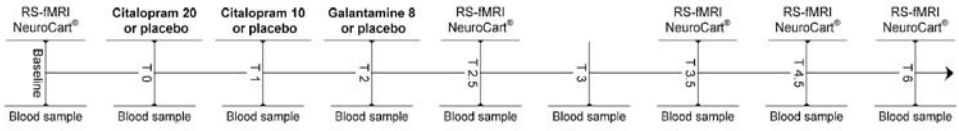

Figure 6.1. Schematic overview of a study day. Each subject received citalopram, galantamine and placebo on three different days. At baseline, two RS-fMRI scans were acquired, followed by the NeuroCart® CNS test battery. After drug administration, four more RS-fMRI scans were acquired at time points T = 2.5, 3.5, 4.5 and 6 h post dosing, each time followed by the NeuroCart® test battery. During the day, nine blood samples were taken to measure the concentrations of citalopram, desmethylcitalopram, galantamine, cortisol and prolactin. On each study day there were three moments of administration. The second administration only took place when subjects tolerated the first dose well (did not vomit or feel too nauseous):

Galantamine study day:	T = 0) placebo	T = 1) placebo	T = 2) galantamine 8 mg
Citalopram study day:	T = 0) citalopram 20 mg	T = 1) citalopram 10 mg	T = 2) placebo
Placebo study day:	T = 0) placebo	T = 1) placebo	T = 2) placebo

Outcome measures

Pharmacokinetics

PK parameters for citalopram, galantamine and citalopram's active metabolite desmethylcitalopram were calculated using a non-compartmental analysis to validate the choice of time points of pharmacodynamic endpoints (RS-fMRI, NeuroCart®, neuroendocrine measures). Blood samples were collected in 4 mL EDTA plasma tubes at baseline and 1, 2, 2.5, 3, 3.5, 4.5 and 6 h post dosing,

centrifuged (2000 *g* for 10 min) and stored at -40°C until analysis with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Neuroendocrine variables

Blood samples were obtained to determine cortisol and prolactin concentrations. Serum samples were tak en in a 3.5 mL gel tube at baseline (twice) and 1, 2, 2.5, 3.5, 4.5 and 6 h post dosing, centrifuged (2000 *g* for 10 min) and stored at -40°C until analysis. Serum concentrations were quantitatively determined with electrochemiluminescence immunoassay.

NeuroCart® test battery

Each RS-fMRI scan was followed by functional CNS measures in a separate room using the computerized NeuroCart® test battery measuring alertness, mood and calmness (Visual Analogue Scales (VAS) Bond & Lader), nausea (VAS Nausea), vigilance and visual motor performance (Adaptive Tracking task), reaction time (Simple Reaction Time task), attention, short-term memory, psychomotor speed, task switching and inhibition (Symbol Digit Substitution Test and Stroop task), working memory (N-back task) and memory imprinting and retrieval (Visual Verbal Learning Test) [95-103]. The Visual Verbal Learning Test was only performed once during each day (at 3 and 4 h post dosing) as the test itself consists of different trials (imprinting and retrieval). Duration of each series of NeuroCart® brain function tests was approximately 20 min. To minimize learning effects, training for the NeuroCart® tasks occurred during the screening visit within 3 weeks prior to the first study day.

MR imaging

Scanning was performed at the LUMC on a Philips 3.0 Tesla Achieva MRI scanner (Philips Medical System, Best, The Netherlands) using a 32-channel head coil. All subjects were asked to close their eyes while staying awake prior to each RS-fMRI session on all study days. T1-weighted anatomical images were acquired once per visit. To facilitate registration to the anatomical image, each RS-fMRI scan was followed by a high-resolution T2*-weighted echo-planar scan.

RS-fMRI data were obtained with T2*-weighted echo-planar imaging (EPI) with the following scan parameters: 220 whole brain volumes, repetition time (TR) = 2180 ms; echo time (TE) = 30 ms; flip angle = 85°; field-of-view (FOV) = 220 x 220 x 130 mm; in-plane voxel resolution = 3.44 x 3.44 mm, slice thickness = 3.44 mm, including 10% interslice gap; acquisition time 8 min. For 3D T1-weighted MRI the following parameters were used: TR = 9.7 ms; TE = 4.6 ms; flip angle = 8°; FOV = 224 x 177 x 168 mm; in-plane voxel resolution = 1.17 x 1.17 mm; slice thickness = 1.2 mm; acquisition time 5 min. Parameters of high-resolution T2*-weighted EPI scans were set to: TR = 2200 ms; TE = 30 ms; flip angle = 80°; FOV = 220 x 220 x 168 mm; in-plane voxel resolution = 1.96 x 1.96 mm; slice thickness = 2.0 mm; acquisition time 30 s.

Statistical analysis

Pharmacokinetics

Maximum plasma concentrations (C_{max}) and time of C_{max} (T_{max}) were obtained directly from the plasma concentration data. The area under the plasma concentration vs. time curve was calculated from time zero to the time of the last quantifiable measured plasma concentration ($AUC_{0\text{-}last}$). To investigate differences between groups, PK parameters were analysed using a mixed effects model with group as fixed effect (SAS for Windows V9.4; SAS Institute, Inc., Cary, NC, USA).

Neuroendocrine variables and NeuroCart® test battery

Treatment (drug vs. placebo) x group (AD patients vs. elderly controls) interaction effects on cortisol and prolactin concentrations and Neurocart® measures were investigated using a mixed effects model with treatment, time, group, visit, treatment by time, treatment by group and treatment by group by time as fixed effects, subject, subject by treatment and subject by time as random effects and the average of the period baseline (pre-dose) values as covariate (SAS for Windows V9.4; SAS Institute, Inc., Cary, NC, USA). The neuroendocrine data and data of the Simple Reaction Time task were not normally distributed and therefore log-transformed before analysis and back transformed after analysis. The data of the Visual Verbal Learning Test were analysed using a mixed effects model with treatment, group, visit and treatment by group as fixed effects and subject as random effect.

MR imaging

All fMRI analyses were performed using the Functional Magnetic Resonance Imaging of the Brain (FMRIB) Software Library (FSL, Oxford, United Kingdom) version 5.0.7 [119-121].

Data preprocessing

Each individual functional EPI image was inspected, brain-extracted and corrected for geometrical displacements due to head movement with linear (affine) image registration [122, 123]. Images were spatially smoothed with a 6 mm full-width half-maximum Gaussian kernel. Registration parameters for non-smoothed data were estimated to transform fMRI scans into standard space and co-registered with the brain extracted high resolution T2*-weighted EPI scans (with 6 degrees of freedom) and T1 weighted images (using the Boundary-Based-Registration method) [124]. The T1-weighted scans were non-linearly registered to the MNI 152 standard space (the Montreal Neurological Institute, Montreal, QC, Canada) using FMRIB's Non-linear Image Registration Tool. Registration parameters were estimated on non-smoothed data to transform fMRI scans into standard space after Automatic Removal of Motion Artifacts based on Independent Component Analysis (ICA-AROMA vs0.3-beta). ICA-AROMA attempts to identify and remove motion related noise components by investigating its temporal and spatial properties. As recommended, high

pass temporal filtering (with a high pass filter of 150 s) was applied after denoising the fMRI data with ICA-AROMA [207, 208].

Estimation of network connectivity

RS-fMRI networks were extracted from each individual denoised RS-fMRI dataset (24 subjects x 3 days x 6 scans = 432 datasets) with a dual regression analysis [36, 125] based on 10 predefined standard network templates [199, 275]. These standard templates have been identified using a data-driven approach [10] and comprise the following networks: three visual networks (consisting of medial, occipital pole, and lateral visual areas), default mode network, cerebellar network, sensorimotor network, auditory network, executive control network and left and right frontoparietal networks. Time series of white matter, measured from the centre of the corpus callosum, and CSF, measured from the centre of the lateral ventricles, were added as confound regressors in this analysis to account for non-neuronal signal fluctuations [126].

With the dual regression method, spatial maps representing voxel-to-network connectivity were estimated for each dataset separately in two stages and used for higher level analysis. First, the weighted network maps were used in a spatial regression into each dataset. This stage generated 12 time series per dataset that describe the average temporal course of signal fluctuations of the 10 networks plus 2 confound regressors (CSF and white matter). Next, these time series were entered in a temporal regression into the same dataset, resulting in a spatial map per network per dataset with regression coefficients referring to the weight of each voxel being associated with the characteristic signal change of a specific network. The higher the value of the coefficient, the stronger the connectivity of this voxel with a given network.

For an overall impression of connectivity alterations during study days, mean *z*-values of these regression coefficients within networks were calculated for each group and study day separately. By comparing the average of the four post measurements with the average of the two baseline measurements it was semi-quantitatively inspected how the average connectivity within each network changed (increased vs. decreased) during study days. Fisher's exact test was applied to investigate differences between groups in the number of networks with a specific direction of this global connectivity change.

Higher level analysis

Local group x treatment interaction effects of citalopram and galantamine were investigated with non-parametric combination (NPC) as provided by FSL's Permutation Analysis for Linear Models tool (PALM vs94-alpha) [129, 209, 210]. NPC is a multivariate method that offers the possibility to combine data of separate, possibly non-independent tests, such as our multiple time points, and investigate the presence of joint effects across time points, in a test that has fewer assumptions and is more powerful than repeated-measurements ANOVA or multivariate ANOVA (MANOVA).

First, tests were performed for each time point using 1000 synchronized permutations, followed by the fit of a generalized Pareto distribution to the tail of the approximation distribution, thus refining the *p*-values at the tail further than otherwise possible with a small number of permutations [318]. More specifically, to investigate group x treatment interaction effects on voxelwise functional connectivity with each of the 10 functional networks, four two-sample t-tests (AD patients: drug - placebo vs. controls: drug - placebo) were performed for all post-dose time points (T = 2.5, 3.5, 4.5 and 6 h), with average heart rate (beats/m) per RS-fMRI scan as confound regressor [127]. The average of the two baseline RS-fMRI scans was used as covariate as well, by adding the coefficient spatial map as a voxel-dependent regressor in the model. This will control for the confounding influence of possibly systematic individual differences and group differences at baseline level as recently analysed and described in Klaassens et al. [319]. The same method was applied for additional investigation of treatment effects (drug vs. placebo) on the DMN within the group of AD patients and within the control group as was previously done for a group of young adults [275]. To that end, four one-sample t-tests (drug vs. placebo) were performed for all post-dose time points (T = 2.5, 3.5, 4.5 and 6 h), with average heart rate (beats/m) per RS-fMRI scan as confound regressor.

Second, to analyse effects across time, the tests for the four time points were combined non-parametrically via NPC using Fisher's combining function [211] and the same set of synchronized permutations as mentioned above. A liberal mask was used to investigate voxels within the MNI template, excluding voxels belonging to CSF. Threshold-free cluster enhancement was applied to the tests at each time point and after the combination, and the resulting voxelwise statistical maps were corrected for the familywise error rate using the distribution of the maximum statistic [128, 129]. Voxels were considered significant at $p < 0.05$, corrected.

RESULTS

Pharmacokinetics

PK parameters (T_{max}, C_{max} and $AUC_{0\text{-}last}$) in AD patients and elderly controls are summarized in Table 6.2. There were no PK differences between AD patients and controls. Figure 6.2 shows the individual and median citalopram and galantamine PK time profiles.

Neuroendocrine variables and NeuroCart® test battery

There were no significant group x treatment interaction effects of citalopram and galantamine on cortisol and prolactin. See Supplementary Figure S6.1 for cortisol and prolactin levels in AD patients and controls. For an overview of all NeuroCart® results, we refer the reader to Supplementary Table S6.1. No significant group x treatment interaction effects were observed for citalopram or galantamine.

Table 6.2. Pharmacokinetics of citalopram, desmethylcitalopram and galantamine in young and older adults

	Citalopram			Desmethylcitalopram			Galantamine		
	Mean ± SD		Contrasts (*p*-value)	Mean ± SD		Contrasts (*p*-value)	Mean ± SD		Contrasts (*p*-value)
PK parameters	AD patients	Controls		AD patients	Controls		AD patients	Controls	
T_{max}	3.6 ± 1.2	3.4 ± 1.1	0.527	4.3 ± 1.4	4.0 ± 1.3	0.491	5.0 ± 0.9	4.5 ± 1.1	0.306
C_{max}	38.8 ± 4.5	41.8 ± 11.7	0.147	3.0 ± 1.3	3.5 ± 1.8	0.395	36.4 ± 8.0	41.8 ± 12.2	0.324
$AUC_{0\text{-last}}$	153.0 ± 19.0	165.0 ± 43.6	0.150	11.1 ± 5.3	13.3 ± 7.1	0.366	84.7 ± 35.7	104.0 ± 40.2	0.151

Abbreviations: AD = Alzheimer's disease; PK = pharmacokinetic; T_{max} = time point (h) of maximum concentration; C_{max} = maximum concentration (ng/mL); $AUC_{0\text{-last}}$ = area under the plasma concentration vs. time curve (ng*h/mL).

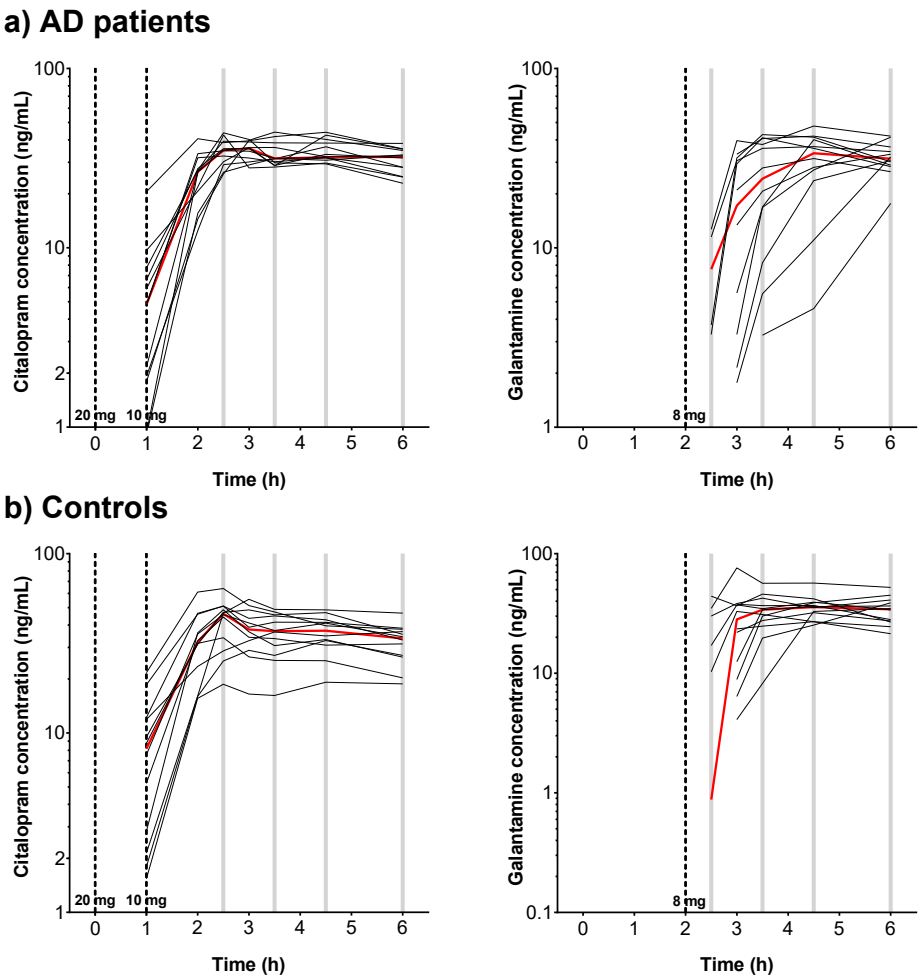

Figure 6.2. Pharmacokinetic profiles. Median (red line) and individual (black lines) PK profiles for citalopram (left) and galantamine (right) concentrations in AD patients (a) and controls (b). Vertical bars illustrate the timing of RS-fMRI acquisition post drug administration. Observations below limit of quantification were dismissed.

Imaging

Global connectivity changes

Calculations of the pre and post treatment average connectivity (mean *z*-values) per network, group and treatment are summarized in Table 6.3. Delta scores show that on placebo days connectivity reduced from pre to post measurement for 6 of the 10 networks in patients with AD and for 4 of the 10 networks in elderly controls. Fisher's exact test did not lead to a significant difference in prevalence in number of networks that showed a decrease in average connectivity (6/10 vs. 4/10).

Table 6.3. Mean *z*-scores within networks per group, per treatment, pre (average of 2 baseline measurements) and post (average of 4 measurements) drug administration, and delta scores of the difference between pre and post measurements

Group	AD patients									Controls								
Treatment	Placebo			Citalopram			Galantamine			Placebo			Citalopram			Galantamine		
Measurement	pre	post	Δ	pre	post	Δ	pre	post	Δ	pre	post	Δ	pre	post	Δ	pre	post	Δ
Visual network (medial)	7.13	7.07	-0.06	6.78	7.27	0.49	6.06	6.38	0.32	4.58	5.71	1.13	5.64	4.92	-0.72	4.97	4.32	-0.65
Visual network (occipital)	5.18	5.40	0.22	4.82	5.56	0.74	4.77	4.72	-0.05	3.69	4.25	0.56	4.50	4.35	-0.15	4.03	3.74	-0.29
Visual network (lateral)	4.64	4.81	0.17	4.57	4.96	0.39	4.32	4.16	-0.16	3.82	4.13	0.31	4.57	4.14	-0.43	3.51	3.91	0.4
Default mode network	6.66	5.91	-0.75	6.42	6.55	0.13	6.76	6.20	-0.56	6.82	6.59	-0.23	6.98	6.70	-0.28	6.93	6.29	-0.64
Cerebellar network	3.55	3.94	0.39	3.01	3.61	0.6	3.11	2.85	-0.26	3.50	2.87	-0.63	2.83	2.78	-0.05	2.69	3.03	0.34
Sensorimotor network	4.23	4.71	0.48	4.68	4.46	-0.22	4.01	4.32	0.31	3.92	4.04	0.12	4.56	3.54	-1.02	3.72	3.69	-0.03
Auditory network	4.60	4.34	-0.26	4.27	4.29	0.02	4.36	4.24	-0.12	4.20	4.27	0.07	4.75	4.07	-0.68	4.28	4.01	-0.27
Executive control network	4.61	4.09	-0.52	4.42	3.80	-0.62	3.63	4.06	0.43	3.74	4.11	0.37	3.98	3.50	-0.48	3.67	3.96	0.29
Frontoparietal network right	4.55	4.38	-0.17	4.59	4.27	-0.32	4.62	3.96	-0.66	4.83	4.29	-0.54	4.82	4.61	-0.21	5.17	4.48	-0.69
Frontoparietal network left	4.94	4.18	-0.76	4.60	4.35	-0.25	4.78	4.57	-0.21	5.12	4.87	-0.25	5.45	5.21	-0.24	5.60	5.15	-0.45

Table 6.3 also presents the pre-post changes in global connectivity during treatment days. The diurnal patterns of network alterations after galantamine administration were similar between groups as well. The prevalence in number of networks that showed a decrease in connectivity in elderly controls (3/10) vs. patients with AD (7/10) did not lead to a significant difference.

In contrast to placebo and galantamine study days, group differences were observed during citalopram occasions. After citalopram administration, reduced connectivity was consistently observed for all 10 networks in elderly controls, but only in 4 out of 10 networks in patients with AD. A prevalence of 10/10 vs. 4/10 networks that showed a decrease in connectivity was tested significant ($p < 0.05$).

Local differences in drug effects between AD patients and controls

A significant group x treatment interaction effect of galantamine was found for connectivity within the cerebellar network (see Table 6.4 for specifications and extent of significant effects). In AD patients, galantamine induced a decrease in connectivity of the cerebellar network with the cerebellum, thalamus and brain stem (interaction and main effects are shown in Figure 6.3). In controls, galantamine did not induce connectivity alterations with the cerebellar network.

There were no significant differences in network effects of citalopram vs. placebo between AD patients and elderly controls. Within-group analyses showed that citalopram significantly increased connectivity between the DMN and precuneus/posterior cingulate cortex (PCC) compared to placebo in AD patients, but not in controls (Figure 6.4). Table 6.4 shows specifications and extent of significant effects.

DISCUSSION

We investigated functional network alterations after a serotonergic and cholinergic challenge to gain insight into disruptions of neurotransmitter pathways in AD. Comparing AD patients with controls, we found a significant group x treatment interaction effect after administration of the AChEI galantamine on cerebellar network connectivity. Galantamine induced a local decrease in cerebellar connectivity in AD patients, but not in controls. The SSRI citalopram did not alter regional connectivity differently between groups. However, after citalopram intake, the observed overall effect of lowered connectivity among all networks in controls was absent in AD. In addition, although there was no local interaction effect, a citalopram intensified DMN-precuneus/PCC connection was only observed in the AD group. To guarantee appropriate comparison between groups, PK properties and neuroendocrine effects of both compounds were investigated as well, and reassuring of equal absorption rates and hormone fluctuations [216], that might otherwise have led to spurious group x treatment interactions.

Table 6.4. Overview of significant citalopram and galantamine effects on functional connectivity as estimated with threshold-free cluster enhancement ($p < 0.05$, corrected)

Network effect	Region (Harvard-Oxford or Cerebellar atlas)		*z**	x	y	z	# voxels
Cerebellar network (galantamine: AD patients > controls)	R	Cerebellum (lobules I-VI)	4.25	20	-42	-38	414
	L	Cerebellum (lobule VI)	3.91	-26	-48	-34	106
	R	Cerebellum (crus I and II)	3.91	20	-84	-26	9
Cerebellar network (AD patients: galantamine < placebo)	R	Cerebellum (lobules I-VI, IX, crus I), middle and inferior temporal gyrus, fusiform gyrus, temporal occipital fusiform cortex, parahippocampal gyrus	4.22	50	-34	-10	3108
	L	Cerebellum (lobules IX, V, VI, crus I)	3.95	-12	-56	-34	540
	L	Thalamus	4.00	-12	-12	6	168
	R	Inferior frontal gyrus, pars opercularis; precentral gyrus	4.50	36	14	22	110
	M	Brain stem	3.66	-4	-26	-24	66
	L	Thalamus, brain stem	4.08	-8	-30	-2	22
	R	Thalamus	4.03	18	-8	10	7
	L	Cerebellum (lobule VIIb, crus II)	2.85	-26	-72	-50	6
	R	Caudate	4.05	16	10	4	1
Default mode network (AD patients: citalopram > placebo)	L/R/M	Precuneus, PCC	4.34	-6	-72	-26	685
	R	Intracalcarine cortex, precuneus	3.54	4	-64	14	153

Abbreviations: AD = Alzheimer's disease; L = left; R = right; M = midline; PCC = posterior cingulate cortex. Voxel dimension = 2 mm x 2 mm x 2 mm (voxel volume 0.008 mL). * = standardized *z*-value of the uncorrected peak Fisher-statistic (NPC) within regions.

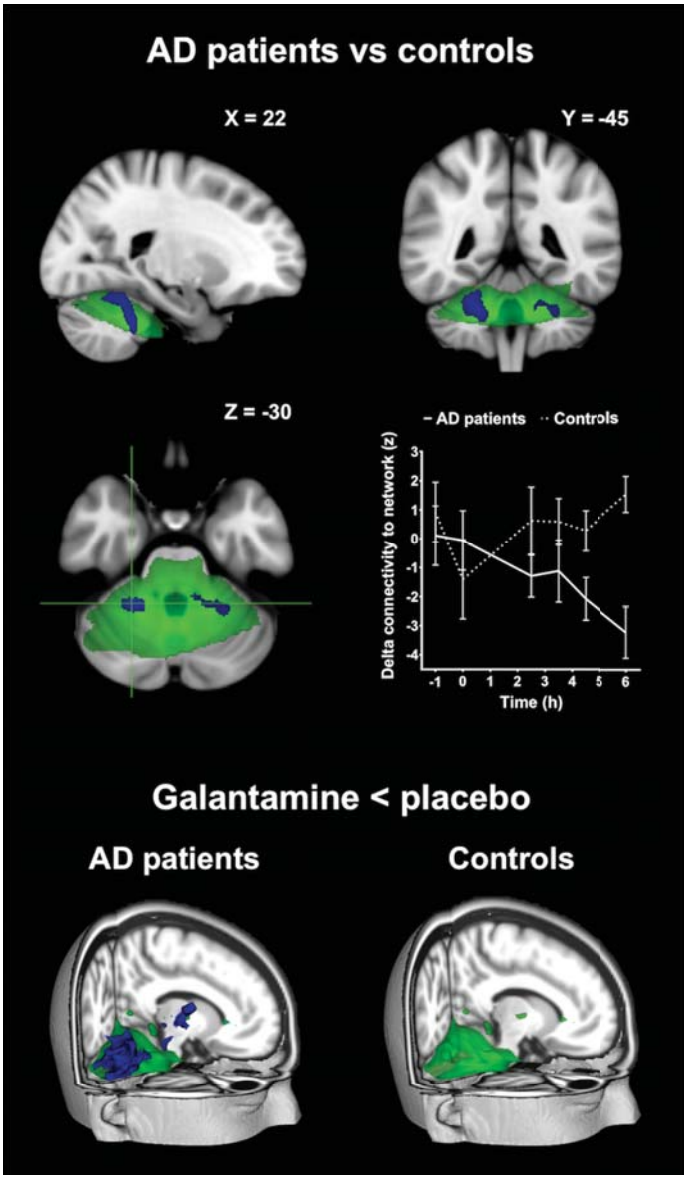

Figure 6.3. Galantamine effects on functional network connectivity. A different effect on connectivity in AD patients compared to elderly controls after galantamine vs. placebo within the cerebellar network (shown in green) for regions as shown in blue (top). The included plot visualizes the corresponding average time profiles of changes in functional connectivity per group for galantamine - placebo conditions (delta *z*-values with standard errors of the mean as error bars). The 3D images (bottom) show main galantamine effects per group. In AD patients connectivity between the cerebellar network (green) and regions in blue was decreased, whereas no effect was found within the group of elderly. Coronal and axial slices are displayed in radiological convention (left = right).

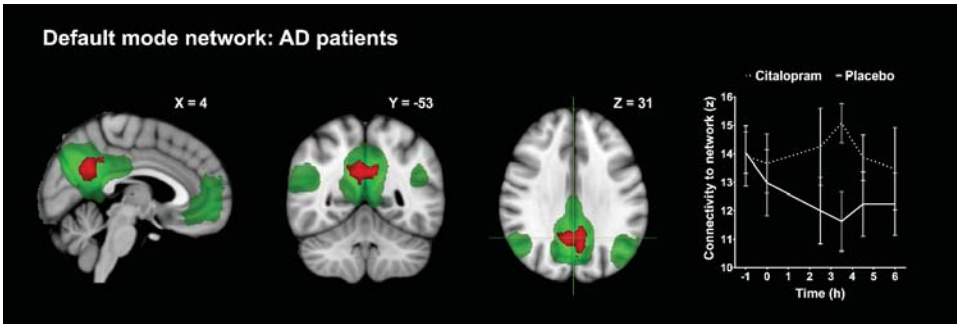

Figure 6.4. Citalopram effects on functional network connectivity. Increased connectivity in AD patients after citalopram vs. placebo was observed within the DMN (shown in green) for the precuneus/PCC (shown in red). The plot visualizes the corresponding average time profiles of changes in functional connectivity for citalopram (dotted line) and placebo (continuous line) conditions (*z*-values with standard errors of the mean as error bars). Coronal and axial slices are displayed in radiological convention (left = right).

Galantamine effects

This study is the first to investigate single-dose galantamine effects on resting state functional connectivity in AD, providing novel information on acute cholinergic alterations of related neural circuits that might underlie the cognitive improvements during chronic treatment. Acute AChEI administration usually does not lead to cognitive enhancement in healthy subjects or AD [214, 215]. Correspondingly, we did not find convincing effects of galantamine on any NeuroCart® task. However, galantamine did result in a diminished cerebellar network response in AD patients compared to elderly controls. Most studies in the literature describe enhanced resting state connectivity after AChEI intake in AD patients [89-94]. Contrary to our single-dose administration these studies all pertain to long-term cholinergic treatment. It is possible that neuroplasticity and modulation of cholinergic pathways over a longer period of AChEI treatment result in opposite findings. For example, increases in posterior DMN connectivity of AD patients as described by Blautzik et al. [92] were prevalent after 12 but not after 6 months of galantamine treatment, which was interpreted as indicating an insufficient time delay of 6 months to measure cholinergic effects. Solé-Padullés et al. [90] demonstrated significant increased DMN connectivity with the right-hemispheric parahippocampal gyrus in treated compared to untreated AD patients after 12 weeks of AChEI treatment but were not able to find longitudinal effects on connectivity with the DMN within treated patients. Of their 8 treated subjects, 5 even showed stable or increased connectivity when they used this area as region of interest.

Galantamine and the cerebellar network

The reduction of cerebellar-thalamic connectivity in patients with AD was partly due to an increase in cerebellar connectivity after placebo as opposed to a decrease after galantamine.

This observation underlines the importance of implementing a placebo-controlled design to investigate drug effects in comparison to diurnal fluctuations that are observed on placebo days and, as is the case for the cerebellar network, might show opposite patterns. Similarly, we found the average cerebellar network connectivity to decrease after placebo and to increase after galantamine administration. The average change in global cerebellar network connectivity during placebo days in the control group also indicates a normalizing effect of galantamine in AD patients, since the mean connectivity after galantamine in patients (mean z = 2.85) equals the mean connectivity after placebo days in controls (mean z = 2.87) instead of after placebo in patients with AD (mean z = 3.94).

It is increasingly recognized that the cerebellum is involved in cognitive and affective processes that are affected in neurodegenerative diseases [342-344]. Certain parts of the cerebellum have extensive fibre connections with specific cerebral areas [345, 346] and previous studies have demonstrated robust structural cerebellar-cortical atrophy connections [347] and lower functional connectivity within a network consisting of the basal ganglia and cerebellum [23] in dementia. It has also been suggested that the cerebellum contributes to the DMN, salience and executive control networks, indicating that cortico-cerebellar pathways are involved in executive and salience functioning, episodic memory and self-reflection [348], and might therefore play a role in symptoms as seen in AD.

The results of our study imply that a relation exists between cholinergic pathways and cerebellar connections in AD. Despite a lack of dense cholinergic innervation of the mammalian cerebellum, the cerebellum is known as a region with high acetylcholinesterase activity and acetylcholine seems to excite the cerebellum's muscarinic Purkinje cells and mossy fibres that are rich in choline acetyltransferase [349-355]. The observed depletion of dendritic Purkinje neurons in AD [356] possibly accounts for altered cholinergic projections after galantamine as shown in our study, which is also supported by delayed loss of Purkinje cells after AChEI treatment [349, 357]. Apart from cortical cholinergic input originating in the nucleus basalis of Meynert, a prominent cholinergic cell group in the brain stem projects towards the thalamus [243, 358]. The thalamus receives input from cerebellar nuclei, which in turn sends signals to all association areas of the cerebrum, including the prefrontal cortex [359]. In line with these pathways the observed decreased functional connections between the cerebellum, thalamus and brain stem in our mild AD group might represent diminished cholinergic trajectories in AD, which may be related to neuronal loss [347].

Citalopram effects on cognitive functions

We did not find any citalopram induced network differences between patients with AD and controls. Likewise, citalopram did not affect any behavioural or cognitive state as measured with the NeuroCart® battery differently between both groups. SSRIs are traditionally not used

as medication for cognitive symptoms, but have been proposed to treat emotional disturbances and agitation which in many AD patients are an integral part of the disease [360-362]. The included participants, motivated to comply with our intensive study program, were perhaps not representative of patients with AD with additional neuropsychiatric impairment, lowering the chance on a differentiated responsiveness of their serotonergic systems. Potentially, 5-HT hypofunction is also involved in cognitive disturbances of AD, although studies on the effect of SSRI administration on these aspects in AD patients are scarce [363]. Combining AChEI treatment with an SSRI seems to improve global cognitive functioning in AD compared to AChEI treatment alone [364], indicating a beneficial interaction between cholinergic and serotonergic stimulation, which is in line with observations on the receptor level [365]. At any rate, our findings confirm the limited cognitive effects of single-dose SSRI administration [167, 314]. A slight worsening of performance on two subtests of the N-back in the elderly was most likely due to chance. It may also be a reflection of a non-linear dose-response, as small immediate memory improvements are most consistently observed in a low (therapeutic) dose range of SSRIs [167].

Connectivity change after citalopram

Since single-dose serotonergic stimulation in non-AD subjects mainly shows effects on DMN connectivity, and DMN coherence is most often found to be altered in AD, we examined drug effects on DMN connectivity within each group separately. An increase in DMN-precuneus/PCC connectivity after citalopram was found in the AD group which could not be detected within the group of elderly subjects. We also observed a significant difference between AD patients and controls in the number of networks that showed a decrease vs. increase in connectivity after citalopram. The control group showed a reduction in connectivity after citalopram compared to baseline for all 10 networks, whereas this was only the case for 4 networks in the AD group. It is remarkable that we found this global connectivity to be enhanced after serotonergic stimulation in AD because previous studies almost uniformly show diminished network coherence after SSRI administration in healthy [83, 85-87, 199, 275] and depressed subjects [84].

Notably, depression is mainly characterized by increased connectivity [88], which may explain a lowering in connectivity after SSRI intake as antidepressant effect. AD however, is defined by decreased DMN-precuneus/PCC connectivity [23, 282, 285, 286]. The precuneus and PCC, both part of the DMN, are specifically implicated in symptomatology of AD such as impaired episodic memory retrieval, self-consciousness and visual-spatial imagery [159, 160, 287-289] and opposite findings after pharmacological enhancement in this study might therefore be regarded as beneficial neurochemical effects in AD. Our observations are concordant with the effects of memantine, an N-methyl-d-aspartate (NMDA) receptor antagonist, which is used to treat moderate and severe cases of AD. Similar to our results, memantine has been shown to strengthen connectivity of the DMN with the precuneus in AD, which was interpreted as

representing regularization of glutamatic levels that, in effect, leads to increased brain metabolic activity [291]. Although evidence on the efficacy of SSRIs as a treatment for cognitive symptoms of dementia is limited, several studies have demonstrated that serotonin might be an important target of pharmacological intervention. The serotonin antagonist and reuptake inhibitor trazodone hydrochloride has recently been discovered as a potential new disease-modifying treatment for dementia by arresting the unfolded protein response, and thereby neurodegenerative cell loss, in mice [366]. Another promising feature of SSRIs is the ability to suppress generation of beta-amyloid in CSF of mice and human volunteers [367], which implies the potential to prevent accumulation of beta-amyloid, which has also been found in the precuneus of AD patients [290]

Conclusions

Whether serotonin dysregulation in AD mostly contributes to behavioural or cognitive symptoms, or both, has yet to be sorted out. The absence of group x treatment interaction effects after administering citalopram points to relatively similar serotonergic systems in AD patients and controls. Nevertheless, our results suggest that SSRI administration has an enhancing effect on DMN-precuneus/PCC connectivity, which has been shown to be decreased in AD [20]. This opposite finding indicates that SSRIs might have an improving effect on memory, self-referential processes and/or visual-spatial functions. We also confirm the significance of a cerebellar network in AD [347], that has been largely neglected within dementia research, but might be an important component associated with cholinergic decline. A challenge for the future is to unravel how the acute response to these compounds develops over a longer treatment period and if this response could be predictive for treatment efficacy in AD.

Acknowledgements

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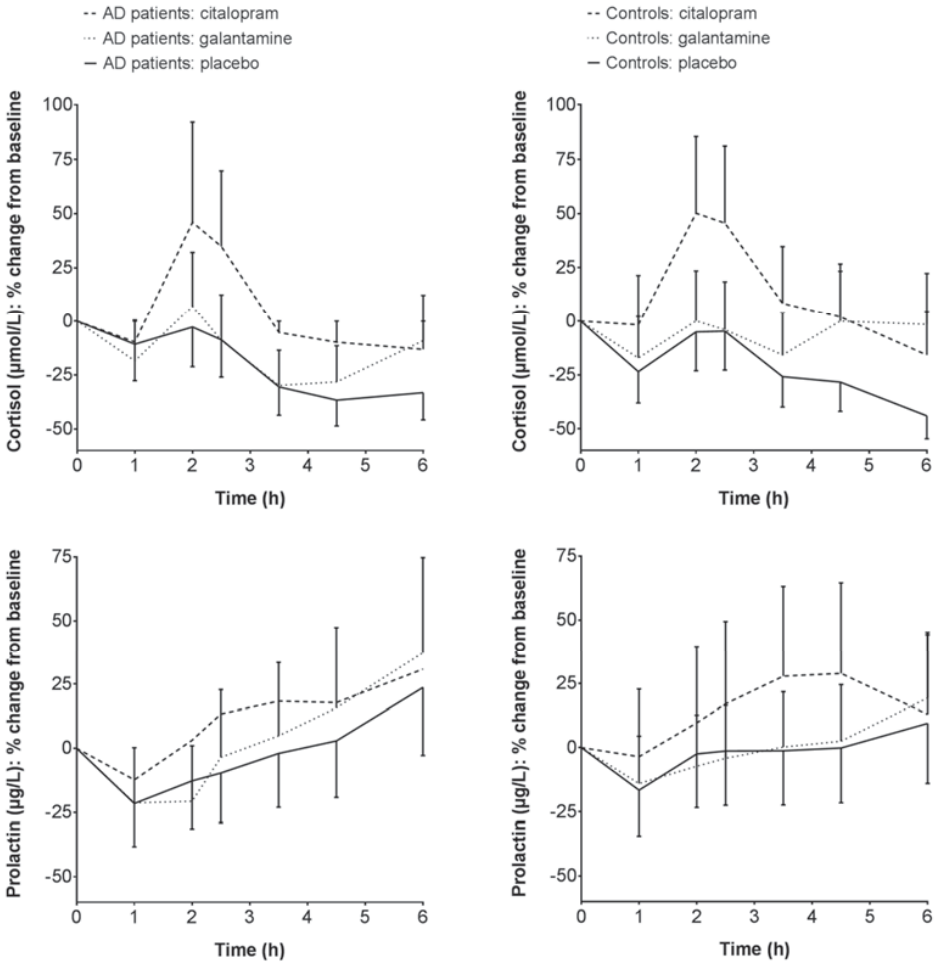
SUPPLEMENTARY MATERIAL

There was no significant group x treatment interaction effect of citalopram nor galantamine on cortisol and prolactin. In both groups, citalopram increased the level of cortisol ($p < 0.0001$) and prolactin ($p < 0.005$), relative to placebo. Galantamine did not affect the level of prolactin in any group. Cortisol was increased after galantamine vs. placebo in the elderly controls ($p < 0.005$), but not in AD patients, although this did not lead to a significant difference between groups.



Supplementary Figure S6.1. Least squares means percent change from baseline profiles of cortisol and prolactin concentrations (with standard errors of the mean as error bars).

Supplementary Table S6.1. Summary of group x treatment interaction effects and treatment effects per group (N-back task and VVLT) of citalopram and galantamine vs. placebo on the NeuroCart® test battery

	Least Squares Means						Contrasts interaction effects (difference, 95% CI, *p*-value)	
Parameter	AD patients: placebo	AD patients: citalopram	AD patients: galantamine	Controls: placebo	Controls: citalopram	Controls: galantamine	AD patients (citalopram vs. placebo) vs. controls (citalopram vs. placebo)	AD patients (galantamine vs. placebo) vs. controls (galantamine vs. placebo)
VAS Alertness (mm)	56.9	51.7	54.9	53.6	53.3	51.0	-4.9 (-10.9, 1.1), $p = 0.106$	0.6 (-5.0, 6.1), $p = 0.833$
VAS Calmness (mm)	57.5	57.1	56.4	54.5	54.0	53.3	0.1 (-4.3, 4.5), $p = 0.977$	0.2 (-4.0, 4.3), $p = 0.938$
VAS Mood (mm)	60.1	56.2	59.4	56.8	55.7	54.2	-2.9 (-9.0, 3.1), $p = 0.332$	1.9 (-3.7, 7.5), $p = 0.500$
VAS Nausea log (mm)	0.40	0.49	0.57	0.42	0.46	0.62	0.053 (-0.147, 0.252), $p = 0.595$	-0.031 (-0.230, 0.168), $p = 0.757$
Adaptive tracking (%)	12.74	13.27	13.42	12.66	13.86	12.89	-0.67 (-2.57, 1.23), $p = 0.479$	0.46 (-1.45, 2.36), $p = 0.632$
Simple reaction time task (s)	348.57	366.38	371.72	342.56	340.53	345.04	5.7 (-3.7, 16.1), $p = 0.231$	5.9 (-3.7, 16.5), $p = 0.230$
Stroop mean RT Incongruent-Congruent (ms)	486.6	470.4	662.7	222.9	197.9	198.6	8.8 (-240.1, 257.7), $p = 0.942$	200.5 (-49.7, 450.7), $p = 0.110$
Stroop Correct Congruent-Incongruent	2.2	4.1	3.3	1.6	1.1	1.5	2.4 (-0.3, 5.1), p = 0.073[2]	1.2 (-1.5, 3.9), $p = 0.347$
SDST Correct Responses	47.3	47.2	49.2	48.6	48.7	47.9	-0.2 (-5.3, 5.0), $p = 0.945$	2.6 (-2.5, 7.8), $p = 0.308$
SDST Average Reaction Time (ms)	3072.9	3042.0	2929.3	2840.6	2895.3	2947.7	-85.59 (-520.3, 349.15), $p = 0.675$	-250.8 (-616.3, 114.75), $p = 0.157$

Supplementary Table S6.1 continues on next page

Supplementary Table S6.1. *Continued*

	Least Squares Means						Contrasts main treatment effects (estimate of difference, 95% CI, *p*-value)			
Parameter	**AD patients: placebo**	**AD patients: citalopram**	**AD patients: galantamine**	**Controls: placebo**	**Controls: citalopram**	**Controls: galantamine**	**AD patients: citalopram vs. placebo**	**AD patients: galantamine vs. placebo**	**Controls: citalopram vs. placebo**	**Controls: galantamine vs. placebo**
N-back mean RT 0 back (ms)	542	542	527	490	495	482	0 (-31, 31), *p* = 0.998	-15 (-47, 18), *p* = 0.359	5 (-7, 16), *p* = 0.428	-9 (-22, 4), *p* = 0.167
N-back mean RT 1 back (ms)	645	653	631	579	575	556	8 (-41, 57), *p* = 0.728	-14 (-64, 36), *p* = 0.559	-4 (-39, 31), *p* = 0.814	-23 (-58,11), *p* = 0.172
N-back mean RT 2 back (ms)	814	878	815	713	692	677	64 (-50, 179), *p* = 0.244	1 (109, 112), *p* = 0.978	-21 (-95, 52), *p* = 0.555	-37 (-110, 37), *p* = 0.310
N-back correct-incorrect/total 0 back	5.74	5.84	5.90	0.97	0.94	0.95	0.10 (-0.18, 0.38), *p* = 0.448	0.16 (-0.13, 0.44), *p* = 0.254	-0.03 (-0.10, 0.04), *p* = 0.391	-0.02 (-0.10, 0.05), *p* = 0.522
N-back correct-incorrect /total 1 back	5.11	4.93	5.22	0.95	0.88	0.96	-0.18 (-1.13, 0.77), *p* = 0.649	0.11 (-0.87, 1.08), *p* = 0.790	**-0.07 (-0.12, -0.01), *p* = 0.023**[1]	0.01 (-0.04, 0.07), *p* = 0.612
N-back correct-incorrect /total 2 back	3.12	3.07	3.13	0.88	0.78	0.84	-0.05 (-1.07, 0.97), *p* = 0.922	0.01 (-1.04, 1.06), *p* = 0.981	**-0.10 (-0.20, -0.00), *p* = 0.042**[1]	-0.03 (-0.13, 0.06), *p* = 0.477
VVLT Recall 1 correct	2.2	1.9	2.3	7.5	7.2	7.4	-0.3 (-1.1, 0.5), *p* = 0.413	0.1 (-0.6, 0.9), *p* = 0.757	-0.3 (-1.6, 1.1), *p* = 0.704	-0.1 (-1.4, 1.3), *p* = 0.899
VVLT Recall 2 correct	3.6	3.4	3.5	10.7	9.8	9.8	-0.2 (-1.2, 0.8), *p* = 0.675	-0.1 (-1.1, 0.9), *p* = 0.808	-0.8 (-2.6, 0.9), *p* = 0.340	-0.8 (-2.6, 0.9), *p* = 0.340
VVLT Recall 3 correct	3.9	4.5	4.4	12.8	11.6	13.3	0.6 (-0.3, 1.4), *p* = 0.190	0.5 (-0.4, 1.3), *p* = 0.262	-1.3 (-3.5, 1.0), *p* = 0.269	0.5 (-1.8, 2.8), *p* = 0.654
VVLT Delayed Recall correct	0.8	1.5	1.1	8.0	8.2	7.7	0.7 (-0.1, 1.5), p = 0.093[2]	0.3 (-0.5, 1.2), *p* = 0.421	0.3 (-2.1, 2.6), *p* = 0.829	-0.3 (-2.6, 2.1), *p* = 0.829
VVLT Delayed Recognition correct	8.0	6.9	7.6	23.5	23.0	20.5	-1.1 (-2.7, 0.5), *p* = 0.179	-0.4 (-2.1, 1.4), *p* = 0.652	-0.5 (-3.0, 2.0), *p* = 0.682	**-3.0 (-5.5, -0.5), *p* = 0.022**[1]
VVLT Delayed Recognition RT correct (ms)	1012.8	1096.3	1076.4	1015.0	1047.8	1074.6	83.5 (-56.8, 223.8), *p* = 0.222	63.6 (-95.9, 223.2), *p* = 0.408	32.8 (-40.6, 106.3), *p* = 0.362	59.6 (13.8, 133.0), *p* = 0.106

[1]Significant at $p < 0.05$; [2]non-significant trend ($0.05 < p < 0.1$). Abbreviations: AD = Alzheimer's disease; CI = confidence interval; VAS = Visual Analogue Scale; SDST = Symbol Digit Substitution Test; VVLT = Visual Verbal Learning Test; RT = reaction time. The N-back task and the VVLT for AD patients is an adapted (easier) version. It was therefore not possible to compare performance on these tasks between AD patients and elderly controls.