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

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Chapter 5

Serotonergic and cholinergic modulation of functional brain connectivity: a comparison between young and older adults

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ABSTRACT

Aging is accompanied by changes in neurotransmission. To advance our understanding of how aging modifies specific neural circuitries, we examined serotonergic and cholinergic stimulation with resting state functional magnetic resonance imaging (RS-fMRI) in young and older adults. The instant response to the selective serotonin reuptake inhibitor citalopram (30 mg) and the acetylcholinesterase inhibitor galantamine (8 mg) was measured in 12 young and 17 older volunteers during a randomized, double blind, placebo-controlled, crossover study. A powerful dataset consisting of 522 RS-fMRI scans was obtained by acquiring multiple scans per subject before and after drug administration. Group x treatment interaction effects on voxelwise connectivity with ten functional networks were investigated ($p < 0.05$, FWE-corrected) using a non-parametric multivariate analysis technique with cerebrospinal fluid, white matter, heart rate and baseline measurements as covariates. Both groups showed a decrease in sensorimotor network connectivity after citalopram administration. The comparable findings after citalopram intake are possibly due to relatively similar serotonergic systems in the young and older subjects. Galantamine altered connectivity between the occipital visual network and regions that are implicated in learning and memory in the young subjects. The lack of a cholinergic response in the elderly might relate to the well-known association between cognitive and cholinergic deterioration at older age.

INTRODUCTION

During the process of aging, there is a decline in brain function [255]. Reduced synaptic plasticity, transmitter release and receptor availability in the central nervous system (CNS) might affect cognitive and behavioral performance [44, 306]. Impaired cholinergic transmission has been associated with age-related disruptions in attention and memory storage and retrieval, [53, 307-309], whereas serotonin (5-hydroxytryptamine; 5-HT) dysregulation may contribute to the increased prevalence of depressive symptoms in the elderly [45, 47, 310].

Magnetic resonance imaging (MRI) of resting state functional connectivity cannot be used to measure neurotransmission directly, but is commonly applied to improve insight into neurotransmitter function by studying brain networks after a pharmacological intervention [76, 79, 118, 311-313]. With regard to aging, the effects of serotonergic and cholinergic challenges on brain connectivity are especially relevant as compounds acting on these systems are used to treat depression and dementia [50, 189].

Acute or short-term dosing of drugs that prevent the presynaptic reuptake of serotonin seems to counteract the observed increased connectivity patterns in depression [88], showing reduced connectivity with several cortical and subcortical areas in healthy and depressed young subjects [82-87, 199, 275]. Cholinesterase inhibitors (AChEIs) cause connectivity enhancement of regions that are important for learning, memory and executive control after long-term treatment in patients with Alzheimer's disease [89-93] and immediately after administration in young subjects [275].

Despite evidence of cholinergic and serotonergic alterations with aging, it is unknown how the corresponding connectivity pathways are altered at older age. There is little proof of differentiated effects of selective serotonin reuptake inhibitors (SSRIs) and AChEIs on subjective and cognitive measures between healthy young and older subjects [167, 215, 314]. However, during senescence 5-HT receptor density declines [315] and the cholinergic system has been suggested to have diminished and more variable responsiveness [309, 316]. Given a negative association of aging with neuromodulation and brain function, we anticipate that effects of the SSRI citalopram and the AChEI galantamine on resting state connectivity are more constrained in older compared to young subjects.

MATERIALS AND METHODS

Subjects

Twelve healthy young volunteers (mean age 22.8 ± 2.8; age range 18-27; 6 female/6 male; body mass index range 21-28 kg/m^2) and 17 healthy older adults (mean age 71.2 ± 6.1; age range 61-

79; 9 female/8 male; body mass index range 22-31 kg/m^2) were included in the study. 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 and mental health and were able to refrain from using nicotine and caffeine during study days. Exclusion criteria included a positive drug or alcohol test 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.

Study design

Part of the data, showing drug effects in young adults, have been described previously [275]. This was a single center, double blind, placebo-controlled, crossover study with citalopram 30 mg and galantamine 8 mg. 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 and obtain all pharmacodynamic measures within an equal time frame at around the T_{max} of both compounds, citalopram was administered earlier than galantamine. In addition, since a lower dose of SSRIs is recommended in elderly compared to young subjects [317], it was decided to retain the opportunity of administering a lower dose of citalopram in elderly than in young subjects. Therefore, citalopram 20 mg was administered at T = 0 h, followed by a second dose of 10 mg at T = 1 h (only 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 also 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 resting state fMRI (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). Each scan was followed by performance of computerized cognitive tasks (taken twice at baseline) on the NeuroCart® test battery, developed by the CHDR 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 drug concentrations related to absorption, distribution, metabolism and excretion. 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. An overview of the study design is provided in Figure 5.1.

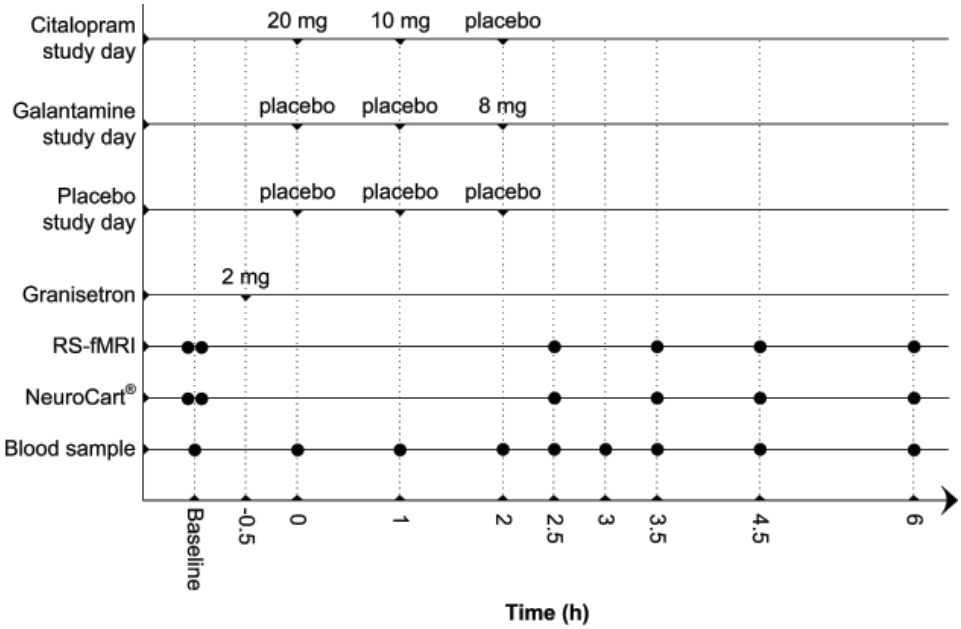

Figure 5.1. Schematic overview of the study design. Each subject received citalopram, galantamine and placebo on three different study days. On each study day there were three moments of administration. The second administration only took place when subjects tolerated the first dose well (did not vomit or feel too nauseous). At baseline, two RS-fMRI scans were acquired, followed by the NeuroCart® test battery. On all study days, subjects received 2 mg of granisetron 30 min before drug administration, to prevent for possible side effects of citalopram and/or galantamine. After drug administration, four 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.

Outcome measures

Pharmacokinetics

Pharmacokinetic 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 and, in case of equal absorption rates, increase confidence in pharmacodynamic outcomes (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 also obtained to determine cortisol and prolactin concentrations. Serum samples were taken 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 outside the scanner 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. During the RS-fMRI scans, all subjects were asked to close their eyes while staying awake. Instructions were given prior to each scan 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 analyzed using a mixed effects model with group as fixed effect (SAS for Windows V9.4; SAS Institute, Inc., Cary, NC, USA).

Neuroendocrine variables

Treatment (drug vs. placebo) x group (young vs. older subjects) interaction effects on cortisol and prolactin concentrations 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 data were not normally distributed and therefore log-transformed before analysis and back transformed after analysis.

NeuroCart® test battery

All post-dose repeatedly measured NeuroCart® measures were analyzed using the same mixed effects model as for neuroendocrine variables. As data of the Simple Reaction Time task were not normally distributed, these data were log-transformed before analysis and back transformed after analysis. The data of the Visual Verbal Learning Test were analyzed using a mixed effects model with treatment, group, visit and treatment by group as fixed effects and subject as random effect.

MR imaging

All 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 (29 subjects x 3 days x 6 scans = 522 datasets) with a dual regression analysis [36, 125] based on 10 predefined standard network templates as used in our previous research [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 two frontoparietal networks (left and right). Time series of white matter (measured from the center of the corpus callosum) and cerebrospinal fluid (measured from the center of 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 (cerebrospinal fluid and white matter). Next, these time series were entered in a temporal regression into the same dataset. This resulted 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.

Higher level analysis

To investigate group x treatment interaction effects of citalopram and galantamine we used 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 analysis of variance (ANOVA) or multivariate analysis of variance (MANOVA). NPC testing was used in two phases to estimate for each network whether drug vs. placebo effects on connectivity were significantly different between young and older subjects.

First, tests were performed for each post-dose time point (T = 2.5, 3.5, 4.5 and 6 h) separately, 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]. To investigate group x treatment

interaction effects on voxelwise functional connectivity with each of the 10 functional networks, four two-sample t-tests (young adults: drug - placebo vs. older adults: drug - placebo) were performed, one per time point, 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 age-related differences at baseline level as recently analyzed and described in Klaassens et al. [319]. The same method was applied for additional investigation of treatment effects (drug vs. placebo) within the group of older adults as was previously done for the 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 and the average of the two baseline RS-fMRI scans as covariates.

Second, to analyze 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 cerebrospinal fluid. 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-values < 0.05, corrected.

RESULTS

Pharmacokinetics

Table 5.1 provides an overview of the PK parameters in young and older subjects, and the statistical outcomes of group analyses. In comparison to the young subjects, the T_{max} of galantamine occured significantly later in the older adults ($p < 0.001$). There were no further pharmacokinetic differences between young and older subjects. Figure 5.2 shows individual and median PK time profiles.

Cortisol and prolactin

There was a significant group x treatment interaction effect of citalopram and galantamine on cortisol ($p < 0.05$). In comparison to the young subjects, the increase in cortisol was significantly larger in the older adults after citalopram and galantamine, relative to placebo. There was no significant group x treatment interaction effect on prolactin. In both groups, there was an increase in prolactin after citalopram vs. placebo ($p < 0.005$). Galantamine did not affect the level of prolactin. See Figure 5.3 for cortisol and prolactin levels in young and older subjects.

Table 5.1. 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	Young adults	Older adults		Young adults	Older adults		Young adults	Older adults	
T_{max}	3.0 ± 1.2	3.4 ± 1.1	0.319	4.9 ± 1.3	4.0 ± 1.3	0.068	2.7 ± 1.1	4.5 ± 1.1	< 0.001
C_{max}	35.8 ± 6.3	41.8 ± 11.7	0.165	3.0 ± 1.1	3.5 ± 1.8	0.332	40.7 ± 10.4	41.8 ± 12.2	0.811
$AUC_{0\text{-}last}$	146.0 ± 25.2	165.0 ± 43.6	0.259	11.7 ± 4.8	13.3 ± 7.1	0.500	95.1 ± 27.7	104 ± 40.2	0.494

Abbreviations: 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).

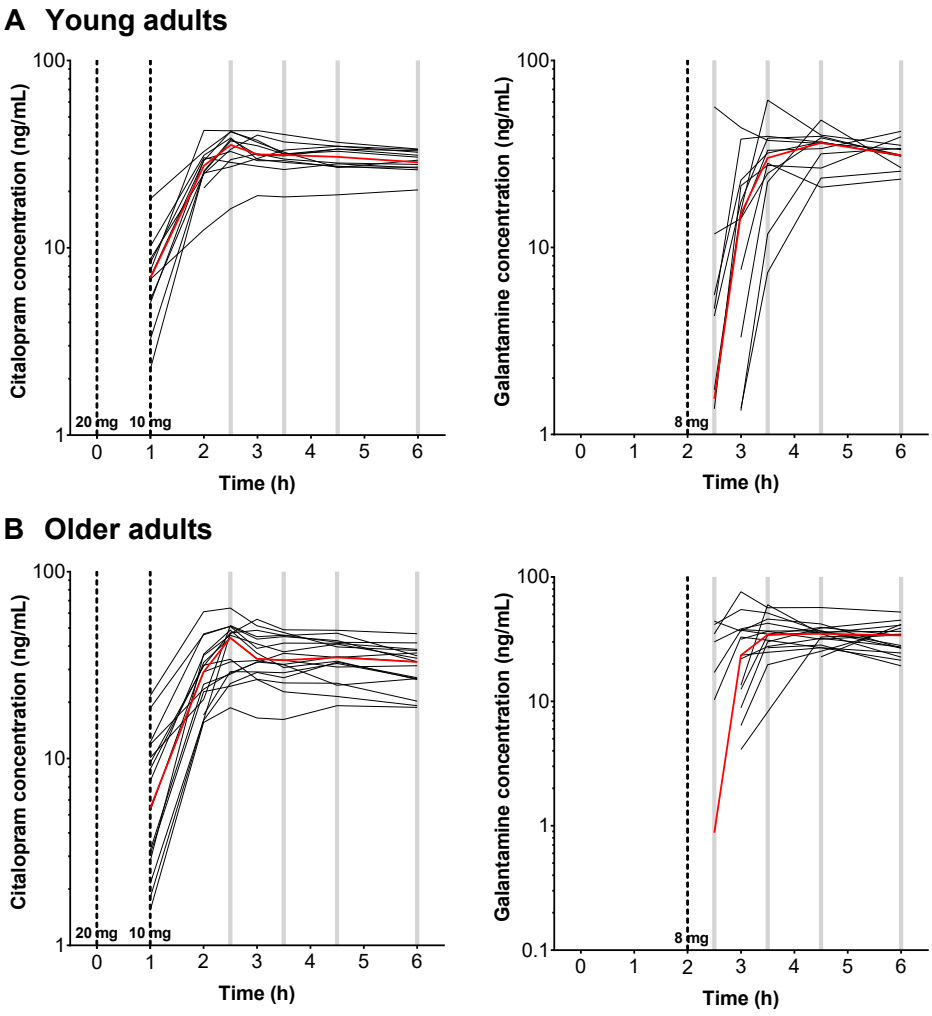

Figure 5.2. Median (red line) and individual (black lines) pharmacokinetic profiles for citalopram (left) and galantamine (right) concentrations in nanograms per milliliter on semi-log scale in young (A) and older (B) subjects. Grey bars illustrate moments of RS-fMRI acquisition post drug administration. Observations below limit of quantification were dismissed.

NeuroCart® test battery

Supplementary Table S5.1 provides an overview of all NeuroCart® results. There were no convincing significant group x treatment interaction effects after galantamine or citalopram vs. placebo on any NeuroCart® measure.

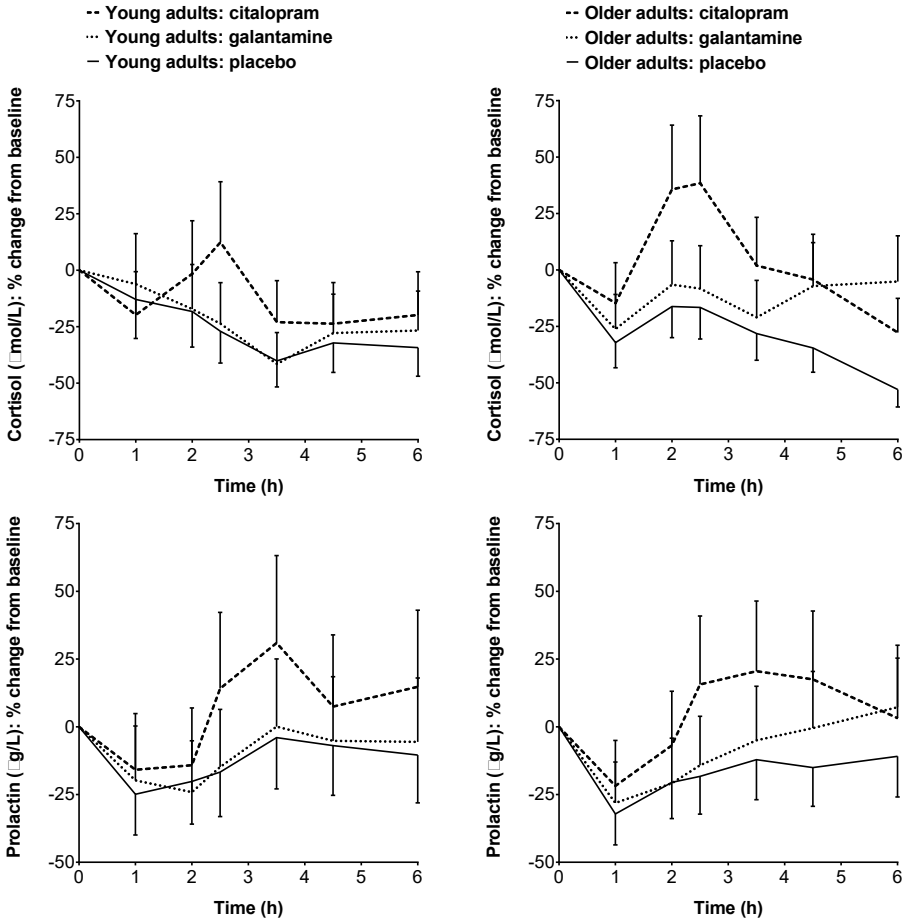

Figure 5.3. Least squares means percent change from baseline profiles of cortisol and prolactin concentrations (with standard errors of the mean as error bars) in young (left) and older (right) subjects.

Functional connectivity

Serotonergic effects

There was no group x treatment interaction effect of citalopram on functional network connectivity. We did find main treatment effects of citalopram on connectivity with the sensorimotor network in both groups. Within the young subjects, citalopram decreased connectivity between the sensorimotor network and supplementary motor area, pre- and postcentral gyrus, anterior and posterior cingulate cortex (ACC and PCC), precuneus, medial and orbital prefrontal cortex, and cerebellum. Within the older adults, citalopram decreased connectivity between the sensorimotor network and supplementary motor area, pre- and postcentral gyrus, ACC, PCC, cingulate gyrus, precuneus, superior frontal gyrus and frontal orbital cortex (Figure 5.4). Specifications and extent of significant citalopram effects are summarized in Table 5.2.

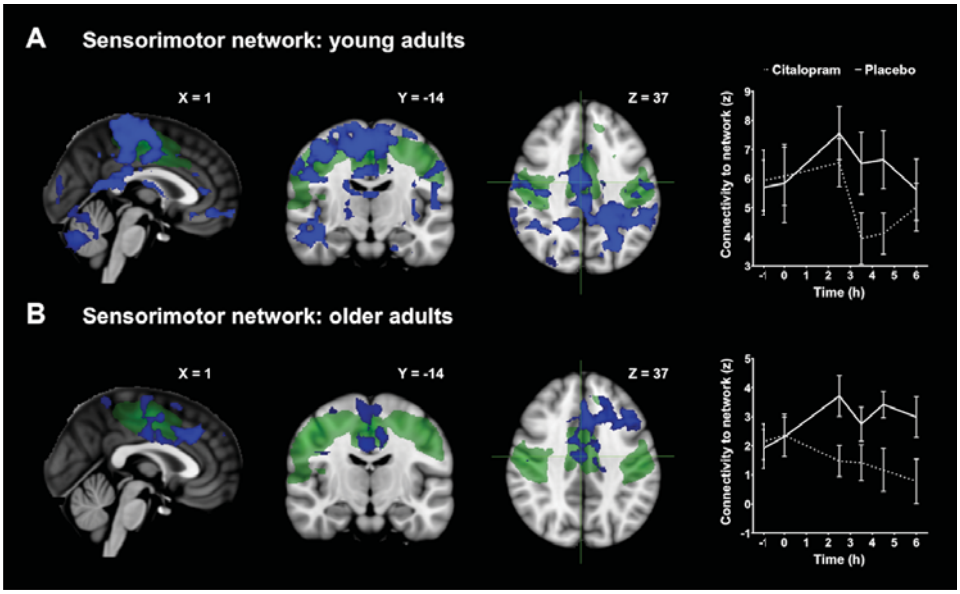

Figure 5.4. Decreased connectivity in young (A) and older subjects (B) after citalopram vs. placebo was observed between the sensorimotor network (shown in green) and regions as shown in blue. Plots visualize the corresponding average time profiles of changes in functional connectivity for citalopram (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).

Cholinergic effects

An interaction effect of galantamine was found for one visual network (occipital pole). An increase in connectivity after galantamine vs. placebo was significantly larger in the young compared to the older adults between the occipital visual network and the precuneus, PCC, postcentral-, angular- and supramarginal gyrus (Figure 5.5). Galantamine led to increased connectivity between the occipital visual network and the left and right hippocampus, precuneus, thalamus, fusiform gyrus, precentral and superior frontal gyrus, PCC and cerebellum in the young subjects, whereas no significant treatment effect of galantamine on this network was detected in the group of older adults. Specifications and extent of significant galantamine effects are summarized in Table 5.3.

DISCUSSION

To study the influence of older age on neurotransmitter pathways, we investigated differences in functional network responsiveness to single-dose serotonergic and cholinergic stimulation between young and older adults, independent of between-group variation in brain connectivity at baseline [319]. We found a significantly weaker pharmacological effect of galantamine on

Table 5.2. Overview of significant citalopram effects on functional connectivity as estimated with threshold-free cluster enhancement ($p < 0.05$, corrected)

Network effect	Region (Harvard-Oxford)		*z**	x	y	z	# voxels
Sensorimotor network (Young adults: drug < placebo)	L/R/M	ACC, PCC, precuneus, SMA, pre- and postcentral gyrus, medial and orbital frontal cortex, cerebellum	5.23	-22	50	-16	36214
	L	Inferior temporal gyrus, inferior and temperooccipital part	3.65	-50	-46	-12	153
	L	Occipital fusiform gyrus	3.58	-40	-74	-18	94
	L	Cerebellum	4.03	-28	-42	-32	52
Sensorimotor network (Older adults: drug < placebo)	L/R/M	SMA, superior and middle frontal gyrus, ACC, PCC, paracingulate gyrus	4.27	-36	26	40	4076
	L/R/M	Precuneus, postcentral gyrus, posterior cingulate gyrus, superior parietal lobule	3.52	0	-48	64	467
	L	Temporal pole, frontal orbital cortex	4.54	-46	14	-18	368
	R	Pre- en postcentral gyrus	3.34	34	-10	30	258
	R	Occipital fusiform gyrus	4.29	30	-64	-16	204
	R	Precentral gyrus	2.76	32	-12	68	41
	R	Occipital fusiform gyrus	3.65	24	-78	-18	32
	R	Middle frontal gyrus, frontal pole	3.43	28	36	34	30
	R	Occipital fusiform gyrus	3.43	16	-82	-26	18
	R	Precentral gyrus	3.33	20	-36	42	15

Abbreviations: L = left, R = right, M = midline, ACC = anterior cingulate cortex, PCC = posterior cingulate cortex, SMA = supplementary motor area. 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 (with # voxels > 10).

Table 5.3. Overview of significant galantamine effects on functional connectivity as estimated with threshold-free cluster enhancement ($p < 0.05$, corrected)

Network effect	Region (Harvard-Oxford)		*z**	x	y	z	# voxels
Occipital visual network (group x treatment interaction effect)	R/M	Precuneus, PCC, pre- and postcentral gyrus, posterior cingulate gyrus, posterior supramarginal gyrus, superior parietal lobule	4.15	10	-36	44	1218
	R	Lateral occipital cortex, superior division	3.83	28	-62	26	224
	R	Frontal pole	5.13	38	50	28	94
	R	Cerebellum	4.05	20	-68	-32	79
	R	Middle and superior frontal gyrus	4.25	34	0	56	57
	M	Cerebellum	3.78	4	-68	-24	55
	R	Cerebellum	3.80	20	-54	-34	52
	R	Lateral occipital cortex, superior division	3.99	18	-60	54	29
Occipital visual network (Young adults: drug > placebo)	L/R/M	Hippocampus, thalamus, amygdala, precuneus, PCC, lateral occipital cortex, brain stem, fusiform gyrus, superior and middle frontal gyrus, superior temporal gyrus, pre- and postcentral gyrus and cerebellum	4.86	2	-62	-26	15341
	L	Precuneus, posterior cingulate gyrus	3.15	-16	-52	20	75
	L	Temporal pole	3.41	-58	10	-10	18
	R	Inferior frontal gyrus	3.81	48	14	14	17
	L	Lateral occipital cortex, superior division	3.98	-46	-70	26	12
	L	Central opercular cortex, insular cortex	3.11	-46	4	-4	12

Abbreviations: 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 (with # voxels > 10).

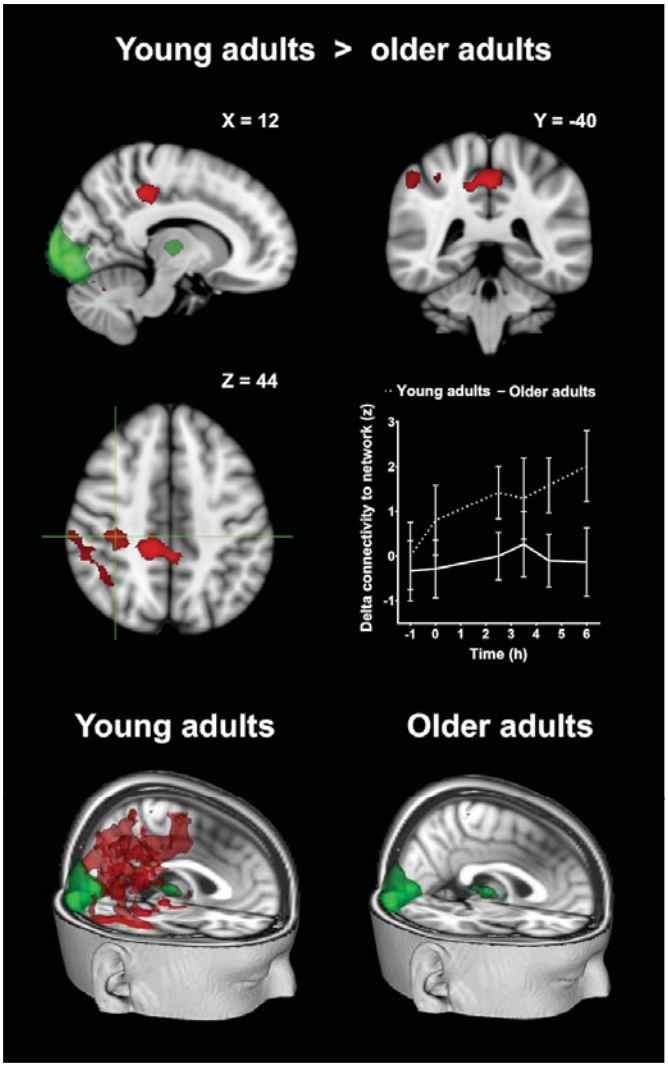

Figure 5.5. A larger effect on connectivity in young compared to older adults after galantamine vs. placebo between the occipital visual network (shown in green) and regions shown in red (top). The plot visualizes the corresponding average time profiles of changes in functional connectivity per group 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. Coronal and axial slices are displayed in radiological convention (left = right).

functional integrity of the occipital visual network in the older compared to the young subjects. Since the effects of AChEIs depend on intact cholinergic synapses, this finding might represent the general tendency towards diminished cholinergic function in the elderly [53, 309]. In contrast, older age had no impact on the response to the SSRI citalopram, which induced a strong decrease in connectivity with the sensorimotor network within both groups.

Citalopram

Reduced connections between multiple regions after citalopram administration is consistent with previous SSRI research in healthy young individuals [83, 199]. Although these effects seemed less pronounced in the older adults compared to young subjects, there was no significant difference in effect between the two groups on all networks. As an altered serotonergic system in the elderly has most often been associated with a higher incidence of depression and sleep disorders [45, 310], one explanation for this lack of differences is that there was no evidence for any abnormality of mood, anxiety or sleep in both groups as assessed during screening. The most prominent finding in both samples was a decrease in connectivity between the sensorimotor network and sensorimotor regions, precuneus, ACC and PCC (Figure 5.4). Similar findings have been detected in healthy young subjects with the SSRI sertraline, in which, amongst other networks, sensorimotor network connectivity with the ACC, PCC, precuneus, central gyri and supplementary motor cortex was decreased after single-dose administration [199]. Aging has been shown to alter motor network connectivity, possibly representing deteriorated motor ability in the elderly [40, 270] and leading to the somewhat smaller response in older compared to young subjects. However, in favor of a rather unaffected serotonergic system in our healthy group of elderly, we did not find significantly different changes in sensorimotor connectivity in the much younger subjects. This relative sparing of serotonergic network responsiveness in elderly subjects suggests that the reduced effects of galantamine are indicative of a selective age-related cholinergic decline. Citalopram caused a decrease in connectivity of cortical midline structures as the precuneus, ACC, PCC and prefrontal areas, related to self-referential mechanisms and emotion regulation, which is in line with opposite observations in (non-elderly) depressed patients [88] and indicates that SSRIs might reverse abnormalities in functional connectivity as seen in depression. The effects on sensorimotor connectivity also denote the well-known involvement of 5-HT pathways in motor behavior [320]. For example, an (uncommon) side effect of SSRIs is the observance of movement disorders (e.g. muscle twitches), possibly due to direct adverse effects on motor neurons or enhancement of serotonergic input on dopaminergic pathways [222, 321, 322]. In the young subjects, citalopram also reduced connectivity between the midbrain and left frontoparietal network [275]. This effect was restricted to a discrete region, and a comparable response could not be detected in the older adults, despite the lack of significant differences between groups on this network.

Additional measures of cognitive functioning and neuroendocrine responses were investigated to confirm the presence of neuropharmacological effects and improve the understanding of underlying changes in brain connectivity. There was no difference between young and older adults in effect of citalopram on performance on the NeuroCart® test battery. Single-dose SSRI administration does not seem to alter behavioral states in young and older subjects differently [167, 314]. Acute SSRI effects in healthy subjects of all ages are limited and variable and our protocol did not include more sensitive measures of SSRI modulation as EEG recordings, REM-

sleep and flicker discrimination tests [167]. Since SSRIs are mainly used as a treatment for depression and anxiety disorders [50, 109], the most likely change in our study would have taken place on mood, alertness or calmness as measured with the VAS. However, such improvements are usually only noticeable after a mood-specific behavioral challenge [176, 177, 323] or after a few weeks [173, 174], and accordingly we did not find these effects. The fact that citalopram had pharmacological effects in both age groups is demonstrated by increasing levels of cortisol and prolactin. Neuroendocrine fluctuations can be regarded as indirect measures of the 5-HT system state [216] and a larger increase in cortisol level in the older compared to young adults might be indicative of some serotonergic disturbances that accompany the process of normal aging. However, this interpretation is complicated by the fact that elevated cortisol has been shown to reduce amygdala-medial prefrontal cortex connectivity [183, 324], which in turn seems to be related to baseline cortisol levels. As cortisol was slightly but not significantly lower at baseline in the elderly, this might lead to a stronger drug effect on cortisol and network connectivity in older compared to young adults. There are multiple factors that can cause a decrease in clearance of antidepressants in the elderly [317, 325]. But most of these affect the terminal part of the concentration-time curve and exposure during multiple dosing. The duration of our study was limited, and our elderly subjects were relatively healthy. Consequently, no differences in pharmacokinetic profiles were observed between groups, ensuring comparable exposure in both age groups over the observation period, with similar and limited variability. Because elderly are known to have an increased expectancy for SSRI-related side effects [317], we wanted to provide them with the opportunity to take a lower dose of 20 instead of 30 mg of citalopram, by skipping the second 10 mg if necessary. However, all young and older subjects were administered the total dose of 30 mg without vomiting or experiencing nausea as measured with the VAS. This might also be due to the 2 mg of granisetron, given 30 minutes prior to drug administration on all three study days. Granisetron was added to suppress nausea and vomiting, which could otherwise adversely affect study procedures or alter network effects. Yet, it cannot be excluded that the selective 5-HT_3 receptor agonist granisetron might have also altered specific functional responses [109].

Galantamine

Whereas galantamine increased brain connectivity in the young subjects with one visual network (Figure 5.5), we could not detect any effects of galantamine on resting state connectivity in older adults. The cholinergic system is chiefly associated with an age- and dementia-related decline in memory, learning and attention with evidence pointing to cholinergic dysfunction in the hippocampus, cortex, the entorhinal area, the ventral striatum and the basal forebrain [228, 229]. In our young subjects, galantamine altered connectivity with the hippocampus, thalamus and the fusiform gyrus, areas that are involved in learning and memory [326-328]. ACh release in the primary visual cortex seems to be relevant for visual processing and learning [233, 234].

The findings in the young group are therefore consistent with studies that show an essential role for cholinergic enhancement in visual attention [235, 236], visual episodic memory and recall [238, 241, 242], processing of novel faces [239, 240], perceptual processing during working memory [246] and visual orientation [247]. The absence of effect in the elderly might point to attenuated activity of the cholinergic system, which accompanies the process of normal aging [329]. The sensitivity of the cholinergic system to aging is emphasized by the detection of clear and very similar serotonergic effects on connectivity in both young and older adults. An age-appropriate decline in cognitive function was also observed in our older group compared to the young subjects by investigating the difference in NeuroCart® performance at baseline level (before pharmacological stimulation), as presented in Klaassens et al. [319]. The elderly performed worse on several tests (% correct on the Adaptive Tracking task, reaction time on the Symbol Digit Substitution Test, 0-back, 1-back and 2-back task, and number of correct responses on the 2-back task), relating to decreased attentional, memory and processing speed capacities. Our observations of reduced connectivity alterations after galantamine in the older adults seem to confirm the cholinergic hypothesis of cognitive decline during aging [53].

Galantamine's mean T_{max} in the elderly (mean T_{max}: 4.52 ± 1.08) occurred significantly later than the mean T_{max} in the young subjects (mean T_{max}: 2.67 ± 1.11). This delay in pharmacokinetics might have caused small shifts in the time course of effects, although it is unlikely that this affected the overall response over the duration of the experiment, which was the basis for all principal analyses and comparisons. Since the T_{max} of galantamine was within the time frame of measurements for both groups, analyzing a combination of data points would be minimally influenced by different PK timing profiles. As the level and variability of plasma concentrations, as determined by C_{max} and $AUC_{0\text{-last}}$, in the elderly was generally similar to those in the younger group, it is implausible that galantamine effects were obscured by pharmacokinetic dispersion. Moreover, the rise in plasma cortisol after galantamine was larger in elderly than in young subjects, which was particularly noticeable at T = 2 and T = 2.5. Because galantamine has been shown to increase cortisol [106], this indicates that galantamine was absorbed well enough in the older adults to induce pharmacodynamic effects. Nevertheless, as described earlier this finding may partly influence the observed network effects [183, 324]. In addition, despite the administration of granisetron, an increase in nausea, a typical side effect of AChEIs [330], after galantamine in both groups provides further support for sufficient drug concentrations in the older adults. AChEIs are commonly used to treat cognitive symptoms of Alzheimer's disease [331] and in healthy subjects there is little evidence for neuroenhancement with this drug [73]. A few studies have been performed on AChEI efficacy in subjects without cognitive disturbances, with inconclusive and contradicting results in both young and elderly subjects [73, 215, 238, 332, 333]. Two measures of the delayed recognition subtest of the Visual Verbal Learning Test, the number of correct responses and reaction time, showed a difference between young and older subjects with $p < 0.05$. However, the number of included NeuroCart® tests was large and

the effects were not clearly related to drug levels. Therefore, this marginal result might likely be due to chance, suggesting that AChEI challenges do not affect cognitive performance differently between young and older subjects.

Conclusions

The outcomes of this study illustrate the use of resting state connectivity to investigate different neurotransmitter systems, and how these selectively change with age. The SSRI citalopram affected sensorimotor network connectivity in both young and older adults, demonstrating that SSRIs consistently reduce the functional integrity of regions that are related to motor function and self-reference, regardless of age. The effect of the AChEI galantamine was restricted to the young subjects, who showed a response that indicates the contribution of acetylcholine to perceptual processing and learning mechanisms. We did not observe any network effects in the elderly, possibly reflecting a diminished cholinergic system that is associated with an age-appropriate decline in memory and attention. Combining RS-fMRI with pharmacological challenges and additional outcome measures offers a useful way to investigate age-related functional processes, which is in line with Geerligs and Tsvetanov [334], who recommend to implement an integrative approach in studying neurocognitive aging instead of merely using fMRI data. Compared to cognitive performance, RS-fMRI seems to serve as a relatively sensitive measure of drug-induced functional change. Our findings support the confidence in RS-fMRI as an important tool in psychopharmacological research, and its potential to measure disease specific alterations in neurotransmission.

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Supplementary Table S5.1. Summary of group x treatment interaction effects of citalopram and galantamine vs. placebo on the NeuroCart® cognitive test battery

	Least Squares Means						Contrasts interaction effects (difference, 95% CI, *p*-value)	
Parameter	Young adults: Placebo	Young adults: Citalopram	Young adults: Galantamine	Older adults: Placebo	Older adults: Citalopram	Older adults: Galantamine	Young (citalopram vs. placebo) vs. older adults (citalopram vs. placebo)	Young (galantamine vs. placebo) vs. older adults (galantamine vs. placebo)
VAS Alertness (mm)	50.4	49.4	48.4	51.7	52.0	49.6	1.3 (-2.4, 5.0), $p = 0.497$	-0.1 (-3.8, 3.6), $p = 0.940$
VAS Calmness (mm)	52.1	52.0	53.6	53.2	52.8	52.7	-0.3 (-3.6, 2.9), $p = 0.839$	-2.0 (-5.3, 1.3), $p = 0.230$
VAS Mood (mm)	53.8	53.9	52.0	54.1	51.7	52.8	1.9 (-1.2, 5.0), $p = 0.227$	-1.1 (-4.3, 2.0), $p = 0.482$
VAS Nausea log (mm)	0.36	0.37	0.50	0.30	0.41	0.56	-0.021 (-0.196, 0.155), $p = 0.813$	-0.002 (-0.176, 0.171), $p = 0.977$
Adaptive tracking (%)	21.28	20.09	20.0	19.71	20.26	19.51	1.75 (-0.34, 3.83), $p = 0.098$	1.08 (-0.99, 3.16), $p = 0.296$
Simple reaction time task (sec)	283.43	293.98	286.57	294.29	291.62	296.47	-4.5 (-11.4, 3.0), $p = 0.225$	-0.4 (-7.5, 7.4), $p = 0.922$
Stroop mean RT Incongruent-Congruent (msec)	80.5	108.7	94.0	175.2	154.8	155.4	-48.7 (-108.0, 10.7), $p = 0.105$	-33.3 (-93.5, 26.9), $p = 0.271$
Stroop Correct Congruent-Incongruent	0.1	0.5	0.2	0.6	0.4	0.4	-0.5 (-1.3, 0.2), $p = 0.159$	-0.3 (-1.0, 0.5), $p = 0.495$
SDST Correct Responses	62.7	62.6	62.5	62.4	61.5	61.7	-0.9 (-2.2, 0.5), $p = 0.206$	-0.5 (-1.8, 0.9), $p = 0.491$
SDST Average Reaction Time (msec)	1966.4	2039.1	2037.1	2083.8	2142.8	2194.6	-13.8 (-144.6, 117.0), $p = 0.830$	40.7 (-91.0, 171.1), $p = 0.536$
N-back mean RT 0 back (msec)	412	414	405	478	485	473	5 (-15, 25), $p = 0.635$	2 (-18, 23), $p = 0.812$
N-back mean RT 1 back (msec)	464	464	449	547	544	526	-2 (-37, 33), $p = 0.907$	-6 (-41, 29), $p = 0.737$
N-back mean RT 2 back (msec)	559	568	552	665	654	640	-19 (-89, 52), $p = 0.596$	-17 (-87, 54), $p = 0.633$
N-back correct-incorrect/total 0 back	0.95	0.96	0.95	0.98	0.98	0.96	-0.01 (-0.06, 0.04), $p = 0.728$	-0.02 (-0.06, 0.03), $p = 0.524$
N-back correct-incorrect /total 1 back	0.96	0.96	0.94	0.94	0.93	0.96	-0.01 (-0.07, 0.04), $p = 0.568$	0.03 (-0.02, 0.09), $p = 0.191$
N-back correct-incorrect /total 2 back	0.91	0.91	0.91	0.88	0.82	0.86	-0.06 (-0.14, 0.02), $p = 0.140$	-0.03 (-0.11, 0.05), $p = 0.430$
VVLT Recall 1 correct	11.4	10.7	12	7.2	7.3	6.4	0.8 (-1.6, 3.2), $p = 0.497$	-1.4 (-3.8, 0.9), $p = 0.231$
VVLT Recall 2 correct	16.9	16.8	16.8	10.5	9.7	9.6	-0.7 (-2.8, 1.4), $p = 0.511$	-0.9 (-3.0, 1.2), $p = 0.401$
VVLT Recall 3 correct	19.8	19.9	19.6	12.6	11.2	12.7	-1.4 (-4.2, 1.3), $p = 0.301$	0.4 (-2.4, 3.2), $p = 0.772$
VVLT Delayed Recall correct	18.4	17.0	18.1	7.3	8.2	7.4	2.4 (-0.4, 5.1), $p = 0.087$	0.4 (-2.3, 3.1), $p = 0.774$
VVLT Delayed Recognition correct	25.7	26.3	26.9	23.0	22.2	20.9	-1.3 (-4.3, 1.7), $p = 0.380$	-3.3 (-6.4, -0.3), $p = 0.032$
VVLT Delayed Recognition RT correct (msec)	894.3	860.8	836.3	1017.0	1030.8	1077.6	47.4 (-40.8, 135.6), $p = 0.286$	118.6 (29.5, 207,8), $p = 0.010$

Abbreviations: VAS = Visual Analogue Scale; SDST: Symbol Digit Substitution Test; VVLT = Visual Verbal Learning Test; RT = reaction time.