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Original Paper

Neuropsychobiology

Neuropsychobiology
DOI: 10.1159/000358840

Received: August 8, 2013
Accepted after revision: February 25, 2014
Published online: ■■■

The Effects of Aging on Changes in Regional Cerebral Blood Flow in Schizophrenia

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Key Words
Schizophrenia · ^{99m}Tc-ethyl cysteinate dimer single-photon emission computed tomography · Aging · Cerebral blood flow · Temporal lobe

Abstract
Aims: Although there have been no conclusive pathophysiological findings in support of the degeneration theory in the etiology of schizophrenia to date, results of our neuroimaging studies suggest functional changes in the brains of schizophrenics. We evaluated age-related changes of brain perfusion in medicated patients with schizophrenia. ***Method:*** In this study, we evaluated age-related changes in brain perfusion in medicated schizophrenia patients (n = 44) and control subjects (n = 37) undergoing ^{99m}Tc-ethyl cysteinate dimer single-photon emission computed tomography. ***Result:*** Although the regional cerebral blood flow (rCBF) was found to be reduced in bilateral frontal lobes by analysis with age in the patients with schizophrenia, significant differences compared to controls in age effects on perfusion were found in the patients with schizophrenia in bilateral temporal lobes. Moreover, in multiple regression analysis including age, total time of treatment and overall neuroleptic dose, rCBF was found to be reduced in bilateral frontal and parietal lobes. As a result, cerebral perfusion in temporal lobes with schizophrenia might be related to age rather than medication. ***Conclusion:*** In this study, the patients with schizophrenia appeared to have significant bilateral temporal hypoperfusion related to age compared with controls. And bilateral temporal rCBF is decreased in patients with schizophrenia and even more in older schizophrenia patients. These changes might be consistent with degenerative changes observed in patients with schizophrenia and be a promising method for the efficient development of a treatment strategy by measuring temporal perfusion in patients with schizophrenia.

Introduction

Although schizophrenia has increasingly been conceptualized as a neurodevelopmental disorder [1], there is mounting evidence on progression not only of cognitive, but also of brain structural and functional pathology [2, 3]. Even though this progression might not be related to classical neuropathological markers of neurodegeneration such as astrogliosis, it has significant relevance for our understanding of the disease course, especially with

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respect to cognitive deterioration and general clinical outcomes [4, 5].

Alterations in brain structure have been observed in patients with schizophrenia at different stages of the disorder, including prodromal, first-episode and chronic stages, and recent meta-analysis comparing the differences in the cross-sectional patterns of patients with healthy subjects suggest that there might be an increase in structural pathology over the course of the disease [6]. In addition, both structural and functional imaging studies suggest changes at later disease stages that are suggestive of accelerated aging compared with healthy individuals [2]. Thus, the present cross-sectional imaging studies of schizophrenia patients as well as the longitudinal MRI studies with follow-up periods of up to 10 years are suggestive of progressive changes exceeding those seen in healthy subjects [7]. These changes seem to occur at different stages of the disease, including the transition to psychosis, the early course of schizophrenia and the senescence process.

In a previous study [8], we revealed that the schizophrenia patients had hypoperfusion of the bilateral temporal lobes and the right frontal lobe compared to control subjects. Moreover, we evaluated age-related changes of brain perfusion in medicated patients with schizophrenia. In this study, we focused on cerebral blood flow and compared age-related changes in patients with schizophrenia with those in healthy controls.

Table 1. Patient and control data

	Schizophrenia	Controls	p value
Subjects, n	44	37	
Men:women	24:20	22:15	n.s.
Age, years	40.0 ± 10.5	37.5 ± 6.3	n.s.
Smokers, n	28	7	<0.001
Subtype			
Paranoid	18	–	
Hebephrenic	20	–	
Catatonic	6	–	
BPRS	40.0 ± 6.2	–	
PANSS	42.3 ± 11.3	–	
Duration of illness, years	5.3 ± 3.5	–	
Duration of neuroleptic therapy, years	4.5 ± 3.0	–	
Users of atypical drugs, n	40	–	
Dose of neuroleptics[a], mg/day	319.82 ± 2.1	–	

BPRS = Brief Psychiatric Rating Scale; PANSS = Positive and Negative Symptom Scale. Comparisons were made by ANOVA or χ^2 tests. The level of statistical significance is $p < 0.05$; n.s. = nonsignificant.

[a] Chlorpromazine equivalents.

Methods

Subjects

Schizophrenia patients (n = 44) were diagnosed according to DSM-IV criteria [9]. Subjects were outpatients or inpatients of the Department of Psychiatry, Shimane University School of Medicine Hospital, without acute psychiatric symptoms. Psychiatric symptoms were rated on the same day as the single-photon emission computed tomography (SPECT) examination by a senior psychiatrist (T.M.) who was unaware of the SPECT findings and diagnosis using the Brief Psychiatric Rating Scale [10] and the Positive and Negative Syndrome Scale [11]. Diagnoses were determined by the consensus of 3 senior psychiatrists (J.H., T.M. and R.W.) based on extended interviews and reviews of the Structured Clinical Interviews for DSM-IV (SCID) in the medical chart. Patients with alcohol and substance abuse or dependence (other than nicotine) or the presence of a severe organic condition were eliminated from this study. Forty patients were taking second-generation antipsychotics (risperidone, n = 14; olanzapine, n = 10; quetiapine, n = 8; aripiprazole, n = 5; blonanserin, n = 3). Patients were matched for age and sex with healthy controls (n = 37; table 1). None of the control subjects had any history of psychiatric disorders as based on the SCID nonpatient edition, organic disease (diabetes mellitus, hypertension, hyperlipidemia, cerebral infarction or cerebral hemorrhage), alcoholism or drug abuse. Control subjects had no abnormal signal intensities on MRI. Pregnancy was excluded in the 20 patient and 15 control women of child-bearing age before perfusion scans. All subjects were right-handed as defined by the Edinburgh inventory [12]. The subjects were given a complete and thorough description of the study and the purpose of measuring blood flow by SPECT, and written informed consent was obtained. The study followed institutional guidelines and the recommendations of the Declaration of Helsinki. The study was approved by the Ethical Committee of Shimane University Faculty of Medicine.

SPECT Analysis Procedure

Brain SPECT with ^{99m}Tc-ethyl cysteinate dimer (^{99m}Tc-ECD) was performed during bed rest with no stimulation. Subjects were injected with 740 MBq of ^{99m}Tc-ECD (Neurolite, BMS) and placed at rest for 1 h in quiet surroundings with their eyes closed. SPECT images were acquired using a double-headed rotating gamma camera (ECAM, Siemens) equipped with a fan beam collimator. In total 64 projections, 32 projections per detector, of 40 s per view were collected in 128 × 128 matrices. Tomographic 3-dimensional reconstruction was performed using a filtered back projection algorithm (Butterworth filter order 4 with a cutoff frequency of 0.4 cm^{-1}) and Chang's attenuation correction. A voxel-by-voxel group study was then performed using SPM 8 (Welcome Department of Cognitive Neurology, University College, London, UK, running on Matlab 7.9.0, Mathworks Inc., Sherborn, Mass., USA). Images were initially converted from the DICOM to the Analyze format using MRIcro, and transferred to SPM 8. The data were then standardized with the Montreal Neurological Institute atlas by using a

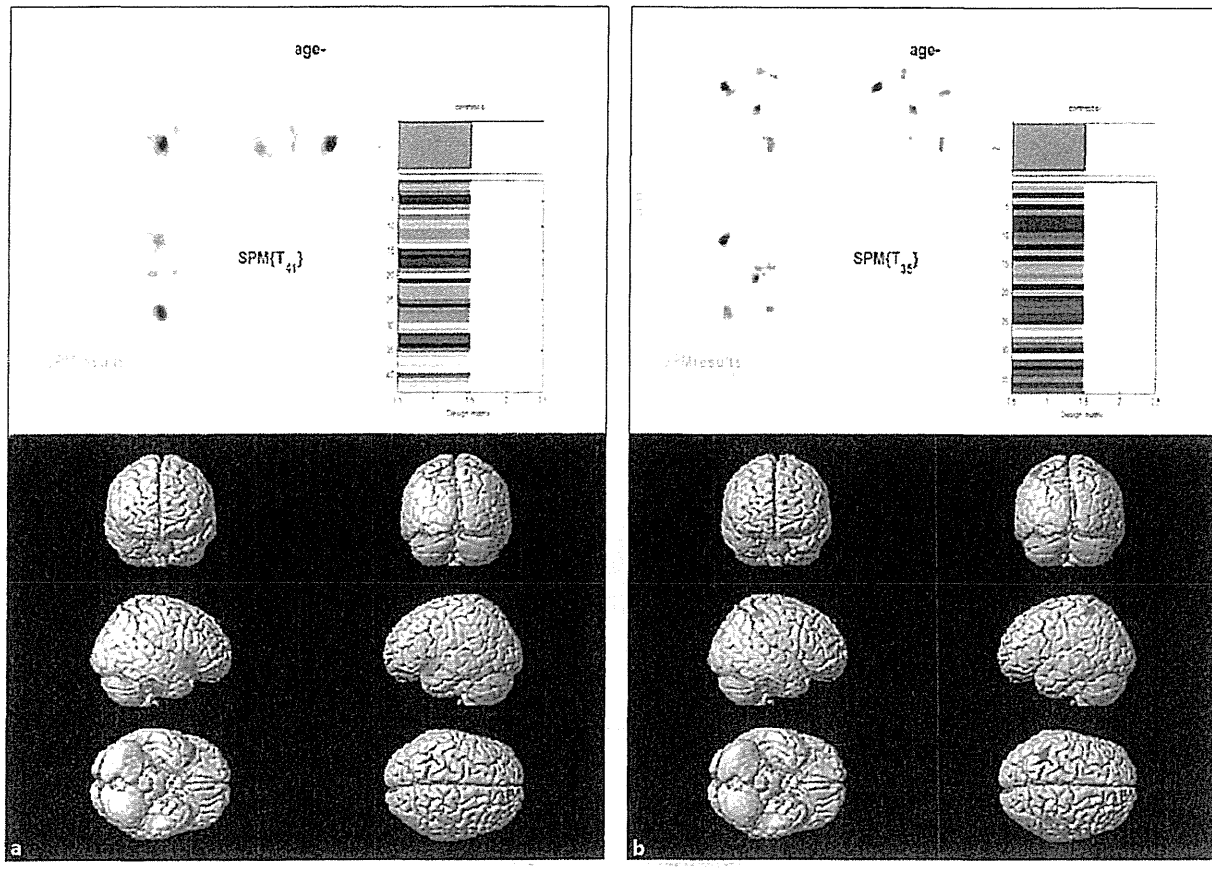

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Fig. 1. SPECT imaging analysis using SPM in schizophrenia patients and controls related to age ($p < 0.001$, uncorrected, extent: 100). **a** Decreased blood flow was observed in the bilateral frontal lobe (the right inferior frontal gyrus, the left inferior frontal gyrus and the left anterior cingulate) in schizophrenia. **b** Decreased blood flow was observed in the bilateral parietal lobes (the left postcentral gyrus, the right inferior parietal lobes, the right cingulate gyrus, the right sublobar extranuclear white matter) in controls.

12-parameter affine transformation, followed by nonlinear transformations and a trilinear interpolation. The dimensions of the resulting voxels were $2 \times 2 \times 2$ mm. Standardized data were then smoothed by a gaussian filter (full width at half maximum 12 mm). Schizophrenia and control groups were compared using the 'compare-populations one scan/subject' routine, which carries out a fixed-effects simple t test for each voxel. Global normalization was performed using proportional scaling. The SPM (T) maps were initially obtained at a height threshold of $p < 0.001$, then an extent threshold uncorrected for multiple comparisons for the cluster was obtained. A complementary analysis was performed using small volume correction with a volume of interest sphere of 10 mm radius, and a height threshold of $p < 0.05$. Montreal Neurological Institute coordinates were finally converted into Talairach coordinates using the Talairach Daemon database. Statistical analysis was performed using SPSS software for Windows version 11.5.1J (SPSS Japan, Tokyo, Japan). The 2-sample t test by SPM 8 was used to determine which areas were affected by the decreasing regional cerebral blood flow (rCBF) between age and schizophrenia (fig. 1a) or control (fig. 1b), between schizophrenia and control with age as covariates (fig. 2a). Statistics were performed using Matlab or SPSS 18 (SPSS Inc., Chicago, Ill., USA). We computed post hoc analyses of covariance in order to investigate the interaction of the regression slopes between rCBF of the most hypoperfused area in schizophrenia, bilateral temporal lobes and age. The noninvasive method of Matsuda et al. [13, 14] (Patlak plot method) was used for the quantitative measurement of rCBF with ^{99m}Tc-ECD SPECT. In bilateral temporal lobes, we calculated a coefficient of determination (R^2 value) with rCBF data (ml/100 g/min) and age (fig. 3). Moreover, step-wise multiple regression analysis was used to determine which areas were affected by the decreasing rCBF in schizophrenia as dependent variable, age, total time of treatment and overall neuroleptics dose as independent variables (fig. 2b).

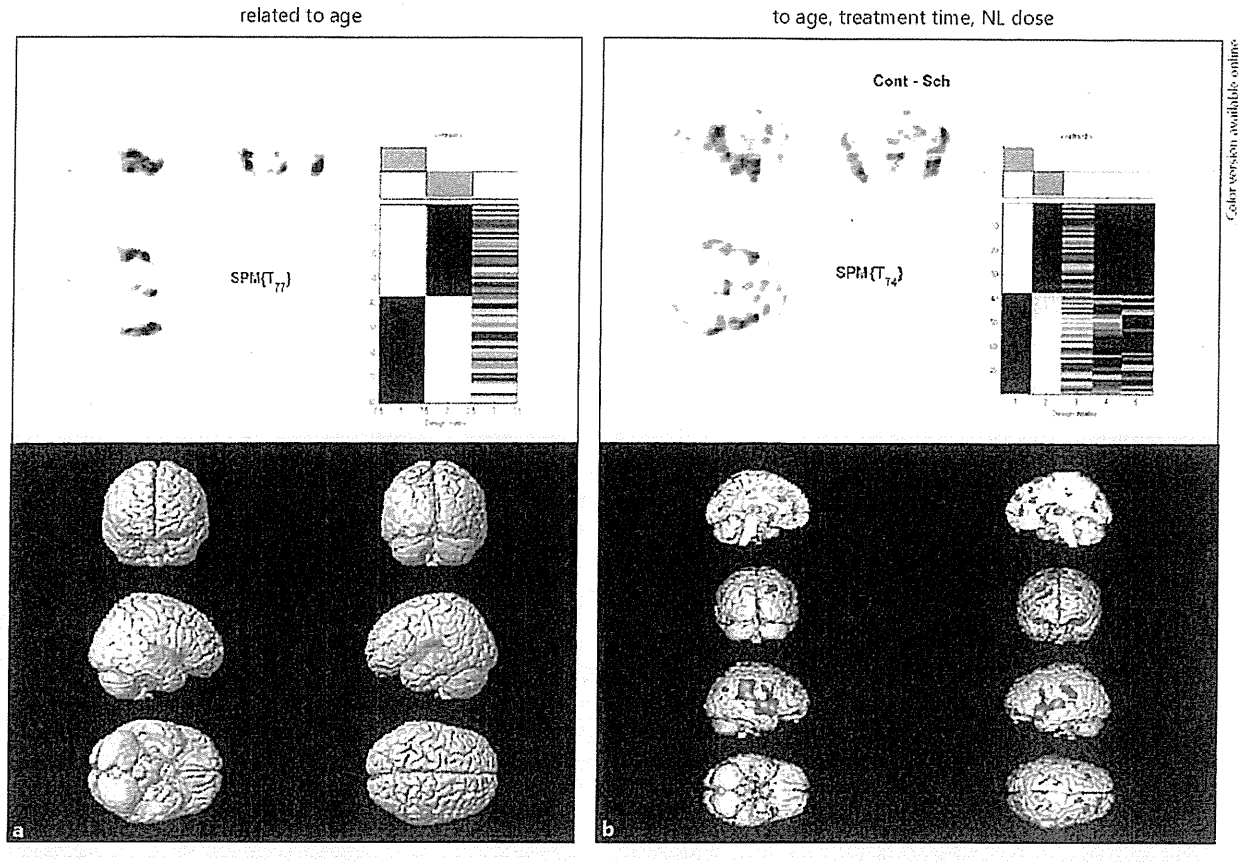

Fig. 2. SPECT imaging analysis using SPM in schizophrenia patients in comparison with control subjects related to age and to age, total time of treatment and neuroleptic dose. **a** Decreased blood flow was observed in the bilateral temporal lobe (the right insular, the left superior temporal gyrus, the anterior cingulate, the thalamus, the lingual gyrus) related to age ($p < 0.001$, uncorrected, extent: 100). **b** Decreased blood flow was observed in the bilateral frontal and parietal lobes (the right thalamus, occipital lobe and bilateral parietal lobes, frontal gyrus, cingulate gyrus) related to age, total time of treatment and neuroleptic dose ($p < 0.001$, uncorrected, extent: 50).

Results

The schizophrenia patients and corresponding normal healthy subjects were matched for age and sex. Statistical group analysis found significant differences in SPECT findings of the 44 schizophrenia patients and 37 control subjects.

The bilateral inferior frontal gyrus and left anterior cingulate of the patients with schizophrenia showed decreasing brain perfusion related to age (fig. 1a, table 2). The right cingulate gyrus, inferior parietal lobes, sublobar extranuclear and left postcentral gyrus in control subjects showed decreasing brain perfusion related to age (fig. 1b, table 2). The area of hypoperfusion related to age in schizophrenia patients and controls was significantly different.

In comparison to controls, the right insular, left superior temporal gyrus, anterior cingulate, thalamus and lingual gyrus of the patients with schizophrenia showed decreasing brain perfusion related to age (fig. 2a, table 3). The graphs in figure 3 showed adjusted response changes with age in perfusion of bilateral temporal lobes, which are the areas with the most decreased rCBF in the patients with schizophrenia. An approximate curve showed that the subject's age was correlated with rCBF change in the bilateral temporal lobes, of control subjects and patients with schizophrenia alike, but that the correlation between age (x) and rCBF (y) in patients with schizophrenia was

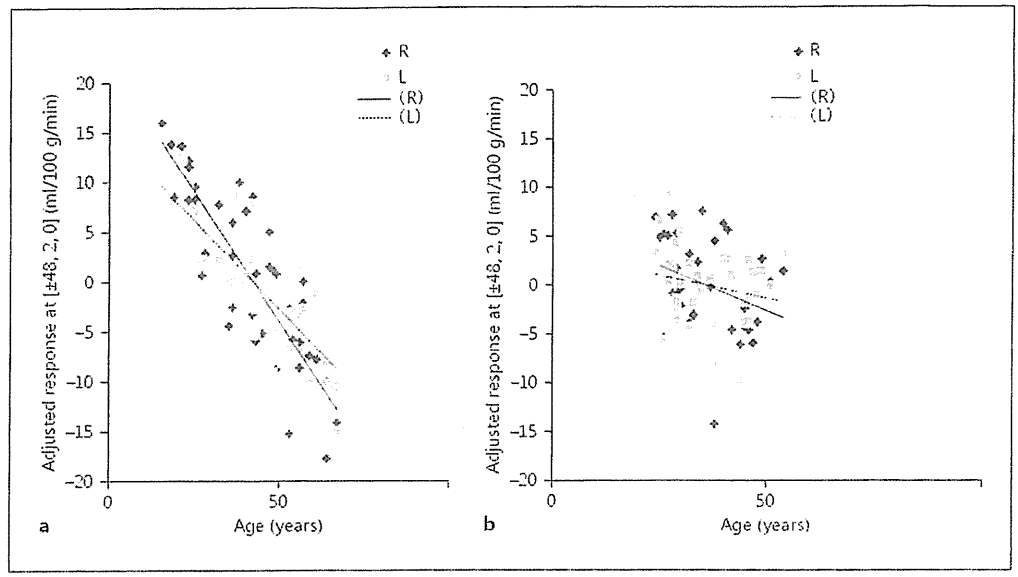

Fig. 3. Correlation between rCBF changes (ml/100 g/min) in the corresponding area related to age in schizophrenia, temporal lobes, and the age. An approximate curve showed that the subject's age (x) was correlated with rCBF (y) change in the most significant area of decreasing blood flow, the bilateral temporal lobes, of control subjects (**b**) and patients with schizophrenia (**a**). The correlation between age (x) and rCBF (y) in patients with schizophrenia (left: $y = -0.3536x + 14.95$, $R^2 = 0.5751$, $p < 0.001$; right: $y = -0.5179x + 21.895$, $R^2 = 0.7217$, $p < 0.001$) was more significant than that of control subjects (left: $y = -0.0935x + 3.3647$, $R^2 = 0.0423$, $p = 0.222$; right: $y = -0.1844x + 6.6327$, $R^2 = 0.1043$, $p = 0.051$) in the effects of age on perfusion.

Table 2. Regions of significant hypoperfusion related to age

k value	Z score	T score	x, mm	y, mm	z, mm	BA	Localization
Schizophrenia							
1,224	7.24	10.41	46	20	−8	47	rt. inf. frontal gyrus
958	6.01	7.68	−40	14	−12	47	lt. inf. frontal gyrus
203	5.42	6.61	26	44	42	25	lt. anterior cingulate
187	5.32	6.43	−2	36	10	32	lt. anterior cingulate
Control							
227	3.47	3.82	−40	−48	62	5	lt. postcentral gyrus
190	3.15	3.41	6	−8	36	24	rt. cingulate gyrus
220	2.93	3.14	44	−44	56	40	rt. inf. parietal lobule
162	2.93	3.14	40	10	−10	13	rt. sublobar extranuclear

Results are listed by clusters. The k value, T score, Z score and Talairach coordinates of peak voxels are provided for each cluster. The k value represents the number of significant voxels in a particular cluster (p voxel level <0.001; p cluster level <0.05, uncorrected for multiple comparisons). BA = Brodman area; rt. = right; lt. = left; inf. = inferior.

Table 3. Regions of significant hypoperfusion in patients with schizophrenia compared with controls

k value	Z score	T score	x, mm	y, mm	z, mm	BA	Localization
Related to age							
1,889	4.67	5.04	−48	2	0	22	lt. sup. temporal gyrus
2,316	4.65	5.02	44	10	−4	13	rt. insula
475	4.48	4.81	0	12	−6	25	lt. anterior cingulate
344	3.87	4.08	−8	−6	14	*	lt. thalamus
161	3.67	3.85	−16	−96	−8	17	lt. lingual gyrus
Related to age, total time of treatment and neuroleptic dose							
3,954	4.79	5.19	58	−30	40	2	rt. postcentral gyrus
1,852	4.41	4.78	−40	14	−12	47	lt. inf. frontal gyrus
596	4.31	4.61	8	−20	10	*	rt. thalamus
526	3.95	4.19	−60	−36	28	40	lt. inf. parietal lobule
285	3.87	4.09	32	30	42	8	rt. middle frontal gyrus
218	3.82	4.03	−6	28	30	9	lt. cingulate gyrus
115	3.74	3.93	14	20	66	6	rt. sup. frontal gyrus
98	3.69	3.88	48	−66	32	39	rt. angular gyrus
212	3.69	3.88	−2	10	−6	25	lt. anterior cingulate

Results are listed by clusters. The k value, T score, Z score and Talairach coordinates of peak voxels are provided for each cluster. The k value represents the number of significant voxels in a particular cluster (p voxel level <0.001; p cluster level <0.05, uncorrected for multiple comparisons). BA = Brodman area; lt. = left; rt. = right; sup. = superior; inf. = inferior.

more significant (left: $y = -0.3536x + 14.95$, $R^2 = 0.575$, $p < 0.001$; right: $y = -0.5179x + 21.895$, $R^2 = 0.7217$, $p < 0.001$) than control subjects (left: $y = -0.0935x + 3.3647$, $R^2 = 0.0423$, $p = 0.222$; right: $y = -0.1844x + 6.6327$, $R^2 = 0.1043$, $p = 0.051$) in neighboring areas where significant differences in the effects of age on perfusion were found in the patients with schizophrenia (fig. 3).

Moreover, in comparison to controls, the brain perfusion related to age, total time of treatment and neuroleptic dose decreased in the right thalamus, occipital lobe and bilateral parietal lobes, frontal gyrus and cingulate gyrus of the patients with schizophrenia (fig. 2b, table 3).

Discussion

In this study we found, first, that the brain perfusion in schizophrenia patients decreased related to age significantly in the bilateral temporal lobes compared to control subjects. Second, hypoperfusion in the most decreased area, the bilateral temporal lobes, in schizophrenia patients tended to be getting severer than in control subjects. Third, hypoperfusion in additional analysis by total time of treatment and neuroleptic dose was found in bilateral frontal and parietal lobes. So, this might suggest that hypoperfusion became enhanced with aging, and that the disease course in the bilateral temporal lobes of schizophrenia patients was related to the enhanced hypoperfusion in these regions. Taken together, these results suggest that hypoperfusion in the brain in schizophrenia may progress in the temporal lobes with aging and the disease course. To our knowledge, this is the first study to investigate rCBF in schizophrenia patients by ^{99m}Tc-ECD SPECT using SPM related to age and the disease course.

In our previous study [8], we found hypoperfusion in the left temporal lobe and the right frontal lobe in the first-episode schizophrenia group compared with the respective control group. These results suggest that hypoperfusion in the brain in schizophrenia may progress mainly in the left temporal lobe earlier in patients than in control subjects and that it might be a characteristic finding of first-episode schizophrenia.

Schizophrenia may be associated with a fundamental disturbance in the temporal coordination of information processing in the brain, leading to dysfunctions in the timing of perceptual, cognitive and motor processes, and disturbances of consciousness [15]. This putative deficit in the temporal coordination of information processing in the brain is sometimes referred to as cognitive dysmetria [16]. 'Disconnection' models [17] posit that deficits in

neural communications arise from aberrations in the modulation of time-dependent changes in synaptic connectivity, which manifest as dysfunctions in the timing or sequencing of mental activity and behavior. Accordingly, classic symptoms of schizophrenia such as thought disorder and disorganized and contextually inappropriate behavior may be manifestations of a timing dysfunction [14]. Support for these conceptualizations is emerging with evidence that brain structures and neurotransmitter systems that are directly linked to neural timing processes are also impaired in schizophrenia [15, 16, 18–20]. Despite the growing interest and centrality of these time-dependent conceptualizations of the pathophysiology of schizophrenia, there remains a paucity of research directly examining overt timing performance in the disorder. Thus, the aim of the present study was to investigate the function of temporal lobes in schizophrenia by age and the disease course.

It has been a matter of interest whether cerebral perfusion decreases with age and/or the disease duration because age and the disease course effects would distort the results of perfusion imaging studies of neuropsychiatric disorders. Several studies using SPECT have shown decreases in perfusion in several cortical and subcortical areas with age, while a characteristic frontotemporal pattern of perfusion decreases has been reported [21]. Our findings have shown a significant correlation between aging and the disease course effect of schizophrenia, and a reduction in temporal perfusion might be in accordance with a report that the rCBF reductions in the frontal lobe tended to extend to posterior brain regions in the chronic stage of schizophrenia [22]. This study used rCBF data by ^{99m}Tc-ECD SPECT to investigate the age-related progression in schizophrenia patients versus healthy controls across an age range. Our findings support the notion of an age-related decline in rCBF in bilateral temporal lobes in the patients with schizophrenia in an age range covering most of the adult lifespan. Although rCBF reduction might relate to not only aging, but also the disease duration and/or its severity, progression of functional brain changes has rarely been assessed by longitudinal studies. Thus, our age-related design took advantage of imaging techniques to discover changes occurring over most of the adult lifespan.

There are several confounding factors in our study. This study has a methodological limitation in the choice of patients, subtype, sex and medication. But the patients are fairly young with a relatively brief duration of illness of 5 years, and treatment was initiated in these patients very rapidly. So results of this study might reflect, particularly, the change of structure and function in middle-aged patients with schizophrenia. The antipsychotics dosage administered to the patient group may have had some effect on rCBF distribution. Different patterns of effects on perfusion have been reported for haloperidol and risperidone [23]. Metabolic and perfusion changes have also been reported for clozapine and risperidone [24, 25]. In our study, hypoperfusion in schizophrenia was found in the bilateral frontal area related to aging and treatment time. Moreover, by the addition of analysis by neuroleptic dose, the hypoperfusion area in schizophrenia spread to bilateral parietal lobes. Although our samples consisted solely of patients who had taken neither haloperidol nor clozapine, the effects caused by medication were unclear in this study. Other biases may also have influenced the results in this study, and further study is necessary.

In conclusion, taken together, our findings show that the patients with schizophrenia appeared to have significant bilateral temporal hypoperfusion related to aging and the disease course. Although some reasons for rCBF reduction might relate to not only aging, but also the disease duration and/or its severity, bilateral temporal rCBF is decreased in patients with schizophrenia and even more in older schizophrenia patients. These changes might be consistent with the degenerative changes observed in patients with schizophrenia. In the future, it would be a very important issue whether there are different characters in changes of structure and function in young, middle-aged and old-age patients with schizophrenia. Thus, this method of analysis permits an objective assessment of the change in rCBF in relation to the diagnosis of schizophrenia and its relationship to rCBF changes. This might be a promising method for the efficient development of a treatment strategy by measuring temporal perfusion in patients with schizophrenia.

Acknowledgments

Part of this work was supported by Grants-in-Aid for Scientific Research on Priority Areas No. 13770544, 15790622 and 20591366 from the Ministry of Education, Science, Sports and Culture of Japan.

Disclosure Statement

There are no potential conflicts of interest.

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RESEARCH ARTICLE

International Journal of Geriatric Psychiatry

The effects of combine treatment of memantine and donepezil on Alzheimer's Disease patients and its relationship with cerebral blood flow in the prefrontal area

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Background: In this study, we evaluated the effect on cognitive function of memantine, behavioral and psychological symptoms of dementia, and the care burden, in patients with moderate-to-severe Alzheimer's disease (AD). Furthermore, with near-infrared spectroscopy (NIRS), we examined the association between effect of memantine and brain blood flow.

Methods: We evaluated the effect of memantine administration from baseline on Clinical Global Impression-Improvement scale, mini mental state examination (MMSE), Clock Drawing Test (CDT), Neuropsychiatric Inventory (NPI), Japanese version of the Zarit Burden Interview (J-ZBI) and NIRS in two groups, donepezil administration memantine combination group (combination group, $n = 19$) donepezil administration memantine non-administration group (control group, $n = 18$) were assessed at weeks 0, 4, 12, and 24.

Results: Significant difference was found between the combination group and the control group in the score variation of Clinical Global Impression-Improvement scale, MMSE, CDT, NPI, and J-ZBI. In the NIRS measurements, trend oxyhemoglobin reduced suppression was observed in some channels centered on the superior frontal gyrus. A significant correlation was observed in the scores of MMSE, CDT, NPI, and J-ZBI. In addition, a significant positive correlation was also observed between the number of words in NIRS and scores of MMSE and CDT.

Conclusions: In this study, by administering memantine in AD patients that inhibit the reduction of cerebral blood flow in the prefrontal area and improve clinical symptoms overall cognitive function, behavioral and psychological symptoms of dementia, thereby reducing the care burden of caregivers was suggested. Copyright © 2014 John Wiley & Sons, Ltd.

Key words: Alzheimer's Disease; memantine; donepezil; prefrontal area; near-infrared spectroscopy (NIRS)
History: Received 5 September 2013; Accepted 11 December 2013; Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI: 10.1002/gps.4074

Introduction

Alzheimer's disease (AD) is one of the most common types of dementia in Japan, followed by cerebrovascular dementia and dementia with Lewy bodies. It is a progressive neurodegenerative disease, of which the chief symptoms include memory loss, impaired judgment, and disorientation. Because patients with advanced AD need care in all aspects of their daily life and sometimes show behavioral and psychological symptoms, referred to as behavioral and psychological symptoms of dementia (BPSD), such as agitation, irritability, and wandering, AD poses an extremely heavy physical and mental burden on the patient and his or her family.

Whereas donepezil treats dementia by increasing brain acetylcholine to ease neural transmission, memantine binds to N-methyl-D-aspartate (NMDA), a receptor of the neurotransmitter glutamic acid, and

acts to prevent neuropathy caused by excess glutamic acid. Because memantine and donepezil have different mechanisms of action, memantine can be used in concomitant therapy with donepezil. The usefulness of concomitant therapy has been investigated in many clinical studies, both in Japan and abroad (Tariot *et al.*, 2004; Schmitt *et al.*, 2006). Although both medications have the effect of improving cognitive function for a certain period, and delay cognitive dysfunction, they still cannot treat the clinical condition. This makes the early identification and management of AD of great importance.

Studies using single photon emission tomography, positron emission tomography, and functional magnetic resonance image are commonly used for early diagnosis of AD and are successful up to a point. However, they require large-scale facilities and have the drawbacks of invasiveness, radiation exposure, and high cost. On the other hand, near-infrared spectroscopy (NIRS) used in this study is a technique for quantifying the state of brain activity by measuring the amount of hemoglobin in the blood of the cerebral cortex.

Memantine is known to lessen agitation and aggression. It is also reported, in some cases, to improve the frontal lobe functions of attention and executive function (Hashimoto, 2012). However, there are few study results on the effects of memantine on the burden of care, and there are no reports on this subject in Japan. A study of AD patients using NIRS reports that AD patients have lower oxygenated hemoglobin levels in the prefrontal area than in healthy individuals (Arai *et al.*, 2006; Herrmann *et al.*, 2008). However, little has been studied about the association between NIRS findings and the therapeutic effects of anti-dementia drugs. In addition, no objective AD diagnosis method has yet been established in other functional brain imaging studies.

In this study, we evaluated the effects of and relationship of memantine to cognitive function, BPSD, and care burden in moderate-to-severe AD patients being treated with donepezil. We also closely examined, using NIRS, the relationship between memantine's effects and cerebral blood flow.

Methods

Subjects

The subjects comprised 37 patients with moderate-to-severe AD (mean age 78.8 ± 7.7 years), who are outpatients being treated at the Department of Clinical Psychiatry and Neurology, Shimane University Hospital. They had been diagnosed with AD using the diagnostic criteria set out in the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV), and the International Classification of Diseases, 10th edition, scoring 3 to 16 points on Hasegawa's dementia scale-Revision, and had been treated with donepezil for 6 months or longer. The subjects were divided randomly, using a random number table, into two groups: a memantine combination donepezil group (combination group, $n = 19$, 77.9 ± 9.8 years) and a non-memantine combination donepezil group (control group, $n = 18$, 79.8 ± 4.6 years) (Table 1).

This study was conducted after a review by and approval of the Ethics Committee of Shimane University's Faculty of Medicine. Patients and their families received an explanation in writing on the purpose of the study and the content of the tests beforehand, and signed a consent form to agree to participation.

Measure methods

The combination group continued donepezil treatment and took oral memantine repeatedly for 24 weeks. Memantine administration started from 5 mg/day, with the dose was increased by 5 mg every week until reaching the maintenance dose of 20 mg/day. Clinical Global Impression - Improvement scale (CGI-I), mini mental state examination (MMSE),

Table 1 Baseline subject characteristics

		Combination group	Control group	Total	*p*-value*1
	Number of cases	19	18	37	—
Gender	Male	11	7	18	0.26
	Female	8	11	19	
Age (years)	Mean ± standard deviation	77.9 ± 9.8	79.8 ± 4.6	78.8 ± 7.7	0.45
HDS-R at enrolment (points)	Mean ± standard deviation	10.5 ± 3.5	12.3 ± 3.0	10.9 ± 3.6	0.10

HDS-R, Hasegawa's dementia scale-Revision.
*1*t*-test

clock drawing test (CDT), Neuropsychiatric Inventory (NPI), Japanese version of the Zarit Burden Interview (J-ZBI) and Near-infrared spectroscopy (NIRS) were measured four times: at enrollment, 4 weeks after enrollment, 12 weeks after enrollment, and 24 weeks after enrollment.

Primary outcome measures

Clinical Global Impression-Improvement scale evaluates how the patient's condition has deteriorated from their condition before intervention on a scale of one to seven: 1-greatly improved, 2-improved, 3-slightly improved, 4-unchanged, 5-slightly deteriorated, 6-deteriorated, and 7-greatly deteriorated. MMSE is a screening test for dementia developed by Folstein *et al.* (1975). It consists of 11 questions with a perfect score of 30 points to evaluate faculty of orientation, memorization faculty, calculating ability, linguistic competence, and graphic competence. CDT scores the dial face and clock hands drawn by the subject: it is used as simple screening test for dementia. In the past, it was thought to reflect the function of the parietal lobes (Tuokko *et al.*, 1992). However, it is now thought to be a test for a wide range of cognitive functions, including executive function. This study adopted the method to draw a clock on a sheet of blank paper according to the verbal instruction, because it is considered to best reflect frontal lobe function (Royall *et al.*, 1998). Freedman's method (Freedman *et al.*, 1994) was used for rating. NPI is based on a structured interview with a caretaker who is fully familiar with the patient's behavior, such as a spouse. NPI is highly regarded as a mental symptom assessment scale for dementia patients, and is frequently used as an indicator to evaluate the effects of anti-AD drugs in clinical trials. J-ZBI was formulated by Professor Steven Zarit at Pennsylvania State University, and is one of the most common scales for burden of care in the world (Zarit *et al.*, 1980). The ZBI is a scale that totals the physical burden, the psychological burden, and the economic burden created by nursing care and measures it as an overall care burden. It consists of 22 items. This study uses the J-ZBI prepared by a group at the National Center for Geriatrics and Gerontology (Arai *et al.*, 1997).

Near-infrared spectroscopy procedure

Near-infrared spectroscopy is a brain function measuring technique using near-infrared light. It is increasingly being employed in clinical applications because of its greater ease of use than other brain function measuring techniques. Measurements can be taken with the patient in any posture or even while in motion. For quantifying brain activity status, changes in oxygenated hemoglobin [Oxy-Hb] and deoxygenated hemoglobin [Deoxy-Hb] concentrations, both of which are dependent on local cerebral blood flow change induced by the cerebrocortical neuron activities, are measured by exposing the head to near-infrared light and measuring the reflected light. It enables non-invasive and easy viewing of changes in blood flow in the cerebral cortex.

This study adopted a standard procedure for NIRS established by Takizawa *et al.* (2008). Using an ETG-4000 (Hitachi Medical, Tokyo, Japan), the bases of the measurement probes for the frontal region were placed at positions Fp1 and Fp2 (International 10–20 system). With the subject wearing a 22-channel probe on the frontal region, changes in the hemoglobin content of the blood in the cerebral cortex were measured while performing a verbal fluency task, which is a frontal cortex-activating task. As an activation task, each subject was instructed to say as many words as they could come up with, starting with the initial letter spoken by the tester. The initial letters were changed every 20 s, and the activation task continued for 60 s. Before and after the activation task, the subjects were instructed to say 'A, I, U, E, O' aloud repeatedly to establish the baseline condition.

Statistical analysis

To examine the effect of memantine on the test results, two-way repeated-measures analysis of variance was performed on the changes in the scores of the combination group and the control group (change in value of the integral of the results of NIRS measurement) at 4 weeks, 12 weeks, and 24 weeks. To examine in detail the differences between the groups, a multiple comparison using Bonferroni's method was performed. Channels including artifacts and noise (low S/N) because of body motion were excluded from the results of NIRS measurement, and inter-channel influence was corrected using the false discovery rate method (two-tailed; we set the value of q specifying the maximum false discovery rate to 0.05, so that there are no more than 5% false positives on average) because we performed 22 paired t-tests. For each endpoint of effectiveness and safety, the significance level of the two-sided test was set to 0.05. We then compared each of the two groups by using Student's t-test, and the significance level in the analysis was set to $p = 0.05$. The Pearson correlation coefficient was used to examine the relationship between the tests.

Results

There were 37 patients enrolled in total. The full analysis set for efficacy analysis included 25 cases, except for the patients excluded as shown in Figure 1. The individual reasons for excluding 12 patients are listed in Figure 1. Change from the baseline for each endpoint is shown in Table 2.

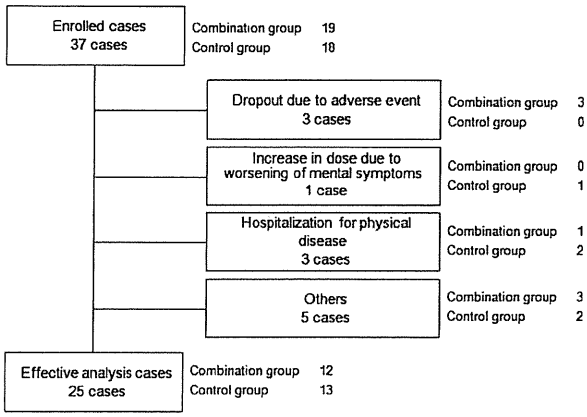


Figure 1 Flow chart of analysis case. A total of 37 subjects were enrolled. The full analysis set for effectiveness analysis included 25 cases with the exception of the excluded cases.

Table 2 Change from the baseline and results of analysis of variance

	Change from the baseline (24 weeks later)				
	Combination group		Control group		*p*-value*1
	(*n* = 12)		(*n* = 13)		
Endpoint	Mean	SD	Mean	SD	
CGI-I	−0.83	1.19	1.77	0.93	<0.001***
MMSE	0.83	2.52	−3.38	3.25	0.002**
CDT	1.67	3.14	−1.92	2.39	0.003**
NPI	−0.33	4.33	23.38	15.54	<0.001***
J-ZBI	−0.33	10.87	18.23	7.89	<0.001***
NIRS (Mean of all channels)	7.85	46.34	−30.12	45.35	0.643

CGI-I, Clinical Global Impression-Improvement scale; MMSE, mini mental state examination; CDT, clock drawing test; NPI, Neuropsychiatric Inventory; J-ZBI, Japanese version of the Zarit Burden Interview ; NIRS, near-infrared spectroscopy.
*1 *t*-test
** $p < 0.01$,
*** $p < 0.001$.

Combinational effect of memantine on global symptoms and cognitive function

As concerns the evaluation of global symptoms, there was significant interaction between the control group and the combination group in CGI-I ($F = 39.22$ (1, 23), $p < 0.001$). Regarding cognitive function, there was significant interaction between the combination group and the control group in MMSE ($F = 14.65$ (1,23), $p = 0.001$) and in CDT ($F = 12.61$ (1,21), $p = 0.002$) (Figure 2). Figure 2 shows examples of CDT drawings before and after administration of memantine.

Combinational effect of memantine on behavioral and psychological symptoms of dementia

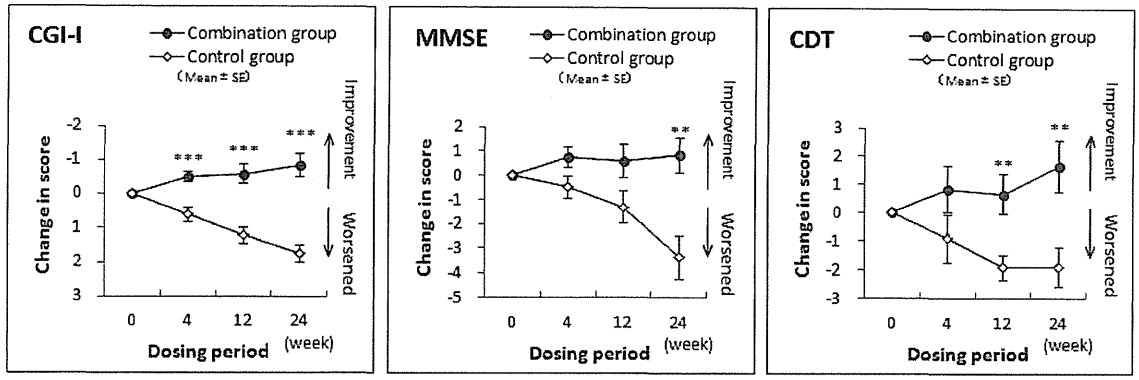
Regarding BPSD assessment, there was significant interaction between the combination group and the control group in NPI ($F = 22.24$ (1,23) , $p < 0.001$). As to item-by-item score of NPI, a significant difference between the combination group and the control group was observed in delusion, agitation, depression and dysphoria, anxiety, inaction and apathy, irritability and instability, and abnormal behavior (Figure 3).

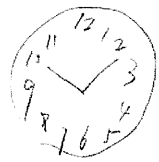
Combinational effect of memantine on care burden

Regarding care burden assessment, there was significant interaction between the combination group and the control group in J-ZBI ($F = 14.77$ (1,23), $p < 0.001$). A significant difference was observed in 8 out of 22 sub-items of J-ZBI (Figure 3).

Change in near-infrared spectroscopy measurements

Regarding NIRS measurement, change in value of Oxy-Hb integral from week 0 was compared between the combination group and the control group. There was no significant interaction in total value of Oxy-Hb integral from of all channels. However, a significant difference between the administered group and the control group was observed at the 24th week in CH5 (right middle frontal gyrus), CH7, and CH8 (left middle frontal gyrus) (*t*-test). Figure 4 show the changes at the 24th week from the baseline (week 0). A significant positive correlation was observed between the mean of the three channels and MMSE score and CGI-I score.



Case 1	Baseline	24 weeks after administration
Drawings		
CDT Score	8 points	15 points

Case 2	Baseline	24 weeks after administration
Drawings		
CDT Score	7 points	14 points

Figure 2 Assessment of global symptoms (Clinical Global Impression-Improvement scale (CGI-I)) and cognitive function (mini mental state examination (MMSE) and clock drawing test (CDT)). *: $p < 0.0125$, **: $p < 0.01$, ***: $p < 0.001$ by a multiple comparison using Bonferroni's method. A significant difference in CGI-I was observed between the combination group and the control group from the fourth week. A significant difference in MMSE was observed between the combination group and the control group from the 24th week. A significant difference in CDT was observed between the combination group and the control group from the 12th week. Examples of drawings for the CDT before and 24 weeks after memantine administration are shown. Before administration, Case 1 was not able to draw a round frame smoothly and only drew two number 10s, in spite of being asked to draw the clock's hands showing 10 minutes past 10. Case 2 before administration drew a circle that was small relative to the size of the paper and with the clock's hand pointing in the wrong direction. Cases 1 and 2 were both able to draw a clock correctly 24 weeks after administration.

Relationship among the measurements

A study of the relationships among the tests revealed a significant and relatively strong positive correlation between MMSE score and CDT score ($r = 0.614$, $p < 0.001$). A significant and relatively strong positive correlation was also observed in NPI score and J-ZBI score ($r = 0.666$, $p < 0.001$). A significant positive correlation was observed between mean change in values of Oxy-Hb integral in CH5, CH7, and CH8, and MMSE and CGI-I scores (MMSE: $r = 0.433$, $p = 0.003$, Pearson correlation coefficient, CGI-I: $r = -0.300$, $p = 0.045$, Pearson correlation coefficient). A significant positive correlation was also observed between the number of words spoken while performing the verbal fluency task and MMSE score and CDT score ($r = 0.498$, $p < 0.001$, and $r = 0.409$, $p = 0.001$, respectively).

Safety

Of the adverse events that occurred during the study, a causal relationship with memantine could not be ruled out in three cases and thus led to discontinuation of the drug. The most frequent adverse event was gait instability (2 cases, 10.5% of the combination group).

Regarding changes in laboratory data, alanine aminotransferase elevated from 14 to 51 in one case in the control group. Its relationship with this study is not known. There were no other abnormal values.

Discussion

Memantine, a non-competitive NMDA receptor antagonist with an adamantine skeleton, was launched in 2011. Because it forms a low-affinity bond with NMDA receptors, it acts to protect nerve cells from excessive glutamate stimulation; however, it exerts little influence on normal glutamate-mediated neurotransmission (Schmidt *et al.*, 2008). Memantine has a preventive and improving effect on wandering, stereotypic behavior, and agitation/aggression, in addition to a suppressive action on deterioration of cognitive function (Cummings *et al.*, 2006). It is reported that

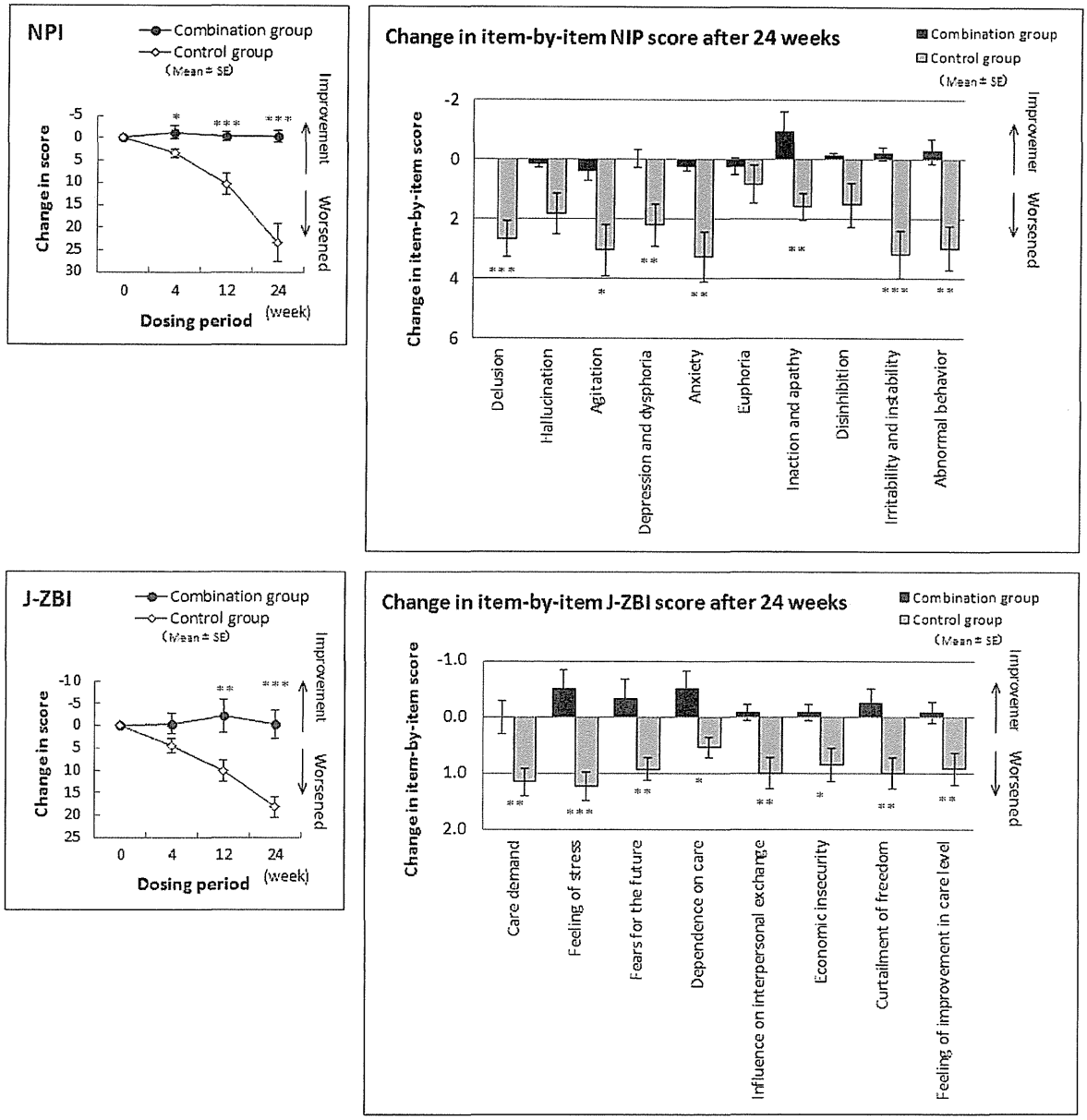
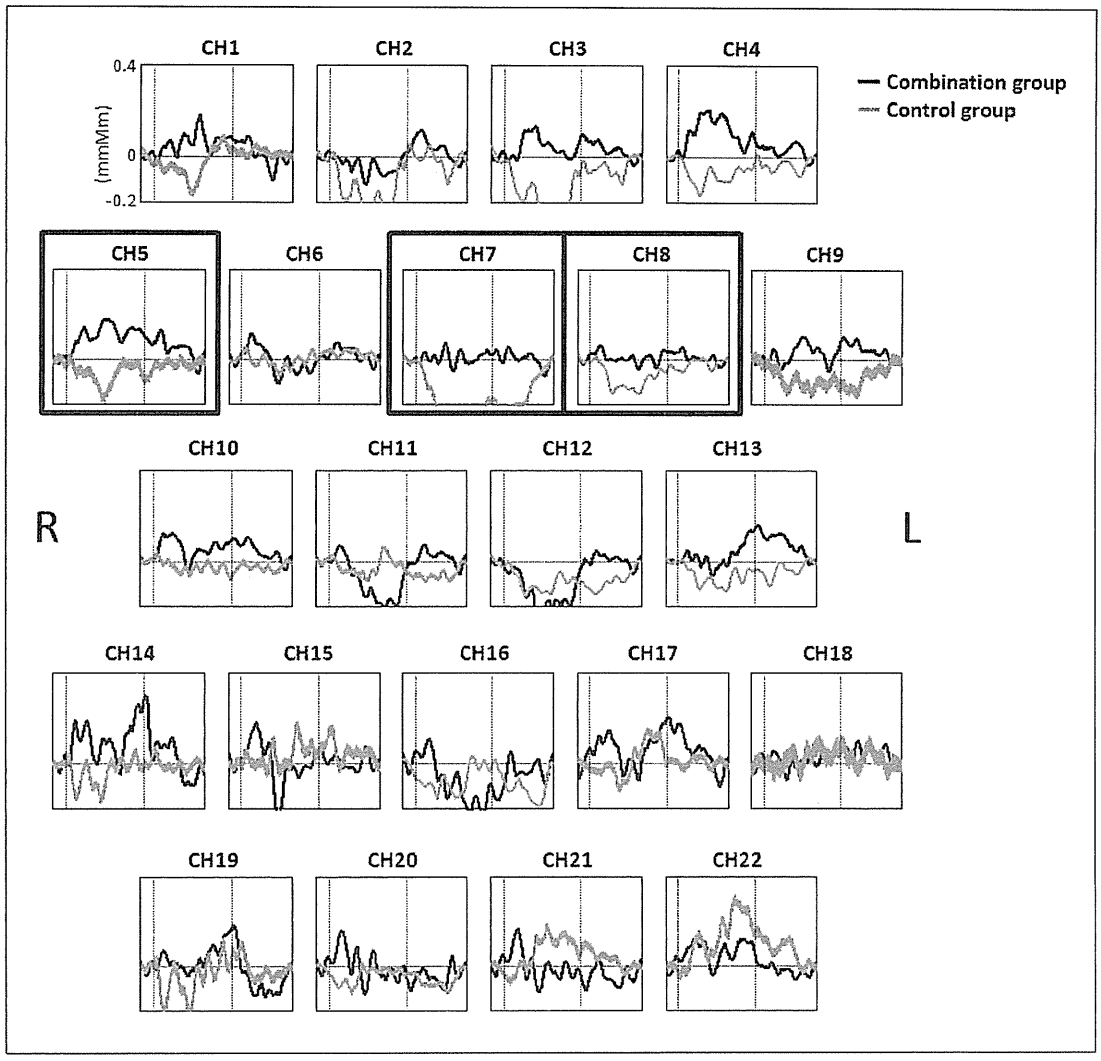


Figure 3 Assessment of behavioral and psychological symptoms of dementia (Neuropsychiatric Inventory (NPI)) and Care burden (Zarit Burden Interview (J-ZBI)). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$: by a multiple comparison using Bonferroni's method. A significant difference in NPI was observed between the combination group and the control group from the 12th week. Regarding item-by-item scores for NPI, a significant difference between the combination group and the control group was observed at the 24th week after administration in delusion, agitation, depression and dysphoria, anxiety, inaction and apathy, irritability and instability, and abnormal behavior. A significant difference in J-ZBI was observed between the combination group and the control group from the 12th week by Bonferroni's comparison. A significant difference was observed between the combination group and the control group in eight of 22 J-ZBI sub-items.

memantine exerts these effects even in combination with cholinesterase inhibitors such as donepezil (Tariot *et al.*, 2004; Atri *et al.*, 2013). Memantine is often used in concomitant therapy with cholinesterase inhibitors, because it has an entirely different mechanism of action. In this study, we evaluated the efficacy of memantine when given concomitantly with a cholinesterase inhibitor, donepezil, by use of rating scales for cognitive function, BPSD, and care burden.

Regarding change over time in CGI-I score, in assessment of global symptoms, a significant difference between the combination group and the control group



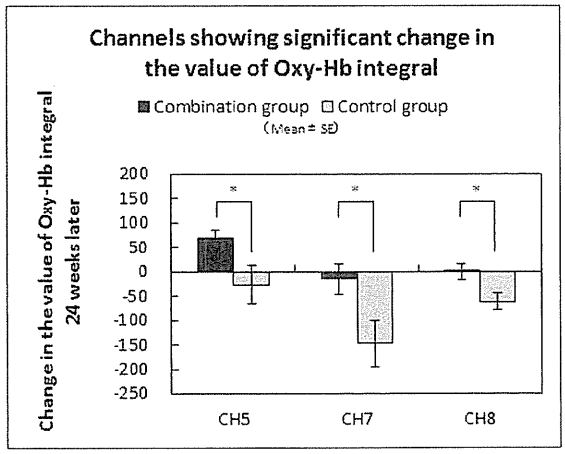


Figure 4 Change in value of Oxy-Hb integral in NIRS (Change from 0 to 24th week). Changes in value of Oxy-Hb integral during task interval of 0 to 24th week compared between the combination group and the control group. NIRS measurements showed a significant difference between the administered group and the control group in CH5 (right middle frontal gyrus), CH7 and CH8 (left middle frontal gyrus) (CH5: $p = 0.026$, CH7: $p = 0.040$, CH8: $p = 0.025$) by a multiple comparison using Bonferroni's method.

was observed from the 4th week. In other words, worsening of global symptoms was more significantly controlled in the combination group than in the control group.

Next, in cognitive function assessment, significant differences in MMSE and in CDT were observed between the combination group and the control group from the 24th week and 12th week, respectively. Memantine appears to inhibit overactivation of NMDA receptors, thus protecting neurocytes and limiting memory loss and learning disorders. Although it is known that, in early-stage AD, working memory is impaired by a loss of neuronal cells in the hippocampus, several reports note that MRI revealed a slowing of hippocampal atrophy after 1 year of administration of memantine (Schmidt *et al.*, 2008; Weiner *et al.*, 2011). Our study also suggests the efficacy of memantine with respect to cognitive dysfunction, one of the core symptoms of AD. The fact that memantine can inhibit worsening of cognitive function in patients with AD has an important clinical implication.

Regarding change over time of NPI score used to assess BPSD, a significant difference was observed between the combination group and the control group from the 12th week. As to item-by-item score of NPI, a significant difference between the combination group and the control group was observed at the 24th week after administration in delusion, agitation, depression and dysphoria, anxiety, inaction and apathy, irritability and instability, and abnormal behavior. Major neuropathological changes in AD are neuritic plaque and neurofibrillary tangle (NFT), and it has been pointed out that NFT is related to the occurrence of psychological symptoms (Farber *et al.*, 2000). NFT is composed of abnormally phosphorylated Tau protein. Because memantine inhibits abnormal phosphorylation, it is thought to lower the amount of NFT with relation to mental symptoms and to be effective against BPSD. Memantine is also reported to have a more preferential action on BPSD associated with frontal lobe function14. There are many reports from clinical investigations on the effectiveness of memantine in BPSD, especially for agitation and irritability. The results of this study support those reports. Because inaction and apathy in sub-items were significantly inhibited in the combination group, memantine is thought to have a possible involvement in improvement of frontal lobe function.

Regarding change over time in J-ZBI score for assessing care burden, a significant difference was observed between the combination group and the control group from the 12th week. Regarding item-by-item scores, significant differences from the control group were observed in the following items: care demand, feeling of stress, fears for the future, dependence on care, influence on interpersonal exchange, economic insecurity, influence on freedom in life, and improvement in care level. It has been pointed out that BPSD sometimes causes family dysfunction and wrecks home nursing care (Asada, 1991). The correlation found between NPI and J-ZBI in this study suggests that reduced BPSD feeds through to a lower care burden. Based on these findings, it appears that dementia medications such as memantine lighten the burden of care because of improvement of cognitive function, increased activity, and stabilization of emotion.

Regarding cerebral blood flow in frontal cortex, significant inhibition of reduction of cerebral blood flow was observed at several channels, although chiefly localized with the middle frontal gyrus. We might infer from those findings that a slower decline in cognitive function results in a lighter care burden. The correlation between the channels showing a significant increase in NIRS and MMSE score and CGI-I score suggests that NIRS measurement might be useful for determining therapeutic efficacy.

Regarding the association between these tests, the significant positive correlation between the number of words spoken while performing verbal fluency tasks and MMSE and CDT scores suggests the potential usefulness of verbal fluency tasks as dementia screening tests. Verbal fluency tasks are incorporated into frontal lobe function testing in many countries and are used as ancillary tests to diagnose or determine the severity of dementia. Because these tests are very easy to apply and have other benefits, we conclude that measuring NIRS while the subject is undergoing a verbal fluency task may be a useful dementia screening test.

Limitations

The results of this study must be viewed in light of some limitations. First, because the analysis was based on cross-sectional data, causality cannot be determined. And we should pay attention to the statistical significance corresponding to the clinical importance. So, longitudinal studies are needed to assess cause-and-effect relationships. Second, our sample size was not large, and although the significant difference by the correction for multiple comparisons was possibly interpretable as a treatment effect, the physiological findings in which only 3 of 22 channels differentiated the two groups might be a type 1 error. Further studies with larger number of patients are required. Finally, regarding the safety of memantine, no adverse events with a 10% or higher incidence are observed,

according to a report examining memantine that refers to the package inserts of overseas anti-AD drugs (Jones RW, 2010). However, gait instability occurred in 10.5% of the subjects in our study. It is possible, however, that differences in assessment procedures and the small number of patients in this study may have influenced the results. As our next step, we regard it necessary to carry out further examinations of the clinical characteristics of memantine as an anti-dementia drug using a larger test population.

Conclusion

Moderate to severe AD is not only agonizing to the patient but also places a heavy burden on his or her caretakers. The results of this study suggest that memantine administered to patients with moderate-to-severe AD could inhibit the reduction of cerebral blood flow in the prefrontal area, improve global clinical symptoms, cognitive function, and BPSD; and lighten the burden of care placed on caretakers. The next step will be to conduct a large-scale study with an increased number of cases.

Conflict of Interest

None declared.

Key points

- In this study, we evaluated the effect of memantine on cognitive function, BPSD and the care burden, in patients with moderate-to-severe Alzheimer's disease (AD).
- Furthermore we examined the association between effect of memantine and brain blood flow with Near-Infrared Spectroscopy (NIRS).
- By administering memantine in AD patients, it was suggested to be improved clinical symptoms overall, cognitive function and BPSD, thereby reducing the care burden of caregivers.
- Moreover the reduction of cerebral blood flow in the prefrontal area might to be inhibited by the combination of memantine.

Acknowledgements

Funding for this study came from a Junior Scientist from the Shimane University Faculty of Medicine, and Health and Labour Sciences Research Grants, Japan.

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Progress in Neuro-Psychopharmacology & Biological Psychiatry 50 (2014) 184–190

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Minocycline improves recognition memory and attenuates microglial activation in Gunn rat: A possible hyperbilirubinemia-induced animal model of schizophrenia

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ARTICLE INFO

Article history:
Received 11 September 2013
Received in revised form 17 December 2013
Accepted 18 December 2013
Available online 2 January 2014

Keywords:
Activated microglia
Behavioral test
Gunn rat
Minocycline
Schizophrenia
Unconjugated bilirubin

ABSTRACT

Background: Accumulating evidence indicates that neuroinflammation plays a significant role in the pathophysiology of schizophrenia. We previously reported evidence of schizophrenia-like behaviors and microglial activation in Gunn rats. We concluded that the Gunn rat, which exhibits a high concentration of unconjugated bilirubin, may be useful as an animal model of schizophrenia. On the other hand, there have been numerous reports that minocycline is effective in treating schizophrenia.

Methods: In the present study, we investigated the effects of minocycline on performance of behavioral tests (prepulse inhibition (PPI) and novel object recognition test (NORT)) after animals received either 40 mg/kg/d of minocycline or vehicle by intraperitoneal (i.p.) injection for 14 consecutive days. Furthermore, we examined the effects of minocycline on microglial activation in the hippocampal dentate gyrus of Gunn rats and Wistar rats.

Results: We found that administration of minocycline for 14 days significantly increased the exploratory preference in retention sessions and tended to improve the PPI deficits in Gunn rats. Immunohistochemistry analysis revealed that microglial cells in the minocycline-treated Gunn rat group showed less expression of CD11b compared to vehicle-treated Gunn and Wistar groups.

Conclusions: Our findings suggest that minocycline improves recognition memory and attenuates microglial activation in the hippocampal dentate gyrus of Gunn rats. Therefore, minocycline may be a potential therapeutic drug for schizophrenia.

1. Introduction

Schizophrenia is one of the most mysterious and costliest mental disorders in terms of human suffering and societal expenditures (van Os and Kapur, 2009). Despite extensive research and remarkable advances in the neurobiological, neurochemical and genetic aspects of this disabling mental illness, the underlying etiological processes remain a challenge for clinicians and basic researchers alike (Meyer, 2013). Moreover, this imperfect understanding of the etiology has been associated with poor treatment outcomes for many individuals (Dean et al., 2012). Therefore, a novel insight into the underlying mechanism of this illness is an urge to allow new treatment targets to be explored.

An accumulating body of evidence points to the significant role of neuroinflammation and the immune system in the pathophysiology of schizophrenia (Takahashi and Sakurai, 2013). There are also numerous reports that support the hypothesis that infection/inflammation is a risk for developing schizophrenia later in life (Kneeland and Fatemi, 2013; Mednick et al., 1988; Miller et al., 2013). Moreover, evidence from genomic (Ripke et al., 2011; Schwab et al., 2002), blood (Erbagci et al., 2001; Miller et al., 2011), postmortem (Fisman, 1975; Radewicz et al., 2000; Steiner et al., 2008), and in vivo imaging (Doorduin et al., 2009; van Berckel et al., 2008) studies are leading toward a greater consensus that immune activation is involved in the pathophysiology of

Abbreviations: UCB, unconjugated bilirubin; PPI, prepulse inhibition; NORT, novel object recognition test; i.p., intraperitoneal; Iba1, ionized calcium binding adaptor molecule 1; ITGAM, integrin alpha M; CD11b, cluster of differentiation molecule 11B; DG, dentate gyrus; SGZ, subgranular zone; GL, granular layer; ML, molecular layer; PB, phosphate buffer; PBS, phosphate buffer saline; ABC, avidin–biotin peroxidase complex; ANOVA, analysis of variance.

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0278-5846/$ – see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.pnpbp.2013.12.017

schizophrenia. Interestingly, some anti-psychotics may have anti-inflammatory effects (Bian et al., 2008; Kato et al., 2007) and some anti-inflammatory agents may have anti-psychotic effects (Laan et al., 2010; Muller and Schwarz, 2008).

Minocycline is a second generation of the antibiotic, tetracycline, and is one of the most promising neuroprotective and anti-inflammatory agents currently in clinical and experimental trials (Miyaoka, 2012). Our previous clinical studies showed the potential of minocycline as an adjunctive treatment in schizophrenia patients (Miyaoka et al., 2007, 2008). Similar results were also found in various animal models of psychosis or schizophrenia. Minocycline was reported to attenuate behavioral impairment as well as neurotoxicity after administration of methamphetamine (Mizoguchi et al., 2008; Zhang et al., 2006a), 3,4-methylenedioxymethamphetamine (MDMA) (Zhang et al., 2006b), N-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine (Levkovitz et al., 2007; Zhang et al., 2007), and viral mimetic polyriboinosinic-polyribocytidilid acid (Poly I:C) (Juckel et al., 2011).

Our previous studies generated Gunn rat (Gunn, 1944) as a possible animal model of schizophrenia. First, we reported behavioral abnormalities, deficits in prepulse inhibition (PPI), and neuropathological changes in Gunn rats that are similar to the characteristics of schizophrenia (Hayashida et al., 2009). Then, we demonstrated evidence of activated microglial cells in the hippocampal dentate gyrus (DG) of Gunn rats (Liaury et al., 2012). Furthermore, anti-psychotic medications affected Gunn rat behaviors in a way similar to their effect on schizophrenic behaviors (Tsuchie et al., 2013). We proposed that hyperbilirubinemia (a high level of unconjugated bilirubin, UCB) leads to chronic neuroinflammation and has a pathogenic effect on the development of the brain and concluded that the Gunn rat may be used as an animal model of schizophrenia.

In the present study, we sought to investigate the effects of minocycline on Gunn rat, a possible hyperbilirubinemia-induced animal model of schizophrenia. We hypothesized that minocycline may improve behavioral disturbances and attenuate microglial activation in Gunn rats. First, we performed behavior tests (prepulse inhibition (PPI) and novel object recognition test (NORT)). Then, using immunohistochemistry analysis, we examined the microglial activation in hippocampal DG of Gunn rats.

2. Methods

2.1. Animals

The animals were male homozygous (j/j) Gunn rats (6 weeks old, 160–200 g body weight) and male Wistar rats (6 weeks old, 200–240 g body weight) at the beginning of the experiment (Japan SLC, Inc., Shizuoka, Japan). Animals were housed under standard conditions with a room temperature of 23 ± 2°C, humidity of 55 ± 5%, and 12 h light/12 h dark cycle (light phase 7:00 to 19:00) with free access to food and water. All procedures were performed with the approval of the Shimane University Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Japan.

2.2. Drug administration

Minocycline hydrochloride (Sigma-Aldrich, St. Louis, Mo., USA) was freshly prepared as a stock solution of 5 mg/ml (10 mM) in filtered PBS and stored frozen at −80 °C every 7 days. We adjusted the acidic pH (pH 4.0) of minocycline hydrochloride to neutrality by adding sodium hydroxide [as described by (Ferretti et al., 2012)]. As the vehicle, we use the filtered PBS, pH 7.4.

Animals were divided into four groups: Wistar + vehicle (WV) group, Wistar − minocycline (WM) group, Gunn + vehicle (GV) group, and Gunn − minocycline (GM) group. Each animal received either 40 mg/kg/d of minocycline or vehicle by intraperitoneal (i.p.) injection for 14 consecutive days. The dose (40 mg/kg) of minocycline was selected based on the fact that this dose was effective in improving the methamphetamine-induced impairment of recognition memory (Mizoguchi et al., 2008) as well as prepulse inhibition deficits after the administration of the NMDA receptor antagonist dizocilpine (Zhang et al., 2007). To avoid stress or pain due to the intraperitoneal injection, animals were anesthetized by putting them into a halothane inhalation chamber for less than 1 min before the daily injection.

2.3. Prepulse inhibition (PPI) of startle response

A startle response system was used (SR-LAB, San Diego Instrument) as reported previously (Hayashida et al., 2009). Briefly, each rat was placed in a Plexiglass cylinder where it was exposed to white background noise at 65 dB for a 5 min acclimatization period. This was followed by four types of trials: (1) pulse (P) alone, consisting of a 20 ms burst of white noise at 120 dB; (2) a 20 ms burst of white noise at 70 dB followed by a 20 ms white noise at 120 dB (70 PP − P); (3) a 20 ms burst of white noise at 80 dB followed by a 20 ms white noise at 120 dB (80 PP − P); and (4) background noise only (no stimulus). Our pre-experiment (unpublished data) found that Gunn rat showed a sensitive response and behavior, therefore the use of three or four different prepulse intensities affected the behavior test and biased the result. In consequence, we decided to use just 2 prepulse intensities (70 dB and 80 dB). The interval between prepulse and pulse was set at 100 ms. Trials were given in a pseudo-random order with variable intervals (20 s–60 s, average 40 s) between each trial. Startle response was measured in sessions consisting of 50 trials. The percentage of PPI (%PPI) was defined as the magnitude of inhibition due to the startle amplitude that was induced by the prepulse. %PPI = (1 − (startle magnitude after prepulse–pulse pair / startle magnitude after pulse-only)) × 100.

2.4. Novel object recognition test (NORT)

The NORT procedure consisted of three sessions: habituation, training, and retention. The experimental apparatus consisted of a Plexiglas open field box (42 × 42 × 42 cm) with a sawdust-covered floor located in a sound attenuated room. Each animal was individually habituated to the box, with 15 min of exploration in the absence of objects for two consecutive days (habituation session, days 1–2). During the training session (day 3), two identical objects were symmetrically fixed to the floor of the box, 5 cm from the walls, and each animal was allowed to explore freely for 2 × 5 min. An animal was considered to be exploring the object when its head was facing the object (a distance between the head and object of approximately 1 cm or less) or it was touching or sniffing the object. In the retention session (day 4), one of the familiar objects used during the training session had been replaced with a novel object. The novel object was different in shape and color, but similar in size to the familiar object. The animal was then allowed to explore freely for 5 min. All sessions were videotaped for later examination. The exploratory preference index in the retention session, a ratio of the amount of time spent exploring the novel object over the total time exploring both objects, was used to measure cognitive function. In the training session, the exploratory preference index was calculated as the ratio of time spent exploring the object that was replaced by the novel object in the retention session over the total exploration time.

2.5. Immunohistochemistry for light and confocal laser scanning microscopy

Soon after finishing the behavior test, animals underwent deep intraperitoneal anesthesia with sodium pentobarbital (80 mg/kg body weight) and were perfused transcardially with 500 ml of physiological