the 18 patients with no mutation were 218.5 ± 16.1% (1 month) and 235.7 ± 16.2% (2 months). The Werner patient's relative responses were similar (261.6%, 1 month; 224.1%, 2 months). Serum adiponectin multimer concentrations in response to 15 mg/day of pioglitazone in the diabetic patients without an adiponectin mutation were as follows: 3.11 ± 0.60 μg/ml (pretreatment), 7.75 ± 1.60 μg/ml (1 month), and 7.75 ± 1.94 μg/ml (2 months); in the Werner patient, these, respectively, were 0.57, 2.34, and 2.12 μg/ml (Fig. 2B). For the multimeric form, relative responses in the18 patients were 284.8 ± 25.9% (1 month) and 326.4 ± 35.7% (2 months). In the Werner patient, these, respectively, were 410.5% and 371.9%.

3. Discussion

The adiponectin I164T mutation has been reported to interfere with adiponectin secretion in transfected cultured cells [7,8]. Kadowaki et al. reported that I164T adiponectin could not assemble into trimers, resulting in impaired secretion from the cell [7]. Another study using gel filtration reported that oligomerization was similar to that seen in wild-type adiponectin, but secretion from adipocytes into plasma was disrupted [8]. In our patient's response to pioglitazone, the serum adiponectin concentration was only half that seen in diabetic patients without mutation of the adiponectin gene, suggesting that secretion of mutant adiponectin from adipose tissues into plasma might be disturbed, and with only the wild-type adiponectin responding. The absolute change in serum concentration of adiponectin multimer, measured in response to pioglitazone, was slightly less than that of the monomer in the Werner patient compared with the other 18 diabetic patients, suggesting that processing of mutatnt adiponectin monomer to high-molecular-weight multimer might be compromised.

Here, we first reported a Werner syndrome patient with an additional mutation involving the adiponectin gene. Our study suggested that despite some differences between monomeric and multimeric forms, serum concentrations of both forms of adiponectin could be increased by treatment with thiazolidine derivatives in patients with hypoadiponectinemia resulting from a heterozygous adiponectin gene mutation. These and future data concerning long-term effects on atherosclerosis in this patient may be informative concerning the pathogenesis and treatment of atherosclerosis associated with hypoadiponectinemia and insulin resistance.

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VII. 特　　論

Werner症候群とメタボリックシンドローム

Werner syndrome and metabolic syndrome

横手幸太郎

Key words：Werner症候群，早老症，ヘリカーゼ，lamin A，老化

1. Werner症候群とは

Werner症候群は，1904年ドイツの眼科医Otto Wernerにより‘強皮症を伴う白内障の一例’として初めて報告された代表的な遺伝的早老症である．臨床症状として，①低身長，②皮膚の萎縮・角化・潰瘍，③四肢の筋・脂肪組織の萎縮，④毛髪の変化(白髪・禿頭)，⑤音声の変化(高調性嗄声)，⑥白内障，⑦高インスリン血症を伴う耐糖能異常，⑧性腺機能低下，⑨軟部組織石灰化，⑩悪性腫瘍合併，⑪骨粗鬆症，などが知られ[1]，毛髪変化をはじめとする早老様徴候が20歳頃からみられるようになる．本症候群は常染色体劣性の遺伝形式をとり，第8染色体短腕に位置するRecQ型DNAヘリカーゼ(WRNヘリカーゼ)のホモ接合型遺伝子変異が原因である[2]．DNAヘリカーゼの異常に起因する疾患としては，ほかにBloom症候群，Cockayne症候群，Rothmund-Thomson症候群，色素性乾皮症などがある．

我が国におけるWerner症候群の頻度は100万人に1-3人といわれ，これまでは主に近親婚の多い地域で報告されてきた．しかし，神奈川県内で行われた研究によると，対象となった一般住民1,000人のうち少なくとも6人がWRN遺伝子の変異をヘテロ接合体として保有しており，単純に計算すれば，毎年少なくとも23人のホモ接合体(すなわちWerner症候群患者)が我が国で出生することが予測される[3]．

2. Werner症候群と早発性粥状動脈硬化

Werner症候群患者の平均寿命は47歳といわれている．その二大死因は間葉系細胞に由来する悪性腫瘍と心筋梗塞であり[1]，本症候群では同年代の健常者に比べて粥状動脈硬化が進みやすいと考えられている[4]．その機序についてはこれまでに様々な報告がある．例えば，本症候群の患者では低比重リポ蛋白(LDL)受容体の活性低下により動脈硬化の主要危険因子である高LDL血症を伴いやすく[5]，血栓形成を促進するPAI-I(plasminogen activator inhibitor-I)や白血球接着分子ICAM-1(intercellular adhesion molecule-1)の可溶型[6]，細胞の遊走や増殖に関与するフィブロネクチンなどがいずれも血中で高値を示す[7]．

また，Werner症候群はインスリン抵抗性を伴いやすいことから，代償性の高インスリン血症やそれに引き続いて生じる糖尿病[8]もまた動脈硬化の進展に寄与すると考えられる．一方，Werner症候群ではない心筋梗塞患者を対象とした検討から，ある種のWRN遺伝子多型が，糖尿病の合併とは無関係に心筋梗塞のリスク増加と関連することも示されており[9]，未知の機序を示唆する成績として興味がもたれる．

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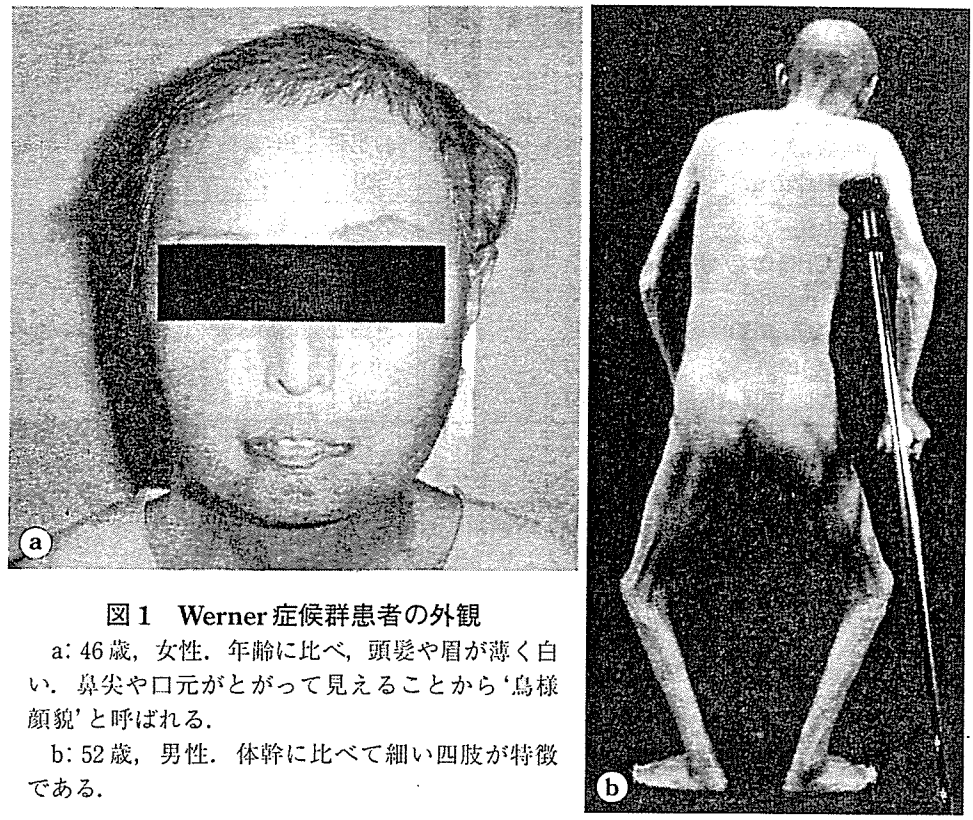

図1 Werner症候群患者の外観

a: 46歳，女性．年齢に比べ，頭髪や眉が薄く白い．鼻尖や口元がとがって見えることから'鳥様顔貌'と呼ばれる．

b: 52歳，男性．体幹に比べて細い四肢が特徴である．

3. Werner症候群にみられるメタボリックシンドローム様の病態

a. 腹部に限局した脂肪の蓄積

内臓型肥満とインスリン抵抗性を基盤に耐糖能障害，血圧高値，脂質代謝異常など複数の代謝性危険因子を合併し，粥状動脈硬化や糖尿病の発症リスクが高い病態としてメタボリックシンドロームが注目されている．一般にWerner症候群患者は枝のように細い上下肢を呈し(図**1-b**)，体格的には'肥満(body mass index: BMI ≧25)'に該当しないことが多い．しかし，体幹部の脂肪組織は通常保たれているため，著者らは，代謝性危険因子の重積と内臓脂肪蓄積の観点からWerner症候群患者を改めて評価することにした．

遺伝子検索により確定診断を得た当院外来通院中のWerner症候群患者5人を対象に検討したところ[10](**表1**)，全例に高トリグリセリド血症，高インスリン血症とヘモグロビンA_{1c}の高値を認め，うち2例は高血圧を合併していた．BMIは5例中3例が正常範囲，2例が18.5以下の低値(すなわち'やせ')を示し，肥満はみられなかった．ところが，臍高部のX線CT撮影による腹部脂肪の評価では，5例中3例が内臓脂肪面積100 cm^2以上と内臓型肥満に相当し，残る2例も内臓脂肪/皮下脂肪面積比(V/S比)が0.4を大きく上回った．したがって，これらの患者は絶対的もしくは相対的な内臓脂肪蓄積状態にあると推察された．

b. 血中アディポサイトカイン異常とチアゾリジン誘導体の効果

Werner症候群における代謝性危険因子の重積に，腹部内臓脂肪蓄積との関連が示唆されたため，病態を理解する目的で，Werner症候群患者の血中アディポサイトカイン濃度を検討した．すると，本症候群患者では年齢をマッチさせた健常対照者に比べてTNF-α値が有意に高いこと(**図2-a**)，糖尿病を発症した本症候群の患者ではアディポネクチン値が著しい低値を示すことがわかった[11]．すなわちWerner症候群は，インスリン抵抗性と内臓脂肪の蓄積，耐糖

表1 Werner症候群患者の糖・脂質プロファイルと内臓脂肪

症 例	1	2	3	4	5
年齢(歳)/性別	52/男性	57/女性	54/男性	39/女性	46/女性
BMI(kg/m²)	21	17 ↓	20	20	17 ↓
高血圧	あ り	な し	な し	あ り	な し
T-CHO(mg/dl)	353 ↑	297 ↑	163	210	270 ↑
TG(mg/dl)	530 ↑	340 ↑	180 ↑	410 ↑	300 ↑
FPG(mg/dl)	92	98	128 ↑	210 ↑	198 ↑
HbA_{1c}(%)	6.0 ↑	7.2 ↑	6.8 ↑	7.4 ↑	8.4 ↑
空腹時 IRI(μU/ml)	20 ↑	28 ↑	70 ↑	14 ↑	28 ↑
内臓脂肪面積(cm²)	175 ↑	96	75	134 ↑	112 ↑
V/S比(基準値<0.4)	1.5 ↑	2.2 ↑	2.6 ↑	0.9 ↑	0.7 ↑

BMI: body mass index, V: 内臓脂肪面積, S: 皮下脂肪面積.
(文献[10]および未発表データより作成)

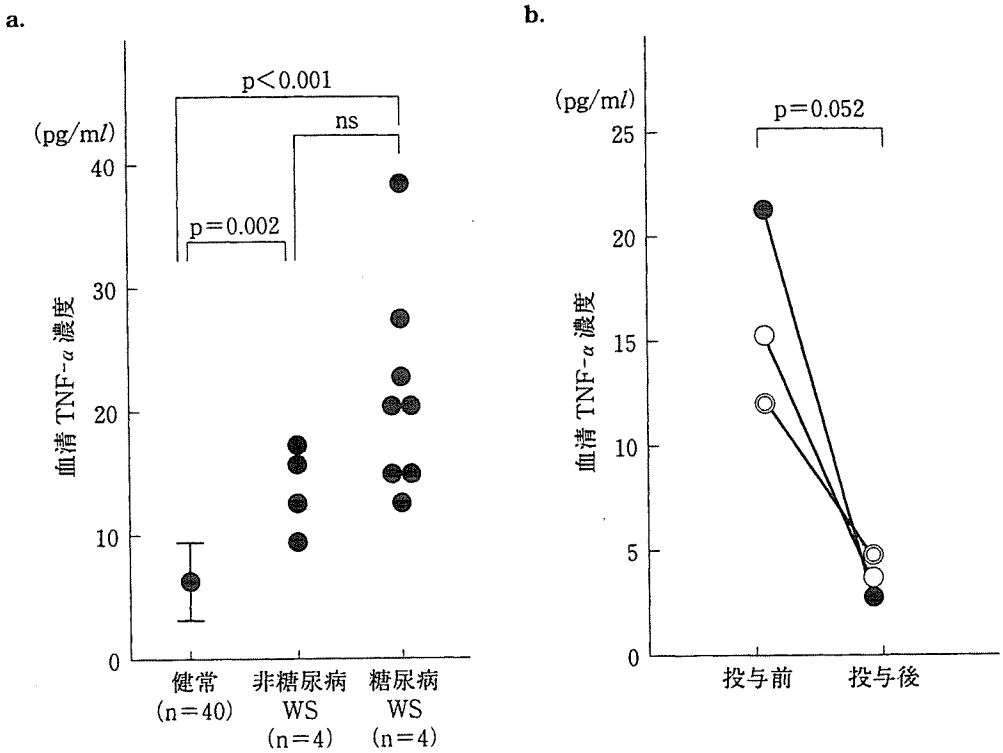

図2 Werner症候群患者の血中TNF-α濃度(文献[11]および未発表データより作成)
a: Werner症候群患者(WS)では，糖尿病の有無にかかわらず，健常コントロール(健常)に比べ血中のTNF-α濃度が有意に高値を示した.
b: 糖尿病を合併するWerner症候群患者3症例にピオグリタゾンを16週間投与したところ，血中TNF-α濃度の低下を認めた.

能障害，脂質代謝異常と高血圧を合併しやすく，メタボリックシンドロームに類する病態を呈することが明らかとなった.

脂肪細胞の分化を促し，インスリン感受性を改善させる薬剤としてPPARγ(peroxisome proliferator-activated receptor γ)のアゴニストであるチアゾリジン誘導体が用いられている．糖尿病を合併したWerner症候群患者にピオグリ

タゾンを投与し，メタボリックシンドロームの各種コンポーネントに及ぼす影響を検討したところ，インスリン感受性の増加と耐糖能の改善に加え，血清トリグリセリド値，総コレステロール値の低下ならびにHDLコレステロール値の増加がみられた[12]．またピオグリタゾンは，これらの患者において血清アディポネクチン値の上昇とTNF-α値の低下(**図2-b**)をもたらすことも明らかとなった[11]．

4. 今後の展望

最近著者らは，画像上内臓脂肪の蓄積は認めないが軽度のインスリン抵抗性を示すWerner症候群の若年例(29歳，女性)に遭遇した．最終結論は今後の経過観察に委ねられるが，Werner症候群にみられる複合代謝異常の一次的要因はインスリン感受性の低下にあり，内臓脂肪の増加は代償的高インスリン血症に伴う二次的な変化であることが推察される．WRNヘリカーゼの変異がどのような機序でインスリン抵抗性をもたらすかは未解明だが，実験的観察事実としてはWerner症候群患者の線維芽細胞において，グルコーストランスポーターGLUT1の細胞膜への移行の障害が報告されている[13]．

内臓脂肪量の増加とインスリン感受性の低下は，一般的な加齢においても観察される変化である[14]．近年，Werner症候群と並ぶ代表的な早老症であり，10歳代で心筋梗塞を発症するHutchinson-Gilford症候群の原因がlamin A遺伝子の変異にあると特定された[15]．lamin Aの変異は，インスリン抵抗性と関連の深い部分的脂肪萎縮症(partial lypodystrophy)の原因としても知られていることから，早老症発生の分子機序に関する研究は，老化-インスリン抵抗性-動脈硬化を結ぶ新たな手がかりを与えてくれることが期待される．

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Original Research Article

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The Clock Drawing Test as a Valid Screening Method for Mild Cognitive Impairment

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Key Words
Clock drawing · Early diagnosis of dementia · Screening test · Cutoff point · Cahn's scoring protocol

Abstract
To validate the Clock Drawing Test (CDT) as a screening method for detecting mild cognitive impairment (MCI) and to find the appropriate scoring protocol and its cutoff point, we compared the sensitivity and specificity of three CDT protocols. Subjects included 219 outpatients with memory complaints, who were attending the geriatric memory clinic. Cahn's protocol, with a cutoff point of 7, was more successful at differentiating clinically diagnosed MCI subjects from normal elderly individuals, with higher sensitivity (74.7%) and specificity (75.6%), than were the other protocols. The CDT, as a handy screening method, may be useful for clinicians to reliably identify subjects with MCI, and it may contribute to early detection of dementia.

Introduction

Early detection of dementia is an issue of growing concern because of improved clinical outcomes expected as a result of early therapeutic interventions or preventive approaches [1]. In terms of care, the diagnosis of cognitive deficits at an early stage, when the patient is still competent enough to make important decisions, can give the patients and their caregivers the opportunity to prepare for situations expected to occur as the symptoms progress (e.g. making environmental arrangements or educating the family), and also facilitate autonomic future planning (e.g. writing a living will, assigning durable power to an attorney or composing advanced directives) [2]. The term 'mild cognitive impairment' (MCI) was originally used to describe a transitional state between normal condition and Alzheimer's disease (AD) [3] and was first defined by Petersen et al. [4]. Recently, a revised and extended definition of MCI has been proposed that covers a broader range of cognitive impairment. It categorizes MCI into the following three subtypes: purely amnestic syndrome, impairment of a single nonmemory domain of cognition, and slight cognitive impairment in multiple domains of cognition [5]. It has also been suggested that enlarging the definition would allow the screening of more subjects at

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risk of dementia [6]. Although many detailed neuropsychological tests evaluating executive functions are available as screening instruments to quantify the degree of cognitive impairment, most of them are impractical for general physicians to administer in their clinical settings [2, 7]. To our knowledge, none of these neuropsychometric tests can contribute to the accurate diagnosis of MCI with reliable sensitivity and specificity.

The Clock Drawing Test (CDT) has been arousing the interest of clinicians and researchers as a convenient screening instrument for dementia, either by itself or as a part of a brief neuropsychological test battery. The CDT takes less than 2 min to administer, and it is easy to comprehend the instructions, making it suitable for elderly patients who may not be able to maintain concentration [8]. Previous replication studies, which applied various scoring systems, demonstrated that the CDT is a reliable method for the detection of dementia [9–12]. The CDT is relatively less affected by the level of education, language, and cultural background than are the other cognitive tests such as the Mini-Mental State Examination (MMSE) [13, 14]. The protocols of the CDT in published studies are various. They differ not only in the instructions (with or without predrawn circle, different time setting) but also in their scoring criteria. To obtain acceptable reliability for screening MCI among a target population, the cutoff points of the CDT applied in previous studies must be reexamined, since they may not be appropriate in populations with lower prevalence rates of dementia [9]. To date, there has been a dearth of studies examining the utility of the CDT in detecting cognitive deficits in their early stages, in particular MCI. Previous studies yielded conflicting results with limitations in terms of the criteria used for group assignment, the sample size, and the optimal cutoff points for different types of cognitive status [15, 16]. Besides, most previous studies emphasized the use of quantitative analyses of the CDT results, leaving detailed qualitative analyses of the results somewhat neglected. Despite the existence of reports regarding qualitative analyses of the CDT results in AD and vascular dementia (VD) patients, those focusing on MCI subjects are still lacking [17, 18]. The purpose of this study was to warrant the validity of the CDT as a screening method for detecting MCI by determining an appropriate scoring protocol with an optimal cutoff point and qualitative features.

Table 1. The neuropsychological test battery

Function	Test
Global cognitive function	MMSE
Orientation	MMSE-1, 2
Memory	MMSE-5
	Verbal recall (ADAS, paragraphs)
Verbal fluency	Initial letter
	Category
Visuospatial praxis	CDT, MMSE-11
	ADAS-7
Psychomotor speed	Digit symbol
Attention	Stroop test
	Digit Span

ADAS = The Alzheimer's disease assessment scale; ADAS-7 = constructional ability.

Methods

Participants

Subjects were recruited from outpatients at the geriatric memory clinic in the Nagoya University Hospital. A total of 219 subjects (male: 75, female: 144) aged 60 years and older who had either subjective memory complaints or memory loss reported by their informants participated in this study. Informed consent was obtained from all the participants or their primary caregivers after complete description of the study. The age of the participants ranged from 60 to 93 years (mean = 75.1 years, SD = 6.7 years). Years of education ranged from 3 to 24 years (mean = 10.2 years, SD = 2.8 years). None of the participants had a history of neurological or psychiatric disorders, and none had been diagnosed as having reversible causes of cognitive impairment. Routine physical examinations and neurological examinations had been carried out in all subjects. Subjects with receptive aphasias or visual impairment and those who had abnormal thyroid functions or serum vitamin B_{12} or folate levels in laboratory studies were excluded from the study. Magnetic resonance imaging of the brain was performed on all subjects. The Geriatric Depression Scale (GDS)-15 was applied as a screening test for excluding subjects with possible depression at a cutoff point of 8 [19]. Subjects were administered a neuropsychological test battery including the CDT, as shown in table 1 [20–22]. General cognitive impairment was assessed by the MMSE with a score <24 [23]. Information derived from a series of diagnostic evaluations, except for the CDT in the neuropsychological test battery, was reviewed by a team of experienced geriatricians at a case conference, and all the participants were categorized into five groups: normal elderly (NE), MCI, AD/senile dementia of Alzheimer's type (AD/SDAT) and mixed dementia, VD, and unclassified demented. The distribution of subjects by diagnostic category and sex is shown in table 2. Consensus diagnosis was made at the conference, using the Diagnostic and Statistical Manual of Mental Disorders Revised Third Edition (DSM-III-R) for dementia [24] as well as the National Institute of Neurological and

Table 2. Participant characteristics

	Whole sample	Nondemented		Demented		
		NE	MCI	SDAT[1]	VD	others
n; M/F	219; 75/144	41; 10/31	48; 21/27	102; 33/69	14; 6/8	14; 5/9
Age	75.1 (6.7)	72.7 (6.3)	74.7 (6.2)	76.0 (6.7)	76.9 (6.0)	74.9 (8.3)
Education	10.2 (2.8)	10.2 (1.8)	11.5 (3.7)	9.7 (2.4)	10.3 (3.0)	8.5 (2.5)
MMSE	24.4 (5.0)	28.4 (1.8)	27.2 (2.1)	22.2 (5.1)	20.5 (5.8)	20.6 (3.7)
CDT (Sunderland)	7.1 (2.4)	9.2 (1.1)	7.8 (2.1)	6.3 (2.3)	5.6 (2.5)	5.4 (2.3)
CDT (Rouleau)	7.0 (2.3)	8.7 (1.0)	8.0 (1.2)	6.3 (2.5)	5.6 (2.2)	5.6 (2.2)
CDT (Cahn)	6.1 (2.8)	8.4 (1.4)	7.1 (2.0)	5.2 (2.8)	4.1 (2.5)	4.1 (2.4)

Figures indicate means, with SD in parentheses.
[1] SDAT includes SDAT, AD and mixed dementia.

Communicative Disorders and Stroke and the Alzheimer's Disease and Related Association Work Group (NINCDS-ADRDA) criteria for probable AD to determine patients with AD/SDAT [25], and using the National Institute of Neurological and Communicative Disorders and Stroke and Association Internationale pour la Recherche et l'Enseignement en Neuroscience Work Group (NINCDS-AIREN) criteria for probable VD to determine VD patients [26]. Mixed dementia was diagnosed as probable mixed dementia, when there was a clinical indication that dementia was likely to be attributable to both conditions. In this study, patients with mixed dementia were incorporated into the AD/SDAT group as stated above. The diagnosis of MCI was made according to the following criteria: (1) not demented, (2) subjective memory complaint, (3) normal general cognitive functioning assessed by the MMSE (score, ≥ 24), (4) objective memory impairment and/or impairment in other cognitive domains as evidenced by scores > 1.5 SD below the age-appropriate mean score of at least one or more neuropsychological tests examined, (5) autonomy in the basic activities of daily living [7, 27].

Measurements

The subjects were given a blank piece of paper and asked to follow a two-step instruction: 'First, draw a 10-cm diameter clock face with all numbers on it. Second, put hands on the clock to make it read 10:10.' The CDT was scored by a psychologist according to the rating scales of Sunderland et al. [28], Rouleau et al. [29] and Cahn et al. [30]. These three sets of scoring criteria were chosen because they are characterized by concrete scoring instructions with presentations of actual error types, unlike other scoring methods, which contain vague or equivocal expressions in their scoring criteria. The psychologist who was the CDT rater of the present study was not given any information about the participant, including performance on other cognitive tests or clinical diagnosis.

The scoring methods used in the present study are as follows. (1) The CDT by Sunderland et al. [28] (Appendix 1): this method is based on the assumption that the representation of the hands is the first and solely affected item (score 6–10 points), and additional errors in the representation of numbers and the clock face occur later (score 1–5 points), so that a 10-point scale is used, with higher numbers indicating better performance. (2) The CDT by Rouleau et al. [29] (Appendix 2): three components of the drawing (integrity of the clock face, 0–2 points; presence and sequencing of the numbers, 0–4 points, and presence and sequencing of the hands, 0–4 points) are independently assessed. The scoring method supplies 0–10 points, with higher numbers indicating better performance. (3) The CDT by Cahn et al. [30] (Appendix 3): this is considered to be a modified version of the method by Rouleau et al. [29]. The difference in the Cahn scoring method is that while Rouleau's scoring method is regarded as a quantitative scoring of 0–10 points, the administrator notes the presence of qualitative errors shown in Appendix 3 and adds the error numbers up as a qualitative score with a maximum number of 8. The global CDT score is calculated by subtracting the qualitative score from the quantitative score. A 10-point scale is used, with higher numbers indicating better performance.

Statistical Analysis

All statistical analyses were performed using SPSS 11.0J. for Windows. Differences in age and years of education among the diagnostic groups were tested using the Kruskal-Wallis test. To examine the relationships between the CDTs and other variables (age, years of education, GDS, MMSE score), correlations and their p values were calculated using the Spearman rank order correlation coefficients. Distributions of Cahn's qualitative errors were examined using χ^2 analyses and Ryan's procedure for multiple comparisons.

Results

The five diagnostic groups shown in table 2 did not differ in terms of age ($p = 0.0811$). As for the educational years, except for the unclassified demented group, the four definite diagnostic groups did not differ ($p = 0.0183$). Distributions of the MCI subtypes are shown in table 3. The three groups (amnestic, single nonmemory and multiple domains) did not differ in age and years of education ($p = 0.8623, 0.3575$, respectively).

Table 3. MCI characteristics

	MCI		
	amnestic	single nonmemory	multiple domains
n; M/F	10; 2/8	10; 4/6	28; 15/13
Age	74.6 (7.2)	74.0 (7.2)	75.0 (5.6)
Education	11.5 (3.0)	9.0 (3.5)	12.4 (3.0)
MMSE	26.8 (2.8)	28.9 (1.2)	26.9 (2.0)
CDT (Sunderland)	9.4 (0.5)	7.3 (1.8)	7.4 (2.3)
CDT (Rouleau)	9.3 (0.7)	7.5 (1.1)	7.7 (1.2)
CDT (Cahn)	9.3 (0.7)	6.1 (2.0)	6.7 (1.8)

Figures indicate means, with SD in parentheses.

Table 4. Correlation matrix

	Sunderland	Rouleau	Cahn
Age	0.146	0.182	0.180
Education, years	0.210	0.220	0.201
GDS	0.281	0.312	0.312
MMSE	0.459 *	0.492*	0.490*
Sunderland		0.836*	0.857*
Rouleau			0.979*

* $p < 0.001$.

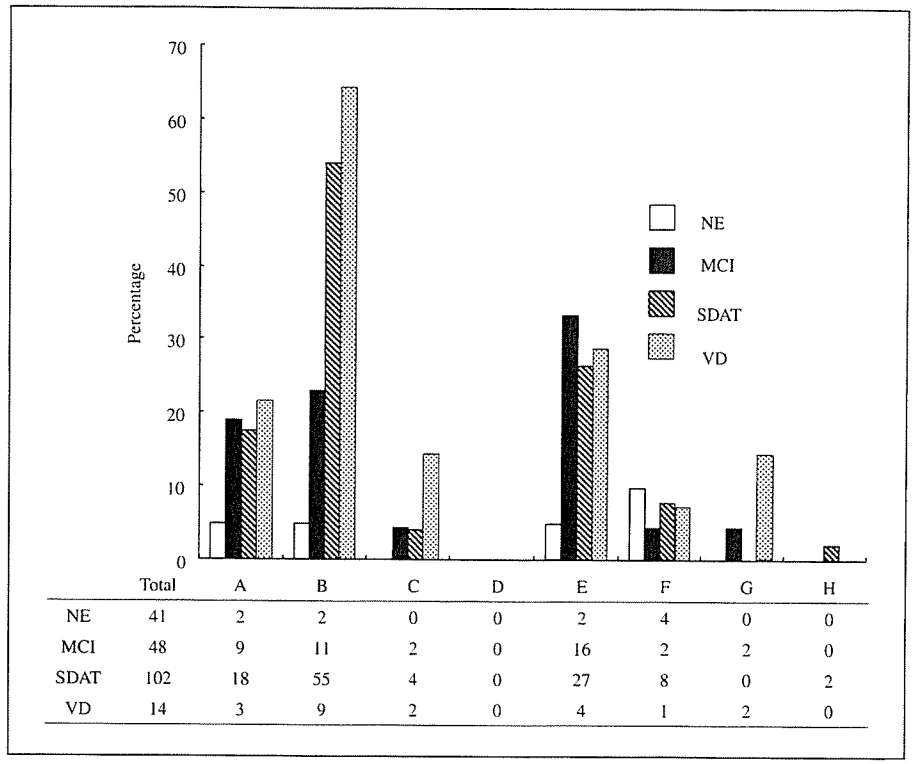
Correlations between the three CDT scores and other variables are presented in table 4. None of the CDTs correlated significantly with age, years of education, or the GDS score. However, all the CDTs correlated significantly with the MMSE score. Within the three CDTs, the scores correlated significantly with each other. In particular, the Cahn and Rouleau scores correlated with the highest correlation coefficient ($r = 0.979$, $p < 0.0001$).

We calculated the sensitivities and specificities with different CDT cutoff points to examine the discriminatory power of the CDTs for differentiating MCI and demented subjects from NE (table 5). The analyses demonstrated that Cahn's protocol had the highest discriminatory power at a cutoff point of 7, with a sensitivity of 74.7% and specificity of 75.6%.

Observed error types in the four definite diagnostic categories using Cahn's criteria for qualitative analysis are presented in figure 1. The four definite diagnostic groups did not differ in terms of age and years of education. The letters 'A' to 'H' represent Cahn's qualitative error types, which are described in Appendix 3. Regarding the distribution of error types in each group, only a few errors were noted in the NE group according to Cahn's criteria. In the MCI group, E (planning deficit) was the most frequent (16 of 48 cases), followed by B (conceptual deficit) and A (stimulus-bound response; 11 and 9 of 48 cases, respectively). In the AD group (SDAT/AD/MIXED), B was the most frequent (55 of 102 cases, 53.9%), followed by E (27 of 102 cases, 26.5%) and A (18 of 102 cases, 17.6%). In

Table 5. Sensitivities and specificities (%)

Cutoff point	Sunderland		Rouleau		Cahn	
	sensitivity	specificity	sensitivity	specificity	sensitivity	specificity
10	100.0	0.0	100.0	0.0	100.0	0.0
9	93.8	48.8	96.1	17.1	96.1	17.1
8	66.0	90.2	78.1	65.9	82.6	65.9
7	61.2	95.1	56.7	92.7	74.7	75.6
6	42.7	95.1	36.5	95.1	60.1	92.7
5	38.2	95.1	24.7	97.6	44.4	95.1
4	26.4	100.0	18.5	100.0	33.7	97.6
3	10.0	100.0	11.8	100.0	23.0	100.0
2	5.1	100.0	0.8	100.0	14.6	100.0
1	1.1	100.0	0.5	100.0	10.6	100.0



	Total	A	B	C	D	E	F	G	H
NE	41	2	2	0	0	2	4	0	0
MCI	48	9	11	2	0	16	2	2	0
SDAT	102	18	55	4	0	27	8	0	2
VD	14	3	9	2	0	4	1	2	0

Fig. 1. Comparison of error types among the four diagnostic groups: NE, MCI, SDAT, (represents SDAT, AD and mixed dementia), and VD. The horizontal scale (A–H) represents Cahn's qualitative error types: A = Stimulus-bound response; B = conceptual deficit; C = perseveration; D = neglect of hemispace; E = planning deficit; F = nonspecific spatial error; G = numbers written on the outside of the clock, and H = numbers written counterclockwise. The bottom table is the matrix showing participants' actual number of errors in the four diagnostic groups.

the VD group, B was the most frequent (9 of 14 cases), followed by E and A (4 and 3 of 14 cases, respectively). The χ^2 analysis comparing the frequency of Cahn's error types made by the four diagnostic groups revealed that there was a significant effect of diagnosis in all error types ($p < 0.0001$).

Discussion

While various CDT scales are available for detecting dementia, few studies have examined the adequate scoring protocol and optimal cutoff point for screening MCI. With the aim of detecting dementia at an early stage, particularly MCI, the present study compares the three scoring methods, all of which were found to be independent of years of education and depression scale, which is in keeping with the findings by Shulman et al. [8]. In addition, the CDT scores determined using the three scoring methods correlated with MMSE scores with high statistical significance, as confirmed in previous studies [12, 31, 32]. In most of the former studies, the cutoff points of CDTs were provided with the aim of distinguishing a demented state or AD from normal cognition. Recently, Powlishta et al. [16], choosing 6 different scoring criteria of the CDT for comparing subjects without altering the cutoff points determined for dementia in each original CDT criterion, have reported that the CDT was a poor screening method for very mild dementia. The sensitivity and specificity for detecting MCI by the CDT obtained in the current study were satisfactory. The discrepancy between the results of the study by Powlishta et al. [16] and those of the current study may be due to different cutoff points. Comparison of sensitivities and specificities among the three CDT protocols revealed that the Cahn scale had the best discriminatory power at the cutoff point of 7. Thus, the results may indicate that Cahn's protocol is the most suitable method for screening MCI in general practice. As shown in table 3, the analyses based on MCI subtypes suggest that subjects with amnestic MCI cannot be screened by the cutoff point we consider optimal for differentiating MCI subjects from normal individuals. This may simply imply that MCI subjects without deficits in the cognitive domain do not lose scores on the CDT, but we need further investigation to warrant this notion, given the limited number of participants included in this study. However, as we acknowledge the significance of including MCI subtypes other than the amnestic type, we believe that the

present findings would provide useful information for clinicians for screening subjects at risk of dementia in earlier stages.

We also examined the error types in MCI subjects using Cahn's qualitative criteria and compared them with the results in the NE and subjects with dementia. In what follows, impairment underlying each type of frequent error is disclosed [11, 17, 30]:

(A) stimulus-bound response: disturbance of inhibition in executive control functioning, an aspect of the frontal cortical function;

(B) conceptual deficit: loss of semantic memory usually evoked by the word 'clock';

(C) perseveration: an aspect of frontal dysfunction, and

(E) planning deficit: suggested to be associated with visuospatial constructional/frontosubcortical dysfuntion.

As shown in figure 1, error type E (planning deficit) could be a distinctive feature of MCI, which is represented by imprecise gaps before 12, 3, 6, or 9 of the numbers arranged in the clock face, or by clock hands drawn not from the center of the clock face. This type of error is considered to represent the inability to form a strategy for drawing a clock, presumably due to frontosubcortical dysfunction. The frequency of conceptual deficit in the MCI group was significantly lower than that in the SDAT/AD/MIXED and VD group. The conceptual deficit reflects a loss or deficit in accessing knowledge of the attributes, features, and meaning of a clock, and this category includes misrepresentation of the clock itself and the time on the clock [30]. Eleven out of 41 MCI subjects made this type of error; the difference in frequency between the MCI and the other groups' subjects did reach statistical significance. The SDAT/AD/MIXED and VD groups made this type of error with a frequency of 50% or above, which was statistically higher than that in the MCI and NE group, and that in the MCI being again higher than that in the NE group. Further investigations with increased number of subjects may clarify detailed characteristics of the clock drawing in MCI or its subgroups.

Between SDAT and VD subjects, no significant difference in error types was identified. This might be because of the relatively small number of patients in the VD subgroup in the current study, which may thus have influenced the statistical analysis. The previous study showed that the frequency of spatial and/or planning deficit was significantly higher in patients with mild VD than mild AD, and in patients with moderate VD, the frequency of graphic difficulties was significantly higher than in moderate AD [17]. These assumptions derived from the observations in this study may help to guide and benefit from future studies with larger numbers of subjects.

Although the CDT cannot be used solely for clinical diagnoses, the CDT, as a simple screening method, provides objective and graphic documentation of cognitive deficits that can be shared by a wide range of clinicians. In conclusion, among the three scales examined in this study, the Cahn scoring method at a cutoff point of 7 is the most likely indicator for MCI. Petersen et al. [7] recommended the CDT using Cahn's protocol as an optional instrument for brief cognitive assessment, as an addition to a general cognitive screening test, e.g. MMSE. We believe that the results obtained in the current study provide important evidence of the validity of the CDT as one of the useful screening method for discriminating MCI from normal cognition.

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Appendix 1
Sunderland's protocol: a priori criteria for evaluating clock drawings

10–6	Drawing of clock face with circle and numbers is generally intact
10	Hands are in correct position
9	Slight errors in placement of the hands
8	More noticeable errors in the placement of hour and minute hands
7	Placement of hands is significantly off course
6	Inappropriate use of clock hands
5–1	Drawing of clock face with circle and numbers is not intact
5	Crowding of numbers at one end of the clock or reversal of numbers
4	Further distortion of numbers sequence; integrity of clock face is now gone
3	Numbers and clock face no longer obviously connected in the drawing; hands are not present
2	Drawing reveals some evidence of instructions being received but only a vague representation of a clock
1	Either no attempt or an uninterpretable effort is made

10 = Best, and 1 = worst.

Appendix 2
Rouleau's protocol: the score is calculated by a sum of three components (I, II, III)

I Integrity of the clock face (maximum: 2 points)
- 2 Present without gross distortion
- 1 Incomplete or some distortion
- 0 Absent or totally inappropriate

II Presence and sequencing of the numbers (maximum: 4 points)
- 4 All present in the right order and at most minimal error in the spatial arrangement
- 3 All present but errors in spatial arrangement
- 2 Numbers missing or added but no gross distortions of the remaining numbers; numbers placed in counterclockwise direction or all present but gross distortion in spatial layout (i.e. hemineglect, numbers outside the clock)
- 1 Missing or added numbers and gross distortions
- 0 Absence or poor representation of numbers

III Presence and placement of the hands (maximum: 4 points)
- 4 Hands are in correct position and the size difference is respected
- 3 Slight errors in the placement of the hands or no representation of size difference between the hands
- 2 Major errors in the placement of the hands
- 1 Only one hand or poor representation of two hands
- 0 No hands or perseveration on hands

Appendix 3
Cahn's protocol: the global score is calculated by subtracting qualitative score (II) from quantitative score (I)

I Quantitative CDT score = maximum 10 points: assesses the presence and correctness of the clock; the clock face (0–2 points), the placement of the hands (0–4 points) and the placement of the numbers (0–4 points)

II Qualitative CDT score = maximum 8 points: summary of the following errors
- 1 Stimulus-bound response: the tendency of the drawing to be dominated or guided by a single stimulus
- 2 Conceptual deficit: this error type reflects a loss or deficit in accessing knowledge of the attributes, features and meaning of a clock
- 3 Perseveration: the continuation or the recurrence of activity without an appropriate stimulus
- 4 Neglect of left hemispace: all attributes of the clock are written on the right half of the clock face
- 5 Plannning deficit: this error type is represented by gaps before 12, 3, 6 or 9
- 6 Nonspecific spatial error: a deficit in the spatial layout of numbers, without any specific pattern in spatial disorganization
- 7 Numbers written on the outside of the clock: numbers written either around the perimeter of the circle or the circle itself
- 8 Numbers written counterclockwise: arrangement of the numbers with '12' at the top of the clock face and then continuing around in a counterclockwise fashion

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ORIGINAL INVESTIGATION

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Dopamine D_2 receptor plays a role in memory function: implications of dopamine–acetylcholine interaction in the ventral hippocampus

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Abstract *Rationale:* The role of the hippocampal dopaminergic system in mnemonic function has not been clarified yet. *Objective:* We previously reported that the dopamine D_2 receptor (D_2R) is involved in the regulation of acethylcholin (ACh) release in the hippocampus. In this study, we further investigated ACh–dopamine (DA) interaction in the hippocampus and its involvement in mnemonic function. *Methods:* For experiment 1, rats fed with Cholin (Ch)-deficient chow were used. We examined the effects of D_2R antagonist, raclopride, on cognitive performance using a passive avoidance task. We further carried out in vivo microdialysis to assess the effect of infusion of D_2R agonist, quinpirole, into the ventral hippocampus on its capacity to release ACh. For experiment 2, rats fed with normal chow were used. The performance of a radial arm maze task was assessed to examine the effects of hippocampal injection of D_2R agonist, quinpirole, on memory impairment induced by scopolamine, a muscarinic ACh antagonist. *Results:* In experiment 1, rats fed with Ch-deficient chow showed impaired performances indicated by prolonged latency on retention trials of a passive avoidance task following the hippocampal injection of D_2R antagonist, and showed reduced capacity to release ACh following the injection of D_2R agonist compared with rats fed with normal chow. In experiment 2, memory impairment induced by the intraperitoneal injection of scopolamine was ameliorated by the injection of D_2R agonist into the ventral hippocampus. *Conclusion:* These results indicate the possible involvement of hippocampal ACh–DA interaction in mnemonic processing.

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Abbreviations D_2R: dopamine D2 receptor · ACh: acetylcholine · DA: dopamine · Ch: Choline · AChE: Acetylcholine esterase · AChEIs: Acetylcholine esterase inhibitors · ACSF: Artificial cerebrospinal fluid · HPLC: High-performance liquid chromatography · HPLC–ECD: high-performance liquid chromatography system with electrochemical detection · PBS: phosphate buffer saline · ANOVA: analysis of variance · DH: dorsal hippocampus · VH: ventral hippocampus

Introduction

It is well known that cholinergic basal forebrain neurons projecting to the cerebral cortex and hippocampus play a crucial role in learning and memory (Mufson et al. 2003; Paul 2003), and a number of studies have shown that disturbances in the cholinergic system induce learning and memory impairment (Day et al. 1991; Wilson and Cook 1994; Walsh et al. 1996; Mishima et al. 2001; Rogers and Kesner 2003). We previously demonstrated that the dietary restriction of Choline (Ch), which is a precursor of acetylcholine (ACh), induces reduced capacity to release ACh in the hippocampus, as confirmed by in vivo microdialysis, and impairs performance of a passive avoidance task in rats (Nakamura et al. 2001).

On the other hand, attention has been paid to the involvement of hippocampal dopamine (DA) systems, especially the dopamine D_2 receptor (D_2R), in mnemonic function. Hippocampal DA neurons project from the ventral tegmental area, with some DA fibers in the posterior hippocampus originating from the substantia nigra (Verney et al. 1985). In fact, several studies have shown that disturbances in dopaminergic systems induce learning and memory impairment in rats. For example, Gasbarri et al. (1996) revealed that direct hippocampal injection of 6-hydroxydopamine causes selective lesions of the mesencephalic dopaminergic system, which in turn induce learning

and memory impairment. Using a radial arm maze, Wilkerson and Levine (1999) demonstrated that D_2R in the ventral hippocampus is involved in spatial memory. In addition, an electrophysiological study showed that a D_2R antagonist leads to disturbances of long-term potentiation, a key phenomenon involved in memory consolidation, in the rat hippocampus (Frey et al. 1990; Yanagihashi et al. 1991).

The ACh–DA interaction in the striatum has been thoroughly investigated (Acquas and Chiara 2002; Kurotani et al. 2003). However, only a few studies have addressed the ACh–DA interactions in the hippocampus. Although it has been well documented that ACh–DA interactions play a role in mnemonic processing by studies with systemic drug injection (Levin et al. 1989; McGurk et al. 1989), to date, there has not been a study using focal injections to investigate the direct involvement of the hippocampal D_2R in this interaction. We previously demonstrated that a D_2R agonist dose-dependently stimulates acetylcholine (ACh) release in the ventral hippocampus; in that study, in vivo microdialysis and the results of a 14-unit T-maze (Stone maze) task suggested that D_2R in the ventral hippocampus was involved in mnemonic function via ACh release (Umegaki et al. 2001).

In the present study, we carried out a series of experiments to further investigate ACh–DA interactions in the hippocampus. In our first experiment using a passive avoidance task, rats were fed with Ch-deficient chow, which was confirmed to show a reduced capacity in releasing ACh in the hippocampus (Nakamura et al. 2001), and the effects of hippocampal injection of a dopamine D_2R antagonist on cognitive performance were observed. We also carried out in vivo microdialysis in rats fed with Ch-deficient chow, and examined the effects of the dietary manipulation of Ch on the capacity to release ACh from the ventral hippocampus induced by the infusion of a D_2R agonist into the ventral hippocampus (experiment 1). Then, by assessing the performance of rats fed with normal chows on a radial arm maze task, we examined whether memory impairment induced by scopolamine, a muscarinic ACh antagonist, could be alleviated by the intrahippocampal administration of a D_2R agonist (experiment 2).

Materials and methods

Experiment 1

Subjects

Male 9-week-old Wistar rats, housed in cages maintained at an appropriate temperature (20°C) and a 12-h light/dark cycle (7 A.M./7 P.M.), were used. The animals were assigned at random to one of two dietary groups. Rats assigned to the control group were fed a diet of chow containing 0.2% Ch. The remaining rats were fed a diet deficient in Ch that contained less than 0.03% (i.e., below the level of detection). Each diet was administered to the animals for 12 weeks. Both diets had the same composition of ingredients, with the exception of the Ch content, as follows: (casein 6.0%, starch 71.5%, sugar 10.0%, soybean oil 0.5%, fatty oil 4.0%, cellulose 3.0%, United States Pharmacopoeia-vitamin: 1.0%, Funabashi Farm, Chiba, Japan). The rats were allowed free access to food and water ad libitum, and they were housed in a temperature-controlled (20°C) facility.

All experiments were conducted according to the protocols approved by the Animal Care and Use Committee of Nagoya University. All efforts were made to minimize the number of animals used and their suffering.

Cannula implantation

The rats were anesthetized with pentobarbital (45 mg/kg) and fixed to a stereotaxic frame. The skull was exposed, and guide cannulae (31-90100, BAS, Tokyo, Japan) were bilaterally placed into the ventral hippocampus at the following coordinates: AP −5.0 mm, ML±4.8 mm, DV 7.0 mm, with reference to bregma and the dural surface in accordance with the atlas of Paxinos and Watson (1986). A recovery period of 3 days was allowed before the test. All rats underwent the cannulae implantation, and were then divided into two groups. The first group (n=42) was subjected to a behavioral study, and the second group (n=12) was subjected to an in vivo microdialysis study.

Histology

After completion of the experiment, the animals were injected 1 μl of cresyl violet dye to confirm cannula placement. The animals were anesthetized with ether and decapitated. After the brain was removed, it was frozen and sliced to a thickness of 40 μm. The injected sites were confirmed by macroscopic examination. Only data from animals in which the injections were made into the desired sites were analyzed.

Behavioral study

Passive avoidance task Both the Ch-deficient rats and controls were habituated to a two-compartment shuttle box on 2 successive days before being subjected to a step-through passive avoidance task. The apparatus used for the task was composed of two compartments of equal size (25×25×25 cm), separated by a common wall. One of the two compartments was brightly illuminated from above by two 12-W bulbs. The other compartment was not illuminated and had an electrifiable grid floor. A guillotine-type door (15×10 cm) was located in the center of the common wall in order to allow rats to move about freely between the compartments (Nakamura et al. 2001). When habituation was completed, the rats were placed in the dark compartment and were then tested for the acquisition of passive avoidance. In brief, the rat was first placed in the light compartment. When the rat entered the dark com-

partment, the door was closed, and an electric shock to the foot (AC 1 mA) was immediately applied via the floor grids for 3 s. The rat remained in the dark chamber for 30 s after the foot shock had been administered, and was then removed from the apparatus and temporarily placed in a holding cage for 120 s. The rat was then returned to the light compartment and tested for the assessment of memory acquisition. The successful acquisition of passive avoidance was recorded when the rat remained in the light compartment for 300 s without entering the dark compartment. If this criterion was not met, upon entering the dark compartment, the rat received the same foot shock. The number of trials required for meeting the criterion was recorded for comparison between the four groups. Retention of passive avoidance was tested 72 h after the acquisition trial. The time until the rats moved to the dark compartment was recorded as latency, for up to a maximum of 300 s (Eidi et al. 2003).

Drug administration A 27-gauge stainless steel injection cannula was used for infusion of the drug. The cannula was connected to a CMA/100 microinjection pump (BSA, Tokyo, Japan) via polyethylene tubing. Either phosphate buffer saline (PBS) or raclopride dissolved in PBS (500 μg/ml in concentration) was infused into the bilateral ventral hippocampi at an injection speed of less than 1.0 μl/min for 10 min in order to avoid tissue destruction. The total volume used for the infusion depended on the body weight of rats (80 μl/kg). The infusion was implemented 20 min prior to both the acquisition and retention of passive avoidance trials. Four different groups were thus created according to diet content and agents administered into the ventral hippocampus. The following groups were used: control diet–PBS (control+PBS), control diet–raclopride (control+raclo), Ch-deficient diet–PBS (Ch-d+PBS) and Ch-deficient diet–raclopride (Ch-d+raclo). After eliminating data from two rats with missed cannula placement and one rat with brain bleeding, the final four group sizes were: control+PBS, n=10 (329.6± 21.3 g); control+raclo, n=11 (337.4±18.5 g); Ch-d+PBS, n=10 (339.3±17.2 g); Ch-d+raclo, n=8 (322.7± 34.6 g). There was no statistically significant difference in body weight among the four groups during the experiments. The injection cannulae were left in place for 2 min following administration to allow the drug to diffuse away from the tip.

In vivo microdialysis study

Microdialysis procedure A perfusion procedure was carried out 72 h after the operation in order to avoid the effects of anesthesia and surgery. A microdialysis probe (31-9503; BAS) was inserted through the guide cannula and a perfusion pump was connected to the probe, according to a procedure devised to allow the rats to move freely in a hemispheric Plexiglass box. We used artificial cerebrospinal fluid (ACSF), which had the following composition: glucose 4 mmol/l, NaCl 126 mmol/l, KCl 4.0 mmol/l, KH_2PO_4 1.4 mmol/l, $MgSO_4$ 1.3 mmol/l, $CaCl_2 2H_2O$ 2.4 mmol/l, $NaHCO_3$ 26 mmol/l. Physostigmine (eserine hemisulfate; 100 μmol/l) (Sigma, St. Louis, MO, USA) was added to the solution, and was refluxed at a flow rate of 2 μl/min. The collected perfusates were sampled every 20 min. At 80 min after the initiation of the perfusion, the perfusate was exchanged for ACSF containing quinpirole (100 μmol), a D_2R agonist. The perfusion was continued for another 80 min. The collected samples, mixed with 1 pmol of ethylhomocholine (BAS) as an internal standard, were immediately subjected to measurement of ACh levels using an HPLC assay system (BAS). After eliminating data from one rat with missed cannula placement, the final group sizes for the two dietary manipulation studies were: control diet group (n=6, 334.3± 19.8 g) and Ch-deficient diet group (n=5, 317.0±9.0 g). There was no statistically significant difference in body weight between two diet groups during the experiment.

High-performance liquid chromatographic assay Twenty microliters of sample perfusate was injected into a high-performance liquid chromatographic system with electrochemical detection (HPLC–ECD) without further purification (BAS). Two separate HPLC–ECD systems were used. Each contained a pump (PM-60), a degasser (CD-22p), a heating box (FT-1), and an electrochemical detector (LC-4C). Acetylcholinesterase and choline oxidase were immobilized on an enzyme cartridge column (BAS). The potential of the platinum electrode was kept at +450 mV vs a Ag/AgCl reference electrode. The mobile phase consisted of 50 mM $Na_2HPO_2 \cdot 12H_2O$ containing 1 mM EDTA 2Na. The pumping rate of the mobile phase was 80 μl/min.

Statistical analysis

The number of trials required for the acquisition of passive avoidance and the latency in retention trials were compared using the Kruskal–Wallis test followed by the Mann–Whitney U-test. The data obtained from the HPLC assay for the determination of ACh levels were expressed as a percentage of the baseline concentrations in the perfusates. Differences in ACh output between groups (controls and Ch-deficient groups) were statistically analyzed using a repeated-measures analysis of variance (ANOVA).

Experiment 2

Subjects

Male 9-week-old Wistar rats, weighing 250–300 g and housed in a cage at an appropriate temperature (20°C) and a 12-h light/dark cycle (7 A.M./7 P.M.), were used. Body weight was maintained at approximately 80% of the initial weight, with dietary restrictions imposed during the experimental period.

All experiments were conducted according to protocols approved by the Animal Care and Use Committee of Nagoya University. All efforts were made to minimize the number of animals used and their suffering.

Apparatus

We used a modified eight-arm radial maze originally developed by Olton and Samuelson (1976) for behavioral testing. The maze was placed 50 cm above floor level. It consisted of a central platform, 40 cm in diameter, with eight arms (12 cm in width, 80 cm in length) extending radially. Each arm was surrounded by opaque plastic side walls (5 cm in height). Guillotine-type doors were located between the central platform and each extending arm. Food cups that had food pellets in the middle, used as reinforcers, were placed near the end of each arm. The experimental room contained extra-maze visual cues that surrounded the maze.

Preparation of animals for the eight-arm radial maze

Before the trial, all rats were allowed to freely explore the maze in order to enable them to become habituated to the apparatus. Habituation was carried out three times a day (10 min each) for 1 day prior to the training session. One day following habituation, training sessions were carried out three times a day, up to a maximum of 4 days. In each training session, the animal was placed on a platform in the middle of the eight radial arms. After 10 s, the doors were lifted, and the animal was allowed to move freely about the maze. Each time the rat returned to the platform, the doors were closed for 10 s and then lifted again. The performance of a given animal on each trial was assessed on the basis of three parameters: the number of correct choices among the eight arms initially chosen, the number of errors (defined as the choice of arms that had already been visited), and the time elapsed before the animal ate all eight pellets. If the animals made seven or eight correct choices and less than one error in three successive sessions within 5 min, they were subjected to no further trials.

Surgery

In the training sessions, if the rats meet the above criteria, they were subjected to the operation for cannulation. They were anesthetized with pentobarbital (45 mg/kg) and were fixed to a stereotaxic frame. The skull was exposed, and guide cannulae (31-90100; BAS) were placed into the bilateral ventral hippocampus at the following coordinates: AP −5.0 mm, ML±4.8 mm, DV 7.0 mm, with reference to the bregma and the dural surface in accordance with the atlas of Paxinos and Watson (1986). A recovery period of 3 days was allowed. Before the drug administration trials, the rats underwent the training procedure to confirm that they again met the above criteria following surgery for a maximum of 2 days.

Histology

After completion of the experiment, the animals received injections of 1 μl cresyl violet dye to confirm cannula placement. The animals were anesthetized with ether and decapitated. After the brain was removed, it was frozen and sliced to a thickness of 40 μm. The injected sites were confirmed by macroscopic examination. Only data from animals in which the injections were made into the desired sites were analyzed.

Drug administration

A 27-gauge stainless steel injection cannula was used for infusion of the drug. The cannula was connected to a CMA/100 microinjection pump (BSA, Tokyo, Japan) via polyethylene tubing. Drugs were administered once in a single trial for each rat. At 30 min before the trial, either scopolamine (0.125 mg/kg) or PBS was administered intraperitoneally. Rats that were administered PBS served as controls. As regards the rats that were given scopolamine, either quinpirole (8 or 16 μg/kg), quinpirole+raclopride (simultaneously 16 μg/kg each), or PBS was injected into the ventral hippocampus bilaterally 20 min prior to the trial. Thus, five different groups were created, depending on the agents administered both intraperitoneally and intracerebrally: PBS–PBS (controls), scopolamine–PBS, scopolamine–quinpirole (8 μg/kg), scopolamine–quinpirole (16 μg/kg), and scopolamine–quinpirole+raclopride (16 μg/kg each). The total injection volume depended on body weight (16 μl/kg each) and the rate of injection was less than 1.0 μl/min over a 10-min period avoiding mass effects. The injection cannula was left in place for 2 min in order to allow the drug to diffuse away from the tip. After eliminating data from two rats with missed cannula placement, the final five group sizes were: controls (n=7, 246.8±15.7 g); scopolamine–PBS (n=7, 242.1±19.3 g); scopolamine–quinpirole (8 μg/kg) (n=8, 246.8±21.3 g); scopolamine–quinpirole (16 μg/kg) (n=8, 231.3±15.7 g); scopolamine–quinpirole+raclopride (16 μg/kg each) (n=5, 228.0±15.2 g). There was no statistically significant difference in body weight among the five groups during the experiment.

Statistical methods

The number of correct choices in the eight arms initially chosen (i.e., the initial response number) was compared using the Kruskal–Wallis test followed by Mann–Whitney U-test to examine significant differences. The average time spent by each rat visiting each arm among four groups statistically was compared using one-way ANOVA.

Results

Experiment 1

Behavioral studies

There was no significant difference noted between groups with respect to the number of trials required for the acquisition of passive avoidance behavior (Fig. 1). Regarding the retention of nociceptive memory, which was assessed by the latency period until the animal stepped into the dark compartment of the shuttle box, Fig. 2 shows the effect of PBS or raclopride on the respective diet groups. The Ch-deficient diet–raclopride (Ch-d+raclo) group had a shorter latency on retention trials than the other three groups ($P<0.01$, Kruskal–Wallis test followed by Mann–Whitney U-test). There was no significant difference among the three groups (control+PBS, control+raclo, and Ch-d+PBS). Therefore, neither dietary manipulation of Ch intake nor focal injection of raclopride alone affected the latency time in this retention trial.

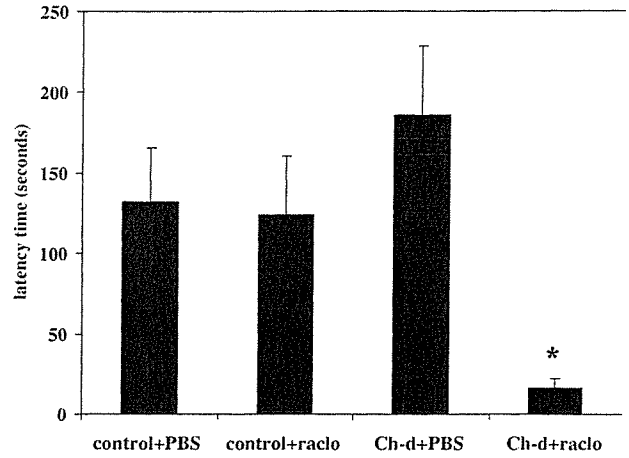

Fig. 2 The latency time (mean±SEM) in retention by the effect of PBS or raclopride on the respective diet groups. Only Ch-deficient diet–raclopride (Ch-d+raclo) group had shorter latency on retention trials than the other three groups (*$P<0.01$, Kruskal–Wallis test followed by Mann–Whitney U test). There was no significant difference among the following three groups: control+PBS, control+raclo, and Ch-d+PBS (control+PBS, $n=11$; control+raclo, $n=10$; Ch-d+PBS, $n=10$; Ch-d+raclo, $n=8$)

Microdialysis studies

As shown in Fig. 3, the increase in ACh release in the ventral hippocampus induced by quinpirole was reduced in the Ch-deficient diet group compared with the control group ($P<0.05$, repeated-measures ANOVA).

Experiment 2

As shown in Fig. 4, the number of correct choices from among the eight arms initially visited differed significantly in the scopolamine–PBS injected group and the scopolamine–quinpirole+raclopride (16 μg/kg each) injected group compared with controls (PBS–PBS injected) ($P<0.01$, Mann–Whitney U-test). Both doses (8 and 16 μg/kg) of scopolamine–quinpirole significantly increased the number of correct choices compared with the scopolamine–PBS injected group and the scopolamine–quinpirole+raclopride (16 μg/kg each) injected group (each $P< 0.05$, $P<0.01$, Mann–Whitney U-test). On the other hand, there was no significant difference between the scopolamine–PBS group and the scopolamine–quinpirole+raclo-pride group (16 μg/kg each). A focal injection was administered into the ventral hippocampus in order to avoid the other DA-related effects (e.g., motivation or locomotion). No statistically significant difference was observed between the four groups that received scopolamine (i.p.) with respect to their locomotor

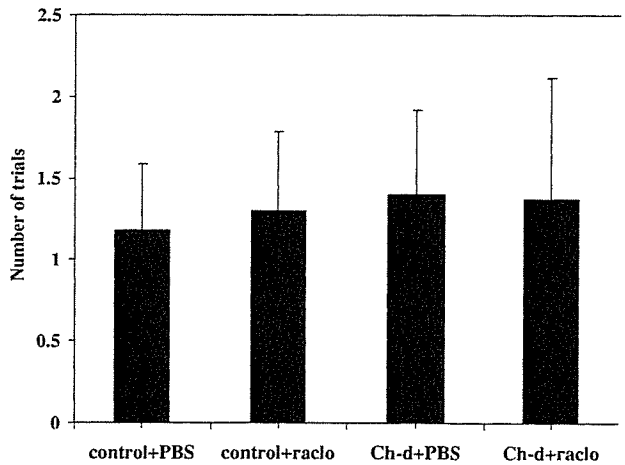

Fig. 1 The number of trials (mean±SEM) required for the acquisition of passive avoidance behavior. There was no statistically significant difference between the four groups (control+PBS, $n=11$; control+raclo, $n=10$; Ch-d+PBS, $n=10$; Ch-d+raclo, $n=8$)

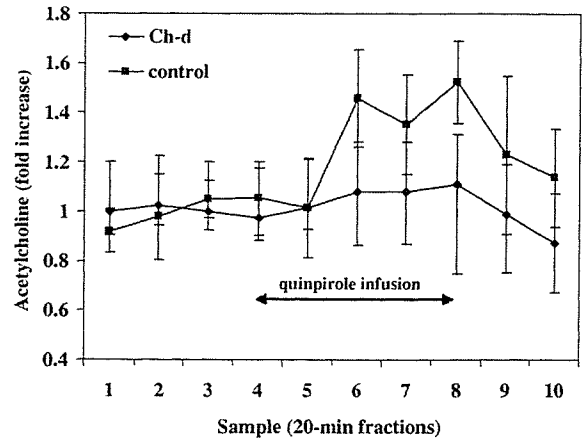

Fig. 3 Acetylcholine release induced by quinpirole injection in the ventral hippocampus. The *data points* represent group means±SEM. A significant difference was observed between Ch-deficient and control rats (control, $n=6$; Ch-d, $n=5$; $P<0.05$, a repeated measures ANOVA)

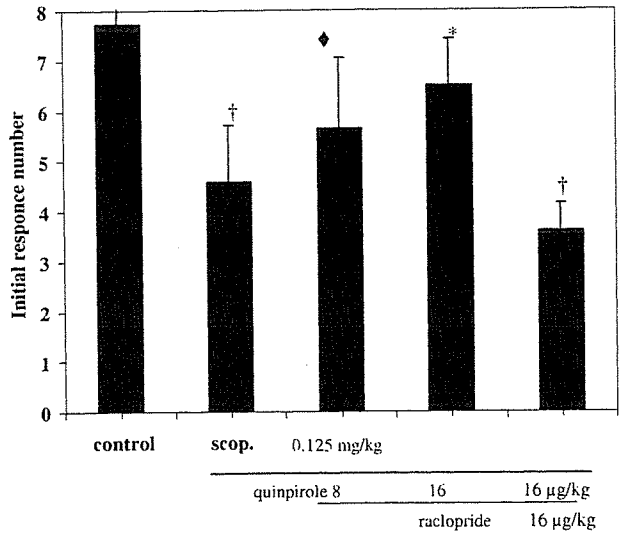

Fig. 4 Effects of intrahippocampal injection of quinpirole and raclopride on scopolamine-induced impairment of memory in the eight-arm radial maze. Scopolamine (scop.; 0.125 mg/kg) and quinpirole (+raclopride) were injected 30 min, intraperitoneally, and 20 min, intracerebrally, prior to the test, respectively (†$P<0.01$ vs control; *$P<0.01$. ◦$P<0.05$ vs scop.+PBS, scop.+quinpirole 16 μg +raclopride 16 μg; Kruskal–Wallis test followed by Mann–Whitney U test). PBS+PBS (control), n=7; scop.+PBS, n=7; scop.+quinpirole 8 μg, n=8; scop+quinpirole 16 μg, n=8; scop.+quinpirole 16 μg +raclopride 16 μg, n=5

speed as they visited each arm of the maze (Table 1). There was no significant difference between the group administered with the intrahippocampal PBS injection and the other groups, according to Scheffe's post-hoc test.

Discussion

Using a 14-unit T maze (Stone maze), we previously demonstrated that maze learning was impaired in rats following ventral hippocampal injection of raclopride, a D_2R antagonist; this impairment was reversed when quinpirole, a D_2R agonist, was administered simultaneously (Umegaki et al. 2001). These findings suggest the possible involvement of hippocampal D_2R dopaminergic systems in mnemonic function. This behavioral study showed the detrimental effects of a hippocampal injection of raclopride on mnemonic performance only when the rats were subjected to a chronic deficiency of dietary Ch, i.e., when the ability to release ACh upon increased neuronal demand was presumably reduced, as confirmed in our previous report (Nakamura et al. 2001). Neither dietary manipulation of Ch intake nor focal injection of raclopride alone affected behavioral performance, while Nakamura et al. (2001) showed that rats fed with Ch-deficient diet had deteriorated retention ability. This inconsistent result is attributable to a difference in behavioral assessments. They assessed the number of days it took for the rats to move into the dark compartment within maximum latency, but not latency time 72 h after the acquisition trial. Assessment of the duration of retaining nociceptive memory would probably be more sensitive to dietary manipulation. We previously demonstrated that quinpirole stimulated ACh release dose-dependently in the ventral hippocampus (Umegaki et al. 2001), and the present in vivo microdialysis study revealed that the increased ACh release in the ventral hippocampus induced by the focal administration of quinpirole was reduced under conditions of chronic dietary Ch deficiency. We believe that these findings, when taken together with the results from the current behavioral experiments, provide evidence of the involvement of ACh–DA interaction in mnemonic processing in the ventral hippocampus of rats.

As confirmed in experiment 2, quinpirole ameliorated scopolamine-induced spatial memory impairment in a dose-dependent manner, and co-injection with raclopride attenuated the ameliorative effect of quinpirole. Scopolamine has been widely known to produce acute cholinergic dysfunction, which is reflected in behavioral deficits in the performance of eight-arm radial maze observed after the intrapenitoneal administration of scopolamine (Zhang and O'Donnell 2000; Mishima et al. 2001; Daniel et al. 2003). In another study using an eight-arm radial maze task, Mishima et al. (2000) demonstrated a correlation between the behavioral changes (initial response number) induced

Table 1 The average time spent until rats entered each arm

Hippocampal injection agents	Time (seconds)	
PBS	100.60 ± 24.8	
Quinpirole (8 μg/kg)	82.85 ± 37.2	N.S.
(16 μg/kg)	73.01 ± 19.5	
Quinpirole+Raclopride (16 μg/kg each)	141.1 ± 42.2	

No significance difference using a one-way ANOVA followed by Scheffe's post hoc test

(n=5-8; mean± SEM)

No significant difference was observed among the four groups administered (i.p.) with scopolamine. No significant difference was found using one-way ANOVA followed by Scheffe's post-hoc test (n=5–8; mean± SEM)