Muscarinic Cholinergic Receptors in Human Narcolepsy

A Positron Emission Tomography Study

ナルコレプシーのムスカリン性受容体に関する研究

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(1)

論文題目

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TITLE: Muscarinic Cholinergic Receptors in Human Narcolepsy: A Positron Emission Tomography Study

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Abstract

Objective: To investigate the function of the muscarinic cholinergic receptor (mAchR) in narcolepsy and the effects of pharmacotherapy on mAchR.

Background: Muscarinic neural transmission serves as the main executive system in rapid eye movement (REM) sleep. Studies in canine narcolepsy reported an increase in mAchR in the pons.

Methods: mAchR of 11 drug naïve/free patients with narcolepsy and 21 normal controls were investigated using positron emission tomography (PET) with [^{11}C]N-methyl-4-piperidylbenzilate (NMPB). Measurements were done in the pons, thalamus, striatum and cerebral cortex. Seven of the 11 patients also underwent an additional PET scan after the alleviation of symptoms by pharmacotherapy.

Results: There were no differences in [^{11}C]NMPB binding between the control and drug naïve/free patients in all areas analyzed. At the time of on-medication PET scan, [^{11}C]NMPB binding tended to be inhibited only in the thalamus ($p<0.1$), but to a small degree.

Conclusions: We did not confirm the occurrence of increased mAchR density in human narcolepsy. The present results do not support the notion that mAchR is the main site of action of pharmacotherapy in the marked clinical improvement of human cataplexy.

Introduction

Narcolepsy is a rather rare sleep disorder characterized by recurrent daytime sleep episodes and symptoms of dissociated REM (rapid eye movement) sleep such as cataplexy, sleep paralysis, and hypnagogic hallucinations. Although its pathophysiology is largely unknown, the discovery of the canine model of narcolepsy and the subsequent establishment of a multi-litter colony has provided invaluable information about this disorder. Currently, it appears that several neural transmitter systems are involved in canine narcolepsy (for review, see ref 1). Among these, the pontine muscarinic cholinergic system has been one of the most thoroughly investigated.

Normally in the regulation of awake-sleep cycles, pontine cholinergic cells constitute part of the so-called "brainstem reticular formation" and serve as cortical activation systems. In addition, considerable attention has centered on the role of pontine cholinergic systems in the regulation of REM sleep. Large amounts of cells in this region behave as REM-on cells, which are specifically active during REM sleep. Cholinergic stimulation in this region induces the signs of REM sleep (for review, see ref 2). In canine narcolepsy, the microinjection of muscarinic antagonist into this area ameliorates, and agonist aggravates cataplexy,[3, 4] and the amount of endogenous acetylcholine released is particularly high during cataplectic attacks.[5] With regard to receptor binding sites, an increase in the number of brainstem M2 muscarinic cholinergic receptors (mAchR) was also reported in canine narcolepsy.[6, 7] In addition, Nitz et al.[8] recently reported that the number of cholinergic cells increased in the brainstem, although Tafti et al.[9] were unable to confirm this observation.

In contrast, little is known about human narcolepsy. To our knowledge, only one post mortem autoradiographic study has reported a non-significant increase of mAchR in the

striatum and amygdala.[10] Post mortem studies, however, are susceptible to a variety of confounding factors, such as previous medication[11] and storage conditions after autopsy,[12] and the number of subjects (n=2)[10] was certainly too few to draw any conclusions. So, the hypothesis that the elevation of mAchR is a fundamental abnormality in narcolepsy remains unproven.

Current pharmacological treatment of narcolepsy includes the use of central nervous system stimulants and antidepressants.[13] Stimulants are used to treat the excessive daytime sleepiness but have no effect on cataplexy.[14] Although antidepressants, especially clomipramine, can alleviate REM-related symptoms in narcolepsy, they are potent serotonin (5HT) or noradrenaline (NA) uptake inhibitors and have many binding sites other than mAchR.[15,16] Previous studies on canine and human narcolepsy showed that 5HT or NA transmission was also involved in cataplexy[1] and it is uncertain where the therapeutic property of antidepressants is derived from. Verifying mAchR abnormalities is of crucial importance in understanding the pathogenesis of narcolepsy and developing more effective anti-narcolepsy drugs.

The aim of this study was to explore the function of mAchR in narcolepsy. We investigated the mAchR of drug naïve/free patients with narcolepsy and the effects of drug therapy on mAchR using positron emission tomography (PET) and [^{11}C]N-methyl-4-piperidylbenzilate ([^{11}C]NMPB), a non-selective muscarinic cholinergic ligand.[17,18]

Methods

Patients and control. Eleven patients with narcolepsy, meeting the minimum diagnostic criteria for narcolepsy (recurrent daytime sleep episodes and cataplexy) of the International Classification of Sleep Disorders,[19] and 21 healthy people participated in this study. All were men, and the mean age was 42.6 years for the patients and 32.7 years for the control. All patients were HLA-DR2 positive.[20] Eight of the 11 patients had no previous antipsychotic drug exposure and were termed the drug naïve subgroup. The remaining three were free from antipsychotic medication for more than one year and termed the drug free subgroup. Data of the patients are shown in Table 1. Written informed consent was obtained. This study was approved by the Ethical Committee and the Radiation Safety Committee of the National Institute of Radiological Sciences, Chiba, and the Ethical Committee of the University of Tokyo, Japan.

PET scan. PET scans were performed using a Siemens ECAT47 system (Siemens, Knoxville, TN, USA), which images 47 contiguous slices 3.375mm (center-to-center) apart with an in-plane resolution of 6 mm.

The participants took a supine position on the scanner bed with eyes closed and ears unoccluded. To minimize head movements during each scan, head fixation devices (Fixster Instruments, Stockholm, Sweden) and thermoplastic attachments made to fit the individuals were used. During PET scanning of patients, scalp electrodes were placed on Cz and bilateral Os using the international 10-20 system. During the procedure, patients were monitored by electroencephalogram (EEG) and encouraged to keep awake for at least the first 20 minutes of the dynamic scan.

After 10 minutes of transmission scan with a 3 mCi ^{68}Ge-^{68}Ga rod source, data acquisition commenced with a bolus injection of 15-20mCi [^{11}C] NMPB, a radiolabelled

compound of non-selective mAchR antagonist.[17,21] The radiochemical purities were more than 95 %, and the specific radioactivities ranged from 0.7 to 8.5 Ci/μmol. Dynamic imaging was performed for 60 minutes -- 2 minutes x 30 scans.

After the first PET scan, the pharmacological treatment was initiated, and seven of the 11 patients continued their regular visits to the outpatient clinic. They additionally underwent on-medication PET scans. For these scans, blood samples were collected on injection of the tracer. Samples were centrifuged and plasma was frozen at –20°C until analyzed. Plasma concentrations of each drug were determined by high performance liquid chromatography.

Data Analysis. In each PET study, circular regions of interest (ROIs) were manually delineated on reconstructed images with reference to the brain atlas. All samples taken were averaged to provide the mean count density for a given region. The location and volume of ROIs to be analyzed are shown in Figure 1 and Table 2. To examine the effects of pharmacotherapy, bilateral ROIs in the striatum were combined, and all the ROIs in the cortex were also combined to increase the signal-to-noise ratio. Then, these two ROIs were used as the representative measure for each region.

To quantify [^{11}C]NMPB binding in the brain, a compartment model for irreversible binding was applied.[22,23] As the cerebellum contains negligible mAchR,[24] the normalized graphical method using the cerebellum as a reference region was used.[25] The ratio of the radioactivity in the specific binding region ($C_{ROI}(t)$) to that in the cerebellum ($C_{cer}(t)$) can be expressed by the following equation:

$$\frac{C_{ROI}(t)}{C_{cer}(t)} = \frac{k_2 k_3}{k_2 + k_3} \frac{\int_0^t C_{cer}(\tau) d\tau}{C_{cer}(t)} + \left(\frac{k_2}{k_2 + k_3}\right)^2 + \frac{k_3}{k_2 + k_3}$$

where the constant k_2 is the efflux rate constant from tissue to blood, and k_3 is the association rate constant of ligand to receptor. The magnitude of k_3 is an index of the density of mAchR available for the binding of [^{11}C]NMPB (B'_{max}),[26] and was used for the quantification of [^{11}C]NMPB binding in this study.

If $k_2 >> k_3$, then this equation can be written as follows:[21, 27]

$$\frac{C_{ROI}(t)}{C_{cer}(t)} = k_3 \frac{\int_0^t C_{cer}(\tau)\,d\tau}{C_{cer}(t)} + 1$$

The plot of the C_{ROI}/C_{cer} ratio against the normalized integral of the cerebellum (the ratio of integrated cerebellar radioactivity by actual cerebellar radioactivity for each point) yields a straight line with the slope of k_3. The validation of test-retest data with [^{11}C]NMPB have been described elsewhere.[21]

Data from the control group (n=21) were used to correct the k_3 value of both control and narcoleptic patients for the significant effects of age on k_3.[28,29] Firstly, linear regressions of the control group were calculated to assess the age dependent decline of k_3. The regressions were used to calculate the k_3 values of each group at the age of twenty. Student's *t* test was used to measure the significance between the age-corrected k_3 value of each group.

Results

Although the overall mean [^{11}C]NMPB binding in narcoleptic patients was lower in all areas analyzed, this discrepancy was considered to be due to the differences in age between the two groups. In agreement with the earlier PET studies[28,29], a decrease in mAchR binding with age was observed in this study. The relationship between age and k_3, and the Pearson coefficients and *P* values for each region are given in Table 3. And Figure 2 shows the results of [^{11}C]NMPB binding in representative regions. After age-correction, we did not detect any differences between drug naïve/free narcoleptic patients and normal controls (Table 4; unpaired *t*-test: α=0.05).

Seven of the 11 patients underwent both pre- and on-medication PET scans. At the time of the on-medication PET scans, all participants were relieved from cataplexy and showed detectable levels of clomipramine in plasma. The mean duration of medication at the time of the second PET scans was 35 ± 23 weeks. The plasma concentration of clomipramine was 7-33ng/ml for the patients taking 25mg daily and 20-37ng/ml for those taking 50mg daily. The medication and plasma concentration of drugs at the time of on-medication PET scans are shown in Table 5. Table 6 shows the effects of medication on [^{11}C]NMPB binding. Only in the thalamus was there a tendency of a decrease in [^{11}C]NMPB binding (p<0.1), but this reduction did not correlate with the plasma concentration of clomipramine (Figure 3). No measurable effects were observed in other regions.

Discussion

In this study, we did not detect any change in [^{11}C]NMPB binding in human narcolepsy. We carried out dynamic scan for 60 minutes. Special attention was paid to the control of the scanning conditions, and in the case of the patients, EEG monitoring was used to assess the level of alertness. Patients were encouraged to remain awake.

mAchRs are pharmacologically classified into M1/M2 subtypes according to their affinity for pirenzepine.[30] We used [^{11}C]NMPB, which is a non-selective mAchR antagonist, and could not rigorously differentiate M1/M2 subtypes. However, several in vitro studies have shown that each subtype exhibits characteristic patterns of distribution in the human brain; in the cerebral cortex and the striatum, approximately 80 % of mAchRs are the M1 subtype, and in the thalamus and the pons, approximately 80 % are M2.[31] Therefore, [^{11}C]NMPB binding might indicate mainly M1 mAchR binding in the cerebral cortex and striatum, and M2 mAchR binding in the thalamus and pons.

There is considerable evidence that M2 mAchR in the upper brainstem is essential for the REM sleep elicited by cholinomimetic stimulation.[32-34] Studies of canine narcolepsy demonstrated that cataplexy is also mediated through M2 mAchR,[4] and putative M2 mAchR increased in the nucleus reticularis gigant celluraris of the brainstem,[6, 7] which is one of the sites for cataplexy induced by cholinomimetric stimulation.[3,4]

The present results were not in agreement with previous studies of canine narcolepsy. The cataplexy of human narcolepsy is less severe than that of canine narcolepsy. Further, the spatial resolution of current PET systems (6 mm) does not allow for the quantification of each nucleus in the pons separately. The measurements in this study were inevitably limited to the gross mAchRs in the pons. Even if such symptomatological differences or technical limitations

did affect the current results, the result of the medication still casts doubt on the mAchR hypothesis.

In this study, clomipramine was used in the treatment of cataplexy. Other drugs including stimulants or hypnotic have no effects on cataplexy. Although there was a tendency of a decrease of [^{11}C]NMPB binding in the thalamus, the magnitude remained small (8%; Table 6). Using the same methods, [^{11}C]NMPB binding was reported to be inhibited by about 30% in both M1- and M2– rich regions on oral administration of 4mg of trihexyphenidyl.[35] However, all clinical trials of anticholinergic compounds to date, including trihexyphenidyl, have proven unsuccessful for narcolepsy.[1] In contrast, all the patients in this study were relieved from cataplexy and other REM-related symptoms without a major decrease of [^{11}C]NMPB binding. These results strongly suggest that the anti-cataplectic effects of clomipramine are derived from transmitter systems other than mAchR.

Since the proposal of the reciprocal model by Hobson,[36] many studies have supported the idea that pontine cholinergic neurons interact with other transmitter systems, especially with 5HT and NA systems. Recent *in vivo* studies have demonstrated that both 5HT[37, 38] and NA[37, 38] systems exert inhibitory effects on pontine cholinergic systems. As shown in the present results, the effects of clomipramine on [^{11}C]NMPB binding are minimal. Clomipramine is, however, potent at blocking [^{3}H]serotonin uptake and relatively potent at blocking [^{3}H]noradrenaline uptake in the rat cerebral cortex[15]. A recent PET study has confirmed the high affinity of clomipramine for 5HT transporter in the living human brain as well.[41] Antagonistic effects of clomipramine[15] against these transporters increase their neural transmission and likely inhibit the pontine cholinergic neurons. Consistent with this view, a microinjection of 5HT into the brainstem suppresses REM sleep in rats ,[42] and several types of

selective serotonin reuptake inhibitors such as fluvoxamine[43] and fluoxetine[44] have shown modest effects on cataplexy in human narcolepsy. In addition, prazosin, an α1 NA antagonist, aggravates cataplexy.[45]

Clomipramine has proven be the most effective drug to date in the treatment of cataplexy, but its therapeutic efficacy is not through the direct inhibition of mAchR. It is likely that the presynaptic modulation of 5HT or NA transmission is the substantial pharmacological property of clomipramine. To develop a drug with more specific action for cataplexy, further studies on 5HT or NA will be needed.

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Figure Legends

Figure 1.

Circular regions of interest (ROIs) were drawn on PET slices. For each region, four to six slices were selected from a total of 47 axial slices. The left image shows ROIs in the cerebellum (black) and pons (red), the right image shows ROIs in the frontal cortex (blue), temporal cortex (black), occipital cortex (green), striatum (yellow) and thalamus (red).

Figure 2.

Scatter plots of age against the [^{11}C]NMPB k_3 value, and the regression lines for normal control in the pons (2-1), thalamus (2-2), left temporal cortex (2-3) and left frontal cortex (2-4). (■ : normal control, △ : narcolepsy)

Figure 3.

Scatter plots of the plasma concentration of clomipramine against the inhibition of the [^{11}C]NMPB k_3 value.

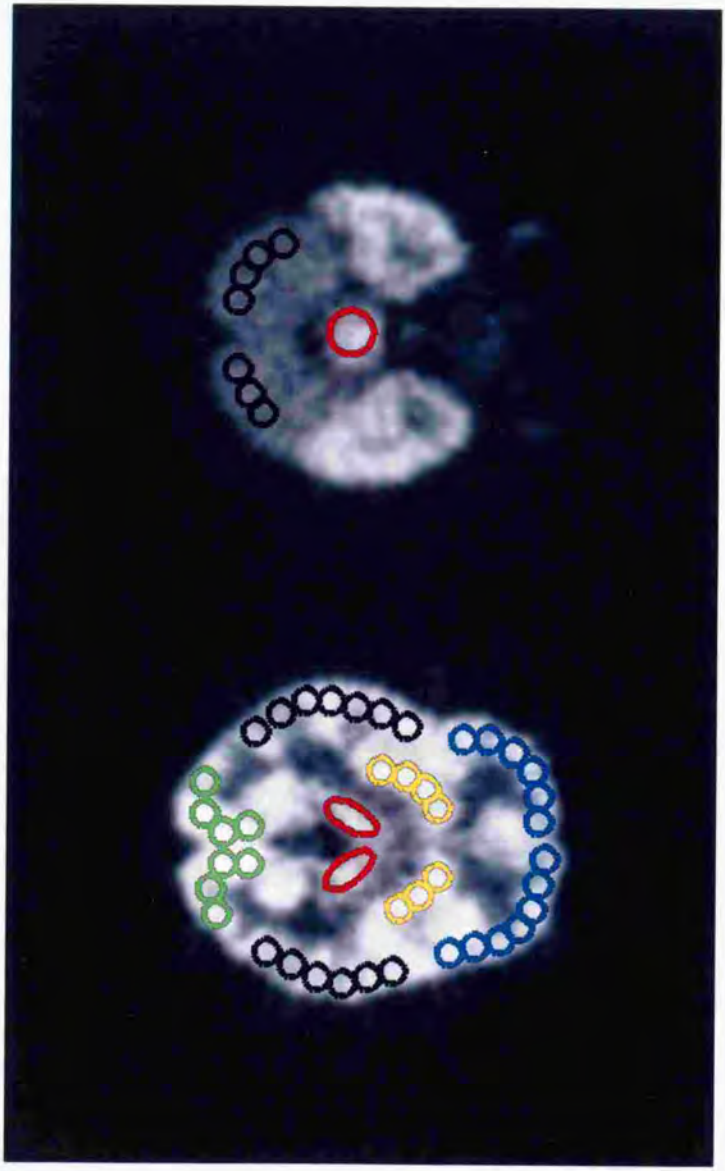

Figure 2-1

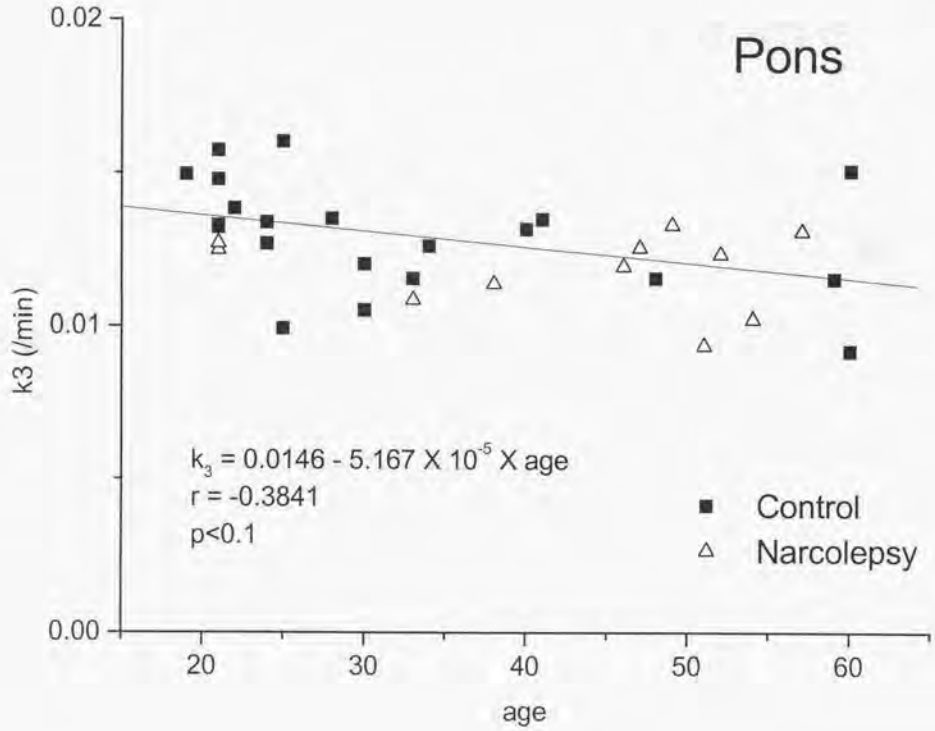
Pons
0.02
0.01
0.00
k3 (/min)
20
30
40
50
60
age
k_3 = 0.0146 - 5.167 X 10^{-5} X age
r = -0.3841
p<0.1
Control
Narcolepsy

Figure 2-2

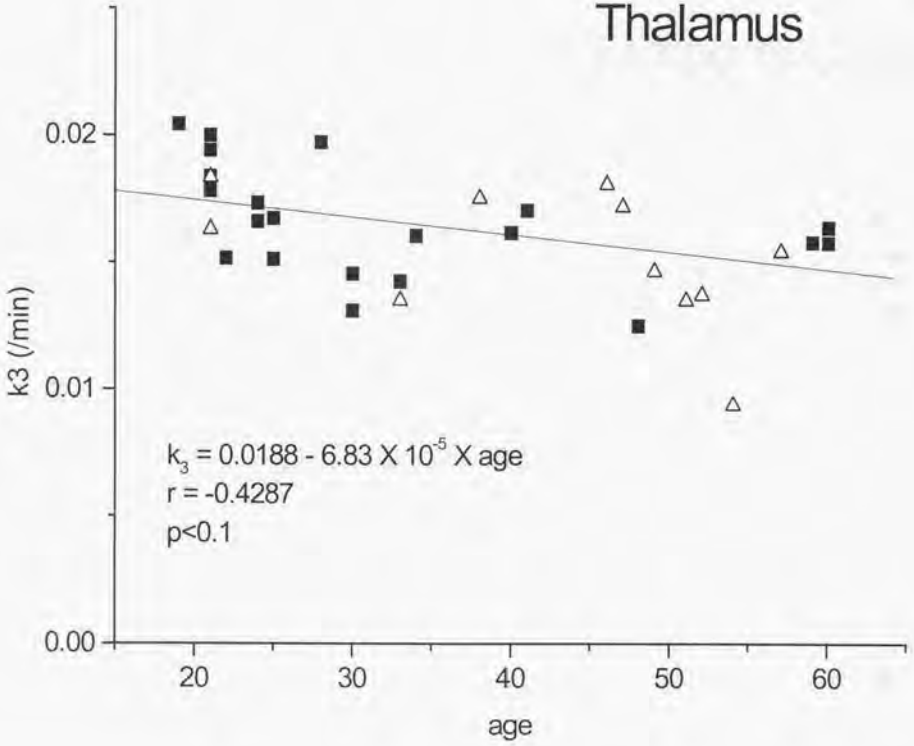
Thalamus
0.02
0.01
0.00
k3 (/min)
$k_3 = 0.0188 - 6.83 \times 10^{-5} \times age$
r = -0.4287
p<0.1
20
30
40
50
60
age

Figure 2-3

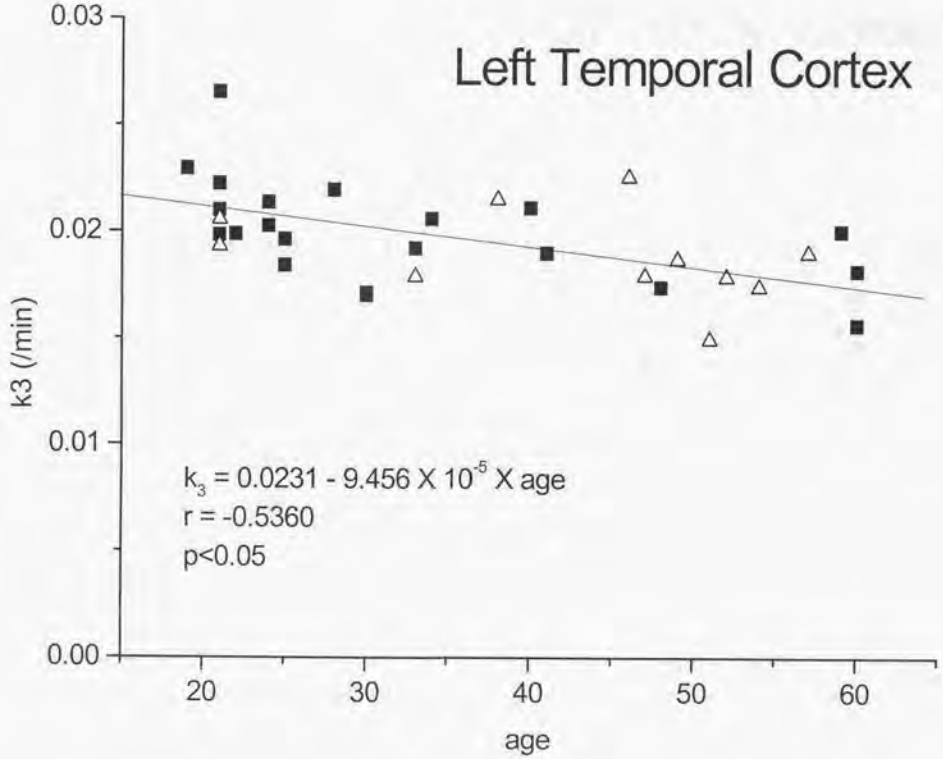
Left Temporal Cortex
0.03
0.02
0.01
0.00
k3 (/min)
$k_3 = 0.0231 - 9.456 \times 10^{-5} \times age$
r = -0.5360
p<0.05
20
30
40
50
60
age

Figure 2-4

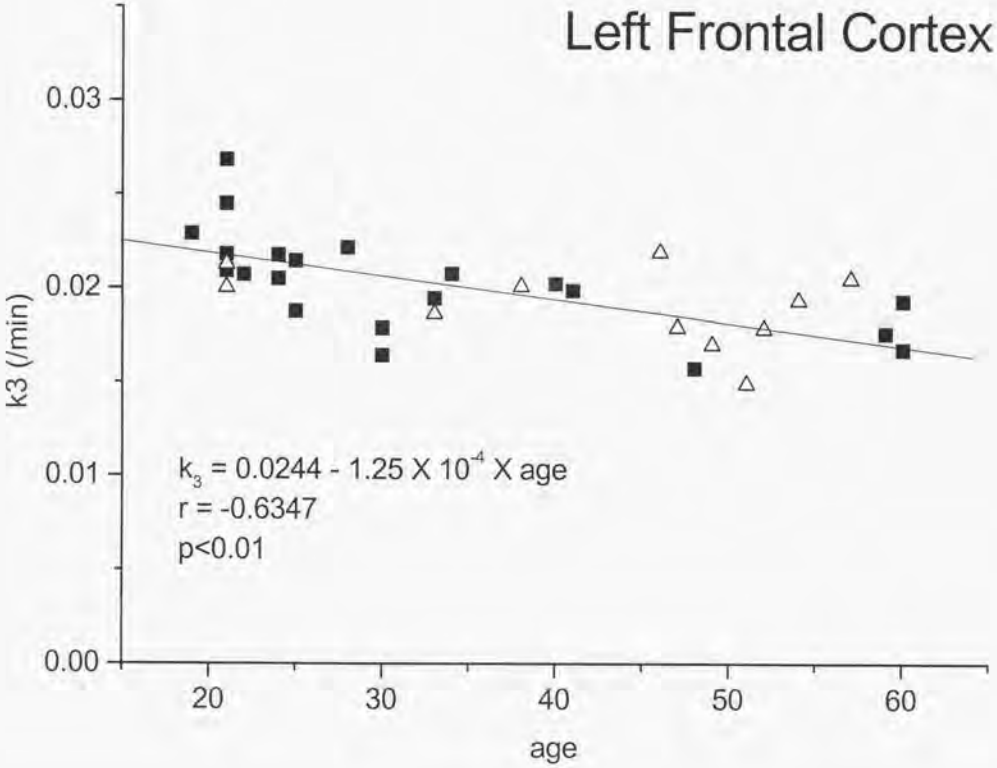
Left Frontal Cortex
0.03
0.02
0.01
0.00
k3 (/min)
k_3 = 0.0244 - 1.25 X 10^{-4} X age
r = -0.6347
p<0.01
20
30
40
50
60
age

Figure 3

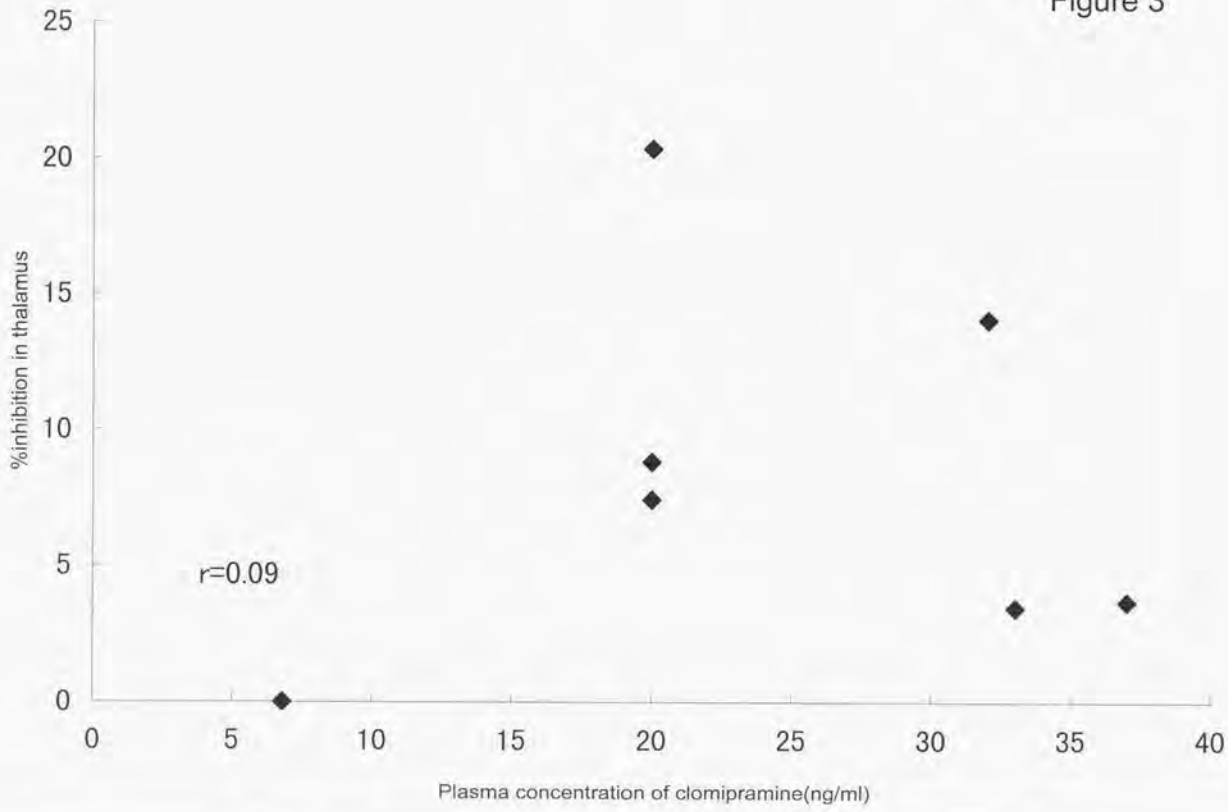
25
20
15
10
5
0
%inhibition in thalamus
r=0.09
0
5
10
15
20
25
30
35
40
Plasma concentration of clomipramine(ng/ml)

Table 1. Demographic data of patients

Patient number	Age	
1	46	Drug naïve
2	21	Drug Free (18 months)
3	54	Drug free (40 months)
4	57	Drug Free (18 months)
5	49	Drug naïve
6	21	Drug naïve
7	47	Drug naïve
8	52	Drug naïve
9	51	Drug naïve
10	33	Drug naïve
11	38	Drug naïve

Table 2. Definition of ROI and mean volume

ROI	Volume (cc)
Cerebellum	19.75
Pons	4.41
Thalamus	7.62
Left Striatum	7.63
Right Striatum	7.62
Left Frontal Cortex	24.05
Right Frontal Cortex	24.56
Left Temporal Cortex	20.73
Right Temporal Cortex	21.01
Occipital Cortex	25.43

Table 3. Aging effects on k3 in the control group

Region of Interest	% decrease[1)]	r-value[2)]
Pons	3.53	-0.38
Thalamus	3.63	-0.42
Left Striatum	2.71	-0.34
Right Striatum	4.69	-0.55**
Left Frontal Cortex	5.11	-0.63**
Right Frontal Cortex	4.79	-0.59**
Left Temporal Cortex	4.10	-0.54*
Right Temporal Cortex	4.32	-0.59**
Occipital Cortex	3.37	-0.49*

1) % decrease per decade relative to the k3 value at the age of zero
2) the Pearson correlation coefficients for the effects of k3 value and age (*: $p<0.05$, **: $p<0.01$)

Table 4. k3 values (/min) of narcolepsy and control subjects before and after correction for age. Data are presented as mean ± SD.

Region of Interest	Before correction for age		After correction for age	
	Normal Control (n=21)	Narcolepsy (n=11)	Normal Control (n=21)	Narcolepsy (n=11)
Pons	0.0130 ± 0.0018	0.0118 ± 0.0012	0.0136 ± 0.0013	0.0130 ± 0.0013
Thalamus	0.0166 ± 0.0021	0.0152 ± 0.0027	0.0175 ± 0.0024	0.0168 ± 0.0024
Left Striatum	0.0227 ± 0.0026	0.0222 ± 0.0025	0.0236 ± 0.0023	0.0237 ± 0.0023
Right Striatum	0.0223 ± 0.0030	0.0218 ± 0.0036	0.0239 ± 0.0036	0.0246 ± 0.0036
Left Frontal Cortex	0.0203 ± 0.0027	0.0190 ± 0.0020	0.0219 ± 0.0020	0.0219 ± 0.0020
Right Frontal Cortex	0.0206 ± 0.0027	0.0191 ± 0.0023	0.0220 ± 0.0025	0.0217 ± 0.0025
Left Temporal Cortex	0.0200 ± 0.0024	0.0189 ± 0.0021	0.0212 ± 0.0020	0.0210 ± 0.0020
Right Temporal Cortex	0.0200 ± 0.0023	0.0189 ± 0.0024	0.0213 ± 0.0025	0.0212 ± 0.0025
Occipital Cortex	0.0232 ± 0.0024	0.0223 ± 0.0025	0.0243 ± 0.0026	0.0243 ± 0.0026

Table 5. Dose of medication and plasma concentration of each drug at the time of on-medication PET scans. The patient number is the same as shown in Table 1. (- : not measured)

Patient number	Medications	Plasma Concentration	Duration of Treatment(Weeks)
1	Pemoline 25mg	-	22
	Clomipramine 25mg	20ng/ml	
	Zopiclone 10mg	-	
	Brotizolam 0.25mg	-	
2	Pemoline 75mg	-	13
	Clomipramine 50mg	37ng/ml	
	Nitrazepam 5mg	-	
	Estazolam 2mg	-	
	Chlorpromazine 12.5mg	-	
5	Pemoline 50mg	1.7 μ g/ml	15
	Clomipramine 25mg	33ng/ml	
	Nitrazepam 5mg	-	
7	Pemoline 75mg	1.9 μ g/ml	59
	Clomipramine 25mg	20ng/ml	
	Triazolam 0.25mg	<1ng/ml	
8	Pemoline 75mg	1.6 μ g/ml	71
	Clomipramine 25mg	6.8ng/ml	
	Nitrazepam 5mg	40ng/ml	
9	Pemoline 50mg	1.5 μ g/ml	22
	Clomipramine 50mg	32ng/ml	
	Nitrazepam 10mg	170ng/ml	
11	Pemoline 100mg	2.7 μ g/ml	40
	Methylphenidate 20mg	<2ng/ml	
	Clomipramine 50mg	20ng/ml	
	Estazolam 2mg	144ng/ml	

Table 6. k_3 values (/min) of patients before and after drug therapy.
Data are presented as mean ± SD.
(*: $p<0.1$, paired t-test (two-tailed))

Region of Interest	Pre-medication	On-medication
Pons	0.0119 ± 0.0013	0.0126 ± 0.0014
Thalamus	0.0159 ± 0.0019	0.0147 ± 0.0017 *
Cortex	0.0196 ± 0.0025	0.0203 ± 0.0017
Striatum	0.0222 ± 0.0036	0.0232 ± 0.0025

ポジトロン・エミッション・トモグラフィ(PET) を利用した局所脳コリン受容体測定に関する研究（研究代表者：須藤康彦）への参加同意書

この検査の目的は PET スキャンと呼ばれる技術により、脳の局所のコリン受容体を測定することにあります。この検査は、あなた御自身の治療にすぐ役立たない場合もありますが、神経の働き方の病気における脳のコリン受容体分布の特徴を測定し、将来的には治療効果の判定や新しい治療、よりよい診断を発展させる際に重要です。

検査は次の手順でおこなわれます。

まず、検査当日は、科学技術庁放射線医学総合研究所（千葉県千葉市稲毛区穴川 4-9-1）に行っていただきます。

そのあと、医師とともに同研究所ポジトロンカメラ室へと移動します。

入室後、検査のための補助的装置（脳波計など）を装着します。

次に、検査のために微量の放射能を有する薬剤（^{11}C-N メチルピペリジルベンジレート）の溶液を右前腕の静脈から注射します。注射する薬剤の放射能は最大 20mCi(ミリキューリー)です。注射後 60 分の間 PET による撮影がおこなわれます。

検査は開始から終了まで約 1 時間 30 分かかります。

この検査に伴う危険や不快については以下のものがあります。

1. 注射の際の不快および局所出血: 注射針の刺入設置の際に不快を感じるかもしれません。また、注射針の設置に伴い少量の出血を見ることがあり、青黒い内出血の跡が残る可能性があります。しかし、これらの操作は熟練した医師によっておこなわれますので、そのようなことはほとんど起こりません。
2. 放射線による発癌危険率: この検査に使用される放射性核種 ^{11}C は半減期も短く(約 20 分)、使用する放射線の量は極めて少なく、問題となることはありません。

検査の結果は医師から直接説明されます。
検査は求めればいつでも中止することができます。

この検査を受けることを拒否なさったとしても治療上不利益を被ることはありません。
この検査を受けて何らかの支障が生じた際は、下記の連絡先に速やかに連絡してください。

私は、以上の説明を平成　　年　　月　　日　医師　　より受け、検査の趣旨に賛同し、検査の内容も十分理解したので、この検査を受けることに同意いたします。

平成　　年　　月　　日

署名:　　印

以上の説明は平成　　年　　月　　日　私がおこないました。

担当医:　　印

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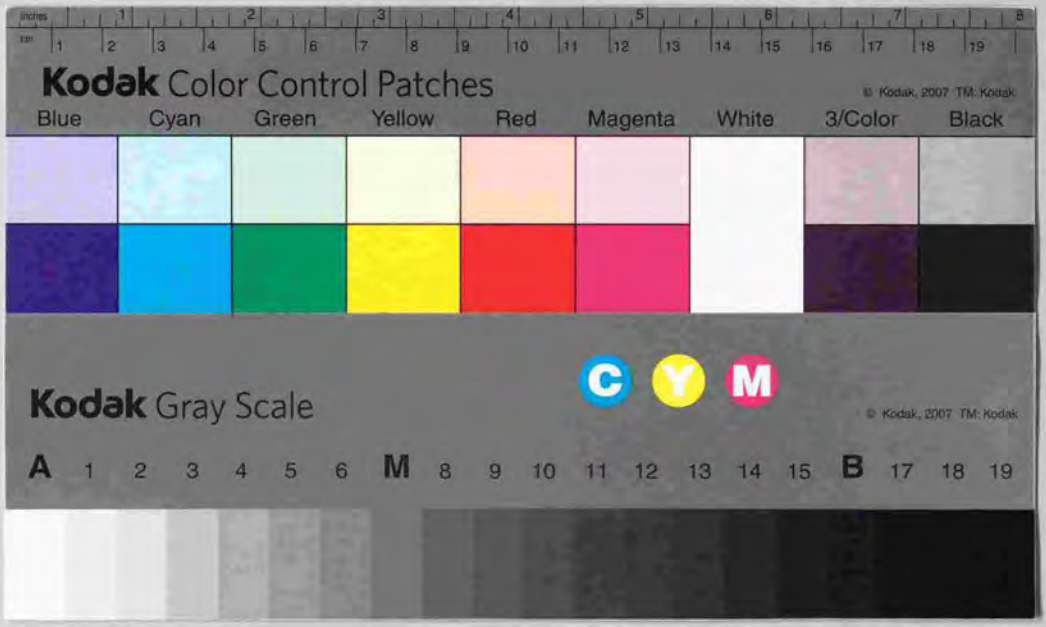
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