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Cholinergic and dopaminergic mechanisms involved in the recovery of circadian anticipation by aniracetam in aged rats

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Abstract

We have reported that repeated administration of aniracetam (100 mg/kg po) for 7 consecutive days recovers mealtime-associated circadian anticipatory behavior diminished in aged rats. The present study examines the mode of action underlying the restoration by aniracetam with various types of receptor antagonists. Coadministration of scopolamine (0.1 mg/kg ip) or haloperidol (0.1 mg/kg ip) for the last 3 days significantly reduced the restorative effects of aniracetam without affecting the timed feeding-induced anticipatory behavior by each receptor antagonist itself. The other receptor antagonists, mecamylamine (3 mg/kg ip), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)quinoxaline (NBQX, 1 μg/rat icv) had no effect on either the basal or aniracetam-elicited circadian anticipation. In contrast, ketanserin (1 mg/kg ip) itself recovered the diminished anticipatory behavior as aniracetam did, but it did not alter the restorative effects of aniracetam. Among the receptor antagonists tested, NBQX reduced appetite and haloperidol induced circadian hypoactivity. These results suggest that the food-entrainable circadian oscillations or the temporal regulatory system of behavior is modulated by cholinergic, dopaminergic and serotonergic systems. Furthermore, aniracetam may restore the aging-diminished behavioral anticipation by activating muscarinic acetylcholine (ACh) and/or dopamine (DA) D_2 receptors through the enhanced release of ACh and/or DA in the brain.

Keywords: Aniracetam; Mealtime-associated circadian anticipation; Temporal regulation of behavior; Mode of action; Cholinergic and dopaminergic systems; Aged rats

1. Introduction

Functional loss of a time-keeping system would appear as sleep disorders, circadian rhythm disorders and nocturnal behavioral problems in the geriatric and demented patients with cerebrovascular or neurodegenerative diseases (Mishima and Hishikawa, 1997; Otomo et al., 1991; Witting et al., 1990). The irregular or severely fragmented sleep–waking rhythm is closely associated with behavioral disturbances, including wandering, nocturnal delirium, agitation and aggressiveness (Mishima and Hishikawa, 1997).

Central cholinergic systems have been proposed to be involved in the temporal regulation of behavior in animals (Ono et al., 1995). In addition, there have been successive reports of a neurochemical correlation between central cholinergic neuronal activity and anticipation. For example, extracellular concentrations of acetylcholine (ACh) in the rat hippocampus and frontal (prefrontal) cortex increased during the anticipation phase preceding meal presentation (Ghiani et al., 1998; Inglis et al., 1994). The ACh release in the frontal cortex could be selectively enhanced by the animal's past training experience, perhaps from the anticipation of the predicted reward, indicating the clear implication of cortical cholinergic function in the temporal memory mechanism (Inglis et al., 1994). Clinically, stroke or Alzheimer's patients with a loss of diverse cholinergic markers exhibit chronobiological impairments and behavioral abnormalities (Gottfries et al., 1994; Mishima and Hishikawa, 1997; Otomo et al., 1991; Perry and Perry, 1995; Tohgi et al., 1996; Witting et al., 1990).

Similarly, there appears to be a close association between central dopaminergic systems and food-anticipatory rhythm. Dopamine (DA) activity in the nucleus accumbens increased prior to feeding time in food-entrained rats (Heffner et al., 1980) and chronic treatment

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of methamphetamine induced a reappearance of circadian food-entrainable rhythm in arrhythmic, suprachiasmatic nuclei (SCN)-ablated rats (Honma et al., 1989a). Moreover, the impairment of methamphetamine-induced behavioral anticipation by aging resulted from reduced or disturbed DA transmission (Shibata et al., 1994b). These results indicate that central DA signals are involved in the entrainment pathway to a food-entrainable oscillator and in the regulation of timed feeding-induced circadian anticipatory rhythm.

Aniracetam, a cognition enhancer, has been demonstrated to ameliorate or improve experimentally induced hypoattention and hypovigilance (Nakamura and Kurasawa, 2000; Nakamura et al., 1998a,b), motivation (driving force) reduction (Nakamura and Kurasawa, 2001a), impulsive behavior (Nakamura et al., 2000), impaired rapid-eye movement sleep (Kimura et al., 2000), depression-like state (Nakamura and Tanaka, 2001) and also different types of anxiety (Nakamura and Kurasawa, 2001b) in rodents. Concerning the mode of action, we have neurochemically clarified the preferential activation of the reticulothalamic cholinergic pathway by enhancing ACh release via Group II metabotropic glutamate (mGlu) receptors and by increasing choline acetyltransferase (ChAT) activity (Nakamura and Shirane, 1999; Shirane and Nakamura, 2000), and also found the regionally specific release of DA and serotonin (5-HT) in the mesocorticolimbic pathway (Nakamura et al., 2001).

In a recent report, we have shown that mealtime-associated anticipatory activity rhythm, which was measured as an index of the circadian time-keeping or temporal regulation of behavior, was diminished with aging and repeated administration of aniracetam and physostigmine restored the impaired circadian anticipatory behavior in aged rats (Tanaka et al., 2000). Although we presently speculate that aniracetam resumes the diminished anticipatory activity by ameliorating the circadian oscillatory function or temporal regulatory system through the facilitation of the cholinergic neurotransmission, the details remain to be solved. Additionally, there are the latest findings that systemic administration of aniracetam selectively releases DA and 5-HT in the dorsal hippocampus, basolateral amygdala and prefrontal cortex by a cholinergic–monoaminergic interaction via nicotinic ACh receptors in the ventral tegmental area and dorsal raphe nucleus (Nakamura et al., 2001; Shirane and Nakamura, 2001). These results prompted us to clarify the mode of action of aniracetam that restored the mealtime-associated circadian anticipatory behavior impaired in aged rats.

The mode of action involved in the aniracetam-elicited recovery of mealtime-associated anticipatory behavior in aged rats was therefore examined through the interaction with scopolamine, a muscarinic ACh receptor antagonist, mecamylamine, a nicotinic ACh receptor antagonist, 2,3-dihydroxy-6-nitrosulfamoyl-benzo(*F*)quinoxaline (NBQX), an α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist, haloperidol, a DA D_2 receptor antagonist and ketanserin, a preferential 5-HT_{2A} receptor antagonist.

2. Method

2.1. Animals

Male Wistar rats were obtained from Charles River Japan and housed in groups of about three rats per cage. They were kept in a room with a controlled temperature of 22 ± 2 °C, relative humidity of 55 ± 10% and illumination from 0730 to 1930 h. The animals had free access to water and food (CRF-1, Charles River Japan) until the start of the experiment. The study was carefully performed according to the "Principles of Laboratory Animal Care," which were dictated by the Animal Care and Use Committee at Nippon Roche Research Center and approved by the Japanese authorities.

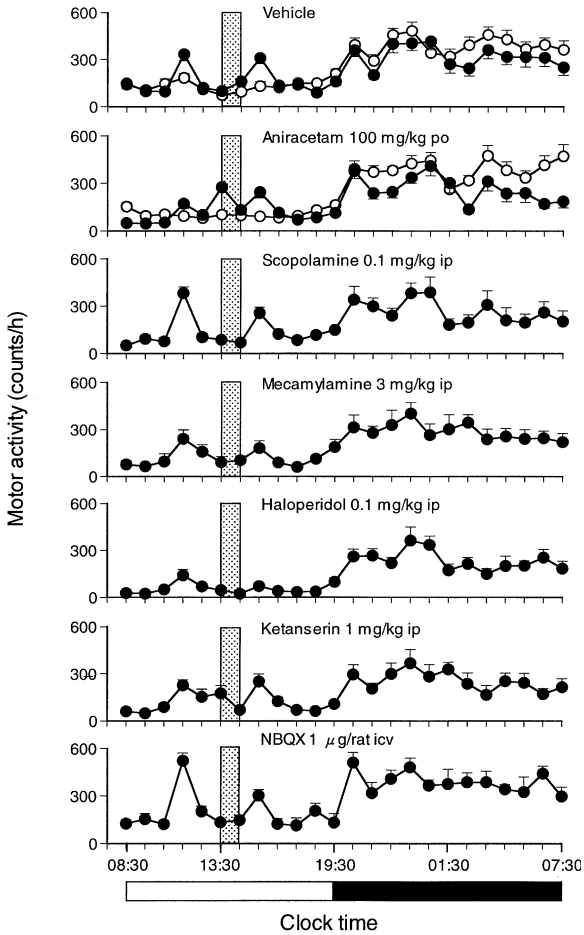

Fig. 1. Effects of aniracetam and various types of receptor antagonists on circadian motor and mealtime-associated anticipatory activity rhythms on Day −1 (○) and Day 7 (●) in aged rats. Data show means ± S.E.M. (n = 6–8 rats/group) of locomotor activity measured every 1 h. Dotted columns indicate a daily feeding period.

2.2. Mealtime-associated motor activity

Mealtime-associated circadian anticipation is known to be diminished with aging (Mistlberger et al., 1990; Shibata et al., 1994a; Tanaka et al., 2000). Therefore, aged (around 25–30 months old) rats were used. The experiment was performed by the method described previously (Tanaka et al., 2000). Briefly, each rat was individually housed in a plastic cage (21 × 32 × 26 cm) to become habituated to the novel circumstances for 1 week before the experiment. Motor activity in the cage was measured with the AB system (Neuroscience, Tokyo, Japan), which was designed to detect the infrared radiation from rats, and the activity counts were recorded at 1-h intervals. After the rats were fasted for 24 h (Day 0), they were fed ad lib for only 1 h from 1330 h on 6 consecutive days (Days 1–6). On Day 7, food was withheld again. The circadian anticipatory behavior emerged during the anticipatory phase from 1130 h to the beginning (1330 h) of feeding on Day 7. Animals had free access to water throughout the experiment. Daily food intake was measured during the last 3 days (Days 5–7) of the restriction periods and averaged. The anticipatory behavior was shown as the motor activity (counts) during the 2-h period or the proportion of activity counts during the anticipatory phase to the total activity counts for 24 h on Day 7. Light and dark periods commenced at 0730 and 1930 h, respectively.

2.3. Drugs

The compounds used here were aniracetam (Ro13-5057), which was synthesized at F. Hoffmann-La Roche (Basel, Switzerland), scopolamine hydrobromide (Tokyo Kasei Kogyo, Japan), NBQX disodium (Research Biochemicals Int., Natick, MA, USA), ketanserin tartrate (Research Biochemicals Int.), haloperidol (Serenace® Injection, Dainippon Seiyaku, Japan) and mecamylamine hydrochloride (Sigma, St. Louis, MO, USA). Aniracetam suspended in 0.25% carboxymethyl cellulose (CMC) solution containing a few drops of Tween 80 was administered orally in a volume of 5 ml/kg, and scopolamine, haloperidol, ketanserin and mecamylamine dissolved in 0.9% saline were given intraperitoneally in a volume of 1 ml/kg. NBQX dissolved in phosphate-buffered saline (pH 7.4) was injected into the left lateral ventricle (AP: − 1.4 mm relative to the bregma, ML: 2.0 mm, DV: 5.0 mm) through a chronically implanted cannula in conscious rats at a volume of 3 μl as described previously (Nakamura et al., 2000). The dose of each compound was calculated as the salt form. Aniracetam or vehicle was administered to rats immediately after the termination of the feeding time, once daily for 7 consecutive days from Days 1 to 7. Each receptor antagonist or vehicle given three times, immediately after the aniracetam treatment on Days 5 and 6, and also at 1030 h on Day 7. Control

Table 1
Effects of various types of receptor antagonists on the aniracetam-elicited circadian anticipatory behavior on Day 7 in aged rats

		Anticipatory motor activity	
Treatment	Dose (mg/kg)	Counts	% Counts
Vehicle	–	210 ± 87.6	3.4 ± 0.9
Aniracetam	100 po	435 ± 32.0*	8.6 ± 0.5***
Scopolamine	0.1 ip	170 ± 35.2	3.7 ± 0.7
+ Aniracetam	100 po	317 ± 54.0	5.8 ± 0.8#
Vehicle	–	173 ± 75.5	2.8 ± 0.7
Aniracetam	100 po	420 ± 55.5*	8.3 ± 1.3**
Mecamylamine	3 ip	249 ± 68.3	5.0 ± 1.1
+ Aniracetam	100 po	280 ± 48.8	7.2 ± 0.9**
Vehicle	–	183 ± 28.6	3.2 ± 0.6
Aniracetam	100 po	304 ± 65.3	7.6 ± 1.5**
Haloperidol	0.1 ip	114 ± 21.1	3.4 ± 0.6
+ Aniracetam	100 po	183 ± 40.1	4.2 ± 0.6#
Vehicle	–	170 ± 27.4	3.1 ± 0.5
Aniracetam	100 po	322 ± 47.0*	8.0 ± 0.6***
Ketanserin	1 ip	328 ± 35.6*	8.2 ± 1.1***
+ Aniracetam	100 po	374 ± 42.3*	7.2 ± 0.6***
Vehicle	–	241 ± 37.8	4.3 ± 0.8
Aniracetam	100 po	439 ± 37.5*	8.5 ± 0.6***
NBQX	1 μg/rat icv	335 ± 46.2	4.9 ± 0.5
+ Aniracetam	100 po	429 ± 51.9	8.8 ± 0.9***

Aniracetam or vehicle was administered to rat once daily for 7 consecutive days (Days 1–7). Scopolamine, mecamylamine, haloperidol, ketanserin, NBQX or vehicle was given once daily for 3 consecutive days (Days 5–7). Anticipatory behavior was expressed as motor activity counts or the proportion of activity counts during a 2-h anticipatory period from 1130 h to the total activity counts for 24 h on Day 7. Data show means ± S.E.M. ($n = 5–9$ rats/group).

* $P < .05$, compared with corresponding vehicle (one-way ANOVA followed by Dunnett's t test).

** $P < .01$, compared with corresponding vehicle (one-way ANOVA followed by Dunnett's t test).

*** $P < .001$, compared with corresponding vehicle (one-way ANOVA followed by Dunnett's t test).

\# $P < .05$, compared with aniracetam alone (Student's t test).

rats were given 0.25% CMC solution, 0.9% saline or phosphate-buffered saline as a vehicle under the same administration schedule.

The doses of the compounds were decided according to the past reports by other investigators and our experience. For example, the 100-mg/kg dose of aniracetam corresponded to one that effectively restores the circadian anticipatory behavior diminished in aged rats (Tanaka et al., 2000). The dosage of each receptor antagonist completely or almost blocked certain pharmacological actions in animals induced by respective agonists or stimulants without causing serious toxic symptoms (Nakamura et al., 1998b, 2000; Schreiber et al., 1995; Semba et al., 1998; Shannon et al., 1990; Shibata et al., 1995a). Additionally, the doses of the receptor antagonists were approximately compatible with those that have been consistently used to examine the mode of action of aniracetam in the recent behavioral pharmacological studies (Nakamura and Kurasawa, 2001b; Nakamura and Tanaka, 2001).

2.4. Data analysis

Differences between groups were statistically analyzed with one-way analysis of variance (ANOVA) for repeated-measures designs followed by a multiple comparison of the Ryan–Einot–Gabriel–Welsch range test for repeatedly measured data, or with one-way ANOVA followed by Dunnett's t test and Student's t test. The percentage data were modified by angular transformation for one-way ANOVA. A P value of $<.05$ was considered statistically significant.

3. Results

3.1. Effects of ACh antagonists

As shown in Fig. 1, vehicle-treated aged rats exhibited a typical nocturnal motor activity rhythm before (Day −1) and after (Day 7) the food restriction. A similar pattern was also seen on Day −1 in aniracetam-treated group. During the food restriction period, mealtime-associated anticipatory activity was not generated in vehicle-treated group and there was only 1.5% increase of the percentage counts on Day 7 as compared with that on Day −1 (Fig. 1). In contrast, repeated administration of aniracetam (100 mg/kg po) for 7 consecutive days gradually emerged the anticipatory activity and probably reached the maximum levels on Day 7 [+302% as % counts, $F(6,2)=44.1$, $P=.0001$ vs. Day −1 value; +253%, $F(3,24)=8.91$, $P=.0004$ vs. vehicle] (Fig. 1 and an interaction experiment with scopolamine in Table 1). The aniracetam treatment significantly ($P<.05$ or .01) and reciprocally altered the diurnal and nocturnal motor activities on Day 7 as compared with the values on Day −1

Table 2
Effects of aniracetam in combination with or without scopolamine and mecamylamine on diurnal and nocturnal motor activity in aged rats

Treatment	Dose (mg/kg)	Period	Motor activity (counts)		
			Day −1	Day 6	Day 7
Vehicle	–	Light	1515 ± 295	1913 ± 429	2131 ± 481
		Dark	4308 ± 760	3381 ± 565$^{\#\#}$	3244 ± 548$^{\#\#}$
		Total	5823 ± 1035	5295 ± 924	5376 ± 953
Aniracetam	100 po	Light	1220 ± 100	1906 ± 240$^{\#\#}$	1693 ± 146$^{\#}$
		Dark	4762 ± 448	3674 ± 362$^{\#\#}$	3401 ± 306$^{\#\#}$
		Total	5982 ± 514	5579 ± 491	5095 ± 313$^{\#}$
Scopolamine	0.1 ip	Light	1263 ± 299	2022 ± 182$^{\#}$	1569 ± 176
		Dark	3852 ± 599	3646 ± 482	3225 ± 627
		Total	5116 ± 807	5668 ± 606	4794 ± 634
+ Aniracetam	100 po	Light	1511 ± 181	2214 ± 228$^{\#\#}$	1747 ± 176
		Dark	4204 ± 270	3379 ± 349$^{\#}$	3753 ± 261
		Total	5715 ± 350	5593 ± 512	5499 ± 348
Vehicle	–	Light	1394 ± 221	1832 ± 381	1875 ± 425
		Dark	4023 ± 537	3464 ± 491	3429 ± 518
		Total	5417 ± 665	5297 ± 809	5304 ± 840
Mecamylamine	3 ip	Light	1314 ± 232	1783 ± 351	1458 ± 285
		Dark	3741 ± 589	2679 ± 506$^{\#\#}$	3430 ± 582
		Total	5054 ± 791	4462 ± 819	4888 ± 782
+ Aniracetam	100 po	Light	1194 ± 158	1796 ± 184$^{\#\#}$	1269 ± 99.6
		Dark	4032 ± 387	2847 ± 339$^{\#\#}$	2558 ± 344$^{\#\#}$
		Total	5227 ± 508	4643 ± 488$^{\#}$	3828 ± 431$^{\#\#}$

After fasting for 24 h (Day 0), feeding was restricted to only 1 h from 1330 h for 6 consecutive days (Days 1–6). Food was withheld again on Day 7. Aniracetam or vehicle was administered immediately after the termination of feeding time once daily for 7 consecutive days (Days 1–7). Scopolamine, mecamylamine or vehicle was given immediately after the aniracetam treatment on Days 5 and 6, and also 1030 h on Day 7. Data show means ± S.E.M. ($n=5$–9 rats/group).

$^{\#}$ $P<.05$, compared with corresponding Day −1 values (repeated measures one-way ANOVA followed by the Ryan–Einot–Gabriel–Welsch range test).

$^{\#\#}$ $P<.01$, compared with corresponding Day −1 values (repeated measures one-way ANOVA followed by the Ryan–Einot–Gabriel–Welsch range test).

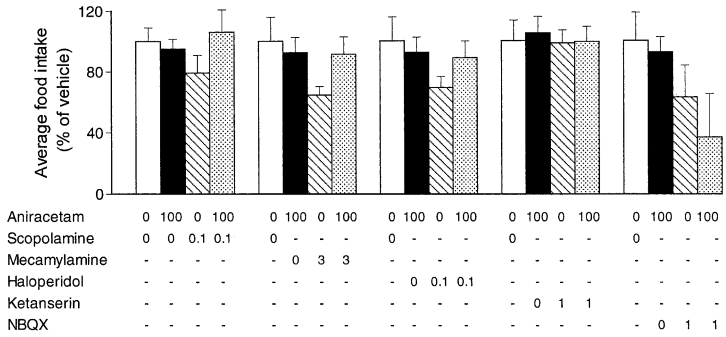

Fig. 2. Effects of aniracetam, various types of receptor antagonists and their combined treatments on food intake during the food-restricted period in aged rats. Aniracetam (100 mg/kg po) or vehicle was administered to rats once daily for 7 consecutive days (Days 1–7). Each receptor antagonist (mg/kg or μg/rat) or vehicle was given once daily for 3 consecutive days (Days 5–7). Average food intake was measured before (Days −6 to −2) and during (Days 2–6) the food restriction and the proportion of the food restriction to the prerestriction was calculated. Data show means ± S.E.M. ($n = 6–9$ rats/group).

(Table 2). However, these changes were similarly observed in vehicle-treated group and there was no significant difference in such variables on Day 7 between vehicle-and aniracetam-treated groups. Since the daily motor activity greatly varied between individuals, the percentage counts was thought to be a better index of anticipatory behavior than the motor activity counts. Indeed, the effects of aniracetam in the scopolamine experiment were observed more clearly in the percentage counts than the nonsignificant counts (Table 1). Coadministration of scopolamine (0.1 mg/kg ip) significantly reduced the restoring effects of aniracetam (−33% as % counts, $P < .05$), whereas the combined treatment with mecamylamine (3 mg/kg ip) had no effect on it (−13% as % counts) (Table 1). Both receptor

Table 3
Effects of the combined treatment of aniracetam with haloperidol, ketanserin and NBQX on diurnal and nocturnal motor activity in aged rats

			Motor activity (counts)		
Treatment	Dose (mg/kg)	Period	Day −1	Day 6	Day 7
Vehicle	–	Light	1650 ± 144	1711 ± 179	1759 ± 147
		Dark	4338 ± 578	3575 ± 447	4074 ± 444
		Total	5988 ± 622	5286 ± 573	5834 ± 437
Haloperidol	0.1 ip	Light	1103 ± 120	1073 ± 164	657 ± 110*,##
		Dark	4973 ± 438	2712 ± 289##	2818 ± 324##
		Total	6077 ± 483	3785 ± 393##	3475 ± 385*,##
+ Aniracetam	100 po	Light	1524 ± 252	1596 ± 302	1178 ± 307
		Dark	4936 ± 347	2887 ± 238##	3041 ± 353##
		Total	6460 ± 524	4483 ± 514#	4220 ± 649#
Vehicle	–	Light	1586 ± 138	1650 ± 163	1679 ± 148
		Dark	4413 ± 495	3602 ± 379	3923 ± 405
		Total	5998 ± 526	5252 ± 486	5602 ± 436
Ketanserin	1 ip	Light	1456 ± 155	1706 ± 156	1432 ± 143
		Dark	4558 ± 320	3093 ± 288###	3055 ± 482###
		Total	6014 ± 427	4799 ± 419##	4486 ± 598##
+ Aniracetam	100 po	Light	1399 ± 88	1573 ± 154	1505 ± 171
		Dark	4799 ± 308	3474 ± 387##	3743 ± 316##
		Total	6197 ± 329	5047 ± 293#	5248 ± 420#
Vehicle	–	Light	1529 ± 197	2532 ± 185##	1891 ± 251
		Dark	4958 ± 333	4906 ± 976	4183 ± 884
		Total	6487 ± 460	7438 ± 1013	6074 ± 1089
NBQX	1 μg/rat icv	Light	1481 ± 257	2415 ± 225##	2275 ± 255##
		Dark	5556 ± 667	4394 ± 577	4654 ± 408
		Total	7038 ± 876	6808 ± 706	6929 ± 623
+ Aniracetam	100 po	Light	1227 ± 91.2	1818 ± 223*	1795 ± 227
		Dark	5076 ± 621	3464 ± 504#	3318 ± 431#
		Total	6303 ± 567	5282 ± 617	5113 ± 636

Each receptor antagonist or vehicle was given immediately after the aniracetam treatment on Days 5 and 6, and also 1030 h on Day 7. NBQX or vehicle was injected intracerebroventricularly. Data show means ± S.E.M. ($n = 6–8$ rats/group).

* $P < .05$; compared with vehicle (one-way ANOVA followed by Dunnett's t test).

# $P < .05$, compared with corresponding Day −1 values (repeated-measures one-way ANOVA followed by the Ryan–Einot–Gabriel–Welsch range test).

$P < .01$, compared with corresponding Day −1 values (repeated-measures one-way ANOVA followed by the Ryan–Einot–Gabriel–Welsch range test).

$P < .001$, compared with corresponding Day −1 values (repeated-measures one-way ANOVA followed by the Ryan–Einot–Gabriel–Welsch range test).

antagonists given alone, however, did not significantly affect the diminished anticipatory behavior (Table 1 and Fig. 1). In addition, scopolamine and mecamylamine showed the normal circadian motor activity rhythm without greatly altering the diurnal, nocturnal and total daily activity as aniracetam itself did (Table 2 and Fig. 1). Daily food intake during the food-restricted period was unaffected by aniracetam, scopolamine or the combined treatment, whereas mecamylamine alone tended to decrease it (Fig. 2).

3.2. Effects of a DA or 5-HT antagonist

When haloperidol (0.1 mg/kg ip) was coadministered with aniracetam, it significantly inhibited the anticipatory behavior recovered by aniracetam (-45% as % counts, $P<.05$) (Table 1). Haloperidol itself well maintained the typical nocturnal motor activity rhythm (Fig. 1) but significantly decreased all of the diurnal [$F(5,2)=12.8$, $P=.0018$ vs. Day -1; $F(2,17)=4.95$, $P=.0203$ vs. vehicle], nocturnal [$F(5,2)=33.6$, $P=.0001$ vs. Day -1] and total daily [$F(5,2)=29.0$, $P=.0001$ vs. Day -1; $F(2,17)=4.43$, $P=.0283$ vs. vehicle] motor activity on Day 7 (Table 3). However, haloperidol alone showed no effect on the diminished anticipatory activity (Table 1 and Fig. 1). On the other hand, although the effects of aniracetam were neither inhibited nor potentiated by the cotreatment of ketanserin (1 mg/kg ip), ketanserin alone significantly ameliorated the diminished anticipatory behavior [$F(3,25)=11.9$, $P=.0001$] to the same degree as aniracetam did (Table 1). However, ketanserin had no significant effect on the circadian motor activity rhythm, and diurnal, nocturnal and total daily activity through the experimental period in comparison with that in the vehicle-treated group (Table 3 and Fig. 1). With respect to appetite, haloperidol weakly reduced the food intake, but ketanserin had no effect on it (Fig. 2).

3.3. Effects of an AMPA antagonist

The NBQX (1 μg/rat icv) that did not alter the circadian motor activity rhythm, diurnal and nocturnal motor activity by itself neither influenced basal nor aniracetam-elicited anticipatory behavior ($+3.5\%$ as % counts) (Tables 1 and 3; Fig. 1). However, NBQX alone or in combination with aniracetam greatly reduced the food intake during the food-restricted period (Fig. 2).

4. Discussion

The food-anticipatory activity is thought to be regulated by a self-sustaining, food-entrainable pacemaker, which physically and functionally differs from a light-entrainable circadian pacemaker. Lines of evidence indicate that food-entrainable and psychostimulant-induced oscillators are located outside the SCN, evidenced by the functional presence of these two oscillators in SCN-ablated rats (Honma et al., 1989a,b). Those oscillators have been reported to be impaired by aging (Shibata et al., 1994a).

In the present study, we well confirmed that aniracetam restored the mealtime-associated circadian anticipatory behavior diminished with aging, a useful animal model of circadian rhythmicity or temporal regulation of behavior (Mistlberger et al., 1990; Shibata et al., 1994a; Tanaka et al., 2000) and demonstrated the underlying mechanism that the recovery of the circadian anticipation by aniracetam was mediated by central cholinergic and dopaminergic activation.

Aging disturbs the temporal regulation of circadian anticipatory behavior, possibly as a result of the dysfunction of the central nervous systems. Among them, the cholinergic system is believed to be one of the most important components to influence such regulation (Mistlberger et al., 1990; Ono et al., 1995; Shibata et al., 1994a). Recent neurochemical studies indicate a close correlation between central cholinergic neuronal activity and anticipation (Ghiani et al., 1998; Inglis et al., 1994). Although we did not examine the effects of other cholinergic agonists in the present study, the previous reports from ours and others showed that arecholine and physostigmine attenuated the impairment of mealtime-associated anticipatory activity rhythm in aged rats (Ono et al., 1995; Tanaka et al., 2000). In the present study, scopolamine alone at the dose to effectively block muscarinic ACh receptors (Shannon et al., 1990) did not affect the mealtime-associated anticipatory activity but it significantly reduced the restoration of the anticipatory behavior by aniracetam. These results suggest that muscarinic ACh receptors positively regulate the food-entrainable circadian anticipation when the receptors are stimulated endogenously or exogenously. However, muscarinic and nicotinic ACh receptor densities and ACh release were decreased in the rat brain with aging (Nabeshima et al., 1994; Uchida et al., 1997; Wu et al., 1988). Systemic administration of aniracetam or local perfusion of its metabolites has been proven to enhance ACh release in the hippocampus, nucleus reticularis thalami and prefrontal cortex of rats (Giovannini et al., 1993; Nakamura and Shirane, 1999). Moreover, there are some further results showing the central cholinergic activation by aniracetam, as evidenced by a down-regulation of muscarinic ACh receptors and reversal of ACh levels decreased by scopolamine (Martin and Haefely, 1993), and increase of ChAT activity (Egashira et al., 1996; Nakamura and Shirane, 1999). Taken together, aniracetam may, therefore, contribute to the recovery of the aging-diminished anticipatory behavior by stimulating muscarinic ACh receptors through the activation of central cholinergic systems (i.e., enhanced ACh release and ChAT activation). On the other hand, it seemed unlikely that nicotinic ACh receptors were involved in the effects of aniracetam, because the coadministration of mecamylamine did not prevent the effects. In contrast, we have recently found that aniracetam acts to be antidepressive in a forced swim test in aged rats (Nakamura and Tanaka, 2001) and anxiolytic in a social interaction test in mice (Nakamura and

Kurasawa, 2001b) with a mecamylamine-sensitive mechanism. Thus, aniracetam may represent its different biological effects via diverse cholinergic mechanisms.

Haloperidol has been reported to have no effect on the emergence of food-entrained rhythm (Mistlberger and Mumby, 1992). In contrast, methamphetamine reappeared anticipatory rhythm in SCN-ablated rats (Honma et al., 1989a) and induced behavioral anticipation in intact rats that was completely blocked by haloperidol at 0.1 mg/kg ip (Shibata et al., 1994b, 1995a), although we have no data on known dopaminergic agonists. The present results also showed that haloperidol at the same dose did not affect the aging-impaired mealtime-associated anticipatory activity. Nevertheless, the receptor antagonist almost attenuated the aniracetam-elicited restoration of the circadian anticipatory behavior in aged rats. Therefore, these results suggest that the central dopaminergic system is actively involved in the basal and aniracetam-elicited regulation of the food-entrainable oscillations. No aggravation of the impairment by haloperidol seen in the present study appeared to result from the complete loss of the anticipatory behavior by aging. On a neurochemical basis, we recently observed that oral treatment of aniracetam (100 mg/kg po) specifically enhanced DA release in the prefrontal cortex, basolateral amygdala and dorsal hippocampus of freely moving stroke-prone spontaneously hypertensive rats (SHRSP) (Nakamura et al., 2001). Further studies clearly indicate the presence of the ventral tegmental area as one of the target sites of aniracetam (actually its metabolites) (Shirane and Nakamura, 2001). In addition, the dopaminergic system in the rat brain exhibits age-associated declines both presynaptically (turnover) and postsynaptically (DA D_2 receptor density) (Moretti et al., 1987; Morgan, 1987). The behavioral evidence that aniracetam improved apomorphine-induced performance impairments in a two-lever choice reaction task in middle-aged rats is supportive to those neurochemical findings (Nakamura et al., 1998b). However, it seemed unlikely that haloperidol-evoked hypoactivity inhibited the emergence of anticipatory behavior elicited by aniracetam, since haloperidol did not prevent the emergence of food-entrained rhythm (Mistlberger and Mumby, 1992). Overall, these results indicate that aniracetam may restore the aging-diminished circadian anticipatory behavior by enhancing DA release and by stimulating DA D_2 receptors, especially in the mesocorticolimbic pathway (Armstrong, 1980).

It is unclear whether mammalian clock genes are expressed in relation to the emergence of circadian anticipatory behavior. However, Wakamatsu et al. (2001) recently revealed that food-entrainable oscillations are associated with a phase-shift of expression of mPer 1 and mPer 2 mRNA in the cerebral cortex and hippocampus of mice. Thus, the restorative process of circadian anticipation by aniracetam, which is based on the cholinergic and dopaminergic mechanisms, may include an increase in the daytime expression of mPer genes, especially in the cerebral cortex, since aniracetam commonly enhanced the release of ACh and DA in the prefrontal cortex and exerted a cholinergic–dopaminergic interaction in the mesocortical DA pathway (Inglis et al., 1994; Nakamura and Shirane, 1999; Nakamura et al., 2001; Shirane and Nakamura, 2001).

Although serotonergic destruction influences endogenous circadian rhythms and light entrainment (Morin, 1994), the role of the central serotonergic system involved in the mealtime-associated anticipatory activity rhythm remains to be answered. In the present study, ketanserin improved the diminished anticipatory behavior, suggesting a tonic temporal regulation of the behavior by central 5-HT_{2A} receptors and an altered 5-HT neurotransmission in aged rats. Indeed, Shibata et al. (1995b) have reported that other 5-HT_2 (mianserin) or preferential 5-HT_{2A} (ritanserin) antagonist attenuated the impaired mealtime-associated anticipatory activity rhythm in old rats. Previous studies indicate age-related declines in 5-HT_{2A} receptor densities and increases in 5-HT transporter sites and 5-HT turnover (Brunello et al., 1988; Moretti et al., 1987; Morgan, 1987; Nabeshima et al., 1994) in rats. If the decrease in 5-HT_{2A} receptors also occurs presynaptically, 5-HT neuronal firing in the raphe nuclei and terminal 5-HT release would be increased (Wright et al., 1990). Since aniracetam has been found to selectively enhance 5-HT release in the prefrontal cortex, basolateral amygdala and dorsal hippocampus of freely moving SHRSP (Nakamura et al., 2001), the aniracetam-elicited restoration of the circadian anticipation may be due to further acceleration of 5-HT transmission in those regions. Ketanserin may mainly act on the presynaptic 5-HT_{2A} receptors. It seems likely that the appearance of the anticipatory behavior reaches the plateau levels by either treatment. As contrasting examples, we have experienced that aniracetam effectively attenuates 5-HT_{2A} receptor-stimulated head-twitch response and attention deficit in the two-lever choice reaction task (Nakamura and Kurasawa, 2000; Tanaka et al., 1998). However, there was no possibility that aniracetam and its metabolites directly interact with 5-HT_{2A} receptors, since those did not affect [^{3}H]ketanserin binding in the rat frontal cortex (Tanaka et al., 1998) and ketanserin had no effect on the aniracetam-elicited response in the present study.

Aniracetam is accepted to be a dual positive allosteric modulator of both ionotropic AMPA and mGlu receptors (Lu and Wehner, 1997; Martin and Haefely, 1993; Nakamura et al., 2000; Pizzi et al., 1993). Furthermore, *N*-anisoyl-γ-aminobutyric acid, one of the actually active metabolites of aniracetam, has been found to enhance ACh release in the prefrontal cortex of SHRSP by mediating Group II mGlu receptors but not AMPA receptors whereas aniracetam itself was devoid of such effect (Shirane and Nakamura, 2000). Accordingly, as the present results also show, it could be ruled out that systemically administered aniracetam (consequently its metabolites) acts as an ACh releaser by modulating AMPA receptors like AMPA (Giovannini et al., 1993; Kendrick et al., 1996) and stimulates muscarinic ACh receptors. In addition, the present study

suggests that central AMPA receptors are not essential for the mealtime-associated anticipatory behavior in aged rats. Although NBQX suppressed the food intake during the food-restricted periods, the appetite-lowering effects did not prevent the recovery of the anticipatory behavior elicited by aniracetam, suggesting the functional involvement of central AMPA receptors in the regulation of appetite (Stanley et al., 1993) and the differential regulation of appetitive and food-entrainable anticipatory behaviors.

As described above, aniracetam exerts multiple mechanisms in the brain. In the present animal model of circadian anticipation, the restorative effects of aniracetam may be mediated by modulating neuronal circuits. For example, it seems likely that the ACh released by aniracetam (or its metabolites) stimulates postsynaptic muscarinic ACh receptors leading to an increase in DA release (Xu et al., 1989), and then activates DA D_2 receptors. The increase in [^{3}H]pirenzepine binding in the pons–midbrain by the aniracetam metabolite 2-pyrrolidinone may, at least in part, be involved in the muscarinic ACh receptor stimulation (Nakamura et al., 1998a). There may be another possible interaction between glutamatergic and dopaminergic systems. Recently, it has been shown that aniracetam modulates *N*-methyl-D-aspartate (NMDA) receptor function in vitro and in vivo (Healy and Meador-Woodruff, 2000; Pittaluga et al., 1999). The activation of NMDA receptors in the ventral tegmental area leads to DA release in the prefrontal cortex (Takahata and Moghaddam, 1998). Therefore, aniracteam may activate NMDA receptors on DA neuronal cells and result in an increase in DA release and stimulation of DA D_2 receptors. Similar events may occur in the dorsal raphe nucleus and consequently enhance 5-HT release in the prefrontal cortex.

In conclusion, the restoration of the aging-diminished mealtime-associated circadian anticipatory behavior by aniracetam may be mediated by facilitating cholinergic (muscarinic) and/or dopaminergic (DA D_2) neurotransmissions through the enhanced release of ACh and/or DA. Central 5-HT receptors appeared to tonically regulate the behavioral anticipation but the involvement of nicotinic ACh or AMPA receptors was minimal or little.

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