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Anxiolytic effects of aniracetam in three different mouse models of anxiety and the underlying mechanism

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Abstract

The anxiolytic effects of aniracetam have not been proven in animals despite its clinical usefulness for post-stroke anxiety. This study, therefore, aimed to characterize the anxiolytic effects of aniracetam in different anxiety models using mice and to examine the mode of action. In a social interaction test in which all classes (serotonergic, cholinergic and dopaminergic) of compounds were effective, aniracetam (10–100 mg/kg) increased total social interaction scores (time and frequency), and the increase in the total social interaction time mainly reflected an increase in trunk sniffing and following. The anxiolytic effects were completely blocked by haloperidol and nearly completely by mecamylamine or ketanserin, suggesting an involvement of nicotinic acetylcholine, 5-HT_{2A} and dopamine D_2 receptors in the anxiolytic mechanism. Aniracetam also showed anti-anxiety effects in two other anxiety models (elevated plus-maze and conditioned fear stress tests), whereas diazepam as a positive control was anxiolytic only in the elevated plus-maze and social interaction tests. The anxiolytic effects of aniracetam in each model were mimicked by different metabolites (i.e., *p*-anisic acid in the elevated plus-maze test) or specific combinations of metabolites. These results indicate that aniracetam possesses a wide range of anxiolytic properties, which may be mediated by an interaction between cholinergic, dopaminergic and serotonergic systems. Thus, our findings suggest the potential usefulness of aniracetam against various types of anxiety-related disorders and social failure/impairments.

Keywords: Aniracetam; Anxiety; Social interaction; Elevated plus-maze; Conditioned fear stress; Mode of action

1. Introduction

Impaired or abnormal serotonin (5-hydroxytryptamine, 5-HT) and/or dopamine neurotransmission in the brain is implicated in the neurobiology of various types of anxiety, such as generalized anxiety disorder (Millet et al., 1999), panic disorder (Gorman et al., 2000), social anxiety disorder (Nutt et al., 1998), obsessive–compulsive disorder (Millet et al., 1999), and post-traumatic stress disorder (Pearlstein, 2000). Neuroimaging studies emphasize functional abnormalities in the prefrontal cortex and amygdala as critical components in anxiety (Davidson et al., 1999). In particular, consistent activation of the amygdala is found in patients with different anxiety disorders.

In contrast, the etiology of post-stroke anxiety is poorly understood, despite its being one of the most common neuropsychiatric disorders related to cerebral infarction. Post-stroke anxiety includes almost all the individual symptoms of generalized anxiety disorder and is neuroanatomically associated with right-hemisphere lesions and with both cortical and subcortical atrophy (Chemerinski and Robinson, 2000).

Besides the strong involvement of central γ-aminobutyric acid (GABA) and 5-HT systems in the regulation of fear and anxiety, there is growing evidence that cholinergic and dopaminergic systems also modulate emotional behavior. Although various experimental models of anxiety have been proposed to measure different types or states of anxiety, there is some uncertainty as to whether anxiety mechanisms and anxiolytic drugs are uniformly active within and between animal models (Handley and MaBlane, 1993). They often yield variable or contradictory effects, probably as a result of differences in the target receptors or subtypes, animal models, dose range and routes of administration (Griebel, 1995).

Aniracetam is a cognition enhancer and its therapeutic effects on emotional disturbances (anxiety, agitation and depressed mood) have been demonstrated, without it in-

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ducing serious side effects in stroke patients (Otomo et al.,1991). In support of its clinical usefulness, we have demonstrated its anti-impulsive activity on impulsive behavior induced by 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (*F*) quinoxaline (Nakamura et al., 2000), and its anti-depressive action on forced swim-induced immobility as a behavioral despair model (Nakamura and Tanaka, 2001) in rats. Concerning the mode of action of aniracetam, we have also recently reported that aniracetam preferentially activates the reticulothalamic cholinergic pathway by enhancing acetylcholine release through group II metabotropic glutamate receptors and by increasing choline acetyltransferase activity (Nakamura and Shirane, 1999; Shirane and Nakamura, 2000). The latest findings are region-specific concomitant releases of dopamine and 5-HT in the mesocorticolimbic pathway (Nakamura et al., 2001).

However, there have been few studies of anxiety (Petkov et al., 1987). Due to the lack of an available animal model of post-stroke anxiety, the present study was designed to experimentally characterize the anxiolytic-like activity of aniracetam in three different animal models of anxiety (social interaction, elevated plus-maze and conditioned fear stress tests). The anxiolytic mechanisms were examined through the interaction with haloperidol, a dopamine D_2 receptor antagonist, mecamylamine, a nicotinic acetylcholine receptor antagonist, and ketanserin, a preferential 5-HT_{2A} receptor antagonist, in the social interaction test. The anxiolytic effects were compared with those of known anxiolytics, anti-depressants and several types of stimulants and inhibitors. The test compounds examined in the social interaction test, which partly differed from those used in the two other tests, were used to obtain information to improve our understanding of the mode of action of aniracetam and to explore the relevance of several drugs that are prescribed clinically or that are under development. Moreover, three major metabolites (*N*-anisoyl-GABA, *p*-anisic acid and 2-pyrrolidinone) were evaluated to identify the active substance(s).

2. Materials and methods

2.1. Animals

Male ICR (for the elevated maze test) and ddY (for the social interaction and conditioned fear stress tests) mice aged 4 or 6 weeks were obtained from Charles River Japan or SLC Japan. They were housed in groups of five in a room with a controlled temperature (22 ± 2°C), relative humidity (55 ± 10%) and illumination from 07:00 to 19:00. The animals had free access to food (CRF-1, Charles River Japan) and water. The study was carefully performed in accordance with guidelines dictated by the Animal Care and Use Committee of Nippon Roche Research Center and approved by the Japanese authorities. All experiments were performed between 10:00 and 15:30. The age of animals was 5 (social interaction test) or 7 weeks at the beginning of the experiments.

2.2. Social interaction test

The general design was essentially as reported by File (1980). A plastic arena ($30 \times 20 \times 13.5$ cm) located in a wooden soundproof box was used for monitoring the animal's social interaction. The light intensity of the arena floor was 380 lx. The mice used as a test pair were separately housed in a group in non-adjacent holding cages.

Experiments were conducted under the high light (380 lx), unfamiliar test condition. Members of each pair of mice, having no prior experience of the test arena, were placed in opposite corners of the arena, one pair at a time, and then left undisturbed for 5 min. Their behaviors were recorded with a video camera for behavioral assessment. The arenas were replaced after each trial to keep them clean. The behavioral measures were the duration and frequency of non-aggressive, active social behaviors (genital investigation, neck/tail licking, trunk sniffing, facing and following) and their sum, and locomotor activity. Passive body contact was not regarded as a social interaction.

2.3. Elevated plus-maze test

The test procedure was performed based on the method of Lister (1987). Briefly, the plus-maze apparatus was made of plywood and had two opposite open arms (white floor with no wall, 25×8 cm) and two opposite enclosed arms (black floor with black walls, $25 \times 8 \times 20$ cm) mounted 50 cm above the floor. The floor of the arms was smooth.

Mice were placed on the central platform (white floor, 8×8 cm) of the apparatus facing either of the open arms. A video camera was used to monitor the animal's behaviors. The number of entries into the open and enclosed arms and the time spent there were measured for 5 min. Arm entry was defined as all four paws on the arm. Data are presented as the percentage of entries into and time spent on the open arms. The maze apparatus was cleaned after each trial.

2.4. Conditioned fear stress test

The procedure was basically identical to that described by Bouton and Bolles (1980). Briefly, conditioned stress training and testing were conducted in an electric foot-shock chamber in a wooden sound-proof box.

Mice were placed in the center of an acrylic chamber ($24 \times 23 \times 22$ cm) with a stainless grid floor (2 mm in diameter, 1-cm intervals) on day 1, and an inescapable

electric footshock (1.0 mA, 0.3 s) produced with a shock generator/scrambler (SGS-002, Muromachi Kikai, Tokyo) was repeatedly delivered to mice every 10 s for 6 min through the grid floor of the chamber. Thereafter, the mice were returned to their home cages. Between each training session, the apparatus was cleaned and wiped. Twenty-four hours later (day 2), the mice were again placed in the same chamber, but without delivery of a footshock. A video camera was used to measure the duration of the freezing behavior of the animal for 6 min. A "frozen" mouse is one that has all four paws on the grid floor and remains in a fixed, rigid posture, without any visible movement of the body and vibrissae, except for movements related to respiration.

2.5. Locomotor activity

Locomotor activity was measured with the AB system (Neuroscience, Tokyo). A sensor mounted vertically above the plastic arena for the social interaction test or on the ceiling of the footshock chamber for the conditioned fear stress test recorded infrared beam breaks as the mouse moved in the arena or chamber.

2.6. Drugs and treatment

Aniracetam (10–100 mg/kg), *N*-anisoyl-GABA (10 and 30 mg/kg), diazepam (0.03–3 mg/kg), fluoxetine (1–10 mg/kg), lazabemide (1–30 mg/kg) and moclobemide (3–30 mg/kg) were synthesized by Hoffman-La Roche (Basel). *p*-Anisic acid (10 and 30 mg/kg) and 2-pyrrolidinone (10 and 30 mg/kg) were purchased from Tokyo Kasei Kogyo (Tokyo) and Wako (Osaka), respectively. Bromocriptine mesylate (1–10 mg/kg), 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) hydrochloride (0.03–1 mg/kg), mecamylamine hydrochloride (1 and 3 mg/kg), (−)-nicotine hydrogen tartrate (0.01–1 mg/kg) and physostigmine hemisulfate (0.01–0.1 mg/kg) were obtained from Sigma (St. Louis, MO), and 5-(propargyloxycarbonyl)-1,4,5,6-tetrahydropyrimidine (CDD-0097) hydrochloride (Messer et al., 1997) (0.1–1 mg/kg), 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) hydrobromide (0.03–3 mg/kg), ketanserin tartrate (0.1–1 mg/kg) and nortriptyline hydrochloride (10–50 mg/kg) were obtained from RBI (Natick, MA). Donepezil hydrochloride (Aricept®) (0.3 and 3 mg/kg), fluvoxamine maleate (Luvox®) (20 and 50 mg/kg), haloperidol (Serenace®) (0.03 and 0.1 mg/kg), methylphenidate hydrochloride (Ritalin®) (1 and 3 mg/kg) and tandospirone citrate (Sediel®) (3–30 mg/kg) were purchased from Eisai (Tokyo), Fujisawa (Osaka), Dainippon (Osaka), Novartis Pharma (Tokyo) and Sumitomo (Osaka) Pharmaceuticals, respectively. Compounds given parenterally were dissolved in physiological saline, and compounds administered orally were suspended in 0.25% carboxymethyl cellulose solution containing a few drops of Tween 80. The test compound or vehicle was administered parenterally in a volume of 10 ml/kg 0.5 h, or orally in a volume of 10 ml/kg 1 h, prior to behavioral testing, while bromocriptine was administered 2 h before testing. All compound solutions were freshly prepared for each experiment.

2.7. Statistical analysis

All results were analyzed using either a one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test or Student's *t*-test. *P* values lower than 0.05 were considered statistically significant.

Table 1
Effects of aniracetam and known compounds on total social interaction time and interaction frequency under high light, unfamiliar test conditions in mice
Compounds were administered orally 1 h, or parenterally 30 min, before the test. All behavioral components of genital investigation, licking, sniffing, facing and following were included in the total social interaction score. Data show means ± S.E.M.

Compound	Dose (mg/kg p.o.)	*N* (pairs)	Total social interaction	
			Time (s/5min)	Frequency (times/5min)
Vehicle	0 i.p.	9	10.6 ± 0.92	12.1 ± 0.90
Diazepam	0.3 i.p.	9	11.3 ± 1.40	13.9 ± 1.37
	3 i.p.	9	12.2 ± 1.44	11.8 ± 1.09
Vehicle	0 s.c.	10	11.7 ± 1.31	13.1 ± 0.92
8-OH-DPAT	0.3 s.c.	9	15.3 ± 2.88	16.9 ± 2.29
	1 s.c.	9	26.4 ± 3.33[b]	21.9 ± 2.09[b]
	3 s.c.	8	33.9 ± 3.40[b]	25.9 ± 2.07[b]
(−)-Nicotine	0.01 i.p.	8	13.5 ± 1.86	14.9 ± 1.73
	0.1 i.p.	10	12.3 ± 1.67	14.4 ± 1.83
	1 i.p.	10	16.2 ± 2.31	17.8 ± 1.82
Vehicle	0 i.p.	16	9.23 ± 0.65	11.9 ± 0.80
DOI	0.03 i.p.	9	13.6 ± 1.30	16.2 ± 1.34
	0.1 i.p.	9	21.9 ± 2.51[b]	23.6 ± 1.74[b]
	0.3 i.p.	9	30.3 ± 3.28[b]	28.0 ± 2.39[b]
	1 i.p.	8	37.8 ± 5.48[b]	30.5 ± 3.13[b]
Vehicle	0	9	8.63 ± 0.99	11.9 ± 1.17
Fluvoxamine	20	8	10.8 ± 1.79	14.8 ± 1.39
	50	8	8.90 ± 1.69	11.1 ± 0.83
Donepezil	0.3	10	14.1 ± 1.81[a]	15.6 ± 1.67
	3	9	14.5 ± 1.81[a]	15.1 ± 1.43
Methylphenidate	1	9	14.5 ± 2.25[a]	17.8 ± 1.65[b]
	3	9	10.9 ± 1.25	13.4 ± 0.87
Vehicle	0 i.p.	10	8.58 ± 0.75	11.2 ± 0.83
Bromocriptine	1 i.p.	9	11.5 ± 1.69	14.8 ± 1.22[a]
	3 i.p.	9	9.21 ± 1.24	13.3 ± 1.15
Vehicle	0	10	8.35 ± 1.01	9.50 ± 0.83
Aniracetam	10	10	16.9 ± 1.60[b]	16.4 ± 1.51[b]
	30	11	12.4 ± 1.11	14.3 ± 1.12[a]
	100	10	15.9 ± 2.38[b]	16.1 ± 1.46[b]

[a] $P < 0.05$ compared with vehicle control.
[b] $P < 0.01$ compared with vehicle control.

3. Results

3.1. Social interaction test

The total social interaction scores are shown in Table 1. Diazepam (0.3 and 3 mg/kg), a benzodiazepine, did not increase either interaction time or interaction frequency, but the higher dose caused sedation and reduced locomotor activity by 43%. 8-OH-DPAT (0.3–3 mg/kg), a 5-HT_{1A} receptor agonist, significantly increased both scores [$F(3,32) = 13.3$, $P < 0.0001$ for interaction time and $F(3,32) = 8.89$, $P < 0.001$ for interaction frequency] in a dose-dependent manner, despite the fact that the higher doses caused hypoactivity (−24% at 1 mg/kg and −32% at 3 mg/kg) as well as 5-HT syndrome such as hypothermia and flat body posture. DOI, a preferential 5-HT_{2A} receptor agonist, dose dependently increased both interaction time [$F(4,46) = 21.0$, $P < 0.0001$] and interaction frequency [$F(4,46) = 21.7$, $P < 0.0001$], but induced hyperthermia and head-twitch at the two higher doses and dose-independent weak hyperactivity. Fluvoxamine, a selective 5-HT reuptake inhibitor, did not produce any effect. Among the cholinergic compounds, (−)-nicotine, a nicotinic acetylcholine receptor agonist, tended to increase social interaction only at the highest dose, and donepezil, an acetylcholinesterase inhibitor, significantly increased interaction time [$F(2,25) = 4.10$, $P < 0.05$] to a similar extent at both doses, accompanied by a non-significant increase in interaction frequency. Methylphenidate, a psychostimulant that inhibits dopamine reuptake and enhances dopamine release, was effective on interaction time [$F(2,24) = 3.64$, $P < 0.05$] and interaction frequency [$F(2,24) = 5.77$, $P < 0.01$], having a significant effect ($P < 0.05$ or $P < 0.01$) only at the lower dose without affecting locomotor activity. Bromocriptine (1 and 3 mg/kg), a dopamine D_2 receptor agonist, while not changing locomotor activity, significantly increased the interaction frequency ($P < 0.05$) only at the lower dose. Anirac-

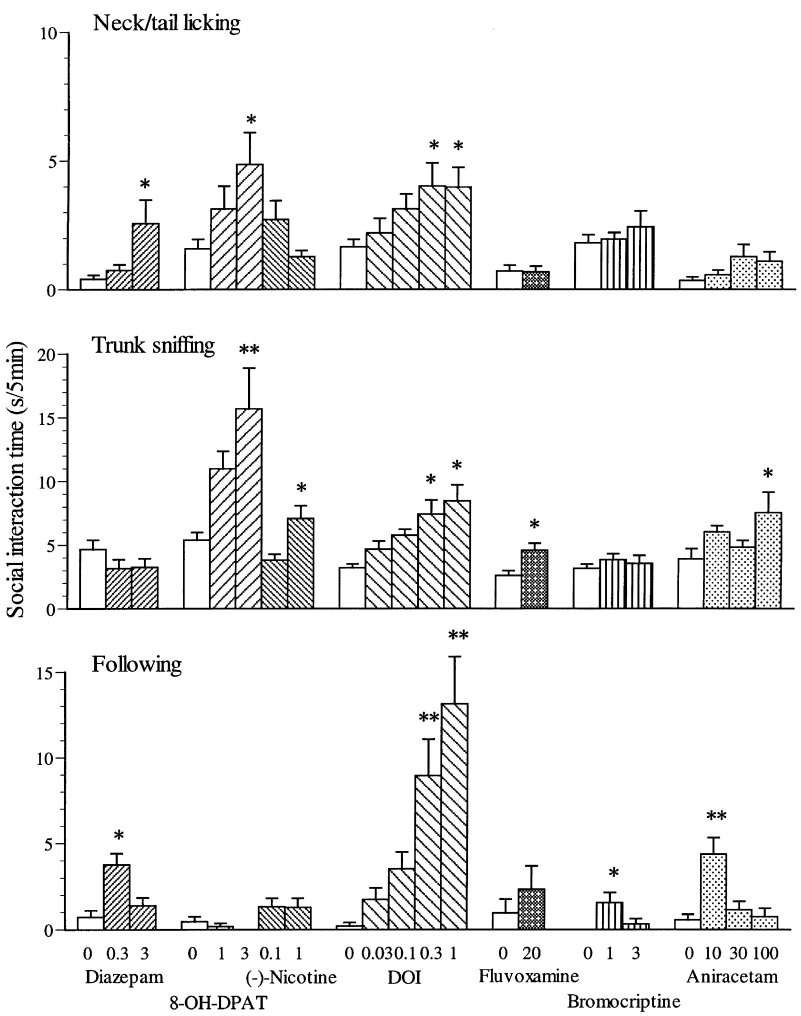

Fig. 1. Effects of aniracetam and known compounds on social interaction time in mice. Compounds (mg/kg) were parenterally administered 30 min before the test, except for fluvoxamine and aniracetam (orally, 1 h prior). Data show means ± S.E.M. ($n = 8$–11 pairs/group but $n = 16$ for vehicle-treated group in the DOI experiment). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with vehicle control.

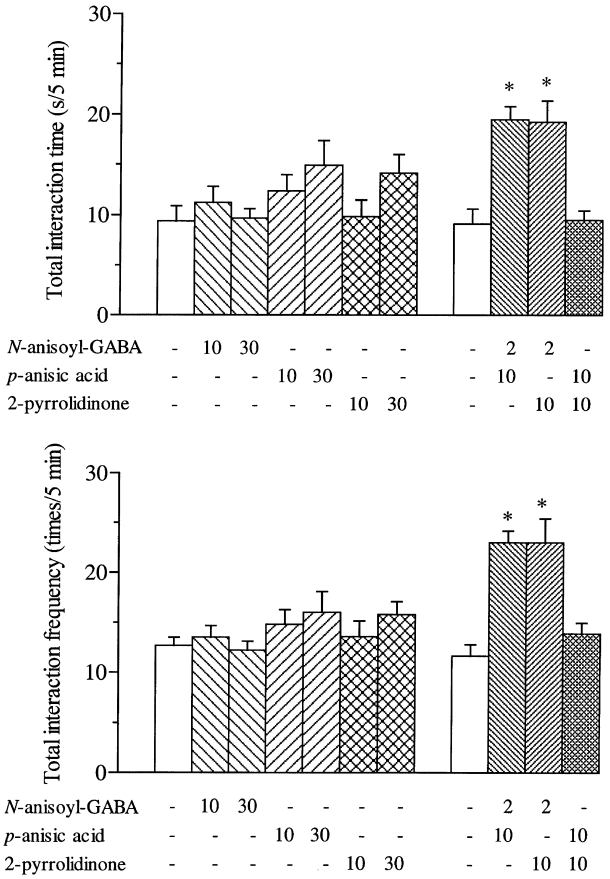

Fig. 2. Effects of aniracetam metabolite alone and different combinations of the two metabolites on total social interaction time and interaction frequency in the social interaction test in mice. Metabolites were orally administered 1 h before the test. Data show means ± S.E.M. ($n = 8$ pairs/group). * $P < 0.01$ compared with vehicle control.

etam also showed anxiolytic effects in the social interaction test (Table 1). Increases in both interaction scores were observed at all tested doses [$F(3,37) = 5.74$, $P < 0.01$ for interaction time and $F(3,37) = 6.29$, $P < 0.01$ for interaction frequency], although they were dose-independent.

Detailed analysis of the interaction time of each social behavior revealed interesting results that were not evident from the total social interaction score (Fig. 1). Among the compounds that had no significant effect on the total score, diazepam significantly increased tail/neck licking [$F(2,24) = 4.45$, $P < 0.05$] and following [$F(2,24) = 4.58$, $P < 0.05$], whereas (−)-nicotine (0.1 and 1 mg/kg) and fluvoxamine (20 mg/kg) increased trunk sniffing ($P < 0.05$ and $P < 0.01$, respectively). 8-OH-DPAT (1 and 3 mg/kg) dose-dependently increased licking [$F(2,24) = 3.64$, $P < 0.05$] and sniffing [$F(2,24) = 7.65$, $P < 0.01$] as well as genital investigation [$F(2,24) = 4.57$, $P < 0.05$] and facing [$F(2,24) = 5.20$, $P < 0.05$] (data not shown). DOI also produced a dose-dependent and significant increase in licking [$F(4,46) = 3.56$, $P < 0.05$], sniffing [$F(4,46) = 9.18$, $P < 0.0001$], following [$F(4,46) = 15.2$, $P < 0.0001$], genital investigation [$F(4,46) = 2.70$, $P < 0.05$] and facing [$F(4,46) = 8.90$, $P < 0.0001$] (data not shown). Donepezil significantly increased sniffing [$F(2,25) = 8.01$, $P < 0.05$] about equally at both doses (data not shown). Methylphenidate increased sniffing [$F(2,25) = 4.56$, $P < 0.05$] with a tendency to increase licking and genital investigation (data not shown). Bromocriptine also increased following [$F(2,25) = 4.89$, $P < 0.05$]. Aniracetam exhibited significant effects on sniffing [$F(3,37) = 2.70$, $P < 0.05$] and following [$F(3,37) = 8.43$, $P < 0.001$] and tended to increase the other social behaviors (Fig. 1).

None of the major metabolites of aniracetam produced a significant effect on the total interaction score by themselves; however, the combined treatment with *N*-anisoyl-GABA (2 mg/kg) and *p*-anisic acid (10 mg/kg) or with *N*-anisoyl-GABA and 2-pyrrolidinone (10 mg/kg) significantly increased both interaction time and interaction frequency ($P < 0.01$) (Fig. 2). The active combinations of the metabolites similarly increased all individual behaviors except for facing (data not shown). The combination of *p*-anisic acid and 2-pyrrolidinone had no effect.

We examined the mechanism of action underlying the anxiolytic effects of aniracetam seen in the social interaction test through an interaction study with some receptor

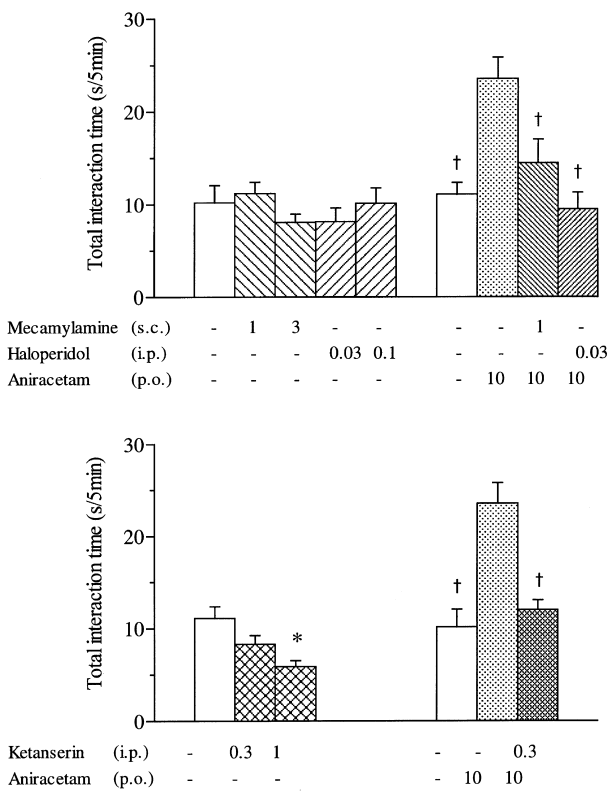

Fig. 3. Inhibition by mecamylamine, haloperidol and ketanserin of the anxiolytic action of aniracetam in the social interaction test in mice. Aniracetam (10 mg/kg) was administered 1 h before the test and each receptor antagonist (mg/kg) was given 30 min after the treatment. Data show means ± S.E.M. ($n = 9$–10 pairs/group). * $P < 0.01$ compared with vehicle control; † $P < 0.01$ compared with aniracetam alone.

Table 2
Effects of known compounds on percentage of entries into and time spent on the open arms by mice in the elevated plus-maze test
Compounds were administered parenterally 30 min, or orally 1 h, before the test. Bromocriptine was given 2 h prior to the test. Data show means ± S.E.M.

Compound	Dose (mg/kg i.p.)	N	%Entries	%Time spent
Vehicle	0	6	33.7 ± 3.78	17.8 ± 4.87
Diazepam	0.3	7	37.8 ± 3.81	30.3 ± 2.64
	1	7	51.0 ± 3.49[a]	35.8 ± 5.53[a]
	3	7	67.8 ± 8.42[b]	67.7 ± 9.59[b]
Moclobemide	3	6	31.9 ± 6.96	20.8 ± 4.52
	10	7	32.4 ± 5.47	25.5 ± 5.17
	30	8	37.4 ± 4.54	20.3 ± 1.26
Vehicle	0	7	27.8 ± 3.27	13.2 ± 2.74
Nortriptyline	10	7	40.4 ± 4.49[a]	32.7 ± 4.54[a]
	20	9	46.4 ± 1.74[b]	25.6 ± 6.03
	50	7	57.4 ± 4.17[b]	48.1 ± 7.35[b]
Ketanserin	0.1	7	38.4 ± 2.78[b]	25.3 ± 4.19[a]
	0.3	8	42.3 ± 1.74[b]	28.7 ± 3.99[a]
	1	8	41.5 ± 2.20[a]	20.8 ± 3.68
Vehicle	0	8	30.5 ± 3.03	16.4 ± 2.80
Fluoxetine	1	8	35.9 ± 2.01	22.8 ± 3.42
	3	7	36.1 ± 2.25	29.4 ± 4.47
	10	8	32.9 ± 2.45	16.6 ± 2.01
Vehicle	0 p.o.	9	29.8 ± 2.89	11.1 ± 1.23
Tandospirone	3 p.o.	7	38.0 ± 3.77	26.5 ± 4.31[b]
	10 p.o.	8	36.5 ± 2.29	18.4 ± 3.34
	30 p.o.	8	37.8 ± 2.94	21.7 ± 2.60[a]
Vehicle	0 s.c.	8	30.6 ± 3.44	16.8 ± 3.19
Physostigmine	0.01 s.c.	8	39.5 ± 2.10	26.4 ± 4.34
	0.03 s.c.	9	44.1 ± 2.51[b]	34.5 ± 3.51[b]
	0.1 s.c.	7	45.1 ± 3.58[b]	9.70 ± 1.01
CDD-0097	0.1	8	38.7 ± 2.20	30.9 ± 3.89[a]
	0.3	8	32.6 ± 2.90	16.5 ± 3.65
	1	8	30.8 ± 1.58	17.8 ± 2.84
Bromocriptine	1	8	34.8 ± 1.99	15.6 ± 3.99
	3	8	33.4 ± 3.62	17.1 ± 3.85
	10	9	41.0 ± 3.23	14.7 ± 2.51
Vehicle	0	7	33.0 ± 2.85	24.4 ± 2.93
Lazabemide	1	8	32.9 ± 3.46	27.1 ± 4.07
	3	8	33.0 ± 3.00	24.6 ± 4.47
	10	8	39.9 ± 2.19	29.0 ± 2.42

[a] $P < 0.05$ compared with vehicle control.
[b] $P < 0.01$ compared with vehicle control.

antagonists. The anxiolytic effects were completely blocked by haloperidol at a non-sedative and non-cataleptic dose of 0.03 mg/kg ($P < 0.01$) (Fig. 3). This complete blockade was observed for all types of social behavior (data not shown). Mecamylamine (1 mg/kg) inhibited the effects of aniracetam by 73% ($P < 0.01$), reflecting the complete reversal especially of sniffing ($P < 0.01$) and facing ($P < 0.01$). Ketanserin (0.3 mg/kg) also inhibited the effects by 86% ($P < 0.01$), as evidenced by a marked decrease in licking ($P < 0.05$), sniffing ($P < 0.05$) and following ($P < 0.01$). Haloperidol and mecamylamine by themselves caused no change in total social interaction time and locomotor activity. In contrast, ketanserin alone (0.3 and 1 mg/kg) reduced social interaction and locomotor activity in a dose-dependent manner [$F(2,26) = 6.30$, $P < 0.01$ and $F(2,26) = 15.5$, $P < 0.0001$, respectively] with a significant effect at the higher dose ($P < 0.01$).

3.2. Elevated plus-maze test

As shown in Table 2, systemic administration of diazepam (0.3–3 mg/kg) increased the percentage of entries into the open arms [$F(3,22) = 6.99$, $P < 0.01$] and the percentage of time spent on the open arms [$F(3,22) = 9.12$, $P < 0.001$] in a dose-dependent manner. One out of seven mice fell from the open arm at the highest dose. Among the serotonergic compounds, nortriptyline, a tricyclic antidepressant, and ketanserin had a significant effect on entries [$F(3,26) = 12.4$, $P < 0.0001$ for nortriptyline and $F(3,26) = 6.92$, $P < 0.01$ for ketanserin] and time spent on the open arms [$F(3,26) = 6.18$, $P < 0.01$ for nortriptyline and $F(3,26) = 3.82$, $P < 0.05$ for ketanserin]. Tandospirone, a 5-HT_{1A} receptor agonist, significantly elevated only time spent on the open arms [$F(3,28) = 5.00$,

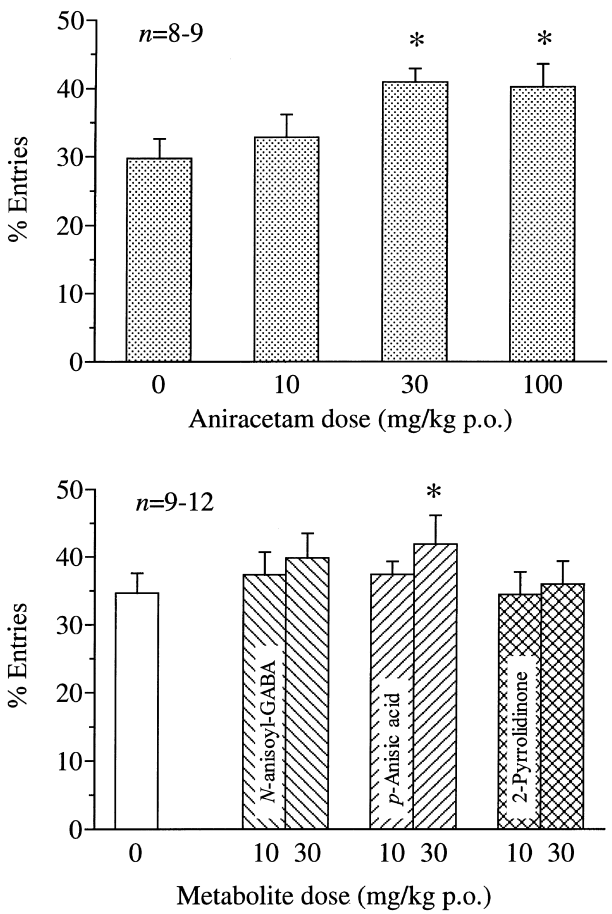

Fig. 4. Effects of aniracetam and its major metabolites on percentage of entries into and time spent on the open arms by mice in the elevated plus-maze test. Data show means ± S.E.M. * $P < 0.05$ and ** $P < 0.01$ compared with vehicle control.

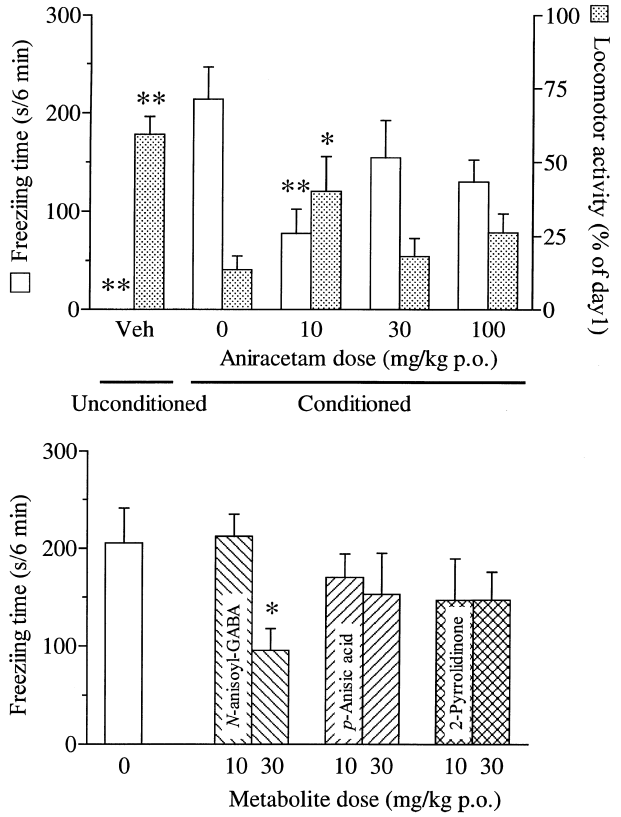

Fig. 5. Effects of aniracetam and its major metabolites on freezing time and locomotor activity in the conditioned fear stress test in mice. Data show means ± S.E.M ($n = 7–8$/group). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with vehicle-treated stress control.

$P < 0.01$], whereas fluoxetine, a selective 5-HT reuptake inhibitor, failed to produce an anxiolytic effect. Animals given the highest dose of nortriptyline (three out of seven) and fluoxetine (six out of eight) stopped their movements on the open arms or showed weak writhing and shrinkage of the hind legs until the start of the test period, respectively. Physostigmine, an acetylcholinesterase inhibitor, concomitantly increased the percentage of entries [$F(3,28) = 5.14$, $P < 0.01$] and time spent on the open arms [$F(3,28) = 12.3$, $P < 0.001$], but reduced time spent on the open arms in spite of an increase in the percentage of entries ($P < 0.01$) at the highest dose. CDD-0097, a muscarinic M_1 acetylcholine receptor agonist (Messer et al., 1997), significantly increased time spent on the open arms ($P < 0.05$) with a tendency to increase the percentage of entries only at the lowest dose. Bromocriptine, moclobemide, a monoamine oxidase A inhibitor, and lazabemide (1–10 mg/kg), a monoamine oxidase B inhibitor, affected neither the percentage of entries nor time spent on the open arms.

Aniracetam significantly increased both the percentage of entries [$F(3,29) = 3.58$, $P < 0.05$] and time spent on the open arms ($P < 0.01$) only at 30 mg/kg (Fig. 4). Of its three major metabolites, only *p*-anisic acid showed a significant anxiolytic effect ($P < 0.05$) at 30 mg/kg.

3.3. Conditioned fear stress test

The conditioned fear stress induced freezing behavior (conditioned: 214 ± 32.5 s, $n = 8$ versus unconditioned: 0 ± 0 s, $n = 8$, $P < 0.01$) in mice during the observation period of 6 min and markedly reduced locomotor activity

Table 3
Effects of known compounds on freezing time in the conditioned fear stress test in mice
Compounds were administered parenterally 30 min, or orally 1 h, before the test on day 2. Bromocriptine was given 2 h prior to the test. Data show means ± S.E.M.

Compound	Dose (mg/kg i.p.)	*N*	Freezing time (s/6 min)
Vehicle	0	10	232 ± 27.1
Diazepam	0.03	10	199 ± 26.9
	0.1	10	208 ± 30.8
	0.3	10	209 ± 19.5
Vehicle	0	7	147 ± 45.8
Diazepam	1	8	261 ± 18.1[a]
	3	8	307 ± 9.49[b]
Vehicle	0	8	185 ± 22.4
Moclobemide	3	8	161 ± 43.9
	10	8	244 ± 36.8
	30	8	253 ± 25.5
Vehicle	0	6	201 ± 41.7
Ketanserin	0.1	8	205 ± 43.2
	0.3	8	216 ± 28.2
	1	8	182 ± 48.7
Fluoxetine	1	8	148 ± 39.2
	3	8	174 ± 40.3
	10	6	52.4 ± 12.1[a]
Vehicle	0	8	242 ± 37.3
Nortriptyline	10	8	263 ± 35.2
	20	8	304 ± 20.5
Vehicle	0 s.c.	8	203 ± 27.4
8-OH-DPAT	0.03 s.c.	8	209 ± 17.3
	0.1 s.c.	8	117 ± 27.4
	0.3 s.c.	8	29.5 ± 14.6[b]
Vehicle	0 s.c.	8	203 ± 27.4
Physostigmine	0.01 s.c.	7	280 ± 34.0
	0.03 s.c.	8	138 ± 39.4
	0.1 s.c.	7	344 ± 5.64[b]
Vehicle	0	8	242 ± 37.3
CDD-0097	0.1	8	205 ± 47.9
	0.3	8	227 ± 30.0
	1	8	219 ± 32.2
Bromocriptine	1	8	183 ± 32.9
	3	8	160 ± 43.1
	10	8	68.8 ± 29.3[a]
Vehicle	0	8	185 ± 22.4
Lazabemide	3	8	190 ± 40.9
	10	8	218 ± 36.1
	30	8	231 ± 28.0

[a] $P < 0.05$ compared with vehicle control.
[b] $P < 0.01$ compared with vehicle control.

($13.1 \pm 4.85\%$ of day 1 versus $59.4 \pm 6.08\%$, $P < 0.01$) (Fig. 5). Diazepam at non-sedative doses (0.03–0.3 mg/kg) did not affect the freezing time, but significantly increased it [$F(2,20) = 8.94$, $P < 0.01$] at sedative doses (1 and 3 mg/kg) (Table 3). Among the serotonergic compounds, only fluoxetine and 8-OH-DPAT (0.03–0.3 mg/kg) significantly shortened the freezing time [$F(3,24) = 2.66$, $P < 0.05$ for fluoxetine and $F(3,28) = 14.2$, $P < 0.0001$ for 8-OH-DPAT]. 8-OH-DPAT induced no 5-HT behavioral syndrome. Physostigmine increased the freezing time [$F(3,26) = 8.73$, $P < 0.001$], probably as a result of sedation with a reduction of 85% ($P < 0.01$) in locomotor activity at 0.1 mg/kg, whereas CDD-0097 did not affect it. Bromocriptine significantly shortened the freezing time [$F(3,27) = 3.05$, $P < 0.05$] in a dose-dependent manner, whereas moclobemide and lazabemide (3–30 mg/kg) failed to reduce it. Although bromocriptine induced sedation until 30 min following the effective dose, this symptom disappeared 2 h later. All effective compounds, fluoxetine, 8-OH-DPAT and bromocriptine, at their respective effective dose did not alter locomotor activity in the test chamber in unconditioned mice (data not shown).

Aniracetam significantly shortened the freezing time [$F(3,28) = 3.55$, $P < 0.05$] with a parallel increase in locomotor activity in the test chamber [$F(3,28) = 2.91$, $P < 0.05$] (Fig. 5). These effects were not due to general motor activation, since the locomotor activity in the chamber of mice naive to electric footshock was unaffected by aniracetam (data not shown). Of the major metabolites, *N*-anisoyl-GABA showed a significant anxiolytic effect ($P < 0.05$) at 30 mg/kg, and other metabolites (*p*-anisic acid and 2-pyrrolidinone) also had a tendency to decrease the freezing time at both doses.

4. Discussion

In this study, we demonstrated that aniracetam was evidently anxiolytic in three different anxiety models (elevated plus-maze, conditioned fear stress and social interaction tests) using mice, and the effects in each model were mimicked by different metabolites or specific combinations of metabolites. The interaction study with some receptor antagonists in the social interaction test indicates a triple mechanism of action via nicotinic acetylcholine, 5-HT_{2A} and dopamine D_2 receptors.

The social interaction test is a useful animal model for evaluating anxiolytic compounds, which are prescribed for treating social phobia, social failure/impairments and emotional immaturity. The positive results of diazepam, 8-OH-DPAT and fluvoxamine in the social interaction test were consistent with the results of previous reports (Olivier et al., 1989; Corbett et al., 1993). The intraraphe (dorsal or median part) injection of 8-OH-DPAT increased social interaction in rats (Higgins et al., 1992; File et al., 1996). However, to our knowledge, this is the first report to experimentally demonstrate that DOI, donepezil, methylphenidate and aniracetam have an anxiolytic-like activity in the social interaction test. Other acetylcholinesterase inhibitors, heptylohysostigmine and tacrine, enhanced the rate of decrease of olfactory investigation on repeated confrontations, indicating a role of acetylcholine in the development of social recognition (Winslow and Camacho, 1995). Nicotinic and muscarinic M_1 acetylcholine receptors, but not M_2 receptors, in the dorsal hippocampus may be functionally important in the anxiety induced by social interaction (File et al., 1998a). (−)-Nicotine showed weak anxiolytic effects, in accord with the results of File et al. (1998b). The anxiolytic effects of bromocriptine were paradoxically supported by the fact that dopamine D_2 receptor antagonists are anxiogenic (Corbett et al., 1993). Thus, the anxiety produced by social contact may depend on serotonergic, dopaminergic and cholinergic mechanisms. In particular, the results obtained with nicotine, DOI and bromocriptine strongly suggest a phasic regulation of social behaviors by nicotinic acetylcholine, 5-HT_{2A} and dopamine D_2 receptors.

The anxiolytic effects of aniracetam in the social interaction test were greatly or completely blocked by either mecamylamine, ketanserin or haloperidol. These results indicate that the effects of aniracetam are mediated by three (cholinergic, serotonergic and dopaminergic) neuronal systems. Recently, we demonstrated that systemic administration of aniracetam enhanced the synaptic release not only of acetylcholine in certain brain regions, including the prefrontal cortex, of rats (Nakamura and Shirane, 1999), but also of dopamine and 5-HT in the prefrontal cortex, basolateral amygdala and dorsal hippocampus, but not in the nucleus accumbens shell and striatum (Nakamura et al., 2001). Moreover, the acetylcholine release was due to the stimulation of group II metabotropic glutamate receptors (Shirane and Nakamura, 2000), whereas the dopamine and 5-HT release resulted from the activation of nicotinic acetylcholine receptors (Shirane and Nakamura, unpublished). Judging from the present results, together with these neurochemical findings, aniracetam appears to exert its anti-anxiety effects via the sequential facilitation of nicotinic acetylcholine, 5-HT_{2A} and dopamine D_2 receptors. The cholinergic mechanism may contribute to the drug's restoration of the impairment of recognition of novel objects, hypoattention and reduced vigilance caused by aging or cholinergic hypofunction (Bartolini et al., 1996; Nakamura et al., 1998a). Serotonergic and dopaminergic mechanisms may be involved in the amelioration of hypoarousal states by aniracetam (Nakamura et al., 1998b; Nakamura and Kurasawa, 2000).

As widely accepted, the basolateral amygdala and the central amygdala are critical structures in the modulation of various types of fear and anxiety responses (Davis et al., 1994; Graeff et al., 1996). In addition, there exists a neural circuit between the amygdala and the prefrontal cortex in response to novel and emotionally arousing events (fear

and anxiety) (Davis et al., 1994; Cahill and McGaugh, 1998). The inhibition of ascending serotonergic pathways from the dorsal raphe nucleus leads to consistent anxiolytic effects in several animal models of anxiety, including the social interaction test (Higgins et al., 1992; Griebel, 1995; File et al., 1996; Graeff et al., 1996). The dorsal raphe nucleus also sends its serotonergic projections to dopamine cells in the ventral tegmental area (Vertes, 1991). Dopamine neurons in the ventral tegmental area express 5-HT_{2A} receptors in rats and in humans (Doherty and Pickel, 2000; Ikemoto et al., 2000). Electrical stimulation of and microinjection of 8-OH-DPAT into the dorsal raphe nucleus enhances 5-HT_{2A}-mediated dopamine release and decreases dopamine release in the projection area, respectively (Yoshimoto and McBride, 1992; De Deurwaerdere and Spampinato, 1999). Therefore, aniracetam may increase social interaction by enhancing 5-HT release in the ventral tegmental area, and consequently by stimulating post-synaptic dopamine D_2 receptors via somatodendritic 5-HT_{2A} receptor-mediated dopamine release in the basolateral amygdala and/or prefrontal cortex (Nakamura et al., 2001). The dorsal raphe nucleus may be triggered by synaptic acetylcholine release and stimulation of somatodendritic nicotinic acetylcholine receptors in the nucleus, elicited by the activation of mesopontine cholinergic nuclei (Nakamura and Shirane, 1999). The 5-HT release in the dorsal hippocampus and basolateral amygdala induced by aniracetam appears not to be involved in the anxiolytic action of aniracetam (File et al., 1996; Gonzalez et al., 1996; Becker et al., 1999). This hypothesis is supported by previous reports that systemic (−)-nicotine increases dopamine and 5-HT release in the nucleus accumbens and frontal cortex of rats, respectively, by activating nicotinic acetylcholine receptors on the respective dopamine and 5-HT cells in the ventral tegmental area and dorsal raphe nucleus (Ribeiro et al., 1993; Nisell et al., 1994). More recently, we have found that dopamine and 5-HT release in the prefrontal cortex elicited by systemically administered aniracetam is completely blocked by intrategmental and intraraphe perfusion of mecamylamine, respectively (Shirane and Nakamura, unpublished), results which evidently support the hypothesis.

Aniracetam has been reported to have no anxiolytic-like action in modified Vogel and Geller–Seifter conflict tests in rats (Petkov et al., 1987; Martin, J.R., unpublished data). However, in the present study, aniracetam exerted significant anxiolytic effects in the elevated plus-maze and conditioned fear stress tests in addition to the social interaction test. The elevated plus-maze test is thought to produce unconditioned fear (clinically compatible with panic anxiety) by single exposure to open spaces (Graeff et al., 1996). Diazepam and most serotonergic compounds, except for fluoxetine, were anxiolytic, in agreement with previous results reported by others (Critchley and Handley, 1987; Griebel et al., 1997). In contrast, the anti-anxiety effects of tandospirone are a new finding. The activation of the dorsal raphe nucleus–periventricular serotonergic pathway may play an important role in the inhibition of innate (unconditioned) fear by serotonergic compounds (Graeff et al., 1996). However, the significant contribution of the median raphe nucleus, but not the dorsal hippocampus and basolateral amygdala, is also suggested (File et al., 1996; Gonzalez et al., 1996; De Almeida et al., 1998). Interestingly, physostigmine and CDD-0097 showed biphasic anxiolytic effects. It has been reported that scopolamine is anxiogenic in the elevated plus-maze test with mice and in humans (Curran et al., 1991; Rodgers and Cole, 1995), and nicotine and a nicotinic receptor agonist are anxiolytic in the same plus-maze test (Brioni et al., 1993). These results clearly indicate the significant involvement of nicotinic and/or muscarinic mechanisms in the anxiety elicited by the elevated plus-maze. However, muscarinic M_1, M_2 and nicotinic acetylcholine receptors located in the dorsal hippocampus may be excluded (File et al., 1998a). In contrast to the cholinergic involvement, it seems unlikely that this type of anxiety results from a dopaminergic mechanism, since both bromocriptine and lazabemide failed to affect the variables investigated. Based on previous neurochemical findings, aniracetam may inhibit unconditioned fear by facilitating a cholinergic–serotonergic interaction (Nakamura and Shirane, 1999; Nakamura et al., 2001), probably via nicotinic acetylcholine or metabotropic glutamate receptors (Maione et al., 1998; Shirane and Nakamura, 2000, unpublished).

Conditioned fear stress-induced freezing behavior is regarded as an animal model of anticipatory/generalized anxiety and also of panic disorder (Inoue et al., 1996a; Cavazzuti et al., 1999). In this study, diazepam not only failed to produce anti-freezing effects, but also augmented the conditioned freezing. These results are partly consistent with previous results for male ddY mice that showed the ineffectiveness of diazepam at 0.2–5 mg/kg i.p. (Nagasaka and Kameyama, 1983) and a tendency to increase immobility time at 1 mg/kg i.p. (Miyamoto et al., 2000). The augmented freezing behavior in the present study may be due to sedation and/or muscle relaxation induced at higher doses. However, Miyamoto et al. (2000) also reported a significant decrease in freezing behavior at lower doses, although there was a difference in the time of injection between their study and ours. Recent work suggests that conditioned aversive stimuli selectively activate the mesofrontal and mesoamygdaloid serotonergic neurons in the dorsal raphe nucleus, and mesoprefrontal dopaminergic neurons in the ventral tegmental area for the normal expression of fear behavior (Graeff et al., 1996; Grahn et al., 1999; Guarraci and Kapp, 1999; Morrow et al., 1999). Fluoxetine and 8-OH-DPAT responded as reported previously (Inoue et al., 1996b), presumably by suppressing serotonergic neurons in the dorsal raphe nucleus. The dose-dependent attenuation of freezing behavior by bromocriptine was similar to that elicited by apomorphine (Nagasaka and Kameyama, 1983). However, selective in-

hibition of monoamine oxidase A or B by moclobemide or lazabemide, respectively, showed no anti-freezing activity, consistent with the results of Maki et al. (2000). Interestingly, physostigmine, but not CDD-0097, augmented freezing behavior. No study of this cholinergic influence has been reported. It may be concluded that conditioned fear stress-induced freezing behavior appears to be regulated mainly by both serotonergic and dopaminergic mechanisms. Thus, the anti-freezing effects of aniracetam may be mediated by the enhanced release of dopamine and/or 5-HT (Nakamura et al., 2001).

The anxiolytic effects of aniracetam were surprisingly mimicked by different metabolites or specific combinations of the metabolites in each model. For example, the effects resembled those elicited by the combination of *N*-anisoyl-GABA with *p*-anisic acid or 2-pyrrolidinone in the social interaction test, *p*-anisic acid in the elevated plus-maze and *N*-anisoyl-GABA in the conditioned fear stress test. These results indicate that the anxiolytic properties of aniracetam are mediated by one or more of its metabolites, but not aniracetam itself. It has been demonstrated that direct application of aniracetam into the rat brain has no effect on the release of acetylcholine, dopamine and 5-HT, whereas certain metabolites enhances the release of these neurotransmitters (Nakamura and Shirane, 1999; Shirane and Nakamura, unpublished).

In conclusion, aniracetam showed a wide range of anxiolytic effects in different anxiety models, although the active metabolite in each model was different. The anxiolytic effects in the social interaction test were mediated by an interaction between cholinergic, serotonergic and dopaminergic systems via nicotinic acetylcholine, 5-HT_{2A} and dopamine D_2 receptors. The present results may, at least in part, suggest the potential usefulness of aniracetam for the treatment of various types of anxiety-related disorders and social failure/impairments.

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