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Activation of the reticulothalamic cholinergic pathway by the major metabolites of aniracetam

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Abstract

The aim of the study was to further investigate the effects of aniracetam, a cognition enhancer, and its metabolites on the brain cholinergic system. We measured choline acetyltransferase activity and acetylcholine release using in vivo brain microdialysis in stroke-prone spontaneously hypertensive rats (SHRSP). The enzyme activity in the pons–midbrain and hippocampus, and basal acetylcholine release in the nucleus reticularis thalami were lower in SHRSP than in age-matched Wistar Kyoto rats, indicating central cholinergic deficits in SHRSP. Repeated treatment of aniracetam (50 mg/kg p.o.× 11 for 6 days) preferentially increased the enzyme activity in the thalamus, whereas decreased it in the striatum. Among the metabolites of aniracetam, local perfusion of *N*-anisoyl-γ-aminobutyric acid (GABA, 0.1 and/or 1 μM) and *p*-anisic acid (1 μM) into the nucleus reticularis thalami, dorsal hippocampus and prefrontal cortex of SHRSP produced a significant but delayed increase of acetylcholine release. We failed, however, to find any effect of aniracetam itself. A direct injection of *N*-anisoyl-GABA (1 nmol) into the pedunculopontine tegmental nucleus of SHRSP enhanced the release in the nucleus reticularis thalami. Thus, these data prove that aniracetam can facilitate central cholinergic neurotransmission via both metabolites. Based on its pharmacokinetic profile, *N*-anisoyl-GABA may contribute to the clinical effects of aniracetam, mainly by acting on the reticulothalamic cholinergic pathway.

Keywords: Choline acetyltransferase; Acetylcholine; Microdialysis; Stroke-prone spontaneously hypertensive rat (SHRSP); Aniracetam; Metabolite

1. Introduction

The reticulothalamic cholinergic pathway, which originates from the pedunculopontine tegmental nucleus and laterodorsal tegmental nucleus (Mesulam et al., 1983; Woolf and Butcher, 1986), is generally considered to be part of the ascending reticular activating systems (Mesulam et al., 1989). Many studies have revealed that the pathways play a functionally important role in the regulation and maintenance of motivation, sleep and arousal, vigilance and attention (Steriade et al., 1990a; Steckler et al., 1994).

Central cholinergic deficits have been reported in patients with ischemic vascular dementia of multi-infarct or Binswanger type. Those include markedly reduced choline acetyltransferase activity, decreased numbers of muscarinic acetylcholine receptors in the hippocampus (Sakurada et al., 1990), and significantly lower concentrations of the enzyme in several brain regions (Gottfries et al., 1994) and of acetylcholine in the cerebrospinal fluid (CSF) (Tohgi et al., 1996). Posterior cholinergic neurons (especially the pedunculopontine tegmental nucleus) were also damaged in Alzheimer's disease, Parkinson's disease and progressive supranuclear palsy (Jellinger, 1988). Such diseases are frequently accompanied by neuropsychiatric syndromes, including cognition deficits, emotional disturbances, sleep disorders and behavior abnormalities.

Aniracetam, a cognition enhancer, has therapeutic efficacies towards emotional disturbances, sleep disorders and behavior abnormalities (delirium and nocturnal wandering) occurring as sequelae of cerebral infarction (Otomo et al., 1991). So far, it has been reported that aniracetam enhanced synaptic efficacy by facilitating long-term potentiation (Martin and Haefely, 1993), activated energy metabolism by increasing ATP production (Himori et al., 1992), and attenuated neuronal damage via metabotropic glutamate receptors (Pizzi et al., 1993). It is especially

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noteworthy that the drug enhances cholinergic neurotransmission, as evidenced by a down-regulation of muscarinic acetylcholine receptors and reversal of acetylcholine levels decreased by scopolamine (Martin and Haefely, 1993), facilitation of acetylcholine release (Giovannini et al., 1993) and recovery of damaged cholinergic transmission (Kondoh et al., 1997). Indeed, aniracetam ameliorated scopolamine-induced delirium-like state (attention deficits and low vigilance) in middle-aged rats (Nakamura et al., 1998) and restored a reduced responding speed in stroke-prone spontaneously hypertensive rats (SHRSP) (Martin and Haefely, 1993). However, aniracetam administered orally has been known to be promptly metabolized and converted to several metabolites in rats and humans (Fig. 1) (Roncari, 1993; Ogiso et al., 1998). The active substance(s) in the brain is obscure and the identification of the target site(s) still remains to be resolved. In the previous study to explore the central action sites, aniracetam restored the decrease of 2-deoxy-D-glucose utilization rate induced by scopolamine in many cerebral nuclei and areas in rats. The authors suggested that the reticulo–thalamo-neocortex pathway is one of the main action sites of aniracetam. Nevertheless, there is no evidence to positively connect the action and its target site of aniracetam with the mesopontine cholinergic neurons.

In the present study, we, therefore, aimed to clarify which metabolite(s) is actually responsible for the cholinergic activation by peripherally administered aniracetam, where is the clinically relevant target site(s), and whether the target site is on cell bodies or on nerve terminals. To address these questions, we examined the effects of aniracetam and its three major metabolites on choline acetyltransferase activity and extracellular acetylcholine concentration (release) as cholinergic presynaptic markers in cerebral regions of SHRSP. The SHRSP are now considered to be a good animal model for multiple cerebral infarction and vascular dementia in humans (Saito et al., 1995), behaviorally (Wultz et al., 1990; Martin and Haefely, 1993; Fujishima et al., 1995; Togashi et al., 1996; Gattu et al., 1997) and neurochemically (Yamada et al., 1984, 1987; Kang et al., 1990; Togashi et al., 1994, 1996; Gattu et al., 1997).

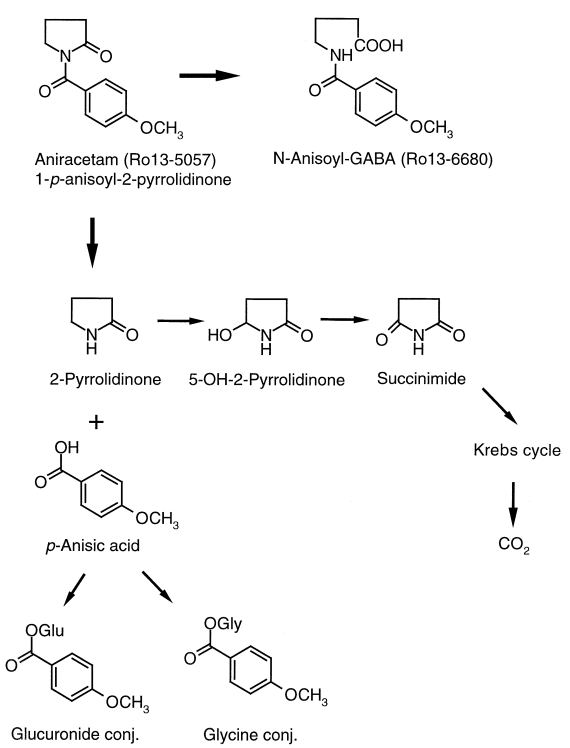

Fig. 1. Chemical structure of aniracetam and its metabolic pathways in rats and humans.

2. Materials and methods

2.1. Animals

Male SHRSP at 7 weeks of age and age-matched Wistar Kyoto rats WKY were obtained from SLC Japan. Animals were housed in groups of three or four in conditions of constant temperature (22 ± 2°C) with a relative humidity of 55 ± 10% and lights on 0730 h to 1930 h. They were given free access to food (CRF-1, Charles River, Japan) and water. To accelerate the early development of hypertension and stroke, SHRSP received 1% NaCl solution instead of water for 5 weeks (Chen et al., 1997). Both strains of animals were used for the following experiments at 13 weeks of age.

2.2. Measurement of choline acetyltransferase activity

Rats were decapitated quickly, and five cerebral regions (pons–midbrain, thalamus, striatum, hippocampus and frontal cortex) were dissected from the brain onto ice-cold glass plates and stored at −80°C until assayed. Choline acetyltransferase activity was measured according to the method of Kaneda and Nagatsu (1985) with a slight modification.

Briefly, cerebral regions were homogenized in 25 volumes (v/w) of 25 mM sodium phosphate buffer containing 0.5% Triton X-100, pH 7.4, using a Teflon homogenizer and left in ice-bath for 30 min, followed by centrifugation at $20{,}000 \times g$ for 60 min at 4°C. The incubation mixture consisted of the following components in a volume of 200 μl: 50 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, 0.5 mM acetyl-CoA (Sigma, St. Louis, MO, USA), 1 mM choline chloride, 0.1 mM eserine hemisulfate (Sigma), 10 mM EDTA and supernatant as an enzyme source. Incubation was performed at 37°C for 20 min, and the reaction was stopped by adding 50 μl of 1 M

$HClO_4$ in ice-bath. After that, 10 μl of 1 mM isopropylhomocholine (Eicom, Kyoto, Japan) as an internal standard was added, and the reaction mixture was centrifuged at $1600 \times g$ for 10 min at 4°C. The supernatant was then filtered with Samprep-LCR (Millipore, USA) and a 5-μl aliquot was injected into a high-performance liquid chromatography (HPLC) system, the conditions of which were detailed in the in vivo microdialysis experiments. For the blank, the enzyme solution was boiled at 95°C for 5 min or replaced with 25 mM sodium phosphate buffer, pH 7.4. An external standard mixture for HPLC consisted of 20 μM acetylcholine perchlorate (Sigma), 10 μM choline chloride and 20 μM isopropylhomocholine, and a 10-μl aliquot was injected.

Protein concentration in the enzyme solution was determined with a Bio-Rad Protein Assay Kit (Bio-Rad Labs., CA, USA) using bovine serum albumin (Sigma) as a standard.

2.3. Surgery for in vivo microdialysis

Rats were anesthetized with sodium pentobarbitone (50 mg/kg i.p.) and then placed in a stereotaxic apparatus. A guide cannula was implanted just above the left thalamus [AP: −1.4 mm, ML: 4.8 mm, DV: 5.8 mm; nucleus reticularis thalami, relative to the bregma and dura surface, at a 20° angle against the cross section (diagonally)], the right prefrontal cortex [AP: 3.3 mm, ML: 3.3 mm, DV: 1.5 mm], the left dorsal hippocampus [AP: −4.5 mm, ML: 4.8 mm, DV: 2.5 mm], and the pedunculopontine tegmental nucleus [AP: −8 mm, ML: 2 mm, DV: 6.6 mm], according to the Brain Atlas of Paxinos and Watson (1986). A stainless steel obdurator was inserted into the guide cannula to prevent occlusion. The rats were allowed to recover for at least 3 days before the experiment. At the end of dialysis experiment, the brain was removed, fixed in 30% formaline containing 20% sucrose, and then sectioned at a thickness of 50 μm. The slices were stained with cresyl violet and the probe location was confirmed. Data from probes that were located inappropriately were discarded (9 out of 94 total locations).

2.4. HPLC determination of extracellular acetylcholine concentration

A concentric microdialysis probe (A-I-4-02 for the prefrontal cortex and dorsal hippocampus or A-I-8-02 for the nucleus reticularis thalami: Eicom) was inserted into the guide cannula and perfused with normal Ringer solution (147 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) containing 10 μM eserine at a constant flow rate of 2 μl/min. After a 3-h equilibration period, the dialysates for every 20 min were collected into 200-μl test tubes containing 5 μl of 0.5 mM HCl to prevent hydrolysis of acetylcholine and ethylhomocholine (Eicom) as an internal standard, and injected into the HPLC system (Eicom). The injected dialysates were separated by Eicompack AC-Gel column (6 mm × 150 mm; Eicom) and an enzymatic reactor (AC-Enzympak, Eicom) with immobilized acetylcholine esterase and choline oxidase to form H_2O_2 from acetylcholine and choline. The resultant H_2O_2 was determined by an electrochemical detector (ECD-100, Eicom) with a platinum electrode at 450 mV. The column was kept at 33°C. The mobile phase, consisting of 0.1 M sodium phosphate buffer, pH 8.2, containing 0.02% sodium 1-decanosulfate and 0.0065% tetramethylammonium chloride, was delivered at a flow rate of 1.0 ml/min. Acetylcholine release was expressed as percent change over the average (basal acetylcholine level) of the first three samples before compound perfusion.

2.5. Drugs and treatment

Compounds used were aniracetam (Ro13-5057, Hoffman-La Roche, Basel, Switzerland), *N*-anisoyl-GABA (Ro13-6680, Hoffman-La Roche), *p*-anisic acid (Tokyo Kasei, Japan) and 2-pyrrolidinone (Wako, Japan). When compounds were administered orally, those were suspended in 0.25% carboxymethylcellulose solution containing a few drops of Tween 80. In the in vivo microdialysis experiments, test compounds were dissolved in Ringer solution and perfused into each region and nucleus at a final concentration of 1 μM for 20 min through the microdialysis probe. For the injection into the pedunculopontine tegmental nucleus, 1 nmol of each compound was applied directly to the nucleus through an inserted injection needle. One microliter was injected over a 1-min period and the needle was kept in place for a further 1 min.

2.6. Statistical analysis

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

3. Results

3.1. Effects of aniracetam on choline acetyltransferase activity in cerebral regions of SHRSP

Of cerebral regions examined, choline acetyltransferase activity in the pons–midbrain and hippocampus were significantly lower in SHRSP than that in age-matched WKY (Table 1). Repeated oral administration of aniracetam to SHRSP for 6 consecutive days (totaling 11 doses) increased choline acetyltransferase activity in those regions in which the activity was lower than that in WKY: e.g., a dose-dependent (30 and 100 mg/kg/day) and significant

Table 1
Effects of aniracetam on choline acetyltransferase activity (pmol ACh/min/mg protein) in cerebral regions of SHRSP
Aniracetam was orally administered at 15 or 50 mg/kg twice daily for 5 consecutive days, and animals were sacrificed 1 h after the 11th dosage on Day 6. Experiments were repeated two times and the data from each experiment were pooled ($n = 7–10$ in each group). Data represent means ± S.E.M.

Region	WKY	SHRSP		
		Vehicle	30 mg/kg/day	100 mg/kg/day
Pons–midbrain	1872 ± 67.2** + 24%	1509 ± 59.2	1569 ± 72.5	1580 ± 31.3
Thalamus	2117 ± 60.1 + 9%	1919 ± 73.8	2057 ± 89.3	2231 ± 105* + 16%
Striatum	3657 ± 213	3706 ± 120	3145 ± 126* − 15%	3112 ± 127* − 16%
Hippocampus	1795 ± 66.3* + 12%	1602 ± 42.2	1599 ± 48.3	1716 ± 45.5
Frontal cortex	1129 ± 35.0	1275 ± 45.8	1241 ± 58.1	1217 ± 34.6

*$P < 0.05$ and **$P < 0.01$ compared with SHRSP control.

increase in the thalamus and an increasing tendency at the higher dose (100 mg/kg/day) in the pons–midbrain and hippocampus (Table 1). On the contrary, the drug decreased significantly choline acetyltransferase activity in the striatum, which showed approximately the same activity as that in WKY. Aniracetam treatment of WKY under the same protocol produced no alterations in choline acetyltransferase activity in the five cerebral regions examined (data not shown).

3.2. Effects of aniracetam and its major metabolites on acetylcholine release in the nucleus reticularis thalami of freely moving SHRSP

In the in vivo microdialysis experiments, we selected three regions of the thalamus, dorsal hippocampus and prefrontal cortex, because those were known to be the major areas receiving dense innervation from cholinergic cell bodies in the central nervous system (Mesulam et al., 1983). For the thalamus, the nucleus reticularis thalami was chosen as noted in Section 4. Aniracetam and its three major metabolites, *N*-anisoyl-GABA, 2-pyrrolidinone and *p*-anisic acid (Fig. 1), were perfused through the dialysis probe used for the collection of dialysates. The tip positions of the dialysis probes inserted into the respective brain regions are illustrated in Fig. 2. In the nucleus reticularis thalami, the basal acetylcholine release was 0.35 ± 0.03 pmol/20 min ($n = 24$). After compound perfusion at 1 μM for 20 min, *N*-anisoyl-GABA and *p*-anisic acid caused a significant but somewhat delayed increase of acetylcholine release (Fig. 3B). The maximum increasing effects were 32% for *N*-anisoyl-GABA and 22% for *p*-anisic acid at 120–140 min after the termination of the

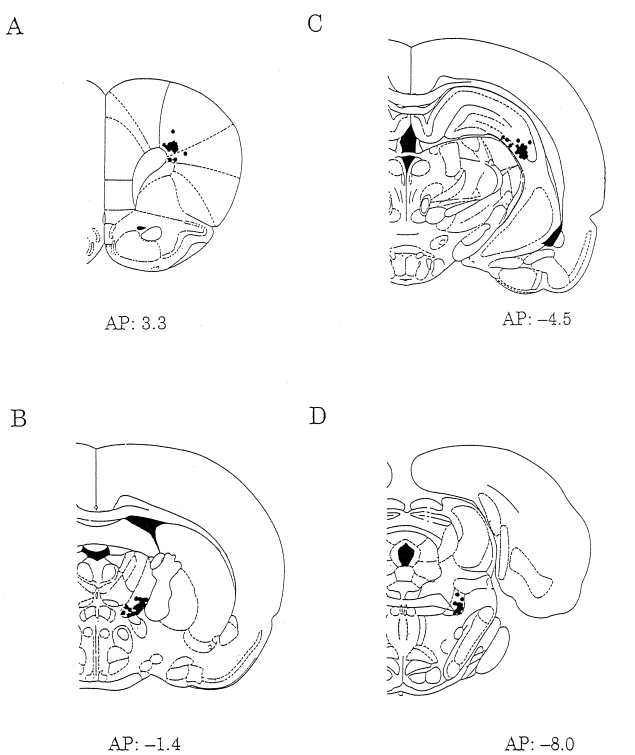

Fig. 2. Schematic drawing of the sites where the microdialysis probe or microinjection needle were inserted into the rat brain. (A) Prefrontal cortex, (B) nucleus reticularis thalami, (C) dorsal hippocampus, (D) pedunculopontine tegmental nucleus.

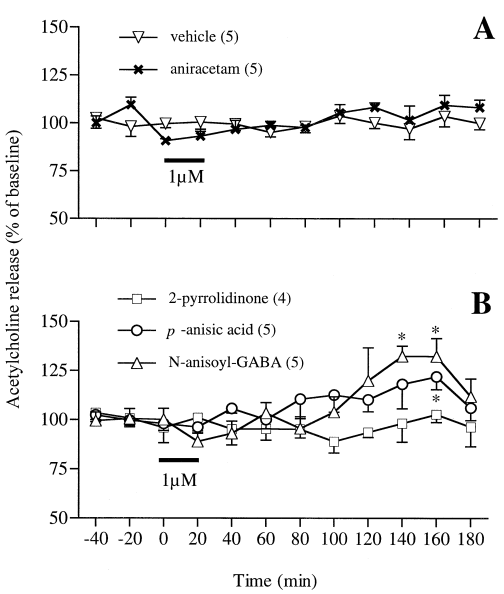

Fig. 3. Effects of aniracetam (A), 2-pyrrolidinone, *p*-anisic acid and *N*-anisoyl-GABA (B) on acetylcholine release in the nucleus reticularis thalami of freely moving SHRSP. Compounds were perfused for 20 min (solid bar). Data represent means ± S.E.M. Basal acetylcholine release was 0.35 ± 0.03 pmol/20 min ($n = 24$). *$P < 0.05$ compared with vehicle control.

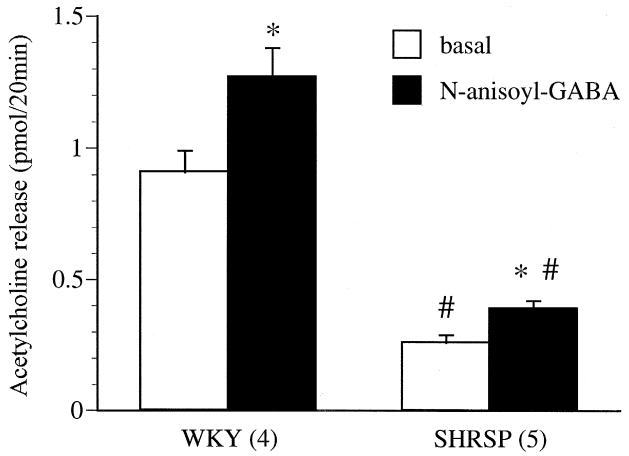

Fig. 4. Comparison of basal and *N*-anisoyl-GABA-stimulated acetylcholine release in the nucleus reticularis thalami between SHRSP and WKY. *N*-anisoyl-GABA at 1 μM was perfused into the nucleus for 20 min. The compound-induced maximum stimulatory effect was assessed individually. Data represent means ± S.E.M. $^{*}P < 0.05$ and $^{\#}P < 0.01$ compared with basal value and corresponding WKY value, respectively.

perfusion. Neither aniracetam nor 2-pyrrolidinone, however, affected basal acetylcholine release (Fig. 3). When the basal and *N*-anisoyl-GABA-stimulated acetylcholine release were compared between SHRSP and WKY with a limited number of animals, the basal release in SHRSP was 0.26 ± 0.03 pmol/20 min ($n = 5$), which was significantly much lower than that in WKY (0.91 ± 0.08 pmol/20 min, $n = 4$) (Fig. 4). *N*-anisoyl-GABA also enhanced acetylcholine release in WKY with the same delayed manner as that in SHRSP (data not shown). The stimulatory effect of *N*-anisoyl-GABA was nearly similar for both SHRSP (51 ± 8%, calculated as percent increases at the peak time, $n = 5$) and WKY (39 ± 4%, $n = 4$), indicating that the intact nerve terminals respond normally (Fig. 4).

3.3. Effects of aniracetam and its major metabolites on acetylcholine release in the dorsal hippocampus and prefrontal cortex of freely moving SHRSP

There was no difference in the basal acetylcholine release between the dorsal hippocampus (0.63 ± 0.04 pmol/20 min, $n = 15$) and prefrontal cortex (0.58 ± 0.03 pmol/20 min, $n = 24$). The values were 1.7–1.8-fold higher than that in the nucleus reticularis thalami. In the dorsal hippocampus of SHRSP, the perfusion of *N*-anisoyl-GABA and *p*-anisic acid at 1 μM significantly enhanced acetylcholine release (48% at 100–140 min later for the former and 51% at 140 min for the latter) (Fig. 5B). Here again, neither aniracetam nor 2-pyrrolidinone affected the release (Fig. 5). In the prefrontal cortex, as shown in Fig. 6C, *N*-anisoyl-GABA significantly in-

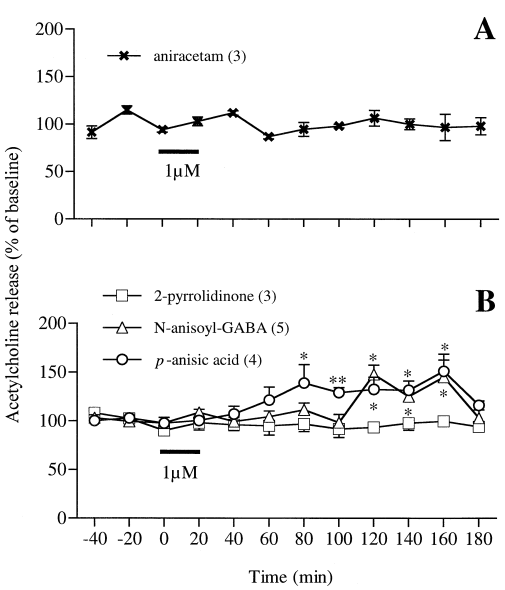

Fig. 5. Effects of aniracetam (A), 2-pyrrolidinone, *p*-anisic acid and *N*-anisoyl-GABA (B) on acetylcholine release in the dorsal hippocampus of freely moving SHRSP. Compounds were perfused for 20 min (solid bar). Data represent means ± S.E.M. Basal acetylcholine release was 0.63 ± 0.04 pmol/20 min ($n = 15$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with aniracetam.

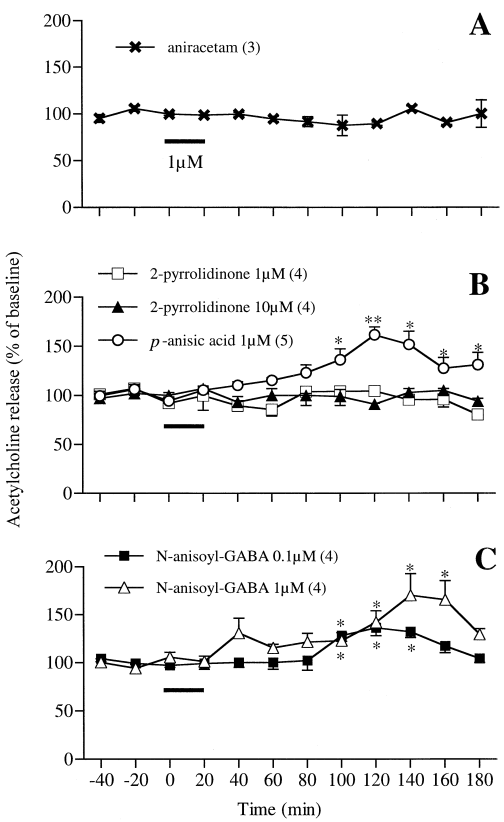

Fig. 6. Effects of aniracetam (A), 2-pyrrolidinone and *p*-anisic acid (B), and *N*-anisoyl-GABA (C) on acetylcholine release in the prefrontal cortex of freely moving SHRSP. Compounds were perfused for 20 min (solid bar). Data represent means ± S.E.M. Basal acetylcholine release was 0.58 ± 0.03 pmol/20 min ($n = 24$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with aniracetam.

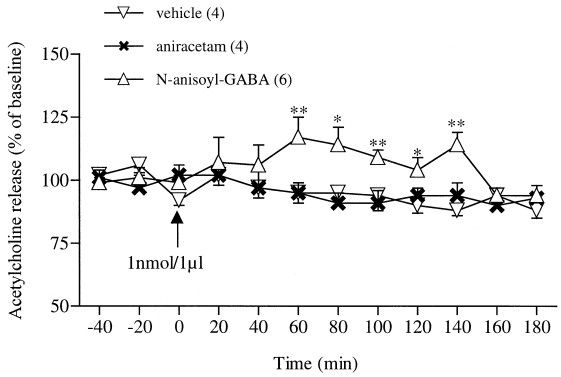

Fig. 7. Effects of a direct injection of aniracetam and *N*-anisoyl-GABA into the pedunculopontine tegmental nucleus on acetylcholine release in the nucleus reticularis thalami of freely moving SHRSP. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle control.

creased acetylcholine release in a dose-dependent manner (0.1 and 1 μM). The stimulatory effects peaked at 80–120 min (36%) at 0.1 μM and at 120–140 min (70%) at 1 μM after the completion of compound perfusion. *p*-Anisic acid at 1 μM showed a significant and delayed increase in acetylcholine release (61%, 100–120 min later) (Fig. 6B). Both aniracetam at 1 μM and 2-pyrrolidinone at 1 and 10 μM had no effect on basal acetylcholine release (Fig. 6).

3.4. Thalamic acetylcholine release after a microinjection of N-anisoyl-GABA into the pedunculopontine tegmental nucleus

To clarify whether the active metabolites also act on the cholinergic cell bodies, we investigated acetylcholine release in the nucleus reticularis thalami after the microinjection of *N*-anisoyl-GABA into the pedunculopontine tegmental nucleus. As shown in Fig. 7, *N*-anisoyl-GABA evoked a significant increase in acetylcholine release during the periods of 60 to 140 min after the injection into the nucleus. As observed in the perfusion experiments into the nerve terminal areas, the intact drug, aniracetam, caused no effect.

4. Discussion

4.1. Comparison between SHRSP and WKY

In the present study, cholinergic presynaptic activity (acetylcholine release and choline acetyltransferase activity) was decreased in SHRSP as compared with that in age-matched WKY, consistent with other reports, including the results from SHR (Yamada et al., 1984, 1987; Kang et al., 1990; Togashi et al., 1994, 1996). Although the comparison for acetylcholine release was restricted to only the thalamus, there was a great 3.5-fold difference between the two strains. As suggested previously, the central cholinergic deficits may lead to behavioral abnormalities, including attention deficits and task performance impairments (Martin and Haefely, 1993; Togashi et al., 1996; Nakamura et al., 1998). In a similar line of studies, it has been reported that nicotinic receptor density was reduced in the thalamus, midbrain and cerebral cortex of SHRSP (Yamada et al., 1987), and in many discrete areas and nuclei of SHR, including the nucleus reticularis thalami (Gattu et al., 1997), which may be associated with a lower cerebral blood flow in the thalamus and cortex of SHR (Fujishima et al., 1995). Clinically relevant observations were reported in patients with vascular dementia (Sakurada et al., 1990; Gottfries et al., 1994; Tohgi et al., 1996).

4.2. Ascending reticulothalamic cholinergic pathway

Activation in cortical electroencephalography and its functional correlates (arousal and attention) are modulated via the reticulothalamic cholinergic and thalamocortical non-cholinergic projections (Mesulam et al., 1983; Steriade et al., 1990a). The reticulothalamic cholinergic pathway particularly regulates the states of the thalamus, which is thought to be crucial in the control of behavioral state (Steriade et al., 1990a). Activation of the ascending brainstem cholinergic neurons inhibits thalamocortically generated neocortical high-voltage spindles, which occur during states of low arousal and low vigilance (drowsiness) (Jäkälä et al., 1996). Although almost all thalamic nuclei are innervated by the efferent fibers from either nucleus, or both nuclei (Mesulam et al., 1983; Woolf and Butcher, 1986), the nucleus reticularis thalami receives a dual cholinergic innervation from the pedunculopontine tegmental nucleus and nucleus basalis of Mynert, and functions as a pacemaker of the high-voltage spindles (Hallanger et al., 1987; Steriade et al., 1990b). In addition, the GABAergic nucleus is considered to be a functional interface between the thalamic nuclei or in the thalamocortical and corticothalamic pathways (Houser et al., 1980; Steriade et al., 1990b). Martin and Haefely (1993) previously showed that the nucleus responded to aniracetam with the highest sensitivity among the tested thalamic nuclei in the amelioration of 2-deoxy-D-glucose utilization reduced in scopolamine-treated rats.

In the present study, local perfusion of *N*-anisoyl-GABA and *p*-anisic acid enhanced basal acetylcholine release in the nucleus reticularis thalami of SHRSP. Moreover, *N*-anisoyl-GABA directly injected into the pedunculopontine tegmental nucleus elicited thalamic acetylcholine release. These findings show that *N*-anisoyl-GABA can act on both nerve terminals and cell bodies in the reticulothalamic cholinergic connections, presumably by a common mechanism, and indicate that the reticulothalamic cholinergic pathway may be activated by *N*-anisoyl-GABA in SHRSP with central cholinergic deficits. The increase in choliner-

gic presynaptic activity in the thalamus may be, at least in part, involved in the amelioration of behavioral abnormalities in SHRSP through the activation of the nicotinic vasodilative system (Uchida et al., 1997).

4.3. Actual responsible metabolites in rats and humans

We clearly demonstrated the definitely important role of *N*-anisoyl-GABA and *p*-anisic acid in the central cholinergic activation elicited by aniracetam. Local perfusion of both metabolites enhanced basal acetylcholine release in all tested regions and nucleus. Aniracetam is known to be rapidly metabolized mainly into 2-pyrrolidinone and *p*-anisic acid in rats (Ogiso et al., 1998) and into *N*-anisoyl-GABA in humans (Fig. 1) (Roncari, 1993). However, the concentration of *N*-anisoyl-GABA in the human CSF after the treatment with aniracetam was 0.02–0.16 μM and that of 2-pyrrolidinone was 1.6–2.9 μM, as a result of relatively poor penetration through the blood–brain barrier of *N*-anisoyl-GABA and no binding to plasma proteins of 2-pyrrolidinone (Roncari, 1993). Nevertheless, we proved that *N*-anisoyl-GABA even at the low concentration of 0.1 μM, which corresponded to the concentration detected in the human CSF, elicited acetylcholine release in the prefrontal cortex, whereas 2-pyrrolidinone even at the high concentration of 10 μM failed to enhance it. Based on these pharmacokinetic data, it is reasonable to consider that *p*-anisic acid is a responsible metabolite in rats, whereas *N*-anisoyl-GABA is the main one contributor to the clinical efficacy of aniracetam mediated by cholinergic activation. *p*-Anisic acid and *N*-anisoyl-GABA given orally, virtually, mimicked the improvement by aniracetam of scopolamine-induced delirium-like state in middle-aged rats (Nakamura et al., 1998).

4.4. Possible underlying mechanisms

There is considerable neurochemical evidence that aniracetam facilitates central cholinergic neurotransmission in normal, aged or gerbil rats (Martin and Haefely, 1993; Giovannini et al., 1993; Egashira et al., 1996; Kondoh et al., 1997; Nakamura et al., 1998). In the present study using SHRSP with cholinergic deficits and behavioral abnormalities, we could find more disease-related and further extended evidence.

In the acute experiment using in vivo microdialysis, *N*-anisoyl-GABA and *p*-anisic acid evoked acetylcholine release with a latent period in all tested regions and nucleus, as Giovannini et al. (1993) have observed partially. They found that aniracetam evoked acetylcholine release with a similar latent period in the hippocampus but not in the parietal cortex when given at 100 mg/kg p.o. to normotensive rats, though there was no examination in the thalamus. The increase in acetylcholine release by the metabolites was not so large (22–70%), especially in the nucleus reticularis thalami (22–32%), but it seemed to be adequate in consideration to its clinical use for long-term periods in the chronic phase of cerebrovascular diseases. Although the underlying mechanism is unclear at present, we think that the delayed response may be an indirect outcome caused by the interaction with other neurotransmitter systems. For example, aniracetam has been reported to act as a positive allosteric modulator of both ionotropic (α-amino-3-hydroxy-5-methylisoxazole-4-propionate; AMPA) and metabotropic glutamate receptors (Martin and Haefely, 1993; Pizzi et al., 1993). Indeed, an intrahippocampal or intrastriatal perfusion of AMPA and/or quisqualate increased acetylcholine release in respective perfusion areas of rats (Kendrick et al., 1996; Giovannini et al., 1998), indicating that the acetylcholine release is regulated through glutaminergic receptors. On the contrary, there was no evidence to support a direct activation of choline acetyltransferase by the metabolites and aniracetam (Egashira et al., 1996).

In the repeated treatment experiment, aniracetam preferentially increased choline acetyltransferase activity in the thalamus of SHRSP. One possible mechanism for the enzyme activation would be explained by an increase of cerebral energy metabolism by aniracetam (Himori et al., 1992). 2-Pyrrolidinone is converted to succinimide and then to succinate; and it is incorporated into the Krebs cycle and accelerates it (Fig. 1) (Mayersohn et al., 1993). The high production of acetyl-CoA from pyruvate seems to increase the supply of a substrate necessary for choline acetyltransferase activity. Consequently, enzyme activity may be increased to ameliorate the reduced level in SHRSP, but did not in WKY. The selective enhancement of [^{3}H]pirenzepine binding (muscarinic M_1 receptor) to the pons–midbrain by 2-pyrrolidinone might afford the other mechanism (Nakamura et al., 1998). It is possible to assume that aniracetam functionally enhances behavioral states (vigilance, arousal, attention) by cortical activation via the reticulothalamic cholinergic and thalamocortical non-cholinergic projections. These mechanisms would serve to account for the clinical usefulness towards cognitive impairments and neuropsychiatric symptoms in patients with cerebrovascular diseases (Otomo et al., 1991; Katsunuma et al., 1998), Alzheimer's and Parkinson's diseases (Senin et al., 1993; Honma et al., 1995; Katsunuma et al., 1998), and progressive supranuclear palsy (Nagasaka et al., 1997).

In conclusion, we used SHRSP as an animal model for multiple cerebral infarction and vascular dementia (Saito et al., 1995) and found that central cholinergic deficits occurred in SHRSP. In the SHRSP, aniracetam or its major metabolites (*N*-anisoyl-GABA and *p*-anisic acid) ameliorated the reduced cholinergic activity, as revealed by a preferential increase of choline acetyltransferase activity in the thalamus, and enhancement of acetylcholine release in all of the tested cerebral regions. Moreover, a direct injection of *N*-anisoyl-GABA into the pedunculopontine tegmental nucleus increased acetylcholine release in the

nucleus reticularis thalami. These data demonstrate that aniracetam can facilitate cholinergic neurotransmission throughout the brain via *N*-anisoyl-GABA and *p*-anisic acid. The pharmacokinetic data and its behavioral efficacy indicate that *N*-anisoyl-GABA may primarily contribute to the therapeutic effects in humans, mainly by acting on the reticulothalamic cholinergic pathway.

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