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TRH and Its Novel Analog (DN-1417): Antipentobarbital Action and Involvement of Cholinergic Mechanisms

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MIYAMOTO, M., Y. NAGAI, S. NARUMI, Y. SAJI AND Y. NAGAWA. *TRH and its novel analog (DN-1417): Antipentobarbital action and involvement of cholinergic mechanisms.* PHARMAC. BIOCHEM. BEHAV. 17(4) 797–806, 1982.—Possible neuroanatomical loci and the mode of action of thyrotropin-releasing hormone (TRH) or its analog, γ-butyrolactone-γ-carbonyl-histidyl-prolinamide citrate (DN-1417), in reducing the pentobarbital-induced sleeping time were investigated by using an intracerebral microinjection technique in rats. Intravenous, intraperitoneal or intracerebroventricular (ICV) injection of TRH or DN-1417 produced a dose-related reduction of the sleeping time induced by pentobarbital. TRH or DN-1417 given into the posterior hypothalamic regions including the dorsal premammillary nucleus, lateral hypothalamic area and posterior nucleus of hypothalamus had a significant pentobarbital sleep shortening action in low doses. Injection of these peptides into the dorsomedial nucleus of thalamus, mesencephalic reticular formation, medial septal nucleus or hippocampus was also effective, in comparatively low doses. However, higher doses were required to elicit the effect when the injections were made into the nucleus accumbens, lateral preoptic area or caudate nucleus. In this respect, the parietal cortex was insensitive to TRH or DN-1417. The pentobarbital sleep shortening action of TRH or DN-1417 injected peripherally or into the hypothalamic regions was markedly antagonized by ICV or intrahypothalamic pretreatment with atropine methyl bromide. On the contrary, ICV injection of atropine methyl bromide had a weak or no antagonizing action on the effect of TRH injected ICV or into the reticular formation, medial septal nucleus or hippocampus. These results suggest that possible neuroanatomical sites mediating the pentobarbital sleep shortening action of TRH or DN-1417 may be posterior hypothalamic regions, dorsomedial nucleus of thalamus, reticular formation, medial septal nucleus or hippocampus. A cholinergic mechanism may also be involved in the effect of TRH on the hypothalamus.

Thyrotropin-releasing hormone (TRH) γ-Butyrolactone-γ-carbonyl-histidyl-prolinamide citrate (DN-1417) Pentobarbital sleep antagonism Hypothalamus Cholinergic mechanisms

THYROTROPIN-releasing hormone (TRH) is a hypothalamic factor which releases thyrotropin and prolactin from the anterior pituitary. However, the radioimmunohistochemical studies provided evidence that TRH is widely distributed in the extrahypothalamic brain tissues of mammals [7,27] and even in the head or other neural tissues of some inframammals lacking the pituitary or thyrotropin [5,8]. These findings suggest that TRH may have played some role in neurotransmission, phyrogenetically antecedent to the acquisition of its endocrine function. In support to this concept, the peptide was pharmacologically shown to have a number of central nervous system effects independent of its hypophysiotropic action, such as behavioral excitations, potentiation of DOPA-induced excitation and antagonism of various central depressants [1, 3,23].

Involvement of cholinergic mechanisms and the septal region in antagonism of TRH on pentobarbital-induced sleep has also been implicated [1, 10, 11, 19]. The present study was an attempt to acquire detailed information on possible neuroanatomical loci and the mode of action in antagonism of pentobarbital-induced sleep in rats. The effects of a TRH analog, γ-butyrolactone-γ-carbonyl-histidyl-prolinamide citrate (DN-1417) were also examined.

METHOD

Animals

Male Sprague-Dawley (Jcl:SD) rats weighing 240–330 g, 7–9 weeks old, were housed in group cages in a temperature- and light-controlled room (22±2°C, a 12-hr illumination starting at 7:00 a.m.), and were maintained on laboratory-chow and tap-water, ad lib, except during the experiments which were performed between 9:00 a.m. and 6:00 p.m.

Implantation of Guide Cannulae

Rats (weighing 280–330 g, 8–9 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg IP) and fixed on a David Kopf stereotaxic instrument. Stainless steel

TABLE 1
STEREOTAXIC COORDINATES OF INJECTION SITES

Injection site	Stereotaxic coordinates A	L	H
Mesencephalic reticular formation	1.0	1.5	−2.5
Dorsal premammillary nucleus	4.6	0.4	3.5
Lateral hypothalamic area	4.6	1.8	3.2
Posterior nucleus of hypothalamus	4.6	0.4	2.8
Dorsomedial nucleus of thalamus	4.4	1.0	0.5
Hippocampus	3.2	2.5	2.0
Lateral preoptic area	7.6	2.5	−2.0
Medial septal nucleus	7.8	0.0	0.8
Lateral septal nucleus	7.4	0.7	1.8
Nucleus accumbens	9.4	1.5	0.2
Caudate nucleus-Putamen	8.2	3.0	1.5
Parietal cortex	7.0	3.0	5.2
Lateral cerebral ventricle	5.2	2.0	3.2

Coordinates are from the rat brain atlas of Pellegrino and Cushman [21].

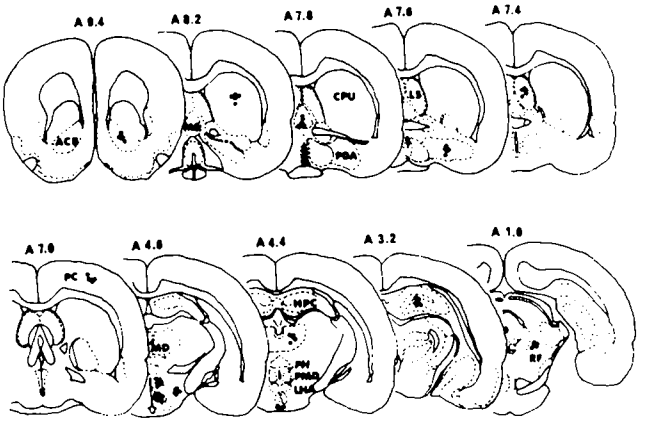

FIG. 1. Diagrams representing the locations of the cannulae tips for intracerebral injection, according to the atlas of Pellegrino and Cushman [22]. Only the unilateral sites are shown here from the histological data obtained from 60 rats. Abbreviations: RF: mesencephalic reticular formation; PMD: dorsal premammillary nucleus; LHA: lateral hypothalamic area; PH: posterior nucleus of hypothalamus; MD: dorsomedial nucleus of thalamus; HPC: hippocampus; POA: lateral preoptic area; MS: medial septal nucleus; LS: lateral septal nucleus; ACB: nucleus accumbens; CPU: caudate nucleus-Putamen; PC: parietal cortex.

guide cannulae (23 gauge, 0.63 mm outer and 0.33 mm inner diameters) were inserted into various brain regions through a small burr hole on the skull, according to the atlas of Pellegrino and Cushman [22]. In order to avoid the sagittal sinus, an angle of 10° to the mid sagittal plane was employed for the implantation into the dorsal premammillary nucleus, posterior nucleus of hypothalamus, medial septal nucleus and lateral septal nucleus. Guide cannulae were lowered to a point of 1.5 mm above the injection sites, whereas, in case of the parietal cortex, lowered to 1 mm above the injection site. The cannulae were anchored to the skull with screws and acrylic dental cement. When not in use, the cannulae were occluded with stainless steel stylets. The rats were used two or three times at intervals of at least 5 days after a 7-day recovery period from surgery.

Measurement of Sleeping Time

After each rat had been given sodium pentobarbital (40 mg/kg) intraperitoneally, TRH, DN-1417, dopamine (DA) or carbachol was injected peripherally or intracerebrally 10 min after onset of the loss of righting reflex. Sleeping time was taken as the time from the time of drug injection until the righting reflex had been spontaneously regained. Percent shortening was calculated by the following formula: [1−(sleeping time in drug-treated group/sleeping time in control group)]×100.

Drugs and Drug Injection

The following drugs were used: TRH (L-pyroglutamyl-L-histidyl-L-prolinamide L-tartrate monohydrate, Takeda), DN-1417 (γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate, Takeda), dopamine hydrochloride (DA, Tokyo Kasei), sodium pentobarbital (Mintal®, Tanabe), carbamylcholine chloride (carbachol, Tokyo Kasei), atropine sulfate (Merck), atropine methyl bromide (Tropin®, Takeda), mecamylamine hydrochloride (Sigma), pimozide (Orap®, Fujisawa), ^{14}C-DN-1417 (specific activity, 9.8 mCi/mmol, Takeda) and 2-deoxy-D-^{14}C-glucose (specific activity, 50–56 mCi/mmol, New England Nuclear Corp.). Pimozide was suspended in a 5% gum arabic solution, and the other drugs were dissolved in physiological saline. The doses are expressed in terms of the salts given above.

Intracerebral injection was made by means of a microsyringe with a 30 gauge needle (0.31 mm outer and 0.13 mm inner diameters), which was extended 1 or 1.5 mm below the tip of the guide cannulae. The stereotaxic coordinates of injection sites are shown in Table 1. The injection volume was 0.5 μl on each side except for 10 μl for the intracerebroventricular (ICV) injection. Infusion rates of drug were 0.5 μl/30 sec for intracerebral injection and 1 μl/sec for ICV injection, respectively. The volume for peripheral administration was 0.2 ml/100 g body weight. Control rats were given an equal volume of saline. Doses shown in the microinjection studies refer to amounts on each side.

Autoradiographic Investigation of Diffusion of ^{14}C-DN-1417

Using an autoradiographic technique, diffusion of ^{14}C-DN-1417 injected ICV or into the lateral hypothalamic area was determined, because the radioactivity of ^{3}H-TRH was too low for detection. ^{14}C-DN-1417 was applied ICV (0.68 μCi/25 μg/10 μl) or into the lateral hypothalamic area (0.34 μCi/1.25 μg/0.5 μl) unilaterally or bilaterally, respectively. In case of the lateral hypothalamic area injection, 2-deoxy-^{14}C-glucose (10 μCi/0.3 ml/rat) was given intravenously 15 min before ^{14}C-DN-1417 injection in order to clarify the outline of the brain structures. The rat was decapitated 15 min after ^{14}C-DN-1417 injection, and the brain was immediately frozen in liquid N_2-chilled Freon XII maintained at −65°C. The brain was fixed to object-holders by means of matrix M1 (Lipshaw Manufacturing Co.) and then sectioned

TABLE 2

EFFECTS OF SYSTEMIC AND ICV INJECTION OF TRH OR DN-1417 ON PENTOBARBITAL-INDUCED SLEEP IN RATS

Drug	Dose	Route	Sleeping time (min, Mean+SE)	% Shortening	MED (μg)‡
Saline	—	IV	44.3 ± 3.3	— (8)	
Tartaric acid	1.4 mg/kg§	IV	50.1 ± 3.9	−13.1 (7)	
TRH	0.5 mg/kg	IV	39.6 ± 1.7	10.6 (8)	250
	1 mg/kg	IV	30.5 ± 2.6	31.2† (8)	
	2 mg/kg	IV	28.1 ± 2.0	36.6† (8)	
	5 mg/kg	IV	25.1 ± 2.1	43.3† (8)	
Saline	—	IV	43.1 ± 1.2	— (7)	
Citric acid	1.7 mg/kg§	IV	45.7 ± 3.2	−6.0 (7)	
DN-1417	0.2 mg/kg	IV	39.9 ± 5.1	7.6 (7)	125
	0.5 mg/kg	IV	34.8 ± 2.1	19.3† (8)	
	1 mg/kg	IV	31.0 ± 2.6	28.3† (7)	
	5 mg/kg	IV	25.4 ± 1.8	47.7† (8)	
Saline	—	IP	43.5 ± 3.7	— (8)	
Tartaric acid	5.6 mg/kg§	IP	46.8 ± 3.9	−7.6 (6)	
TRH	5 mg/kg	IP	35.4 ± 2.6	18.6 (8)	2500
	10 mg/kg	IP	28.5 ± 2.7	34.5† (8)	
	20 mg/kg	IP	22.8 ± 1.5	47.7† (8)	
Saline	—	ICV	64.1 ± 2.7	— (7)	
Tartaric acid	11.3 μg§	ICV	69.5 ± 3.6	-8.4 (6)	
TRH	5 μg	ICV	62.3 ± 3.7	2.8 (7)	20
	20 μg	ICV	47.7 ± 5.6	25.6* (6)	
	40 μg	ICV	38.3 ± 3.5	40.2† (6)	
Saline	—	ICV	63.4 ± 3.8	— (7)	
Citric acid	1.7 μg§	ICV	65.0 ± 3.8	2.5 (7)	
DN-1417	0.2 μg	ICV	54.9 ± 4.8	13.4 (7)	1
	1 μg	ICV	47.9 ± 3.9	24.4* (7)	
	5 μg	ICV	38.6 ± 3.7	39.1† (8)	

Drugs were administered 10 min after the loss of righting reflex.
Number of rats is shown in parentheses.
*$p<0.05$, †$p<0.01$ compared with saline control.
‡Minimum effective dose per rat.
§Since TRH and DN-1417 were used as tartrate and citrate, respectively, effects of these acids in equimolar doses to the maximum doses of TRH and DN-1417 were also tested for reference.

into slices 20 μm in thickness in a cryostat (A. O. Cryocut, American Optical Co.) maintained at −21°C. Autoradiographs were prepared from these sections directly in the X-ray cassette with a Kodak single-coated blue sensitive Medical X-ray film (Tyep SB-5). After exposure for 1 week in a dark room, the film was developed with an automatic developer (Fuji X-ray Processor RN).

Histology

At the end of the experiments, the rats were anesthetized with 50 mg/kg IP of sodium pentobarbital, and perfused with 30 ml of saline following 50 ml of 10% formalin solution through the left cardiac ventricle. The brains were removed and kept in 10% formalin solution for at least 7 days and then sectioned to verify the site of injection (Fig. 1).

Statistics

Statistical comparisons between different treatments were made using Student's *t*-test (two-tailed).

RESULTS

Effects of Systemic or ICV Injection of TRH or DN-1417 on Pentobarbital-Induced Sleep in Rats

Intravenous (0.2–5 mg/kg) or intraperitoneal (5–20 mg/kg) administration of TRH produced a dose-related reduction of pentobarbital (40 mg/kg IP)-induced sleep in rats. A TRH analog, DN-1417 given intravenously showed similar pentobarbital sleep shortening action and the effect was about twice as potent as TRH. Both TRH and DN-1417 injected ICV also produced significant antipentobarbital effects, and the effect of DN-1417 was much more potent than that of TRH. The salt constituents of TRH and DN-1417, tartaric acid and citric acid had no effect (Table 2).

Effects of Intracerebral Injections of TRH and DN-1417 on Pentobarbital-Induced Sleep in Rats

Effects of intracerebral injections of TRH on pentobarbital sleep are shown in Table 3. TRH given into the posterior

TABLE 3

EFFECTS OF TRH INJECTED INTO VARIOUS BRAIN SITES ON PENTOBARBITAL-INDUCED SLEEP IN RATS

Injection site	Drug	Dose (μg)‡	Sleeping time (min, Mean±SE)	%Shortening	MED (μg)§
Dorsal premammillary nucleus	Saline	—	64.7 ± 2.7	— (10)	
	Tartaric acid	1.4¶	62.2 ± 3.0	3.9 (6)	
	TRH	0.1	64.3 ± 6.4	0.6 (6)	1
		1	49.5 ± 5.3	23.5* (6)	
		5	43.3 ± 4.3	33.1† (6)	
Lateral hypothalamic area	Saline	—	47.0 ± 2.7	— (7)	
	Tartaric acid	5.6¶	41.3 ± 2.0	12.1 (6)	
	TRH	1	37.0 ± 3.9	21.3 (7)	5
		5	33.9 ± 3.7	27.9* (7)	
		20	16.0 ± 0.6	66.0† (6)	
Posterior nucleus of hypothalamus	Saline	—	78.4 ± 2.7	— (8)	
	TRH	1	66.1 ± 5.3	15.7 (7)	5
		5	42.6 ± 4.2	45.7† (7)	
		20	36.3 ± 4.1	53.7† (7)	
Dorsomedial nucleus of thalamus	Saline	—	75.4 ± 3.3	— (8)	
	TRH	1	65.4 ± 4.6	13.3 (8)	5
		5	58.5 ± 3.3	22.4* (8)	
		20	50.8 ± 4.9	32.6† (8)	
Mesencephalic reticular formation	Saline	—	47.0 ± 2.6	— (7)	
	TRH	1	40.3 ± 3.5	14.3 (6)	5
		5	32.0 ± 2.1	31.9† (7)	
		20	30.0 ± 3.5	36.2† (6)	
Medial septal nucleus	Saline	—	75.5 ± 3.6	— (6)	
	TRH	1	76.7 ± 4.4	1.6 (6)	5
		5	57.0 ± 4.9	24.5* (6)	
		20	53.2 ± 3.8	29.5† (6)	
Hippocampus	Saline	—	67.6 ± 2.6	— (7)	
	TRH	1	63.3 ± 6.6	6.4 (6)	5
		5	54.3 ± 5.1	19.7* (6)	
		20	44.9 ± 5.9	33.6† (7)	
Nucleus accumbens	Saline	—	47.8 ± 1.7	— (8)	
	TRH	5	42.5 ± 2.8	11.1 (6)	20
		20	35.5 ± 2.4	25.7† (8)	
Lateral septal nucleus	Saline	—	55.1 ± 2.9	— (8)	
	TRH	5	42.9 ± 5.6	22.1 (7)	20
		20	35.6 ± 3.5	35.4† (7)	
Caudate nucleus Putamen	Saline	—	47.4 ± 2.3	— (8)	
	TRH	20	40.6 ± 3.0	14.7 (7)	50
		50	39.8 ± 1.9	16.4* (8)	
Lateral preoptic area	Saline	—	67.0 ± 5.9	— (8)	
	TRH	50	48.3 ± 7.7	27.9 (6)	—
Parietal cortex	Saline	—	56.9 ± 3.5	— (10)	
	TRH	50	48.5 ± 3.5	14.8 (8)	—

Drugs were injected 10 min after the loss of righting reflex.

*$p<0.05$, †$p<0.01$ compared with saline control.

Number of rats is shown in parentheses.

‡Unilateral dose, although drugs were injected bilaterally into the brain regions other than the medial septal nucleus.

§Minimum effective dose (unilateral dose).

¶Equimolar dose to the maximum dose of TRH used.

hypothalamic regions including the dorsal premammillary nucleus, lateral hypothalamic area and posterior nucleus of hypothalamus had a significant pentobarbital sleep shortening action in low doses (1–5 μg). Microinjection into the dorsomedial nucleus of thalamus, mesencephalic reticular formation, medial septal nucleus or hippocampus also reduced the pentobarbital-induced sleep, in comparatively low doses. Similar applications of DN-1417 into these brain regions also produced profound antipentobarbital effects in very low doses (0.02–0.1 μg), and the effects were particularly marked in cases of injection into the posterior hypothalamic regions (Table 4). In contrast, when given into the nucleus accumbens, lateral septal nucleus, caudate-putamen or lateral preoptic area, the action of TRH or DN-1417 was less potent, since high doses of these peptides were required to evoke the effect. Furthermore, TRH or DN-1417 injected into the parietal cortex had no effect, even at high doses. Microinjection of tartaric acid or citric acid into the dorsal premammillary nucleus or lateral hypothalamic area produced no significant changes in the pentobarbital-induced sleeping time.

The effect of DA applied ICV or intracerebrally on pentobarbital-induced sleep was also studied. Fifty and 100 μg of DA injected ICV causes a dose-related potentiation of pentobarbital-induced sleep and the effect of a higher dose of DA was significant (72.4±4.0 min compared with 47.4±2.3 min in saline control). Injection of DA into the lateral hypothalamic area, dorsomedial nucleus of thalamus, reticular formation or nucleus accumbens tended to potentiate the pentobarbital-induced sleep rather than to reduce it. Only intracaudate injection of DA had a significant shortening action, at high doses: the sleeping time in cases of the saline control and in 50 μg and 100 μg of DA was 47.4±2.3, 34.3±3.5, 30.8±4.3 min, respectively.

Cholinergic Involvement in Pentobarbital Sleep Shortening Action Induced by TRH or DN-1417

Table 5 shows that effects of anticholinergic drugs and a DA receptor blocker on TRH- or DN-1417-induced pentobarbital sleep shortening action. Antipentobarbital action of TRH (20 mg/kg IP) or DN-1417 (5 mg/kg IV) was markedly antagonized by ICV pretreatment with atropine methyl bromide (20 μg) which produced no significant changes in pentobarbital sleep *per se*. However, a similar ICV application of atropine methyl bromide had no significant inhibitory action on the effect of TRH injected ICV. The shortening action of TRH given IP was markedly attenuated by systemic administration of mecamylamine but not by systemic administration of atropine, even at high doses. Pimozide did not affect the TRH action.

Effects of intracerebral pretreatment with atropine and atropine methyl bromide on the activity of TRH (20 mg/kg IP) were also investigated. The shortening action was significantly suppressed by pretreatment with atropine or atropine methyl bromide into the posterior hypothalamic regions, including the dorsal premammillary nucleus and lateral hypothalamic area (Fig. 2). However, a similar application into the reticular formation did not alter the effect of TRH.

Pentobarbital sleep reducing action of TRH (10 μg) applied into the lateral hypothalamic area was significantly inhibited by pretreatment with 5 μg of atropine or atropine methyl bromide into the same site, and the inhibitory action of atropine methyl bromide was more potent than that of atropine (Fig. 3). On the other hand, the effect of TRH injected into the reticular formation in a dose of 10 μg was not or only slightly affected by the 5 μg of atropine or atropine methyl bromide injected earlier. ICV injection of atropine methyl bromide, however, partially antagonized the effect of TRH injected into the reticular formation. Similar partial antagonizing action by atropine methyl bromide was also observed on the effect of TRH (20 μg) applied into the medial septal nucleus. However, intrahippocampal injection of TRH (20 μg) caused a marked pentobarbital sleep reducing action in rats pretreated with atropine methyl bromide, such as was seen in rats pretreated with saline (Fig. 3).

Effects of Intracerebral Injection of Carbachol on Pentobarbital-Induced Sleep in Rats

Table 6 shows the effects of carbachol applied intracerebrally on pentobarbital-induced sleep. When 1 μg of carbachol was injected into the posterior hypothalamic regions including the dorsal premammillary nucleus, lateral hypothalamic area and posterior nucleus of hypothalamus, a marked pentobarbital sleep shortening action was elicited. On the other hand, cholinergic stimulation of the reticular formation with carbachol potentiated rather than reduced, pentobarbital-induced sleep in doses 1–5 μg. Furthermore, when microinjected into the hippocampus or medial septal nucleus, carbachol did not produce a significant shortening action. The pentobarbital sleep reducing action of carbachol applied into the posterior nucleus of hypothalamus was markedly antagonized by pretreatment with atropine methyl bromide (5 μg) injected into the same site.

Diffusion of ^{14}C-DN-1417 in the Brain

Using an autoradiographic technique, we observed the diffusion of ^{14}C-DN-1417 given into the lateral hypothalamic area or ICV. As shown in Fig. 4, when ^{14}C-DN-1417 (0.034 μCi/1.25 μg/0.5 μl) was injected into the lateral hypothalamic area, the highest density of grains in the autoradiographs was present in the lateral hypothalamic area. A moderate density was also found in the ventral thalamic regions. Figure 5 is a typical autoradiograph representing diffusion of ^{14}C-DN-1417 (0.68 μCi/25 μg/10 μl) at 15 min after the application into the lateral ICV. The highest density of grains was found in the periventricular structures including the septal regions, hippocampus, central gray substance, etc. In the hypothalamus, medial areas around the third ventricle showed a relatively high density.

DISCUSSION

Systemic administration of TRH shortened pentobarbital-induced sleep in rats, thus supporting data in the literature [1,24]. When TRH was given ICV, similar effects were produced. A TRH analog, DN-1417 also possessed antipentobarbital action, as previously described in the case of mice [4,17].

In the case of intracerebral injections, the sensitive sites to TRH or DN-1417 in the antipentobarbital action were the posterior hypothalamic regions including the dorsal premammillary nucleus, lateral hypothalamic area and posterior nucleus of hypothalamus. Application of TRH or DN-1417 into the dorsomedial nucleus of thalamus, reticular formation, medial septal nucleus or hippocampus also had a significant action, in comparatively low doses. In contrast, the nucleus accumbens, lateral septal nucleus, caudate nucleus or lateral preoptic area was insensitive to TRH or DN-1417. Microinjection into the parietal cortex did not affect the

TABLE 4

EFFECTS OF DN-1417 INJECTED INTO VARIOUS BRAIN SITES ON PENTOBARBITAL-INDUCED SLEEP IN RATS

Injection site	Drug	Dose (μg)‡	Sleeping time (min, Mean ± SE)	% Shortening	MED (μg)§
Lateral hypothalamic area	Saline	—	66.2 ± 5.4	— (6)	
	Citric acid	0.035¶	67.7 ± 3.3	−2.3 (7)	
	DN-1417	0.004	54.0 ± 4.3	18.4 (6)	0.02
		0.02	43.3 ± 4.5	34.6† (6)	
		0.1	37.0 ± 5.6	44.1† (6)	
Dorsal premammillary nucleus	Saline	—	76.6 ± 3.8	— (6)	
	Citric acid	0.035¶	79.3 ± 4.8	−4.6 (6)	
	DN-1417	0.004	63.5 ± 5.6	16.8 (6)	0.02
		0.02	55.2 ± 3.1	27.6† (6)	
		0.1	49.5 ± 5.8	35.4† (6)	
Dorsomedial nucleus of thalamus	Saline	—	75.4 ± 3.3	— (8)	
	DN-1417	0.004	67.3 ± 5.6	10.7 (8)	0.02
		0.02	60.5 ± 3.1	19.8† (8)	
		0.1	49.5 ± 5.8	34.3† (8)	
Mesencephalic reticular formation	Saline	—	64.4 ± 4.7	— (7)	
	DN-1417	0.02	59.0 ± 6.0	8.7 (6)	0.1
		0.1	44.0 ± 3.5	31.9† (7)	
		0.5	42.8 ± 3.7	33.7† (6)	
Medial septal nucleus	Saline	—	85.1 ± 3.5	— (7)	
	DN-1417	0.02	75.9 ± 4.2	10.2 (7)	0.1
		0.1	66.3 ± 4.1	22.1† (7)	
		0.5	56.7 ± 4.6	33.4† (7)	
Hippocampus	Saline	—	69.6 ± 5.4	— (7)	
	DN-1417	0.02	60.6 ± 4.7	12.9 (7)	0.1
		0.1	46.7 ± 3.9	32.9† (7)	
		0.5	43.7 ± 3.8	37.2† (8)	
Nucleus accumbens	Saline	—	71.9 ± 5.2	— (8)	
	DN-1417	0.1	62.6 ± 6.1	12.9 (7)	0.1
		0.5	56.6 ± 4.0	21.3* (6)	
		2	53.5 ± 4.6	27.0* (7)	
Lateral preoptic area	Saline	—	75.0 ± 4.3	— (7)	
	DN-1417	0.1	65.2 ± 8.0	13.1 (6)	0.5
		0.5	56.0 ± 5.1	25.3* (6)	
		2	53.5 ± 4.6	27.7† (6)	
Caudate nucleus Putamen	Saline	—	62.9 ± 6.0	— (7)	
	DN-1417	0.5	53.5 ± 2.3	15.0 (6)	2
		2	45.5 ± 3.5	27.7* (6)	
Parietal cortex	Saline	—	75.6 ± 4.3	— (7)	
	DN-1417	2	70.7 ± 4.5	6.5 (7)	—

Drugs were injected 10 min after the loss of righting reflex.

*$p<0.05$, †$p<0.01$ compared with saline control.

Number of rats is shown in parentheses.

‡Unilateral dose, although drugs were injected bilaterally into the brain regions other than the medial septal nucleus.

§Minimum effective dose (unilateral dose).

¶Equimolar dose to the maximum dose of DN-1417 used.

TABLE 5

EFFECTS OF SYSTEMIC OR ICV INJECTION OF DRUGS ON PENTOBARBITAL SLEEP SHORTENING ACTION OF TRH OR DN-1417 GIVEN PERIPHERALLY OR ICV TO RATS

Pretreatment							
Drug	Dose	Route	Drug	Dose (mg/kg)	Route	Sleeping time (min, Mean ± SE)	% Shortening
Saline	—	ICV	Saline	—	IP	74.8 ± 4.2	— (10)
Saline	—	ICV	TRH	20	IP	49.8 ± 4.3	34.0† (10)
ATMB	20 μg	ICV	Saline	—	IP	70.7 ± 3.0	— (10)
ATMB	20 μg	ICV	TRH	20	IP	64.2 ± 3.2	9.2 (10)
Saline	—	ICV	Saline	—	IV	65.6 ± 3.7	— (7)
Saline	—	ICV	DN-1417	5	IV	38.0 ± 2.7	42.1† (7)
ATMB	20 μg	ICV	Saline	—	IV	63.3 ± 4.9	— (7)
ATMB	20 μg	ICV	DN-1417	5	IV	54.7 ± 7.2	14.6 (6)
Saline	—	IP	Saline	—	IP	46.8 ± 5.0	— (8)
Saline	—	IP	TRH	20	IP	29.1 ± 3.0	37.8† (8)
AT	10 mg/kg	IP	Saline	—	IP	52.6 ± 2.5	— (7)
AT	10 mg/kg	IP	TRH	20	IP	40.9 ± 4.0	22.2* (7)
AT	20 mg/kg	IP	Saline	—	IP	66.3 ± 3.9	— (8)
AT	20 mg/kg	IP	TRH	20	IP	46.6 ± 4.8	29.7† (8)
MCA	10 mg/kg	IP	Saline	—	IP	59.6 ± 5.4	— (7)
MCA	10 mg/kg	IP	TRH	20	IP	53.6 ± 5.3	10.3 (7)
Saline	—	ICV	Saline	—	ICV	76.2 ± 6.1	— (6)
Saline	—	ICV	TRH	20	ICV	49.0 ± 4.2	35.7† (7)
ATMB	20 μg	ICV	Saline	—	ICV	79.2 ± 5.4	— (6)
ATMB	20 μg	ICV	TRH	20	ICV	54.0 ± 3.8	30.8† (6)
Saline	—	IP	Saline	—	IP	44.6 ± 2.5	— (8)
Saline	—	IP	TRH	20	IP	27.4 ± 3.8	38.6† (8)
Pimozide	0.5 mg/kg	IP	Saline	—	IP	46.9 ± 2.1	— (8)
Pimozide	0.5 mg/kg	IP	TRH	20	IP	28.5 ± 2.9	39.2 (8)

Atropine methyl bromide (ATMB) was pretreated ICV 5 min before pentobarbital administration. Mecamylamine (MCA), atropine (AT) and pimozide were pretreated peripherally 15 min, 30 min and 4 hr before pentobarbital administration, respectively. TRH and DN-1417 were given 10 min after the loss of righting reflex.

Number of rats is shown in parentheses.

*$p<0.05$, †$p<0.01$ compared with respective saline control.

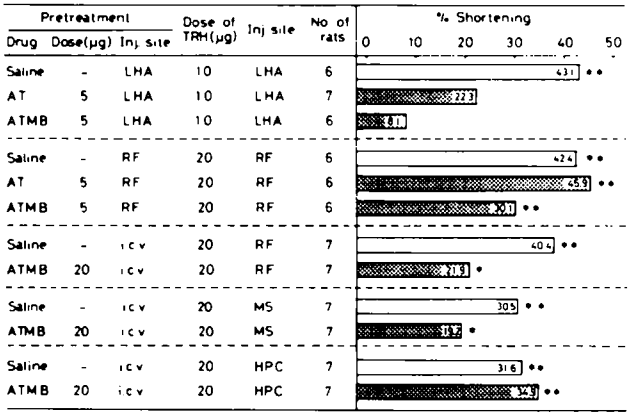

FIG. 2. Effects of intracerebral injection of anticholinergic drugs on reduction of pentobarbital sleep induced by TRH (20 mg/kg IP) in rats. Atropine (AT) and atropine methyl bromide (ATMB) were pretreated 5 min before pentobarbital administration and TRH was given 10 min after the loss of righting reflex. *$p<0.05$, **$p<0.01$ compared with saline control. AT and ATMB produced no significant changes sleeping time *per se*. Abbreviations: LHA: lateral hypothalamic area; RF: mesencephalic reticular formation; MS: medial septal nucleus; HPC: hippocampus.

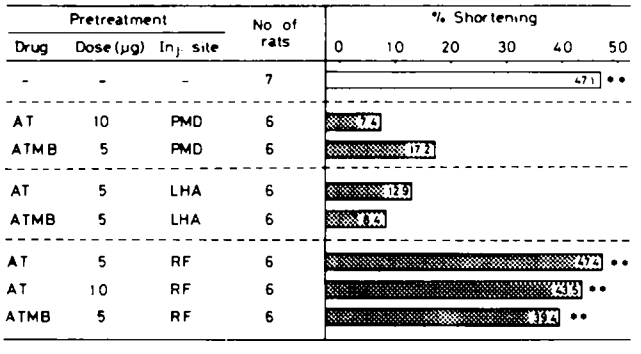

FIG. 3. Effects of intracerebral or ICV injection of anticholinergic drugs on pentobarbital sleep reducing action of TRH injected intracerebrally in rats. Atropine (AT) and atropine methyl bromide (ATMB) were pretreated 5 min before pentobarbital administration, and TRH was injected 10 min after the loss of righting reflex. *$p<0.05$, **$p<0.01$ compared with saline control. AT and ATMB produced no significant changes in sleeping time *per se*. Abbreviations: PMD: dorsal premammillary nucleus; LHA: lateral hypothalamic area; RF: mesencephalic reticular formation.

FIG. 4. Autoradiograph representing diffusion of ^{14}C-DN-1417 injected into the lateral hypothalamic area. ^{14}C-DN-1417 (0.034 μCi/1.25 μg/0.5 μl) and 2-deoxy-^{14}C-glucose (10 μCi/0.3 ml/rat, IV) were injected 15 and 30 min before the decapitation, respectively. Abbreviations: LHA: lateral hypothalamic area; VTR: ventral thalamic regions.

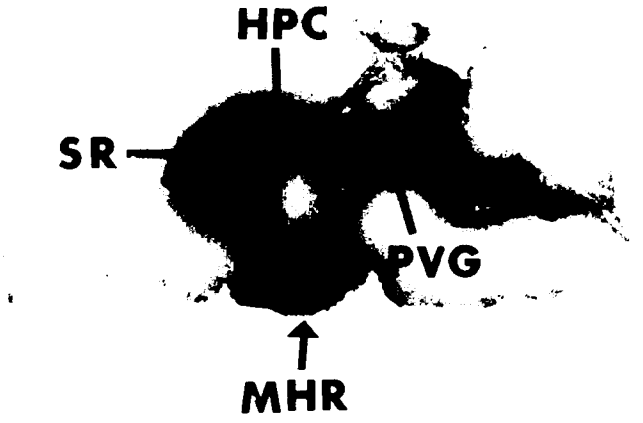

FIG. 5. Autoradiograph representing diffusion of ^{14}C-DN-1417 injected ICV. ^{14}C-DN-1417 (0.68 μCi/25 μg/10 μl) was injected 15 min before the decapitation. Abbreviations: SR: septal regions; HPC: hippocampus; PVG: central gray substance (substantia grisea periventricularis); MHR: medial hypothalamic regions.

TABLE 6

EFFECTS OF INTRACEREBRAL INJECTION OF CARBACHOL ON PENTOBARBITAL-INDUCED SLEEP IN RATS

Drug	Dose (μg)	Injection site	Sleeping time (min, Mean ± SE)	% Shortening
Saline	—	LHA	55.1 ± 3.5	— (7)
Carbachol	1	LHA	36.6 ± 3.7	33.6† (7)
Saline	—	PMD	67.7 ± 5.2	— (6)
Carbachol	1	PMD	42.9 ± 4.5	36.6† (7)
Saline	—	PH	89.0 ± 7.3	— (8)
Carbachol	1	PH	46.8 ± 5.0	47.7† (9)
Saline	—	RF	61.8 ± 2.7	— (8)
Carbachol	0.2	RF	55.8 ± 2.3	9.7 (6)
	1	RF	73.7 ± 5.0	- 19.8* (6)
	5	RF	102.0 ± 5.8	65.0† (6)
Saline	—	HPC	82.4 ± 5.4	— (8)
Carbachol	1	HPC	74.0 ± 6.3	10.2 (9)
	5	HPC	67.8 ± 3.8	17.7 (6)
Saline	—	MS	79.2 ± 4.7	— (6)
Carbachol	1	MS	79.8 ± 5.8	−0.8 (6)

Carbachol was injected 10 min after the loss of righting reflex.
Number of rats is shown in parentheses.
*$p<0.05$, †$p<0.01$ compared with saline control.
Antipentobarbital effect of carbachol (1 μg) injected into the posterior nucleus of hypothalamus was markedly antagonized by pretreatment with atropine methyl bromide (5 μg) into the same site: the sleeping times in saline- and carbachol-treated groups were 93.6 ± 4.9 and 85.7 ± 6.5 min, respectively.
Abbreviations: LHA: lateral hypothalamic area; PMD: dorsal premamillary nucleus; PH: posterior nucleus of hypothalamus; RF: mesencephalic reticular formation; HPC: hippocampus; MS: medial septal nucleus.

sleeping time. Thus, the brain sites sensitive to TRH or DN-1417 with regard to the antagonism of pentobarbital-induced sleep are proposed to be various areas in the posterior hypothalamic regions, dorsomedial nucleus of thalamus, reticular formation, medial septal nucleus and hippocampus. Most of these structures are closely related to the ascending activating system which plays important roles in control of sleep-wakefulness [9, 12, 13, 21]. In particular, the posterior hypothalamic regions are well known as the most important sites in the limbic activating system or as "waking center" [13,21].

An attempt to elucidate the neuroanatomical substrate mediating the analeptic action of TRH in pentobarbitalized animals was also made by other investigators who used similar microinjection procedures. Carino *et al.* [2] reported that TRH causes a potent analeptic action when given into the cortex, caudate nucleus, thalamus, hypothalamus and preoptic area in rabbits. According to reports of Kalivas and Horita [10,11] concerning the antipentobarbital action of TRH in rats, the most sensitive site proved to be the medial septal nucleus, followed by the interpeduncular nucleus, medial thalamus, thalamic periventricular gray and nucleus accumbens. Mesencephalic periventricular gray, locus coeruleus, medial hypothalamus and preoptic/anterior hypothalamus are also comparatively sensitive to TRH. Although there is some discrepancy between our present data and that obtained by others [10,11] regarding the sensitive sites to TRH, the hypothalamus, thalamus, a limited area of septal region, reticular formation and probably hippocampus appear to be important for the antipentobarbital action of TRH or DN-1417. Involvement of the cortex in this action of TRH or DN-1417 could not be excluded on the basis of the present results, because the applied area may be too limited to observe the true effect.

Although the antipentobarbital action of DN-1417 given peripherally was about twice as potent as that of TRH, the action of DN-1417 injected ICV or intracerebrally was 20–50 times as potent as that of TRH. These data indicate that incorporation of DN-1417 into the brain tissues may be relatively less than that of TRH. Another possibility is that the difference in pentobarbital-induced sleeping time between

the cannulae implanted rats and the rats without implantation may induce this discrepancy in potency, therefore DN-1417 with a longer-lasting action, compared with short-acting TRH [17], may exert a more potent effect in the cannulated rats which showed longer sleeping time after pentobarbital alone.

The present study also revealed that ICV injection of atropine methyl bromide markedly suppressed the shortening action of TRH given peripherally, as already described by other investigators in the case of mice or rats [1,19]. A nicotinic receptor blocker, mecamylamine given IP also reduced the TRH action although atropine did not or only slightly antagonized the effect. Microinjection of atropine or atropine methyl bromide into the hypothalamic regions also markedly attenuated the effect of TRH given peripherally or into the hypothalamus. However, ICV application of atropine methyl bromide produced only a partial antagonizing action on the effect of TRH injected ICV. In addition, the effect of TRH applied into the reticular formation, medial septal nucleus or hippocampus was not or only partially suppressed by atropine methyl bromide given ICV, as a pretreatment. These results suggest that the antipentobarbital action of TRH may be mediated mainly via cholinergic, muscarinic and more probably nicotinic mechanisms in the hypothalamus and partly via non-cholinergic neural mechanisms in other regions. In support of this concept, a cholinergic stimulation with carbachol to the hypothalamic but not the other sites, produced a significant reduction in the pentobarbital-induced sleeping time. A DA receptor blocker, pimozide did not modify the antipentobarbital action of TRH, in contrast to involvement of the DA system in the motor stimulatory action of TRH [6, 15, 16]. The lack of participation of the DA mechanism in the effect of TRH is supported by findings that DA microinjected ICV or intracerebrally had no significant action on pentobarbital sleep.

Santori *et al.* [25] demonstrated that the antipentobarbital action of TRH applied ICV was not inhibited by muscarinic antagonists given either peripherally or ICV, as was also shown in the present study. It is not surprising that ICV injection of atropine methyl bromide attenuated the antipentobarbital action of TRH administered peripherally. We considered that this discrepancy may be due to differences in the distribution of TRH in the brain when it is injected ICV or given peripherally. Nagai *et al.* [18] have shown in radioimmunoassay work that TRH given IV was distributed in the highest concentration in the hypothalamus, an area where the cholinergic mechanisms may be involved in the effect. In contrast, ICV applied ^{14}C-DN-1417 was highly distributed in the periventricular structures including the septal regions, hippocampus, central gray substance and some areas of the hypothalamus, and TRH in these sites other than hypothalamus may exert its action via a non-cholinergic mechanism. Therefore, the septum, hippocampus and probably reticular formation rather than hypothalamus may contribute to the effect of TRH given ICV.

Yarbrough [28,29] studied the interaction between TRH and acetylcholine in the acetylcholine sensitive neurons, and showed that TRH potentiates the excitatory response to iontophoretically applied acetylcholine. Also, TRH counteracts the depressed Na^+-dependent, high affinity choline uptake by pentobarbital [20, 26, 30]. Nagai *et al.* [19] found that TRH causes a reversal of the depression in local glucose utilization produced by pentobarbital. In addition, TRH accelerates the turnover rate of acetylcholine in the parietal cortex [14]. These findings may to some extent support our present data.

In conclusion, the main action sites of TRH or DN-1417 in antagonism of pentobarbital-induced sleep may be the posterior hypothalamic regions, dorsomedial nucleus of thalamus, medial septal nucleus, reticular formation and hippocampus, and cholinergic mechanisms may be involved in the effect of TRH or DN-1417 in the hypothalamus.

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