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EFFECT OF MILACEMIDE, A GLYCINAMIDE DERIVATIVE, ON THE RAT BRAIN γ-AMINOBUTYRIC ACID SYSTEM

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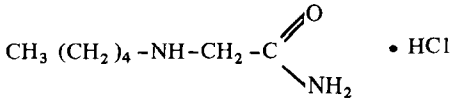
Abstract—Milacemide (CP 1552 S, 2-*n*-pentylaminoacetamide), a drug with anti-epileptic potency, increases the γ-aminobutyric acid (GABA) content specifically in the substantia nigra of rat brain. The effect is dose-related from 25 to 100 mg/kg p.o. The time course shows that at 100 mg/kg p.o. after 2, 3 and 4 hr the substantia nigra GABA content is significantly increased by 28, 33 and 38%, respectively. After 6 hr the GABA contents return to the control value. After repeated oral administration of milacemide a comparable effect to acute administration is obtained. After degeneration of the striato-nigral GABA-ergic pathway, milacemide no longer enhances the content of GABA in the substantia nigra. GABA-transaminase activity measured *ex vivo* in rat brain homogenate is not influenced by milacemide. On the other hand, the glutamate decarboxylase activity measured *ex vivo* 3 hr after 100 mg/kg of milacemide is significantly increased by 11% in homogenates of the whole rat brain. The results show that milacemide increases the GABA content in the GABA pool which is associated with the striato-nigral neurons. This increase is not due to GABA-transaminase inhibition but might be the result of an enhanced synthesis, possibly through glutamate decarboxylase activation.

A dysfunction of γ-aminobutyric acid (GABA) neurons in the brain is related to at least certain types of epilepsy and has been reviewed in depth [1–6]. In addition, inhibition of GABA neurotransmission results in convulsive state. In man, a deficiency of pyridoxine, the precursor of pyridoxal phosphate which is necessary for GABA synthesis, produces convulsions which are rapidly terminated by pyridoxine administration [6]. Furthermore, in animals, drugs that inhibit the synthesis of GABA [7, 8] or antagonize GABA receptors [1, 9] usually induce convulsions. Therefore drugs that elevate GABA levels in the brain may be of interest in preventing convulsions. However, the GABA degradative enzyme GABA-transaminase and presumably also GABA itself are present not only in neurons which utilize GABA as a neurotransmitter but also in other neurons and glial cells [10].

For this reason, we determined if milacemide, 2-*n*-pentylaminoacetamide (Fig. 1), which is active without sedative effect in different animal models of epilepsy (van Dorsser *et al.* to be published) and in the treatment of different epileptic patients (unpublished observations), has an effect on GABA levels in different brain areas.

It appears that this drug produces a selective



2-*N*-Pentylaminoacetamide • HCl

Fig. 1.

GABA increase in the substantia nigra. Therefore, we investigated whether a GABA increase is related to a change in the nerve terminals originating in the neostriatum or is produced locally by nigral neurons and/or glial cells [11, 12].

MATERIALS AND METHODS

Drugs and animals. Milacemide (CP 1552 S, 2-*n*-pentylaminoacetamide) is a product from Continental Pharma Research Laboratories (Belgium); sodium valproate (VPA) was obtained as Depakin®, γ-acetylenic-GABA (GAG) was a gift from Merrell (Strasbourg, France) and aminooxyacetic acid (AOAA) was provided by Aldrich-Europe. Other reagents were of analytical grade. Drugs suspended in a 1% tragacanth gum mucilage were administered by gavage through a gastric tube. Corresponding controls received the tragacanth gum mucilage only. In all cases, the volume administered was 1 ml per 100 g of body weight.

AOAA and VPA were suspended in saline (NaCl 9 g/l) and injected i.p. in a volume of 0.5 ml per 100 g of body weight. Male Sprague–Dawley (Charles River) or Wistar (TNO) rats (120–150 g body weight) were fasted overnight before use.

Hemitransection. Rats were anaesthetized with chloral hydrate (400 mg/kg) and placed in a stereotaxic frame as indicated by König and Klippel [13]. An incision was made on one side of the calvarium along a transverse line parallel and approximately 4.5 mm anterior to the interaural line. A stainless-steel blade (width 3 mm) was lowered twice through the incision to transect the base of the brain approximately 2 mm anterior to the substantia nigra. Ten days after surgery, when the GABA-containing

Table 1. Effect of milacemide on the GABA content in various rat brain areas

Brain areas	GABA content (μmole/g wet tissue) Controls	*N*	Milacemide	*N*	% of the control
Bulbus olfactorius Tuberculum Olfactorium	1.18 ± 0.08	9	1.23 ± 0.09	10	105
Frontal cortex	1.69 ± 0.08	8	1.77 ± 0.06	7	105
Cerebellar cortex	0.69 ± 0.02	8	0.71 ± 0.03	8	103
Striatum	0.84 ± 0.03	10	0.84 ± 0.07	10	100
Hippocampus	1.38 ± 0.06	9	1.53 ± 0.06	10	111
Hypophysis	1.17 ± 0.07	10	1.19 ± 0.05	10	100
Substantia nigra	0.34 ± 0.02	10	0.34 ± 0.04	10	100
	6.86 ± 0.24	8	8.45 ± 0.37	8	123***

Milacemide (100 mg/kg) or vehicle was administered orally 3 hr before sacrifice. *N* = number of rats studied. Results are expressed as the mean ± S.E.
***Significantly different from the control values for $P < 0.001$ following Student's *t*-test.

nerve fibres were degenerated [13, 14], various anti-epileptic drugs were administered.

GABA-assay. In order to avoid post mortem changes in GABA contents, 2 mmole per kg of 3-mercaptorproprionic acid was injected in a lateral vein of the tail 2 min before sacrifice [14]. After decapitation, the brain was rapidly removed and placed on an ice-cold plate; various brain areas were dissected, frozen on dry ice and weighed. To dissect the substantia nigra, we used a binocular microscope with a 10 × enlargement. GABA contents were measured by a semi-automatic method [14]. The GABA was detected fluorimetrically with a continuous flow system (Autoanalyzer AII of Technicon Inc.) equipped with a Perkin–Elmer 3000 fluorimeter. Possible intereferences by milacemide or some of its metabolites (glycine, glycinamide, *N*-(2-amino-2-oxoethyl)pentanamide] were checked. There was no fluorescent interference.

GABA-transaminase activity (GABA-T) determination. For the GABA-transaminase assay, we used the method of Jung *et al.* [15] slightly modified as follows: the brain homogenate was frozen and thawed, then centrifuged at 105,000 *g* during 75 min at 4° using a Beckman L5-65 ultracentrifuge. By this procedure, the turbidity is diminished and a better baseline in the GABA-T assay is obtained. The protein content of the tissue extracts was determined by the method of Bradford [16].

Glutamate decarboxylase (GAD) preparation and assay. Preparation of the enzymatic solution was performed using the method of Miller *et al.* [17] modified as follows: (a) Triton X-100 0.2% (v/v) was added to the preparation; (b) the whole brain homogenates were frozen, thawed and bubbled with nitrogen before centrifugation at 2000 *g* at 4° for 20 min. The use of Triton X-100 is necessary for studies of drug effects on GAD activity since the enzyme is membrane-bound and an artefactual increase of GAD activity may be observed with detergent drugs. Arsenite was added to avoid a possible $^{14}CO_2$ release from the Krebs cycle. The reaction was allowed to proceed for 60 min under nitrogen at 37° in a stirring bath (200 periods per min). Then, 0.5 ml of 40% trichloroacetic acid was added through the stopper; 30 min later the stopper was removed and the $^{14}CO_2$ was absorbed on paper and counted. The results are expressed in dpm $^{14}CO_2$ trapped by hyamine hydroxide per mg of proteins per 60 min. This assay was tested for different parameters such as enzyme and substrate concentrations and time. D, L-[1-^{14}C]glutamate was stored under nitrogen in order to avoid degradation.

RESULTS

Oral or intraperitoneal administration of milacemide did not modify the GABA content in the whole

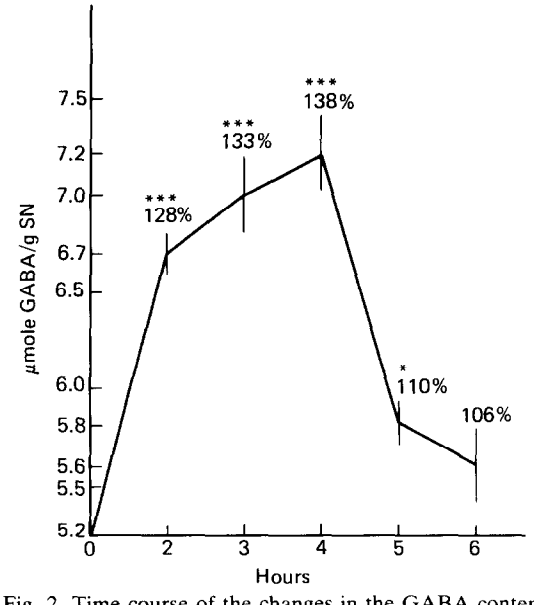


Fig. 2. Time course of the changes in the GABA content in the substantia nigra (SN) after 100 mg/kg p.o. milacemide. In control rats the GABA content was 5.24 ± 0.15 μmole per g of SN (mean ± S.E. — 22 rats). After 2, 3 and 4 hr the values were increased (***$P < 0.001$, when compared to controls, Student's *t*-test) to respectively 6.7 ± 0.1, 7.0 ± 0.2 and 7.2 ± 0.2 μmole per g SN (10 rats per group). After 5 hr, the GABA content was still significantly higher (5.8 ± 0.1 μmole per g SN, 6 rats, *$P < 0.05$ when compared to controls), but at 6 hr the level returned to the control (5.6 ± 0.2 μmole per g SN, 3 rats).

Table 2. GABA content in the substantia nigra (SN) after acute and repeated administration of milacemide

Treatment	GABA content (μmole/g SN)	% of the control
Controls	5.5 ± 0.5 (10)	100
Milacemide (acute)	7.2 ± 0.9 (10)***	131
Milacemide (8 days)	7.5 ± 0.7 (10)***	137

Control groups were administered with vehicle p.o. for 8 days, milacemide acute group received vehicle during 8 days but the last administration was 100 mg/kg p.o. of the drug, and milacemide 'chronic' group was administered with 100 mg/kg/day p.o. milacemide for 8 days.
In each case, the last administration occurred 3 hr before decapitation. The results represent the mean ± S.E. The number of rats is given in parentheses.
***Indicates significant differences from the controls at $P < 0.001$ following Student's *t*-test.

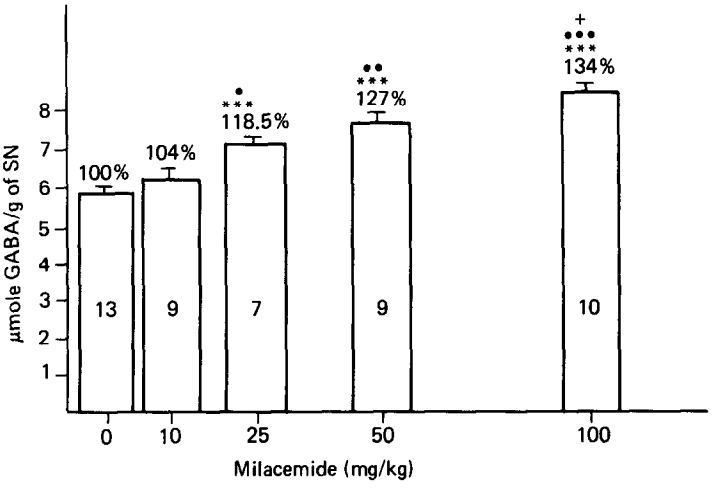


Fig. 3. Dose effect of milacemide on the GABA content in the substantia nigra of rat brain. Milacemide was administered p.o. 4 hr prior to sacrifice. Bars represent mean ± S.E. Numbers in the bars represent number of rats. ***$P < 0.001$ when compared to control (Student's *t*-test). *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$ when compared to milacemide 10 mg/kg group. + $P < 0.05$ when compared to milacemide 25 mg/kg group.

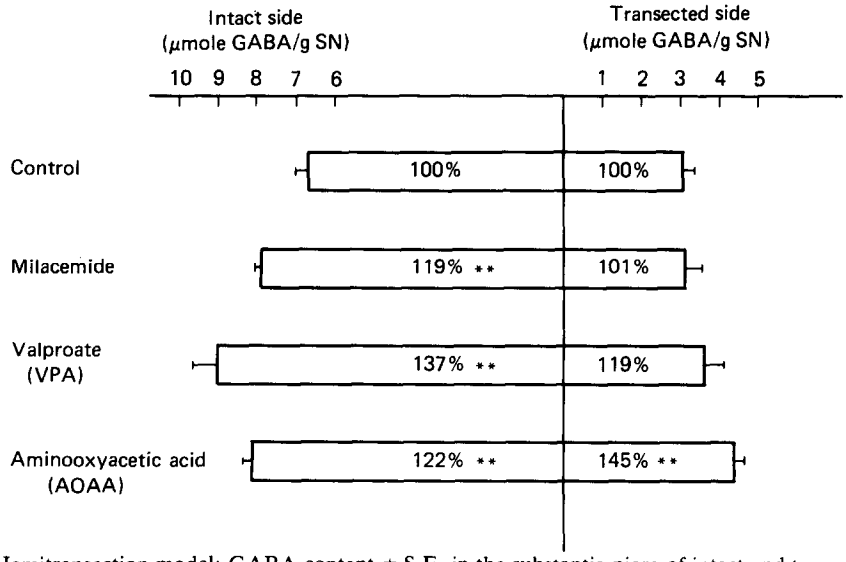


Fig. 4. Hemitransection model: GABA content ± S.E. in the substantia nigra of intact and transected sides in rats. Ten days after hemitransection, rats (5 animals per group) were treated with milacemide 100 mg/kg p.o. 3 hr before death, VPA 300 mg/kg i.p. 30 min before death or AOAA 30 mg/kg i.p. 2 hr before death. ** Indicates significant difference from the corresponding controls at $P < 0.01$ following Student's *t*-test.

Table 3. Effect on GABA-T activity in rat brain homogenate following various drug treatments

Treatment	% of GABA-T activity
Controls	100 (24)
Milacemide	96 ± 4 (10)
GAG	20 ± 8 (6)***
VPA	75 ± 5 (6)**

Drugs were given as follows: milacemide (200 mg/kg p.o. 4 hr before death), γ-acetylenic GABA (GAG; 200 mg/kg p.o. 3 hr before death) and valproate (VPA; 50 mg/kg i.p. 45 min before death). The result represent the mean ± S.E. and the number of rats is given in parentheses.

***$P < 0.001$; **$P < 0.01$ when compared to controls (Student's t-test).

brain (2 μmole/g wet tissue). When administered orally, the drug significantly increased the GABA content by 23% in the substantia nigra (Table 1). In the striatum, an increase by 11% compared to control values was noted in milacemide-treated rats. This change, however, was not statistically significant. There was no difference in the GABA content between control and treated rats in the other brain regions studied. The time course of the changes in the GABA content in the substantia nigra after milacemide acute treatment (100 mg/kg p.o.) is shown in Fig. 2. The GABA content was significantly increased after 2, 3 and 4 hr by 28, 33 and 38%, respectively. After 6 hr the GABA content was no longer significantly increased.

After an oral treatment of 100 mg/kg per day of milacemide for 7 days we obtained the same effect as in the acute treatment (Table 2). The GABA content after 25, 50 and 100 mg/kg of milacemide administered per p.o. was significantly increased (18, 27 and 34%, respectively), but not at 10 mg/kg (Fig. 3). Taken together, these results show a dose-dependent increase of GABA in the substantia nigra. Figure 4 summarizes the results on the GABA content in the substantia nigra of transected animals. Milacemide, VPA and AOAA significantly increased the GABA content in the intact side by 19, 37 and 22, respectively. On the other hand, AOAA was the only drug tested which increased the GABA content in the transected side.

GABA-T activities measured *ex vivo* in rat brain after acute administration of various drugs are indicated in Table 3. GAG (200 mg/kg p.o. 3 hr before death) and VPA (50 mg/kg i.p. 45 min before death) inhibited the GABA-T activity by 80 and 25%, respectively, as found by others [15]. In this procedure milacemide (200 mg/kg p.o. 4 hr before death) did not alter GABA-T activity.

GAD activities have been measured in whole rat brain homogenates 3 hr after the oral administration of 100 mg/kg of milacemide or vehicle. Under these conditions GAD activity was significantly increased by 11% after milacemide treatment (Table 4).

Table 4. Effect of milacemide on GAD activity *ex vivo* in rat brain homogenates

GAD activity (dpm $^{14}CO_2$ per mg protein) mean ± S.E.	
Control (vehicle)	milacemide 100 mg/kg p.o. 3 hr before sacrifice
9399 ± 290 (5) (100%)	10459 ± 123 (5) (111%)**

** Significantly different from the control values at $P < 0.01$ following Student's t-test.
The number of rats is given in parentheses.

DISCUSSION

The present results indicate that milacemide selectively raises the GABA content of the substantia nigra. This increase is dose-dependent, is sustained after repeated administration, and occurs only when the striato-nigral pathway is intact. At 100 mg/kg, the effect increases from 1 to 4 hr post-administration and has vanished at 6 hr. *Ex-vivo* experiments show no decrease in GABA-T activity but an increase in GAD activity in whole brain homogenates following drug administration. These changes in enzymatic activities are consistent with the increased GABA content measured in the substantia nigra, even though a similar increase was not detected by measurements performed on the whole brain. Regional differences in the distribution of the enzyme and drug as well as in GABA turnover could explain these apparent discrepancies. A similar increase of GAD activity in the whole brain has also been observed with sodium valproate [18, 19]. Several drugs are known to increase the GABA content in brain. Among these are the GABA-T inhibitors, e.g. AOAA, GAG, GVG, gabaculine [20], and drugs such as valproate which may also affect not only the GABA-T but also the succinic semialdehyde dehydrogenase activity [20]. The potent GABA-T inhibitors increase the GABA content in virtually all brain areas. Valproate, however, produces a less general effect on the GABA content; for example, notable increases were found at high dosages (300 mg/kg i.p.) in the superior colliculus, substantia nigra and frontal cortex but not in other areas of the brain [21].

The present study shows that the main effect of milacemide given orally at moderate dosages is to increase the GABA content in the substantia nigra. The hemitransection experiment shows that in contrast to the GABA increase observed after AOAA, the effects produced by VPA or milacemide are dependent upon the integrity of the striato-nigral pathway. These results confirm those obtained by Iadarola and Gale [22] for AOAA and valproate. The present results obtained with milacemide suggest a selective action on nerve terminals of the striato-nigral pathway, and that milacemide seems to have no effect on local neurons in the substantia nigra. In contrast, as AOAA elevates GABA whether or not the nerve terminals are intact, dosages of AOAA causing a 2–4-fold increase of GABA may be required to exert an anticonvulsant effect [23]. Iadarola and Gale also suggested that the GABA remaining in the substantia nigra after hemitransec-

tion is exclusively confined to glial cells. This, however, seems unlikely since after the lesion procedure GABA is still synthesized (increases after AOAA treatment) demonstrating the existence of GAD activity since this enzyme is confined exclusively to neuronal element [24, 25]. The localization of the GABA increase is indeed the most important fact for its effect [3, 22], and recently the substantia nigra was identified as an important site for GABA-mediated anticonvulsant activity [26]. Although milacemide affects the GABA levels only in the substantia nigra, there is no evidence to conclude that this brain area is the exclusive site of action. Indeed, the effect on GAD is found in a whole brain extract to which the substantia nigra does not contribute significantly. In a separate study (unpublished data), milacemide was shown to increase GAD activity in several specific areas of the brain, besides the substantia nigra. In addition, milacemide administered under the same conditions as in this study does not alter the glutamic acid content in rat brain, kidney, liver and plasma (J. Christophe, personal communication). In addition to an effect on GAD, other modes of action should also be considered.

Milacemide has no effect on the GABA uptake into synaptosomes *in vitro* at concentrations from 1 to 100 μmole/l. Similarly, no change in [^{3}H]muscimol binding has been observed *in vitro* with milacemide and several of its metabolites [glycine, gylcinamide, and *N*-(2-amino-2-oxoethyl)pentamide]. On the other hand, milacemide at high concentration (0.5 mmole/l.) and glycine (10 μmole/l. = IC_{50}) inhibited the *in vitro* [^{3}H]strychnine binding (results to be published).

Being a derivative of glycine, the possibility exists that milacemide and its metabolites potentiate or mimic some central action of this neurotransmitter amino acid. Indeed, an enhancement of glycine contents in the brain has been found with this drug (J. Christophe, to be published). It is of interest to mention here that recently evidence has appeared that glycine affects neurotransmission processes in the substantia nigra [27] and that glycine binding sites were found in this brain area [28]. Because of its action in the substantia nigra it should be considered for other diseases where basal ganglia are or are supposed to be involved, such as Huntington's chorea or tardive dyskinesia.

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