*Pharmacology Biochemistry & Behavior*, Vol. 32, pp. 993–1001 © Maxwell Pergamon Macmillan plc, 1989. Printed in the U.S.A. 0091-3057/89 $3.00 + .00

# Milacemide Increases 5-Hydroxytryptamine and Dopamine Levels in Rat Brain—Possible Mechanisms of Milacemide Antimyoclonic Property in the p,p′-DDT-Induced Myoclonus

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Received 22 July 1988

TRUONG, D. D., M. P. GALLOWAY, G. PEZZOLI, Z. JAMROZIK AND S. FAHN. *Milacemide increases 5-hydroxytryptamine and dopamine levels in rat brain—Possible mechanisms of milacemide antimyoclonic property in the p,p′-DDT-induced myoclonus.* PHARMACOL BIOCHEM BEHAV **32**(4) 993–1001, 1989.—Milacemide, a glycine prodrug that is able to enter the brain readily, has been shown to have an antimyoclonic property in the p,p′-DDT-induced myoclonus syndrome. Milacemide increased regional 5-HT and dopamine and decreased 5-HIAA, DOPAC and HVA levels in naive rats. In p,p′-DDT-treated rats, 5-HT levels were unchanged at the time the rats experienced spontaneous myoclonus in all brain regions except in the striatum, where it increased. 5-HIAA levels increased but did not reach significant levels except in the striatum. Dopamine, DOPAC, HVA and norepinephrine were unchanged. When rats were treated concurrently with both p,p′-DDT and milacemide, regional 5-HT levels were increased and NE levels in the brainstem and cerebellum decreased. Depletion of brain serotonin by pretreatment with PCPA or with 5,7-DHT, or blocking 5-HT receptors with different 5-HT antagonists, failed to eliminate the antimyoclonic property of milacemide. This antimyoclonic effect of milacemide may be mediated through other mechanisms besides its ability to increase brain 5-HT levels. Possible mechanisms to be considered are its antiepileptic property, and its ability to increase brain glycine levels. Milacemide may have potential for therapeutic trials in patients with myoclonus.

Myoclonus p,p′-DDT Glycine Milacemide Regional brain serotonin levels Dopamine 5,7-DHT

THE serotonin precursor 5-hydroxytryptophan (5-HTP) has been beneficial in the treatment of patients with myoclonus (3, 9, 13, 22, 36, 56). Although the results were not uniform, a number of patients improved with a combination of carbidopa and 5-HTP. Decreased levels of 5-HT and 5-HIAA have been reported in CSF from some patients with myoclonus (13,23). In addition, experimental myoclonus in rats could be precipitated by concurring treatment of 5-HTP and MAO inhibitors, or exposure to 5-HTP after development of receptor hypersensitivity by previous lesioning of the serotonin neurons with the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) (15,48). In an experimental model of myoclonus induced by 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane (p,p′-DDT), the involvement of serotonin is supported by the antimyoclonic effect of 5-HTP (28,43). Furthermore, clonazepam, which is an effective antimyoclonic drug in human disorders (13) and suppressed p,p′-DDT-induced myoclonus (29), has been shown to up-regulate 5-HT receptors after chronic treatment in rats (58). Thus, myoclonus may be mediated by the serotoninergic system.

Recently, glycine was also purported to be involved in myoclonus (5, 6, 27, 53, 54). The work from Chung and Van Woert showed that unilateral stereotaxic infusion of strychnine, a glycine receptor antagonist, into rat medullary reticular formation induced generalized stimulus sensitive myoclonus (5). In urea-induced myoclonus, urea displaced glycine at its receptor sites in the medullary reticular formation (6). Although regional determination showed fluctuation of the glycine level, the hypothesis that glycine levels are central to the induction of myoclonus is unproven (54). Intracerebroventricular glycine had protective

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effects against p,p'-DDT-induced myoclonus, but was not able to reverse full-blown myoclonus or seizures. A therapeutic trial with glycine in human myoclonus was ineffective probably due to inadequate penetration of the blood-brain barrier (55). Milacemide, a glycine precursor, has been shown to have antiepileptic as well as antimyoclonic properties (10,54). It prevented myoclonus and was also able to reverse p,p'-DDT-induced myoclonus when administered after the onset, although the effects were weaker and required more frequent dosing. In this paper, we have examined the effects of milacemide on the serotonin system to determine if the antimyoclonic effect of milacemide could be mediated through increased serotonin levels. For a better understanding of the p,p'-DDT-induced myoclonus, we also undertook a detailed study of the effect of p,p'-DDT on regional monoamine levels at the time the rats were experiencing spontaneous myoclonus.

## METHOD

### *Animals and Drugs*

Male Sprague-Dawley rats weighing 180–200 g were used in all experiments. The animals had free access to food and water. They were housed under standard laboratory conditions under 12-hour light-dark cycles. Animals were fasted overnight before being treated with p,p'-DDT 600 mg/kg or 400 mg/kg mixed in warmed corn oil and administered PO in volume of 2 ml/kg. Milacemide was administered IP. p,p'-DDT was obtained from Aldrich Pharmaceuticals (Milwaukee, WI), milacemide from Continental Pharma (Belgium), ketanserin, pirenperone and pipamperone from Janssen Pharmaceutics Co. (New Brunswick, NJ), methysergide from Sandoz (East Hanover, NJ), metergoline from Farmatalia (Milan, Italy), para-chlorophenylalanine and 5,7-DHT from Sigma Chemical Co. (St. Louis, MO), haloperidol from McNeil Company (Ft. Washington, PA) and desipramin HCl (DMI) from Merrell Dow Pharmaceuticals.

### *Biochemistry*

To measure the effects of milacemide on neurotransmitters, rats were treated with milacemide 400 mg/kg IP and sacrificed 1 hour later. For p,p'-DDT experiments, rats were treated with p,p'-DDT 600 mg/kg in warmed corn oil or p,p'-DDT and milacemide 400 mg/kg at 0 and 2 hours due to its short half life. They were killed at 3 hours, at this point all rats displayed spontaneous myoclonus without any seizure activity. Control rats received corn oil and saline. After decapitation, rat brains were dissected rapidly on ice and stored at −80°F. Frozen tissue was weighed then sonicated (Branson Sonic Power Co., Danbury, CT) in 0.4 ml of ice-cold 0.1 M perchloric acid containing dihydroxybenzamine or N-methylserotonin as internal standards to quantitate recovery. After centrifugation, 350 μl of the clear supernatant was brought to pH 8.5 by the addition of 25 μl of 3 M Tris base (pH 11). This solution was then poured over miniature alumina columns and elution assisted by centrifugation. The effluent of the initial spin, containing the indoles and HVA, was collected in a tube containing 33 μl of 6 M HCl to acidify the samples. Catechols were eluted from $Al_2O_3$ with 150 μl 0.1 M oxalic acid. Separation and quantification of catechols and indoles with reverse phase high performance liquid chromatography was performed as previously described (14,44), using an SSI HPLC pump, 5 μm C-8 reverse phase column (Rainin Instrument, Woburn, MA) and an LC-3 or LC-4 electrochemical transducer using a glassy carbon with an applied potential of 0.7 V (vs. Ag/AgCl reference electrode; Bioanalytical Systems, West Lafayette, IN). Ratios of sample peak height to internal standard were used for quantifications.

### *Lesioning Techniques*

For further testing of the effect of decreased brain serotonin level on p,p'-DDT-induced myoclonus, rats were lesioned with intracisternal injection of 5,7-DHT, a 5-HT neurotoxin, which destroys serotonin neurons and depletes brain serotonin levels (1,2). The rats were anesthetized with pentobarbital and were pretreated with DMI 25 mg/kg IP approximately 20 minutes prior to lesioning to protect the noradrenergic neurons (1). 5,7-DHT was prepared on the day of use in 0.1% vitamin C normal saline solution and protected from light. Intracisternal injection of 5,7-DHT (200 μg free base in 25 μl) was performed using a microsyringe with rats fixed on a custom designed wood block. They were allowed to recover for 48 hours and experiments were performed on the third day prior to development of behavioral hypersensitivity (31,52). Control animals received intracisternal injection of ascorbate in normal saline solution. In a concurrent experiment, rats were sacrificed and whole brain analyzed by HPLC to confirm our lesioning techniques.

### *Behavioral Assessment*

On testing day, animals were housed in individual Plexiglas cages and their behaviors were rated according to a previously validated rating scale (54): 0: normal; 1: myoclonus of body parts such as forepaws, ear twitches and wetdog shakes; 2: stimulus sensitive axial myoclonus but irregular following external stimulation; 3: marked stimulus sensitive axial myoclonus; 4: intermittent myoclonus without external stimulation; 5: continuous myoclonus without external stimulation; 6: intermittent convulsion or death during the evaluation period. External acoustic stimulation was delivered with a General Electric tape recorder (Model 3-5151B) which produced a sound taped from a metronome at 1 tick/second interval as previously described (54). Each observation lasted two minutes, the first one without acoustic stimulation, the second one with acoustic stimulation prior to intragastric treatment with p,p'-DDT and in subsequent 30-minute intervals for 5 hours to 6 hours afterward depending on experiments and p,p'-DDT dose.

### *Statistical Analysis*

Statistical analysis was performed using the CLINFO software program, version 4.3, on a Digital Computer VAX/VMS at the General Clinical Research Center at the Columbia University College of Physicians and Surgeons. The effects of drugs on p,p'-DDT-induced myoclonus were analyzed using the analysis of variance. When the null hypothesis was rejected, the Student-Newman-Keuls test was used to analyze the differences between groups. Comparison at particular neurotransmitter levels was made using the unpaired Student *t*-test.

## RESULTS

### *Biochemistry*

*Effect of milacemide on monoamine levels of naive rats.* Milacemide significantly increased 5-HT levels in all areas of the brain except the striatum, in which the increase did not reach a level of significance (Table 1). 5-HIAA was significantly decreased in the cortex, cerebellum and nucleus accumbens. 5-HIAA also decreased in other brain areas but did not reach statistical significance. Milacemide administration increased dopamine and decreased both DOPAC and HVA levels in the striatum (Table 2). Norepinephrine levels were unchanged in all areas measured (results not shown).

*Effects of p,p'-DDT on monoamine levels.* p,p'-DDT did not change 5-HT levels at the time the rats experienced spontaneous myoclonus except in the striatum where it increased. 5-HIAA levels increased but reached a significant level only in the striatum

TABLE 1

LEVELS OF 5-HT AND 5-HIAA IN MILACEMIDE-TREATED RATS

| | 5-HT | 5-HIAA |
|---|---|---|
| Striatum | | |
| Control | 877 ± 39 | 778 ± 37 |
| Milacemide | 983 ± 28 | 662 ± 36 |
| Cortex | | |
| Control | 195 ± 6 | 176 ± 8 |
| Milacemide | 277 ± 5* | 128 ± 4* |
| Diencephalon | | |
| Control | 344 ± 8 | 451 ± 13 |
| Milacemide | 594 ± 19* | 421 ± 21 |
| Brainstem | | |
| Control | 966 ± 49 | 1076 ± 30 |
| Milacemide | 1598 ± 138* | 1122 ± 44 |
| Cerebellum | | |
| Control | 78 ± 3 | 134 ± 4 |
| Milacemide | 129 ± 8* | 140 ± 7* |
| Nucleus Accumbens | | |
| Control | 857 ± 33 | 542 ± 15 |
| Milacemide | 988 ± 40* | 367 ± 27* |

Rats were treated with milacemide 400 mg/kg IP and sacrificed 1 hour later. Control rats received saline only. Milacemide increased 5-HT levels significantly except in the striatum, in which 5-HT levels increased but did not reach significance. 5-HIAA decreased significantly in the cortex, cerebellum and nucleus accumbens. 5-HIAA also decreased but did not reach statistically significant levels in other brain areas dissected. *Denotes $p<0.05$. Amount in nanogram per gram of wet tissue ±S.E.M.

at this time point (Fig. 1). p,p'-DDT did not change dopamine levels in any area except in the cortex, where they decreased. DOPAC and HVA also did not change in the areas determined (Fig. 2). There were no changes in the norepinephrine levels of the p,p'-DDT-treated rats when compared with controls at the time all of them experienced spontaneous myoclonus (Fig. 3).

*Effect of Milacemide on Monoamine Levels of p,p'-DDT-Treated Rats*

In p,p'-DDT-treated rats, milacemide increased 5-HT levels in the cortex, diencephalon, brainstem and nucleus accumbens, and it decreased 5-HIAA level in the striatum. When compared with vehicle-treated rats, milacemide and p,p'-DDT increased 5-HT levels in the cortex, diencephalon, brainstem, cerebellum, nucleus accumbens and spinal cord. 5-HIAA decreased in the nucleus accumbens and diencephalon only (Fig. 1).

TABLE 2

EFFECT OF MILACEMIDE ON CATECHOLAMINE LEVELS IN THE STRIATUM

| | Dopamine | DOPAC | HVA |
|---|---|---|---|
| Control | 5211 ± 173 | 1054 ± 73 | 1262 ± 52 |
| Milacemide | 6031 ± 196* | 244 ± 18* | 561 ± 32* |

Rats were treated with milacemide 400 mg/kg IP and sacrificed 1 hour afterward. Milacemide increased dopamine and decreased both DOPAC and HVA levels in the striatum. *Denotes $p<0.05$. Amount in nanogram per gram of wet tissue ± S.E.M.

TABLE 3

EFFECTS OF DEPLETION OF BRAIN SEROTONIN LEVELS ON THE ANTIMYOCLONIC PROPERTY OF MILACEMIDE

| Drugs | p,p'-DDT Dose mg/kg | Numbers of Rats | Mean Myoclonus Scores Between 2–5 Hours ± SEM |
|---|---|---|---|
| | Pretreatment With PCPA | | |
| Saline | 600 | 6 | 4.29 ± 1.29 |
| Milacemide 400 mg/kg q. 2 hr | 600 | 6 | 0.62 ± 0.13* |
| | Lesioning With 5,7-DHT | | |
| Saline | 400 | 6 | 4.98 ± 0.33 |
| Milacemide 400 mg/kg q. 2 hr | 400 | 6 | 3.37 ± 0.48* |

After pretreatment with PCPA 400 mg/kg IP 24 hours prior to experiment, rats were treated with p,p'-DDT 600 mg/kg and either saline or milacemide IP 400 mg/kg every 2 hours. Myoclonus scores were mean myoclonus scores between 2–5 hours. The myoclonus scores of milacemide-treated rats decreased significantly compared to control animals. Intracisternal injection of 5,7-DHT did not reverse the antimyoclonic property of milacemide. Myoclonus scores were computed as the mean between 2–6 hours because here a lower p,p'-DDT dose was given. Milacemide-treated rats differ significantly from lesioned rats that received saline only. *Indicates $p<0.05$.

In the group treated with both milacemide and p,p'-DDT, when compared with p,p'-DDT-treated rats only, dopamine levels increased in the hippocampus and diencephalon and decreased in the spinal cord. HVA decreased significantly in the striatum. DOPAC decreased in the striatum, nucleus accumbens, diencephalon and brainstem (Fig. 2).

When compared with control rats treated with vehicle, dopamine levels increased in the hippocampus, and decreased in the cortex, brainstem and spinal cord. HVA decreased in the striatum. DOPAC decreased in striatum, n. accumbens, diencephalon and brainstem (Fig. 2).

In the group that received both p,p'-DDT and milacemide, norepinephrine levels decreased in the cerebellum and increased in the spinal cord when compared with p,p'-DDT-treated rats. They decreased both in the brainstem and cerebellum when compared with control rats (Fig. 3).

*Behaviors*

Depletion of whole brain serotonin levels with parachlorophenylalanine (PCPA) 400 mg/kg 24 hours and 200 mg/kg 6 hours prior to testing (33) did not affect the antimyoclonic property of milacemide (Table 3). Intracisternal injection with 5,7-DHT did not affect milacemide's antimyoclonic property (Table 3). In a parallel experiment the levels of 5-HT depletion with PCPA and 5,7-DHT in whole brain were measured by HPLC, which showed a 50% depletion when compared with control rats (Table 4). Pre- and cotreatment with methysergide and metergoline, which block both 5-$HT_1$ and 5-$HT_2$ receptors and at a dose known to be central acting (19,21), did not modify the milacemide effect (Table 5). p,p'-DDT-induced myoclonus was suppressed by milacemide despite cotreatment with 5-$HT_2$ receptor blockers pipamperone, pirenperone and ketanserin (Table 6) (7, 20, 37, 61). Haloperidol

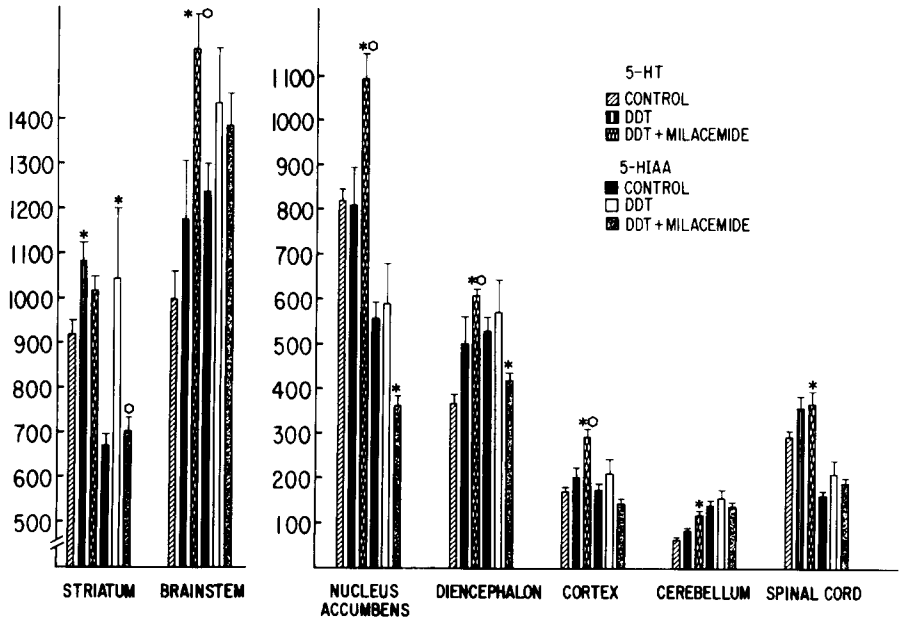


FIG. 1. Levels of 5-HT and 5-HIAA in p,p′-DDT- and milacemide-treated rats. Rats were treated with p,p′-DDT 600 mg/kg in warmed corn oil or p,p′-DDT and milacemide 400 mg/kg at 0 and 2 hours due to its short half life. They were killed at 3 hours; at this time point all rats displayed spontaneous myoclonus without any seizure activity. Control rats received corn oil and saline only. p,p′-DDT did not change either 5-HT or 5-HIAA levels except in the striatum, where it increased. When compared with the p,p′-DDT-treated rats, milacemide increased 5-HT levels in the cortex, diencephalon, brainstem and nucleus accumbens and it decreased 5-HIAA level in the nucleus accumbens. When compared with vehicle-treated rats, milacemide and p,p′-DDT increased 5-HT levels in the cortex, diencephalon, brainstem, cerebellum, nucleus accumbens and spinal cord, 5-HIAA decreased in the nucleus accumbens and diencephalon only. Asterisks denotes $p<0.05$ against vehicle control. Hexagons denote $p<0.05$ against p,p′-DDT-treated rats. Amount in nanogram per gram of wet tissue ± S.E.M.

also did not alter milacemide's effects (Table 5).

## DISCUSSION

### *Effects of p,p′-DDT on Neurotransmitters*

Our study of the effect of p,p′-DDT on the monoamines and metabolites did not show any change in the levels of 5-HT, dopamine, norepinephrine, DOPAC or HVA in any area examined except an increase in 5-HT and 5-HIAA levels in the striatum. We were unable to confirm the results of Hrdina *et al.* who reported that norepinephrine levels decreased in the brainstem (26). In agreement with a previous report, dopamine levels were unchanged in the p,p′-DDT-treated rats (25). Although 5-HIAA levels increased uniformly, they did not reach statistical significance at the time when myoclonus was prominent except in the striatum. Other investigators have reported an increase in 5-HIAA after p,p′-DDT (26,28). Hwang and Van Woert found 5-HIAA increased in all brain regions (28). Hrdina *et al.* measured only striatal 5-HIAA (26). The differences in our data may reflect the fact that different times of killing were chosen. Hwang and Van Woert sacrificed the animal at 4 hours after p,p′-DDT intake and Hrdina *et al.* at 5 hours. p,p′-DDT-treated rats experienced further symptoms besides myoclonus at this time, such as hyperthermia, running seizures and dystonic features. Hyperthermia has been shown to increase 5-HT turnover (47). We therefore conducted our experiments at 3 hour, where only spontaneous myoclonus is obvious. Interestingly, Hrdina *et al.* also did not find an increase in 5-HIAA with a lower dose of p,p′-DDT at 5 hour. At this time "hyperexcitability and tremor" were present but with only a mild increase in body temperature. Although 5-HTP decreased myoclonus in the p,p′-DDT model (28,43), an increase in 5-HT levels in the cerebellum and midbrain has been reported (28). We, however, in agreement with Hrdina *et al.*, did not find any change in the brainstem 5-HT as well as other areas dissected. Similarly, Pratt *et al.* reported that whole brain 5-HT level was unchanged (43). Although steady state evaluation of the major monoamine neurotransmitters is not the most sensitive measure of neuronal dysfunction, these results suggest that a deficiency of norepinephrine, dopamine, and 5-HT may not be the mechanism of p,p′-DDT-induced myoclonus. Since 5-HT does not have to be released before it is metabolized to 5-HIAA (16,59), p,p′-DDT may

TABLE 4

5-HT AND 5-HIAA LEVELS IN PCPA- AND 5,7-DHT-PRETREATED RATS

| | 5-HT | % Control | 5-HIAA | % Control | (n) |
|---|---|---|---|---|---|
| Control | 370 ± 43 | | 158 ± 20 | | (6) |
| PCPA-pretreated | 156 ± 53* | 42% | 35 ± 6.7* | 22.15% | (4) |
| Control-lesioned | 507 ± 16 | | 255 ± 16 | | (8) |
| 5,7-DHT-lesioned | 277 ± 40* | 54% | 131 ± 21* | 51% | (9) |

Pretreatment with PCPA or lesioning with 5,7-DHT depleted 5-HT levels in whole brain to 42% and 54% of control animals. 5-HIAA levels decreased to 22% and 51% of control respectively. Amount in nanogram per gram of wet tissue ± S.E.M. *Indicates $p<0.05$ by unpaired Student *t*-test.

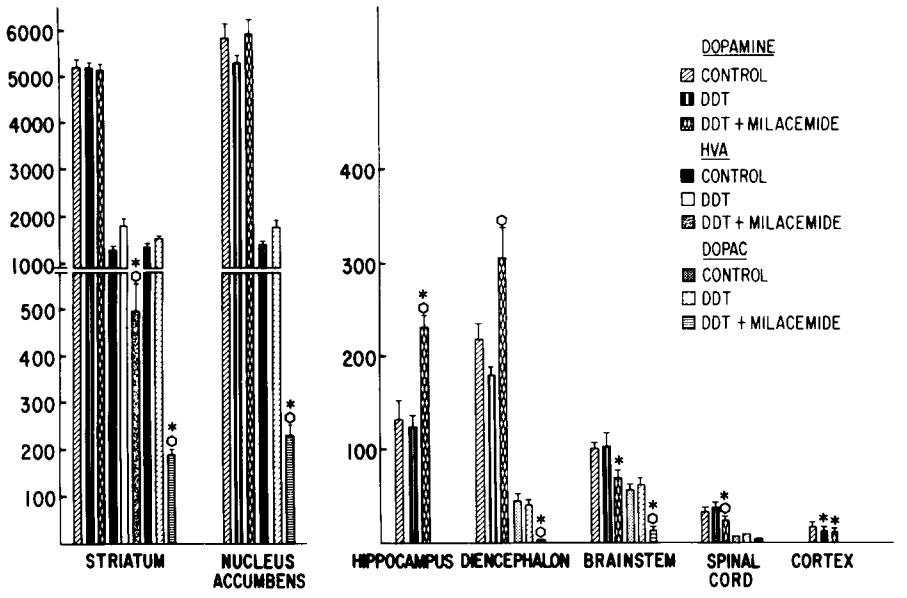


FIG. 2. Dopamine levels in the p,p'-DDT- and milacemide-treated rats. Rats were treated with p,p'-DDT 600 mg/kg PO at 0 hour. p,p'-DDT- and milacemide-treated rats received p,p'-DDT 600/mg at 0 hour, as well as milacemide 400 mg/kg IP at 0 and 2 hours. They were sacrified at 3 hours when all the rats had spontaneous myoclonus. p,p'-DDT did not change dopamine levels in all the areas dissected except in the cortex, where they decreased. DOPAC and HVA also did not change in the two areas determined. In the group treated with both milacemide and p,p'-DDT, when compared with p,p'-DDT-treated rats only, dopamine levels increased in the diencephalon, hippocampus and decreased in the spinal cord. HVA and DOPAC decreased significantly in the areas determined, DOPAC level became not measurable in the diencephalon. When compared with control rats treated with vehicle, dopamine levels increased in the hippocampus and decreased in the cortex, brainstem and spinal cord. HVA and DOPAC levels decreased in the areas determined. Asterisks denote $p<0.05$ against vehicle control. Hexagons denote $p<0.05$ when compared with p,p'-DDT-treated rats. Amount in nanogram per gram of wet tissue ± S.E.M.

interfere with 5-HT release into the synaptic cleft as suspected by Hwang and Van Woert (28) so that a deficiency at the receptor sites exists and 5-HIAA is metabolized intraneuronally without being released and reuptaken.

*Effects of Milacemide on Monoamines*

Our results showed that milacemide at the doses used increased brain dopamine and decreased DOPAC and HVA levels in the striatum of naive rats. It also increased 5-HT and decreased 5-HIAA levels in all areas studied except in the striatum. An increase in 5-HT or DA levels can be achieved either through increased synthesis, decreased degradation, or decreased release. A concurrent decrease of the metabolites 5-HIAA and DOPAC, coupled with an increase in dopamine and 5-HT levels, suggests the possibility of inhibition of monoamine oxidase, since this enzyme is involved in both the degradation of 5-HT and dopamine (11, 17, 18, 24). Milacemide, however, did not alter norepinephrine levels as reported with other MAO inhibitors, such as pargyline, chlorgyline and tranylcypromine (18, 26, 60). Interestingly, it has been shown to increase brain glycine levels (4,54). The interaction between glycine and serotonin metabolism is currently not well known.

*Effects of Both p,p'-DDT and Milacemide on Monoamines: Possible Mechanism of Milacemide's Antimyoclonic Property*

In the p,p'-DDT-treated rats, milacemide increased dopamine levels in the hippocampus and diencephalon, but decreased dopamine level in the spinal cord. Its effects on the norepinephrine levels differed, with an increase in the spinal cord, decrease in the cerebellum and no change in other brain areas studied. Hrdina *et al.* reported the increase in NE level in the brainstem by the MAO inhibitor pargyline was potentiated by treatment with p,p'-DDT (26). We, however, obtained an opposite effect with milacemide. Concurrent application of both milacemide and p,p'-DDT decreased NE in brainstem and cerebellum. Experiments by Hwang and Van Woert have shown that pretreatment with alpha-MPT or blockade of alpha receptors enhanced the antimyoclonic property of 5-HTP (30). They postulated that a noradrenergic inhibitory synapse may be located in the neural circuit connecting a serotoninergic neuron to the final motor response of myoclonus (30). Whether the antimyoclonic property of milacemide is mediated through a noradrenergic mechanism remains to be determined.

In p,p'-DDT rats, milacemide decreased 5-HIAA and DOPAC levels and increased 5-HT levels. At the dose used, the increase in 5-HT was most marked in the brainstem, cortex, cerebellum and nucleus accumbens. This raised the question that the previously reported antimyoclonic property of milacemide could be mediated through an enhancement of 5-HT neurotransmission since 5-HTP and drugs that increased serotonin neurotransmission improved myoclonus in the p,p'-DDT model (28, 38, 43). However, pretreatment with drugs that decreased serotoningeric function, such as PCPA (33), 5,7-DHT or serotonin receptor blockers failed to eliminate the antimyoclonic property of milacemide.

In the serotonin syndrome, which shows forepaws treading, hyperactivity, head waving, snake tails and seizures besides

TABLE 5

EFFECT OF METHYSERGIDE, METERGOLINE AND HALOPERIDOL ON THE ANTIMYOCLONIC PROPERTIES OF MILACEMIDE

| Drugs | Drugs | p,p'-DDT Dose mg/kg | Mean Myoclonus Scores Between 2–5 hr ± S.E.M. | (n) |
|---|---|---|---|---|
| | Pretreatment With Methysergide | | | |
| Saline q. 2 hr | Vehicle 1 hr prior to DDT and at 3 hr | 600 | 4.47 ± 0.32 | (6) |
| Milacemide 400 mg/kg q. 2 hr | Methysergide 10 mg/kg 1 hr prior to DDT and at 3 hr | 600 | 1.00 ± 0.27* | (6) |
| | Cotreatment With Methysergide | | | |
| Saline q. 2 hr | Methysergide 10 mg/kg at time 0 | 600 | 4.90 ± 0.36 | (6) |
| Milacemide 400 mg/kg q. 2 hr | Methysergide 10 mg/kg IP at time 0 | 600 | 0.90 ± 0.29* | (6) |
| | Pretreatment With Metergoline | | | |
| Saline q. 2 hr | Metergoline 2 mg/kg 3 hr prior to DDT | 400 | 4.85 ± 0.34 | (5) |
| Milacemide 400 mg/kg q. 2 hr | Meterogoline 2 mg/kg 3 hr prior to DDT | 400 | 1.34 ± 0.36* | (6) |
| | Cotreatment With Haloperidol | | | |
| Saline q. 2 hr | Haloperidol 1 mg/kg IP at time 0 | 600 | 3.86 ± 0.40 | (5) |
| Milacemide 400 mg/kg q. 2 hr IP | Haloperidol 1 mg/kg IP at time 0 | 600 | 1.10 ± 0.26* | (5) |

Methysergide 10 mg/kg IP was given prior to and at the same time as p,p'-DDT. Metergoline 2 mg/kg IP was given 3 hours prior to experiment. Methysergide and metergoline have no effect on the antimyoclonic property of milacemide. Haloperidol did not alter milacemide's effectiveness. *Indicates $p<0.05$ by ANOVA.

myoclonus, 5-$HT_2$ receptor blockers have been reported to block the head twitches, wet dog shakes and myoclonus (7, 20, 37, 41, 61). Based on these observations, we suspected that 5-$HT_2$ receptors may play a role in p,p'-DDT-induced myoclonus and tested different 5-$HT_2$ receptor blockers such as ketanserin, pipamperone and pirenperone in the presence of milacemide. Nevertheless, pre- or cotreatment with these 5-$HT_2$ receptor blockers, at doses known to be centrally active as reported by others, failed to abolish the antimyoclonic effects of milacemide. Pretreatment with metergoline and methysergide, which block both 5-$HT_1$ and 5-$HT_2$ receptors (8, 12, 19), did not eliminate milacemide's antimyoclonic effects.

These data, however, need to be interpreted with caution since serotonin is normally synthesized in excess of functional need and excessive serotonin may be degraded by monoamine oxidase without release into the interneuronal clefts (16,59). Indeed, there is evidence that different pools of 5-HT exist. The large compartment is thought to be a storage and reserve pool, and the smaller pool is often referred to as the "functional" pool and consists of newly synthesized 5-HT (34). This functional pool may not have been depleted. Although methysergide and metergoline have been shown previously to worsen p,p'-DDT-induced myoclonus in mice (28) and counteracted the antimyoclonic property of clonazepam (29) which increases cerebral 5-HT and 5-HIAA levels (42). These assertions have been disputed by Pratt *et al.*, who reported that neither depletion of serotonin with PCPA (up to 35% of control) nor blocking 5-HT with methysergide and metergoline worsens p,p'-DDT-induced myoclonus in rats (43). It is further not known what relationship exists between the antagonist effects of these blockers and the increased serotonin levels by milacemide. Milacemide also has been shown to have antiepileptic activities in different seizures models (10) and increased brain glycinamide and glycine levels (4,54). The antimyoclonic properties of milacemide, therefore, could be mediated through other mechanisms than increased serotonin transmission alone, such as through increased brain glycine levels or its antiepileptic property. Pretreatment with the antiepileptic agent diphenylhydantoin has been reported to attenuate tremor induced by a lower dose of p,p'-DDT (49). Hunter *et al.* also reported the effect of milacemide in mice and suspected the involvement of a glycinergic mechanism in p,p'-DDT-induced myoclonus (27). The marked effectiveness of milacemide could be due to the synergistic effects of glycine on serotonin's antimyoclonic property. Indeed, glycine has been known to potentiate the anticonvulsant actions of diazepam, phenobarbital and other anticonvulsant drugs (40, 45, 46, 50, 51).

Since milacemide also increases dopamine levels in the striatum, we tested the antimyoclonic activity of milacemide in the presence of haloperidol. Rats treated concurrently with haloperidol and milacemide did not differ from rats treated with milacemide only. Milacemide has also been reported to increase GABA synthesis (57) and although GABA levels are decreased in the p,p'-DDT models (32,39), ICV GABA, GABA agonists such as

TABLE 6

EFFECT OF THE 5-$HT_2$ RECEPTOR BLOCKERS PIPAMPERONE, KETANSERIN AND PIRENPERONE ON THE ANTIMYOCLONIC PROPERTY OF MILACEMIDE

| Drugs | Drugs | p,p'-DDT Dose mg/kg PO | Mean Myoclonus Scores Between 2–5 hr ± SEM | (n) |
|---|---|---|---|---|
| Saline | Saline | 600 | 5.11 ± 0.36 | (5) |
| Pipamperone 1 mg/kg IP q. 3 hr | Milacemide 400 mg/kg q. 2 hr | 600 | 0.78 ± 0.26* | (6) |
| Vehicle | Saline | 600 | 4.40 ± 0.41 | (6) |
| Ketanserin 10 mg/kg IP | Milacemide 400 mg/kg q. 2 hr | 600 | 1.05 ± 0.14* | (5) |
| Ketanserin 2 mg/kg IP | Milacemide 400 mg/kg q. 2 hr | 600 | 0.71 ± 0.17* | (5) |
| Vehicle | Saline | 600 | 4.50 ± 0.37 | (5) |
| Pirenperone 500 μg/kg IP | Milacemide 400 mg/kg IP q. 2 hr | 600 | 0.34 ± 0.09* | (5) |

Rats were treated with p,p'-DDT 600 mg/kg PO and either pipamperone, ketanserin or pirenperone. Rats treated with milacemide were protected from myoclonus despite 5-$HT_2$ blocking effect. *Indicates $p<0.05$ by ANOVA.

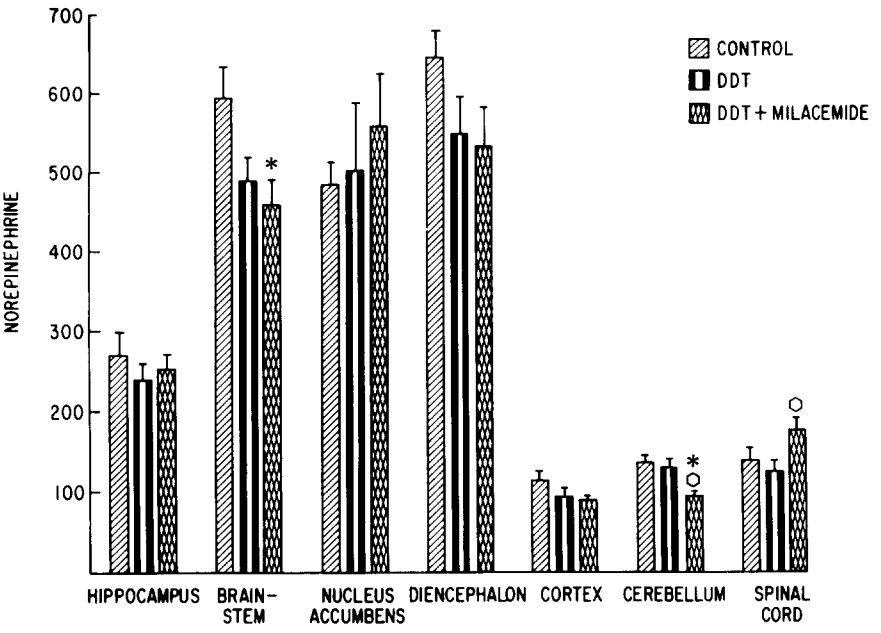


FIG. 3. Effect of p,p'-DDT and milacemide on norepinephrine levels. Rats were treated with p,p'-DDT 600 mg/kg or oil vehicle as control. Rats treated with both p,p'-DDT and milacemide received p,p'-DDT 600 mg/kg at 0 hour and milacemide 400 mg/kg IP at 0 and 2 hours. They were sacrificed at 3 hours. There were no changes in the norephinephrine levels of the p,p'-DDT-treated rats when compared with control, at the time all of them experienced spontaneous myoclonus. In the group that received both p,p'-DDT and milacemide, norepinephrine levels decreased in the cerebellum and increased in the spinal cord when compared with p,p'-DDT-treated rats. They decreased both in the brainstem and cerebellum when compared with control rats. Asterisks denote $p<0.05$ when compared with vehicle control. Hexagons denote $p<0.05$ when compared with p,p'-DDT-treated rats. Amounts in nanogram per gram of wet tissue ± S.E.M.

muscimol, acetylenic GABA, amino-oxyacetic acid or progabide and GABA antagonists bicuculline and isoniacid have no effect on p,p'-DDT-induced myoclonus (28, 29, 54). The mechanism of milacemide's antimyoclonic effect may not be mediated through GABA.

In summary, milacemide increased brain serotonin and dopamine as well as decreased their acid metabolites 5-HIAA, HVA and DOPAC, consistent with the predicted effects of a MAO inhibitor. Although p,p'-DDT-induced myoclonus improved with drugs that increased serotonin transmission, detailed regional studies of different neurotransmitters at the time of myoclonus failed to show alteration of either serotonin, dopamine or norepinephrine levels. A deficiency in 5-HT is therefore not central for the induction of myoclonus. Concurrent administration of both milacemide and p,p'-DDT decreased NE in brainstem and cerebellum. Whether the antimyoclonic property of milacemide is mediated through a noradrenergic mechanism remains to be elucidated. Milacemide also increased serotonin levels in p,p'-DDT-treated rats, but neither depletion of brain serotonin levels with PCPA, lesioning with the 5-HT neurotoxin 5,7-DHT or blocking with different $5\text{-HT}_1$ or $5\text{-HT}_2$ receptors blockers eliminate the beneficial effects of milacemide on myoclonus. Milacemide's antimyoclonic property may not require endogenous serotonin or its effects on $5\text{-HT}_1$ and $5\text{-HT}_2$ receptors. The antimyoclonic effect of milacemide may be mediated through other actions of milacemide such as increased brain glycine levels or its antiepileptic effects besides increased brain serotonin levels. Since milacemide can prevent p,p'-DDT-induced myoclonus in rodents, which is considered to be an animal model of human myoclonus, it may have potential for human therapeutic trials.

ACKNOWLEDGEMENTS

D. D. Truong was supported by a grant from the Myoclonus Research Fund. We are grateful to our technicians Rosario Amato, David Balch and Vernice Jackson-Lewis for their dedicated assistance and Donald McMahon from GCRC Columbia Presbyterian Hospital for statistical advice.

## REFERENCES

1. Bjorklund, A.; Baumgarten, H. G.; Rensch, A. 5,7-dihydroxytryptamine: Improvement of its selectivity for serotonin neurons in the CNS by pretreatment with desipramine. J. Neurochem. 24:833–835; 1975.
2. Bjorklund, A.; Nobin, A.; Stenevi, U. The use of neurotoxic dihydroxytryptamines as tools for morphological studies and localized lesioning of central indoleamine neurons. Z. Zellforsh. Mikrosk. Anat. 145:479–501; 1973.
3. Chadwick, D.; Hallett, M.; Harris, R.; Jenner, P.; Reynolds, E. H.; Marsden, C. D. Clinical, biochemical and physiological features distinguishing myoclonus responsive to 5-hydroxytryptophan, tryptophan with a monoamine oxidase inhibitor, and clonazepam. Brain 100:455–487; 1977.
4. Christophe, J.; Kutzner, R.; Nguyen-Bui, N. D.; Damien, C.; Chatelain, P.; Gillet, L. Conversion of orally administered 2-n-pentylaminoacetamide into glycinamide and glycine in the rat brain. Life Sci. 33:533–541; 1983.
5. Chung, E.; Van Woert, M. H. DDT myoclonus: Sites and mechanism of action. Exp. Neurol. 85:273–282; 1984.

6. Chung, E.; Yocca, F.; Van Woert, M. H. Urea-induced myoclonus: Medullary glycine antagonism as mechanism of action. Life Sci. 36:1051–1058; 1985.
7. Colpaert, F. C.; Janssen, P. A. The head-twitch response to intraperitoneal injection of 5-hydroxytryptophan in the rat: Antagonist effects of purported 5-hydroxytryptamine antagonists and of pirenperone, an LSD antagonist. Neuropharmacology 22:993–1000; 1983.
8. Cox, B.; Ennis, C. Characterization of 5-hydroxytryptaminergic autoreceptors in the rat hypothalamus. J. Pharm. Pharmacol. 34: 438–441; 1982.
9. De Lean, J.; Richardson, J. C.; Hornykiewicz, O. Beneficial effects of serotonin precursors in postanoxic action myoclonus. Neurology 26:863–868; 1976.
10. Dorsser, W. V.; Barris, D.; Cordi, A.; Roba, J. Anticonvulsant activity of milacemide. Arch. Int. Pharmacodyn. 266:239–249; 1983.
11. Ekstedt, B. Substrate specificity of the different forms of monoamine oxidase in rat liver mitochondria. Biochem. Pharmacol. 25:1133–1138; 1976.
12. Ennis, C.; Cox, B. Pharmacological evidence for the existence of two distinct serotonin receptors in rat brain. Neuropharmacology 21: 41–44; 1982.
13. Fahn, S. Posthypoxic action myoclonus: Review of the literature and report of 2 new cases with response to valproate and estrogen. Adv. Neurol. 29:26–84; 1979.
14. Galloway, M. P.; Wolf, M. E.; Roth, R. H. Regulation of dopamine synthesis in the medial prefrontal cortex is mediated by release modulating autoreceptors: Studies in vivo. J. Pharmacol. Exp. Ther. 236:689–698; 1986.
15. Gerson, S. C.; Baldessarini, R. J. Motor effects of serotonin in the central nervous system. Life Sci. 27:1435–1451; 1980.
16. Grahame-Smith, D. R. Studies in vivo on the relationship between brain tryptophan, brain 5-HT synthesis and hyperactivity in rats treated with a monoamine oxidase inhibitor and L-tryptophan. J. Neurochem. 18:1053–1066; 1971.
17. Green, A. R.; Youdim, M. B. H. Effects of monoamine oxidase inhibition by clorgyline, deprenil or tranylcypromine on 5-hydroxytryptamine concentrations in rat brain and hyperactivity following subsequent tryptophan administration. Br. J. Pharmacol. 55:415–422; 1975.
18. Green, A. R.; Mitchell, B. D.; Tordoff, A. F. C.; Youdim, M. B. H. Evidence for dopamine deamination by both type A and type B monoamine oxidase in rat brain in vivo and for the degree of inhibition of enzyme necessary for increased functional activity of dopamine and 5-hydroxytryptamine. Br. J. Pharmacol. 60:343–349; 1977.
19. Green, A. R.; Hall, J. E.; Rees, A. R. A behavioural and biochemical study in the rats of 5-hydroxytryptamine receptor agonists and antagonists with observations on structural-activity requirements for the agonists. Br. J. Pharmacol. 73:703–719; 1981.
20. Green, A. R.; O'Shaughnessy, K.; Hammond, M.; Schächter, M.; Grahame-Smith, D. G. Inhibition of 5-hydroxytryptamine-mediated behavior by the putative 5-$HT_2$ antagonist pirenperone. Neuropharmacology 22:573–578; 1983.
21. Green, A. R.; Heal, D. J. The effects of drugs on serotonin-mediated behavioural models. In: Green, A. R., ed. Neuropharmacology of serotonin. Oxford: Oxford University Press; 1985:326–365.
22. Growdon, J. H.; Young, R. R.; Shahani, B. T. L-5-Hydroxytryptophan in the treatment of syndromes in which myoclonus is prominent. Neurology 26:1135–1140; 1976.
23. Guilleminault, C.; Tharp, B. R.; Cousin, D. HVA and 5-HIAA CSF measurements and 5-HTP trials in some patients with involuntary movements. J. Neurol. Sci. 18:435–441; 1973.
24. Houslay, M. D.; Tipton, K. F. Multiple forms of monoamine oxidase: Fact and artifact. Life Sci. 19:467–477; 1976.
25. Hrdina, P. D.; Singhal, R. L.; Peters, D. A.; Ling, G. M. Role of brain acetylcholine and dopamine in acute neurotoxic effects of DDT. Eur. J. Pharmacol. 15:379–382; 1971.
26. Hrdina, P. D.; Singhal, R. L.; Peters, D. A.; Ling, G. M. Some neurochemical alterations during acute DDT poisoning. Toxicol. Appl. Pharmacol. 25:276–288; 1973.
27. Hunter, C.; Chung, E.; Van Woert, M. H. Antimyoclonic action of milacemide in p,p′-DDT-induced myoclonus. Soc. Neurosci. Abstr. 12:1243; 1986.
28. Hwang, E. C.; Van Woert, M. H. p,p′-DDT-induced neurotoxic syndrome: Experimental myoclonus. Neurology 28:1020–1025; 1978.
29. Hwang, E. C.; Van Woert, M. H. Antimyoclonic action of clonazepam: The role of serotonin. Eur. J. Pharmacol. 60:31–40; 1979.
30. Hwang, E. C.; Van Woert, M. H. p,p′-DDT-induced myoclonus: Serotonin and alpha-noradrenergic interaction. Res. Commun. Chem. Pathol. Pharmacol. 23:257–266; 1979.
31. Jacobs, B. L. An animal behavioral model for studying central serotoninergic synapses. Life Sci. 19:777–786; 1976.
32. Kar, P. P.; Matin, M. A. Possible role of cerebral amino acids in acute neurotoxic effects of DDT in mice. Eur. J. Pharmacol. 25:36–39; 1974.
33. Koe, B. K.; Weissman, A. p-Chlorophenylalanine: A specific depletor of brain serotonin. J. Pharmacol. Exp. Ther. 154:499–516; 1966.
34. Kuhn, D. M.; Wolf, W. A.; Youdim, M. B. H. 5-hydroxytryptamine release in vivo from a cytoplasmic pool: Studies on the 5-HT behavioural syndrome in reserpinized rats. Br. J. Pharmacol. 84: 121–129; 1985.
35. Laduron, P. M.; Janssen, P. F. M.; Leysen, J. E. In vivo binding of [$^3$H]ketanserin on serotonin $S_2$-receptors in rat brain. Eur. J. Pharmacol. 81:43–48; 1982.
36. Lhermitte, F.; Marteau, R.; Degos, C. F. Analyse pharmacologique d'un nouveau cas de myoclonies d'intention et d'action postanoxique. Rev. Neurol. (Paris) 126:107–114; 1972.
37. Lucki, I.; Nobler, M. S.; Frazer, A. Differential actions of serotonin antagonists on two behavioral models of serotonin receptor activation in the rat. J. Pharmacol. Exp. Ther. 228:133–139; 1984.
38. Magnussen, I. p,p′-DDT-induced myoclonus in mice: The effect of enhanced 5-HT neurotransmission. Acta Pharmacol. Toxicol. 56: 87–90; 1985.
39. Matin, M. A.; Kar, P. P.; Hasan, M. Z.; Anand, M. Comparison of the chronic effects of alpha-chlordane and p,p′-DDT on the level of cerebral amino acids and free ammonia in mice. Pharmacol. Res. Commun. 9:613–619; 1977.
40. Peterson, S. L. Glycine potentiates the anticonvulsant action of diazepam and phenobarbital in kindled amygdaloid seizures of rats. Neuropharmacology 25:1359–1363; 1986.
41. Pranzatelli, M. R.; Snodgrass, S. R. Antimyoclonic properties of $S_2$ serotonin receptor antagonists in the rat. Neuropharmacology 25: 5–12; 1986.
42. Pratt, J.; Jenner, P.; Reynolds, E. H.; Marsden, C. D. Clonazepam induces decreased serotoninergic activity in the mouse brain. Neuropharmacology 18:791–799; 1979.
43. Pratt, J. A.; Rothwell, J.; Jenner, P.; Marsden, C. D. Myoclonus in the rat induced by p,p′-DDT and the role of altered monoamine function. Neuropharmacology 24:361–373; 1985.
44. Reinhard, G. F.; Roth, R. H. Noradrenergic modulation of serotonin synthesis and metabolism. Inhibition by clonidine in vivo. J. Pharmacol. Exp. Ther. 221:541–546; 1982.
45. Seiler, N.; Sarhan, S. Synergistic anticonvulsant effects of GABA -T inhibitors and glycine. Naunyn Schmiedebergs Arch. Pharmacol. 326:49–57; 1984.
46. Seiler, N.; Sarhan, S.; Krogsgaard-Larsen, P.; Hjeds, H.; Schousboe, A. Amplification of glycine of the anticonvulsant effect of THPO, a GABA uptake inhibitor. Gen. Pharmacol. 16:509–511; 1985.
47. Squires, R. F. Hyperthermia- and L-tryptophan-induced increases in serotonin turnover in rat brain. Adv. Biochem. Psychopharmacol. 10:207–211; 1974.
48. Stewart, R. M.; Growdon, J. H.; Cancian, D.; Baldessarini, R. J. Myoclonus after 5-hydroxytryptophan in rats with lesions of indoleamine neurons in the central nervous system. Neurology 26: 690–692; 1976.
49. Tilson, H. A.; Hudson, P. M.; Hong, J. S. 5,5-diphenylhydantoin antagonizes neurochemical and behavioral effects of p,p′-DDT but not of chlordecone. J. Neurochem. 47:1870–1878; 1986.
50. Toth, E.; Lajtha, A.; Sarhan, S.; Seiler, N. Anticonvulsant effects of some inhibitory neurotransmitter amino acids. Neurochem. Res. 8:291–302; 1983.
51. Toth, E.; Lajtha, A. Glycine potentiates the action of some anticonvulsant drugs in some seizure models. Neurochem. Res. 9:1711–1718; 1984.
52. Trulson, M. E.; Jacobs, B. L. Behavioral evidence for denervation supersensitivity after destruction of central serotoninergic nerve terminals. Ann. NY Acad. Sci. 305:497–509; 1978.

53. Truong, D. D.; Pranzatelli, M.; Jackson-Lewis, V.; Fahn, S. Intracerebroventricular glycine blocks myoclonus induced by DDT in rats. Ann. Neurol. 20:149; 1986.
54. Truong, D. D.; de Yebenes, J. G.; Pezzoli, G.; Jackson-Lewis, V.; Fahn, S. Glycine involvement in DDT-induced myoclonus. Movement Disord. 3:77–87; 1988.
55. Truong, D. D.; Fahn, S. Therapeutic trial with glycine in myoclonus. Movement Disord. 3:222–232; 1988.
56. Van Woert, M. H.; Rosnaum, D.; Howieson, J.; Bowers, M. B. Longterm therapy of myoclonus and other neurologic disorders with L-5-hydroxytryptophan and carbidopa. N. Engl. J. Med. 296:70–75; 1977.
57. Varebeke, J. D.; Niebes, P.; Paulwels, G.; Roba, J.; Korf, J. Effect of CP 1552S, glycinamide derivative, on the rat brain GABA-ergic system. Biochem. Pharmacol. 32:2751–2755; 1983.
58. Wagner, H. R.; Reches, A.; Fahn, S. Clonazepam-induced upregulation of serotonin$_1$ binding sites in frontal cortex of rat. Neuropharmacology 24:953–956; 1985.
59. Wolf, W. A.; Youdim, M. B. H.; Kuhn, D. M. Does brain 5-HIAA indicate serotonin release or monoamine oxidase activity? Eur. J. Pharmacol. 109:381–387; 1985.
60. Yang, H. Y. T.; Neff, N. H. The monoamine oxidases of brain: Selective inhibition with drugs and the consequences for the metabolism of the biogenic amines. J. Pharmacol. Exp. Ther. 189:733–740; 1974.
61. Yap, C. Y.; Taylor, D. A. Involvement of 5-HT$_2$ receptors in the wet-dog shake behaviour induced by 5-hydroxytryptophan in the rat. Neuropharmacology 22:801–804; 1983.