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Perioral Behaviors Induced by Cholinesterase Inhibitors: A Controversial Animal Model[1]

L. A. RODRIGUEZ, D. E. MOSS,[2] E. REYES* AND M. L. CAMARENA

Psychobiochemistry Laboratory, Department of Psychology
University of Texas at El Paso, El Paso, TX 79968
*and *Department of Pharmacology*
University of New Mexico School of Medicine, Albuquerque, NM 87131

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RODRIGUEZ, L. A., D. E. MOSS, E. REYES AND M. L. CAMARENA. *Perioral behaviors induced by cholinesterase inhibitors: A controversial animal model.* PHARMACOL BIOCHEM BEHAV **25**(6) 1217–1221, 1986.—Perioral behaviors induced by neuroleptic drugs have been interpreted as an animal model of tardive dyskinesia. However, these behaviors have also been induced or enhanced by physostigmine, a cholinesterase inhibitor. The latter result is contradictory to the clinical effect of physostigmine in human tardive dyskinesia. In view of this contradiction and other considerations, perioral behaviors have also been interpreted as a model of acute dystonia. The present experiments replicated an earlier failure to observe spontaneous perioral behaviors after long-term neuroleptic treatment in rats as well as the paradoxical effect of physostigmine. The effect of physostigmine was also compared to phenylmethanesulfonyl fluoride and methanesulfonyl fluoride, irreversible CNS active cholinesterase inhibitors. There were significant differences between the effects of the various cholinesterase inhibitors and their interactions with perioral behaviors and neuroleptic treatment. It is concluded that the effects of cholinesterase inhibitors on perioral behaviors in rodents may not be accounted for entirely by cholinesterase inhibition. Further experiments using additional agonists and antagonists will be required to clarify the behavioral effects of these cholinesterase inhibitors.

Tardive dyskinesia Animal model Perioral behaviors Physostigmine Sulfonyl fluorides Cholinesterase inhibitors

TARDIVE dyskinesia is an iatrogenic disorder that develops in some patients treated chronically with drugs that block central dopamine receptors (i.e., neuroleptics). Tardive dyskinesia is characterized primarily by perioral behaviors including involuntary tongue and chewing movements. According to classical theory, these were thought to be induced by dopamine supersensitivity in the extrapyramidal system [10, 11, 13]. These ideas are, however, in the process of refinement [1].

Because of the clinical significance of tardive dyskinesia, there has been considerable interest in establishing animal models of the disorder by long-term dopamine antagonist administration. Abnormal orofacial movements have been observed in primates undergoing chronic neuroleptic treatment and these appear to resemble human tardive dyskinesia [9,12], although an alternative hypothesis is that this is an animal model of acute dystonia [24].

In rodents, long term neuroleptic treatment has been reported to produce perioral behaviors that resemble tardive dyskinesia. For example, Waddington, Cross, Gamble and Bourne [21] observed spontaneous orofacial dyskinesias in rats after 6 months of neuroleptic treatment and suggested that these were similar to the clinical syndrome observed in schizophrenic patients with tardive dyskinesia. However, Rupniak, Jenner and Marsden [17] discovered that treatment with physostigmine, a CNS active cholinesterase inhibitor, produced a surprising increase, rather than the predicted decrease, in the occurrence of orofacial movements in this rodent model.

Because of the strong and well established antagonistic interaction between dopaminergic and cholinergic function in the extrapyramidal system [23], it is not only theoretically possible to suppress tardive dyskinesia with physostigmine, but this effect is also actually observed in human patients [3,5]. Therefore, a major feature of the animal model contradicts the clinical syndrome of tardive dyskinesia. A recent

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[2]Requests for reprints should be addressed to Dr. Donald E. Moss, Laboratory of Psychobiochemistry, Department of Psychology, University of Texas at El Paso, El Paso, TX 79968.

review of oral dyskinesias in rodents after neuroleptic treatment suggests that these behaviors bear a close pharmacological similarity to acute dystonia in primates [18]. It is also possible that neuroleptic-induced perioral behaviors in rodents are a unique species specific response unrelated to clinical syndromes observed in humans.

In a further evaluation of this controversial rodent model of tardive dyskinesia, rats were treated over several months with fluphenazine decanoate similar to the original model developed by Clow, Jenner and Marsden [2] and later used by Waddington *et al.* [21]. The purpose of the experiments was to replicate the effect of physostigmine observed by Rupniak *et al.* [17] and compare the effect of physostigmine with sulfonyl fluorides, other CNS active cholinesterase inhibitors.

METHOD

Subjects

Forty female Sprague-Dawley rats reared in the animal colony at the University of Texas at El Paso from Holtzman stock served as subjects. Females were used because they show more reliable methylphenidate-induced stereotyped gnawing at lower doses (30–40 mg/kg) than do males (60 mg/kg or more) [14]. This difference is important because methylphenidate-induced gnawing was used as a behavioral measure of the dopamine-blocking effect produced by the fluphenazine treatment. The animals were raised in the UTEP animal colony in order to insure that they were never exposed to cholinesterase inhibiting pesticides (e.g., parathion, malathion, etc.) because such exposure would have invalidated the results obtained with physostigmine and other cholinesterase inhibitors. The subjects were maintained on ad lib food and water in a 12 hour light/dark cycle (on at 1800) throughout the experiment. The animals were 7 months of age at the beginning of the experiment (230 to 270 g).

Drug Treatments

The experiment was originally begun with a sample of 24 animals randomly divided into two groups. The experimental group was treated with 0.2 ml IM injections (15 mg/kg) of fluphenazine decanoate (FPZd, 25 mg/ml in sesame oil; Squibb and Sons, Princeton, NJ) once per week for 3 months. Thereafter, the animals received a 3 month drug holiday to check for behavioral effects. After the holiday, the animals received four additional injections, one per month. The control animals received similar injections of oil vehicle according to the same schedule.

In the absence of observable spontaneous orofacial behaviors in the original group, the sample size was increased by sixteen additional subjects. These animals were also randomly divided into drug-treated and control groups. The experimental animals were treated with 0.2 ml IM injections of FPZd according to a schedule of one per week for five weeks, one every other week for four weeks, and finishing with two more injections at one per month. At the end of the drug treatment schedule, the additional subjects were included with the original sample for all further tests.

In order to test the degree to which FPZd reduced CNS dopamine function, the appearance of stereotypic gnawing induced by methylphenidate HCl (MEPH; 30 mg/kg IP; gift from CIBA-Geigy, Summit, NJ) was tested in the entire original sample of 24 rats once during the drug holiday. Twenty days after the test with MEPH, samples of 4 experimental and 4 control rats were further tested for dopamine receptors sensitivity by measuring stereotyped gnawing induced by a challenge with apomorphine HCl (APO; 1.25 mg/kg SC; Sigma Chemical, St. Louis, MO). The APO was prepared in 0.15 M NaCl containing 1 mg/ml ascorbate.

The behavioral effects of cholinesterase inhibitors were measured after IP injections of physostigmine salicylate (PHYSO; 0.2 mg/kg; Sigma Chemical), methanesulfonyl fluoride (MSF; 1.5 mg/kg; Aldrich, Milwaukee), and phenylmethanesulfonyl fluoride (PMSF; 85 mg/kg; Cal-BioChem, San Diego). These doses of these compounds produce between 30 and 60% inhibition of brain cholinesterase [15]. The sulfonyl fluorides were prepared in sesame oil.

Behavioral Tests

Perioral behaviors were generally assessed according to the procedures of Rupniak, Jenner and Marsden [17]. Chewing, tongue protrusions, yawning, tooth grinding were observed in clear plastic observation chambers 23×23 cm square, one rat per chamber. After a 2 min acclimation period, the frequency of occurrence of each behavior was recorded for 5 min.

Stereotypic gnawing induced by the direct (APO) and indirect (MEPH) dopamine agonists as a measure of the functional level of blockade produced by the FPZd treatment was measured by the method of Moss *et al.* [14].

RESULTS

Spontaneous Orofacial Behaviors

No increase in spontaneous orofacial movements that could be attributed to FPZd treatment was ever observed. The original sample of animals was treated with FPZd at weekly intervals which was more frequent than the schedule used by Waddington *et al.* [21]. More complete blockade of CNS dopamine function may have been the reason for the absence of increased spontaneous orofacial movements. In view of the absence of this important behavior, the original sample of 24 subjects was given the 3 month drug holiday and observations were continued. The second sample of subjects was treated with FPZd more in accordance with the procedures of Waddington *et al.* [21] but increased spontaneous orofacial movements were also not observed.

After the completion of all FPZd treatments in both groups, a 3 month observation period was initiated. At this time, the original group had been treated for a total of 7 months (not counting the 3 month drug holiday) while the second group was treated continuously for 4 months. No increased spontaneous orofacial movements were observed.

Psychostimulant-Induced Stereotyped Gnawing

To test the functional level of the FPZd dopamine-blocking effect, the original sample was tested with methylphenidate during the drug holiday. In this experiment, the animals were first injected with 30 mg/kg MEPH six weeks after the beginning of the drug holiday and gnawing was measured for the following 1 hr. As expected, this dose of MEPH was sufficient to induce gnawing in approximately 75% of the control animals with an average count of 851 (SEM 285). At this time, no experimental animals showed gnawing behavior.

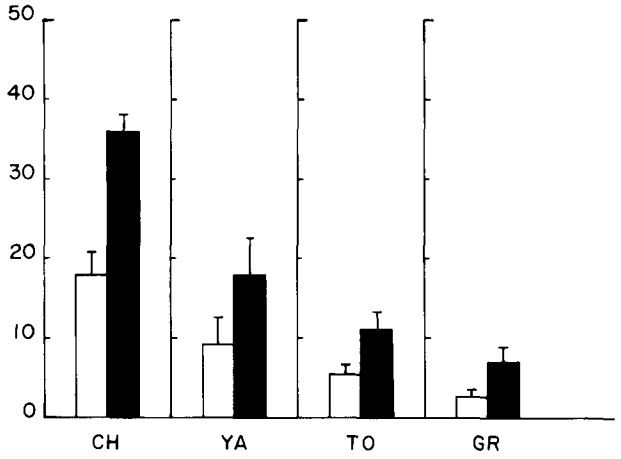

FIG. 1. Physostigmine-induced chewing (CH), yawning (YA), tongue (TO), and tooth grinding (GR) behaviors observed in control (open) and FPZd-treated (filled) rats. The error bars represent one SEM.

The MEPH test for gnawing was again conducted one month later in the drug holiday to determine if dopamine function was returning. The second test was identical to the first and the results were the same. The control animals had a mean gnawing score of 1070 (SEM 364) and no gnawing was observed in the experimental animals. As more direct test of dopamine receptor function, the animals were also tested for stereotyped gnawing after a challenge with APO, a direct receptor agonist. Three weeks after the second MEPH test, a subsample of 4 FPZd and 4 control rats was also treated with 1.25 mg/kg APO and gnawing was again measured. The 4 control rats showed a mean gnawing level of 989 (SEM 465) while no gnawing was observed in the FPZd group.

These results are consistent with the observations of Waddington *et al.* [22] who found prolonged dopamine receptor blockage in rats as measured by APO-induced behaviors after termination of treatment with FPZd. These behavioral results demonstrated that FPZd treatment was producing the predicted long-lasting dopamine-blocking effect.

Behavioral Tests With Cholinesterase Inhibitors

Two months after the termination of FPZd treatment, all subjects were tested for physostigmine-induced orofacial movements. The results obtained from vacuous chewing, tongue thrusting, yawning, and tooth grinding are shown in Fig. 1.

Analysis of variance computed on the data shown in Fig. 1 confirmed that the main effect of FPZd treatment was highly significant, $F(1,37)=18.04$, $p<0.01$. Not surprisingly, the frequency of occurrence of the different behaviors was also highly significant, $F(3,111)=53.74$, $p<0.01$.

At least two weeks after all subjects were tested with PHYSO (above), the subjects were divided into three groups such that the groups were equated for behaviors shown in Fig. 1. The 20 FPZd-treated animals were divided into one group of 7 to receive PMSF, one group of 7 to receive MSF, and one group of 6 to receive a second test with PHYSO. Similarly, the control animals (not FPZd treated) were divided to receive the same cholinesterase inhibitors except that

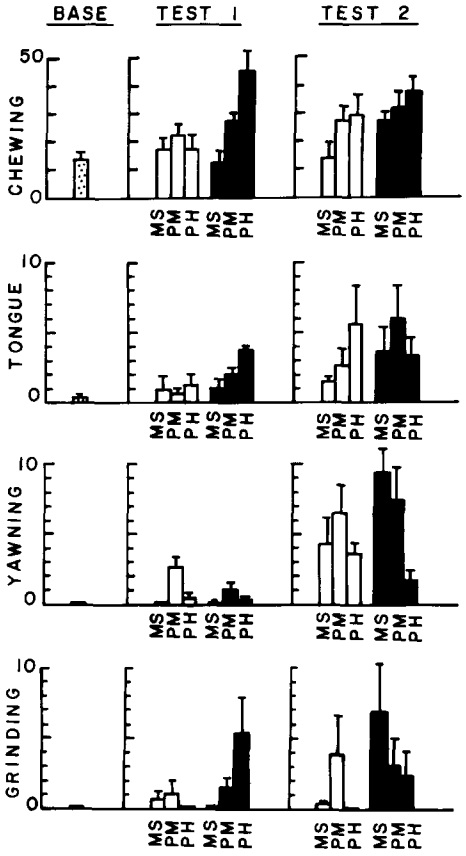

FIG. 2. Cholinesterase inhibitor-induced orofacial movements. FPZd-treated groups are shown with filled bars, control groups with open bars. Cholinesterase inhibitors are methanesulfonyl fluoride (MS), phenylmethane-sulfonyl fluoride (PM), and physostigmine salicylate (PH). The baseline (BASE) frequencies are shown with stippled bars and represent a pooled summary of pretreatment behaviors.

the PHYSO group contained only 5 animals because one of the control subjects died earlier in the experiment.

The animals were first observed 20 minutes after the identified cholinesterase inhibitor was injected IP (Test 1). Twenty four hours later, the animals were again observed before further treatment with a cholinesterase inhibitor. Following the second observation period, all subjects, regardless of initial cholinesterase inhibitor treatment, were treated with PHYSO and observed again 20 min later (Test 2). The results from these tests are shown in Fig. 2.

In contrast to the behavioral effects that might be expected from the sulfonyl fluorides, long-term irreversible cholinesterase inhibitors [15], the behavioral frequencies observed 24 hours after sulfonyl fluoride administration had returned to baseline levels. Therefore, it is clear that in spite of the fact that the cholinesterase inhibiting effect of these compounds remained unabated throughout the 24 hour period, the behavioral effect disappeared.

Multiple analysis of variance comparing the behavioral frequencies observed in Test 1 with those in Test 2 indicated several differences. First, the main effect of FPZd treatment was highly significant, $F(1,33)=10.69$, $p<0.01$. Second, there was also a highly significant difference in the effects of

the three cholinesterase inhibitors tested, $F(2,33)=8.10$, $p<0.01$. As would also be predicted from the initial test with PHYSO conducted earlier, there was a big difference in the overall frequency with which the different behaviors were observed, $F(2,66)=407.62$, $p<<0.01$. Also, the overall frequency with which the behaviors occurred on the second test when all animals were treated with PHYSO was significantly greater than on the first test when the animals were treated with either PHYSO, MSF, or PMSF, $F(1,33)=7.34$, $p<0.025$.

A detailed analysis of the results obtained in Test 1 alone indicated that the effect of FPZd treatment, $F(1,33)=7.47$, $p<0.05$, cholinesterase inhibitor type, $F(2,33)=7.57$, $p<0.01$, and the frequency with which the different behaviors were observed, $F(3,99)=159.07$, $p<0.01$, were significant. In addition, the interactions between FPZd and cholinesterase inhibitor type, $F(2,33)=7.83$, $p<0.01$, FPZd treatment and type of behavior, $F(3,99)=6.33$, $p<0.01$, and cholinesterase inhibitor and type of behavior, $F(6,99)=6.40$, $p<0.01$, were all significant.

Because vacuous chewing occurred with significant frequency in the baseline condition (i.e., without pretreatment with any cholinesterase inhibitor), one additional analysis was computed to compare baseline behavior with that observed in Test 1. This analysis of vacuous chewing confirmed that there was a highly significant difference between the frequency of behaviors observed in baseline and Test 1 conditions, $F(1,33)=18.27$, $p<0.01$. In addition, the effect of FPZd pretreatment was highly significant, $F(1,33)=12.81$, $p<0.01$. The effects of the three cholinesterase inhibitors were also significantly different with regard to vacuous chewing, $F(2,33)=9.69$, $p<0.01$.

DISCUSSION

The purpose was to evaluate perioral behaviors observed after long-term treatment with dopamine antagonists. In sharp contrast to the behavioral results obtained by Waddington, Cross, Gamble and Bourne [21], however, no enhancement of spontaneous perioral behaviors was observed after prolonged treatment with FPZd. The results obtained when all animals were treated with PHYSO alone (Fig. 1) also replicated the results of Rupniak *et al.* [17]. Specifically, treatment with PHYSO increased the frequency of all orofacial behaviors and this effect was enhanced by pretreatment with FPZd.

In addition, in a supplementary experiment conducted to clarify the interpretation of these results, we confirmed that a single injection of 2.5 mg/kg of fluphenazine HCl, a short acting preparation of fluphenazine, in otherwise untreated animals was sufficient to enhance the response to 0.2 mg/kg PHYSO given 8 hr later. This latter result replicates the observations of Yamada and Furukawa [26] who reported an increase in tongue protrusions and chewing produced by cholinergic agonists after only one injection of fluphenazine enanthate.

In view of the above results, perioral behaviors observed after treatment with dopamine antagonists do not appear to be an adequate model of tardive dyskinesia. Specifically, spontaneous perioral behaviors are not reliably observed after prolonged treatment. In addition, the orofacial behaviors are enhanced by cholinergic agonist which contradicts clinical observations in tardive dyskinesia. Lastly, only one treatment with a dopamine antagonist immediately prior to the test with a cholinergic agonist is sufficient to produce the effect. None of these features parallel the clinical profile of tardive dyskinesia.

A second major concern of the present experiments was to compare the effects of three CNS active cholinesterase inhibitors, PHYSO, PMSF, and MSF. The results in Fig. 2 show that treatment with any of the three cholinesterase inhibitors increased the frequency of orofacial behaviors, especially vacuous chewing and tongue protrusions (cf., Fig. 2, Test 1). The effects of the cholinesterase inhibitors were, however, significantly different from one another and there were significant interactions between cholinesterase inhibitors, behaviors, and FPZd treatment. PHYSO, PMSF, and MSF have also been found to produce different effects on extrapyramidal motor behaviors [15].

Even though cholinesterase inhibition produced by PMSF and MSF is irreversible and continues without significant change over periods longer than 24 hr [15,16], no behavioral effects were observed at 24 hr after these drugs. The frequency of all orofacial behaviors had returned to baseline levels. A strikingly similar result has been reported by Fernando, Hoskins and Ho [6] who observed a prominant increase in vacuous chewing, sometimes preceded by yawning, that peaked during the first 2 hr after treatment with diisopropylfluorophosphate (DFP, an irreversible organophosphate inhibitor). However, these behaviors were not different from control levels at 25 hr even though cholinesterase inhibition produced by DFP, like that produced by PMSF and MSF, was virtually unchanged during that time. If the frequency of orofacial behaviors was enhanced because of the direct effect of cholinesterase inhibition, the increase in frequency produced by PMSF, MSF, and DFP would be expected to persist much longer than 24 hr.

One interpretation of this inconsistency is that tolerance to cholinesterase inhibition developed within 24 hr. However, this is not satisfactory insofar as a second treatment with PHYSO (Fig. 2, Test 2) produced a response that was generally greater than the first (Test 1).

Another more compelling interpretation is that PMSF, MSF, and possibly PHYSO, or their metabolites, act as direct ligands on muscarinic, nicotinic, or other neurotransmitter receptors. In this case, these compounds would remain active only as long as significant concentrations of free compound remained in the body fluids. Radioactivity in rat blood after the administration of labelled PMSF, the only compound tested, was present within 15 min, peaked between 1 and 8 hr, and was virtually absent at 24 hr [16]. This time course is consistent with the interpretation that a direct receptor effect might produce the behaviors.

Considering only the inhibition of cholinesterase, there is no logical reason for the significant, but small, differences in perioral behaviors produced by the various inhibitors. The noncholinesterase actions of these compounds that might affect the complex dopaminergic-cholinergic interaction with other neurotransmitters require further study. It has already been demonstrated, for example, that various cholinesterase inhibitors have direct actions of peripheral muscarinic and nicotinic receptor functions [7,8] and CNS choline acetyltransferase activity [19].

If orofacial behaviors produced by cholinesterase inhibitors result from direct receptor or other noncholinesterase effects, then it may be possible to interpret the interactions between various inhibitors and specific behaviors. It has been shown, for example, that vacuous chewing and yawning may be mainly muscarinic in character [4, 6, 20, 25].

Tongue protruding, on the other hand, may involve activation of nicotinic receptors [20]. Therefore, if PHYSO, MSF, and PMSF have different direct receptor ligand effects on muscarinic, nicotinic, or other receptors in the CNS, this could explain the differences in the effects of these inhibitors on orofacial behaviors.

Although understanding orofacial behaviors in rodents will require additional research, a few conclusions are clear. One is that cholinesterase inhibitors in general appear to increase the frequency of orofacial behaviors after treatment with dopamine antagonists. Because of this paradoxical result, orofacial behaviors observed in rats after long-term neuroleptic treatment do not appear to be a suitable animal model of tardive dyskinesia. Other explanations should be studied. For example, Rupniak *et al.* [18] have suggested that these behaviors are pharmacologically more similar to acute dystonia. Secondly, not all cholinesterase inhibitors produce the same quantitative and qualitative effects on behaviors. Therefore, some behavioral results do not appear to be caused directly by cholinesterase inhibition. Further research using direct agonists and antagonists is clearly necessary in order to define the effects of cholinesterase inhibitors on these behaviors.

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