Inhibition of Grooming by Pilocarpine Differs in High- and Low-Yawning Sublines of Sprague–Dawley Rats

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EGUIBAR J. R. AND A. MOYAHO. *Inhibition of grooming by pilocarpine differs in high- and low-yawning sublines of Sprague–Dawley rats.* PHARMACOL BIOCHEM BEHAV **58**(2) 317–322, 1997.—A comparative study of the effect of pilocarpine, a muscarinic receptor agonist, on grooming, scored during 45 min via a time-sampling procedure, was carried out on two sublines of male rats selectively bred for high-(HY) and low-yawning (LY) frequency. In one condition, we introduced rats in a novel cage and observed them immediately after receiving an IP injection of pilocarpine (0.5-3.75 mg/Kg) or an equivalent volume of saline. Besides grooming, the occurrence of yawns was continuously recorded. In the other condition, we immersed rats in water for 60 s, then they received an IP injection of pilocarpine (3.75 mg/Kg) or an equivalent volume of saline and we placed them in an open field, in which we recorded the number of crossed squares. Grooming scores were significantly higher in the condition after water immersion than in the novel situation; in both conditions HY had a grooming response higher than that of LY rats. Pilocarpine produced a dose-dependent inhibition of novelty-induced grooming in HY rats, whereas LY grooming was reduced only with the highest dose. In contrast, yawning increased in a dose-dependent manner with HY rats curve over that of LY animals, except for the highest dose. Pilocarpine inhibited water immersion-induced grooming in both sublines of rats, but it did not reduce grooming as much as it did in the novel condition. Pilocarpine affected distinctly each of the components of grooming, without inhibiting animals locomotor activity. The results indicate that HY rats also have a higher number of grooms than LY rats, and because grooming and yawning can appear after stressful circumstances, HY rats may be used to study the role that both behaviors could have in counteracting the effects of stress. Similarly, HY animals might be utilized to study the underlying neurochemical mechanisms of grooming. This study also indicates that the cholinergic systems exert an inhibitory influence on grooming which contrasts with the excitatory effect on yawning. © 1997 Elsevier Science Inc.

Novelty-induced grooming   Water immersion-induced grooming   Yawning   Muscarinic receptors
Genotypic variations   Rats

GROOMING occurrence in several species of animals, as a result of environmental manipulations, has generated an increasing interest in the study of this behavior, and many authors have suggested that grooming serves a variety of adaptive functions (27). In rodents, grooming can be elicited by either exposure to a novel environment, including handling and transportation of the animal to an observation room (3, 18), or immersion in water (3), which allows the recording of repetitive and natural sequences of grooming for long periods. Grooming and other behaviors, particularly yawning, which is less susceptible to modification by environmental manipulations, are increased by the i.c.v. administration of ACTH, α-MSH and related neuropeptides (10–12) suggesting that

these behaviors might share some of their underlying neurochemical mechanisms.

The fact that the prior administration of naloxone, a nonselective opioid antagonist, and other neurotransmitter substances prevent environmental or pharmacological induced grooming, suggests a complex interaction among several neural systems (3,13). For instance, the muscarinic receptor antagonists atropine and scopolamine can specifically antagonized, in a dose-dependent manner, ACTH-induced grooming, (6). Similarly, a previous injection of atropine into the ventral tegmental area inhibits grooming induced by the i.c.v. administration of α-MSH (29). A prior injection of muscarinic antagonists also suppressed the effect of bombesin-stimulated groom-

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ing (20). Spontaneous and drug-induced yawning have also been related to a cholinergic influence (10,32), especially to an excitatory action by the administration of cholinomimetic substances (34,38).

These findings indicate that grooming and yawning are under the influence of the muscarinic cholinergic system. However, there are no studies that reveal a direct relation between grooming frequency and cholinergic drugs, as there are for yawning. This is because most pharmacological studies have focused on the ability of cholinergic antagonists to reverse grooming induced by neuropeptides (13,27). In addition, most studies have been carried out either on grooming or yawning, but rarely considering the relationship between both behaviors, probably because in contrast to grooming, yawning occurs with a low spontaneous frequency (15) which restricts its analysis to pharmacological manipulations.

In our laboratory we have developed two sublines of Sprague–Dawley rats, selectively bred for high-(HY) and low-yawning (LY) frequency (33), and it has been shown that they differ in their responses to cholinergic and dopaminergic drugs (34) as well as in emotional reactivity and hierarchical composition of grooming elements (21). In this study we tested whether novelty- and water immersion-induced grooming frequency differ between HY and LY rats in a way that it parallels the difference in yawning between both groups of rats. We also examined whether pilocarpine increases grooming as it does with yawning. The results indicate a genotypic variation between HY and LY rats that supports the first hypothesis of the study, but not the latter.

METHOD

Subjects

We used male Sprague–Dawley rats of both sublines HY and LY, from our own colony, aged 2.5–3.5-mo, weighing 280–386 g (HY) and 310–394 g (LY). All subjects were maintained under a controlled light-dark cycle with light between 0700 and 1900 hours. We housed the rats after weaning (30 days) in collective transparent acrylic cages (four to a cage measuring $46 \times 32 \times 20$ cm) with the floor covered with wood shavings. Tap water and rodent food pellets (Purina, México) were freely available. We used animals only once and tested randomly between 0900 and 1300 h.

Procedure

For the novelty-induced grooming test, we divided a transparent acrylic cage ($46 \times 32 \times 20$ cm) in the middle with a Plexiglas partition, with wood shavings on the floor. During 15 min., the animals habituated to the experimental room, then we put them singly in each compartment and observed them immediately after receiving an IP injection of pilocarpine (0.5–3.75 mg/Kg) or an equivalent volume of saline. We scored grooming during 45 min. via a time-sampling procedure (11). In brief, an observer recorded the behavior of each rat at 15 s intervals and we gave a positive score if the rat displayed grooming. We recorded separately the following components of grooming: face washing, body grooming, genital grooming, paw licking, and scratching. For the water immersion-induced grooming test, rats were also habituated to the observation room during 15 min. After this period, we immersed each rat into a swimming tank which consisted of a plastic box (70 $\times$ 40 $\times$ 30 cm) filled with tap water (22°–24°C). After 60 s, we removed the rat and administered an IP injection of pilocarpine or an equivalent volume of saline. A single dose of 3.75

mg/Kg was used as we detected that it was the most effective to reduce novelty-induced grooming. We placed rats into an open field cage, a test commonly used for measuring locomotor activity, to eliminate the possibility that pilocarpine was inhibiting motor activity (36), and we recorded simultaneously their ambulatory and grooming behaviors. The open field was a wooden cage ($60 \times 60 \times 50$ cm) with a glass front and the floor divided into nine squares (20 cm^2 each). We put a wooden right angle (20×50 cm each side) in the left front corner and we introduced each rat into that compartment to ensure a departure from an initial placement square. After 60 s, we removed the angle leaving the rat free to circulate during 45 min. Two observers recorded grooming and distance traveled by each animal (number of crossed squares $\times$ 20 cm) according to the method previously mentioned. After each observation, we thoroughly cleaned the open field cage.

Drug

We dissolved pilocarpine hydrochloride (Sigma, St. Louis, MO, USA) in saline (0.9% NaCl) solution and injected it IP in a volume of 2 ml/Kg body weight. Control animals received the same volume of saline.

Data Analysis

We used Students t-test to make overall comparisons of grooming score. We determined analysis of grooming components and the effect of pilocarpine by analysis of variance (ANOVA) followed either by Duncan's multiple range test or Student's t-test. We analyzed comparisons of duration of grooming episodes using chi-square test. $p < 0.05$ was considered statistically significant.

RESULTS

Novelty-Induced Grooming

The average grooming score for HY rats ($n = 22$) was 23.32 ± 11.23 ($\pm$ SD), whereas for LY ($n = 22$) it was 13.95 ± 7.8; a significant difference between both groups, $t(42) = 3.21$, $p < 0.01$. In relation to individual components, a two-way ANOVA indicated significant differences between sublines (Fig. 1), $F(1, 210) = 15.09$, $p < 0.01$, across grooming component groups, $F(4, 210) = 24.31$, $p < 0.01$, but there was no significant subline $\times$ grooming component groups interaction, $F(4, 210) = 1.25, p > 0.05$. The difference in grooming between both sublines was due to face washing, $t(42) = 2.14$, $p < 0.05$ and scratching, $t(42) = 2.88$, $p < 0.01$ which were higher in HY than in LY rats (Fig. 1). Face washing, body grooming, and genital grooming, which follow that cephalo-caudal progression, decreased quantitatively in that order whereas paw licking and scratching, which usually interrupt grooming sequences, did not.

We carried out a detailed analysis following a method previously reported to establish whether these differences were due to the number of grooming episodes, their duration or both (13). A bout or episode was defined as a 15 s sampling interval(s) in which grooming was scored without a response having been scored in the observation intervals immediately prior to or after the scored response(s). The total amount of grooming episodes defined in this way was higher in HY than in LY rats, $t(22) = 3.207$, $p < 0.01$. An analysis of empirically defined duration of episodes did not show any statistical difference between both sublines of rats, $\chi^2 (4) = 2.12$, $p > 0.05$; thus, the difference depended on the number of times that an
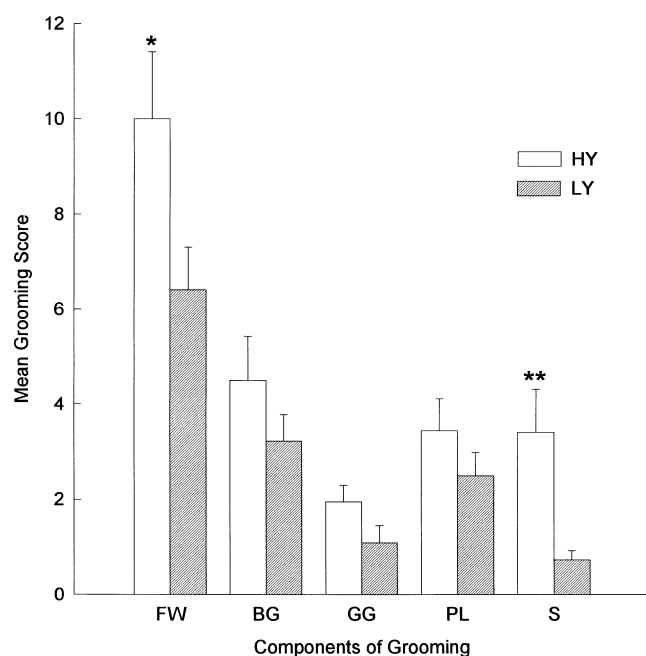
FIG. 1. The influence of a novel environment on distinct components of grooming behavior. FW = face washing, BG = body grooming, GG = genital grooming, PL = paw licking, S = scratching. Values expressed are means ± SE. * $p < 0.05$, ** $p < 0.01$; significance of difference between sublines by applying ANOVA followed by Student's t-test.

FIG. 2. Dose-dependent inhibitory effect of pilocarpine (IP) administration on total grooming score. Values expressed are means ± SE. * $p < 0.05$ compared to respective saline (Sal) groups, applying ANOVA followed by Duncan's test.

episode started however long it was. In both sublines of rats, most episodes (75%) were of a short duration, between 1 and 2–3 consecutive scores, whereas longer bouts were infrequent.

A two-way ANOVA indicated differences in grooming score, after the administration of pilocarpine (Fig. 2), between sublines, 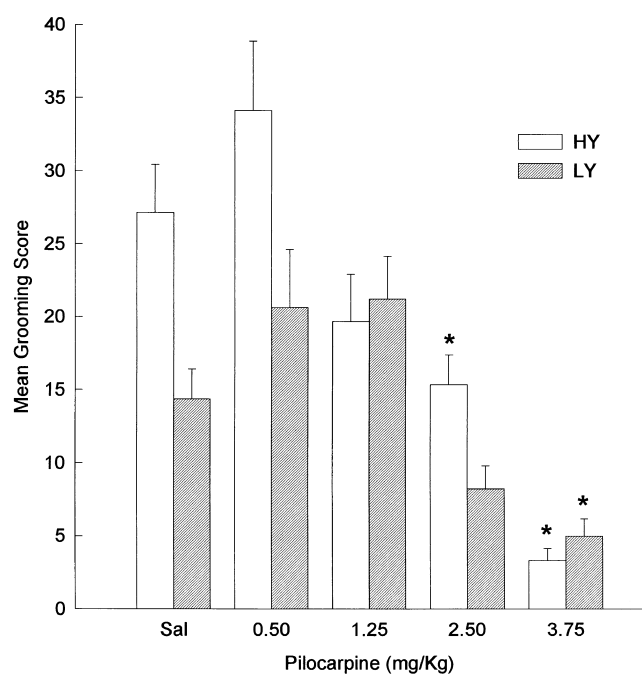$F(1, 79) = 9.04$, $p < 0.005$, across treatments, $F(4,$ 79) $= 16.15$, $p < 0.001$ and also a subline × treatment interaction, $F(4, 79) = 2.72$, $p < 0.05$, suggesting a differential behavioral effect of pilocarpine on each subline. The interaction corresponded to 1.25 and 3.75 mg/Kg doses, and except for these doses, HY mean grooming score was above LY grooming. One-way ANOVAs indicated significant differences among treatments for both HY, $F(4, 38) = 9.89$, $p < 0.001$, and

TABLE 1

SUMMARY OF VARYING PILOCARPINE DOSE ON GROOMING COMPONENTS IN HIGH- (HY) AND LOW-YAWNING (LY) RATS

HY grooming	Pilocarpine (mg/Kg)				
	Sal (14)	0.5 (8)	1.25 (6)	2.5 (9)	3.75 (6)
FW	8.0 ± 3.2	9.7 ± 5.1	11.0 ± 3.7	6.7 ± 4.3	1.7 ± 1.2*
BG	5.0 ± 3.5	6.7 ± 3.2	1.2 ± 1.0*	0.7 ± 0.7	2.0 ± 0.4*
GG	2.1 ± 1.7	5.1 ± 4.1	1.7 ± 3.6	2.0 ± 2.4	0.3 ± 0.8
PL	7.2 ± 5.3	7.7 ± 5.4	3.0 ± 3.2	1.9 ± 1.7	1.0 ± 1.7*
S	4.4 ± 4.6	4.7 ± 4.3	2.8 ± 2.7	4.1 ± 2.1	0.7 ± 0.8

LY grooming	Pilocarpine (mg/Kg)				
	Sal(14)	0.5(8)	1.25(9)	2.5(9)	3.75(6)
FW	6.9 ± 3.7	8.9 ± 3.1	12.7 ± 4.7*	5.2 ± 3.2	4.0 ± 2.3
BG	2.3 ± 1.9	2.7 ± 1.9	2.1 ± 3.4	0.6 ± 0.7	0.2 ± 0.4
GG	0.8 ± 1.0	1.5 ± 1.7	0.9 ± 1.2	0.0 ± 0.0	0.0 ± 0.0
PL	3.3 ± 3.4	4.1 ± 4.2	3.6 ± 2.2	1.9 ± 1.6	0.5 ± 1.2
S	1.1 ± 2.0	3.4 ± 3.9	2.0 ± 2.3	0.6 ± 0.9	0.3 ± 0.5

Number of rats for each dose in parentheses. Values expressed are means ± SE. *$p < 0.05$ compared to saline (Sal) treated animals, applying ANOVA followed by Duncan's test. For abbreviations see legend of Fig. 1.
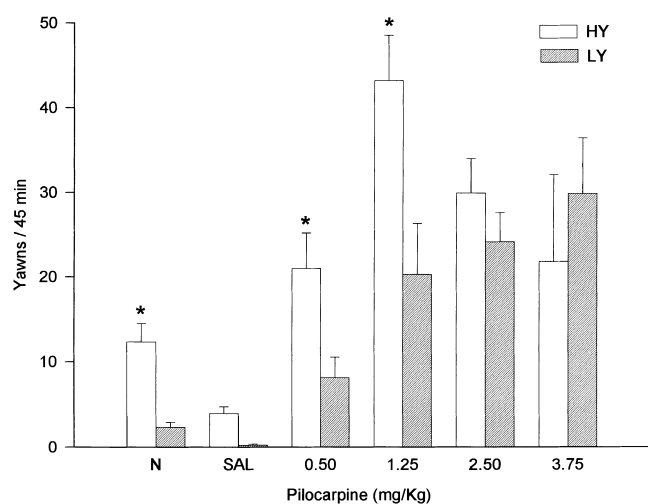
FIG. 3. Dose-dependent effect of pilocarpine (IP) administration on yawning scores on 45 min observation period. N = novel cage. Values expressed are means ± SE. * $p < 0.05$ compared to respective saline (SAL) groups, applying ANOVA followed by Duncan's test.
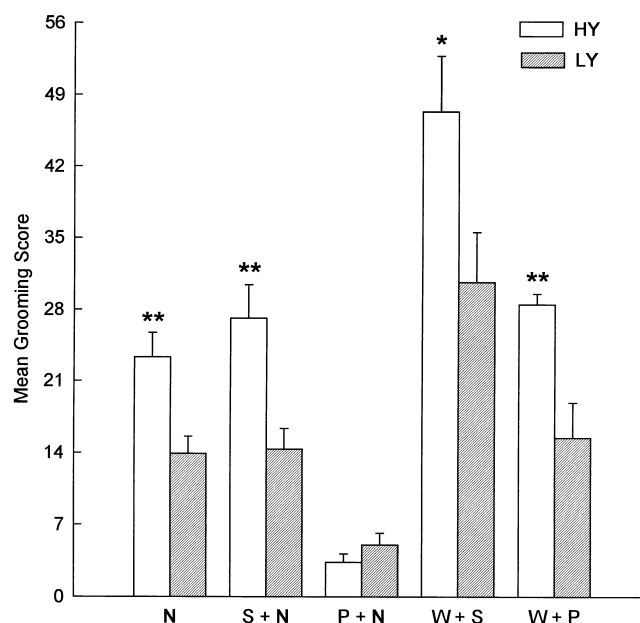

FIG. 4. Summary of the influence of different conditions on total grooming score. N = novel cage, S + N = saline + novel cage, P + N = pilocarpine (3.75 mg/Kg) + novel cage, W + S = wet + saline, W + P = wet + pilocarpine (3.75 mg/Kg). Values expressed are means ± SE. * $p < 0.05$, ** $p < 0.01$; significance of difference between sublines by Student's t-test.

LY rats, $F(4, 41) = 6.34$, $p < 0.001$. Subsequent comparisons, relative to saline-treated animals, revealed that 2.5 and 3.75 mg/Kg doses significantly reduced HY mean grooming score (Duncan's multiple range test, $\alpha = 0.05$). LY mean grooming score was reduced with only a 3.75 dose (see Fig. 2). With the highest drug-dose, several animals showed piloerection and chewing, but not an apparent diminution of motor activity.

To detect which components of grooming were affected by the administration of pilocarpine, we applied individual ANOVAs for each of them. We carried out an adjustment to the acceptance level of statistical significance because grooming components were not independent measures and the same test was applied several times (24). With this correction, Type I error was diminished taking differences significant at $\alpha = 0.05$ level only if the test detected a difference of $p \leqslant 0.01$. Table 1 summarizes the effect of pilocarpine on the different grooming components of HY and LY rats. HY face washing, $F(4, 38) = 5.73$, $p = 0.001$; body grooming, $F(4, 38) = 11.09$, $p = 0.0005$, and paw licking, $F(4, 38) = 4.78$, $p = 0.003$ were significantly affected, but not so genital grooming, $F(4, 38) = 3.22$, $p = 0.02$ and scratching, $F(4, 38) = 1.55$, $p = 0.21$. With regard to LY rats, pilocarpine only affected face washing, $F(4, 41) = 7.65$, $p = 0.0001$, and not body grooming, $F(4, 41) = 2.47$, $p = 0.059$; genital grooming, $F(4, 41) = 3.05$, $p = 0.027$; paw licking, $F(4, 41) = 1.84$, $p = 0.14$, and scratching, $F(4, 41) = 2.44$, $p = 0.06$.

A two-way ANOVA revealed differences in yawning scores between both sublines (Fig. 3), $F(1, 121) = 14.97$, $p < 0.001$, across treatments, $F(5, 121) = 22.93$, $p < 0.001$ and a subline × treatment interaction $F(5, 121) = 4.25$, $p < 0.01$. Note that LY curve is shifted to the right suggesting a different responsiveness to pilocarpine between both sublines of rats.

Water Immersion-Induced Grooming

A two-way ANOVA showed significant differences between sublines, $F(1, 20) = 13.45$, $p < 0.005$, across treatments, $F(1, 20) = 17.29$, $p < 0.001$, but there was no significant subline × treatment interaction $F(1, 20) = 0.18$, $p > 0.1$ (Fig. 4, the last two groups of columns on the right, n = 6 per treat-

ment). *Post hoc* analyses revealed that pilocarpine significantly reduced mean water immersion-induced grooming score of both HY and LY rats, $t(10) = 3.42$, $p < 0.01$ and $t(10) = 2.53$, $p < 0.05$, respectively. HY rats had a higher mean grooming score than LY relative to the wet + saline treatment, $t(10) = 2.29$, $p < 0.05$, and also to the wet + 3.75 mg/Kg pilocarpine-dose group, $t(10) = 3.6$, $p < 0.01$ (see Fig. 4). Although the amount of grooming in the water + saline condition was higher than in the saline + novel situation, $t(18) = 3.29$, $p < 0.005$ and $t(18) = 3.7$, $p < 0.005$ for HY and LY rats respectively, pilocarpine did not reduce water immersion-induced grooming as much as it did in the novel condition. An analysis of the distinct components of grooming, with an adjustment to the acceptance level of statistical significance as previously mentioned, revealed that pilocarpine diminished HY body grooming, $t(10) = 3.15$, $p = 0.01$, but not the other components. In the case of LY grooming, none of the components were significantly reduced, though body grooming was in the border of statistical significance, $p = 0.02$. Finally, pilocarpine did not affect the locomotor activity of HY and LY rats which traveled similar distances in the open field as the saline groups, HY (saline: 35.83 ± 20.11 vs pilocarpine: 22.6 ± 12.94 m), $t(10) = 1.35$, $p > 0.1$ and LY (saline: 20.1 ± 10.97 vs pilocarpine: 11.63 ± 5.79 m), $t(10) = 1.67$, $p > 0.1$. The differences between sublines are due to distinct emotional reactivities (21).

DISCUSSION

The present results show that HY rats groom more than LY when they are exposed to a novel environment or after immersion in water. The latter produces a higher amount of grooming than the novel testing condition, which is in accordance with other experiments (3). The difference in grooming

between HY and LY rats, which parallels that of spontaneous yawning frequency shown by both sublines, indicates a positive correlation between yawning and grooming and suggests that the selection for yawning frequency also affected grooming. Previous results (21) showed that HY and LY rats also differ in open field activity and the hierarchical organization of grooming elements, which supports the findings of this report and demonstrates that the differences between both strains of rats go beyond yawning frequency. Whether these behavioral differences are a direct or secondary consequence of the inbred selection is still not clear, although it is consistent with other inbred selection studies that have revealed that genetic influences are ubiquitous for animal behavior (22). It is possible that yawning and grooming have a motivational or functional relationship, other than that concerning their pharmacological induction (9). It has previously been suggested that these behaviors are after responses to stressful stimuli or circumstances (4,17,35). If this were the case, it would follow that HY rats are more sensitive to stressful circumstances, a conclusion partially supported by preliminary results (9), and to the pituitary-adrenal system, which is activated as an animal's reaction to exposure to novel stimuli (1). Indeed, it has been reported that the central release of ACTH can be involved in the increased grooming observed in a novel environment (5), and probably in that after immersion in water. The finding that HY animals maintain a high level of grooming in a novel condition and after immersion in water, supports the idea that both kinds of grooming response might be similar with respect to the neurochemical mechanisms of their generation. Stressors such as fur moistening may differ from exposure to a novel condition (35) and from immersion in water, which not only disturbs the fur of the animal but also makes it too dry. Therefore, drying after water immersion seems to overcome grooming caused by solely moistening the fur. It is interesting that the difference in novelty-induced grooming between HY and LY rats is restricted to face washing and scratching, which have been considered as components of two sub-branches in the grooming system (25), suggesting that HY and LY rats differ in both of them.

Although pilocarpine diminished novelty- and water immersion-induced grooming of both strains of rats, with a higher sensitivity to this drug shown by HY rats, the inhibition was more marked in the novel than in the water immersion condition. This indicates that the inhibitory influence of this drug depends on the manipulation to which the animals are previously subjected. In addition, pilocarpine seems to inhibit grooming by disrupting mainly body washing, which is a transitional element between rostro and caudal components and also a crucial element in HY grooming structure (21). Conversely, pilocarpine appears not to affect individual LY grooming components, but all of them to a similar degree. This is probably because LY grooming structure as a whole is more resistant to modification even after immersion in water, in which we observed greater number of grooms, this leaves out any possibility of statistical bias due to the occurrence of few events. The pharmacological effect of pilocarpine was due to a direct influence on grooming response rather than to a generalized inhibition of locomotor activity, as reflected by the finding that activity in open field was not affected by this drug suggesting a specific effect on grooming. The fact that pilocarpine at doses of 3.75 mg/Kg induced chewing response, which has been proposed as a reliable index of central muscarinic agonist activity in rats (26), indicates that it affected

mostly central cholinergic activity. In addition, the doses of pilocarpine used in this study are within those (0.5–10 mg/Kg) frequently found in other reports on yawning (2,14,31,34,38) and are below those currently used to produce massive effects.

The genotypic variation in grooming and yawning between HY and LY rats may be the result of differences in the expression of cholinergic neurotransmission and its interaction with other systems, as several neurotransmitters have been evidenced in other comparative studies in inbred strains of rodents (7). Results from our laboratory raise the possibility that HY may have a higher cholinergic tone than LY rats (34). However, the differences in grooming and yawning between HY and LY rats cannot be dependent only on the effect of pilocarpine, because in contrast with the inhibitory effect on grooming, that on yawning is a dose-dependent increase in both sublines of animals. The cholinergic system affects these behaviors in opposite directions and probably along distinct pathways. In fact, the cholinergic neurons involved in yawning are thought to be influenced by dopaminergic neurons (38). The results presented here are not conclusive on the type of muscarinic receptors, which pilocarpine is mostly affecting, and it is likely that other neurotransmitter systems are involved in the production of grooming.

There is evidence that ACTH, in vitro, inhibits the quinuclidinylbenzilate (muscarinic antagonist) receptor-binding (30). This might account for the increase of the acetylcholine turnover rate after central administration of ACTH (37). This is consistent with the finding that inhibition of muscarinic receptor binding in the brain may lead to an increased release of acetylcholine (16,28). Therefore, we expected pilocarpine to increase grooming, which contrasts with the results reported here. On the basis of these experimental findings, it appears that the effects of pilocarpine and ACTH on grooming differ, a hypothesis that has been suggested for yawning too (38). The situation is even more intricate than it may appear since the sole i.c.v. administration of pirenzepine or AFDX-116, selective antagonists of M1 and M2 muscarinic receptors respectively, does not modify grooming behavior in rats (23), but decreases yawning (14). Furthermore, the administration of scopolamine in hamsters receiving artificial cerebrospinal fluid did not change grooming (19). However, some authors have reported that not only muscarinic but also nicotinic receptors are involved in ACTH-induced grooming (23). We are currently attempting to test whether the differences in grooming between HY and LY rats also involve peptidergic influences. Initial results indicate that HY and LY rats differ in their grooming response to the administration of ACTH (8).

In conclusion, these results have revealed evidence for a significant genotypic variation in grooming response between HY and LY rats as well as differences in sensitivity to the behavioral effects of pilocarpine. This difference parallels that of yawning which makes HY rats to be an excellent tool for studying neurochemical mechanisms involved in the generation of both behaviors, and their implications in stressful circumstances.

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