Sildenafil and a Compound Stimulating Endothelial NO Synthase Modify Sexual Incentive Motivation and Copulatory Behavior in Male Wistar and Fisher 344 Rats

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ABSTRACT

Introduction. Earlier studies have shown that sildenafil may modify some aspects of male rat sexual behavior and sexual incentive motivation. Stimulation of endothelial nitric oxide synthase (eNOS) has also been reported to affect sexual motivation in old rats.

Aim. To determine the effects of sildenafil and a compound stimulating eNOS on copulatory behavior and sexual incentive motivation in young adult Fisher 344 and Wistar male rats.

Methods. The rats were selected for a low intromission ratio, and then treated with Impaza (stimulator of eNOS), sildenafil, or Impaza + sildenafil for 28 days. Tests for copulatory behavior and sexual incentive motivation were performed before the beginning of treatment and at days 7, 14, and 28 of treatment.

Main Outcome Measures. Standard parameters of copulatory behavior and sexual incentive motivation. Measurements of penis length at mount, intromission, and ejaculation.

Results. The Fisher 344 rats displayed a higher level of sexual incentive motivation than the Wistar rats, while the copulatory behavior was similar in both strains. Impaza and sildenafil enhanced the sexual incentive motivation after 28 days of treatment in the Wistar rats, but failed to do so in the Fisher 344 rats. The copulatory behavior was unaffected in the Wistar strain, while the Fisher 344 males had an enhanced intromission ratio after treatment with Impaza and sildenafil for 28 days.

Conclusions. The nitric oxide-guanylyl cyclase pathway seems to be of importance for sexual incentive motivation in animals with a modest baseline level. The different drug effects in the Wistar and Fisher 344 rats can be attributed to baseline differences. The importance of eNOS for sexual functions should not be overlooked. **Chu X, Zhavbert ES, Dugina JL, Kheyfets IA, Sergeeva SA, Epstein OI, and Ågmo A. Sildenafil and a compound stimulating endothelial NO synthase modify sexual incentive motivation and copulatory behavior in male Wistar and Fisher 344 rats. J Sex Med 2008;5:2085–2099.**

Key Words. Sexual Incentive Motivation; Sexual Behavior; Endothelial NO Synthase; Sildenafil; Erection

Introduction

A common consequence of the clinical use of proerectile drugs is that the frequency of penile-vaginal intercourse is increased in the men taking such drugs (e.g., [1]). An equally common explanation for this fact is that the enhanced intercourse frequency is a result of improved erection, making this kind of sexual activity possible. It could also be maintained that proerectile drugs enhance sexual motivation, which would facilitate erection as well as the desire to use it for intercourse. However, a majority of clinical data suggest that proerectile drugs do not enhance sexual motivation. This assertion applies both to the most commonly used treatments for erectile deficiency, the phosphodiesterase type 5 (PDE5) inhibitors, as well as to the centrally acting agent apomorphine (e.g., [1,2]). To the contrary, results from experimental studies in rodents suggest that

sildenafil may enhance motivation [3–5]. Further evidence for a stimulatory action of sildenafil on male sexual motivation has been obtained in the goats [6]. Animal data with apomorphine are conflicting, but there is some evidence showing that the compound enhances sexual motivation in animals displaying an unusually low sexual activity (reviewed in [7]). One likely explanation for the discrepancy between animal and human data is that the clinical studies have focused on evaluating improvements in erectile function. In fact, the evaluation of potential actions on sexual desire (the term frequently used for sexual motivation in the human literature) has not been a major goal of any clinical study. Thus, motivational actions of proerectile drugs in humans cannot be excluded. Such actions could, in fact, contribute to the therapeutic actions of these drugs, because erectile deficiency frequently is associated with reduced motivation to engage in sexual activity [8,9].

We recently reported that sildenafil and a compound stimulating endothelial nitric oxide synthase (eNOS), Impaza, showed a tendency to stimulate the sexual incentive motivation in old Fisher 344 rats displaying a very low level of copulatory behavior [10]. Despite the incentive motivational effect of the drug treatments, the 20-month-old rats continued to display little copulatory behavior. The reason for employing the Fisher 344 strain, rarely used in studies of sexual behavior, was that it is one of the strains where old rats are commercially available.

Aims

In the present experiments, we evaluated the effects of sildenafil and Impaza on sexual incentive motivation and on copulatory behavior in young adult Fisher 344 rats. In order to determine the drug effects in a strain more commonly used, we also administered the drugs to Wistar rats. Furthermore, some clinical data suggest that a combination therapy with sildenafil and Impaza is more efficient than any of these compounds administered separately [11]. Thus, a group of animals receiving both drugs was included in the present experiments. Prior to drug treatment, animals with a low erectile capacity were selected from a larger pool.

Materials and Methods

Animals

Male (about 300 g upon arrival) Wistar and Fisher 344 rats were purchased from Scanbur, Sollentuna, Sweden. They were housed in pairs in Macrolon cages under a reversed light/dark cycle (12:12 hours, lights on 2,300) in a room with controlled temperature (21 ± 1°C) and relative humidity (55 ± 10%). Rodent pellets (RM1(E), Special Diets Services, Witham, Essex, UK) and tap water were freely available.

Female Wistar rats (about 250 g upon arrival) were obtained from the same provider and were kept under conditions identical to those of the males. They were ovariectomized under isoflurane anesthesia at least 2 weeks before the beginning of experiments. Prior to each testing session, estrus was induced by administration of estradiol benzoate, 25 μg/rat, followed by progesterone, 1 mg/rat, 48 hours later. Females were used between 4 and 8 hours after the progesterone injection. The steroids were from Sigma (St. Louis, MO, USA). They were dissolved in peanut oil and were injected subcutaneously in a volume of 0.2 mL/rat.

The experimental procedures employed were approved by the Norwegian Committee for Ethics in Research on Animals, and were in agreement with the European Union Council directive 86/609/EEC.

Apparatus

Test for Sexual Incentive Motivation

The arena used for determining the intensity of sexual incentive motivation consists of a rectangular open field (100 × 50 cm) with rounded corners. On each long side, there is an opening (25 × 25 cm), at floor level, in the 45-cm-high arena wall. The openings are diagonally opposed. Outside each opening, a cage (15 × 25 × 25-cm high) can be fitted. These outside cages are equipped with a double wire mesh wall (mesh size, 12 mm; the distance between the meshes was 10 mm) facing the arena. The arena wall, as well as the external cages, is made of sheet-steel covered with a black plastic surface. A dark gray polyvinylchloride was used for the floor. The apparatus was located in a room adjacent to the animals' room. A video camera, connected to a computer, was installed above the arena. The experimental subject's position in the arena was determined online every 200 msec with a videotrack system (Ethovision Pro, Noldus, Wageningen, the Netherlands). An incandescent light bulb provided a dim white light (about 5 lux in the arena). Detailed descriptions of the apparatus can be found in [12,13].

In front of each external cage, a virtual zone of 30 × 21 cm was defined. The videotrack system calculated the time the experimental animal spent

in each of these virtual zones and the number of visits to them. In addition, the distance moved, the mean velocity of movement while moving, and the time spent moving were determined.

Mating Test Cages

Black sheet-steel cages ($40 \times 60 \times 40$-cm high) with Plexiglas front and glass floor were positioned over a mirror inclined at 45 degrees. This allowed for a simultaneous side and ventral view of the copulating male. Tests were recorded on videotape with a two-camera system connected to a video cassette recorder (VCR) via a multiplexer. These cages were identical to those employed in an earlier experiment [10]. The tests for copulatory behavior were made in a different room under a dim white light (about 25 lux).

Procedure

Screening for Sexual Behavior and Selection of the Experimental Subjects

About 3 weeks after arrival to the laboratory, screening tests for copulatory behavior were initiated. At these tests, the male was placed in the mating cage, and a sexually receptive female was introduced 5 minutes later. The male was allowed to copulate with the female until the first postejaculatory intromission. However, the test was terminated before that event if the male failed to perform an intromission within 15 minutes of the introduction of the female, or if more than 30 minutes elapsed between the first intromission and ejaculation, or if the interval between ejaculation and the following intromission exceeded 15 minutes.

During the test, the mount latency (time from the introduction of the female until the first mount), the intromission latency (time from the introduction of the female until the first mount with vaginal penetration, intromission), the ejaculation latency (time from the first intromission until ejaculation), the postejaculatory interval (time from the ejaculation until the following intromission), as well as the number of mounts and intromissions, were recorded with the help of an in-house software. The software also calculated the intromission ratio (number of intromissions/[number of mounts + number of intromissions]) and the interintromission interval (ejaculation latency/number of intromissions).

The length of the part of the erect penis protruding from the prepuce during mount and/or following withdrawal after intromission or ejaculation was estimated from the video record. The video image was projected on a screen with a liquid crystal display (LCD) projector. Image size, measured diagonally, was 150 cm. From the beginning of a mount with or without intromission/ejaculation until withdrawal, the video was advanced frame by frame. The frame where the erection was maximal was always chosen for measurement of the protruding penis. Measurement was not possible at every copulatory event because of an unsatisfactory view. Nevertheless, in most sexually active animals, at least five erections at mount and another five at intromission were measurable. In case that the subject displayed more than five mounts and five intromissions at a test, only the first five of each were measured. The erection observed at ejaculation was measured whenever possible.

Three screening tests separated by 48–72 hours were performed. Intertest intervals of this length have been reported to assure a stable level of sexual activity (see [14,15]), something that was desirable for the selection of experimental subjects. One hundred Wistar and 100 Fisher 344 males were screened in this way. After screening, all animals that had failed to display sexual activity at all three tests were eliminated. The mean intromission ratio for the three screening test was determined for each of the remaining animals, and the 50 Wistar males and the 50 Fisher 344 males with the lowest ratios were selected for inclusion in the experiment. The reason for making this selection was that we wanted to include only males with an erectile capacity below average. Clinical use of proerectile drugs is limited to men with low (deficient) erectile capacity, and by selecting animals with suboptimal erectile performance, we obtained a sample sharing an important characteristic with the clinical population. It may be noted that the intromission ratio is an exquisitely sensitive measure of erectile function (see [14] and references therein). The animals selected for the drug treatments had an intromission ratio of 0.16 ± 0.01 (mean $\pm$ standard error of the mean [SEM]), while the discarded animals had a ratio of 0.24 ± 0.04. The difference is significant ($t[198] = 2.06$, $P < 0.05$), suggesting that the experimental subjects indeed had a reduced erectile capacity.

Familiarization to the Sexual Incentive Motivation Test

The selected males were familiarized to the sexual incentive motivation test arena at three sessions of 10 minutes each, separated by 48–72 hours. Familiarization was initiated 2 days after the last screening test for copulatory behavior. Before every

session, the arena was carefully washed with a 0.1% solution of glacial acetic acid in water. No washing was performed between the animals within a session. During familiarization, one external cage contained an intact male rat, and the other external cage contained a sexually receptive female. The presence of the incentive animals already during familiarization assures a more stable future performance than familiarization without the incentive animals with regard to indices of ambulatory activity (unpublished results). Because of the double wire mesh, no physical contact was possible between the animal in the external cages and the experimental subject in the arena. Nevertheless, the animals could see, hear, and smell each other. The position of the cages containing the male and the female was semirandomly changed within every session, so that about half the animals had the male on one side of the arena, and the other half on the opposite side. This means that any spontaneous position preferences were balanced out. Experimental tests were identical to the familiarization, i.e., the test duration was 10 minutes and the incentives were a sexually receptive female and an intact male.

Drugs

Sildenafil citrate was obtained as commercial tablets (Viagra, Pfizer, New York, NY, USA) containing 25 mg. The tablets were crushed in a mortar and then dissolved in physiological saline. The reason for using commercial tablets rather than the pure compound was that we wanted to make the drug treatment as similar to the clinical use of sildenafil as possible. Antibodies to C-terminal fragment of eNOS (mixture of homeopathic dilutions C12, C30, and C200; Impaza, OOO NPF Materia Medica Holding, Moscow, Russia) were provided as a ready-to-use solution in distilled water. The actual concentration of the antibodies is not known, but the solution used here is identical to the one employed in clinical practice.

Design

The following five groups of 10 rats from each strain were employed: (i) distilled water, 3 mL/kg/day during 28 days; (ii) and (iii) Impaza, 3 and 9 mL/kg/day during 28 days, respectively; (iv) Impaza, 3 mL/kg/day for 28 days + sildenafil 3 mg/kg twice weekly during the treatment period. One of the weekly drug administrations was always on a test day. On days when sildenafil was not given, 3 mL/kg of distilled water was administered instead; (v) sildenafil, 3 mg/kg, twice weekly during the 28-day treatment period. On days when sildenafil was not given, distilled water was administered. The reason for administering sildenafil twice weekly rather than every day was that men of the age typical of users rarely perform intercourse more than twice weekly [16,17]. Again, we wanted to make the drug treatment as similar to the clinical use as possible. At difference to sildenafil, the clinical use of Impaza consists of daily administration for long periods. The doses of the compounds coincide with those employed in earlier studies of male rat sexual behavior [3,5,10].

All treatments were given orally by gavage. On test days, the treatments were given about 1 hour before testing. On other days, they were administered in the morning, before the end of the light phase of the light/dark cycle.

Experimental tests were performed before the beginning of the drug treatment (baseline) and on days 7, 14, and 28 of treatment. First, the test for sexual incentive motivation was performed. Immediately thereafter, the subjects were transported to the room where observations of copulatory behavior were performed. The copulation test was started right away with a 5-minute wait for the female. The test was ended at the first postejaculatory intromission, or if one of the criteria employed in the screening tests was satisfied. Although preexposure to a sexually receptive female has been reported to facilitate copulatory behavior [18], the effect is of small magnitude. Furthermore, a repeated preexposure to a male has similar effects [19], suggesting that any stimulus predicting access to a mate may facilitate copulation. This proposal is in agreement with data showing that cues present during copulation will enhance future copulatory behavior [20,21]. Thus, the simple exposure to the mating cage and/or the testing room environment may facilitate copulatory behavior as much as preexposure to a female does. In fact, we have not been able to detect any difference in copulatory behavior between the animals tested immediately after the test for sexual incentive motivation and the animals tested 24 hours after that test (unpublished results).

Data Preparation and Statistics

Sexual motivation was evaluated in two ways. The most important for determining changes in the sexual incentive value of the receptive female are the *preference score* (time spent in the female incentive zone/[time spent in the female incentive zone + time spent in the male incentive zone]) and

the *time spent in the female incentive zone*. There needs to be a statistically significant change on both parameters if an effect on sexual motivation is to be considered. A double criterion is needed in order to avoid false positive effects. An increased preference score may be a result of either increased time in the female zone or reduced time in the male zone, or a combination of both. However, reduced time in the male zone without a concomitant increase in time in the female zone does not necessarily indicate an enhanced sexual incentive motivation. At the same time, an increase in the time spent in the female zone could be a consequence of increased attractivity of any incentive animal and is therefore not a sufficient indicator of an increased sexual incentive motivation. Similar arguments could be made for reduced sexual incentive motivation. The use of both criteria (change in preference score *and* a corresponding change in the time spent in the female zone) avoids the pitfalls of them when used singly.

With regard to penis length, the mean of the five measurements performed at mount and intromission, respectively, was calculated for each rat and test. These means were then used for all data analyses. In case that measurement had been obtained from less than five occasions in a rat at a particular session, the mean of the available observations was used.

Comparisons Between Wistar and Fisher 344 Rats

These were based on the data obtained at the baseline test, before the drug treatments had been initiated. The preference score, indices of ambulatory activity, parameters of copulatory behavior, and penis length were compared with the *t*-test for independent groups. The time spent in the vicinity of the male and the sexually receptive female, as well as the number of visits to them, were analyzed with a two-factor mixed analysis of variance (ANOVA), with incentive animal (male–female) as within-groups factor, and strain as between-groups factor. In case of significant interaction, tests for simple main effects of strain within each incentive and of incentive within each strain were performed. The proportion of subjects displaying mount, intromission, or ejaculation was evaluated with the Fisher exact probability test.

Treatment Effects on Sexual Incentive Motivation

The change from baseline was analyzed rather than the raw data. The value obtained at baseline was simply subtracted from the value obtained at later tests. There were two reasons for this. First, the Fisher 344 and Wistar rats had a different level of sexual motivation and, to a minor degree, copulatory behavior at the baseline test (see the Results section). This precludes any comparison of the magnitude of treatment effects between the two strains based on the raw data. Second, this procedure allows for a sensitive analysis of treatment-induced behavioral changes, corrected for any differences at baseline. It is commonly used in pharmacological and behavioral studies.

The analyses of treatment effects were made separately for the Wistar and Fisher 344 rats. Otherwise, the analyses would have become exceedingly complex and difficult to interpret. The preference score was analyzed with a two-factor ANOVA with repeated measures on one factor, the between-groups factor being the treatment, and the within-groups factor being the test (day of treatment). The time spent in the incentive zones was evaluated by a three-factor ANOVA with repeated measures on two factors, the within-group factors being the incentive (male, female), and the test and the between-groups factor being the treatment. Indices of ambulatory activity at all tests were analyzed as the preference score, while the number of visits to the incentives were analyzed like the time spent in the incentive zones. In case of significant interactions, analyses of simple main effects comparing treatments at a particular day as well as tests within a particular treatment were performed. If these tests established significant effects, the significance of the deviation from baseline in each group was evaluated by a *t*-test.

Treatment Effects on Copulatory Behavior and Penis Length

As was the case with the sexual incentive motivation data, separate analyses were made for the Fisher 344 and Wistar rats. The proportion of subjects displaying mount, intromission, or ejaculation at each test was evaluated with the χ^2 test. The change from baseline in the number of mounts and intromissions was evaluated with mixed two-factor ANOVA, with treatment being the between-groups factor, and test (day of treatment) the within-groups factor. The other measures of sexual behavior could not be obtained from every animal at every test. Clearly, the latencies cannot be recorded if the behavior does not occur, and the intromission ratio cannot be calculated in animals displaying neither mount nor intromission. Likewise, the penis length cannot be measured in the absence of copulatory behavior. Repeated measures analyses of these data would then exclude animals that did

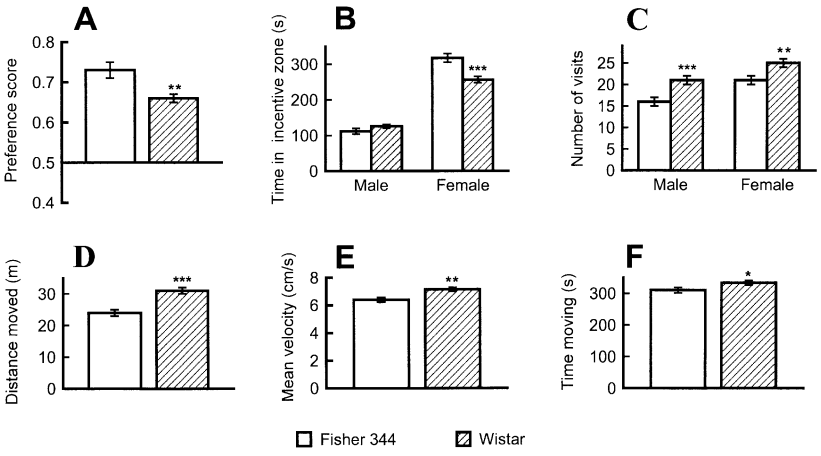

Figure 1 Mean ± SEM preference score (A), time spent in the vicinity of the sexually receptive female or the male incentive (B), number of visits to the incentives (C), distance moved during the 10-minute test (D), mean velocity while moving (E), and time spent moving (F) in male Fisher 344 and Wistar rats at the baseline test. N = 50 for each strain. *Different from Fisher 344, $P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

not display all elements of copulatory behavior at all tests. For a similar reason, the raw data rather than the change from baseline were used in these analyses. It may be observed that neither strains nor treatment groups differed with regard to the latencies or the intromission ratio (see Results) at the baseline test. A separate analysis was performed for each test day, comparing treatments with one-factor ANOVAs. In case of a significant effect, posteriori analyses were performed with Tukey's honest significant difference (HSD) test.

Main Outcome Measures

Intensity of sexual incentive motivation and copulatory behavior as well as penis length during copulation.

Result

Comparison of Fisher 344 and Wistar Rats at the Baseline Test

The Fisher 344 strain had a higher preference score than the Wistar strain ($t[98] = 2.89$, $P < 0.01$). Analysis of the time spent in the vicinity of the incentive animals revealed a significant effect of strain ($F_{1,98} = 14.43$, $P < 0.001$) and of incentive ($F_{1,98} = 217.82$, $P < 0.001$). The interaction strain × incentive was also significant ($F_{1,98} = 10.77$, $P = 0.001$). Tests for simple main effect of incentive within strains showed that there was no difference with regard to the male incentive ($F_{1,98} = 2.13$, nonsignificant [*NS*]) while the strains differed in the time spent in the vicinity of the sexually receptive female ($F_{1,98} = 15.37$, $P < 0.001$). The Fisher 344 strain spent more time in the vicinity of that incentive than the Wistar rats did. This observation suggests a more intense sexual incentive motivation in the Fisher 344 rats. Both strains spent more time close to the female than to the male (Fisher 344, $F_{1,98} = 162.72$, $P < 0.001$; Wistar, $F_{1,98} = 65.86$, $P < 0.001$). There was also a strain difference concerning the number of visits to the incentives ($F_{1,98} = 24.59$, $P < 0.001$). The male and females incentives differed ($F_{1,98} = 34.59$, $P < 0.001$), while there was no interaction strain × incentive ($F_{1,98} = 0.34$, *NS*). The Wistar strain made more visits to both incentives than the Fisher 344 rats. The Wistar also moved a larger distance ($t[98] = 5.81$, $P < 0.001$), moved faster while moving ($t[98] = 3.55$, $P < 0.001$), and spent more time moving ($t[98] = 2.11$, $P < 0.05$) than the Fisher 344 rats. Thus, the larger number of visits to the incentives performed by the Wistar rats seems to be a consequence of a more intense ambulatory activity. The data are illustrated in Figure 1A–F.

While there were marked differences in sexual incentive motivation and ambulatory activity between the Fisher 344 and Wistar males, there were only minor differences in copulatory behavior (see Table 1). In fact, the only significant difference between the strains was found in the number of mounts, where the Wistar rats made more than the Fisher 344 ($t[98] = 3.41$, $P < 0.01$). The larger number of mounts displayed by the Wistar rats, without any accompanying difference in the number of intromissions, means that the intromission ratio should be lower in the Wistar rats. However, the difference was only of borderline significance ($t[87] = 1.98$, $P = 0.051$).

There was a small but significant difference between the Fisher 344 and Wistar rats with regard to penis length at intromission ($t[66] = 2.01$, $P < 0.05$) but not at mount ($t[86] = 0.95$, *NS*) or ejaculation ($t[37] = 1.53$, *NS*) at the baseline test.

Table 1 Comparison of copulatory behavior and protruding penis length between Fisher 344 and Wistar males at the baseline test. All 50 animals from each strain are included

Behavior parameter	Fisher 344	Wistar
Mount latency	152 ± 28	121 ± 26
Intromission latency	133 ± 29	131 ± 25
Ejaculation latency	448 ± 35	500 ± 52
Postejaculatory interval	351 ± 17	318 ± 21
Number of mounts	13 ± 2	32 ± 5*
Number of intromissions	7 ± 1	8 ± 1
Intromission ratio	0.37 ± 0.04	0.26 ± 0.03
Interintromission interval	45 ± 4	42 ± 6
Penis length at mount	3.19 ± 0.10	3.04 ± 0.11
Penis length at intromission	4.03 ± 0.11†	3.68 ± 0.13*†
Penis length at ejaculation	4.97 ± 0.16†‡	4.54 ± 0.24†

*Different from Fisher 344, $P < 0.01$.
†Different from penis length at mount within the same strain, $P < 0.05$.
‡Different from penis length at intromission within the same strain, $P < 0.05$.
Data are mean ± SEM. The latencies as well as the postejaculatory and interintromission intervals are expressed in s. Penis length is expressed in arbitrary units (cm on the projection screen).

When the penis length observed at mounts was compared with that observed at intromission or ejaculation, it was found to be shorter, both in the Fisher 344 (mount-intromission, $t[32] = 5.98$, $P < 0.001$; mount-ejaculation, $t[24] = 8.79$, $P < 0.001$) and Wistar (mount-intromission, $t[33] = 6.01$, $P < 0.001$; mount-ejaculation, $t[12] = 3.07$, $P < 0.01$) rats. In the Fisher 344 rats, the penis length recorded at ejaculation was superior to that recorded at intromission ($t[25] = 4.58$, $P < 0.001$). This was not the case in the Wistar rats ($t[12] = 1.79$, *NS*). The data are found in Table 1.

Effects of Drug Treatment in the Fisher 344 Males

There was no treatment effect on the preference score at day 28 ($F_{4,45} = 0.25$, *NS*). Likewise, there was no difference between the tests ($F_{2,90} = 1.14$, *NS*) and no interaction treatment × test ($F_{8,90} = 0.77$, *NS*). Analyses of the time spent in the male and female incentive zones showed that there was no effect of treatment ($F_{4,45} = 0.97$, *NS*) or of test ($F_{2,90} = 0.49$, *NS*), and no interaction treatment × incentive ($F_{4,45} = 0.35$, *NS*). To the contrary, there was a significant interaction between test and treatment ($F_{8,90} = 2.22$, $P < 0.05$). Further analysis of this interaction with tests for simple main effect of test within treatments revealed that the group treated with Impaza, 3 mL/kg + sildenafil showed a reduction in the time spent in the incentive zones as the treatment progressed ($F_{2,90} = 6.06$, $P < 0.01$), while the tests did not differ in the other groups (*P*s > 0.21). However, the deviation from baseline was nonsignificant for both the male and female incentives. The data are illustrated in Figure 2A,B.

In order to keep the results reasonably short, only the data obtained at the test on day 28 of the treatment are illustrated. This is the time when drug effects should be clearly established according to clinical experience with Impaza [11]. It is also the time when sildenafil have maximal effects in diabetic men suffering from erectile deficiencies [22]. Other effects of sildenafil, including the activation of eNOS, may also require a long-term treatment (see e.g., [23–25]). Thus, maximal drug effects should not be expected until day 28. Consequently, no important information is lost by not illustrating data from the tests performed on days 7 and 14. Nevertheless, it can be mentioned that no significant drug effect was obtained at the test performed on day 7 of the treatment, and few effects were significant at day 14.

Analysis of the number of visits to the incentives showed that there was no treatment effect, no difference between tests, and no interaction (all *P*s > 0.11). These data are not illustrated.

All three indices of general activity showed that there was a reduction over tests. This applies to the distance moved ($F_{2,90} = 14.75$, $P < 0.001$), the velocity of movement ($F_{2,90} = 20.90$, $P < 0.001$), and the time spent moving ($F_{2,90} = 25.59$,

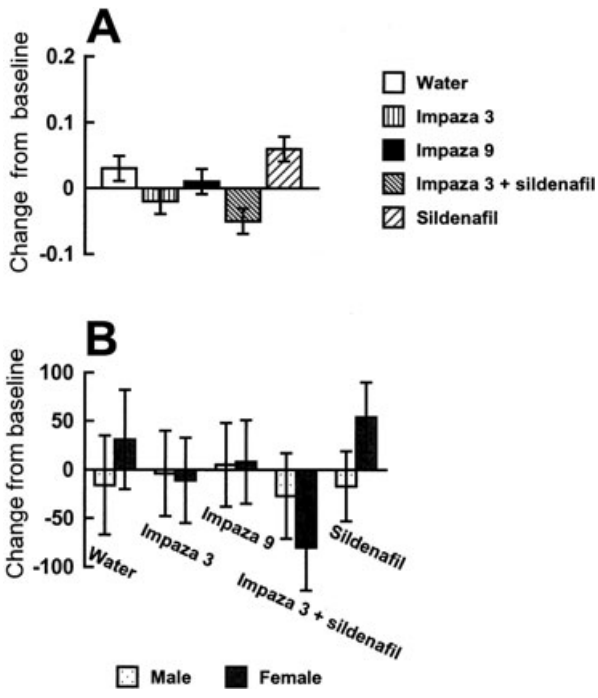

Figure 2 Mean ± SEM change from baseline in the preference score (A) and in the time spent in the vicinity of the female and male incentive (B) at the test performed on day 28 of the treatment in Fisher 344 rats. N = 10 per treatment. For details of drug treatment, see Methods.

Table 2 Copulatory behavior and protruding penis length in Fisher 344 males at the test performed on day 28 of treatment

	Treatment				
Behavior parameter	Water	Impaza 3	Impaza 9	Impaza 3 + sildenafil	Sildenafil
Number of mounts	15 ± 5	10 ± 4	3 ± 1	9 ± 7	5 ± 3
Number of intromissions	5 ± 1	8 ± 2	4 ± 2	2 ± 1	4 ± 1
Intromission ratio	0.25 ± 0.07	0.49 ± 0.06	0.66 ± 0.05*	0.46 ± 0.13	0.61 ± 0.13*

*Different from water, $P < 0.05$.
Data are mean ± SEM. N = 10 per treatment.

$P < 0.001$). There was no treatment effect, and no interaction test × treatment (Ps > 0.36). The reduction in activity was moderate. The distance moved, for example, diminished by 26% between baseline and the test at day 28 in the group given water, and by 35% in the group given Impaza + sildenafil. The reduction in the other groups fell between these values. The diminished activity could not have been produced by the the drug treatments because there was no significant treatment effect at any test. It may be a result of repeated testing.

χ^2 tests revealed that the treatment groups did not differ with regard to the proportion of animals displaying at least one mount, intromission, or ejaculation at any test. There was a significant treatment effect neither on the latencies to mount, intromit, or ejaculate, nor on the postejaculatory and interintromission intervals. The penis length was also unaffected (all Ps > 0.1; data not shown). To the contrary, on the test performed at day 28 of the treatment, there was an effect on the intromission ratio ($F_{4,22} = 3.46$, $P < 0.05$). A post hoc test revealed that the groups treated with Impaza, 9 mL/kg, and sildenafil, 3 mg/kg, had a larger intromission ratio than the control group. The number of mounts and intromissions was not significantly modified, though. The data are illustrated in Table 2.

In conclusion, none of the treatments affected sexual incentive motivation at any test in the Fisher 344 rats. Copulatory behavior was also essentially unaffected, with the exception of the intromission ratio that was enhanced by 28 days of treatment with Impaza, 9 mL/kg, and sildenafil, 3 mg/kg.

Effects of Drug Treatments in the Wistar Rats

The ANOVA of the preference score showed that there was a significant effect of treatment ($F_{4,45} = 2.78$, $P < 0.05$) and of test ($F_{2,90} = 9.38$, $P < 0.001$). The interaction test × treatment was also significant ($F_{8,90} = 2.55$, $P < 0.05$). This prompted tests for simple effects of treatment for each test day. It turned out that there was a significant group difference only at the test performed on day 28 ($F_{4,45} = 3.78$, $P < 0.01$). The animals treated with Impaza, 3 mL/kg, had a higher preference score on day 28 than at baseline ($t[9] = 3.45$, $P < 0.01$). This was also the case for the animals given with sildenafil ($t[9] = 4.27$, $P < 0.01$). None of the other groups differed from baseline (Ps > 0.30). The data are illustrated in Figure 3A. The group treated with sildenafil also had a higher preference score on day 14 than at baseline ($t[9] = 3.52$, $P < 0.01$). To the contrary, Impaza, 3 mL/kg, did not have any effect on day 14 ($t[9] = 0.33$, *NS*). No other treatment effect was obtained on day 14, and none at all on day 7.

The analysis of the time spent in the incentive zones found no main effect of treatment

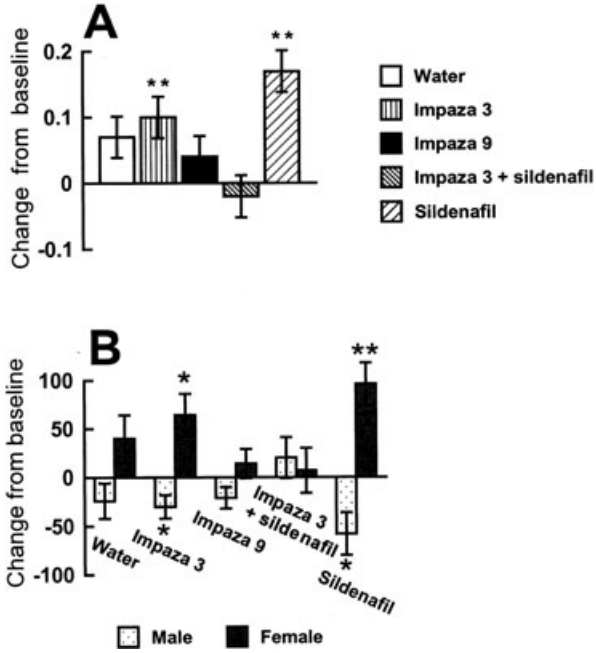

Figure 3 Mean ± SEM change from baseline in the preference score (A) and in the time spent in the vicinity of the female and male incentive (B) at the test performed on day 28 of the treatment in Wistar rats. N = 10 per treatment. *Different from baseline, $P < 0.05$; **$P < 0.01$. For details of drug treatment, see Methods.

($F_{4,45} = 1.38$, *NS*). At difference, both the test × incentive and treatment × incentive interactions were significant ($F_{2,90} = 3.38$, $P < 0.01$, and $F_{4,45} = 3.16$, $P < 0.05$, respectively). When the change from baseline at day 28 were analyzed, it was found that the group treated with Impaza, 3 mL/kg, and the group given with sildenafil had increased the time spent in the vicinity of the female incentive ($t[9] = 3.07$ and 4.47, respectively, $P < 0.05$ and 0.01), while that spent in the vicinity of the male incentive was reduced ($t[9] = 2.78$ and 2.77, respectively, both Ps < 0.05). There were no significant changes in the other groups (Ps > 0.30). On day 14, sildenafil had an effect similar to that obtained on day 28 (female, $t[9] = 3.24$, $P = 0.01$; male, $t[9] = 2.61$, $P < 0.05$), while no effects were found in the other groups (ts < 1.2). The data from day 28 are shown in Figure 2B.

The treatments differed with regard to the number of visits to the incentive animals ($F_{4,45} = 3.72$, $P < 0.05$). The Tukey test revealed that no group differed from control, but the animals treated with sildenafil displayed a lower number of visits than the animals given with Impaza, 3 mL/kg. There was also an effect of test ($F_{2,90} = 3.58$, $P < 0.05$). The number of visits was lower at the test on day 28 than on day 7. There was no interaction effect, and the incentives did not differ with regard to the change from baseline in the number of visits (all Ps > 0.1).

General activity declined during the experiment. There was a significant effect of test on the distance moved ($F_{2,90} = 11.94$, $P < 0.001$) and the velocity of movement ($F_{2,90} = 11.91$, $P < 0.001$) as well as on the time spent moving ($F_{2,90} = 7.59$, $P = 0.001$). The treatment effect and the interaction treatment × test were nonsignificant (Ps > 0.13). Thus, the diminished activity was independent of treatment. With regard to the distance moved, the reduction ranged from 14% from baseline to the test on day 28 in the group given with water, to 17% in the group given with Impaza 3 mL/kg + sildenafil. As was the case for the Fisher 344 rats, this reduction may be a consequence of a repeated exposure to a constant environment.

There was no significant change in the proportion of animals displaying mount, intromission, or ejaculation during the period of the drug treatment (Ps > 0.36). Likewise, the number of mounts was similar in all treatment groups, and there was no difference between tests and no interaction treatment × test (all Ps > 0.59). To the contrary, the number of intromissions changed between tests ($F_{2,90} = 19.32$, $P < 0.001$), but there was no treatment effect ($F_{4,45} = 0.39$, *NS*) and no significant interaction treatment × test ($F_{8,19} = 0.50$, *NS*). Regardless of treatment, the number of intromissions was reduced at the test performed on day 7 of the treatment (change from baseline was different from 0, $t[49] = 2.62$, $P < 0.05$), while the tests performed on days 14 and 28 did not differ from baseline ($t[49] = 1.56$ and 1.62, respectively, both *NS*). There is no evident explanation for the diminished number of intromissions on day 7, but because it is similar in all groups, it must be unrelated to the drug treatments.

There was no effect on the latencies, the postejaculatory interval, the intromission ratio, the interintromission interval, or the penis length at mount, intromission, and ejaculation (all Ps > 0.13). In sum, none of the drug treatments affected the copulatory behavior in the Wistar rats (data not shown).

Discussion

The present data show that the Fisher 344 rats display a higher level of sexual incentive motivation than the Wistar rats. Both parameters indicating intensity of motivation, the preference score and the time spent in the vicinity of the sexually receptive female, coincided in suggesting this. At difference, the Wistar rats had a higher level of general activity. This is in agreement with earlier reports of lower activity in Fisher 344 than in Wistar rats in open-field tests [26,27]. Interestingly, the strains are not different in home cage activity [27]. Inhibited open-field activity is often interpreted as a reaction to fear, and the lower activity in the Fisher 344 rats coincides with reports of increased fearfulness in this strain [28–30]. It may be interesting to note that this fearfulness does not impede the Fisher 344 strain from displaying a higher level of sexual incentive motivation. Despite the strain difference in incentive motivation, the copulatory behavior was essentially similar in the Fisher 344 and the Wistar rats. This is another example of the fact that sexual approach behaviors and the execution of copulatory reflexes are determined by partly independent mechanisms (see [31,32]).

It may be important to remember that the animals used in the present studies had been selected for a low intromission ratio, and it could be argued that this selection may have produced biased samples. This, however, is most unlikely. First, the selection criterion was identical for both

strains, and the initial number of animals was the same. Second, the intromission ratio is determined by the number of mounts and intromissions, and these numbers appear to vary independently of other parameters of copulatory behavior according to factor analytic studies [33,34]. Thus, there is no reason to suppose that the selection procedure would affect one strain more than another, and it is doubtful whether the selection for intromission ratio would affect variables related to sexual incentive motivation.

An important question is whether the observed strain difference is of relevance for the selection of a strain to be used in pharmacological studies of sexual functions. Insofar as the study has the purpose of experimentally analyzing drugs used in clinical practice, it could be useful to employ a strain as similar as possible to the clinical population in relevant characteristics. The users of proerectile drugs can be expected to have a low sexual motivation (see Introduction). Consequently, a strain with high sexual motivation would not be ideal. Thus, it seems that the purpose of an experiment designed to evaluate potential effects of proerectile drugs on sexual behaviors would best be fulfilled by the employment of Wistar rather than Fisher 344 rats. Interestingly, sildenafil has larger effects on sexual behavior in "sluggish" rats, i.e., animals with unusually low sexual activity, than in animals with a "normal" activity [3,4]. This is also the case for other kinds of drugs (see [7] for additional examples).

The present data reinforce the conclusion presented in the preceding paragraph. None of the treatments affected sexual incentive motivation in the highly motivated Fisher 344 rats, while stimulatory actions were detected in the modestly motivated Wistar rats. Interestingly, 28 days of treatment with sildenafil and Impaza, 3 mL/kg, produced an intensity of sexual motivation in the Wistar rats similar to the baseline value in the Fisher 344 animals. The mean ± SEM preference score at day 28 in the Wistar rats treated with sildenafil was 0.80 ± 0.02, while the corresponding value for Impaza, 3 mL/kg, was 0.75 ± 0.02. The baseline preference score in the Fisher 344 rats was 0.73 ± 0.02. A t-test shows that the difference between the Wistar rats treated with sildenafil for 28 days and the Fisher 344 rats at baseline was not significant ($t[58] = 1.53$, *NS*). Clearly, this was also the case for the animals treated with Impaza, 3 mL/kg ($t[58] = 0.39$, *NS*). These data allow for the speculation that the drug treatments fail to affect sexual incentive motivation when it is already high, but enhance it when initially modest. This proposal also coincides with earlier data showing that both sildenafil and Impaza had a stimulatory action in old Fisher rats, with an initially very low level of sexual incentive motivation [10]. This observation suggests that strain difference is an unlikely explanation for the fact that the Wistar, but not the Fisher 344 rats, responded with an enhanced motivation in the present experiment. It rather reinforces the proposal made previously that baseline level of motivation is the crucial factor.

An interesting observation is that only the low dose of Impaza, 3 mL/kg, stimulated sexual incentive motivation in the Wistar males. Moreover, while sildenafil alone was efficient, its effects were lost when combined with Impaza, 3 mL/kg. These observations indicate that there is an optimal level of stimulation of the nitric oxide (NO)–cyclic guanosine monophosphate (cGMP) pathway, and stimulation above that level renders it inefficient. Dose-effect curves having the shape of an inverted U are not rare in behavioral pharmacology, and a common explanation is that doses above the optimal level activate incompatible behaviors interfering with the expression of the target behavior. In the present case, we have to conclude that we do not know anything about any potential incompatible behavior. However, there is a possible biochemical explanation for the lack of effect on sexual motivation of the combined treatment with Impaza and sildenafil.

A few studies [23,35] have shown that long-term treatment with sildenafil durably enhances erection in response to cavernous nerve stimulation in aged, but not in the young Fisher 344 rats. The effect involved Akt-dependent phosphorylation of eNOS (Ser-1177) in the penis. The lack of erectile ability enhancement in young rats was explained as a possible decrease in NO signaling by PDE5 and Rho-pathway upregulation. In addition, the high basal levels of phosphorylated Akt and eNOS typical of young rats might not be further increased by the treatment. Supposing that these observations also apply to eNOS outside of the penis, they may explain the fact that the combination of sildenafil and Impaza resulted in a loss of effect. Impaza (ultra-low doses of antibodies to C-terminal regulatory region comprising Ser-1177) may arrest Akt-dependent phosphorylation of Ser-1177 by sildenafil. However, this proposal is weakened by the fact that clinical trials have revealed that addition of Impaza to PDE5 inhibitor monotherapy

in patients with erectile dysfunction increased the overall efficacy of the treatment [36].

Impaza, 3 mL/kg, and sildenafil not only increased the time spent in the vicinity of the sexually receptive female in the Wistar rats, but they also reduced the time spent in the vicinity of the male incentive. The superior preference score reflects a combination of these two effects. Identical effects were found in the old Fisher 344 rats [10], showing that these drug effects are reliable. We have earlier reported that treatments modifying sexual incentive motivation, like castration or immediately preceding intense sexual activity, fail to affect the time spent in the vicinity of the male incentive [12,13]. This fact was interpreted as indicating that approach to the male is essentially determined by social motivation, which is known to vary independently of sexual motivation [37]. Consequently, the reduced time spent in the vicinity of the male incentive after Impaza, 3 mL/kg, and sildenafil suggests that social motivation had been reduced by the drugs. One possible explanation is that Impaza and sildenafil increased fearfulness, thereby reducing approach to the male incentive. Sildenafil has been found to have anxiogenic properties [38,39], while the effects of Impaza on anxiety are unknown. Nevertheless, any anxiogenic action of the compounds should have been more evident in the already fearful Fisher 344 rats than in the Wistar strain. Indeed, anxiogenic compounds are known to reduce social approach in Fisher 344 rats [40]. It seems improbable, then, that the effect on approach to the male incentive obtained in the present experiment is a result of enhanced anxiety (fear). A viable, alternative explanation is not available at present.

With regard to sexual behavior, the drugs employed here enhanced the intromission ratio in the Fisher 344 rats after 28 days of treatment. The slow onset of effect coincides with clinical data with regard to Impaza [11]. Sildenafil may also require a long-term treatment for full effect in patients with comorbidity ([22–24], see also [41,42]), although the drug generally is effective after acute treatment. Both compounds failed to have any significant effect in the Wistar strain. Lack of effect of sildenafil on ex copula penile erection in Wistar rats has been reported previously [43]. The strain difference cannot be explained as a consequence of a baseline difference, because both strains had a similar intromission ratio at the baseline test. It seems more likely that differences in the responses to the drugs can account for the lack of effect in the Wistar strain. Before addressing that issue, it is necessary to present some notions concerning the possible mechanisms of action of Impaza and sildenafil on erection. Their site of action can be supposed to be the penis [44,45]. Penile erection is initiated by the release of NO from afferent nerves. Neuronal nitric oxide synthase (nNOS) is crucial for this process. Subsequently, NO activates soluble guanylyl cyclase in the penile smooth muscle cells, and the production of 3,5-cyclic guanosine monophosphate (cGMP) is enhanced. The action of cGMP is terminated by PDE5. However, full erection, as well as erection maintenance, requires additional NO supplied by eNOS [46,47]. Indeed, mice lacking nNOS show normal erection. Administration of a NOS inhibitor produces severe erection deficiency, prompting the conclusion that eNOS is crucial [48]. This conclusion might have been premature, though, because eNOS-knockout mice do not show deficient erection [49]. It rather seems that NO is essential for erection regardless of its origin. The drugs employed in the present experiments affect different parts of the NO–guanylyl cyclase pathway. While sildenafil blocks PDE5, thereby prolonging the action of cGMP, Impaza has been reported to stimulate the activity of eNOS [11,36].

Some recent data have shown that a cytochrome P450 metabolite of arachidonic acid, 11,12-epoxyeicosatrienoic (EET) acid, produces potent relaxation of the corpus cavernosum mediated by the NO–cGMP pathway and adenosine triphosphate (ATP)-sensitive K^+ sensitive channels [50]. Injection of an EET antagonist into the rat penis produced diminished cavernosal pressure increases in response to electrical stimulation of the major pelvic ganglion [51]. Moreover, P450 may itself have NOS activity [52]. In physiological conditions, NO produced by P450, eNOS, or nNOS might interact with EET to produce erection. Considering that Fisher 344 have a larger P450 activity than the other strains [53,54], it is possible that they are more sensitive to the actions of sildenafil and Impaza, and therefore show enhanced erection after these treatments, while the Wistar rats, with lower P450 activity, fail to do so. Unfortunately, this explanation for the different effects of the drugs on intromission ratio in the Wistar and Fisher 344 rats is entirely speculative.

The actions of the drugs on sexual incentive motivation are most likely of central origin. NO-dependent guanylyl cyclase and PDE5, as well as nNOS and eNOS, are present in the brain [55–57]. Although eNOS, as its name suggests, is

mainly located in blood vessels, it has also been described in neurons [58–60]. Furthermore, NO derived from vascular eNOS has been found to diffuse to adjacent neurons where it acts in a cGMP-dependent way [61]. Behavioral and neurochemical data have established a functional role for central eNOS. For example, long-term potentiation is dependent on eNOS as well as on nNOS [58,62]. Studies in mice lacking the gene for eNOS have shown that they display a low level of aggression [63] as well as altered sexual behavior [49]. The present results make it possible to add sexual incentive motivation to the list of behaviors modified by eNOS.

Stimulation of the NO–guanylyl cyclase pathway is most likely one of the main actions of NO derived from both eNOS and nNOS in the central nervous system. No wonder, then, that sildenafil and other PDE5 inhibitors have been found to affect the actions of antidepressant drugs [64,65], modify anxiety responses [38,39], and improve performance in some learning tasks [66,67] as well as stimulating aspects of sexual behavior (see Introduction). PDE5 inhibition also enhances sexual incentive motivation in animals where it is low, according to present and earlier data [10].

Conclusions

In addition to its well-established role in erection, the NO–guanylyl cyclase pathway has been implicated in several other sexual functions, including female hypoactive sexual desire and sexual arousal disorders, as well as in premature ejaculation [68]. Data obtained in the present experiments show that it may also be a determinant of a nonhuman animal's response to sexually relevant stimuli. One of the most evident effects of sildenafil in men is to enhance erection in response to such stimuli, be they in the form of a partner or of a video sequence (e.g., [69,70]). In rats, the main effect seemed to be to enhance the response of approach to a sexually relevant stimulus. The failure to detect a consistent effect on erection, reflected in the intromission ratio as well as penis length, may be due to a lack of sensitivity of these measures. Perhaps that female-induced enhancement of spontaneous erection would be a more sensitive model [71]. Likewise, the absence of convincing data demonstrating motivational effects of sildenafil and other agents affecting the NO–guanylyl cyclase pathway in the human may be a consequence of the lack of studies adequately designed for evaluating such effects. It is our hope that such studies will soon be undertaken.

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