In search of animal models for male sexual dysfunctions

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In search of animal models for male sexual dysfunctions

Op zoek naar diermodellen voor mannelijke seksuele disfuncties

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 22 november 2010 des middags te 12.45 uur

door

Johnny Song Wing Chan

geboren op 29 juli 1975
te New York, Verenigde Staten

Promotoren:	**Prof. dr. B. Olivier** **Prof. dr. M.D. Waldinger**
Co-promotor:	**Dr. R. S. Oosting**

Hundreds and thousands of times, for her I searched in chaos; suddenly, I turned by chance, to where the lights were waning, and there she stood.

Chinese poem of the Song Dynasty

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Chapter 1: General Introduction

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The Diagnostic and statistical manual of mental disorders (DSM-IV-TR [1]) characterizes sexual dysfunctions as a disturbance in the sexual response cycle. The sexual response cycle is divided into four phases:

1. The Desire phase consists of fantasies about and desires to have sexual activity
2. The Excitement phase consists of the senses of sexual pleasures and in tow, the bodily responses. For males, this pertains to erection.
3. The Orgasm phase consists of the climax of sexual pleasure. For males, this leads to the expulsion of semen.
4. The Resolution phase consists of muscle relaxation and feeling of well-being. During this time, males have a refractory period to further erections and orgasms.

One or more of these phases may be affected in sexual dysfunctions and should be "persistent or recurrent" and cause distress in order to be clinically relevant. Sexual dysfunctions could be further classified as spontaneous (i.e. lifelong or from the onset of sexual activities) or acquired types (i.e. drug-induced). The main complaints and prevalence (in a sample population) for males are orgasmic problems (10%), premature ejaculation (27%) and erectile difficulties (10%)[1].

There is a need for a reliable animal model since clinical experiments in humans can be long-lasting or unethical. In the search for animal models of male sexual dysfunctions, the male rat is an ideal testing medium with a large literature reference base for sexual behaviors [2] and pharmacological data. Men and rats find sex rewarding [3, 4]. Based on such and other findings, it can be postulated that sexual behavior in rats shows great face validity to the human situation. When we began testing male rats for sexual behaviors, differences in individual male rats were revealed. Repeated testing of these rats exposed the stability of sexual behaviors and allowed us to categorized rats in groups of low, normal and high sexual activities. These low sexually active rats may model low desire, anorgasmia, or delayed ejaculation in humans while the high sexually active rats models

premature ejaculation. The average sexually active rat represent a group that can be tested for stimulating and inhibiting effects of various situations (i.e behavioral or pharmacological). The repeated inhibitory effects of chronic administration of SSRIs on sexual behaviors of male rats provide face and predictive validity to the use of those rats to model human sexual responses. By theorizing (and then proving) the effects of various drugs on sexual response, based on its neurochemical activity and brain mechanisms involved, construct validity is also presented.

Aim and outline of the thesis

The aim of this thesis is to describe an animal paradigm that predicts sexual side effects of psychoactive compounds. The focus lies on antidepressant drugs, the underlying mechanisms, in particular the serotonin transporter, and putative pro-sexual drugs.

The thesis is divided into two main parts. Part one deals with the description of the paradigm and the existence of sexual endophenotypes in a population of male Wistar rats. A thorough description of the methods used in our experiments is presented in Chapter 2. This method deals with the induction of sexual dysfunctions by SSRIs. In Chapter 3, a review is provided about slow, normal and fast sexual endophenotypes and the translation into human sexual behaviors. In addition, some pharmacological and genomic results are presented.

Part two of this thesis deals with the detection of sexual side effects of novel and current drugs and the mechanisms of these side effects are discussed. In this part, we discuss the inhibitory and stimulatory side effect profiles of these drugs. In Chapter 4, we compared the effect of various current antidepressants in our animal paradigm. Novel and current drugs acting on the serotonin, noradrenalin, and/or dopamine transporters, and 5-HT_{1A} and 5-HT_{2C} receptors were investigated.
From the data presented in chapter 4 it was clear that inhibition of the serotonin transporter (SERT) leads to sexual side effects. To investigate the function of SERT in

sexual behavior in further detail, we performed experiments in the SERT heterozygous (HET) and homozygous rat (SERTKO) in Chapter 5. The HET rat expresses ± 50% of SERT as compared to wildtypes and as such this animal may model the short/short polymorphism in the SERT promoter as found in the human population. The SERTKO mimics chronic SSRI administration. Upon discovering differences between WT, HET and KO animals, some pharmacological studies were performed to investigate the involvement of the 5-HT_{1A} receptor in sexual behavior. In chapter 6, we investigated antidepressant-like effects and sexual side-effects of a triple monoamine uptake inhibitor, a putative new antidepressant without sexual side effects.. As animal model for depression, we used the OBX rat. Finally in Chapter 7, Clavulanic acid, a beta-lactamase inhibitor, which is normally combined with penicillin to make an antibiotic, is tested for its effects on sexual behavior in male rats. There were preliminary findings suggesting that clavulanic acid had some prosexual activity and we were able to confirm that in our rat model.

In the final summarizing chapter we discuss some implications of the rat model for detecting putative effects of psychoactive drugs on sexual behavior and the role of the 5-HT_{1A} receptor in SSRI –induced or SERT-KO rat models of sexual dysfunction.

Chapter 2: Drug-induced Sexual Dysfunction in Rats

Current Protocols in Neuroscience 9.34.1-9.34.11, October 2010

Abstract: This unit describes the testing of sexual behaviors of male Wistar rats. The described test enables the detection of stimulatory and inhibitory profiles of compounds. The test includes four training sessions to reach a stable sexual performance followed by acute and/or chronic administration of drugs. The main quantifiable sexual behaviors are number of mounts (no vaginal penetration), intromissions (vaginal penetration), and ejaculations. By comparing the test compound to reference compound(s), sexual (side) effects can be determined.

INTRODUCTION

Sexual disturbances and their treatment are extensively studied both in humans and in animal models. A large number of psychotropic drugs exert unwanted sexual side effects [5, 6]. In particular, the antidepressant class of selective serotonin reuptake inhibitors (SSRIs) are notorious for their sexual side effects. SSRI users complain about libido, erection and orgasm problems [7, 8]. Although depression itself is associated with these same problems, it is highly desirable to develop drugs without these cumbersome sexual side effects.

The commercial success of phosphodiesterase type 5 inhibitors for the treatment of erectile dysfunction [9] clearly indicates a high need for drugs to treat non-drug related sexual dysfunctions.

Animal models of sexual behavior have predictive value for the sexual (side) effects of drugs in humans [10]. As in humans, chronic administration of antidepressants, particularly SSRIs, lowers the sexual activity of male rats [11-14].

Over the years we have tested more than 1500 male Wistar rats and we have consistently found that around 10-20% of the animals are sluggish copulators (low number of ejaculations), 60-80% are normal (2–3 ejaculations per test) and again 10-20% are rapid (4–5 ejaculations/test; . In our experiments we try to exploit these endophenotypes. For example, when testing for sexually stimulating drugs, we use sluggish or normal rats, and exclude the fast ejaculators. When the aim is to detect sexual inhibitory or side-effects we exclude the sluggish males.

NOTE: The use of live animals in all protocols requires the approval by an Institutional Animal Care and Use Committee (IACUC) or must meet the governmental regulations installed for the care and use of laboratory animals.

STRATEGIC PLANNING

Animals

The use of female rats as stimulus animals requires some preparation beforehand. Female rats need to be sexually primed in order for male sexual behaviors to be measured reliably. Female rats can either be bilaterally ovariectomized or left sexually intact. Ovariectomized female rats have to be primed with 5-10 μg estradiol benzoate and 500 μg progesterone, 36-42 hrs and 4 hrs, respectively, before testing with a male rat. Intact female rats can be given a single injection of 50 μg of estradiol benzoate 36-42 hrs before testing with a male rat. In addition, the high dose of estradiol prevents pregnancies. All hormones are given subcutaneously under the nape of the neck.

Sexual behaviors are best observed during the active (dark) period of the animals' light/dark cycle. Thus, animals should be housed under reversed light conditions.

Animal sample size should be considered when using this protocol. The initial animal cohort will generate animals with stable low, normal and high ejaculating profiles. Make sure to have enough animals in the initial cohort if a certain ejaculatory profile is desired (see Background in Commentary section).

The Wistar rat strain is used in this protocol. While sexual behaviors of other rat strains are probably comparable, small differences in behavior cannot be excluded. Also differences in sensitivity for certain drugs, e.g. due to differences in metabolism or polymorphisms in certain genes, may exist among different rat strains.

Equipment

Observations of sexual behaviors can be videotaped and the behaviors scored later with basically a pen, paper, and a timer. A trained observer can score up to four animals at one time using a scoring program such as Noldus Observer®. Since four animals are being

observed at one time, be sure that the observer program can distinguish the mounts, intromissions, and ejaculations from each of the animals in a chronological order. In addition, an observation cage with adequate dimensions (enough for the rats to rear and run) should be used for testing. Eight observation cages allow for the habituation of rats and sex behavior observations to occur simultaneously. With two shelves, cages can be positioned over each other in a 2 x 2 format (two cages on the top shelf and two cages on the bottom shelf; Fig. 9.34.1). In our set-up, the eight observation cages are placed in the same room.

Figure 9.34.1. Experimental 2x2 cage set-up to examine four rats at one time.

BASIC PROTOCOL

SEXUAL BEHAVIOR IN RATS

The sexual behavior of male Wistar rats can be measured easily and reliably in the laboratory. Typically, male rats are allowed to copulate with a receptive female for 30 minutes. The ideal testing time is during the rat's active (dark) period and, thus, the animals should be housed in reversed day/night lighting conditions, with the use of red lights as needed

Rats with normal sexual behaviors display consistent patterns of sexual behaviors. The main quantifiable behaviors are mounts (no vaginal penetration), intromissions (vaginal penetration), and ejaculations (Fig. 9.34.2). In the initial period of contact, male rats may display appetitive behaviors such as sniffing the anogenital region of the female rat for receptive cues. Male rats may also immediately mount or intromit the female. When the male approaches a receptive female from behind, the female usually hops and darts away (dashing forward with a sudden stop often with ear wiggling), which are female

proceptive behaviors. As the female darts from an approaching male, it adopts a reflexive freezing posture called lordosis (with the spine arched inwards), raises the head and rump, extends its back legs and deflects its tail to one side; all of which is necessary for copulation. Intromissions can be distinguishable from mounts by the male rat thrusting his pelvis forward and then hopping off of the female with a long duration of self grooming. With mounts, the male will climb on the female's back with no pelvic thrusting. The male may also groom itself after a mount but for a relatively short time (few licks). Ejaculations can be detected by a lower back spasm and the momentary freezing of the male with both forepaws raised over the back of the female (Fig. 9.34.2).

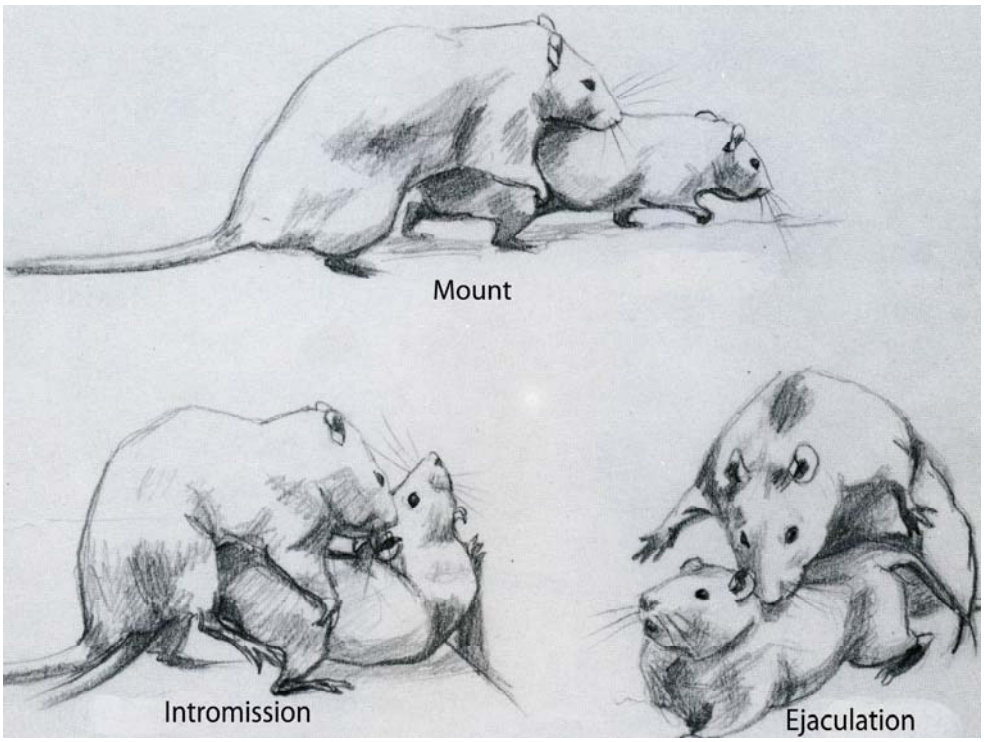

Figure 9.34.2. Rat drawings of mount, intromission, and ejaculation behavior [15].

Following ejaculation, the male rat will groom itself extensively for a much longer period of time. During this time, the male rat emits low frequency 22 kHz ultrasonic vocalizations, which is associated with displeasure in rats [16]. This is a signal for the female to stay away from the male and this also gives the male time to recover from the ejaculation. After around five minutes (this is called the post-ejaculatory latency; PEL), a sexually trained male Wistar will resume pursuit of the female and continue with more mounts, intromissions and another ejaculation. On average, the trained male Wistar rat will ejaculate 2-3 times in a 30 min session.

Materials

Adult Wistar male rats, 300-400 g
Adult Wistar female rats, 200-300 g
Sesame oil saturated with phosphatydylcholine (lecithin; see recipe)
Estradiol benzoate (for female rats; see recipe)
Progesterone (only for ovariectomized females; see recipe)
Observation cage (e.g. 60 x 30 x 40 cm with a Plexiglas front) with bedding material i.e. can be self-built or bought as an aquarium fish tank.
Scoring device with observing program or video recording equipment, i.e Psion Workabout Pro with Noldus Pocket Observer software
Paroxetine as the reference compound (in pure form: Sigma-Aldrich; in tablet form: Paxil® or generic brand from a pharmacy
Test compound solutions (see recipe)

Prepare the animals

1. Order animals for delivery to the laboratory at least one week prior to the beginning of sexual behavior training. Adult males of at least 300 grams are needed. Group-house the animals (four animals per cage) and provide *ad libitum* food and water. Place the animals in a room with reversed light/dark cycle (e.g. lights off from 7 AM till 7 PM), with the use of red light in the dark when needed. Handle and tail mark the rats at least once before the training session.

Perform sexual training of the animals

2. Induce estrus in sexually intact female rats with an estradiol injection 36-42 hours before testing. When using ovariectomized female rats, estrus is induced by estradiol and progesterone injections 36-42 hours and 4 hours before testing, respectively.

3. Place up to four rats in four different observation cages. Training of the male rats begins with a 30 min habituation time in the observation cage. All sex testing occurs between 9:00 and 16:00 hrs during the reversed light/dark cycle with the lights going off at 7:00 and on at 19:00 hrs. Before starting the sexual experiment on these animals place another cohort of four animals in the other four observation cages (in order to save time in habituating the next group of rats).

4. After the habituation period, place the female in the cage and commence with video recording or live scoring with the scoring device. Look for mounts, intromissions, and ejaculations. During the training period, the observer may only look for ejaculations to distinguish between rats with varying sexual activities.

5. Remove the female rats and return male rats to their home cage after 30 min. For sexual training of male rats, repeat steps 2-5, once a week for 4 to 5 weeks. After this period the rats are sexually trained. Sexually trained male rats possess stable sexual activities especially in ejaculation frequencies (see more about sexual endophenotypes in the Commentary section).

Test drug compounds putatively inducing sexual dysfunctions

6. Use the ejaculation frequency over 30 min of the last two training sessions to select endophenotype. Select animals with normal ejaculation frequencies (2-3 ejaculations every 30 min). Low frequency rats with 0-1 ejaculations and high frequency rats with more than 3 ejaculations are excluded. Balance the animals by ejaculation frequencies across treatment groups. Balance the treatments over the observation cages. Run experiments over multiple days if there are multiple treatment groups.

7. Repeat steps 2 to 5 above noting this exception: Depending on the pharmacokinetics of the drug, inject the drug before or during the habituation time. To induce sexual dysfunctions and/or use as a positive reference control, inject 10 mg/kg of the SSRI, paroxetine, either intraperitoneally or orally 30 min (or otherwise depending on the specific pharmacokinetics of the drug under investigation) before the introduction of the female. For non-test days, inject the drug daily between 9:00 and 16:00 (preferably in a fixed period per day - e.g. between 9 and 10 AM each day). Subchronic (day 8 of test) and chronic (day 15 of test) injection of the drugs consists of a total of 8 and 15 injections, respectively. Allow a 7 day post-treatment test (day 22 of test) with no treatments given during this time.

Analyze data and perform statistics

8. For each test period, separate the data for each animal tested. Extracted data can be manually separated using a spreadsheet application software such as Excel. In Excel, a macros command table can be formulated to handle all of the data in a few clicks.

a. Break the data down into ejaculatory series (Fig. 9.34.3) with the first being all events occurring before the first ejaculation. Record the following: mounts (frequency per ejaculation series and time of first mount in series), intromissions (frequency per ejaculation series and time of first intromission in series), number of ejaculations, and time of occurrence of ejaculations.

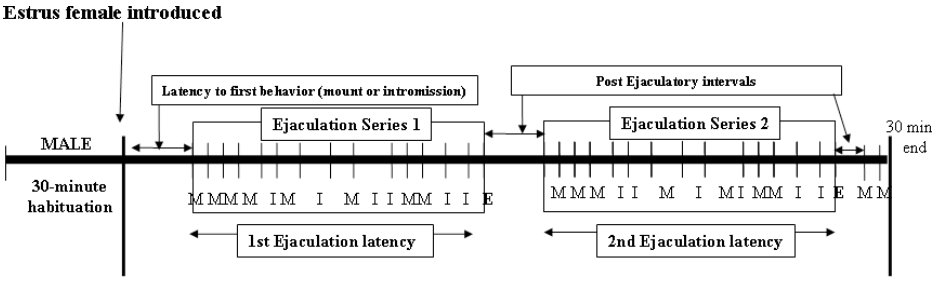

Figure 9.34.3. Sexual behavior parameters with data broken down into the first and second ejaculatory series (M= mount, I= intromission, and E = ejaculation).

b. From these data the following parameters are deduced: number of ejaculations per test; latency to the first mount in the first ejaculation series; latency to the first intromission in the first ejaculation series; number of mounts in the first ejaculation series; number of intromissions in the first ejaculation series; latency to the first ejaculation (time from the first mount or intromission to the first ejaculation); post-ejaculatory latency (PEL,time from the first ejaculation till the first mount or intromission, whichever comes first, from the second ejaculatory series) and the copulatory efficiency (CE = (#I / (#I + #M)) * 100%). All parameters are measured again for the subsequent ejaculatory series.

c. Missing values: A number of issues may develop in the testing of male rat sexual behaviors. Male rats have cyclic sexual behaviors towards a receptive female, ending with an ejaculation. After a rest period, the male resumes sexual activities leading to the next ejaculation, and so forth. The behaviors leading up to the first ejaculation (along with the variable duration of the 1st post-ejaculatory latency) is measured. In order to study drugs, it is important to have comparable pharmacodynamics and kinetics, and thus, a fixed test duration of 30 minutes (1800 seconds) is chosen. If some treatments cause low sexual activities (zero ejaculations), some animals cannot be actually used for the statistics. Artificial maximum values of 1800 sec (i.e. the test duration) for some latencies (e.g. ejaculation latency, mount and intromission latency, post-ejaculatory latency) can be used, although this is questionable. The mount and intromission data from these non-ejaculating rats are also problematic because it is actually not known whether the rat may eventually ejaculate. These data may be considered artificial and, thus, are not *usually* used for statistical comparison. In some experiments where the drug inhibited ejaculatory behaviors, few animals achieve a 2nd ejaculation and thus statistics on the data from these higher ejaculation series could not be performed. The present protocol brings to light these issues: drugs that improve sexual behaviors do not cause any of these problems; whereas drugs that inhibited sexual behaviors do. In cases where the drug blocked ejaculations in a majority of the animals, data values of 1800 seconds are imputed for latency to 1st ejaculation, mount and intromission latency, and post-ejaculatory latency, and include the frequency values for all animals for statistical purposes. In such cases,

the strong drug effect warrants the use of these imputed values. We chose to skip statistical analysis if less than 7 animals in a certain drug-treated group were left for 2nd ejaculatory series parameters.

9. Compare these data points using a statistical software package, such as SPSS. Perform a repeated measure ANOVA to compare drug effects over chronic treatment. Subsequently, use a one way ANOVA to compare drug treatments to one another on each treatment day. If significant effects are found, perform post-hoc analysis using a proper correction method for multiple testing (e.g. Bonferoni) to find the differences between treatment and treatment days. If the assumptions under which the reliability of the ANOVA are not met (normal distribution or homogeneity of variance), use non-parametric statistics such as Kruskal Wallis test and the two-tailed Mann–Whitney U post-hoc correction test. Chi-squared tests, along with Fisher's exact test, are not recommended since these tests deal more with categorical variables, while the data obtained in this protocol deals with interval variables (with units of measurements).

REAGENTS AND SOLUTIONS

Estradiol solution

Heat and stir with a magnetic stir bar 500 ml of sesame oil on a heated stir plate until about 80ºC. Turn off the heat, add 10 grams of lecithin (various vendors), and continue stirring until the preparation has cooled down to handleable temperature. Separate this mixture into 50 ml centrifuge tubes. Centrifuge (i.e. Beckman Coulter Allegra® 6 Benchtop Centrifuge) for 10 minutes at 3000 rpm (1500g) at room temperature to remove all lecithin sediments. Transfer the lecithin-saturated sesame oil into new storage tubes. This is your stock sesame oil and should be stored in the refrigerator until the expiration date of the oil or lecithin . Use this lecithin saturated sesame oil to prepare a 0.5 mg/ml estradiol benzoate solution. Use 0.1ml (50µg) of this solution for inducing estrus in female rats.

Test Compound solution

Paroxetine can be used in its pure form or crushed from pharmacy tablets. In its pure form, it can be dissolved in any vehicle. Use a mortar and pestle to crush pharmacy tablets into a fine powder. Mix the powder with any vehicle to form a suspension. For inducing sexual dysfunctions in rats, inject 10mg/kg at the maximum volume of 10ml/kg.

COMMENTARY

Background Information

Sexual behavior in rodents is studied for many reasons, including the evaluation of new and current drugs for stimulatory and inhibitory effects. Disturbances in normal sexual function affect human quality of life, and drug-induced dysfunctions may cause non-compliance with drugs prescribed to treat a certain disorder.

Sexual behavior studies are most reliable in animals with stable sexual behaviors. This stable sexual behavior is obtained after 3 to 4 training sessions. On the basis of their sexual performance, Wistar rats can be divided into three groups: sluggish, normal and fast ejaculators [17]. The best indicator of the sexual activity or endophenotype is the ejaculation frequency performed in a 30 min test; sluggish rats have 0-1 ejaculations/30 min and fast rats have 4-6 ejaculations/30 min. In between these extremes are the "normal" animals displaying 2-3 ejaculations/30 min. The normal rats represent the largest population of sexually trained rats. The normal animals provide the ideal medium to examine sexually stimulating or inhibiting profiles of drugs. The advantage of studying animals with multiple ejaculations is the possibility to examine facilitating profiles of drugs following the first ejaculation. Animals that return to pursuing the female faster may be more sexually motivated.

As in humans, rats do not respond to acute treatments of SSRIs [11]. With (sub)chronic treatment, we and others observed inhibitions in many aspects of sexual behaviors [18-20]. This provides good predictive validity to this animal model.

A major player in the regulation of sexual behavior is the serotonin (5-HT) system, as is apparent from many neurochemical, pharmacological and neuroanatomic studies [21-23]. SSRIs, compounds that stimulate serotonin turnover and activity, have sexual inhibiting effects and have a bad reputation in depressed patients as they exacerbate existing sexual problems [24].

Similar to humans, after chronic administration of SSRI in rats the inhibitory effects of SSRIs become apparent. Figure 9.34.4 shows the effects of acute, subchronic and chronic administration of our standard reference dose of paroxetine (10 mg/kg intraperitoneal) on the sexual behavior of Wistar rats that had been selected on a normal sexual endophenotype. At this dose, the inhibitory effects are pronounced already after 7 days of treatment and remain present with longer treatment. Stopping of treatment for a week restores the sexual behavior to baseline.

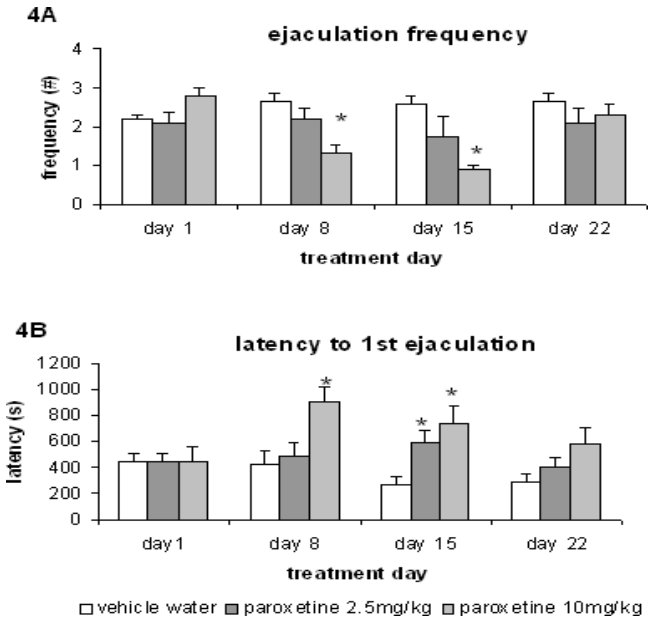

Figure 9.34.4. Effects of paroxetine (2.5 and 10 mg/kg p.o.) on ejaculation frequency (A) and latency to first ejaculation (B) in a 30-min sexual behavior test after acute (day 1), subchronic (day 8), and chronic treatment (day 15). One week after the cessation of treatment (day 22), all animals were tested without treatment. Data are mean ± S.E.M. *Different than vehicle for the same treatment day (ANOVA $p<0.05$).

Systemic administration of 5-HT_{1A} receptor agonists increases the number of ejaculations, including sluggish rats [18]. This prosexual activity already occurs after acute administration. After chronic administration of the 5-HT_{1A} receptor agonist and anxiolytic, buspirone, some tolerance seems to occur for this pro-sexual activity [12] but clearly more drugs have to be tested.

Various other pharmacological mechanisms and drugs have been tested, although the chronic administration test as we describe it here has still limited data. This has to do with the time and space consuming character of these experiments.

Critical Parameters and Troubleshooting

Animals

Male rats will respond optimally towards a fully proceptive and receptive female. Make sure that the female stimulus rats are proceptive and receptive with a quick introduction (one minute suffices) of these females to extra male rats that do not contribute to the experiment. Observe these females for normal hopping, darting and lordosis behaviors. Remove any females that reject the males too vigorously. Male rats may become demotivated, uninterested in sex, or even aggressive with an unreceptive female. Receptive female rats may be reused in a few tests with a rest period of at least 30 minutes. The authors usually use females twice with a one hour interval between uses.

The level of sexual activity varies in different cohorts of rats, certainly at the start of the training. By training the animals, stable sexual endophenotypes can be uncovered. It is possible to have a large number of rats in a group that are sexually inactive or have intermittent sexual behaviors. This may be due to some genetic changes (drift) in the animals or differences in environment and social conditions during rearing and transport. Inbred or outbred animals may possess genes that lead to lower sexual activities [12]. Also inadequate housing and testing conditions (i.e., ventilation, humidity, temperature, cleanliness, etc.) can lead to lower sexual activities. Some males may become sexually active with a change in females or a pinch in the tail (with forceps or digging your fingernail firmly into the tip of the tail without breaking the skin). Unusual mounting

positions (side or head mounts) usually disappear with more training. Aggression towards the female may also be exhibited; a changing of the female may rectify this situation.

For experiments that seek to induce sexual dysfunctions and screen drugs for sexual side effects, even with the use of normal ejaculating rats expect approximately 70% of the males in a cohort to perform normally. Make sure to include enough animals in order to meet the desired number of experimental animals (our cohorts start with 120 animals). Once the sexual endophenotypes are known, try to keep the males in their original housing groups. Re-housing the animals may result in fights and stress to the rats.

Equipment

The 30-min habituation period to the test cages is usually enough for the males to be ready to copulate with a newly introduced female. To reduce possible novelty stress to a clean cage, mix a handful of bedding material from the home cage of all male rats. The bedding in the test cages may remain dirty until unbearable to the experimenters.

Female rats may occasionally jump and escape out of the test cage; a cover with breathing holes can also be added in these instances. Such covers can also be designed to fit microphones for ultrasonic vocalization recordings.

Experimental conditions

Most rat sexual activities occur during the active (dark) period of the light/dark cycle (see figure 9.34.5). Experiments with rats during normal (non-reversed) light/dark cycle show a higher ejaculation frequency in the morning hours with a dramatic decrease in ejaculation frequency in the afternoon. When the lighting system was reversed, and the test was performed in dark phase of the light cycle, these afternoon tests had no significant differences from the morning session. Thus, rats should be housed in reversed light/dark cycle.

The test room should also be kept in the dark with the use of dim red lights to observe the experiment. If the housing rooms are separate from the testing rooms, then an exposure to light during the transport should be limited as much as possible by covering the cages during transport. The habituation time to the test room and cages should be adequate to alleviate any stress endured. In this protocol, we use a standard 30 minutes to habituate the animals.

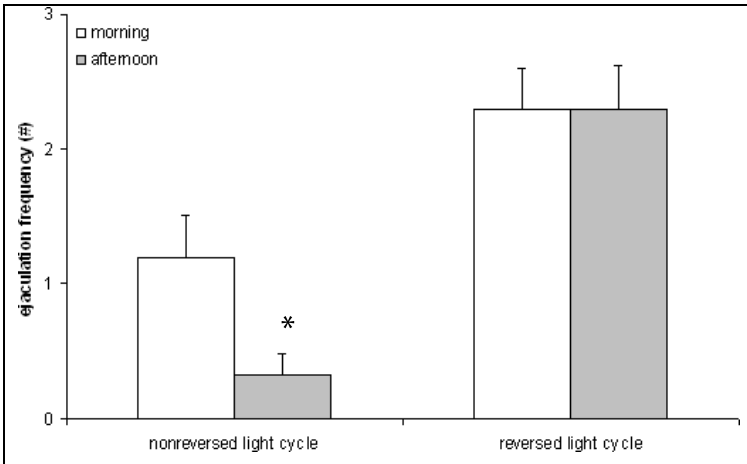

Figure 9.34.5. Effects of housing light conditions on male Wistar rat ejaculation frequencies (n=120). Experiment was performed over four weeks, with two weeks under non-reversed lighting conditions and two weeks under reversed light/dark conditions. All testing was performed from 9:00 to 15:00 hrs. * Difference between morning and afternoon testing (ANOVA $p<0.05$).

Experimental design

For chronic pharmacological experiments a between subject design (i.e., different groups for each drug/dose) is the most favorable. The disadvantage is the need for a large number of rats and thus, housing requirements and costs.

Acute effects of different doses of a drug can be best tested using a within subject design. Because most animals have very stable sexual endophenotypes, a within animal design

reduces the number of animals for an acute experiment. The disadvantage of a within-animal design is the relatively lengthy experimental period needed and the intrinsic difficulty in designing chronic treatment experiments.

Thirty minute observations are ideal to examine effects of drugs and sexual behaviors. While longer periods could be adopted, the rats may become sexually exhausted. This may lead to failed performances in the future. Some experimenters prefer to stop an experiment not after a fixed time but after a certain behavioral event, e.g. the start of the second ejaculation series. A disadvantage of this approach in pharmacology is the absolute incomparability in pharmacokinetics between animals and the uncertainty about the outcome.

Anticipated Results

Sexual behavior is an innate behavior. When a male rat is first introduced to a receptive female, the male usually begins by investigating the anogenital region of the female. Some males may immediately mount the female. The first training sessions may yield low ejaculation frequencies. Subsequent training sessions should result in increased ejaculation frequencies and after 3-4 training sessions almost all males show a very stable level of sexual performance, leading us to postulate that male rats display sexual endophenotypes. Thus, sexually trained rats fall into sexual endophenotypic distribution of slow, normal and fast ejaculators. From among the 1862 Wistar rats we have observed over the course of 6 years], we usually find that 10-20% are slow and 10% are fast ejaculators (Fig. 9.34.6). For our drug studies, we routinely use the normal ejaculating animals (2-3 ejaculations/30 min). These animals show reduced sexual behavior following chronic SSRI treatment (Fig. 9.34.4). There is a dose-dependent response to the SSRIs, where 2.5 mg/kg show a tendency to inhibit some aspects of sexual behavior after chronic treatment (Fig. 9.34.4). We continue to see sexually inhibiting effects at doses of 5 and 10 mg/kg [12]. From the start of drug administration, drug-induced sexual dysfunctions should be evident after seven days of treatment. After 14 days of administration, the rats continue to have lower ejaculation frequencies (Fig. 9.34.4) and

other inhibitions; increases in latencies to first mount; first intromission and first ejaculation; first series intromission frequency; and decrease in copulatory efficiency (data not shown). One week after cessation of two weeks of paroxetine administration (10 mg/kg) the males show their normal sexual behavior again.

There is a difference in sexual side effects between various SSRIs, both in humans and rats. Delayed ejaculation reliably occurs in rats in response to chronic treatment with paroxetine or fluoxetine [5, 13, 19], but less so or not at all in response to citalopram or fluvoxamine [5, 13, 18], which resembles the situation in humans [20, 25]. Also the serotonin–norepinephrine reuptake inhibitor (SNRI) venlafaxine had very limited sexual side effects in rats. Only at the highest dose tested (40 mg/kg) a reduction in the number of ejaculations was found (unpublished data).

Phosphodiesterase type 5 (PDE5) inhibitors are used to treat erection problems. In slow-performing male rats, PDE5 inhibition did not stimulate sexual behavior, suggesting that these animals do not suffer from erection problems.

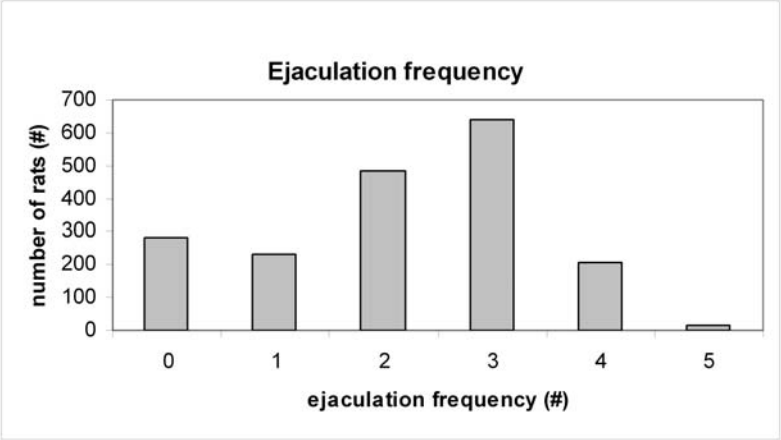

Figure 9.34.6. Histogram of ejaculation frequency of sexually trained male Wistar rats (total N= 1862, obtained from 17 experiments, gathered from the year 2004 to 2009). Based on the fourth mating test, these male rats were classified with the stable copulatory behaviors of: 'normal' (2-3 ejaculations/30 min), 'sluggish' (0-1 ejaculation/30 min), and 'fast' (4-5 ejaculations/30 min) ejaculating.

Time Considerations

Animals require at least a one week habituation time from delivery. Sexually training 120 male rats can be performed over 2-3 days, depending on the experience of the observer. An experienced observer to sexual behaviors can observe up to 10 animals at one time. The untrained observer should start with four animals and increase the number of animals gradually. With a double set of test cages, animals could habituate to the test cage and room while a group of animals is being observed. The drug administration portion of the experiment requires a minimum of one week of administration, although for translational research purposes longer periods of administrations should be adopted. The duration for all sexual behavior tests is 30 minutes. The rats should not be tested for another seven days in order to give the animals time to recover.

Chapter 3: Translational research into sexual

disorders: pharmacology and genomics

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Abstract

The existence of sexual dysfunctions in men, including premature and retarded ejaculation poses challenges to develop translational models in rats that may help in improving treatment and delineate the neural mechanisms of action. Most of our current understanding of the neurobiology, neuroanatomy and psychopharmacology of sexual behavior and ejaculatory function has been derived from preclinical studies in the rat. When large populations of male rats are tested on sexual activity during four successive tests, over time individual rats display a very stable sexual behavior that is either slow, normal or fast as characterized by the number of ejaculations performed. These sexual endophenotypes are postulated as rat counterparts of premature (fast rats) or retarded ejaculation (slow rats). Psychopharmacology in these endophenotypes may help to delineate the underlying mechanisms and pathology. This is illustrated by the effects of serotonergic antidepressants and serotonergic compounds on sexual and ejaculatory behavior of rats. Further unravelling of sexual endophenotypes may benefit from the use of chromosomal substitution strains in mice that enable the localization of relevant chromosomal areas and genes involved in ejaculation processes. These preclinical studies and models contribute to a better understanding of the neurobiology of ejaculation and boost the development of novel drug targets to treat ejaculatory disorders such as premature and retarded ejaculation.

Introduction

Many drugs influence male and female sexual performance and/or behavior. Considering the extremely complex mechanisms involved in the regulation of sexual behavior in humans, but similarly complex in other mammals, it is not surprising that such, often disturbing (side) effects occur. On the other hand, there is an increasing use of drugs promoting different aspects of sexual performance or behavior, including PDE-5 inhibitors for erectile dysfunction [26], antidepressants (selective serotonin reuptake inhibitors—SSRIs) for premature ejaculation [14, 27] and androgens for female libido [28]. Research into the mechanisms involved in all aspects of sexual behavior, including physiological, neurological, pharmacological, neuroanatomical, endocrinological and pathological mechanisms is strongly increasing, and fuels the emerging pharmaceutical market aiming at 'sexual health' products. In this context, is it extremely important to have access to preclinical animal models that have face, predictive and construct validity towards human sexual disorders and dysfunctions. Such translational models are scarce, but vital, to study the underlying neural mechanisms and putative targets for innovative drug treatment. The present paper starts with a short description of animal sexual behavior, followed by the important role of the serotonergic system in sexual and ejaculatory behavior; focusing on the role played by the serotonin transporter in sexual dysfunctions. Also, the effects of other SSRIs and other antidepressants on sexual activities are discussed. The main emphasis of the present paper is on the development of novel rat paradigms modeling premature, normal and retarded ejaculation in humans. Effects of a selected number of psychoactive drugs will be described in these three different models. Finally, a discussion on the relevance and therapeutic use of the findings and their interpretation is performed.

Animal sexual behavior

Increasing understanding of the neurobiology of normal and 'pathological' sexual functioning has been derived from animal studies in which specific brain areas have been manipulated or animals have been challenged pharmacologically [2, 29, 30]. Most of the

current theoretical models of animal sexual functioning—and underlying neurobiology—have been based on sexual and copulatory behavior of laboratory rats. Typically, in these experiments, male rats are exposed to a receptive female and allowed to copulate for a certain period of time, or until ejaculation has occurred. Male rat sexual behavior is characterized by a series of mounts, either with or without vaginal intromission, which eventually will lead to an ejaculation, usually in a couple of minutes [29]. After a certain rest period (post ejaculatory interval), sexual activities resume and over a period of 30 min a rat may display up to 5 ejaculations. A distinction can be made between appetitive and consummatory aspects of copulatory behavior, where latency until the first mount putatively reflects some of the appetitive aspects and sexual motivation [31]. Consummatory aspects of sexual behavior include intromission latencies, ejaculation latencies, mount frequencies and intromission frequencies and may all affect ejaculatory behavior. With regard to male rat ejaculatory behavior, over the last decades numerous pharmacological studies have shown that various neurotransmitters and/or neuropeptides may be involved, e.g. dopamine, serotonin, prolactin and oxytocin [32-36].

Moreover, the neuroanatomical pathways of male rat ejaculatory behaviors are increasingly well understood, both at the supraspinal [37-39] and spinal cord level [40-42]. Nonetheless, little is known with regard to the putative underlying neurobiology of ejaculatory dysfunctions, such as premature and retarded ejaculation or an-ejaculation.

Serotonin, serotonergic receptors and ejaculatory behavior

Over the last decades, an extensive body of research has indicated that central neurotransmitters such as dopamine and serotonin and their receptors play an important role in the regulation of ejaculation. As excellent reviews exists concerning the role of central dopamine and central serotonin in sexual behavior [27, 33, 36, 43-45], here, we only briefly review the most important findings with regard to the role of serotonin in the regulation of ejaculation and sexual activities in human ejaculation [27, 43, 46, 47].

The serotonergic neurotransmitter system is equipped with one endogenous ligand, 5-hydroxytryptamine (5-HT, serotonin) and has at least 14 structurally, functionally and pharmacologically distinct 5-HT receptor subtypes. These receptor subtypes can be assigned to one of seven families, namely 5-$HT_{1–7}$ and each receptor subtype appears to have a distinct and limited distribution in the central nervous system [48]. Nonetheless, whether all these different receptor families and/or subtypes have their own distinct functions is unclear although very likely. Importantly, in addition to their postsynaptical distribution throughout the central nervous system, 5-HT_{1A} receptors are also located presynaptically as autoreceptors, where they regulate the activity of 5-HT neurons in the dorsal raphé nucleus [49]. Moreover, serotonergic transporter molecules (5-HTT) are present on serotonergic neurons (both somatodendritically and on axon terminals) where they facilitate the re-uptake of 5-HT after cell firing-induced 5-HT release. SSRIs inhibit this transporter and cause enhanced serotonin levels in the synaptic cleft leading to enhanced serotonergic neurotransmission. The importance of 5-HT in sexual behavior has been demonstrated by numerous studies showing that, for instance, lesions of the brainstem raphé nuclei [50] and 5-HT depletion [51] facilitate sexual behavior. On the other hand, administration of the 5-HT precursor, 5-hydroxytryptophan , 5-HT itself and 5-HT releasers such as MDMA (ecstacy) and fenfluramine have been shown to inhibit sexual behavior in male rats [52-55]. Altogether these findings suggest that a decrease in 5-HT neurotransmission may be involved in facilitation, whereas an increase in 5-HT neurotransmission may result in inhibition of sexual behavior. Moreover, these findings fit with the idea that spinal genitourinary circuits are under inhibitory control of supraspinal brainstem structures presumably mediated by 5-HT [56-58].

Effects of SSRIs on ejaculation in humans

The frequently reported sexual side effects of SSRIs in men suggest an important role of 5-HT in human ejaculatory behavior [5]. As described previously, in several human studies, we and others have demonstrated that various SSRIs such as paroxetine, sertraline and fluoxetine are able to delay ejaculation in men with premature ejaculation [14, 59, 60].

Moreover, studies with daily treatment with SSRIs in men with premature ejaculation with an intravaginal ejaculation latency time (IELT) of less than 1 min [61, 62], suggest that SSRIs exert a clinically not relevant ejaculation delay in the first week of treatment [14, 60, 63]. Interestingly, in contrast to the antidepressive activity of SSRIs which usually becomes manifest after 4-6 weeks but sometimes also earlier, clinically relevant ejaculation delay in mentally healthy men with lifelong premature ejaculation occurs within 2–3 weeks of daily treatment [14, 60, 63]. Several authors claim, however, that some conventional SSRIs (e.g. sertraline, paroxetine) may have acute effects and can be used for on-demand treatment [64-66]. Their studies, however, suffer from methodological insufficiencies impairing a generalization of the study results [62]. Recently, it has also been suggested that on-demand use of a new SSRI with a short half-life (e.g., dapoxetine) may be a better option than daily use of an SSRI to treat premature ejaculation [67]. However, in contrast to these on-demand SSRI studies it has been argued that based on current psychopharmacological knowledge ejaculation delay by on-demand treatment, either with conventional SSRIs or SSRIs with a short half life, will be very limited and likely exert a delay that is not much higher than a 3 fold-increase of the geometric mean IELT over baseline IELT values [61]. For example, in a double-blind stopwatch study in men with lifelong premature ejaculation, it was found that on-demand treatment with 20 mg paroxetine exerted a fold-increase IELT of only 1.41 (95% CI: 1.22–1.63) at a drug coitus interval time of approximately 5 h [62]. The calculated 1.4-fold increase means that on-demand treatment with 20 mg paroxetine in men with an IELT of less than 1 min would induce only approximately 40% ejaculation delay. Patients and their partners considered the resulting degree of ejaculation delay as clinically not relevant. In contrast to SSRIs, the tricyclic antidepressant clomipramine (25 mg) taken at least 4–6 h before intercourse, may result in clinical relevant ejaculation delay [62, 68]. Moreover, critical analysis of 19 drug treatment curves of various studies with SSRIs, mirtazapine, nefazodone and placebo, have shown that strong ejaculation delaying drugs (like daily paroxetine treatment) give rise to a positively skewed IELT distribution, whereas weak or non-ejaculation delaying drugs (like mirtazapine or placebo) give rise to a minimally skewed and rather normal IELT distribution [69]. Therefore, it is considered mandatory to express

ejaculation delay in the fold-increase of the geometric mean IELT instead of that of the mean IELT, in order to diminish the chance of an overestimation of the ejaculation delaying effect of a drug in case of a positively skewed IELT distribution [69-71]. In contrast to the strong ejaculation delaying effects of daily SSRI treatment, on-demand use of dapoxetine study leads to a very weak ejaculation delay [67, 69-71]. For example, a multicenter study showed that placebo, 30 and 60 mg dapoxetine exerted a 1.9, 3.0, and 3.6 fold increase of the mean IELT from baseline, respectively [67]. The very weak ejaculation delaying properties of on-demand use of dapoxetine may further be confirmed by its normal IELT distribution at study end-point [69]. These very weak ejaculation delaying effects of on-demand use of conventional SSRIs and dapoxetine are in contrast with the data of a systematic review and meta-analysis of all drug treatment studies conducted between 1943 and 2003, in which it was shown that daily paroxetine treatment resulted in the strongest ejaculation delay (geometric mean IELT fold increase of 8.8 [95% CI: 5.9-13.2]) [68]. The very weak and very strong ejaculation delay after acute and daily SSRI treatment in human, mirrors central serotonin neurotransmission. Although, serotonin enhancing effects of conventional SSRIs are already present after acute treatment, they are further enhanced after chronic treatment. Interestingly, administration of dapoxetine leads to immediately similar high levels of synaptic serotonin as occurs after 2 weeks of conventional SSRI administration [72], yet showing hardly any ejaculation delaying effect after acute treatment [69-71]. The relatively slow onset of action of conventional SSRIs in the treatment of premature ejaculation, that is however clearly faster than in depression, and the minimal ejaculation delaying effect of acute treatment with dapoxetine, indicates that chronic elevated activation of certain serotonergic receptors is needed for the effect on ejaculation. Apparently, this effect on ejaculation is not similar to the antidepressant activity of the SSRIs. The more so because, despite the putative similar underlying mechanism of action of SSRIs—briefly, preventing the reuptake of 5-HT, thereby elevating 5-HT levels—not all SSRIs delay ejaculation to the same extent. In humans, for instance, of all the various SSRIs, paroxetine appears to have the strongest ejaculation delaying effects after 4–6 weeks of daily treatment whereas fluvoxamine and citalopram hardly show a clinically relevant inhibition [60, 63, 68].

Acute and chronic SSRI administration

Analogous to the human situation, also in male rats, a distinction can be made between the effects of acute and chronic SSRI administration on ejaculation. Acute administration of various SSRIs, including citalopram, clomipramine, paroxetine, sertraline, fluoxetine and fluvoxamine did not have any delaying effects on ejaculations as shown earlier [19, 73, 74]. On the other hand, chronic administration of fluoxetine [19, 75, 76] and paroxetine [18, 77] did have delaying effects on ejaculation in male rats. Nonetheless, as in humans, not all SSRIs potently delay ejaculation after chronic administration in male rats. For instance, fluvoxamine slightly affected some aspects of copulatory behavior, but did not affect ejaculation even after chronic administration [77]. Similar results were obtained in our laboratory with chronic citalopram that only marginally delays ejaculation in sexually active rats [78].

Until now it is still unclear why the various SSRIs differ in their ability to delay ejaculation after chronic administration. The delay in onset of the therapeutic effect of SSRIs in depression and anxiety disorders has been related to adaptive changes of serotonergic autoreceptors [79, 80]. Therefore, it is conceivable that also the ejaculation-delaying effects of various SSRIs are due to adaptive changes of, for instance, 5-HT receptor subtypes.

Ahlenius and Larsson [73] have studied the mechanism of SSRI-induced delay of ejaculation in more detail and showed that acute treatment with citalopram did not affect ejaculatory behavior. Nonetheless, when the 5-HT_{1A} receptor antagonist WAY-100635 was coadministered with citalopram, ejaculation latencies were strongly delayed, suggesting the involvement of 5-HT_{1A} receptors in effects of citalopram on ejaculation. De Jong et al. [78] also showed that doses of citalopram, acutely or chronically, that did not inhibit sexual behavior on itself, when combined with one sexually inactive dose of WAY-100635, completely abolished sexual behavior. Moreover, Hillegaart and Ahlenius [81] showed that the ejaculation delaying effects of the combination of citalopram and WAY-100635 could be fully blocked by a selective 5-HT_{1B} receptor antagonist,

suggesting a role for this receptor subtype in the delay of ejaculation. Interestingly, a previous study from the same laboratory also suggested a role of the 5-HT_{1B} receptor in the delay of ejaculation as it showed that the 5-HT_{1B} receptor agonist anpirtoline dose-dependently delayed ejaculation in rats [81]. Several other explanations for the differential effects of SSRIs on ejaculation have been postulated, including, next to the inhibition of the serotonin transporters, other mechanisms of action in the various molecules. However, most if not all of these competing mechanisms in SSRIs, are only activated at higher doses of the SSRI than used in the treatment of premature ejaculation. We postulate here that the inhibitory effects of some SSRIs (paroxetine, sertraline, fluoxetine, clomipramine) on ejaculation are mediated via activation of particular serotonergic receptors, probably in specific brain or spinal cord areas. The noneffective SSRIs (citalopram, fluvoxamine) apparently do not (or not enough) activate these particular 5-HT receptors. It is feasible that the inhibiting SSRIs particularly activate 5-HT_{2C} receptors and not, or hardly 5-HT_{1A} receptors, whereas the reverse would hold for the noninhibitory SSRIs.

Interestingly, adaptive changes in 5-HT receptors after chronic SSRI administration affect neuroendocrine systems as well, including the oxytocinergic system, which is generally known to facilitate sexual behavior [82]. Chronic fluoxetine [83] and paroxetine [84] reduced G-protein levels in the hypothalamus and neuroendocrine responses, including oxytocin release, to 5-HT_{1A} receptor agonists were blunted in animals chronically treated with SSRIs compared to controls. It may be possible that the blunted oxytocin responses due to adaptive changes of G-proteins are responsible for the sexual side effects of SSRIs [34]. Supporting evidence comes from a study showing that the sexual side effects of fluoxetine in rats were reversed by coadministration of oxytocin [75].

To summarize, until now the sexual side effects of SSRIs are not well understood. Nevertheless, recent findings suggest that adaptive changes in the 5-HT system and its interactions with neuroendocrine systems may be responsible for their sexual side effects. For the development of novel therapeutic interventions to treat premature ejaculation, it is

important to identify where in the central nervous system and in which—if any—5-HT receptor subtypes these adaptive changes have occurred.

Acute and chronic animal models: SSRIs and other antidepressants

Animal studies have been performed to investigate whether there is a difference in the degree to which various SSRIs influence sexual behavior. One can distinguish acute [74] and chronic animal models [5, 19, 75, 76]. In our own group, we initially used an acute model. Sexually experienced and naive rats were tested 60 min after oral administration of clomipramine, fluvoxamine, fluoxetine, sertraline or paroxetine. At non-sedative doses, clomipramine and the SSRIs did not inhibit male rat sexual behavior [74]. It seems therefore that masculine sexual behavior in rats, using an acute model paradigm, does not constitute a suitable model to investigate the differential mechanisms of sexual inhibition of SSRIs [74]. Using a chronic model, in which the sexual behavior of the rats was tested after 7 and 14 days of daily oral SSRI treatment, we found significant effects of paroxetine, and no or only mildly ejaculation delaying effects of fluvoxamine and citalopram [85, 86]. Both the acute and chronic models resemble the effects of SSRIs in men [59, 77]. Acute treatment of SSRIs has no relevant effect on ejaculation after 1–5 h [46, 47], while chronic (daily) SSRI treatment results in clinically very relevant ejaculation delaying effects [14, 60]. The chronic administration of SSRIs in this male sexual behavior model seems to simulate the human situation. Using this methodology, we tested different drugs, including paroxetine, buspirone and bupropion. In our standard behavior model, we routinely run 10 mg/kg paroxetine as an inhibitory SSRI. At this dosage, sexual behavior is inhibited after (sub)chronic dosing and not, after acute administration.

Figure 1 shows the effects of paroxetine on sexual behavior of normal performing rats. Acutely, paroxetine does not affect sexual behavior (number of ejaculations in 30 min and the 1st ejaculation latency. However, after 7-days, but particularly after 14 days, sexual behavior is affected (decreased number of ejaculations and increased ejaculation latency). Remarkably, the 5 mg/kg dose was equally potent to the 10 mg/kg dose, suggesting that much lower doses of paroxetine might still be sexually inhibitory.

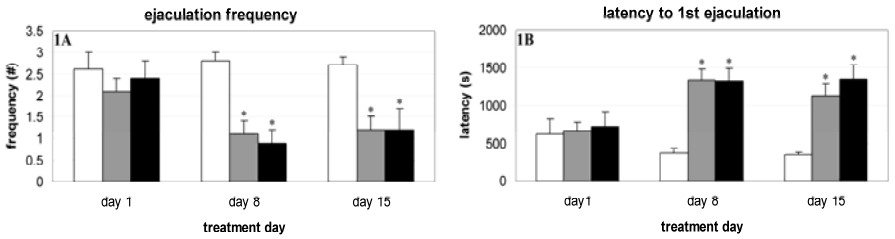

Fig. 1: Effects of paroxetine (5 and 10 mg/kg IP) on the (1A) number of ejaculations and (1B) latency to 1st ejaculation in a 30-min sexual behavior test after acute (Day 1), sub chronic (Day 8) and chronic treatment (Day 15). Data are mean ± S.E.M. * = different from vehicle ($p < 0.05$). White bar = water vehicle; gray bar = paroxetine 5mg/kg; black bar = paroxetine 10mg/kg.

Fig. 2 shows the effects of 15 mg/kg bupropion, a noradrenergic and (weak) dopaminergic reuptake blocker with antidepressant properties. Bupropion has some stimulatory efficacy on sexual behavior particularly after acute and (sub) chronic administration. The number of ejaculations after acute adminstration is significantly higher and the latency to 1st ejaculation shortened. This effect reduced, but still marginally significant after 7-days treatment, and disappears after 14 days treatment. The absence of a serotonergic reuptake blocking component and/or the increase in dopamine and noradrenaline in buproprion's mechanism of action might explain its stimulatory effect on sexual behavior. This effect of bupropion suggests that the drug should also have no sexual inhibitory effects in humans and the available clinical evidence indeed suggests that bupropion is often used as co-medication in SSRI-induced sexual side effects in depressed patients [87, 88].

Fig. 3 shows the effects of the partial 5-HT_{1A} receptor agonist buspirone (1 and 3 mg/kg IP) on the number of ejaculations and latency to the 1st ejaculation. Buspirone exerted some prosexual activity after acute and (sub) chronic and chronic administration,

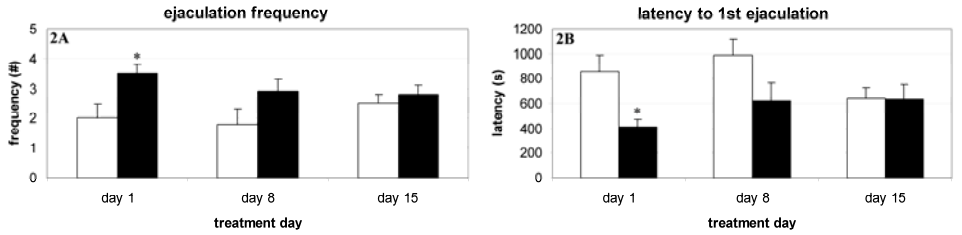

Fig. 2: Effects of bupropion (15 mg/kg IP) on (2A) the number of ejaculations and (2B) latency to 1st ejaculation in a 30-min sexual behavior test after acute (Day 1), sub chronic (Day 8) and chronic treatment (Day 15). Data are mean ± S.E.M. * = different from vehicle of the same treatment day ($p < 0.05$). White bar = water vehicle; and black bar = bupropion 15mg/kg.

although the effect seemed to diminish over time. In contrast the SSRI paroxetine (10mg/kg IP) showed a strong inhibitory effect on sexual activities, even after acute administration. One week after cessation of treatment (extinction) no residual effects of any drug treatment were present showing the reversibility of the treatments. Buspirone is used as treatment for anxiety and depressive co-morbidity and has not a reputation for sexual side effects. Based on the present data buspirone might be used as add-on medication for depressed patients with SSRI-induced sexual side effects.

Effects of various serotonin receptor agonists and antagonists on ejaculation in male rats

As described above, activation of 5-HT_{1B} receptors has been associated with delaying ejaculation in male rats. Other 5-HT receptor subtypes implicated in the inhibition of ejaculation are 5-HT_2 receptors. For instance, in a standard mating paradigm, the nonselective 5-$HT_{2A/2C}$ receptor agonist DOI inhibited sexual behavior including ejaculation [89]. On the other hand, several other studies have shown that 5-$HT_{2A/2C}$

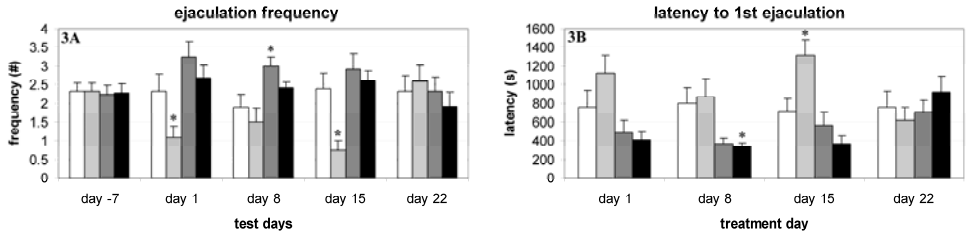

Fig. 3: Effects of buspirone (1 and 3 mg/kg IP) and paroxetine (10 mg/kg IP) on (3A) the number of ejaculations and (3B) latency to 1st ejaculation in a 30-min sexual behavior test after acute (Day 1), sub chronic (Day 8) and chronic treatment (Day 15). One week after cessation of treatment (day 22), all animals were tested without treatment. For the ejaculation frequencies the data of the last training day (day -7) are shown. Data are mean ± S.E.M. * = different than vehicle of the same treatment day ($p < 0.05$). White bar = water vehicle; light grey bar = paroxetine 10mg/kg; dark grey bar = buspirone 1mg/kg; and black bar = buspirone 3mg/kg.

receptor agonists generally inhibit sexual behavior by decreasing the number of animals that initiated copulation, but do not affect ejaculation latencies in animals that do initiate copulation [90-92]. Thus, it appears that 5-HT_2 receptors in general inhibit sexual behavior,but their precise role in the regulation of ejaculation is not entirely clear.

In contrast to 5-HT_2 receptors, a facilitatory role on ejaculation has been ascribed to activation of 5-HT_{1A} receptors and various selective agonists for this receptor, such as 8-OH-DPAT [93], FG-5893 [94] and flesinoxan [95, 96] potently facilitate sexual behavior and decrease ejaculation latencies. Nevertheless, the underlying mechanism of the facilitatory effects of 5-HT_{1A} receptor agonists is still unclear. A possibility for the mechanism of action may be activation of presynaptic 5-HT_{1A} receptors that will lead to an inhibition of 5-HT neuronal firing and consequently results in facilitation of sexual behavior as described above. Alternatively, activation of postsynaptic 5-HT_{1A} receptors may result in facilitation of sexual behavior. Evidence for a postsynaptic mechanism of action is

provided by studies demonstrating that injection of 8-OH-DPAT directly into the medial preoptic area potently facilitated sexual behavior and lowered ejaculatory threshold [97].

Animal models of premature and retarded ejaculation

Most of our current understanding of the anatomy and neurobiology of sexual behavior is based on animal studies using rats that are sexually experienced and display normal sexual behavior. Interestingly, the comparable ejaculation-delaying effects of SSRIs in humans and rats suggest high predictive validity with regard to the regulation of ejaculation. Nevertheless, face validity is low when one tries to extend results obtained in rats that display normal sexual behavior to dysfunctions such as premature and retarded or even (an)-ejaculation. Over the last decades, several groups have studied rats that display hypo-sexual behavior and are referred to, by different investigators, as sexually inactive, sluggish, impotent and noncopulating rats. Recent findings suggest the presence of neurobiological differences associated with the hypo-sexual behavior that these rats display. On the other hand, hypersexual behavior can also be provoked pharmacologically. However, there are only few studies that have studied rats that are hypersexual by nature. Thus, investigating animals that do not display normal sexual behavior may help understanding of the underlying neurobiological mechanisms and hopefully provides further insights in the etiology of ejaculatory dysfunctions.

Studies with rats displaying hypo-sexual behavior

It was already demonstrated in early experiments in the 1940s that rats reared in isolation are either not capable to achieve ejaculation or remain sexually inactive, after repeated exposure to a receptive female. In contrast, rats that were reared in groups with either same-sex or hetero-sex cage mates did not show these clear deficits in copulatory behavior. Importantly, in most but not all of the isolation-reared males sexual performance gradually improved with experience. These early findings suggest that experience and learning play an important role in rat copulatory performance, but apparently do not exclusively determine the ability to successfully copulate until

ejaculation. In early studies focussing on rats displaying different levels of sexual performance, we have tried to create hypo-sexual behavior in male rats by manipulating the level of sexual experience. We studied the sexual behavior of 278 sexually naïve male Wistar rats in tests of 15 min with an estrus female. From those 278 males, 23 showed no sexual activity at all, that is, no intromissions and maximally one mount was scored during the test. From the remaining 255 rats, 211 displayed sexual activity, but failed to ejaculate during the test. The average ejaculation latency of the 44 ejaculating males was 620±28 s. If sexually naïve male rats were treated with 5-HT_{1A} receptor agonists, these males performed quite well. In particular, the two full 5-HT_{1A} receptor agonists (±)-8-OH-DPAT and flesinoxan enhanced sexual behavior to the level of sexually experienced male rats. The partial 5-HT_{1A} receptor agonists buspirone and ipsapirone also facilitated sexual activity although buspirone at a higher dose was sedative. These findings indicate that naïve male rats are able to perform sexual activities reminiscent of sexually 'experienced' rats in a very short time interval. Apparently, sexually naïve rats may be influenced by certain factors that can be overcome by treatment with psychoactive drugs, at least 5-HT_{1A} receptor agonists and a_2-adrenoceptor antagonists like yohimbine and idazoxan [96]. Mos et al. also showed that males treated with 5-HT_{1A} receptor agonists (flesinoxan, gepirone) were more attractive to females than vehicle treated males using a tethered two-choice paradigm, whereas a_2-adrenoceptor antagonist treated males were equally or even less attractive than vehicle-treated males for estrus females under such tethered conditions. It has already been shown earlier that hypo-sexual behavior in sexually inactive rats can be reversed by the opioid receptor antagonist naloxone [98]. Following these findings, numerous other studies have shown that also other pharmacological compounds and certain neuropeptides were able to improve copulatory behavior in sexually inactive rats. Again, the 5-HT_{1A} receptor agonist 8-OH-DPAT potently increased sexual activity in rats that were sexually inactive [99]. Similarly, the erectogenic drug sildenafil [100, 101] and low doses of the hormone melatonin [102] were able to reverse the hyposexual behavior of sexually inactive rats. These pharmacological studies strongly suggest that neurobiological mechanisms underlie the differences observed in

basal sexual behavior. Indeed, in recent years, neurobiological differences have been found between rats that are sexually inactive and rats that display normal sexual behavior.

The neurotransmitter oxytocin appears to play a facilitatory role in ejaculation [82]; however, until now its precise role has not fully been understood. Oxytocin-producing neurons in the brain are primarily located in the paraventricular nucleus of the hypothalamus and projections from these hypothalamic areas reach into the lumbosacral portion of the spinal cord [103]. Arletti et al. [104] showed that expression of oxytocin mRNA was reduced in the paraventricular nucleus of the hypothalamus of sexually inactive rats strongly suggesting a functional role of oxytocin in the expression and execution of copulatory behavior. It would therefore be interesting to measure levels of oxytocin in, for instance, men suffering from retarded or an-ejaculation to determine whether oxytocin levels are decreased.

There is general agreement that brain opioids are involved in the inhibition of copulatory behavior [82, 105]. In line with this view, recent findings indicate that in the hypothalamus of sexually inactive rats levels of the endogenous opioid octapeptide are elevated [106] and mRNA expression of pro-enkephalin and pro-dynorphin is increased [104]. Whether these findings are related to the observed differences in behavior remain to be proven in subsequent experiments, although the findings fit the idea that opioid peptides inhibit sexual behavior.

Beside these findings—to our knowledge—there have been no other reports on neurobiological differences between rats displaying hypo-sexual and normal sexual behavior. In summary, the recent findings obtained in hypo-sexual rats have identified some neurobiological targets that may be responsible for the expression of the hypo-sexual behavior. Of course, more studies are necessary to validate and extend these findings; however, it would already be worthwhile to study these targets in men suffering from retarded ejaculation and an-ejaculation.

Studies with rats displaying hyper-sexual behavior

In contrast to studies focussing on rats that are hypo-sexual by nature, reports of rats that are hypersexual by nature are scarce. Nevertheless, numerous studies have indicated that a variety of selective pharmacological compounds, neurotransmitters and neuropeptides may facilitate sexual behavior [33, 82]. Most interesting are those studies in which male rat sexual behavior is potently facilitated and in which it shares characteristics of human premature ejaculation. Indeed, some of the clinical symptoms of premature ejaculation can be evoked pharmacologically in male rats. For instance, various selective 5-HT_{1A} receptor agonists potently decrease ejaculation latencies and intromission and mount frequencies, although the mechanism of action of these effects is still unclear as discussed above. Beside selective 5-HT_{1A} receptor agonists, a dopamine receptor agonist like SND-919 [107] and apomorphine [108] also decreased ejaculation latencies in rats, although its effects were far less pronounced compared to 5-HT_{1A} receptor agonists.

Besides pharmacological manipulations, 'tactile' stimulation, such as shock and tail-pinching [109, 110] also facilitates ejaculatory behavior. Presumably, these facilitatory effects are mediated by activation of the brain dopaminergic system [111].

Although the experiments described here are certainly not directly comparable to human ejaculatory disorders, the results do provide further support and insight into which neural mechanisms are involved in the facilitation of copulatory behavior. It would therefore be highly interesting to examine these mechanisms in men suffering from premature ejaculation.

Variability in ejaculatory behavior: a putative model for premature, normal and retarded ejaculation?

Waldinger and Olivier [27] hypothesized that lifelong premature ejaculation characterized by IELTs of less than 1 minute, is not a psychological or physiological disorder but part of a biological variation in the ejaculation latency (Intra Vaginal Ejaculation latency: IELT) in men with a possible genetic component as depicted in Fig. 4. According to this

hypothesis there are men who throughout their live always have an early ejaculation, men who always have retarded or even no ejaculation, and men who ejaculate in a range that can be characterized as having an average or 'normal' ejaculation time.

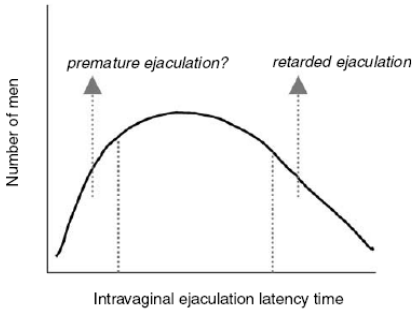

Fig. 4: Hypothetical distribution of intravaginal ejaculation latency times in men, with on either side of the 'Gaussian' distribution men suffering from premature ejaculation (left) and retarded or an-ejaculation (right).

Based on this hypothesis, we investigated whether such a biological variation does exist in male rats. Therefore, we investigated the presence of fast or 'rapidly' and slow or 'sluggishly' ejaculating rats in large populations of Wistar rats, an out-bred laboratory rat strain used standard in our lab in the study of sexual behavior. With regard to the variability in male rat sexual behavior, it appears that during a 'standardized' mating paradigm of 30-min (see Methods [74]), ejaculation frequencies in several experiments are distributed following a kind of Gaussian distribution as depicted in Figure 5, with approximately 10% of the rats displaying 'hypo-sexual' (0 ejaculations) and 10% displaying 'hyper-sexual' behavior (4 or 5 ejaculations) after at least four successive weekly sexual tests of 30-min. Based on this biological continuum in ejaculation frequencies we further investigated whether the by nature 'hyper-' and 'hypo-sexual' rats could be used as a model for human lifelong premature and delayed (an)-ejaculation, respectively.

To this end, we matched rats on either side of the Gaussian distribution into groups of 'sluggish' ejaculators and 'rapid' ejaculators. Interesting differences were found between these groups of rats on a variety of other parameters of sexual behavior, resembling clinical symptoms of men suffering from premature and retarded (an)-ejaculation. These rats displayed, in addition to differences in ejaculation frequencies, significant differences

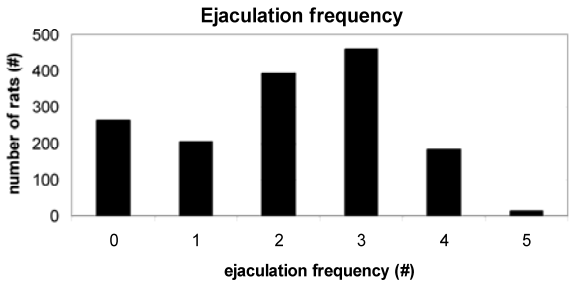

Fig. 5: Histogram displaying number of ejaculations during a 30-min mating test in a pooled population of male Wistar rats (total N=1564, obtained from 14 experiments). The data were collected during the fourth mating test, representing stable copulatory behavior. Male rats on either side of the Gaussian distribution were matched into 'sluggish' (0–1 ejaculation) and 'rapid' ejaculators (4–5 ejaculations) and compared to 'normal' ejaculators (2–3 ejaculations).

in their latencies to achieve ejaculation. Compared to 'normal' ejaculators, ejaculation latency was shortest in 'rapid' and longest in 'sluggish' ejaculators. Also, the number of mounts the animals displayed prior to ejaculation varied between groups. Sluggish ejaculators, although the majority did not achieve ejaculation, displayed the highest number of mounts, whereas 'rapid' ejaculators displayed the lowest number of mounts prior to ejaculation. In other words, the high number of mounts may suggest that these rats needed more vagino-penile sexual stimulation to get an ejaculation. In contrast, the rapid ejaculators ejaculated already after little vagino-penile sexual arousal. The differences in mounting behavior may suggest differences in penile sensitivity between groups as has been shown in men suffering from premature ejaculation [112]. Intromission frequencies and mount latencies, the latter often regarded as a putative index of sexual motivation [31] did not differ between 'sluggish', 'normal' and 'rapid' ejaculators, suggesting no differences in appetitive components of sexual behavior. We consider these different sexual phenotypes as endophenotypes, because they emerge in every population of rats

and are very stable over time. More research is of course needed to prove whether these sexual endophenotypes have particular genetic genotypes (polymorphisms) and are strictly under genetic control or dependent on environmental conditions and/or genotypic/environmental interactions. When the sexually inactive (retarded ejaculation) group was subsequently tested with a prosexual (0.8 mg/kg ip) dose of (±)-8-OH-DPAT, all animals were able to ejaculate, indicating that physical abnormalities do not underlie the lack of sexual activity [113]. When retested under no-treatment conditions 1 week after the 8-OH-DPAT treatment, rats were back to their original endophenotypic behavior, that is, sexually inactive. One could argue that aversive sexual experience during the first sexual tests might cause definitive changes in later sexual level of performance, but treating sexually naïve rats with 8-OH-DPAT before the first sexual test, which led to higher than normal sexual performance, did not change the final distribution (after four successive tests) in approx. 10% sluggish, 10% rapid and 80% normal ejaculators. This strongly suggests that, in normal rat populations, like in the human population, endophenotypes may exist with regard to basal sexual (ejaculatory) performance. Therefore, the behavioral differences found in sluggish and rapid ejaculators in rats strongly suggest commonalities with human lifelong premature and retarded ejaculation, namely differences in tactile stimulation (number of mounts needed to achieve ejaculation) and ejaculation latency. Although normal and rapid ejaculating rats have higher basal levels of sexual activity, they still were sensitive to the prosexual activity of 8-OH-DPAT. Even in the rapid ejaculators, the ejaculation latency is further decreased. This illustrates that presumably 5-HT_{1A} receptors play a role in sexual behavior of all three endophenotypes, although it is unlikely that the basal differences in sexual behavior are due to adaptive changes in this receptor.

We have started research into the pharmacological sensitivity of the various endophenotypic rat models, focussing particularly on the slow performing animals as model for retarded ejaculation in men [46]. There is currently no pharmacological treatment for delayed ejaculation and the animal model might give insight in the underlying mechanisms and help to develop effective treatment [46].

Our first results in slow or sluggish rats show that 5-HT_{1A} receptor agonists might be very effective in this endophenotype [113]. This study used a rather high dose of (±)-8-OH-DPAT and found that sluggish rats that did not ejaculate after at least 4 training tests, displayed more than 3 ejaculations/30-min under drug-conditions. Retesting these animals one week later without treatment showed that these animals returned to their original endophenotype. They were physically able to ejaculate but for some unknown reason were not able to ejaculate under normal conditions even when they learned/experienced the physical and psychological sequela of full sexual performance.

We ran a dose-response study of 8-OH-DPAT in sluggish rats (0.1, 0. 2 and 0.4 mg/k IP) and found a dose-dependent increase in sexual behavior (Fig. 6A), suggesting that 5-HT_{1A} receptors play an important role in the initiation of sexual behavior. This was confirmed by the dose-dependent facilitation of sexual behavior (number of ejaculations) by another (partial) 5-HT_{1A} receptor agonist buspirone (Fig. 6B) that also increased the number of ejaculations. In contrast, bupropion (Fig. 6C) was not able to significantly enhance the number of ejaculations at a broad dose range.

It seems possible to pharmacologically stimulate the sexually sluggish male rats, particularly by 5-HT_{1A} receptor agonist. Whether other mechanisms are also involved is largely unknown and subject for future research.

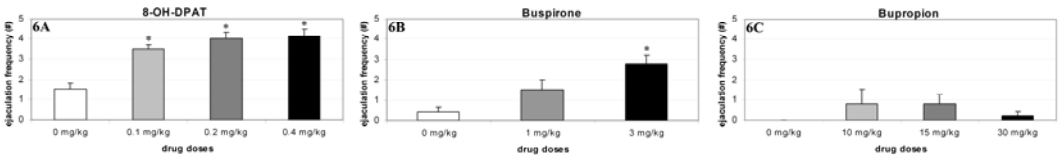

Fig. 6: Effects of (6A) 8-OH-DPAT (0.1; 0.2 and 0.4 mg/kg IP), (6B) buspirone (1 and 3 mg/kg IP) and (6C) bupropion (10, 15 and 20 mg/kg IP) on the number of ejaculations in a 30-min sexual behavior test in sexually sluggish male rats. Data are mean ± S.E.M. of mean. * = different than vehicle ($p < 0.05$).

Genetic influence in sexual behaviors in mice

The mouse is particularly popular for behavioral studies including sexual behavior because of the possibility to generate transgenics, knockouts, and knockdowns [114]. However, male sexual behavior in mice is markedly different from rats. Moreover, the differences in sexual behaviors between strains of mice [115] allow for better research of the influence of genes in sexual function and disorders. Of particular interest is the difference in sexual behaviors between two strains of mice that were used to create chromosomal substitution (consomic) strains; A/J (donor strain) and C57BL/6J (host strain). Female A/J mice display more sexual behavior anomalies than C57BL/6J are older at their first litter (73.3 days vs. 68.6 days), retire earlier from breeding (26 weeks vs. 30 weeks), and show higher percentage of unproductive matings (32.1% vs. 12.6%) (JAX® Mice Database). The generation of consomic strains in these mice [116] allows the search for quantitative trait loci putatively involved in sexual functions and sexual disorders. In these consomic strains, one chromosome of the C57BL/6J strain is replaced, through breeding, by the same chromosome of the A/J origin; generating 22 different strains.

We examined male sexual activities of many of the available consomic strains in a 60 minute test (once a week for 2 weeks) with an estrus-induced, ovariectomized female mouse. In the second testing with a female mouse, there are significant differences between the two parental strains. When compared to the C57BL/6J strain, A/J male mice show significantly less sexual activity with no ejaculations (Fig. 7) and only one of 12 mice performing intromissions (data not shown). Consomic strains 7, 17, and 19 also show significantly less ejaculation than the C57BL/6J mouse (Fig. 7). CS strains 17 and 19 also tend to exhibit less intromissions but this was not significant. These findings suggest that loci on certain chromosomes are involved in ejaculations and may provide a stepping stone in the search for the link between genes and sexual dysfunctions.

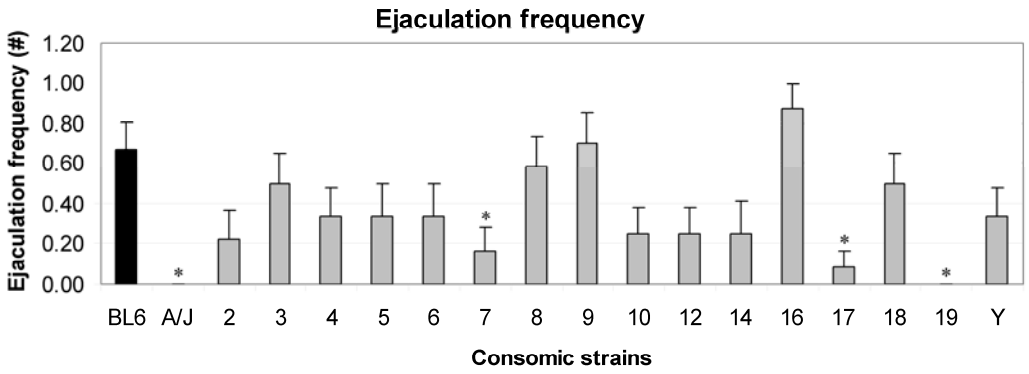

Figure 7. Mean ejaculation frequency in a 60 minute test with an estrus-induced female mouse**.** The different consomic strains are listed on the x-axis; each number represents the chromosome that was substituted in the C57BL/6J (BL6) strain. The data represent the mean ejaculation frequencies + S.E.M of at least 8 mice per strain. * = significantly different from BL6 (Chi-Square test with Bonferroni correction: $p < 0.05$).

Ejaculatory Dysfunctions in men

It should be noted that ejaculatory dysfunctions in men are complex and may arise from a combination of neurobiological, physiological and psychological factors. Recently, a stopwatch study in 491 nonselected men from five different countries (The Netherlands, United Kingdom, Spain, Turkey and USA) demonstrated the existence of an IELT continuum in men. The shape of the IELT distribution was positively skewed, with a median IELT of 5.4 min (range, 33 s to 44 min) [47]. Using the 0.5 and 2.5 percentiles as cutoff points for dysfunction definition, the study demonstrated a prevalence of IELTs less than 0.9 min in 0.5% and less than 1.3 min in 2.5% [61].

Psychopharmacological studies have shown that lifelong premature ejaculation is probably highly neurobiologically determined [117]. In addition, there is no well-controlled evidence-based research confirming a hard psychological basis of and successful

psychological treatment of lifelong premature ejaculation. However, it should be noted that there are men who despite normal and even long IELT durations still perceive themselves of having premature ejaculation. Recently, these men have been distinguished from men with lifelong premature ejaculation as a separate category, entitled premature-like ejaculatory dysfunction [69, 71]. Complaints of self perceived premature ejaculation in these men with normal ejaculation times are related to psychological, relationship and perhaps even cultural factors and should not be considered of neurobiological origin [69, 71]. But also, in lifelong premature ejaculation, a certain influence of psychological factors cannot be fully excluded a priori. The present findings of a natural occurrence of rapid, average (normal) and delayed ejaculation latencies in rats may be used as a model of human ejaculatory dysfunctions. The approach seems worthwhile, because as described earlier, several differences in neurobiology have been shown in rats that are hypo-sexual compared to controls. Also, in other fields of neuroscience, variability in certain behaviors has been focus of research to further elucidate the neurobiological determinants of, for instance, addiction (low grooming versus high grooming rats) [118], schizophrenia (apomorphine susceptible versus unsusceptible rats) [119], aggression (short attack latency versus long attack latency mice) [120] and anxiety and depression (8-OH-DPAT sensitive versus insensitive rats) [121].

Chapter 4: A male

rat paradigm for measuring

the potential sexual side-effects of antidepressants

Journal of Sexual Medicine (In Revision)

Abstract

Introduction. Antidepressant-induced sexual dysfunction adversely affects the quality of life of depressed patients and reduces compliance with treatment. Animal models provide an instructive approach for examining potential sexual side effects of novel drugs.

Aim. To characterize a novel animal procedure for predicting sexual side-effects of currently-used and novel psychopharmacological treatments for depression.

Methods. After 4 sexual trainings over 4 weeks, male Wistar rats displayed stable ejaculatory profiles. Rats displaying an average ejaculatory profile of 2-3 ejaculations in a 30 minute period were selected for acute, subchronic and chronic administration of mechanistically-distinct antidepressants (paroxetine, venlafaxine, bupropion, buspirone, DOV 216,303 and S32006).

Results. Paroxetine and venlafaxine, which inhibit serotonin (5-HT) reuptake and are known to disrupt sexual function in patients, inhibited sexual performance. By contrast, bupropion (a dopamine/noradrenalin reuptake inhibitor) and buspirone (a 5-HT_{1A} partial agonist), which do not cause sexual impairment in patients, did not interfere with sexual behavior. The novel putative antidepressants, DOV 216,303 (a triple monoamine reuptake inhibitor) and S32006 (a 5-HT_{2C} antagonist) likewise did not inhibit sexual behaviors.

Conclusions. Drugs that primarily elevate serotonin levels lead to sexual disturbances, while those that mainly increase levels of dopamine and noradrenalin are devoid of sexual side effects. This animal paradigm may be useful for evaluating the potential sexual side-effects of novel treatments for human depression.

Introduction

Antidepressant-induced sexual dysfunctions may lead to added distress in depressed patients, and if untreated, may exacerbate depression symptoms, affect quality of life, and lead to non-compliance with treatment [122-124]. Since the collection of data and testing in humans can be time-consuming or unethical, the development of predictive animal models would be a valuable tool to examine and predict the effects of currently-used and novel antidepressants on sexual behavior. Most sexual behavioral set-ups involve training a group of male rats and discarding non-copulators. The differences in sexual behaviors between individual male rats may affect the results of such experiments. In training large populations of male Wistar rats, we have observed stable sexual ejaculatory behaviors in individual animals [12, 17, 125]. They could be classified into ejaculatory categories of "slow" (0-1), "normal" (with 2-3), and "fast" (with more than 3) ejaculations in a 30 minute test with a receptive female rat. While slow animals were considered a putative model for retarded ejaculation, and the fast animals as a model for premature ejaculation [125], the "normal" ejaculating rats with *stable* ejaculatory behaviors provide a vehicle for examining either facilitatory or inhibitory profiles of currently-used and novel antidepressants. Since 2003, we have performed such male sexual behavior tests using a design in which we train male rats 4 times weekly in a "sex test" of thirty minutes: in this procedure, all males develop their own, stable "endophenotype" [12]. An experimental drug test consists of 14 days of daily treatment with a drug followed by a wash out test one week after stopping the last treatment. We measure the sexual performance of all rats after acute, 8 days (subchronic), 15 days (chronic) and 22 days (wash-out) of treatment. Using this experimental design, we tested various drugs which are either clinically used as antidepressants, viz. paroxetine (selective serotonin reuptake inhibitor; SSRI), venlafaxine (serotonin and noradrenalin reuptake inhibitor; SNRI), bupropion (dopamine and noradrenalin reuptake inhibitor; DNRI) and buspirone (5-HT_{1A} receptor agonist), as well as putative antidepressants in development for clinical use (DOV-216,303, a triple reuptake inhibitor; TRI) and S32006 (5-HT_{2C} receptor antagonist [126]). Antidepressants in general, and specifically SSRIs (paroxetine, fluoxetine, (es)citalopram, fluvoxamine,

sertraline), are notorious for their sexual side effects [25, 127-129]. Although venlafaxine has been promoted as having less sexual side effects, emerging data indicate no substantial difference from the SSRIs [130, 131]. Bupropion, lacking effects on the serotonergic transporter (SERT), has been suggested to have few sexual side effects, and even to favour sexual function [132-134]. Buspirone, a partial 5-HT_{1A} receptor agonist mainly used in anxious patients but that also possesses antidepressant properties [135-138], has not been associated with sexual side-effects [139, 140]. Further, in animal studies, prosexual effects have been reported [141-143]. The putative antidepressant, DOV-216,303, a triple monoaminergic reuptake blocker [144, 145] may have less or no sexual side effects because of the dopaminergic stimulatory profile inherent in its mechanism of action. 5-HT_{2C} receptor antagonists like S32006 display an antidepressant profile in preclinical models and elevate extracellular levels of dopamine (DA) and noradrenalin (NA) but not serotonin (5-HT) [146-149]. They have been claimed to possess stimulatory sexual effects, or at least not to compromise sexual function [138, 146, 150].

Antidepressants have a delayed onset of therapeutic action [138]. Although some side-effects of antidepressants emerge rapidly (e.g. nausea, dizziness), sexual side effects seem to mirror the antidepressant profile and emerge over time [69]. An ideal animal model predicting sexual side effects should follow such a time course: acutely no or marginal effects, with an inhibitory effect on sexual behavior occurring after subchronic (one week) or chronic (two weeks) treatment. If the model is also able to detect pro-sexual activities of psychotropics, this would further support its use in predicting their putative influence on sexual behavior in humans.

Materials and Methods

Animals

Seven separate cohorts of 120 male Wistar rats each were derived at about 8 weeks old from Harlan Laboratories in The Netherlands. The animals were all group-housed with 4 per cage in reversed day-night lighting conditions (lights off from 6:00AM till 6:00PM). Food and water was provided ad libitum. After habituation to the lighting schedule for at

least a week, males were trained once a week for 4 weeks, without injection, with an oestrus female rat in an observation cage (30*60*40 cm) with a Plexiglas front for 30 minutes. Females were brought into behavioral oestrus (showing both proceptive and receptive behavior) by injecting 50μg estradiol-benzoate in the nape of the neck 36-52h prior to the test. The number of ejaculations over the last 2 training test was used to designate animals either as low, medium (normal) or high ejaculating. In the present experiments, drugs were tested on 'normal-performers' with on average 2-3 ejaculations/test during the last training tests. The drug studies began after this training period. In all drug studies, there were 12 rats per treatment group and each drug was tested against a vehicle and a standard dose of paroxetine (10 mg/kg).
Experiments were performed in accordance with the Dutch guidelines for care and use of laboratory animals, and were approved by the Ethical Committee for Animal research of the Faculties of Veterinary Medicine, Pharmaceutical Sciences, Chemistry and Biology at Utrecht University, The Netherlands.

Drugs

Paroxetine hydrochloride (Hexal Pharma Nederland B.V.) (2.5, 5, 10 mg/kg), Venlafaxine hydrochloride (Efexor®)(5, 10, 20, 40mg/kg) and bupropion hydrochloride (Zyban®)(7.5 and 15mg/kg) were obtained through a pharmacy. The pills were crushed finely and suspended in the vehicle saline and injected PO). Buspirone hydrochloride (Sigma-Aldrich)(1, 3, 10mg/kg) was dissolved in the vehicle saline and injected IP. DOV 216,303 ((+/−)-1-(3,4-dichlorophenyl)-3-azabicyclo[3.1.0]hexane hydrochloride) was obtained through Sepracor Inc. (Marlborough, USA) and dissolved into the vehicle water and injected IP. S32006 (N-pyridin-3-yl-1,2-dihydro-3H-benzo[e]indole-3-carboxamide) was synthesized at the Institut de Recherches Servier (France) and dissolved in the vehicle water with a few drops of Tween 80.

Behavioral test

Seven experiments, lasting 2 months each, were performed over a period of 4 years. In each experiment, the drug was administered for 14 days and the behaviors of the animals

were recorded on days 1, 8, and 15 (drug was administered 30 minutes before the introduction of the female rat). One week after the last treatment, the animals were tested again without any administration of drugs unless noted below:

Exp. 1: Venfaxine (5, 10, and 20mg/kg), paroxetine (5 and 10mg/kg), and vehicle saline (all orally administered) started in December 2004.

Exp. 2: S32006 (0.16, 0.63 and 2.5mg/kg), paroxetine (10 mg/kg) and vehicle (water with Tween 80) (all administered IP) started in August 2005.

Exp. 3: DOV 216,303 (5, 10, and 20mg/kg), paroxetine (10 mg/kg)), and vehicle (water (all administered orally) started in March 2006.

Exp. 4: Buspirone (1, 3, and 10mg/kg), paroxetine (10 mg/kg) and vehicle (saline) (all administered IP) started in September 2006.

Exp. 5: Bupropion (7.5 and 15mg/kg), paroxetine (10 mg/kg) and vehicle (saline) (all administered IP) started in November 2006. The one week washout test was not performed.

Exp. 6: Venlafaxine (40mg/kg), paroxetine (10mg/kg) and vehicle (saline) (all administered orally) started in October 2008.

Exp. 7: Paroxetine (2.5 and 10mg/kg) and vehicle (saline) (all orally administered) started in March 2009.

In Experiments 5, 6 and 7 other treatments groups were tested but not reported here.

Experiments per experimental day (Days 1, 8, 15 and 22) were performed over two successive days. Testing was performed between 9:00AM and 3:00PM in the dark phase of the light-dark cycle under dim red lighting. Males were injected and immediately placed into the experimental cage for habituation. After 30 minutes, an oestrus female was placed into the cage and the behavior of the male was scored for the next 30 minutes. On non test days, the animals were injected between 9:00AM and 2:00PM.

The following parameters were scored using the Noldus Observer® 5.0 program: the times and frequencies of mounts, intromissions, and ejaculations per test of 30 min. From these data files the following parameters were deduced/quantified: ejaculation frequency (#); latency to 1st mount (sec); latency to 1st intromission (sec); mount frequency (#); intromission frequency (#); latency to the 1st ejaculation (sec) - time from the first mount

or intromission to the first ejaculation; post-ejaculatory latency (PEL)(sec) - time from the first ejaculation to the first mount or intromission (whichever comes first) from the second ejaculatory series and the copulatory efficiency (CE) that is calculated as CE = (#Intromissions/(#Intromissions+#Mounts))*100%. All parameters are measured again for each ejaculation series. All data are represented as mean ± S.E.M.

Missing values

Some issues may arise in the testing of male rat sexual behaviors. Male rats have repeated mounts and intromissions with a receptive female, usually ending with an ejaculation. In order to find comparable pharmacodynamics and kinetics profiles in drug studies, a fixed test duration of 30 minutes was chosen. If some treatments cause low ejaculatory behaviors (zero ejaculations), some animals cannot be used for the statistical testing. Maximum values of the test duration (1800 seconds or 30 minutes) for some latencies (e.g. ejaculation latency, mount and intromission latency, post-ejaculatory latency) can be used, although this is dubious. In addition, the use of the mount and intromission data from these non-ejaculating rats is questionable since it is not known if the rat may achieve an ejaculation. These data could be considered artificial and are not *usually* used in statistics. In some experiments where the drug inhibited ejaculations, statistical testing in the data from higher ejaculation series could not be performed since few animals achieve a 2nd ejaculation. These statistical problems were encountered in trials where the drug inhibited sexual behaviors severely. In cases where the majority of the animals had no ejaculations, data value of 1800 seconds was imputed for latency to 1st ejaculation, mount and intromission latency, and post-ejaculatory latency, and include the frequency values for all animals in the statistics. In these cases, the potent inhibitory drug effect necessitates the use of these imputed values. We chose to skip statistical analysis if less than 7 animals in a certain drug-treated group were left for 2nd ejaculatory series parameters.

Statistics: All data are analyzed using one-way ANOVA (SPSSv11.0) of individual test days, followed by Bonferroni-post-hoc tests in case of overall significant effects. In

experiments where paroxetine was used as the negative reference compound, a separate one way ANOVA was perform comparing it to the vehicle to ensure that the experiment was properly controlled. A multivariate repeated measure ANOVA, with the Greenhouse- Geisser correction, was used to compare the treatments over the different test days.

Results:

Since the experiments were performed over a period of 4 years with different routes of administration and slightly different vehicles, we performed an general linear model repeated measures statistical test of all the vehicle treated animals over the 7 experiments to see if there were any differences in the behavior of the animals over acute (30 minutes after introduction of the drug on day 1 of treatment), subchronic (8 days into the treatment), chronic (15 days into the treatment) and washout testing periods (Fig. 1). During each of the testing periods, the vehicle treated animals did not differ significantly in ejaculation frequency ($F_{(18, 171)}$ = 1,253, p= 0.232) and latency to 1st ejaculation ($F_{(15, 168)}$ = 1,360, p = 0.185; data not shown). With a lack of differences found between the vehicle treatments for these important behaviors, we will discuss the results for each drug separately.

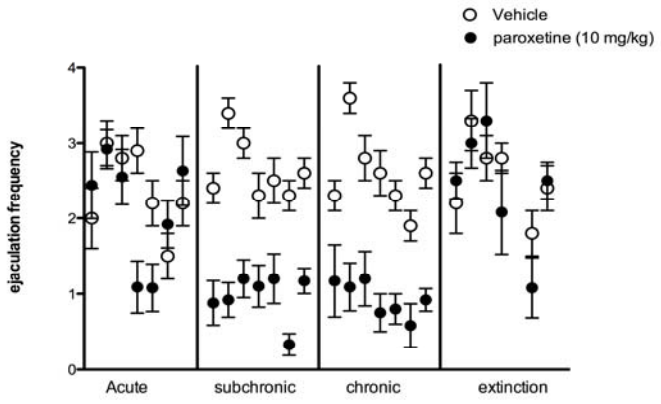

Figure 1: Ejaculation frequencies of the vehicle and paroxetine treated animals over 7 experiments. In each experiment, normal male Wistar rats are treated with the vehicle chronically and tested in a 30 minute session. For each treatment day the experiments 1 to 7 are presented from left to right.

Paroxetine

Paroxetine (10 mg/kg) was the standard reference dose in each of the 7 constructed experiments. We did no extensive dose range study in one single experiment but the dose range study came from data for experiments 1, 6 and 7. During acute treatment of paroxetine, none of the doses tested (2.5; 5 and 10 mg/kg) had an effect in any of parameters examined. Fig. 2 shows the effect on the number of ejaculations the 4 experimental days. Paroxetine significantly reduced the number of ejaculations after subchronic and chronic treatment at the 5 and 10 mg/kg doses, whereas it inhibited it at 2.5 mg/kg only chronically. One week after cessation of treatment, all groups had returned to normal. At the mid and high doses (5 and 10mg/kg), paroxetine strongly inhibited sexual behaviors in many of the parameters examined after subchronic and chronic treatments (Table 1: data not shown for the 2.5 mg/kg dose). After subchronic and particularly after chronic administration of 5 and 10 mg/kg paroxetine the intromission latency, the ejaculatory latency and the 1st post-ejaculatory latencies were enhanced in line with the strong sexually inhibitory effects of paroxetine. The number of animals that reached at least one ejaculation also decreased considerably, making comparisons of the 2nd ejaculation series impossible. The low dose of paroxetine (2.5 mg/kg) only had effects on ejaculation frequency and the latency to 1st ejaculation (data not shown) after chronic administration.

Figure 1 summarizes all the reference (10 mg/kg) dose of paroxetine for all the different experiments at all treatment days. Occasionally, after acute dosing, paroxetine had some inhibiting effects on the number of ejaculations (in two experiments). However, in the other 5 experiments, it did not affect sexual behavior. Overall, this dose did not affect sexual behavior. After subchronic and chronic administration in all groups, paroxetine clearly inhibited the number of ejaculations (and other parameters) compared to the vehicle treatment. One week after cessation of treatment (washout) all groups were back to normal and there were also no differences between the washout and acute treatment data.

Table 1: Effects of paroxetine (5 and 10 mg/kg, PO) on various sexual behavior parameters of normal 'endophenotypic' male Wistar rats in a 30 minute sex test with an estrus female rat. EF= ejaculation frequency, ML= latency to 1st mount, IL= latency to 1st intromission, MF= 1st series mount frequency, IF= 1st series intromission frequency, EL= latency to 1st ejaculation, PEL1= 1st series post-ejaculatory latency, and CE1= 1st series copulatory efficiency. * = $p<0.05$ ANOVA significance when compared to the vehicle.

parameter	VEHICLE	PAR 5	PAR 10	ANOVA
Acute				
EF	2.0 ± 0.4	2.1 ± 0.3	2.4 ± 0.4	0.697
ML	65.7 ± 38.0	7.6 ± 3.3	25.4 ± 15.1	0.295
IL	216.3 ± 176.6	89.3 ± 54.5	28.0 ± 8.8	0.520
MF	11.6 ± 4.7	19.4 ± 4.1	22.6 ± 8.6	0.438
IF	11.1 ± 4.5	8.6 ± 1.0	7.0 ± 0.9	0.586
EL	619.9 ± 200.1	661.7 ± 111.9	715.8 ± 198.4	0.932
PEL1	584.3 ± 203.5	330.0 ± 37.1	636.7 ± 221.0	0.517
CE1	57.0 ± 8.9	38.0 ± 6.3	39.6 ± 8.3	0.173
Subchronic				
EF	2.4 ± 0.2	1.1 ± 0.3*	0.9 ± 0.3*	0.000
ML	10.1 ± 4.0	10.6 ± 3.1	4.2 ± 0.7	0.323
IL	55.9 ± 30.2	265.2 ± 193.4	616.6 ± 290.9	0.127
MF	10.8 ± 2.5	33.4 ± 8.9	32.0 ± 8.3	0.042
IF	6.7 ± 0.7	9.1 ± 2.1	5.9 ± 1.9	0.358
EL	369.8 ± 62.5	1340.5 ±136.6*	1323.0 ±171.5*	0.000
PEL1	305.5 ± 13.1	994.5 ± 255.5	904.7 ± 263.5	0.039
CE1	45.6 ± 6.6	25.2 ± 7.3	20.0 ± 8.5	0.048
Chronic				
EF	2.3 ± 0.2	1.2 ± 0.3*	1.2 ± 0.5*	0.002
ML	40.6 ± 33.4	34.5 ± 14.7	6.6 ± 1.8	0.648
IL	18.7 ± 7.1	451.0 ± 231.3	89.5 ± 35.0	0.109
MF	11.4 ± 3.2	25.2 ± 4.6	32.5 ± 7.7*	0.019
IF	6.5 ± 1.0	6.9 ± 1.5	4.2 ± 1.9	0.449
EL	347.5 ± 38.1	1125.6 ±155.8*	1350.9 ±182.3*	0.002
PEL1	294.2 ± 15.0	1060.5 ±246.9*	1281.9 ±327.9*	0.008
CE1	46.6 ± 10.1	25.6 ± 7.1	17.6 ± 7.6	0.079
Washout				
EF	2.2 ± 0.4	2.6 ± 0.2	2.3 ± 0.3	0.688
ML	14.2 ± 3.2	12.1 ± 6.5	17.1 ± 7.6	0.662
IL	33.5 ± 17.4	70.3 ± 31.7	39.8 ± 23.2	0.489
MF	23.8 ± 4.8	23.5 ± 4.4	17.2 ± 4.1	0.356
IF	8.2 ± 0.7	5.8 ± 0.7	7.9 ± 0.6	0.425
EL	555.9 ± 131.7	583.6 ± 115.5	497.0 ± 86.3	0.802
PEL1	379.1 ± 130.0	433.3 ± 124.9	273.2 ± 14.2	0.638

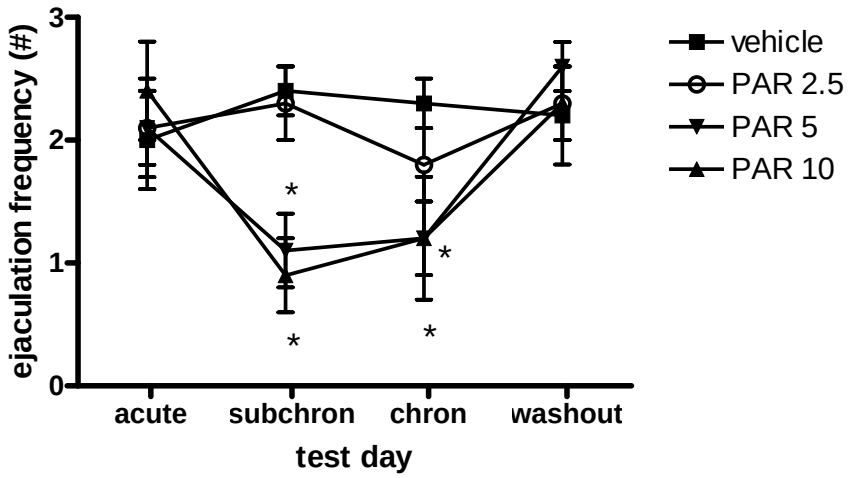

Figure 2: Effects of chronic administration of paroxetine (2.5, 5, and 10 mg/kg, PO) over 4 treatments days on ejaculation frequency of normal 'endophenotypic' male Wistar rats in a 30 minute sex test with an estrus female rat.

Venlafaxine (Fig. 3)

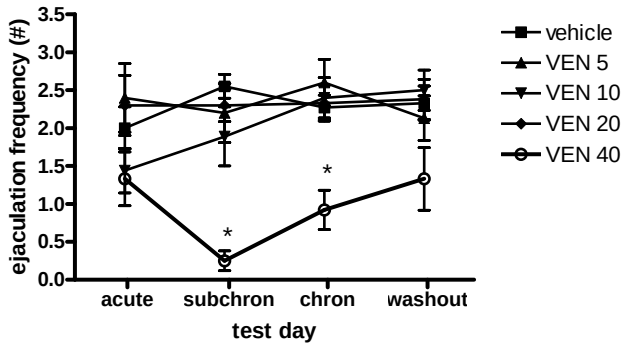

Figure 3: Ejaculation frequency of chronic administration of venlafaxine over 4 experimental days. In each experiment, normal 'endophenotypic' male Wistar rats are tested in a 30 minute session with an estrus female rat.

Venlafaxine dose ranges were performed over experiments 1 and 6. As with paroxetine, venlafaxine did not exert any effects during acute treatment in any doses. The lower doses (5, 10 and 20mg/kg) did not exert any effects during any of the treatment days. The high dose (40mg/kg), however, reduced sexual behaviors during the subchronic and chronic treatments and had a

comparable behavioral profile to paroxetine 5 or 10 mg/kg.

Bupropion (Fig. 4)

Acutely, bupropion showed an increase in ejaculation frequency at the highest dose (15mg/kg) tested. No other behavioral parameters were affected (data not shown). After subchronic and chronic treatment, this initial increase dissipates but no significant improvements or inhibitions are noted in any of the treatment doses.

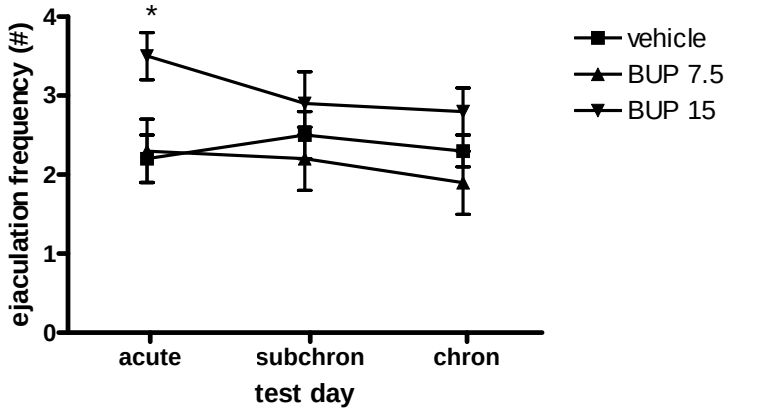

Figure 4: Effects of chronically administered bupropion (7.5 and 15mg/kg, IP) on ejaculation frequencies of normal 'endophenotypic' male Wistar rats, tested in a 30 minute session with an estrus female rat.

DOV 216,303

Vehicle treated animals maintained an average ejaculation frequency between 2.8 and 3.0 during the experimental duration. DOV216,303 showed no effects whatsoever during the entire test when compared to the vehicle (data not shown).

Buspirone

Vehicle treated animals maintained an average ejaculation frequency between 2.3 and 2.9 during the experimental duration. Acutely, buspirone had small stimulating effects on sexual behaviors. All the doses showed less intromissions than the vehicle treatment with significance found in the mid dose (3mg/kg). During subchronic treatment, there were further enhancements in sexual behaviors with all doses showing a decrease in latency to 1st ejaculation. In addition, the mid dose (3mg/kg) and the high dose (10mg/kg) showed significantly lower mount and intromission frequencies which consequently led to higher copulatory efficiency. This profile of activity did however not lead to an increased number of ejaculations within the 30-min test period.

S32006

Vehicle treated animals maintained an average ejaculation frequency between 3.0 and 3.6 during the experimental duration. The only significant result uncovered was an increase in 1st series mount frequencies in all 3 doses of S32006 during the subchronic treatment. This effect was not significant at the chronic dosing point.

Discussion

We have previously shown [17, 151] that male Wistar rats display, when sexually trained 4 times, a stable level of individual sexual behavior. Using this paradigm in large cohorts (N=120) of male Wistar rats [12], we continuously find a distribution in the number of ejaculations of individual rats from zero to 5 ejaculations per 30-min test. We have postulated that rats displaying 0-1 ejaculation/test can be classified as 'slow' ejaculators, whereas animals displaying 4 or 5 ejaculations/test are 'fast' ejaculators. The intermediate group displaying 2-3 ejaculations/test, which is always the vast majority of the animals in a cohort, is classified as "normal" ejaculators. The latter groups have been used over the last decade to study the effects on sexual behavior of various psychotropic drugs, focusing on antidepressants.

In the present study, we present 7 cohort studies performed over a period of 5 years. All rats were similarly trained and in all cohorts we found the typical inverse U-shaped

distribution of 'sexual endophenotypes'. In all separate studies, we were able to select at least 60 stable 'normal' sexually performing (2-3 ejaculations/30min) rats. These animals displayed stable sexual behavior during the individual experiments. All control groups (Fig. 1) showed a relatively constant level during successive vehicle tests (acute; subchronic, chronic and wash-out) emphasizing the stability of individual sexual behavior. Apparently, individual rats do not further improve their sexual performance with increasing sexual experience, supporting the notion of fixed individual "sexual endophenotypes". An additional finding is the stability of sexual behavior over years. In all of our experiments (described in this paper and in previous papers[17, 151]), we have always found - sometimes with variability in the course of a year (seasonal variation?) - the same level of sexual performance within the cohorts. In general, the number of 'slow' ejaculators is larger than the number of 'fast' ejaculators [12], but we always have at least 60 animals in a cohort that, upon training, exhibit at least 2 ejaculations per 30 min-test). Such a paradigm of sexual behavior is ideally suited to test the effects of psychotropic drugs, because both inhibitory and stimulatory effects can be detected. Moreover, the paradigm tests sexual behavior of individual rats both after acute as well as (sub) chronic administration, paralleling the putative onset of action of the drugs tested. In our paradigm, we use the SSRI paroxetine as the standard reference drug and 10 mg/kg as the reference dose: this always has inhibitory effects after (sub) chronic dosing (fig. 1 and 2). In individual experiments, this dose sometimes has minor inhibitory effects after acute administration, but they are inconsistent.

Paroxetine (and most other SSRIs [25, 129, 152]) have strong inhibitory effects on sexual behavior of humans, both in healthy individuals [69] and in depressed patients [152-156]. Paroxetine seems to have sexual inhibitory effects after at least one week [69] or several weeks (in depressed patients). In our rat paradigm, paroxetine has a dose-dependent inhibitory effect on sexual behavior as can best be seen by its effects on the number of ejaculations/test (Fig. 1 and 2). The 5 and 10 mg/kg doses seem to be equipotent, whereas the 2.5 mg/kg dose only inhibits sexual behavior after two weeks of administration. Remarkably, acute administration of paroxetine does not reliably inhibit sexual behavior. This parallels the human situation where at least one week of administration is needed to

affect sexual behavior [8, 14]. One week after cessation of treatment of the sexual performance of all paroxetine treated groups returned to the control level, showing the absence of irreversible or delayed effects. In humans, no data are available as to how fast the sexual side effects of SSRIs wane after cessation of treatment, but data in healthy males with premature ejaculations suggest that after 6 weeks of treatment, sexual inhibitory effects subside within a week [27]. In this aspect, these sex and effects seem to be relatively independent of the antidepressant effects of SSRIs which take time to emerge, but after long term and effective treatment, depression seems to recur only (if at all) after a long period following cessation of treatment [157, 158]. This might also indicate that the sexual inhibitory effects of SSRIs in depressed patients could be blocked by pharmacological treatment independent of therapeutic effects.

Venlafaxine is a serotonin-noradrenalin reuptake inhibitor (SNRI) and its effects on sexual behavior may be different from pure SSRIs. Although at lower doses, venlafaxine did not affects sexual behavior, it had a comparable sexual inhibitory profile as to paroxetine at the highest dose tested (40 mg/kg), suggesting that it may also compromise sexual performance in humans. Human data [130, 131] show that venlafaxine, at doses that exert antidepressant efficacy, induces comparable sexual side effects to SSRIs including fluoxetine and paroxetine. Venlafaxine most potently increases 5-HT levels and only at higher doses acts as a noradrenalin-reuptake blocker [138, 159, 160]. This might explain its profile in our rat paradigm consistent with sexual side-effects of venlafaxine in humans [130]. On the other hand, the limited data available on venlafaxine in premature ejaculation does not suggest that it has a strong sexual inhibiting effect in healthy men [161, 162].

Bupropion, a DNRI [163], exerted a sexual stimulatory profile in our paradigm, particularly after acute dosing. The number of ejaculations/test increased considerably after acute administration of 15 mg/kg. After (sub) chronic dosing there was still some sexual stimulating activity, although less than after acute dosing. The effect of bupropion was only found in an increase in ejaculations; all other parameters showed a trend towards prosexual effects but this never reached significance. This suggests that the relatively weak blockade of the dopamine transporter (DAT) with bupropion [164] might not be strong enough to lead to permanent sexual stimulatory activity and that some tolerance for

prosexual activity had occurred. In a similar experiment [108], the mixed D_2/D_3 dopamine receptor agonist apomorphine showed a comparable behavioral profile, although revealing a somewhat stronger prosexual profile than bupropion. The number of ejaculations was only enhanced acutely, but some other sexual parameters were improved after (sub) chronic administration of apomorphine. This illustrates that dopaminergic mechanisms may exert prosexual activity that potentially antagonizes inhibitory effects induced by SSRIs [165, 166]. Human data on bupropion show that it is not itself associated with sexual inhibitory effects [132], and that it can alleviate SSRI-induced sexual side effects [133, 138, 167].

DOV216,303 is a triple monoaminergic reuptake inhibitor [144, 168] that blocks NE, 5-HT and DA reuptake: it was designed to combine the antidepressant efficacy of SNRIs with dopamine reuptake blockade thereby reversing several side effects, including sexual ones [144, 145]. DOV216,303 has been tested in animal paradigms of depression and shown antidepressant efficacy [144, 169, 170]. Pilot studies in depressed patients have seen some efficacy of DOV216,303 in depression and significantly reduced HAM-D scores similar to citalopram [144]. At doses that exert antidepressant effects in animal models of depression, DOV216,303 had no effects on sexual behavior. This suggests that dopaminergic stimulation (*via* DAT blockade) antagonizes inhibitory effects on sexual behavior induced by SERT blockade. A putative contribution and/or interaction between SERT and noradrenalin transporter (NET) blockade is possible, but requires exploration.

Buspirone, a partial $5\text{-}HT_{1A}$ receptor agonist and weak dopamine D_2 receptor partial agonist, is a clinically effective anxiolytic with antidepressant properties [138, 171]. $5\text{-}HT_{1A}$ receptor agonists, including buspirone, have prosexual activity in rats upon acute administration [17, 35]. However, to our knowledge chronic administration studies of sexual behavior have not been performed. The present data suggest that low doses of buspirone, that are also antidepressant in animal depression paradigms, have mild prosexual activities. Human data on the sexual side-effects of buspirone are scant and do not suggest inhibitory activity [137]. Most human studies that have examined buspirone as a putative treatment for sexual side-effects induced by SSRIs suggest that buspirone is

modestly beneficial [139, 172]. These human data seem to concur with our data; either no effect, or mild stimulatory activity.

S32006 is a novel benzourea derivative and 5-HT_{2C} receptor antagonist, and it has shown antidepressant and anxiolytic properties in various rodent behavioral models after acute and chronic administration [126]. At doses that display antidepressant activity, S32006 does not have any effect on sexual behavior. Because 5-HT_{2C} receptor agonists clearly inhibit the sexual behavior of rats [36, 173], it was thought that an antagonist may have some prosexual activity: though this was not the core, interaction studies with SSRIs would be of interest [138, 146].

Conclusions

The paradigm used here to examine the inhibitory and stimulatory effects of antidepressant drugs on the sexual behavior of male rats relates well to their known and predicted effects in humans. In line with clinical experience, marked blockade of SERT (paroxetine and venlafaxine) interfered with male sexual behavior, in contrast to the DA/NAT reuptake inhibitor, bupropion. Further, other drugs that primarily increase levels of DA and NA *vs.* 5-HT (the 5-HT1A agonist, buspirone; the TRI, DOV216,303 and the 5-HT2C antagonist, S32006) exerted no detrimental influence or a mild stimulatory effect on sexual performance, and they are predicted to have little or no sexual side-effects in men. It is suggested that blockade of DAT or NAT, as well as 5-HT_{2C} receptor blockade, would be a useful avenue to clinically explore for the reduction of SERT-mediated sexual inhibition. Blocking DAT might be particular useful to overcome the sexual inhibition due to disruption of SERT. These possibilities also justify investigation employing the present experimental model and combinations of drugs. More generally, exploration of the present paradigm, if possible in parallel with therapeutic investigations, should provide important insights into the influence of antidepressants and other classes of psychotropic agent on male sexual function.

Chapter 5

The serotonin transporter plays an important role in male sexual behavior: a study in serotonin transporter knockout rats

J Sex Med. 2010 Aug 5

Abstract.

Aim. To investigate the putative role of the serotonin transporter (SERT) in male sexual behavior.

Methods. After extensive sexual training, the effects of the 5-$HT_{1A/7}$ receptor agonist ±8-OH-DPAT, the 5-HT_{1A} receptor antagonist WAY100,635 and a combination of both on sexual behaviors of homozygous ($SERT^{-/-}$) and heterozygous ($SERT^{+/-}$) knockout and wildtype ($SERT^{+/+}$) male Wistar rats were examined.

Results. $SERT^{-/-}$ had lower basal ejaculation frequencies than $SERT^{+/-}$ and $SERT^{+/+}$ animals. ±8-OH-DPAT enhanced sexual performance in all 3 genotypes to the same extent. WAY100,635 dose-dependently inhibited sexual behavior in all 3 genotypes with significant dose to genotype interactions. WAY100,635 exerted the strongest effects in $SERT^{-/-}$ animals. The combination of a dose range of ±8-OH-DPAT and a selected dose of WAY100,635 revealed only partial antagonism by ±8-OH-DPAT of the sexual inhibitory effects of WAY100635.

Conclusions. Absence of the serotonin transporter reduces basal ejaculatory performance in male rats. Pharmacological experiments suggest that separate pools of 5-HT_{1A} receptors regulate different aspects of sexual performance in male rats. 5-HT_7 receptors may play a minor role in the partial recovery of sexual behavior after combination of ±8-OH-DPAT and WAY100,635.
The $SERT^{-/-}$ rat may be a model for chronic SSRI treatment, delayed ejaculation, anorgasmia, and/or low libido.

Introduction

The sexual side effects after long term use of selective serotonin reuptake inhibitors (SSRIs) in humans are well documented [7, 25, 129, 134] with the main complaints being delayed or absent ejaculations, and/or low libido in men. Rats also show altered sexual behavior when given SSRIs chronically with increases in ejaculation thresholds (decreased ejaculation frequencies, and increased mount and intromission frequencies, and latencies to ejaculations) [5, 12]. SSRIs work by blocking the reuptake of 5-HT by SERT, and thus increasing the availability of 5-HT in the synaptic cleft. The sexual side-effects of SSRIs are thought to be attributed to desensitization of serotonin (5-Hydroxytryptamine; 5-HT) receptors, in particular 5-HT_{1A} [174, 175] and 5-HT_{2C} receptors [176]. The availability of genetically modified rats that lack the serotonin transporter (SERT) [177] offers the possibility to study the effects of life long absence of SERT on basal sexual activity and the functional status of 5-HT_{1A} receptors in these animals. We hypothesize these animals have disturbed sexual behaviors, similar to rats chronically treated with SSRIs, and that these animals will show an altered 5-HT_{1A} receptor functioning.

Therefore, we used homozygous (-/-) and heterozygous (+/-) SERT knockout rats, which express 0% and 50% of wild type SERT levels, respectively [177]. These reductions in SERT expression level (and probably function) are within the range of the percentages of occupied SERT binding sites following chronic usage of an SSRI in human studies [178, 179]. $SERT^{-/-}$ rats have lower 5-HT tissue levels, reduced depolarization-induced 5-HT release, and increased basal extracellular 5-HT levels [177]. Despite the reduction in SERT protein, tissue levels of 5-HT in the $SERT^{+/-}$ rats do not differ from wild types [180], although a reduction in the kinetics of 5-HT removal from the extracellular space in these animals is very likely. $SERT^{-/-}$ rats show increased anxiety and depression-like behaviors [180].

In the present paper, we compared the sexual behaviors of $SERT^{-/-}$ and $SERT^{+/-}$ rats with that of wild type ($SERT^{+/+}$) rats. We tested a receptor agonist (±8-OH-DPAT)[181] and a silent receptor antagonist (WAY100,635) to investigate possible adaptive changes in the 5-HT_{1A} receptor system. Because ±8-OH-DPAT is also a weak agonist for 5-HT_7 receptors[182], whereas WAY100,635 is a highly selective 5-HT_{1A} receptor antagonist [183,

[184], without any affinity for the 5-HT_7 receptor, combination of a selected dose of WAY100,635 with a dose range of ±8-OH-DPAT may support the specificity of the 5-HT_{1A} receptor in the sexual changes but also may give clues to a possible contribution of the 5-HT_7 receptor in male sexual behavior.

Materials and Methods

Animals

Through target-selected ENU-induced mutagenesis, the SERT knockout rat on a Wistar background was generated [177, 185]. All animals were bred and reared at the Central Laboratory Animal Institute at Utrecht University. Test animals of the three genotypes (+/+, +/-, -/-) were bred by heterozygous ($SERT^{+/-}$) crosses. After weaning at 21 days of age, small ear tissue samples were collected for genotyping[177]. All animals were housed 4 per cage (with at least one of each genotype) in reversed 12/12-h day/night-cycle (lights off at 7.00 am) and food and water was available *ad libitum*. $SERT^{+/+}$ (n = 16), $SERT^{+/-}$ (n = 16), and $SERT^{-/-}$ (n = 16) rats were used for comparison. Animals were approx. 3 months old when training started. Animals were weighed before every drug experiment. Outbred female Wistar rats (Harlan, Zeist) were used as stimulus rats and oestrus was induced with a single injection of 50µg of estradiol benzoate in sesame oil saturated with lecithin 36-42 hours prior to testing.

Experiments were performed in accordance with the Dutch guidelines for care and use of laboratory animals, and were approved by the Ethical Committee for Animal research of the Faculties of Veterinary Medicine, Pharmaceutical Sciences, Chemistry and Biology at Utrecht University, The Netherlands.

Drugs

±8-hydroxy-2-(di-n-propylamino)tetraline (±8-OH-DPAT) (Sigma-Aldrich) and N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]- N-(2-pyridyl)cyclohexanecarboxamide (WAY100,635) (Sigma-Aldrich) were dissolved in 0.9% NaCl. Doses of WAY100,635 were selected based on previous behaviorally inactive (although active in blocking 8-OH-DPAT effects) doses[186] and a high dose, to ensure total blockade of 5-HT_{1A} receptors.

Doses of ±8-OH-DPAT were selected based on previously active doses[12, 18, 187]. ±8-OH-DPAT (s.c.) and WAY100,635 (i.p.) were injected at a dose volume of 2ml/kg, 5 minutes and 30 minutes, respectively, before the introduction of the female stimulus rat.

Experimental design and testing

Males were trained for 30 minutes once weekly for 7 consecutive weeks against an oestrus female in an observation cage (30 x 40 x 60 cm) with a Plexiglas front. The floor of the test cage was covered with bedding and was not refreshed for each test session. Sex tests were performed as described previously [169, 188]. The following 3 experiments were performed on these 16 males per genotype:

Experiment I: ±8-OH-DPAT in 3 doses of 0.01, 0.1, and 1 mg/kg;

Experiment II: WAY100,635 in 3 doses of 0.1, 0.3, and 1 mg/kg, and

Experiment III: combination of 0.1 mg/kg WAY100,635 and the vehicle and 3 increasing doses of ±8-OH-DPAT (0.01, 0.1, and 1.0 mg/kg).

The experiment lasted 14 weeks with a one week washout period between each experiment. Drug testing was performed using a within animal design (latin square) where each animal received vehicle and 3 doses of the treatment over 4 weeks in a randomized fashion. Per week, the experiments were performed over two successive days, because 48 animals could not be tested within one testing day, with 8 animals of each genotype tested each day (randomized); testing was performed between 9:00 and 15:00h in the dark phase of the light/dark cycle under red light. In the case of WAY100,635, males were injected and immediately placed into the observation cage to habituate for 30 minutes. For ±8-OH-DPAT, the rats were placed first in the test cage and allowed to habituate for 25 minutes and then injected with the drug, followed by placing an oestrus female into the cage. Male sexual behavior was scored over the following 30 min. In all cases, the vehicle saline was used.

Behavioral parameters

The following parameters were scored for each ejaculation series using Observer® 5.0 (Noldus, the Netherlands): amount of mounts and intromissions, time of first mount and intromission, and time of ejaculation.

From these data the following parameters were deduced: number of ejaculations/test, latency to first mount(s), latency to first intromission(s), total number of mounts, total number of intromissions, and latency to the ejaculation(s) (calculated as time of ejaculation minus the time of the very 1st behavior of that ejaculation series) per ejaculation series. After the first ejaculation, the first post ejaculatory latency (PEL1) was calculated, using the time from the first ejaculation and the time of the first mount/intromission (whichever occurred first) of the second ejaculation series. Copulatory efficiency (CE) was calculated as: CE=(#I/(#I+#M)) x 100% (I=intromission, M=mounts) per ejaculation series. Basal sexual behaviors were pulled from the vehicle treatment of the 3 pharmacological tests (table 1).

Missing values

When testing rats on sexual behavior a number of problems emerge. Male rats have sequential sexual cycles in which they start sexual activities towards the female, ending with an ejaculation. After a refractory period, sexual activity is resumed leading to a next ejaculation, and so forth. One can decide to measure sexual behavior up to the first ejaculation (often also the 1st post-ejaculatory latency is added which generates a test with a variable duration. Because we want to study drugs, it was of prime importance to have similar pharmacodynamics and kinetics, and thus, we chose for a fixed test duration of 30 minutes. If, like in the case of the SERT-/-, animals have a low number of ejaculations/30 min, some animals (Table 1: 1 of the 16 animals had no ejaculation during the test) actually cannot be used for the statistical testing. One can resolve this by putting artificially a maximum of 1800 sec (i.e. the test duration) for some latencies (e.g. ejaculation latency, intromission latency, post-ejaculatory latency).although this is disputable. An associated problem is then the mount and intromission frequencies in these non-ejaculating rats because it is actually unknown whether an ejaculation would have

followed. This may lead to artificial data. Therefore, such data are not usually used for statistical comparison.

Because very few SERT-/- reach a 2nd ejaculation (only 5 animals out of 16), no data on these higher ejaculation series can be used.

The present experiments with drugs confronted us with similar problems: 8-OH-DPAT stimulates sexual behavior, and therefore, does not lead to any of these problems; whereas WAY100,635, notably in the SERT-/-, severely reduced sexual behavior, leading to similar problems as described before. In cases where the drug blocked ejaculations in a majority of the animals, we imputed data values of 1800 for latency to 1st ejaculation and include the data values for all animals for statistical purposes. We chose to skip statistical analysis if less than 7 animals in a certain drug-treated genotype group were left for 2nd ejaculatory series parameters. .

Statistics

The data was analyzed by ANOVA using SPSS version 11.0. The difference between genotypes in each treatment was compared using a univariate ANOVA analysis. If significant differences were discovered, a Bonferroni post-hoc analysis was used. Interaction effects between drug doses and genotypes were analyzed using multivariate repeated measure ANOVA, with the Greenhouse-Geisser correction examined. This analysis was also used to determine the stability of ejaculation frequencies over the three successive tests in the training period.

In table 1, the 3 training values for each parameter of the vehicle groups were averaged and a one way ANOVA test was performed on these means.

Results

Basal sexual behaviors

The sexual behavior of the three genotypes stabilized over time and was stable during the last 3 training test. The mean ejaculation frequencies over the last 3 training tests for the $SERT^{+/+}$, $SERT^{+/-}$, and $SERT^{-/-}$ did not differ significantly from one training period to the next; average ejaculation frequency for the three genotypes being 1.6 ± 0.1 ($F_{(2, 29)}$ =

0.496, p=0.608), 1.2 ± 0.2 ($F_{(2, 29)}$ = 0.753, p=0.478), and 0.4 ± 0.1 ($F_{(2, 29)}$ = 3.058, p=0.342), respectively. Remarkably, in the three succeeding drug experiments which lasted 14 weeks, the vehicle treated animals of all three genotypes displayed higher, but again very stable levels of sexual behaviors. The relative differences between the genotypes were still present (Table 1). $SERT^{-/-}$ rats showed significantly less ejaculations than $SERT^{+/-}$ and $SERT^{+/+}$ rats, and took longer to achieve the 1st ejaculation. In addition, the post-ejaculatory latency of the $SERT^{-/-}$ is significantly larger than the wild type animals. In addition, the 1st series mounts were significantly higher between the $SERT^{-/-}$ and $SERT^{+/-}$. The $SERT^{+/-}$ animals showed an improved 1st series copulatory efficiency when compared to the wild type animals, but did not differ in any other parameters.

Table 1 Sexual behaviors of serotonin transporter (SERT)$^{+/+}$, SERT$^{+/-}$, and SERT$^{-/-}$ under the three experiments while receiving vehicle treatment

Parameter	SERT$^{+/+}$	SERT$^{+/-}$	SERT$^{-/-}$	ANOVA significance
EF (#)	2.3 ± 0.2	2.8 ± 0.2	1.2 ± 0.2*†	$P < 0.001$
EL (s)	675.6 ± 69.7	527.8 ± 43.5	1073.3 ± 95.3*†	$P < 0.001$
IF (#)	7.2 ± 0.7	7.8 ± 0.5	8.3 ± 0.6	ns
IL (s)	103.4 ± 56.1	25.7 ± 5.2	54.7 ± 16.6	ns
MF (#)	22.9 ± 2.9	14.7 ± 2.7	31.8 ± 4.1†	$P < 0.05$
ML (s)	38.6 ± 9.1	44.3 ± 12.4	22.6 ± 8.5	ns
PEL (s)	273.4 ± 10.1	260.4 ± 11.6	671.7 ± 125.9*	$P < 0.001$
CE (%)	30.3 ± 3.7	44.7 ± 3.5*	28.2 ± 3.2†	$P < 0.05$
IPS (#)	0.015 ± 0.002	0.018 ± 0.002	0.010 ± 0.001*†	$P < 0.05$
MPS (#)	0.033 ± 0.002	0.025 ± 0.003	0.028 ± 0.002	ns

All data are represented as mean ± standard error of the mean.
*Significantly different than WT.
†Significantly different than HET.
= frequency; ANOVA = analysis of variance; s = seconds; EF = ejaculation frequency over the 30-minute test; first ejaculatory series parameters: EL = ejaculation latency; IF = intromission frequency; IL = intromission latency; MF = mount frequency; ML = mount latency; PEL = post-ejaculatory latency; CE = copulatory efficiency; MPS = first series mounts/latency to first ejaculation (mounts per second); IPS = first series intromissions/latency to first ejaculation (intromissions per second); ns = no significant differences were found.

Pharmacological tests

In the 3 pharmacological test performed, heterozygote ($SERT^{+/-}$) animals did not differ from the wild type animals in any of the parameters studied. Therefore, for simplicity, the graphs only compare $SERT^{-/-}$ and wild type animals. However, the statistics were performed on all 3 genotypes.

± 8-OH-DPAT

±8-OH-DPAT showed a dose-dependent effect in all 3 genotypes with increases in ejaculation frequency ($F_{(3, 114)}$ = 1.151, p < 0.001, figure 1A) and decreases in the latency

to 1st ejaculation ($F_{(3, 121)} = 9.363$, $p < 0.001$, figure 1B), 1st ejaculatory series mount frequency ($F_{(2, 104)} = 17.481$, $p < 0.001$, figure 1C) and 1st ejaculatory series intromission frequency ($F_{(2, 110)} = 62.28$, $p < 0.001$, figure 1D). There were no significant interaction effects between genotype and the effects of ±8-OH-DPAT for any of the parameters measured. However, a trend for an interaction effect between drug treatment and genotype for the 1st ejaculatory series intromission was found ($F_{(6, 110)} = 2.202$, $p = 0.060$).

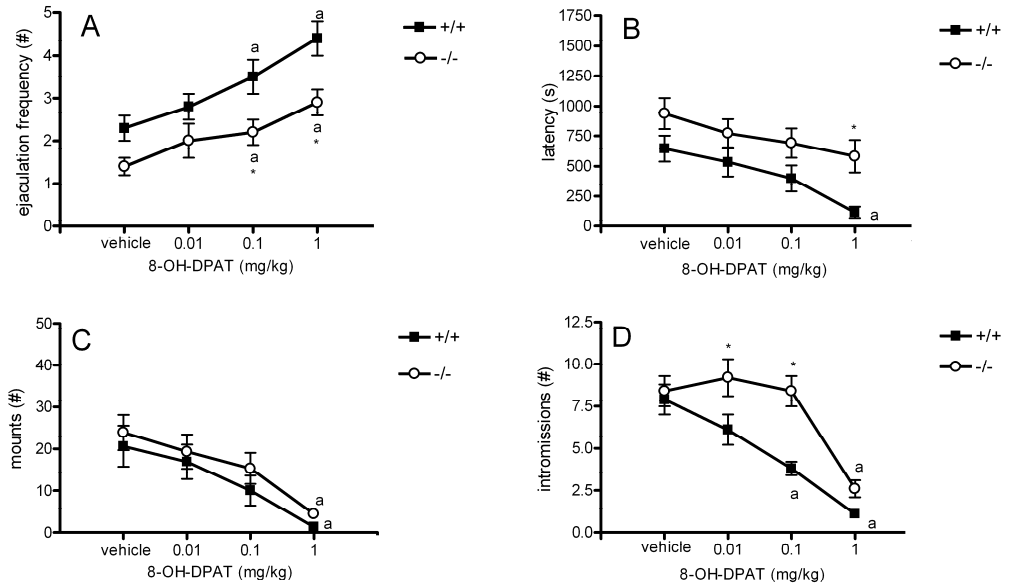

Figure 1. Effects of 8-OH-DPAT (s.c.) on ejaculation frequency over 30 minute test (A), latency to 1st ejaculation (B), 1st ejaculatory series mounts (C), and 1st ejaculatory series intromissions (D) of SERT+/+ (+/+) and SERT -/- (-/-) animals. * = significantly different than +/+. a = significantly different than vehicle treatment.

WAY100,635

WAY100,635 showed overall inhibitory effects on sexual behaviors with decreases in ejaculation frequencies ($F_{(3, 125)} = 31.384$, $p < 0.001$, figure 2A), the 1st ejaculatory series mount frequency ($F_{(2, 92)} = 5.814$, $p < 0.001$, figure 2C), and 1st ejaculatory series intromission frequency ($F_{(2, 110)} = 6.233$, $p < 0.001$, figure 2D), and increased latency to 1st ejaculation ($F_{(3, 110)} = 13.376$, $p < 0.001$, figure 2B). In addition, significant interaction

effects were detected between genotype and dose for ejaculation frequency ($F_{(6, 125)}$ = 5.417, $p < 0.001$) and all 1st ejaculatory series behavioral parameters: mount frequency ($F_{(5, 125)}$ = 6.864, $p < 0.001$), series intromission frequency ($F_{(5, 125)}$ = 4.894, $p < 0.001$). Post Hoc analysis revealed that the inhibition of the total number of ejaculations by all doses of WAY100,635 was only significantly present in $SERT^{-/-}$ rats, while only the highest dose of WAY100,635 (1mg/kg) was active in the $SERT^{+/+}$ and $SERT^{+/-}$ animals. At this dose of WAY100,635, wild type and $SERT^{+/-}$ showed significantly increased 1st series post ejaculatory latencies ($F_{(1, 16)}$ = 9.935, $p < 0.05$ and $F_{(2, 33)}$ = 10.437, $p < 0.001$, respectively) and decreased ejaculation frequencies ($F_{(3, 38)}$ = 6.053, $p < 0.05$) and ($F_{(3, 42)}$ = 25.597, $p < 0.001$, respectively) when compared to the vehicle treatment and the lowest dose of WAY100,635. The $SERT^{+/-}$ animals also showed an increased latency to 1st ejaculation ($F_{(3, 41)}$ = 15.272, $p < 0.001$) when compared to the vehicle treatment.

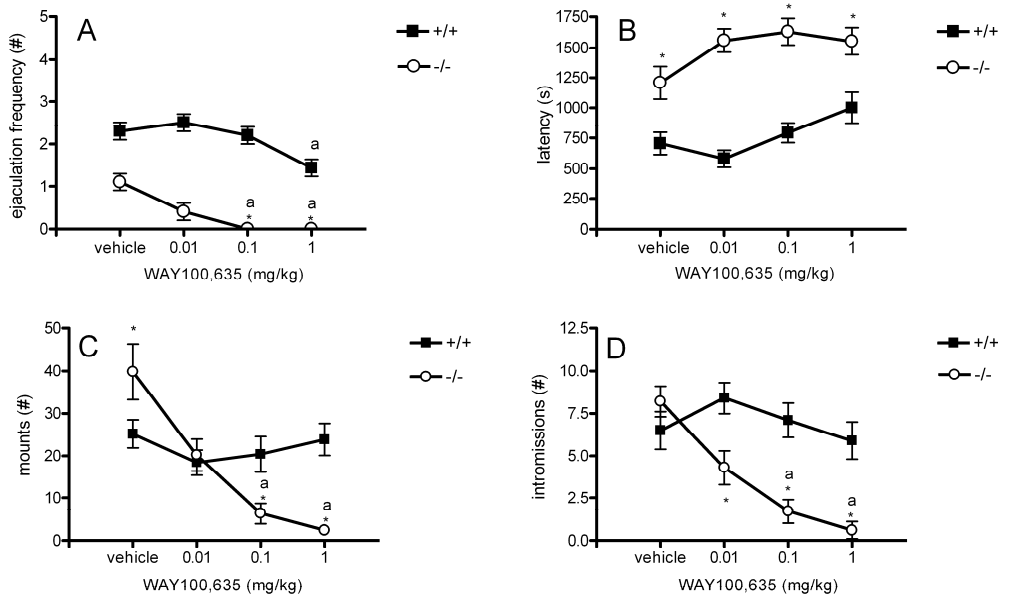

Figure 2. Effects of WAY100,635 (i.p.) on ejaculation frequency over 30 minute test (A), latency to 1st ejaculation (B), 1st ejaculatory series mounts (C), and 1st ejaculatory series intromissions (D) of SERT+/+ (+/+) and SERT-/- (-/-) animals. * = significantly different than +/+. a = significantly different than vehicle treatment.

WAY100,635 and ±8-OH-DPAT interaction

0.1 mg/kg WAY100,635 had comparable effects as found in exp. II; decreased number of ejaculations, increased latency of first ejaculation, and reduced number of mounts and intromissions in the $SERT^{-/-}$ with no significant differences found in the $SERT^{+/+}$ and $SERT^{+/-}$. Increasing doses of ±8-OH-DPAT partially restored the sexual behavior parameters although already at the lowest dose (0.01 mg/kg) the maximal recovery was found and further dose increments of ±8-OH-DPAT did not bring the level of sexual behavior of the $SERT^{-/-}$ to the wild type level.

Significant differences were found between the vehicle plus WAY100,635 treatment and any of the WAY100,635 plus ±8-OH-DPAT treatments in ejaculation frequency ($F_{(3, 118)} = 17.902$, $p < 0.05$) (figure 3A), latency to 1st ejaculation ($F_{(3, 123)} = 9.850$, $p < 0.05$) (figure 3B), and 1st series intromission frequency ($F_{(2, 111)} = 58.969$, $p < 0.05$) (figure 3D). Both

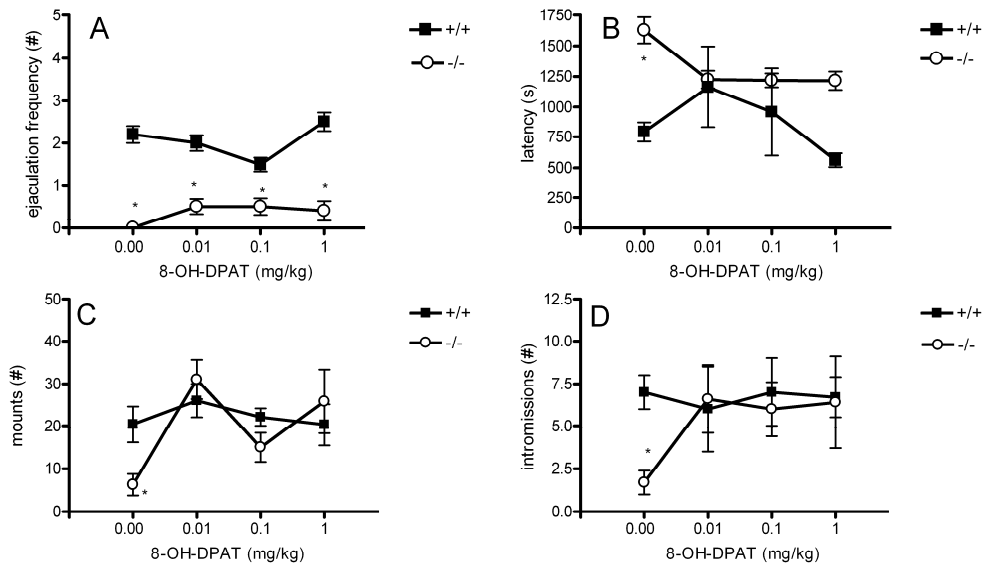

Figure 3. Effects of 3 doses of 8-OH-DPAT (0.01, 0.1, and 1mg/kg; s.c.) and one dose of WAY100,635 (0.1mg/kg; i.p.) on ejaculation frequency over 30 minute test (A), latency to 1st ejaculation (B), 1st ejaculatory series mounts (C), and 1st ejaculatory series intromissions (D) of SERT+/+ (+/+) and SERT-/- (-/-) animals. * = significantly different than +/+. a = significantly different than vehicle treatment.

in wild type and $SERT^{+/-}$ animals, the effects of ±8-OH-DPAT up to 1 mg/kg (figures 3) were completely blocked by 0.1 mg/kg WAY100,635 and no significant differences were observed; i.e. for ejaculation frequency for $SERT^{+/+}$ ($F_{(5, 125)} = 0.499$, p=0.785) and $SERT^{+/-}$ ($F_{(5, 125)} = 0.499$, p=0.610).

Discussion

In the present study in rats homozygous for a null mutation in the SERT coding sequence, we found that the serotonin transporter is important for male sexual behavior. The $SERT^{-/-}$ animals had perturbed sexual behaviors, and it appears that the 5-HT_{1A} receptors play a role in this disruption. $SERT^{-/-}$, but not $SERT^{+/-}$ rats showed a lower number of ejaculations during a 30 minute sex test. This difference became evident after a few training tests and remained stable over the complete duration of the experimental testing period, suggesting that the sexual effects of the SERT null mutation are life-long and can not be compensated by extensive training. Between the training period and experimental testing, all genotypes expressed a comparable increase in ejaculation frequencies.

Previous research has shown that manual stimulation, such as tail pinching, can stimulate sexual behaviors in male rats [110, 111]. It is possible that the experimental conditions of the drug experiments (handling, injection) underlie this increase in ejaculation frequency. Unfortunately, we have not retested the animals after cessation of the drug experiments. The lower sexual behavior of the $SERT^{-/-}$ rats mimics the effects of chronic SSRI treatment in men and rats. In male rats, chronic treatment of the SSRIs citalopram [186], paroxetine [18] and fluoxetine [75, 189] decreases sexual performance. In humans, nearly all SSRIs delay or completely inhibit ejaculation, with reductions in libido and arousal [190]. In particular, the use of paroxetine is notorious for its high rate of induced-sexual dysfunctions[25, 129, 152, 191].

In humans, chronic SSRI usage, at the minimum effective clinical dose, results in approximately 80% blockade of the SERT [192]. Here, we showed that $SERT^{+/-}$ rats, with a 50% reduction in SERT level and function, have no disturbances in sexual behavior,

while rats with a complete absence of SERT ($SERT^{-/-}$ animals) show perturbed sexual behaviors. The basal levels of sexual behavior of $SERT^{-/-}$ rats is comparable to that of male rats chronically treated with SSRIs [5, 18, 19, 186]; in both models, rats are slower in reaching an ejaculation. It seems possible that a critical number (threshold) of available and functional 5-HT transporters is needed to perform normal sexual behavior. Acute administration of SSRI's, at doses that reach >80% transporter occupancy, does not lead to inhibition of sexual behavior[11]. Therefore, adaptations to the transporters must occur after chronic treatment, leading to changes in serotonergic functioning that underlie the changed (disturbed) sexual behavior.

$5\text{-}HT_{1A}$ receptor stimulation with ±8-OH-DPAT in $SERT^{-/-}$ animals led to normal wild type levels in sexual behaviors. $5\text{-}HT_{1A}$ receptor agonists stimulate sexual activity in rats [173, 193, 194] which could be confirmed in the present research by the strong pro-sexual effects of ±8-OH-DPAT in the wild type rats. Although the basal sexual behavior levels of the $SERT^{-/-}$ was much lower than the $SERT^{+/+}$, the pro-sexual effects were comparable, indicating that the pool of $5\text{-}HT_{1A}$ receptors involved in this mechanism is functionally intact in the $SERT^{-/-}$. With increasing doses of 8-OH-DPAT, intromission and mount behaviors decreased while ejaculation frequency increased; thus signaling a lowering of ejaculation threshold. The $SERT^{+/-}$ were in no way different from the $SERT^{+/+}$. In contrast, previous research [34, 195-198] suggests that $5\text{-}HT_{1A}$ receptor desensitization plays an important role in the ejaculation inhibiting effects of SSRI's. The trend for 1st series intromission frequency between 8-OH-DPAT treatment and genotype suggests some desensitization of the $5\text{-}HT_{1A}$ receptor for this parameter. Although the graph suggests a right shift of the dose response curve, the absence of hard statistical support in the other sexual parameters makes such a conclusion disputable. These findings suggest a difference in the functionality of $5\text{-}HT_{1A}$ receptors of $SERT^{-/-}$ rats and chronic SSRI-treated rats. Alternatively, different pools of $5\text{-}HT_{1A}$ receptors may be involved in the underlying mechanisms in these two models.

The 5-HT_{1A} receptor antagonist WAY100,635 was behaviorally inactive in the dose range of 0.01 and 0.1mg/kg used in $SERT^{+/+}$ and $SERT^{+/-}$ rats. These doses have been studied in male sexual behavior of normal rats [186] and were always inactive after acute and chronic dosing. However, WAY100,635, in all doses, strongly and dose-dependently decreased sexual behavior in the $SERT^{-/-}$ rats. Ejaculations were completely blocked in the highest doses of WAY100,635 (0.1mg/kg and 1mg/kg) in the $SERT^{-/-}$. This led to a problem of having too few animals to perform statistics. Since the response of the animals to this drug was so potent, we chose to represent the data with the imputed ceiling value of 1800 seconds for latency to 1st ejaculation (but in reality, the animal could take much longer to ejaculate but the effects of the drug may not be represented there).

WAY100,635 is a silent 5-HT_{1A} receptor antagonist with zero intrinsic activity for activating the receptor and was able to dose dependently block agonistic activities of 5-HT_{1A} receptor agonists[184]. $SERT^{-/-}$ rats have increased basal serotonin levels compared to wild type rats, and in contrast to the absence of effect in the $SERT^{+/+}$ and $SERT^{-/-}$ rats, the effects of WAY100,635 are present at all doses. Only at the highest dose of WAY100,635 (1mg/kg) some inhibitory effects in the $SERT^{+/+}$ and $SERT^{+/-}$ animals are found. It may be possible that this high dose is associated with some adverse, e.g. sedation, side effects. Whether the finding that 5-HT release in the lateral hypothalamus is enhanced after an ejaculation[42], which may be modulated by WAY100,635 in $SERT^{-/-}$ rats leading to further inhibition of sexual behavior, remains highly speculative. The usage of this high dose of WAY100,635 has not been previously reported, since lower doses have shown to be effective in blocking the 5-HT_{1A} receptor. This concurs with the finding that inhibition of sexual behavior after SSRI-treatment can be strongly facilitated by the co-administration of a 5-HT_{1A} receptor antagonist [12, 186]. This may suggest that a sensitized pool of 5-HT_{1A} receptors, different from the one involved in the 5-HT_{1A} receptor activating properties of ±8-OH-DPAT, is involved in the regulation of sexual activities in $SERT^{-/-}$ rats. It appears that there needs to be a complete (100%) blockade of 5-HT_{1A} receptors (affected by the highest dose of WAY100,635) for sexual side effects to be witnessed in the $SERT^{+/+}$ and $SERT^{+/-}$ animals.

When a selected dose of WAY100,635 (0.1 mg/kg) was combined with a dose-range of ±8-OH-DPAT, a number of interesting phenomena was observed. Increasing doses of ±8-OH-DPAT were only partially able to antagonize the inhibiting effects of WAY100,635 in the $SERT^{-/-}$. Remarkably, ±8-OH-DPAT also had no stimulatory effect at any dose in the $SERT^{+/+}$ and $SERT^{+/-}$ rats, suggesting that the blocking of 5-HT_{1A} receptors by WAY100,635 at the dose used was never overcome by the 5-HT_{1A} receptor agonist ±8-OH-DPAT. However, in the $SERT^{-/-}$ rats, ±8-OH-DPAT had a small stimulatory activity in the WAY100,635 pretreated rats, already present at the lowest dose used but not further enhanced at the higher doses.

Other studies have also shown that changes induced by 8-OH-DPAT are not blocked by WAY100,635, namely, prolactin secretion [199] and blockade of decrease in serotonin levels [200], suggesting effects mediated by non-5-HT_{1A} receptors. ±8-OH-DPAT is known to stimulate 5-HT_7 receptors, although much weaker than 5-HT_{1A} receptors. In female rat sexual behavior activation of 5-HT_7 receptors was inhibitory[201], but no studies could be found on male rat sexual behaviors. 8-OH-DPAT also weakly binds to 5-HT_{1D} receptors[202], but since this receptor seems absent in rats[203], no obvious role emerges. This study suggests that the complete absence of SERT leads to alterations in the 5-HT_{1A} receptor functioning, namely, certain receptor pools with different levels of sensitivity. One aggregate mediates the prosexual effects after stimulation of 5-HT_{1A} receptors and is not (de)sensitized. The other, mediating the inhibitory effects of antagonized 5-HT_{1A} receptors, seems to be sensitized in the $SERT^{-/-}$ rats. The notion of two differentially regulating 5-HT_{1A} receptor pools in $SERT^{-/-}$ animals has also been found in autonomic regulation of body temperature and stress [204]. In this study exposure to a mild stressor elicited stress-induced hyperthermia (SIH) [205, 206], which was lower in the $SERT^{-/-}$ than in the $SERT^{+/+}$. The 5-HT_{1A} receptor agonist flesinoxan, which has no 5-HT_7 receptor affinity, reduced basal body temperature in $SERT^{+/+}$ but not in the $SERT^{-/-}$. The lower doses of WAY100,635 were inactive in the $SERT^{+/+}$, but strongly enhanced body temperature and SIH in the $SERT^{-/-}$. WAY100635 was able to antagonize the decrease in

basal body temperature in the $SERT^{+/+}$. These data are in line with our findings about the alterations in functionality of 5-HT_{1A} receptors in sexual behaviors.

Taken together, we hypothesize that there are at least two populations of 5-HT_{1A} receptors involved in male sexual behavior. For normal sexual behavior, activation of a certain group of 5-HT_{1A} receptors is needed and these receptors are desensitized in the $SERT^{-/-}$ rats. The ejaculation stimulatory effects of ±8-OH-DPAT are mediated by a different pool of 5-HT_{1A} receptors, which were not desensitized in $SERT^{-/-}$ rats. The lack of interactive effects on ejaculation frequency between the genotypes and doses of ±8-OH-DPAT and the discovery of interactive effects between the genotypes and doses of WAY100,635 provides support to this idea. With both drugs targeting the 5-HT_{1A} receptor, this theory could explain the different effects observed.

5-HT_{1A} receptors are found throughout the central nervous system in areas associated with sexual behaviors; namely the raphe nucleus, medial preoptic area, lateral hypothalamic area, medial amygdala, bed nucleus of the stria terminalis, nucleus accumbens, paraventricular hypothalamic nucleus, arcuate hypothalamic nucleus, and the spinal cord [198, 207-211]. It could be possible that the receptors are affected differently depending on where they are located. In addition, the location (presynaptic or postsynaptic) and type of receptors (heteroreceptors or autoreceptors) may also play a role.

We recently studied the sexual behavior of female $SERT^{+/-}$ and $SERT^{-/-}$ rats [212]. In contrast to the males, basal proceptive and receptive sexual activities in a paced-mating situation were not different between any genotype. ±8-OH-DPAT inhibited sexual behavior in all genotypes, but a clear right-shift of the dose response curve was found in the $SERT^{-/-}$ suggesting a strong desensitization of 5-HT_{1A} receptors. In contrast to the males, WAY100,635 was, over a broad dose range, inactive in all genotypes. Moreover, it was able to antagonize the ±8-OH-DPAT induced inhibition in all genotypes, indicating that probably only one pool of 5-HT_{1A} receptors was involved in the observed effects. It

can be hypothesized that different mechanisms or differential activation of serotonergic substrates are involved in brain mechanisms of male and female sexual behavior.
In behavioral tests, such as elevated plus maze, novelty suppressed feeding and the forced swim task, SERT$^{-/-}$ rats show anxiety and depression-related phenotypes [180]. The question arises whether the reduced sexual behavior of the male SERT$^{-/-}$ rats is a consequence of their anxiety and/or depression-like behavior or whether it is due to mechanistic differences in brain areas involved in the regulation of sexual behavior. In this study, we trained all rats extensively in the sex test to minimize the amount of perceived stress for the animals. Significant statistical differences in ejaculation frequency were uncovered between the genotypes after the 3rd training test. These results repeat our previous observations of stable ejaculatory phenotypes in ejaculatory behavior after a few training sessions; where animals with low ejaculatory performance remain low and those with higher performances remain high [12, 17]. The inherent rewarding aspects of sexual behavior [213] and the lack of anxiety differences found in these low and high ejaculatory performers [17] suggests that anxiety does not explain the reduced sexual behaviors in the SERT$^{-/-}$ rats. In addition, the absence of basal differences in female sexual behavior between the genotypes [212] also contradicts an explanation in terms of mood differences.

The availability of the SERT knockout rat made this study possible. Nearly all gene-knockout studies are performed in mice, basically because only suitable embryonic stem cells exist for this species. To model sexual dysfunctions in humans, the rat is a preferred species over mice. Rats are one of the most frequently used animals to model sexual behaviors and, thus, has a large literature reference base[214]. Conversely, there are limited psychopharmacological studies in the sexual behaviors of mice. In addition, male rat sexual behaviors are more reliable, with stable sexual behaviors witnessed in individual animals[12]. Male rats can have multiple ejaculations during a 30- minute sex test [12], while mice will have one ejaculation and then resume sexual activities hours later [215]. Multiple ejaculations in a relatively short time frame in rats allow for the examination of ejaculation frequencies (an indication of consummatory behaviors) and post ejaculatory intervals (an indication of precopulatory or arousal behaviors). The data described here

focused on the 1st ejaculatory series due to the low ejaculation frequencies of the $SERT^{-/-}$. The $SERT^{-/-}$ rat showed increased ejaculation latency, decreased ejaculation frequency and increased post-ejaculatory latencies which could provide an animal model to examine the human disorders of delay ejaculation, anorgasm, and low libido, respectively.

The rat SERT knockout was generated using ENU mutagenesis. By sequencing the SERT-coding region in the offspring of ENU treated rats, one animal was found with a mutation that resulted in a stopcodon [185]. It is very likely that the null mutation in the SERT gene was not the only ENU mutation in this animal. However by crossing this rat for multiple generations with $SERT^{+/+}$ rats and selecting those animals with the SERT mutation present, it is very likely that most other ENU-induced mutations (with exception of those that are located very close to the mutation in the SERT gene) are lost from the selected line[216]. In other words, it is unlikely that the observed sexual disturbance observed in the knockout rats, are due to a mutation other than the one in the SERT gene. Nevertheless, these results must be considered with caution since the absence of the SERT gene during development may affect the organization of the serotonergic system. It could be said that differences in sexual behaviors could be a result of deletion of the gene during development. Although possible, there is no current evidence of a difference in serotonergic organization in the central nervous system of the SERT knock out mouse model[217, 218].

Future research should look more specifically at the role of $5\text{-}HT_7$ receptors in sexual behaviors. By targeting this receptor or augmenting current anti-depressant treatments, reductions of drug-induced sexual side effects could be possible. In addition, the reoccurring notion that there are sub-populations of $5\text{-}HT_{1A}$ receptors need to be fully investigated; along with their location in the central nervous system and/or the site of action.

Conclusions

In summary, 5-HT_{1A} receptors are involved in normal sexual function of male rats. Animals with a life long absence of SERT have altered sexual behaviors and 5-HT_{1A} receptor functioning. Desensitization of certain pools of these receptors may hinder sexual performance. Research still needs to be performed on whether the absence of SERT affects the sensitivity of other 5-HT receptors. It would be important to see if humans suffering from long term or life long serotonergic disturbances are affected the same way.

Chapter 6

The triple monoaminergic reuptake inhibitor DOV 216,303 has antidepressant effects in the rat olfactory bulbectomy model and lacks sexual side effects

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Abstract

Current antidepressants have a delayed onset of action and disturbing side effects, including inhibition of sexual behavior. It is hypothesized that novel drugs, hitting multiple disease relevant targets, may yield a new generation of superior antidepressants. One such approach is simultaneous inhibition of serotonin, norepinephrine and dopamine transporters. We tested the triple uptake inhibitor (TUI), DOV 216,303 (5, 10 and 20 mg/kg) after 1, 7 and 14 days administration in the olfactory bulbectomized (OBX) rat depression model, and in a model of rat sexual behavior to detect putative sexual side effects. Chronic, but not acute treatment of DOV 216,303 (20 mg/kg) normalized OBX-induced hyperactivity in the open field, similar to the effect of imipramine (20 mg/kg). None of the doses of DOV 216,303 had any effect on sexual behavior at any time point. The results indicate that DOV 216,303 displays antidepressant efficacy and is devoid of sexual side effects.

Introduction:

Depression is a severe psychiatric disorder with lifetime prevalence as high as 20%. The World Health Organization says it will be the second largest global burden of disease (DALYs) by the year 2020, illustrating the severity and impact of the disorder [219, 220]. Depression is not a unitary disorder and most experts agree that it should be considered a syndrome (DSM-IV 2000) comprised of a spectrum of various symptoms, making animal research into the underlying mechanisms difficult but feasible [221].
The first effective treatments for depression were primarily monoamine modulating drugs [222]. Over the past five decades, the leading theory behind the mechanism of action of these drugs has been known as the monoamine hypothesis of depression, postulating that depressive symptoms are primarily caused by disruptions in serotonin, noradrenaline and/or dopamine neurotransmission [223]. This hypothesis was based on findings that monoamine oxidase inhibitors and tricyclic antidepressants elevated monoamine levels in the central nervous system, and appeared to have antidepressant efficacy [224]. The next generations of antidepressants, selective serotonin reuptake inhibitors and selective serotonin/norepinephrine reuptake inhibitors, were variations on this monoamine theme. Blockade of noradrenaline or dopamine also appeared to have antidepressant effects [163], suggesting that compounds blocking all three monoamine transporters would constitute effective antidepressants.

The involvement of dopamine in depression is thought to be dependent upon dopaminergic reward mechanisms in the limbic system [225]. Several animal studies have shown that dopamine neurons in the ventral tegmental area (VTA) play a role in movement abnormalities associated with Parkinson's disease (PD), and neural projections from the VTA to the frontal lobes may play a role in decreased initiative [226]. Also, homovanillic acid (the major metabolite of dopamine) has been shown to be decreased in the cerebrospinal fluid of depressed patients [227], and hypofunction of the mesolimbic dopamine pathways is thought to be a mediator of anhedonia, a major symptom of

depression [228]. Dopamine also plays a role in sexual behavior, and is thought to mediate ejaculation, although this may be dependent on specific dopamine receptor sub-types [229].

Recently, the development of triple monoamine uptake inhibitors (TUIs) has piqued the interest of various pharmaceutical and biotech companies. SSRIs exert sexually inhibitory effects, but only after chronic treatment [5, 74, 85]. Adding a stimulatory dopaminergic component to a dual serotonin-noradrenaline reuptake inhibitor might compensate for the inhibitory action of chronic serotonin transporter inhibition on sexual behavior without losing the antidepressant efficacy. It has previously been suggested that treatment with dopaminergic agonists may decrease SSRI induced sexual dysfunction [230]. It has been previously shown that patients treated with SSRIs showed sexual improvements when also treated with bupropion, a norepinephrine/dopamine reuptake inhibitor [231]. Addition of a dopaminergic component to the proven antidepressant profile of combined 5-HT and NA inhibitor uptake might have advantages. Clinical and pre-clinical evidence links one of the core symptoms of depression (anhedonia) to deficits in dopaminergic transmission [232-235], and that dopaminergic stimulation may be associated with pro-sexual effects [36]. Removal of the olfactory bulbs (OBX) in rats results in similarities to brain chemistry seen in depressed humans [236, 237], such as altered dopamine [238] and serotonin concentrations in the brain [239]. Olfactory bulb ablation also leads to enlarged lateral and 3rd ventricles, as well as decreased hippocampal volume, phenomena that are also observed in depressed humans [240]. Bulb ablation also results in several behavioral changes, including increased hyperactivity in a novel environment [241], and deficits in passive-avoidance learning and anhedonia [242]. Olfactory bulbectomy is one of the best available models to predict antidepressant activity, as OBX-induced depressive symptoms respond to chronic, but not acute, antidepressant treatment [236, 243, 244].

The present study investigate the behavioral effects of the TUI DOV 216,303 ([(+/−)-1-(3,4-dichlorophenyl)-3-azabicyclo[3.1.0]hexane hydrochloride]) [144, 168] in the OBX depression model, and in sexual behavior in endophenotypically normal rats [113, 125]. It should be noted that two different rat strains were used for this experiment, due to the fact

that the bulk of sexual behavior studies has been done using Wistar rats [12, 125], while most bulbectomy studies have been performed using the Sprague–Dawley strain [236, 241, 245].
DOV 216,303 inhibits NE, 5-HT and DA reuptake in vitro at 20, 14 and 78 nM, respectively [144, 168] and is active in acute antidepressant tests like the forced swim and the tail suspension [144, 168].
DOV 216,303 is also well-tolerated in humans, and significantly reduced the HAM-D scores of depressed patients, similar to citalopram [144]. The goal of this study was to compare the effects of DOV 216,303 in these two animal models reflecting antidepressant activity and sexual side effects, creating an opportunity to predict human therapeutic efficacy and sexual side effects of this putative new antidepressant.

Materials and Methods:

Experiment I: Olfactory Bulbectomy

Animals: One hundred-and-twenty male albino Sprague–Dawley rats (Harlan, Zeist, The Netherlands) weighing between 220 and 250 g were housed four/cage on a 12 h: 12 h light dark cycle, with lights off at 18:00 and on at 6:00. Food and water were available ad libitum. Animals were allowed to acclimate for one week, and were then run in a pre-surgical open field test, four animals at a time, in three equal groups of forty animals each. Animals were then assigned to surgical groups, with regard to their basal open field activity, so that there were equal numbers of more and less active animals in each group. After surgery, animals recovered for two weeks before the rest of the tests were performed. Animals were assigned to treatment groups with regard to their post-surgical open field activity so that there were equal numbers of more and less active animals in each treatment group.

Surgical procedure: Animals were anesthetized using isofluorane gas (3–4%), mixed with oxygen and nitrous oxide. The animals were placed in a stereotaxic instrument (Kopf), and two burr holes were drilled on either side of the skull, 2 mm in diameter, 8 mm anterior to bregma, and 2 mm from the midline of the frontal bone overlying the olfactory

bulbs. The bulb tissue was removed using a blunt hypodermic needle and a vacuum pump. The burr holes were packed with haemostatic sponge to prevent blood loss. Animals receiving sham surgery underwent a similar procedure, but retained their olfactory bulbs. All incisions were closed using 4-0 vicryl suture material (resorbable). After surgery, all animals received 5 ml of saline (subcutaneously), and Rimadyl (5 mg/kg, subcutaneously) for pain. When all animals were awake and moving, they were returned to the colony room.

Behavioral testing: Two weeks after surgery, all animals were tested in the open field for lesion verification (hyperactivity). Animals in both the sham and OBX groups were then assigned to treatment groups (N=12/group) of vehicle, imipramine (20 mg/kg; Sigma Aldrich, Zwijndrecht, The Netherlands), or DOV 216,303 (5, 10 and 20 mg/kg, synthesized by Sepracor Inc., Marlborough USA). Five animals died during surgery and the remaining animals were distributed over the groups. All animals received one injection per day for fourteen days. On days one, seven and fourteen, animals were tested in the open field, 30 min after injection, to observe the effects of acute and (sub) chronic drug administration. All injections were given orally, using an oral gavage needle, with a dose volume of 3 ml/kg. All solutions were made daily, using sterile water as the vehicle. This experiment was performed in accordance with the governmental guidelines for care and use of laboratory animals and was approved by the Ethical Committee for Animal research of the Faculties of Veterinary Medicine, Pharmaceutical Sciences, Chemistry and Biology at Utrecht University.

Open field: The gray open field chambers measured 70×70×45 cm, under fluorescent lighting; activity was measured using Noldus EthoVision®. All testing was done during the light period. After a thirty-minute acclimation period, in which the animals acclimated to the test room while in their home cage, each animal was placed in the center of the open field and allowed to explore for 15 min. There was no acclimation effect over the testing period; previous studies in our lab have shown that bulbectomized animals remain hyperactive in the open field for at least 8 months following the surgery.

When all testing was completed, the animals were sacrificed and the brains were removed olfactory bulb ablation verified. Animals with partial bulbectomies or damaged prefrontal cortices were excluded. Body weights were taken weekly.

Statistics: All data are expressed as mean distance traveled (cm)±SEM. All statistical analyses were done using SPSS version 11.0. Analysis of the pre- and post-surgical open field data for each experiment was done first using repeated measures ANOVA, with surgery (OBX and sham) as the main factor, and distance traveled over time as the repeated measure. Acute, subchronic and chronic time points were analyzed using univariate ANOVA. If any of the ANOVA analyses revealed a significant surgery, treatment, or a surgery/drug treatment interaction, these tests were further analyzed using Fisher's PLSD post-hoc analyses.

Experiment II: sexual behavior

Animals and training: One hundred-and-twenty male and one hundred-and-twenty female Wistar rats (Harlan, Zeist, The Netherlands) of approximately eight weeks of age were group-housed under a reversed day–night schedule (Lights off from 6:00 and on at 18:00). After a one-week habituation period, males were paired once weekly for four consecutive weeks (without injection) with an oestrus female in an observation cage (30×60×40 cm) with a Plexiglas front. Females were brought into oestrus by administering 50 μg estradiol-benzoate (in 0.1 ml of sesame oil saturated with lecithin) in the nape of the neck 36 h prior to testing. The number of ejaculations over the last training test was used to designate animals either as low, medium (normal) or high performers; testing began after this training period.

In the present experiment, drugs were tested on ‘normal performers’; all animals (N=60) with an average of two to three ejaculations/test were included. This experiment was performed in accordance with the governmental guidelines for care and use of laboratory animals and was approved by the Ethical Committee for Animal research of the Faculties of Veterinary Medicine, Pharmaceutical Sciences, Chemistry and Biology at Utrecht University.

Experimental design and testing: The following experiment was performed on five groups of twelve rats each:

Group one received water vehicle, group two received paroxetine (10 mg/kg), and groups three, four and five received DOV 216,303 (5, 10 and 20 mg/kg, respectively). All drugs were dissolved in water and injected orally for fourteen consecutive days, with a dose volume of 3 ml/kg.

On days one, seven and fourteen, animals were injected 30 min before testing. Behavioral experiments were performed over two successive days; testing was performed between 9:00 and 15:00 in the dark phase of the light/dark cycle under red light. Males were injected and immediately placed into the experimental cage to acclimate. After 30 min an oestrus female was placed into the cage and male sexual behavior was scored over the following 30 min.

Behavioral parameters: The following parameters were scored using the Noldus Observer®: mount frequency/ejaculation series, time of first mount/ejaculation series, intromission frequency/ejaculation series, time of first intromission/ejaculation series, number of ejaculations, and time of ejaculation.

From these data the following parameters were deduced for the first ejaculation series: number of ejaculations/test, latency to first mount (s), latency to first intromission (s), number of mounts, number of intromissions, and latency to the first ejaculation (s). After the first ejaculation, the first post ejaculatory latency (PEL1) was calculated, using the time from the first ejaculation and the time of the first mount/intromission (whichever occurred first) of the second ejaculation series. Copulatory efficiency (CE) was calculated as: CE=(#I/(#I+#M)) *100% (I=intromission, M=mounts). All parameters were measured again for the second ejaculation series.

Missing values: The maximum value of 1800 s was placed for latency to first intromission, first mount, first ejaculation, and post ejaculatory latency (PEL) for any animal that failed to display those behaviors. Behavioral parameters in treatment groups with less than 6 data points (n<6) were excluded from statistical analysis. Body weights

of the animals were recorded on days one, seven, fourteen and twenty-one and % change in body weight was always compared to the acute treatment.

Statistics: Due to the unequal data distribution, nonparametric statistical analyses were performed using SPSS version 11.0. The drug effects on each of the treatment days were compared using the Kruskal Wallis test (KW test: vehicle versus the 3 doses of DOV 216,303) and the two-tailed Mann–Whitney U test (MW test: vehicle to paroxetine treatments and 3 doses of DOV 216,303, if significance was found in the KW test). The KW test was also used to compare all test days for each treatment. If a significant value was discovered, a post-hoc MW test was performed comparing individual test days to one another. Percent change in body weight data was calculated from the acute treatment body weights, using a one-way ANOVA with a Bonferroni post-hoc test, comparing all drug doses to vehicle treatment.

Results

Experiment I: olfactory bulbectomy

Repeated measures analysis revealed that there was a significant surgical effect when comparing pre- and postsurgical total distance traveled ($F(1,103)=24.58$, pb0.001). After surgery, bulbectomized animals were hyperactive compared to shams, indicating a significant OBX-lesion effect (Fig. 1).

After acute treatment, there was a significant effect of surgery ($F(1,104)=36.05$, pb0.05), and drug treatment ($F(4,104)= 3.21$, $p=0.01$), but there was no significant interaction between surgical and drug treatments. Further analysis showed that OBX animals in every group were significantly more active compared to all shams in all groups (Fig. 2A). After sub-chronic administration, there was still a significant overall effect of surgery ($F(1,104)=28.79$, $p=28.79$), as well as drug treatment ($F(4,104)=5.46$, $p=0.001$); however, there was no interaction effect between surgery and drug treatment ($F(4,104)=1.25$, NS), perhaps due to the fact that both doses of DOV 216,303 had no effect on OBX-induced hyperactivity at this time point. However, when comparing the difference in activity from baseline (postsurgical activity minus sub-chronic treatment activity), there was a

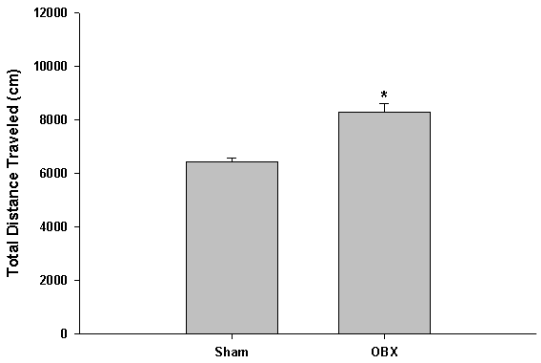

Figure 1: Hyperactivity resulting from olfactory bulbectomy surgery. Bulbectomized animals are significantly more active in the open field compared to shams. * = $p < 0.001$ compared to shams.

significant surgery x drug treatment interaction (F4,95)=2.78, p=0.03. Post-hoc analysis revealed that OBX animals in the imipramine group were no longer significantly different compared to their sham counterparts (Fig. 2B). We found this comparison necessary, as we thought it important to show that imipramine, the positive control, did have an antidepressant effect at this time point.

Analysis of chronic (fourteen days) treatment revealed that there were still significant effects of surgery (F(1,104)=11.57, p=0.001) and treatment (F(4,104)=4.05, p=0.004); there was no interaction effect between surgery and treatment (F(4,95)=1.34, NS), perhaps due to the fact that the low dose of DOV 216,303 was still unable to significantly reduce OBX-induced hyperactivity at this time point. However, when comparing the difference in activity from baseline (post-surgical activity minus chronic treatment activity), there was a significant surgery x drug treatment interaction (F4,95)=2.52, p=0.04. Separate comparisons show that OBX animals treated with either imipramine or DOV 216,303 (20 mg/kg) had similar activity compared to their sham counterparts. We found these comparisons necessary in order to show that imipramine, the positive control, did have an effect at this time point, as well as the highest DOV dose. However, OBX animals treated with DOV216,303 (5 and 10 mg/kg), remained significantly more active compared to their sham counterparts (Fig. 2C). Imipramine treated shams were never significantly less active compared to vehicle shams. OBX vehicle treated animals remained significantly more active compared to shams at all time points. There was no significant effect of surgery or drug treatment on body weight at any time.

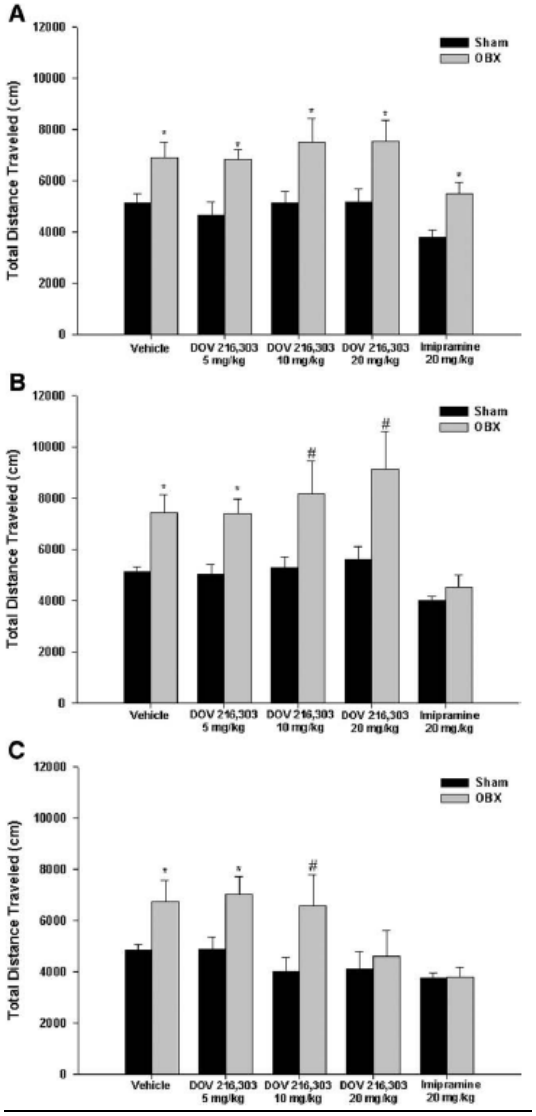

Figure 2A: Activity after acute (1 day) treatment with DOV 216,303 and imipramine. OBX animals in all groups are significantly more active when compared to sham counterparts. All distances are given as mean distance traveled (cm) ± SEM. OBX and sham post-hoc comparisons: Vehicle: p = 0.02, DOV 216,303 at 5 mg/kg: p = 0.005, DOV 216,303 at 10 mg/kg: p = 0.002, DOV 216,303 at 20 mg/kg: p = 0.005, imipramine 20 mg/kg: p = 0.03. * = $p < 0.05$ compared to shams within the same treatment group.

Figure 2B: Activity after sub-chronic (7 days) treatment with DOV 216,303 and imipramine. OBX animals in all groups except imipramine remain significantly more active compared to shams. OBX and sham post-hoc comparisons: Vehicle: p = 0.01, DOV 216,303 at 5 mg/kg: p = 0.01, DOV 216,303 at 10 mg/kg: p = 0.003, DOV 216,303 at 20 mg/kg: p = 0.001, imipramine 20 mg/kg: NS. * = $p < 0.05$ compared to all shams except those in the DOV 216,303 (20 mg/kg) group ** = $p < 0.05$ compared to all shams

Figure 2C: Activity after chronic (14 days) treatment with DOV 216,303 and imipramine. OBX animals in the imipramine and DOV 216,303 groups are no longer significantly different from shams. OBX animals in the vehicle and DOV 216,303 (5 and 10 mg/kg) remain significantly more active compared to shams. OBX and sham post-hoc comparisons: Vehicle: p = 0.03, DOV 216,303 at 5 mg/kg: p = 0.02, DOV 216,303 at 10 mg/kg: p = 0.006, DOV 216,303 at 20 mg/kg: NS, imipramine 20 mg/kg: NS

* = $p < 0.05$ compared to all shams except those in the DOV 216,303 (5 mg/kg) and vehicle groups. ** = $p < 0.05$ compared to all shams

Experiment II: sexual behavior

The average number of ejaculations in the last training test (day seven) and for the vehicle treatment days and drug groups are shown in Fig. 3A. After the last training test, animals were randomly assigned to the various treatment groups; there were no significant effects between groups with regard to the mean number of ejaculations.

There were no significant effects on any aspect of sexual behavior after acute treatment of either paroxetine and all doses of DOV 216,303 when compared to the vehicle (ejaculation frequency and latency only; Z=−0.547=, p=0.584 and KW test: X2 =3.513, p=0.319, respectively) (Fig. 3A and B). All animals displayed on average between 2.3 and 2.8 ejaculations in the 30-minute test (Fig. 3A).

After sub-chronic (seven days) treatment, only paroxetine had significant inhibitory effects on sexual behavior (Fig. 3A and B). This was reflected in the drastic reduction (60%) in the number of ejaculations (Z=−3.837, p=0.001) (Fig. 3A). Paroxetine's inhibitory effects were most clear in the second ejaculatory series, but due to the low number of animals still performing sexual activities these effects could not be statistically determined. None of the DOV 216,303 doses had any effect on sexual behavior (Fig. 3A and B for ejaculation and latency).

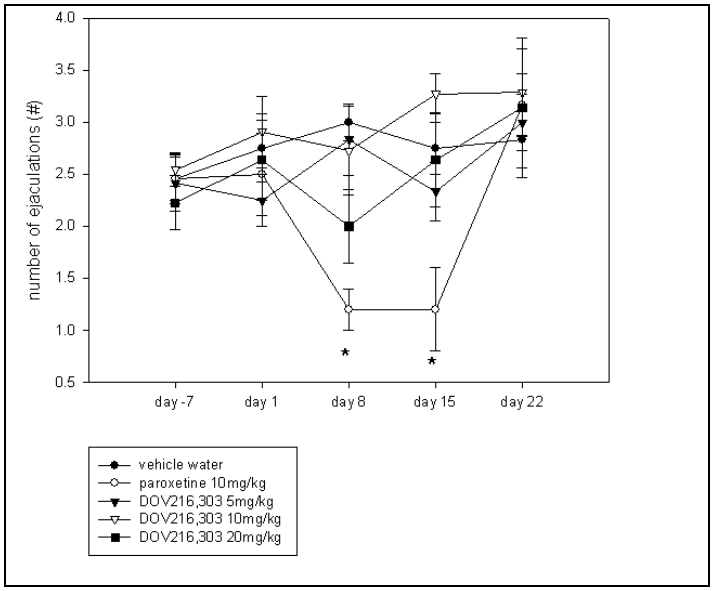

Figure 3A: Number of ejaculations (± SEM) from the last training (Day 7), and the four experimental days. Day 1 reflects acute treatment; Days 8 and 15 reflect data after 7 and 14 days of treatment, respectively. Day 22 is one week after cessation of treatment.
* = p <0.05 compared to vehicle treatment.

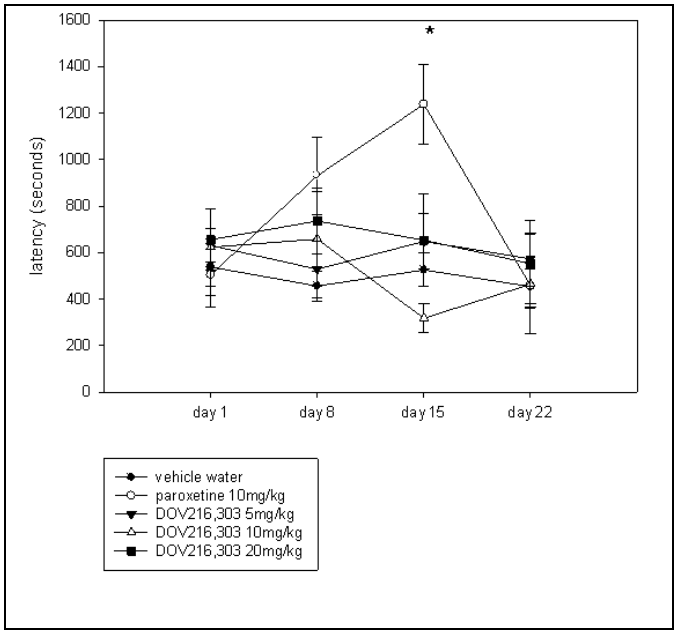

Figure 3B: The ejaculation latency of the first ejaculation series (± SEM) over the four experimental days. Days 8 and 15 reflect data after 7 and 14 days of treatment, respectively. Day 22 is one week after cessation of treatment.
* = $p < 0.05$ compared to vehicle treatment.

After chronic (fourteen days) treatment, paroxetine still exerted inhibition of sexual behavior (Fig. 3A and B). Besides the reduction in the number of ejaculations (Z=−2.826, p=0.005) (Fig. 3A), there were also significant effects on the number of mounts in the first ejaculation series (Table 1; increased; Z=−2.409, p=0.014), and the latency to the first ejaculation (enhanced: Fig. 3B; Z=−2.968, p=0.003). DOV216,303 had no inhibitory effects on sexual activities at any dose after chronic treatment when compared to the vehicle. There was a decrease in the latency to 1st ejaculation at the 10 mg/kg dose of DOV 216,303 (Z=−2.154, p=0.031).

Table 1 Effects of paroxetine versus vehicle in the first ejaculatory series over 4 tests

Parameter	Acute vehicle	Acute paroxetine	Sub-chronic vehicle	Sub-chronic paroxetine	Cronic vehicle	Chronic paroxetine	Extinction vehicle	Extinction paroxetine
EF (#)	2.8±0.3	2.5±0.4	3.0±0.2	1.2*±0.2	2.8±0.3	1.2*±0.4	2.8±0.3	3.2±0.3
EL (s)	238.6±121.5	5051.1±136.2	456.7±63.1	931.0±167.0	525.9±71.9	1238.2*±171.7	454.8±75.7	458.3±97.4
IF (#)	9.5±1.0	7.4±10	8.3±0.5	7.3±1.4	10.3±1.0	8.0±1.6	8.3±0.7	8.7±0.8
IL (s)	14.0±2.8	198.2±160.6	8.4±0.9	90.2±48.1	12.8±3.2	277.1±182.9	16.9±6.6	12.2±4.2
MF (#)	17.2±4.9	22.5±5.1	17.7±5.2	38.5±8.4	16.4±4.6	49.3*±11.5	18.6±5.4	12.5±4.3
ML (s)	10.1±5.1	3.8±0.4	78.7±60.7	13.9±9.4	5.5±0.6	4.1±0.7	19.6±10.6	4.7±0.9
PEL (s)	286.5±21.1	284.2±13.2	289.4±21.6	625.7±196.0	323.6±16.6	939.1*±234.5	252.0±29.6	291.9±18.6
CE (%)	43.5±5.3	29.7±6.0	44.2±6.9	23.7±6.6	49.1±6.8	21.9*±6.0	42.5±6.1	55.7±7.9

EF=ejaculation frequency, EL=ejaculation latency, IF=intromission frequency, IL=intromission latency, MF=mount frequency, ML=mount latency, PEL=post ejaculatory latency, CE=copulatory efficiency.
*=$p<0.05$ compared to vehicle treatment at the same time point.

Nonparametric statistics comparing the different treatment days showed no significant inhibitory effects of DOV 216,303 (5 and 10 mg/kg) and the vehicle treatment across the four experimental days (Table 1). There was an increase in the 2nd CE in the 5 mg/kg dose of DOV 216,303 after chronic treatment (Z=−2.716, p=0.007 and Z=−2.160, p=.031, respectively). Due to low numbers of paroxetine-treated animals achieving more than one ejaculation in the sub-chronic and chronic treatments (n=6 rats with less than 1 ejaculation), only the 1st ejaculatory series data were compared. Sub-chronic and chronic treatment of paroxetine led to significant decreases in ejaculation frequency when compared with acute treatment (Z=−2.658, p=0.008 and Z=−2.296, p=0.022, respectively) and one-week post treatment (Z=−3.107, p=0.002 and Z= −2.869, p= 0.004, respectively). Sub-chronic and chronic paroxetine increased the post ejaculatory latency when compared to acute treatment (Z=−2.256, p=0.024, and Z= −3.048, p= 0.002, respectively). Sub-chronic and chronic paroxetine treatment also significantly inhibited total mounts in the 1st series (Z=−2.043, p=0.041, and Z=−2.459, p=0.014, respectively), the 1st CE (Z=−2.194, p=0.028, and Z=−2.383, p=0.017, respectively), and the PEL1 (Z=−2.722, p=0.006 and Z=−3.263, pb0.001, respectively) when compared to one week post treatment. Chronic treatment also resulted in a significant increase in the 1st ejaculation latency compared to the acute treatment (Z=−2.959, p=0.003) and one-week post treatment (Z=−2.798, p=0.005). There were no statistical differences in any of the parameters between the acute treatment and one-week post treatment (see Table 1 for all paroxetine results).

Vehicle-treated animals gained approximately 2% more weight over the experimental two-week treatment period. During this period, the paroxetine (10 mg/kg) group lost almost 3% of their basal body weight when compared to vehicle ($F(4,10)=5.007$, $p=0.002$), resulting in a body weight difference of approximately 5% at the last treatment day ($F(4,10)=10.07$, $p < 0.001$). One week after cessation of treatment, the paroxetine group still exhibited a significant % difference in body weight compared to vehicles. The 20 mg/kg DOV 216,303 group also showed a reduction in body weight (at day 7: F(4, 5)=

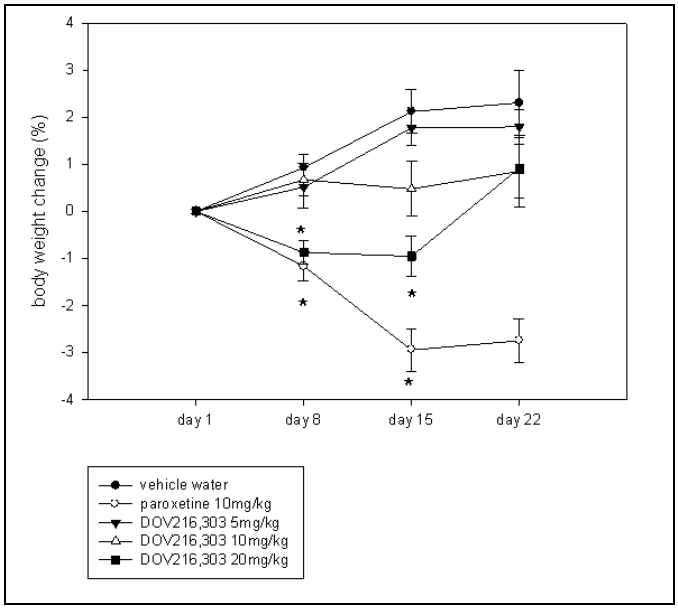

Figure 4: Effects of chronic administration of vehicle, DOV 216,303 and paroxetine on percent body weight change in male Wistar rats. Body weight at the first day has been taken as 100% and body weights have been depicted as percent changes of that initial weight. * $p < 0.05$ compared to vehicle at same time point.

3.633, p=0.011) but after cessation of treatment, these animals regained weight, although at the last measuring point (seven days post treatment) they had not recovered completely (Fig. 4).

Discussion

Chronic, but not acute, administration of the triple reuptake inhibitor (TUI) DOV 216,303 normalized OBX-induced hyperactivity at the highest dose (20 mg/kg). However, the two lower doses of DOV 216,303 (5 and 10 mg/kg) were unable to normalize OBX-induced hyperactivity at any time. This may indicate that these doses were possibly too low to exert an antidepressant effect or that longer treatment periods were needed before an effect could be observed. This is similar to results seen in the forced swim test in rats (unpublished findings), in which only the 20 mg/kg dose was effective. Shaw et al. [246] found that treatment with PRC025 and PRC050, two triple reuptake inhibitors, increased swimming and decreased immobility in rats in the forced swim paradigm at doses of 5–10 mg/kg. Also, these compounds, at the same doses, increased struggling and decreased immobility in mice in the tail suspension paradigm. However, these studies focused on

acute treatment (not chronic), used a different triple reuptake inhibitor, and focused on C57Bl/6J mice as well as Sprague–Dawley rats, differences which may account for the behavioral differences between studies. Also, while DOV 216,303 was active at the 20 mg/kg dose after chronic treatment, it was not so after seven days (subchronic) treatment, similar to results seen with the SSRI paroxetine [247]. In this experiment, imipramine had a faster onset of action compared to DOV216,303, perhaps due to the fact that imipramine does not influence dopamine, a neurotransmitter known to influence activity [248]. Since DOV 216,303 also has an influence on dopamine levels, it may be that treatment with DOV 216,303 initially increased activity in the bulbectomy animals, an effect that was normalized after chronic treatment.

DOV 216,303 did not alter, at a similar dose range, sexual behavior of male rats. Together, these results suggest this TUI might be an effective antidepressant devoid of the sexual inhibitory effects of SSRIs in depressed patients. The control substance, paroxetine, had no significant effects after acute administration, but after seven and fourteen days, paroxetine inhibited sexual behavior, similar to effects seen with other SSRIs and imipramine [18, 85, 249] One week after cessation of treatment, the paroxetine group returned to normal (compared with pretreatment values and values of the vehicle treated group) levels of sexual behavior, showing the reversibility of this SSRI's sexually inhibitory effects. The typical SSRI effects on sexual behavior, including decreased ejaculation latency, increased mount frequency and decreased copulation efficiency [5, 18, 85], were absent in the behavioral profile of the triple reuptake inhibitor DOV 216,303. DOV 216,303 showed no inhibitory effects on sexual behavior. The results of this experiment have therefore shown that the presence of a dopaminergic stimulatory component may have compensated for the inhibitory serotonergic component of DOV 216,303, although a noradrenergic component cannot be excluded.

Data from depressed patients taking TUIs are lacking, although preliminary evidence suggests that TUIs have an antidepressant effect [144]. Whether these compounds have sexual side effects remains to be seen, but bupropion, a norepinephrine/dopamine reuptake inhibitor [163] is suggested to be without sexual side effects [87, 163] and is often prescribed as co-medication with SSRIs to counteract sexual side effects [250, 251]. The

SNRI venlafaxine, however, has comparable sexual side effects to SSRIs [252, 253] suggesting that noradrenergic uptake inhibition does not preclude the inhibitory effects of serotonin reuptake inhibition on sexual behavior. This strongly suggests that the addition of a dopamine reuptake inhibitor to SNRIs may be advantageous with regard to controlling sexual side effects.

There was also body weight loss in Wistars receiving TUI treatment. In a previous study, Tizzano et al. [254] found that treatment with DOV 21,947, a compound similar to DOV216,303, induced body weight loss and altered eating patterns in diet-induced obese Sprague–Dawley animals. However, there was no significant body weight loss in bulbectomized or sham operated animals receiving DOV216,303 treatment; it may be that DOV 216,303 has different effects on the body weights of Wistars compared to Sprague–Dawley rats.

In conclusion, the present data indicate that DOV 216,303, a TUI, exhibited antidepressant-like effects in the OBX model of depression after chronic administration. It did not affect sexual behavior in rats after either acute or (sub)chronic administration (at least up to 20 mg/kg). Therefore, this putative antidepressant may not have inhibitory effects on sexual behavior in humans, a problem commonly seen with today's available treatments.

Chapter 7

Clavulanic acid stimulates sexual behaviour in male rats.

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Abstract

Sexual behavior in rats can be used to predict putative effects on human sexual behavior. Anecdotic reports exist, that the beta-lactamase inhibitor, clavulanic acid exerts sexual stimulating activities in monkeys. To characterize these pro-sexual activities, clavulanic acidwas tested in three doses and compared to one dose of a sexually inhibitory dose of the selective serotonin reuptake inhibitor, paroxetine, in sexually-experienced male rats, selected for a moderate level of sexual performance in a standard 30-min test with an oestrus female. After acute administration, clavulanic acid had minor sexual stimulating effects at the highest dose in the number of intromissions and in the first ejaculation series. After sub-chronic 7-days treatment, clavulanic acid increased the number of ejaculations at all three doses and reduced the number of intromissions in the 1st series at the highest dose. After chronic 14 days treatment, a similar but stronger pro-sexual profile was observed. The sexual side effects of paroxetine were as expected, including slight sexual inhibitory effects after acute administration, but somewhat stronger overall inhibitory effects after 7 and 14-days pretreatment, particularly notable in the decreasing number of animals contributing to the 2nd ejaculation series, which was even stronger after 14-days treatment. One week after cessation of treatment, the paroxetine group had completely recovered, whereas the highest dose-group of clavulanic acid still showed some pro-sexual effects. This remarkable pro-sexual activity of clavulanic acid cannot readily be explained by its mechanism of action as a beta-lactamase inhibitor but could be due to unexpected central activity of the compound.

Introduction

In humans, the area of sexual disturbances and their treatment has become a field of intense research particularly after the emergence of phosphodiesterase type 5 inhibitors for erectile dysfunction [26]. Moreover, quite a number of psychotropic drugs exert unwanted sexual side effects [5, 255]. Notoriously, this holds for many antidepressants, and particularly selective serotonin reuptake inhibitors (SSRIs), where sexual disturbances including libido, erection and orgasm problems belong to the main complaints [7, 8]. Although depression on itself is associated with these same problems, it is highly desirable to develop drugs without these cumbersome sexual side effects. Although it is desirable to develop new psychotropics, including antidepressants without sexual side effects, drugs that treat non-drug related sexual dysfunctions would also be needed. Currently, such drugs are not readily available although some developments are in progress, both for male [255] and female sexual dysfunction [256, 257]. Therefore, research in new drugs aiming at improvement of sexual function, physiology and behavior is absolutely necessary. Animal models of sexual behavior are considered useful in predicting effects of drugs in humans [10] and are used to study the effects of various drugs. Antidepressants and particularly SSRIs, like in humans have sexual inhibiting effects, but only after chronic administration [12, 14, 74, 85].

We have found compelling evidence [12, 113, 125] that male rats display 'endophenotypes' with regard to sexual performance: some animals are sluggish copulators (low number of ejaculations), some are normal (2–3 ejaculations per test) and some are rapid (4–5 ejaculations/test). Our testing hypothesis tries to exploit these endophenotypes. In the present study we used the 'normally' performing rats (around 2–3 ejaculations/test) to be able to observe potential facilitating effects of the drugs on sexual activities.

Using this strategy we tested the compound clavulanic acid. Clavulanic acid is produced by Streptomyces clavuligenus with a chemical structure similar to some β-lactamines, e.g. penicillin and is used clinically and veterinary as a beta-lactamase inhibitor, often in combination with penicillin, e.g. amoxicillin. Clavulanic acid by itself is orally active and

stable. The cerebrospinal fluid/plasma ratio in humans is around 0.25, indicating that clavulanic acid readily passes the blood brain barrier [258]. While testing anti-anxiety effect of clavulanic acid in non-human primates (cotton-top tamarin), it was discovered that it increased sexual arousal as indicated by the increased rate of penile erections (unpublished findings by Rexahn).

The assumption in the present experiment is that this molecule potentially would stimulate sexual performance in male rats. As a reference compoundwe tested a selected dose of paroxetine (10mg/kg) as this dose has reliably and repeatedly been shown to inhibit sexual activities after chronic, but not acute administration [12]. Drugs were administered for 14 days, and effects were measured acutely (Day 1), after 7 Days (Day 7) and after 14 Days (Day 14) of administration. Moreover, one week after cessation of treatment (Day 21) one extra sexual test (no treatment) was performed to study the potential 'after' effects of all chronic treatments.

Materials and methods

Male and female Wistar rats of approx. 8 weeks old were derived from Harlan (The Netherlands) and were group-housed. The day–night schedule was reversed (Lights off from 6:00AM till 6:00PM). After habituation to the lighting schedule, males were trained once weekly for 4 consecutive weeks without injection against an oestrus female in a 30-min test in an observation cage (30x60x40 cm) with a Plexiglas front. Females were brought into behavioral oestrus by injecting 50 µg estradiol benzoate (dissolved in sesame oil) in the nape 36 h prior to the test. The number of ejaculations over the last training test (#4) was used to designate animals either as low, medium (normal) or high performers. In the present experiments, drugs were tested on 'normal performing' animals (n=60) with on average 2 ejaculations. After this training period drug testing started and the 60 animals were randomized over the 5 experimental groups.

The following experiment was performed on 5 groups of 12 rats each: Group 1: Animals were orally injected once daily with the vehicle (distilled water) during 14 days. Groups

2, 3 and 4: clavulanic acid treatment in doses of 0.01 mg/kg (Group 2), 0.1 mg/kg (Group 3) and 1 mg/kg (Group 4). Group 5: Animals were orally injected once daily with 10mg/kg paroxetine (suspended in distilled water) during 14 days.

Clavulanic acid was dissolved in distilled water and orally administered. On non-experimental testing days, all assigned treatments were injected between 10AM and 4PM. On the experimental days (Days 1, 7 and 14) animals were injected with the assigned treatment 60 min before testing. On Day 21, one week after cessation of treatment another sexual behavior test was performed (post- treatment test) and animals did not receive injections before this test. Experimental groups were randomly divided over all treatment groups and over the experimental days.

The behavioral experiments per testing day: acute, sub-chronic (Day 7), chronic (Day 14) and post-treatment (Day 21)) were performed over two successive days per week. Testing was performed between 9:00AM and 3:00PM in the dark phase of the LD-cycle under dim red light conditions. Males were injected with the assigned treatment and 30 min later placed into the experimental cage. After 30 min an oestrus female was placed into the cage and the behavior of the malewas scored over the ensuing 30-min. The female was checked for receptive and proceptive behavior before the actual tests started.

The following parameters were scored using the Noldus Observer 5.0 program: mounts (frequency per ejaculation series and time of first mount in series, intromissions (frequency per ejaculation series and time of first intromission in series), number of ejaculations per test of 30 min, time of occurrence of ejaculations. From these data the following parameters were deduced: number of ejaculations/test; latency to 1st mount (s) in the 1st ejaculation series; latency 1st intromission (s) in the 1st ejaculation series; number of mounts in the 1st ejaculation series; number of intromissions in the 1st ejaculation series; latency to the 1st ejaculation (s) — time from the first mount or intromission to the first ejaculation; post-ejaculatory latency (s) — time from the 1st ejaculation till the first mount or intromission (whichever comes first) from the second

ejaculatory series and the copulatory efficiency that is calculated as (# intromissions/(# intromissions+# mounts))* 100%. All parameters are measured again for the second ejaculation series.

Missing values

The maximum value of 1800 was placed for latency to 1st intromission, 1st mount, 1st ejaculation, and 1st post-ejaculatory latency for any animal that failed to display those behaviors. Behavioral parameters in treatment groups with less than 7 data points ($n<7$) are given in the tables but statistics is not reliable because of the low number of contributing animals.

Statistics

Previous experiments show that the data obtained from sexual behavior experiments are normally distributed. All data are analyzed using ANOVA using SPSSv11.0, followed by Bonferroni–post-hoc tests in case of overall significant effects.

The stability of training ejaculation frequencies were assessed with the non-parametric Kruskal Wallis test. All experiments were approved by the Ethical committee of Utrecht University (DEC GNK/FSB).

Results

The average number of ejaculation for the 60 animals used in these 5 groups was 2.3±0.1 on the last training day (day −7 in Fig. 1). Over the 5 experimental days the number of ejaculations remained unchanged ($\chi 2=1.704$, $P=0.790$) indicating the very stable character of the sexual behavior of the rats.

Day 1: Acute administration

After acute treatment all groups displayed on average between 1.6 and 3.0 ejaculations/30-min. tests (Fig. 1). Clavulanic acid had limited effects on sexual behavior after acute dosing; only after the 1.0 mg/kg dose of clavulanic acid one significant effect was observed, indicating a weak pro-sexual effect: the number of intromissions in the

first ejaculation series was decreased. Paroxetine had some mild inhibitory effects: it increased the latency to the 1st intromission and decreased the number of intromissions in the 1st series (Table 1).

Table 1
Effects of chronic administration of clavulanic acid and paroxetine on sexual behaviour in male Wistar rats – acute treatment Day 1.

Parameter	Vehicle	Paroxetine	CA 0.01 mg/kg	CA 0.1 mg/kg	CA 1 mg/kg	ANOVA sig.
EF (#)	2.6 ± 0.2	1.6 ± 0.3	2.4 ± 0.4	3.0 ± 0.3	2.8 ± 0.4	0.040
LM1 (s)	61.8 ± 25.1	258.9 ± 138.9	23.8 ± 8.2	22.1 ± 13.3	76.3 ± 36.1	0.076
LI1(s)	26.4 ± 9.0	403.2[a] ± 185.9	16.1 ± 3.2	27.1 ± 7.3	49.3 ± 19.0	0.006
MF1 (#)	16.1 ± 3.4	14.7 ± 4.8	12.1 ± 4.4	8.3 ± 1.3	9.8 ± 3.2	0.539
IF1 (#)	12.3 ± 1.4	6.1[a] ± 0.8	9.3 ± 0.8	8.9 ± 0.8	7.9[a] ± 1.1	0.002
LE1 (s)	585.1 ± 69.8	640.1 ± 167.4	410.7 ± 67.2	420.2 ± 51.7	264.2 ± 49.5	0.062
PEL1 (s)	284.5 ± 18.8	802.2 ± 213.2	262.9 ± 20.3	242.6 ± 27.2	246.3 ± 20.8	0.042
CE1 (%)	47.5 ± 6.0	39.1 ± 8.3	54.0 ± 5.7	53.3 ± 4.7	56.8 ± 6.8	0.318
MF2 (#)	12.1 ± 3.3	18.4 ± 8.0	9.3 ± 1.6	7.0 ± 1.7	6.2 ± 1.5	0.121
IF2 (#)	3.9 ± 0.4	4.4 ± 0.6	5.0 ± 1.3	3.0 ± 0.4	4.0 ± 0.4	0.347
LE2 (s)	258.7 ± 43.4	375.5 ± 97.5	336.6 ± 96.8	248.9 ± 48.4	184.8 ± 32.3	0.315
PEL2 (s)	355.2 ± 15.7	409.8 ± 10.3	364.2 ± 19.8	333.0 ± 33.6	332.5 ± 23.5	0.318
CE2 (%)	31.8 ± 4.3	31.4 ± 8.0	35.7 ± 4.5	41.5 ± 8.1	47.3 ± 6.3	0.377

Data is represented as mean ± S.E.M., CA = clavulanic acid, EF= ejaculation frequency, LM1 = latency to 1st mount, LI1 = latency to 1st intromission, MF1 = mount frequency in the 1st ejaculatory series, IF1 = intromission frequency in the 1 st ejaculatory series, LE1 = latency to the 1st ejaculation, PEL1 = 1st post-ejaculatory latency, CE1 = 1st series copulatory efficiency, MF2 = mount frequency in the 2nd ejaculatory series, IF2 = intromission frequency in the 2nd ejaculatory series, LE2 = latency to the 2nd ejaculation, PEL2 = 2nd post-ejaculatory latency, CE2 = 2nd series copulatory efficiency.
[a] $P<0.05$ significantly different than vehicle.

Day 7: Sub-chronic administration

After 7-days treatment, clavulanic acid clearly showed pro-sexual activities at all doses used. The number of ejaculations was considerably enhanced (Fig. 1), although no dose-dependent effect was seen. Concomitant, some other changes were seen supporting the

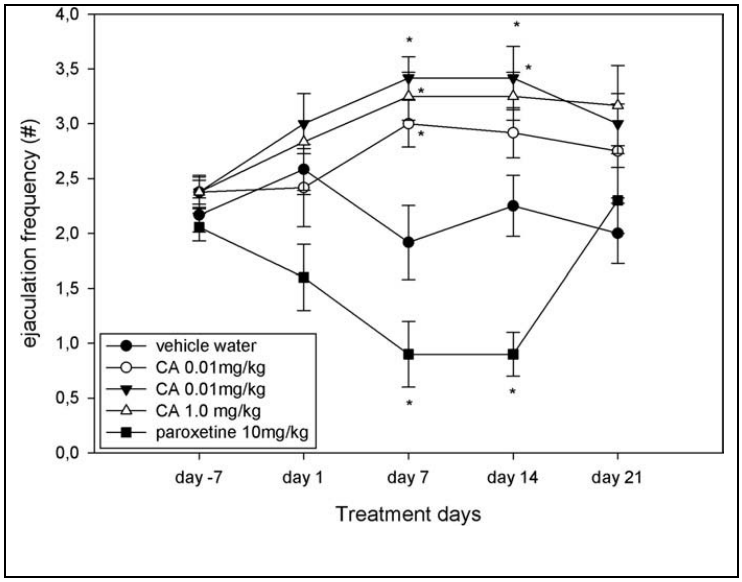

Fig.1. Effects of vehicle, clavulanic acid (CA) and paroxetine on the number of ejaculations in sexual behaviour of male rats. Data (mean±S.E.M.) are given for the last training day (day−7), after acute, sub-chronic, and chronic (Days 1, 7, and 14, respectively) treatment and one week after cessation of treatment (Day 21). * = indicates significant difference ($P<0.05$) from vehicle at the corresponding day.

pro-sexual activity of clavulanic acid: decreases in the number of intromissions in the 1st ejaculation series (Fig. 2). Paroxetine clearly reduced sexual behavior, although not strongly significant in all parameters. Its effect can be most clearly seen in the absence of the completion of the 2nd ejaculation series. Because only one serieswas completed (and not even in all animals) many parameters did not generate enough data points to make statistical evaluation worthwhile (Table 2).

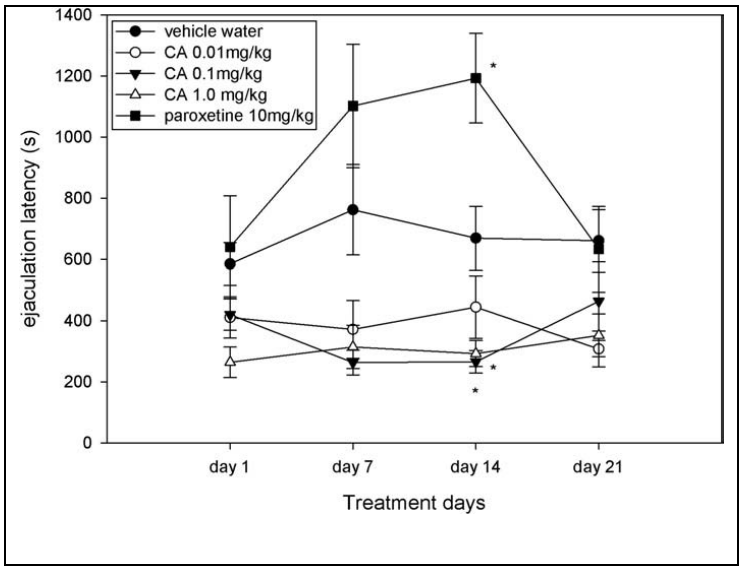

Fig. 2. Effects of vehicle, clavulanic acid (CA) and paroxetine on the latency to the first ejaculation in sexual behaviour of male rats. Data (mean±S.E.M.) are given for the last training day (day−7), after acute, sub-chronic, and chronic (Days 1, 7, and 14, respectively) treatment and one week after cessation of treatment (Day 21). * = indicates significant difference ($P<0.05$) from vehicle at the corresponding day.

Table 2
Effects of chronic administration of clavulanic acid and paroxetine on sexual behaviour in male Wistar rats – sub-chronic treatment Day 7.

Parameter	Vehicle	Paroxetine	CA 0.01 mg/kg	CA 0.1 mg/kg	CA 1 mg/kg	ANOVA sig.
EF (#)	1.9 ± 0.3	0.9 ± 0.3	3.0[a] ± 0.2	3.4[a] ± 0.2	3.3[a] ± 0.2	0.000
LM1 (s)	28.3 ± 9.7	168.5 ± 148.5	17.8 ± 5.9	41.9 ± 24.9	89.0 ± 51.2	0.560
LI1(s)	34.0 ± 14.5	130.0[a] ± 47.8	22.5 ± 5.2	11.7 ± 2.4	32.6 ± 8.6	0.004
MF1 (#)	22.0 ± 4.0	41.7 ± 10.0	12.3 ± 3.8	6.0 ± 1.2	8.7 ± 2.5	0.000
IF1 (#)	9.9 ± 0.8	10.0 ± 1.7	7.7 ± 1.0	7.1 ± 0.6	6.0[a] ± 0.6	0.031
LE1 (s)	761.9 ± 148.1	1101.7 ± 202.1	371.7 ± 94.6	263.6 ± 40.5	314.3 ± 70.6	0.000
PEL1 (s)	265.4 ± 19.1	1035.3 ± 231.3	258.8 ± 20.3	283.2 ± 14.8	240.2 ± 28.9	0.000
CE1 (%)	37.4 ± 5.8	34.4 ± 8.5	45.9 ± 5.5	59.2 ± 4.8	52.6 ± 7.3	0.051
MF2 (#)	15.8 ± 4.8	ND	10.3 ± 2.8	6.1 ± 1.6	8.9 ± 3.0	0.300
IF2 (#)	3.9 ± 0.5	ND	3.9 ± 0.4	3.9 ± 0.3	3.7 ± 0.6	0.902
LE2 (s)	241.4 ± 55.5	ND	245.6 ± 53.1	189.5 ± 37.9	248.1 ± 58.9	0.798
PEL2 (s)	404.7 ± 28.4	ND	351.4 ± 28.9	339.6 ± 25.7	364.9 ± 15.3	0.916
CE2 (%)	30.6 ± 7.7	ND	34.7 ± 5.4	48.0 ± 6.2	43.9 ± 8.2	0.440

Data is represented as mean ± S.E.M., CA = clavulanic acid, EF= ejaculation frequency, LM1 = latency to 1st mount, LI1 = latency to 1st intromission, MF1 = mount frequency in the 1st ejaculatory series, IF1 = intromission frequency in the 1st ejaculatory series, LE1 = latency to the 1st ejaculation, PEL1 = 1st post-ejaculatory latency, CE1 = 1st series copulatory efficiency, MF2 = mount frequency in the 2nd ejaculatory series, IF2 = intromission frequency in the 2nd ejaculatory series, LE2 = latency to the 2nd ejaculation, PEL2 = 2nd post-ejaculatory latency, CE2 = 2nd series copulatory efficiency, ND = data not determined since $n < 7$.
[a] $P<0.05$ significantly different than vehicle.

Day 14: Chronic administration

After 14-days of chronic treatment, clavulanic acid induced a similar behavioral profile (pro-sexual) as after the 7 days subchronic treatment. Although the lowest dose tested showed no significant effects, strong statistical tendencies were present. Additionally, at the higher doses tested, clavulanic acid showed reductions in the latency to the 1st ejaculation (Fig. 2) and reduction in the number of intromissions in the 1st series paroxetine still exerted its strong inhibitory profile: a decrease in the number of ejaculations and strong increases in the latencies to the 1st (Fig. 2) and 2nd ejaculation and the 1st post-ejaculatory latency (Table 3).

Table 3
Effects of chronic administration of clavulanic acid and paroxetine on sexual behaviour in male Wistar rats – chronic treatment Day 14.

Parameter	Vehicle	Paroxetine	CA 0.01 mg/kg	CA 0.1 mg/kg	CA 1 mg/kg	ANOVA sig.
EF (#)	2.3 ± 0.3	0.9[a] ± 0.2	2.9 ± 0.2	3.4[a] ± 0.3	3.3[b] ± 0.2	0.000
LM1 (s)	77.1 ± 34.4	62.6 ± 27.8	13.5 ± 4.8	54.1 ± 18.3	19.6 ± 10.1	0.192
LI1(s)	56.9 ± 22.0	309.2 ± 151.7	21.5 ± 4.8	8.2 ± 1.3	29.6 ± 9.3	0.015
MF1 (#)	17.3 ± 4.3	30.8 ± 5.1	13.6 ± 3.9	8.4 ± 2.8	7.6 ± 2.0	0.000
IF1 (#)	11.6 ± 1.7	10.3 ± 1.8	7.8 ± 0.9	8.1 ± 0.5	6.8[b] ± 0.6	0.050
LE1 (s)	669.1 ± 104.3	1192.8[a] ± 146.8	444.8 ± 102.3	265.8[a] ± 37.1	292.7[b] ± 42.8	0.000
PEL1 (s)	306.0 ± 26.9	1084.8[a] ± 216.3	259.7 ± 23.6	253.7 ± 13.0	271.9 ± 13.3	0.000
CE1 (%)	47.7 ± 6.1	29.4 ± 6.1	46.5 ± 6.1	60.2 ± 6.3	55.2 ± 7.0	0.014
MF2 (#)	11.2 ± 3.3	ND	8.8 ± 1.9	7.6 ± 1.4	3.7 ± 0.9	0.113
IF2 (#)	4.3 ± 0.4	ND	3.7 ± 0.5	3.8 ± 0.2	3.5 ± 0.2	0.145
LE2 (s)	278.2 ± 51.1	ND	244.7 ± 41.6	179.3 ± 20.2	133.9[a] ± 12.9	0.010
PEL2 (s)	336.4 ± 17.4	ND	342.5 ± 20.5	336.9 ± 15.3	310.1 ± 20.7	0.053
CE2 (%)	39.5 ± 8.6	ND	36.7 ± 6.4	39.3 ± 5.6	55.1 ± 6.3	0.307

Data is represented as mean ± S.E.M., CA = clavulanic acid, EF= ejaculation frequency, LM1 = latency to 1st mount, LI1 = latency to 1st intromission, MF1 = mount frequency in the 1st ejaculatory series, IF1 = intromission frequency in the 1 st ejaculatory series, LE1 = latency to the 1st ejaculation, PEL1 = 1st post-ejaculatory latency, CE1 = 1st series copulatory efficiency, MF2 = mount frequency in the 2nd ejaculatory series, IF2 = intromission frequency in the 2nd ejaculatory series, LE2 = latency to the 2nd ejaculation, PEL2 = 2nd post-ejaculatory latency, CE2 = 2nd series copulatory efficiency, ND = data not determined since $n < 7$.

[a] $P<0.05$ significantly different than vehicle.

[b] Marginally different than vehicle $0.05<P<0.10$.

Table 4
Effects of chronic administration of clavulanic acid and paroxetine on sexual behaviour in male Wistar rats – extinction treatment Day 21.

Parameter	Vehicle	Paroxetine	CA 0.01 mg/kg	CA 0.1 mg/kg	CA 1mg/kg	ANOVA sig.
EF (#)	2.0 ± 0.3	2.3 ± 0.3	2.8 ± 0.4	3.0 ± 0.3	3.2 ± 0.4	0.076
LM1 (s)	19.4 ± 9.0	34.6 ± 9.4	72.1 ± 51.7	23.0 ± 10.3	62.5 ± 18.8	0.498
LI1(s)	38.5 ± 11.4	228.6 ± 148.0	19.5 ± 4.5	21.0 ± 11.1	28.4 ± 16.6	0.137
MF1 (#)	19.2 ± 3.2	12.3 ± 2.2	11.8 ± 4.3	13.7 ± 4.0	7.6 ± 2.3	0.189
IF1 (#)	9.6 ± 1.0	9.8 ± 1.3	7.2 ± 0.9	9.8 ± 1.8	6.5 ± 0.7	0.147
LE1 (s)	660.8 ± 101.8	633.0 ± 140.5	308.0 ± 58.7	464.0 ± 128.0	352.3 ± 70.0	0.424
PEL1 (s)	272.7 ± 15.2	260.1 ± 17.4	265.1 ± 15.5	271.6 ± 7.6	265.9 ± 19.2	0.727
CE1 (%)	37.5 ± 5.0	46.6 ± 5.7	52.5 ± 6.9	49.5 ± 4.7	56.7 ± 7.1	0.225
MF2 (#)	11.7 ± 3.0	8.1 ± 2.0	7.3 ± 2.3	8.0 ± 2.2	6.7 ± 2.7	0.671
IF2 (#)	4.4 ± 0.6	4.9 ± 0.7	2.7 ± 0.4	3.9 ± 0.5	3.8 ± 0.2	0.030
LE2 (s)	269.4 ± 30.2	245.2 ± 31.1	188.7 ± 27.8	188.9 ± 27.2	134.0[a] ± 18.0	0.010
PEL2 (s)	371.6 ± 13.0	394.1 ± 18.7	362.3 ± 25.3	329.2 ± 33.4	327.6 ± 22.2	0.274
CE2 (%)	36.4 ± 8.1	43.0 ± 6.9	36.8 ± 6.2	41.0 ± 5.1	55.6 ± 7.9	0.271

Data is represented as mean ± S.E.M., CA = clavulanic acid, EF= ejaculation frequency, LM1 = latency to 1st mount, LI1 = latency to 1st intromission, MF1 = mount frequency in the 1st ejaculatory series, IF1 = intromission frequency in the 1 st ejaculatory series, LE1 = latency to the 1st ejaculation, PEL1 = 1st post-ejaculatory latency, CE1 = 1st series copulatory efficiency, MF2 = mount frequency in the 2nd ejaculatory series, IF2 = intromission frequency in the 2nd ejaculatory series, LE2 = latency to the 2nd ejaculation, PEL2 = 2nd post-ejaculatory latency, CE2 = 2nd series copulatory efficiency.

[a] $P<0.05$ significantly different than vehicle.

Day 21: One week post-treatment

One week after cessation of treatment all effects of clavulanic acid have disappeared except for the latency to the 2nd ejaculation at the highest dose. The paroxetine treated group also completely returned to normal (Table 4).

Discussion

According to Rexahn, preliminary studies in monkeys had indicated that clavulanic acid potentially exerted some pro-sexual activities. To this end we performed a sexual behavior study in male rats that were selected on basis of their sexual performance in a number of training tests. We selected animals with normal sexual behavior (around 2 ejaculations per 30-min)which enables the detection of putative sexual stimulating (pro-sexual) and inhibiting effects. As a reference compound one dose of the inhibitory SSRI antidepressant paroxetine was included. Paroxetine indeed inhibited sexual behavior and did so clearly after (sub) chronic administration in line with our earlier observations [85]. Clavulanic acid has a remarkable sexual stimulating (pro-sexual) activity. This profile is not yet fully present after acute treatment, although some indications of it are already seen (decreased latency to ejaculate), but becomes very evident after 7 and 14 days treatment, and also at all doses (0.01–1 mg/kg), although the effect of the 0.01 mg/kg dose seems to weaken a bit over time. The most intriguing finding is the increased number of ejaculations reached by the clavulanic acid-treated animals. After sub-chronic dosing of 7 and 14 days we see an increase of over 60% in this parameter. This seems primarily due to decreased latencies to the first (and to a lesser extent to 2nd) ejaculations. Because of these shortened latencies the animals seem to be more efficient although the copulatory efficiencies are not significantly improved, but the number of mounts and intromission are clearly diminished. More detailed studies are clearly needed to discern whether clavulanic acid exerts its pro-sexual activities via motivational or sexual execution mechanisms or both. As the cerebrospinal fluid/plasma ratio is 0.25, clavulanic acid apparently readily passes the blood-brain barrier. It might alter brain chemistry in some way to achieve pro-sexual activity. Preliminary in vivo microdialysis studies in rats following clavulanic acid treatment (unpublished data) revealed a time-dependent

enhanced neurotransmission of serotonin and dopamine in the nucleus accumbens. The neurotransmitters serotonin and dopamine coordinate sexual motivation, copulatory behavior and erectile function [36, 259]. Further studies are ongoing to elucidate its mechanism of action in the brain. Our studies document a new property of clavulanic acid demonstrating that clavulanic acid exerts some putative pro-sexual effects. This is, to our knowledge, the first evidence of stimulatory enhancement of sexual activity by clavulanic acid in male rats. Further research is needed to investigate the mechanisms underlying this exciting finding.

Chapter 8

Discussion, Summary and Future perspective

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Discussion, Summary and Future perspective

The present thesis dealt with the development of an animal (rat) model (paradigm) that has face, predictive and construct validity towards human sexual behavior and its disorders and can be used to study the intrinsic effects but also the side effects of drugs on sexual behavior.

To this end, we tested large cohorts of male rats on sexual behavior and found large differences in the individual level of sexual performance. In such cohorts, after extensive sexual training, individual rats show stable levels of sexual behavior over time: rat's individual sexual behavior could be classified as 'sluggish' (slow; 0-1 ejaculation/30 min test), 'normal (2-3 ejaculations/30 min test) or 'rapid' (fast) (>3 ejaculations/30 min test). As we were interested in developing models for lifelong premature ejaculation, normal sexual behavior and retarded ejaculation [125, 151], we postulated that these three classes of animals might represent 'endophenotypes' that can be used to study the effects of drugs. The 'fast' ejaculating animals might model PE, whereas the 'sluggish' (slow) ejaculating animals might model retarded ejaculation [260]. The 'normal' ejaculating animals (2-3 ejaculations per test) are actually the main subject of the present thesis as they can be applied to test the intrinsic effects of all kind of drugs on sexual behavior itself. At this level of sexual performance both inhibitory and stimulatory effects of drugs can be detected.

Endophenotypes

In the five year period covered in this thesis, ranging from June 2004 till the end of 2009, we have run 12 studies with large cohorts of male rats, extending the initial results of Pattij et al. [17]. In each study, male Wistar outbred rats derived from one vendor (see Chapter 2 for extensive methodology) were trained 4 times at weekly sessions of 30 minutes, against female Wistar rats brought into behavioral estrus (receptive and proceptive) by estrogen injections. Under these conditions approx. 1900 rats were tested and the distribution of their ejaculation frequency at the last training test is shown in Fig. 6 of Chapter 2. In this histogram, it can be seen that the majority of rats (approx. 60%) display 2-3 ejaculations/30 min, whereas a considerable number (around 15%) does not

ejaculate, or only once (around 10%). Animals ejaculating 4 times (around 10%) or higher (<1%) are less frequently found. Although initially it looked as if the distribution of the different 'endophenotypes' of male sexual performance was, within a cohort, relatively similar to the overall pattern (Fig. 1 in this discussion), individual portrayal of 12 successive cohort studies we have performed over the last 5 years in Utrecht tell differently.

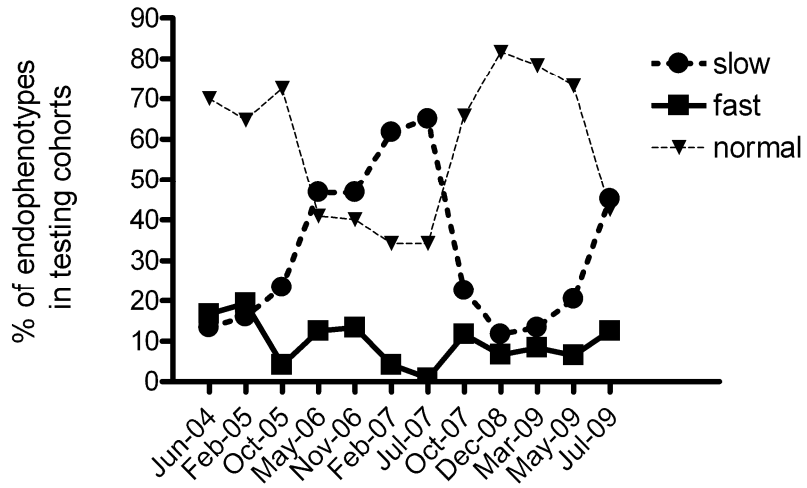

Figure 1. Percent of endophenotypes in separate cohorts of 120 male rats.

The number of 'fast' ejaculating animals fluctuated between 2-20%. However, the number of 'sluggish' animals (0-1 ejaculations/test) fluctuates dramatically between cohorts and over time (between 10 and 60%). Accordingly, the number of 'normal' ejaculating rats (2-3 ejaculations/test) fluctuates (between 35-80%). Although within each individual cohort, animals display a very stable sexual 'endophenotype', between the cohorts and over time, the distribution of the various endophenotypes fluctuates considerably. Combining all experiments, it is clear that normal rats form the majority of the population. The variability in the endophenotypic distribution makes it necessary to start training with large cohorts - we mostly use 120 male rats - in order to generate

sufficient numbers of animals for our drug studies. The latter are composed of groups of animals (N=12/group) of a certain endophenotype. In this Thesis we have performed our drug studies, particularly those that involve chronic dosing, on 'normal' endophenotypes (2-3 ejaculations/test). We always use, next to a vehicle group, a standard reference drug group (paroxetine-10 mg/kg). This dose of paroxetine induces a very reliable and reproducible inhibitory effect on male sexual behavior (see Chapter 2 and 6 for a discussion).

It is unclear to us what causes the large between-cohort variability in the number of sexual endophenotypes. Whether genetic factors play a major role is unclear: the strain of rats we use, Wistar ourbred, has always been obtained from the same vendor (Harlan, Zeist). This outbred strain has been used by us for more than 30 years to study the effects of drugs on male sexual behavior [11] and appeared a very reliable animal in sex research. It seems highly unlikely that 'genetic drift' may cause the variability over time because more recent cohorts seem to 'mimic' our first cohorts and the rapid change in the distribution of the endophenotypes that occurred (i.e. between July and October 2007) (Fig. 1). At this stage, we can only speculate over what caused the sexual endophenotypes. For instance maternal stress and/or care may contribute to the differences in endophenotypic distribution. Never the less, whether an 'individual' endophenotype (i.e. 'sluggish', 'normal' or 'fast' ejaculator) is (strongly) genetically determined is still not clear; selective breeding programs should be developed to study this. Pattij et al. [17] studied the effects of a prosexual drug (8-OH-DPAT) on representative rats from the three endophenotypic classes. 8-OH-DPAT stimulated sexual performance in all three classes equally. When testing their basic sexual behavior one week after the 8-OH-DPAT facilitation, all animals had resumed their original endophenotype; this suggests that the ejaculatory behavior of either endophenotypic ejaculator is not due to a physiological or physical abnormality in their reproductive system. We [17] concluded that the differences in ejaculatory behavior in the various endophenotypes have a neurobiological rather than a psychological origin contrasting the dogma that human life long premature ejaculation has a 'learning' background [261]. Further studies did not find differences in various behaviors (elevated plus maze or open field tests) or dopaminergic

sensitivity (apomorphine-induced gnawing) between the three endophenotypes indicating the specificity of the endophenotypes for sexual performance and not due to some general behavioral trait (e.g. anxiety or stress).

Pharmacology of male sexual behavior

Very little research has been performed thus far in the 'slow' and 'fast' endophenotypes (Chapter 3). For the present thesis, we mainly used the 'normal endophenotypes to study the effects of various drugs, in particular antidepressants (Chapter 4). In early studies by our laboratory [11, 262], we used acute administration of various psychoactive drugs to investigate either their putative mechanism of action, the putative role of that mechanism in male sexual behavior or possible side effects. In chapter 3, we studied the effects of 8-OH-DPAT, a potent and selective 5-HT1A receptor agonist on 'slow' male rats. Similar to previous results [17], 8-OH-DPAT restored sexual behavior to normal or even supranormal levels. Buspirone, also a (partial) 5-HT1A receptor agonist was also able to stimulate sexual behavior although less efficaciously than 8-OH-DPAT, presumably reflecting the partial character of the 5-HT receptor activation or the involvement of DA-D2 receptor blockade inherent in buspirone's mechanism of action [263]. Bupropion, an antidepressant blocking NE and DA transporters, was not able to stimulate significantly sexual activity but was clearly devoid of any inhibitory action.

In an unpublished small pilot study, the 5-HT2C receptor agonist RO60-0175 (0.16, 0.63 and 2.5 mg/kg) dose-dependently reduced the ejaculation frequency in 'fast' rats. In addition, when these rats were treated with the 5-HT1B receptor agonist TFMPP (0.1 mg/kg) we also found a strong inhibition of sexual behavior in line with earlier findings in normal rats [264].

Our standard protocol to test acute, subchronic and chronic effects of compounds, mainly but not exclusively antidepressants, is described in Chapter 2. Basically, trained and stably sexually performing male rats (2-3 ejaculations/test) are treated daily for 14 days with a dose of a compound. On days 1, 7 and 14, the effects of that compound is measured (30-60 min after treatment) in a standard sexual test of 30 min with a receptive

female. On intervening days the compound is administered once per day. One week after treatment, a last sex test is performed to study the putative 'after' effects of the compound and also to check whether the stability of the sexual behavior has been influenced by a putative effect of the compound on the sexual behavior. In all the drug tests performed, the sexual behavior after washout has never been affected. Only in the case of clavulanic acid some positive effects on sexual behavior were still present although the prosexual effects seemed to wane (Chapter 7).

In this design, we both have drug effects after acute (Day 1), subchronic (Day 7) and chronic administration (Day 14). This design was developed to study the effects of SSRI antidepressants that have severe (side) effects in people, although main complaints have been particularly emerged in depressed patients, the target group for SSRIs. Depression itself is accompanied by sexual disturbances [265] and it is not clear, although plausible, whether SSRIs exacerbate those sexual disturbances. This is supported by findings in healthy men with PE showing that SSRIs lengthen the ejaculation time [25]. This therapeutic effects however, is caused by the inhibitory action of SSRIs [266] illustrating the inhibitory action independent from a depressive state.

Paroxetine, a potent and frequently used SSRI antidepressant in humans, had inhibitory effects in 'normal' ejaculating rats. Typically, these inhibitory effects do not occur immediately. i.e. after acute dosing (although we sometimes find some small effects) but emerge after 7 and continue to 14 days of treatment or longer [86]. This pattern, gradually emerging of the inhibitory action, seems to parallel the antidepressant profile of SSRIs and other antidepressants strongly suggesting that adaptational processes have to take place in the CNS before the therapeutic effects appear. It is suggested that 5-HT1A receptors play a pivotal role in these adaptational machinery [174, 175]. By adding a 5-HT1A receptor antagonist to an SSRI, the inhibitory action of the SSRI is strongly facilitated, even after acute administration of the SSRI [186]. The complex inhibition of sexual behavior by adding an on itself inactive dose of WAY100,635 to an inactive dose of paroxetine (unpublished-this thesis) or citalopram [186] suggest that 5-HT1A receptors are becoming desensitized after (sub)chronic treatment with SSRIs which can be mimicked by acute 5-HT1A receptor blockade to the SERT blockade.

The return of sexual behaviors one week after the end of paroxetine treatments mimics the human situation of a drug holiday, where a patient would take a break from the treatment medication to relieve some of the side effects. This return in sexual behaviors makes this paradigm quite distinct from animal models of depression where SSRI effects continue weeks after the end of treatment [267].

We and others have tested several compounds in a standard sexual behavior protocol. The results are summarized in Table 1.

Several conclusions can be drawn from these and literature data.
In general, SSRIs show an inhibitory profile in male sexual behavior in rats, although most pronounced after (sub) chronic dosing. Acutely, sometimes some inhibitory effects have been found, but these findings are often not consistent or reproducible. Therefore, the paradigm developed by us, has considerable face and predictive validity for the sexual (side) effect of SSRIs. There is evidence, mainly from human studies in PE [69, 195, 268], that not all SSRIs have comparable inhibitory activity in PE; notably fluvoxamne and citalopram are not very active in PE [25]. We tend to explain those differences to the degree of 5-HT1A receptor desensitization induced by the various SSRIs [34]. However, both fluvoxamine and citalopram have sexual side effects after chronic treatment in depressed patients [152] suggesting that some 5-HT1A receptor desensitization has occurred.
Apart from the predictive validity for inhibitory actions of drugs, the paradigm also detects sexually enhancing or prosexual effects of drug, e.g for bupropion, buspirone, apomorphine and clavulanic acid. This is in line with human evidence indicating that bupropion can be used to counteract the inhibitory actions of SSRIs [133, 167], and the lack of sexual complaints after buspirone use [139]. Apomorphine is clinically used a prosexual drug [269] although its side effect profile (nausea, vomiting) is not very promising.
Clavulanic acid is not known for its prosexual activities in humans but it increases penile erections in non-human primates (cotton-top tamarins) [188].
The absence of effects on sexual behavior (DOV, S2006) might be predictive for the absence of such effects in the human patient; this has to be proven clinically however.

Table 1: Summary of effects of various drugs on male sexual behavior after acute, subchronic or chronic treatment. ↓ :inhibition; ↑stimulation; (↑↓) trend; 0: no effect; -: not tested. SSRI: Selective serotonergic reuptake inhibitor; SNRI: serotonergic and noradrenergic reuptake inhibitor; NDRI: noradrenergic and dopaminergic reuptake inhibitor; TRI: triple monoaminergic reuptake inhibitor; r: receptor

Drug	Mechanism of Action	Acute	Sub - chronic	Chronic	Washout	reference
Paroxetine	SSRI	0	↓	↓	0	Chapter 2 & 4
Fluvoxamine	SSRI	0	0	↓	-	[86]
Citalopram	SSRI	0 (↓)	↓	↓	-	[186]
Fluoxetine	SSRI	0	↓	↓	-	[19]
Venlafaxine	SNRI	0	0	↓	0	Chapter 4
Bupropion	NDRI	↑	0	0	0	Chapter 4
Buspirone	5-HT1A R Agonist	↑	0	0	0	Chapter 4
DOV216,303	TRI	0	0	0	0	Chapter 6
clavulanic acid	beta-lactamase inhibitor	↑	↑	↑	(↑)	Chapter 7
Apomorphine	DA-D2 R agonist	↑	↑	↑	-	[108]
S32006	5-HT2C R antagonist	0	0	0	0	Chapter 4
WAY100,635	5-HT1A R antagonist	0	0	0	-	[186]

SSRIs, SERT and 5-HT1A receptors

It is generally assumed that chronic treatment with SSRIs leads to desensitization of 5-HT1A receptors in animals and man [270]. The availability of the SERT-knock out (SERT-/-) rat created an opportunity to study the consequences of life long absence of the serotonin transporter, a very important regulator of serotonergic neurotransmission and homeostasis [177]. Seen the importance of certain polymorphisms in the promoter of the SERT [271] in the expression and functioning of the SERT, this animal model potentially could contribute to a better understanding of the role of serotonin and the SERT in sexual behavior, as 5-HT seems a major player in the regulation of sexual behavior [36]. In particular the heterozygous SERT-KO rat might constitute an animal model for chronic SSRI use, whereas the homozygous SERTKO rat could contribute to unravel developmental issues. Chapter 5 describes the basic sexual behavior of the SERT genotypes; WT (SERT+/+), heterozygous KO (SERT+/-) and homozygous KO (SERT-/-). SERT-/-, but not the SERT+/- had a lower basial sexual performance than the Wildtype animals. This implicates that the neurotransmitter serotonin in involved in the expression of sexual performance under normal conditions but that sexual behavior is only affected when more that 50% of the SERT are affected. Apparently, the absence of all SERT from conception on has developmental consequences for normal sexual activity in male rats. SSRI administration, which might mimic (part of) the SERT null mutation,
When given chronically for 30 days during adolescence [86] led to a permanent decrease in sexual behavior in male rats 5 weeks after stopping treatment, indicative that this treatment period led to irreversible changes in the CNS due to SERT blockade. Although not definitely proven, these data suggest that SERT blockade (or null mutation) during a certain critical period in development, might lead to permanent perturbation of adult male sexual behavior. The finding that heterozygous SERTKO rats have no perturbed sexual behavior suggests that 50% reduction in SERT activity is not sufficient to induce permanent changes in 5-HT homeostasis.
Studies on the functional status of 5-HT1A receptors in the 5-HT-/- rat indicated a complex role of different pools of 5-HT1A receptors implicated in different aspects of

sexual performance. 5-HT1A receptor activation by 8-OH-DPAT led in all genotypes to comparable prosexual activity, suggesting that the pool of 5-HT1A receptors mediating this activity is undisturbed in the SERT-KO animals. A different pool of 5-HT1A receptors, and only in the homozygous SERT-/-, appeared hypersensitive as indicated by the (strong) inhibitory action of the 5-HT1A receptor antagonist WAY100,635, which is behaviorallly inert in the WT and SERT+/- animals. The latter pool might be involved in the basal reduction in sexual behavior of the SERT-/- rats (see Chapter 5 for a more extensive discussion).

The SERT-KO rat might be an interesting animal model to study modulating effects of various drugs on a perturbed serotonergic CNS system. Could it model retarded ejaculation and as such be used for testing of prosexual drugs that normalize the disturbed sexual function?

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Nederlandse Samenvatting

In dit proefschrift wordt de ontwikkeling van een diermodel besproken, dat zowel “face validiteit”, “predictieve validiteit” en “construct validiteit” heeft van zowel normaal humaan seksueel gedrag als humane seksuele disfuncties. Het diermodel is ontwikkeld in de mannelijke Wistar rat en kan gebruikt worden om de effecten en bijwerkingen van psychofarmaca op seksueel gedrag te bestuderen en te voorspellen in de mens.

In seksueel getrainde mannelijke Wistar ratten, stabiliseert het seksuele gedrag van de individuele ratten zich in de loop van de tijd en blijkt dat iedere individuele rat zijn eigen zeer stabiele niveau van seksueel gedrag laat zien. Daardoor kunnen de ratten worden ingedeeld in 3 typen aan de hand van het aantal ejaculaties per test van dertig minuten: ‘langzame ratten’ (0-1 ejaculaties per 30 minuten), 'normale ratten’ (2-3 ejaculaties per 30 minuten) en 'snelle ratten' (meer dan 3 ejaculaties per 30 minuten). Aangezien we geïnteresseerd zijn in de ontwikkeling van een diermodel voor zowel levenslange (aanwezige) vroegtijdige zaadlozing, normale zaadlozing en vertraagde zaadlozing, hebben wij gepostuleerd dat elk van deze drie groepen een afzonderlijk 'endofenotype' is, dat gebruikt kan worden om de effecten van psychofarmaca of andere verbindingen te bestuderen. De "snel" ejaculerende ratten staan derhalve model voor levenslange vroegtijdige zaadlozing, terwijl de 'langzame' ratten model staan voor mannen die lijden aan levenslange vertraagde zaadlozing.

Echter, de 'normale' ratten (2-3 zaadlozingen per 30 minuten test) vormen het belangrijkste onderwerp van dit proefschrift, aangezien deze ratten kunnen worden gebruikt om de intrinsieke effecten van allerlei psychofarmaca en andere verbindingen op seksueel gedrag zelf te testen. Op dit niveau van seksuele prestaties kunnen zowel remmende als stimulerende effecten van medicijnen worden gedetecteerd.

In Hoofdstuk 1 wordt een korte maar algemene inleiding gegeven over de opzet van dit proefschrift.
Een gedetailleerde beschrijving van de in onze experimenten gebruikte methoden wordt gepresenteerd in Hoofdstuk 2. Dit hoofdstuk behandelt de wijze waarop seksuele

stoornissen worden veroorzaakt door selectieve serotonine heropname remmers (SSRIs), een bepaalde klasse van serotonerge antidepressiva.

In Hoofdstuk 3 wordt een overzicht gegeven van de langzame, normale en snelle ejaculerende ratten en de vertaling ervan naar het menselijke seksuele gedrag. 8-OH-DPAT, een krachtige 5-HT_{1A} receptor agonist, verbeterde het seksuele gedrag van langzame ratten tot normale of zelfs supranormale niveaus. Buspirone, een (partiële) 5-HT_{1A} receptor agonist, is eveneens in staat om seksueel gedrag te stimuleren, hoewel dit farmacon minder efficiënt werkt dan 8-OH-DPAT. Dit wordt vermoedelijk veroorzaakt door het partiële karakter van de 5-HT receptor activering of door de betrokkenheid van de dopamine (DA) receptor-D2 blokkade die inherent is aan het werkingsmechanisme van buspirone. Bupropion, een antidepressivum dat noradrenaline (NE) en dopamine (DA) transporters blokkeert, heeft geen stimulerende werking op de seksuele activiteit, maar heeft ook geen remmende werking op het seksuele gedrag.

In een ongepubliceerde kleine pilot-studie, verlaagt de 5- HT_{2C} receptor agonist RO60-0175 (0.16, 0.63 en 2.5 mg / kg) dosisafhankelijk de ejaculatiefrequentie van 'snelle' ratten. Daarnaast vinden we een sterke remming op seksueel gedrag, wanneer deze ratten worden behandeld met de 5- HT_{1B} receptor agonist TFMPP (0.1 mg / kg). Dit komt overeen met eerdere bevindingen in normale ratten.
Een pilotstudie in muizen naar de mogelijke erfelijke achtergrond van mannelijk seksueel gedrag maakte gebruik van chromosomale substitutiestammen. De preliminaire gegevens wijzen niet op een sterke betrokkenheid van een (locus op een) bepaald chromosoom bij ejaculatiegedrag in de muis.

In hoofdstuk 4 hebben we het effect van verschillende antidepressiva in normaal ejaculerende ratten met elkaar vergeleken. Nieuwe en bestaande geneesmiddelen die werken op de serotonine (SERT), noradrenaline (NET) en/of DA-transporters (DAT), en 5-HT_{1A} en 5-HT_{2C} receptoren, werden onderzocht. In dit model, komen de resultaten van de remmende en stimulerende effecten van antidepressiva op het seksuele gedrag van

mannelijke ratten overeen met de reeds bekende en verwachte effecten bij de mens. Het diermodel weerspiegelt de humane situatie waarbij de remmende effecten van SSRIs pas na chronische toediening, en niet na acute toediening, zichtbaar worden. In overeenstemming met de humane situatie, zorgt blokkade van SERT (paroxetine en venlafaxine) voor storingen in het mannelijke seksuele gedrag, in tegenstelling tot de DA / NA heropname remmer, bupropion. Andere psychofarmaca die vooral dopaminerge en noradrenerge, maar niet de serotonerge, neurotransmissie laten toenemen (de 5-HT_{1A} receptor agonist, buspirone; de triple reuptake inhibitor, DOV216, 303 en de 5-HT_{2C} receptor antagonist, S32006), hadden geen nadelige effecten of zelfs een mild stimulerend effect op seksueel gedrag, waardoor ze naar verwachting weinig of geen seksuele bijwerkingen veroorzaken bij mannen. De blokkade van DAT of NET, evenals 5-HT_{2C} receptor blokkade, zouden goed gebruikt kunnen worden voor klinisch onderzoek naar de vermindering van SERT-gemedieerde seksuele remming. Vooral de blokkade van DAT zou nuttig kunnen zijn om de seksuele remming, als gevolg van verstoring van SERT, te overwinnen. Deze mogelijkheden rechtvaardigen het gebruik van het huidige diermodel en combinaties van psychofarmaca. Meer in het algemeen moet onderzoek met het huidige model, indien mogelijk parallel met therapeutisch onderzoek, belangrijke inzichten geven in de invloed van antidepressiva en andere klassen psychotrope middelen op mannelijk seksueel gedrag.

De data gepresenteerd in hoofdstuk 4, laten duidelijk zien dat remming van de (SERT) leidt tot seksuele bijwerkingen. Om de rol van de SERT op seksueel gedrag in meer detail te bestuderen, hebben we experimenten met genetische gemodificeerde ratten uitgevoerd waarbij de serotonine transporter (SERT) uitgeschakeld is(Hoofdstuk 5). Naast het Wildtype (normaal), hebben we heterozygote SERT (+/-) en homozygote knockout (SERT-/-) ratten getest. De SERT+/- rat bevat ongeveer 50% van de SERT in vergelijking met het Wildtype (SERT+/+), waardoor deze mogelijk overeenkomen met het kort/ kort polymorfisme in de promoter van het SERT gen zoals bij mensen is gevonden. De SERT-/-Is een mogelijk model waarbij chronische SSRI toediening wordt nagebootst. De knockout ratten bleken duidelijk een gestoord seksueel gedrag te hebben,

terwijk de heterozygote ratten niet afweken van het Wildtype. De mogelijke betrokkenheid van de 5-HT1A receptor werd vervolgens in deze dieren getest. De resultaten suggereren een complexe betrokkenheid van verschillende 'pools' 5-HT_{1A} receptoren in het afwijkende seksuele gedrag van de knockout ratten. Deze resultaten lijken overeenkomstig met de veranderingen in seksueel gedrag en de 5-HT1A receptoren na chronisache SSRI toediening. De SERT-KO rat lijkt dus een interessant diermodel om de modulerende effecten van verschillende psychofarmaca op een verstoord serotonerg systeem in het centraal zenuwstelsel te bestuderen.

In Hoofdstuk 6 onderzochten we antidepressivum-achtige effecten en seksuele bijwerkingen van een triple monoamine opname remmer (DOV216,303). Zo'n stof zou een goed antidepressivum kunnen zijn met geen of minder seksuele bijwerking in depressieve patienten. De antidepressieve werking van DOV216,303 werd getest en bevestigd in een diermodel dat zeer gevoelig is voor het vinden van antidepressieve effecten (het Olfactory Bulbectomy model). De stof bleek geen remmende werking te hebben op seksueel gedrag en voorspeld wordt dat deze nieuwe klasse van antidepressiva een beter bijwerkingenprofiel in depressieve patienten zal hebben.

Tenslotte wordt in Hoofdstuk 7 clavulaanzuur getest. Dit is een bèta-lactamase remmer, die normaliter wordt gecombineerd met een penicilline om als antibioticum te functioneren. Verschillende observaties suggereerden dat clavulaanzuur een proseksuele aktiviteit in verschillende diersoorten had. Wij testten de stof in ons rattenmodel en konden bevestigen dat clavulaanzuur een stimulerend effect op seksuele activiteit heeft. Een mogelijk onderliggend werkingsmechanisme is onbekend maar vervolgonderzoek hiernaar lijkt uiterst interessant.

Uit de data van de in dit proefschrift beschreven studies, gecombineerd met de literatuur, kunnen verschillende conclusies worden getrokken (Hoofdstuk 8). In het algemeen hebben SSRI's een remmend effect op mannelijk seksueel gedrag bij ratten, hoewel dit met name zichtbaar is na een (sub) chronische toediening. Acuut hebben SSRI's soms

remmende effecten, maar deze bevindingen zijn vaak niet consistent of reproduceerbaar. Derhalve heeft het door ons ontwikkelde diermodel een aanzienlijke ‘face’ en ‘predictive’ validiteit voor de seksuele bijwerkingen van SSRI's. Er zijn aanwijzingen, vooral uit studies bij mensen met levenslange vroegtijdige zaadlozing, dat niet alle SSRI's een vergelijkbare remmende werking hebben op vroegtijdige zaadlozing; met name fluvoxamine en citalopram geven geen sterke zaadlozings vertraging bij mannen met vroegtijdige zaadlozing. Deze verschillen worden over het algemeen verklaard door de mate van 5-HT_{1A} receptor desensitisatie die de verschillende SSRI's veroorzaken. Zowel fluvoxamine als citalopram geven seksuele bijwerkingen na chronische behandeling bij depressieve patiënten hetgeen doet vermoeden dat bepaalde 5-HT_{1A} receptor desensitisatie heeft plaatsgevonden.

Naast de ‘predictive’ validiteit voor de remmende werking van medicijnen, detecteert het besproken diermodel ook proseksuele effecten van farmaca, bijv. van bupropion, buspiron, apomorfine en clavulaanzuur. Dit stemt overeen met aanwijzingen dat bupropion bij mensen kan worden gebruikt om de remmende werking van SSRI's tegen te gaan, en het ontbreken van seksuele klachten tijdens het gebruik van buspiron.
Tot slot, het optimale geneesmiddel is er een die het probleem verhelpt zonder bijwerkingen te veroorzaken. Ons diermodel is ontwikkeld met dit adagium in gedachten in de hoop en verwachting dat medicatie geïnduceerde seksuele bijwerkingen kunnen worden voorkomen. Dit proefschrift geeft tevens meer inzicht in enkele mechanismen die ten grondslag liggen aan seksuele functiestoornissen.

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About the author

Johnny was born on 29 July 1975 in lower Manhattan in New York City. He graduated as an animal science major at John Bowne High School in Flushing, Queens, in 1993. He continued his interest in animals and graduated with a Bachelor of Science in animal science from Cornell University in Ithaca, NY in 1997. After his graduation, he worked as a research assistant in the Laboratory of Neurobiology and Behavior at Rockefeller University in NYC. After 3 years, he joined the biotech company, Psychogenics, Inc. in Westchester County, NY. After 3 years of gaining neurobehavioral methods and techniques, he had the opportunity to leave the USA and experience the European life style and pursue a Ph.D. degree under the supervision of dr. Ronald Oosting, prof. Berend Olivier, and prof. Marcel Waldinger. The results are presented and discussed in this thesis.

List of Publications

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