Neuroscience 134 (2005) 1351–1361

EFFECTS OF CHRONIC PAROXETINE PRETREATMENT ON (±)-8-HYDROXY-2-(DI-*n*-PROPYL-AMINO)TETRALIN INDUCED *c-fos* EXPRESSION FOLLOWING SEXUAL BEHAVIOR

T. R. DE JONG,[a,b,c]* T. PATTIJ,[a,c] J. G. VEENING,[a,c] P. J. W. C. DEDEREN,[a] M. D. WALDINGER,[c,d] A. R. COOLS[b] AND B. OLIVIER[c,e]

[a]*Department of Anatomy, Radboud University Nijmegen Medical Centre, Geert Grooteplein 21, Noord, 6500 HB, Nijmegen, The Netherlands*

[b]*Department of Psychoneuropharmacology, Radboud University Nijmegen Medical Centre, Geert Grooteplein 21, Noord, 6500 HB, Nijmegen, The Netherlands*

[c]*Department of Psychopharmacology, Utrecht Institute of Pharmacological Sciences and Rudolf Magnus Institute of Neuroscience, Sorbonnelaan 16, 3583 CA, Utrecht University, Utrecht, The Netherlands*

[d]*Department of Psychiatry and Neurosexology, Leyenburg Hospital, Leyweg 275, 2545 CH, the Hague, The Netherlands*

[e]*Department of Psychiatry, Yale University Medical School, 25 Park Street, New Haven, CT 06519, USA*

Abstract—Chronic treatment with the selective serotonin reuptake inhibitor paroxetine impairs the functioning of 5-HT_{1A} receptors involved in ejaculation. This could underlie the development of delayed ejaculation often reported by men treated with paroxetine. The neurobiological substrate linking the effects of selective serotonin reuptake inhibitor-treatment and 5-HT_{1A} receptor activation with ejaculation was investigated. Male Wistar rats that were pretreated with paroxetine (20 mg/kg/day p.o.) or vehicle for 22 days and had received an additional injection with the 5-HT_{1A} receptor agonist 8-OH-DPAT ((±)-8-hydroxy-2-(di-*n*-propyl-amino)tetralin; 0.4 mg/kg s.c.) or saline on day 22, 30 min prior to a sexual behavior test, were perfused 1 h after the sexual behavior test. Brains were processed for Fos-, and oxytocin immunohistochemistry. The drug treatments markedly changed both sexual behavior and the pattern and number of Fos-immunoreactive cells in the brain. Chronic pretreatment with paroxetine caused delayed ejaculation. Acute injection with 8-OH-DPAT facilitated ejaculation in vehicle-pretreated rats, notably evident in a strongly reduced intromission frequency, whereas 8-OH-DPAT had no effects in paroxetine-pretreated rats. Chronic treatment with paroxetine reduced Fos-immunoreactivity in the locus coeruleus, and prevented the increase in Fos-immunoreactive neurons induced by 8-OH-DPAT in the oxytocinergic magnocellular part of the paraventricular nucleus as well as in the locus coeruleus. Since oxytocin and noradrenalin facilitate ejaculation, the alterations in Fos-IR in these areas could connect selective serotonin reuptake inhibitor treatment and 5-HT_{1A} receptor activation to ejaculation. Chronic paroxetine treatment and 8-OH-DPAT changed *c-fos* expression in a number of other brain areas, indicating that Fos-immunohistochemistry is a useful tool to find locations where selective serotonin reuptake inhibitors and 8-OH-DPAT exert their effects. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: SSRI, ejaculation, 5-HT_{1A} receptor, desensitization, oxytocin, locus coeruleus.

Chronic treatment with the selective serotonin reuptake inhibitor (SSRI) paroxetine causes desensitization of pre- and postsynaptic 5-HT_{1A} receptors (Le Poul et al., 1995; Li et al., 1997; Davidson and Stamford, 1998), which has been implicated in the antidepressant effect of SSRIs (Artigas et al., 1996; Blier et al., 1998). Since 5-HT_{1A} receptor activation is known to accelerate ejaculation in rats (Ahlenius et al., 1981; Fernandez-Guasti and Escalante, 1991; Coolen et al., 1997; Haensel and Slob, 1997; Rehman et al., 1999), and treatment with a SSRI combined with a 5-HT_{1A} receptor antagonist strongly inhibits ejaculation (Ahlenius and Larsson, 1999; de Jong et al., 2005a), desensitization of 5-HT_{1A} receptors may also play a role in the delayed ejaculation induced by chronic paroxetine treatment (Rosen et al., 1999; Waldinger et al., 2001, 2002, 2004). This theory was supported by recent findings that chronic pretreatment with paroxetine attenuated the effects of (±)-8-hydroxy-2-(di-*n*-propyl-amino)tetralin (8-OH-DPAT) on sexual behavior in rats (de Jong et al., 2005b).

It is not yet known in which brain areas SSRIs and 5-HT_{1A} receptors exert their effect on depression and/or ejaculation. One approach to tackle this problem is the staining of Fos, the protein product of the immediate-early gene *c-fos*, which is expressed in neurons in response to intracellular signaling cascades and used as a marker of neural activation (Kovacs, 1998; Hoffman and Lyo, 2002; Sng et al., 2004). Fos-immunoreactivity changes in response to acute and chronic SSRI-treatment (Veening et al., 1998; Lino-de-Oliveira et al., 2001; Jongsma et al., 2002; Thomsen and Helboe, 2003), 5-HT_{1A} receptor agonists (Compaan et al., 1996; Tilakaratne and Friedman, 1996; Hajos et al., 1999; Hajos-Korcsok and Sharp, 1999; Mikkelsen et al., 2004) and copulation and/or ejaculation (Coolen et al., 1996; Greco et al., 1996, 1998; Pfaus and Heeb, 1997; Veening and Coolen, 1998). Taken together, Fos-immunoreactivity can be considered as a useful tool to

*Correspondence to: T. R. de Jong, Department of Anatomy, Radboud University Nijmegen Medical Centre, Intern Mail 230, Geert Grooteplein 21, 6525 EZ, Nijmegen, The Netherlands. Tel: +31-243616687; fax: +31-243613789.
E-mail address: t.dejong@pnf.umcn.nl (T. R. de Jong).

Abbreviations: DAB, 3,3′-diaminobenzidine tetrahydrochloride; PBS, phosphate-buffered saline; PBS-BT, phosphate-buffered saline containing 0.1% bovine serum albumin and 0.5% Triton X-100; SSRI, selective serotonin reuptake inhibitor; 8-OH-DPAT, (±)-8-hydroxy-2-(di-*n*-propyl-amino)tetralin.

0306-4522/05$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.05.012

locate specific brain areas that might link serotonergic neurotransmission to sexual behavior.

In order to trace these brain areas, 24 rats ($n=6$ per experimental group) were selected from a previously reported experiment (de Jong et al., 2005b). The selected rats had been pretreated for 22 days with vehicle or paroxetine (20 mg/kg/day p.o.) and challenged with saline or 8-OH-DPAT (0.4 mg/kg s.c.) 30 min prior to a sexual behavior test. One hour after the test the rats were perfused and the pattern and number of Fos-positive cells were quantified throughout the rat brain.

EXPERIMENTAL PROCEDURES

Animals

Adult male (250–300 g and 3 months of age at the start of the experiment) and female Wistar rats (Harlan, Zeist, the Netherlands) were used. The animals arrived at the laboratory at least 14 days prior to the start of the experiments in order to adapt to the laboratory environmental condition and to a reversed light/dark cycle (12-h, lights off at 6:30 am). Food and tap water were available *ad libitum*. Males were group-housed and 3 weeks before the start of the drug treatment individually housed. They were handled daily from the start of the experiment. The females, which served as stimulus animals, were sterilized by ligation of the oviducts and housed in couples. Their sexual receptivity was fully and reliably induced by s.c. administration of 50 μg estradiol benzoate dissolved in 0.1 ml arachidis oil, 36 h prior to testing. All experiments were approved by the Animal Ethical Committee of the University of Nijmegen and conform the national and international guidelines on the ethical use of animals. All measures were taken in order to minimize the number of rats used in this study and their suffering.

Drugs

Paroxetine tablets (20 mg paroxetine/tablet; Genthon BV, Nijmegen, The Netherlands) were obtained from the local pharmacy and were freshly crushed and dissolved in 1% methylcellulose on every day of the drug treatment. The rats received daily oral injections (5 ml/kg) with vehicle (methylcellulose) or paroxetine (20 mg/kg/day) for 21 consecutive days between 3:30 and 5:00 pm. On the 22nd day, all rats received their paroxetine or vehicle injection 1 h prior to the sexual behavior test. In addition, all rats received a s.c. injection (1 ml/kg) with saline or 0.4 mg/kg 8-OH-DPAT (Sigma-Aldrich Chemie, Steinhein, Germany) dissolved in saline, 30 min prior to the sexual behavior test.

This resulted in four experimental groups ($n=6$ per group): methylcellulose+saline, methylcellulose+8-OH-DPAT, paroxetine+saline and paroxetine+8-OH-DPAT.

Behavioral observations

All rats were trained in at least six sexual behavior tests before the start of the experiment, in order to establish consistent ejaculation frequencies. Individuals with normal ejaculatory behavior (an average ejaculation frequency of $1.33 \leq 4.33$ in the last three training sessions) were selected for the experiment.

All training sessions and experimental sessions were conducted using the same paradigm and always between 10:30 am and 3:30 pm, in a red-lighted room. The rats were placed in a rectangular mating arena (40×50×65 cm) with wood shavings on the floor and a Perspex front. The rats were allowed to habituate to the arena for 15 min. Then, a receptive female was placed in the arena and free contact was allowed for 30 min. In this time frame, the ejaculation frequency (total number of ejaculations), ejaculation latency (time from first mount or intromission to first ejaculation), mount frequency (number of mounts prior to the first ejaculation) and intromission frequency (number of intromissions prior to the first ejaculation) were counted using event recording software The Observer (Noldus Information Technology, Wageningen, the Netherlands).

Immunohistochemistry

One hour after the end of the sexual behavior test, the rats were anesthetized using an overdose of sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.3) followed by fixative (4% paraformaldehyde in PBS, pH 7.2). Brains were removed and postfixed for 1 h at 4 °C before the paraformaldehyde was replaced by 30% sucrose in phosphate buffer. Each brain was cut in coronal sections (40 μm) with a freezing microtome and collected in a series of six PBS-containing tubes per brain. One tube per brain was immunohistochemically stained. The sections were rinsed in PBS, soaked in 30% H_2O_2 for 30 min and rinsed 3×20 min in PBS. After 30 min of preincubation with PBS containing 0.1% bovine serum albumin and 0.5% Triton X-100 (PBS-BT), sections were incubated overnight in the same medium with *c-fos* antiserum raised in rabbit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, dilution 1:20,000). The next day, the sections were rinsed 3×20 min in PBS and incubated for 90 min in donkey anti-rabbit antibody (Biotin SP conjugated, Jackson Immuno Research, West Grove, PA, USA, dilution 1:400) in PBS-BT. Sections were rinsed 3×20 min and incubated for 90 min in ABC-elite (Vector elite 1:800 in PBS, prepared 60 min in advance, Brunschwig Chemie, Amsterdam, the Netherlands) in PBS-BT. The sections were rinsed 3×20 min in PBS and stained with a chromogen solution consisting of 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.03% nickel-ammonium in 0.05 M Tris-buffer (pH 7.6): exactly 10 min of incubation without, and 10 min with 30% H_2O_2. This resulted in a blue-black staining of Fos-immunoreactive cell nuclei. All sections were rinsed 3×20 min in PBS, and the complete process was repeated starting from the pre-incubation with PBS-BT. This time, the overnight incubation was in an oxytocin GP antiserum (Peninsula Laboratories Incorporated, San Carlos, CA, USA, 1:100,000) raised in rabbit. The staining was established using DAB in 0.05 M Tris-buffer (pH 7.6), which resulted in a brown-colored staining of oxytocinergic neurons. All steps of the immunohistochemistry described above were performed at room temperature.

The sections were mounted on gelatin chrome alum-coated glass slides, dried overnight, cleared in xylene, embedded with Entellan (Merck & Co., Darmstadt, Germany) and coverslipped.

Immunoreactive cells were quantified using the software program Neurolucida (MicroBrightfield, Williston, VT, USA). Numbers of Fos-IR nuclei were counted in homologous square fields using a grid size of 200×200 μm (except for the compact part of the nucleus incertus: grid size 100×200 μm) displaying a representative density of stained cells. Fos-immunoreactivity was quantified in areas selected for their involvement in sexual behavior or serotonergic neurotransmission, as well as in areas showing substantial Fos-immunoreactivity in at least one of the experimental groups, namely: the prelimbic cortex, nucleus accumbens core and shell, dorsal lateral and posterior medial bed nucleus of the stria terminalis, ventral lateral septal nucleus, medial preoptic nucleus, medial parvocellular paraventricular hypothalamic nucleus, anterodorsal and posterodorsal medial amygdaloid nucleus, central amygdaloid nucleus, arcuate hypothalamic nucleus, ventral premammillary nucleus, dorsomedial ventromedial hypothalamic nucleus, lateral hypothalamic area, medial parvocellular subparafascicular thalamic nucleus, ventral lateral periaqueductal gray, central lateral parabrachial nucleus, compact part of the nucleus incertus, locus coeruleus and the medial nucleus of the solitary tract. In addition, Fos-positive nuclei in oxytocinergic cell bodies were counted in the posterior magnocellular part of the hypothalamic paraventricular nucleus.