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Short communication

LHRH antagonist attenuates the effect of fluoxetine on marble-burying behavior in mice

Gaikwad Uday, Bhutada Pravinkumar, Wanjari Manish, Umathe Sudhir *

Department of Pharmaceutical Sciences, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur - 440 033, Maharashtra, India

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Abstract

Leuprolide — a luteinizing hormone-releasing hormone (LHRH) agonist, dose dependently (100, 200 and 300 μg/kg, s.c.) inhibited marble-burying behavior in mice, which was comparable to that of fluoxetine (10 and 15 mg/kg, i.p.) — a drug used in the treatment of obsessive–compulsive disorder. Co-administration of sub-effective dose of leuprolide (50 μg/kg) and fluoxetine (5 mg/kg) significantly inhibited marble-burying-behavior. Pre-treatment with parachlorophenylalanine [300 mg/kg, i.p. (×3 days)] — a serotonin depleting agent, reversed the effect of fluoxetine, whereas partially attenuated the effect of leuprolide. Further, LHRH antagonist pre-treatment (2.5 μg/mouse, s.c.) completely blocked the effect of leuprolide and reduced the effect of fluoxetine. Motor activity remained unaffected after all treatments. In conclusion, the findings suggest that fluoxetine also implicates LHRH in its anti-compulsive effect.

Keywords: Leuprolide; Fluoxetine; Marble-burying behavior

1. Introduction

Obsessive–compulsive disorder is characterized by persistent thoughts (obsessions), which are ego-dystonic and associated with seemingly purposeful behaviors (compulsions) (Rasmussen and Eisen, 1992). Its co-morbidity with major depression is often evident, and it is considered as an anxiety disorder (Bartz and Hollander, 2006). Only potent serotonin reuptake inhibitors (SSRIs) are consistently effective in patients of obsessive–compulsive disorder (El Mansari and Blier, 2006).

In one case study, leuprolide — a luteinizing hormone-releasing hormone (LHRH) agonist has been found to benefit a patient with obsessive–compulsive disorder (Eriksson, 2000). Incidentally, *in vitro* studies have shown stimulatory influence of serotonin on the LHRH release (Meyer et al., 1992; Vitale et al., 1986). 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) — an agonist for 5-hydroxytryptamine-1A/7 (5-$HT_{1A/7}$) receptors, stimulated the LHRH release from GT-1 cells, and the effect was mediated through 5-HT_7 receptors (Hery et al., 1997). Treatment with amitriptyline — a non-selective monoamine reuptake inhibitor, increased the immuno-reactivity of LHRH neurons in rat brain (Jain and Subhedar, 1993). Incidentally, LHRH receptors have been identified in amygdala, hippocampus, anterior cingulate cortex, caudate, putamen and thalamus (Jennes et al., 1988; Rance et al., 1994; Reubi et al., 1987; Reubi and Maurer 1984), that are the regions involved in obsessive–compulsive disorder (Aouizerate et al., 2004). An increase in paroxetine-binding sites on platelets in premenstrual dysphoric disorder disappeared on treatment with burserilin — a LHRH agonist treatment, indicating that LHRH agonist modulates 5-HT transmission (Bixo et al., 2001).

These evidences suggest some interplay between serotonergic system and LHRH, which can be clinically meaningful in the control of obsessive–compulsive disorder. Therefore, the influence of fluoxetine and leuprolide was first investigated on the marble-burying behavior of mice — a well-accepted model of obsessive–compulsive behavior, due to its high face and predictive validity (Joel, 2006). Their effects were further studied in mice, pre-treated with either LHRH antagonist or parachlorophenylalanine (PCPA) — a serotonin depleting agent.

* Corresponding author. Tel.: +91 712 2500324; fax: +91 712 2500355.
E-mail address: umathesn@hotmail.com (S. Umathe).

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2. Materials and methods

2.1. Animals

The studies were carried out in adult male albino Swiss mice (22–25 g), group housed ($n=6$), under a standard 12 h light/dark cycle and controlled conditions of temperature and humidity (25±2 °C, 55±2%). They received standard rodent chow (Goldmohar brand, Lipton India Ltd.) and water *ad libitum*. The experiments were carried between 9.00 to 14.00 h in a noise-free room. Separate groups ($n=6$) of mice were used for each set of experiments and each animal was used only once. The animal studies were approved by Institutional Animal Ethics Committee (IAEC) vide sanction number 3 dated 02/08/2005, constituted for the purpose of control and supervision of experiments on animals by Ministry of Environment and Forests, Government of India, New Delhi, India.

2.2. Drugs

Leuprolide, LHRH antagonist (pGlu-D-Phe-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly- NH_2) and PCPA hydrochloride methyl ester were purchased from Sigma-Aldrich Ltd., USA. Fluoxetine was a gift by Reliance Laboratories Ltd., India. All above drugs were dissolved in 0.9% saline. Drug solutions were freshly prepared, and their doses are expressed in terms of free bases.

2.3. Treatments

Leuprolide (50, 100, 200, 300 µg/kg, s.c.) or fluoxetine (5, 10, 15 mg/kg, i.p.) or leuprolide (50 µg/kg, s.c.) plus fluoxetine (5 mg/kg, i.p.) were administered 30 min prior to the assessment of marble-burying behavior and locomotor activity.

In another set of experiment, mice were pre-treated with PCPA (300 mg/kg, i.p.) for 3 consecutive days, and 24 h thereafter leuprolide (300 µg/kg, s.c.) or fluoxetine (15 mg/kg, i.p.) were administered. Thirty minutes later above behavioral tests were conducted.

In another group, LHRH antagonist (2.5 µg/mouse, s.c.) was given 30 min prior to the administration of leuprolide (300 µg/kg, s.c.) or fluoxetine (15 mg/kg, i.p.). Thirty minutes thereafter, mice were subjected to the above behavioral tests.

For each of the above treatment, there was a separate control group, which received vehicle (0.9% saline, 10 ml/kg) by respective routes at corresponding time intervals. Separate groups ($n=6$) of mice were used for each set of experiments. The dose of leuprolide and LHRH antagonist was based on our preliminary observations whereas the dose of PCPA and fluoxetine was based on the previous reports (Chiavegatto et al., 2001; Takeuchi et al., 2002).

2.4. Assessment of marble-burying behavior

The anti-compulsive effect was assessed by widely used model of studying the marble-burying behavior of mice (Njung'e and Handley, 1991a,b). In brief, mice were individually placed in separate plastic cages (21×38×14 cm) containing 5 cm thick sawdust bedding. Twenty small marbles of glass (diameter ~10 mm), were arranged on the bedding evenly spaced in four rows of five. After 30 min exposure to the marbles, mice were removed, and unburied marbles were counted. A marble was considered 'buried' if its two-third size was covered with saw

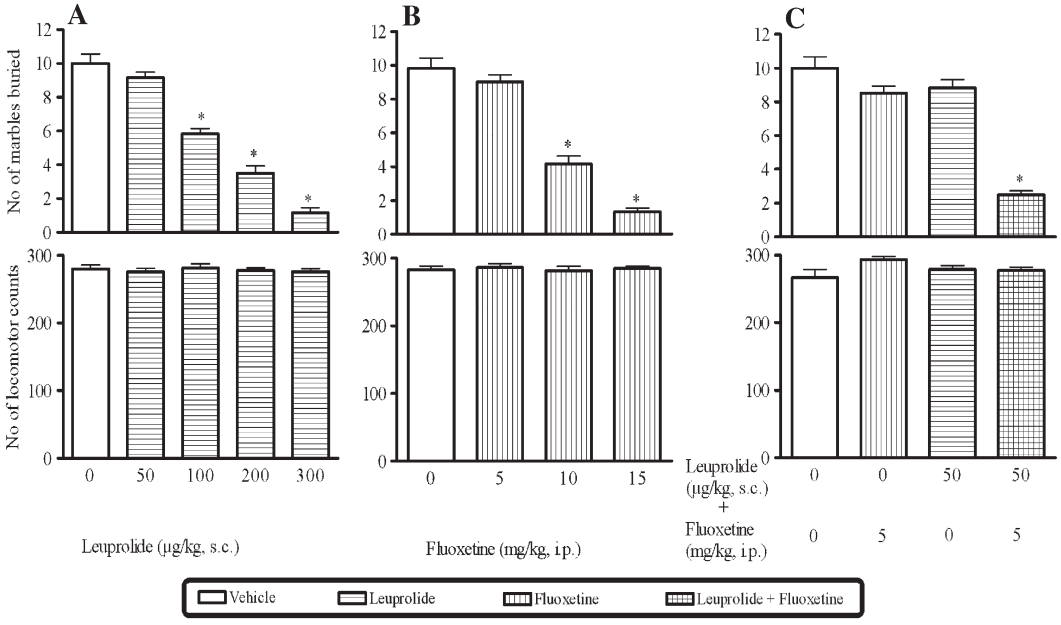

Fig. 1. Influence of various drug-treatments on marble-burying behavior and locomotor activity in mice [(A) leuprolide, (B) fluoxetine, and (C) leuprolide plus fluoxetine]. Separate groups of mice were injected with vehicle (10 ml/kg, i.p. or s.c.) or increasing doses of leuprolide (50–300 µg/kg, s.c.) or fluoxetine (5–15 mg/kg, i.p.), and 30 min thereafter, individual mouse was tested for marble-burying behavior and locomotor activity. For co-administration studies, separate groups of mice were injected with vehicle (10 ml/kg, i.p.) or fluoxetine (5 mg/kg, i.p.) and 5 min later either vehicle (10 ml/kg, s.c.) or leuprolide (50 µg/mouse, s.c.), and 30 min thereafter, individual mouse was tested for marble-burying behavior and locomotor activity. Marble-burying behavior and locomotor activity was tested in separate groups of mice. Each bar represents mean±S.E.M. of data from 6 mice. *$P<0.001$ vs. respective vehicle control and fluoxetine control or leuprolide control (One-way ANOVA followed by Newman–Keuls post hoc test).

dust. The total number of marbles buried was considered as an index of obsessive–compulsive behavior.

2.5. Assessment of motor activity

Motor activity was assessed in separate group of mice using Actophotometer (Techno, Lukhnow), which had a circular arena of 40 cm, equipped with three infrared beams and photo-cells connected to digital counter. Motor activity was assessed in terms of total number of counts of light beams interruptions in 30 min.

2.6. Statistical analysis

The data were analyzed with either one-way ANOVA followed by Newman–Keuls test for multiple comparisons or two-way ANOVA followed by Bonferroni test for multiple comparisons. The results are expressed as mean±S.E.M. of six observations. $P<0.05$ was considered to be statistically significant in all the cases.

3. Results

3.1. Effect of leuprolide and fluoxetine on marble-burying behavior and motor activity in mice

Leuprolide (100, 200, 300 μg/kg) [F (4, 25)=86.94, $P<0.0001$] and fluoxetine (10, 15 mg/kg) [F (3, 20)=78.09, $P<0.0001$] dose dependently reduced marble-burying behavior in mice, without any effect on motor activity [F (4, 25)=0.2164, $P=0.9268$ and F (3, 20)=0.1035, $P=0.9570$, respectively for leuprolide and fluoxetine] (Fig. 1A and 1B). The lower dose of leuprolide (50 μg/kg) and fluoxetine (5 mg/kg) was found to be ineffective ($P>0.05$). It was further observed that co-administration of sub-effective dose of leuprolide (50 μg/kg) and fluoxetine (5 mg/kg) significantly attenuated [F (3, 20)=48.89, $P<0.0001$] marble-burying behavior without affecting motor activity [F (3, 20)=2.170, $P=0.1233$] (Fig. 1C).

3.2. Effect of PCPA on leuprolide and fluoxetine induced inhibition of marble-burying behavior

Pre-treatment of mice with PCPA [300 mg/kg, i.p. (×3 days)], partly but significantly attenuated the effect of leuprolide (300 μg/kg), and completely eliminated the inhibitory influence of fluoxetine (15 mg/kg) on marble-burying behavior; *per se* PCPA had no effect ($P>0.05$). Two-way ANOVA revealed a significant PCPA-drug treatment interaction [F (2, 30)=26.43, $P<0.0001$], PCPA pre-treatment effect [F (1, 30)=104.5, $P<0.0001$] and drug treatment effect [F (2, 30)=123.4, $P<0.0001$]. All these treatments did not influence the motor activity; PCPA-drug treatment interaction [F (2, 30)=1.096, $P=0.3472$], PCPA pre-treatment effect [F (1, 30)=1.906, $P=0.1776$] and drug treatment effect [F (2, 30)=1.166, $P=0.3253$] (Fig. 2A).

3.3. Effect of LHRH antagonist on leuprolide and fluoxetine induced inhibition of marble-burying behavior

LHRH antagonist pre-treatment (2.5 μg/mouse) significantly prevented the inhibitory influence of leuprolide (300 μg/kg) and fluoxetine (15 mg/kg) on marble-burying behavior without any *per se* action ($P>0.05$). Two-way ANOVA revealed a

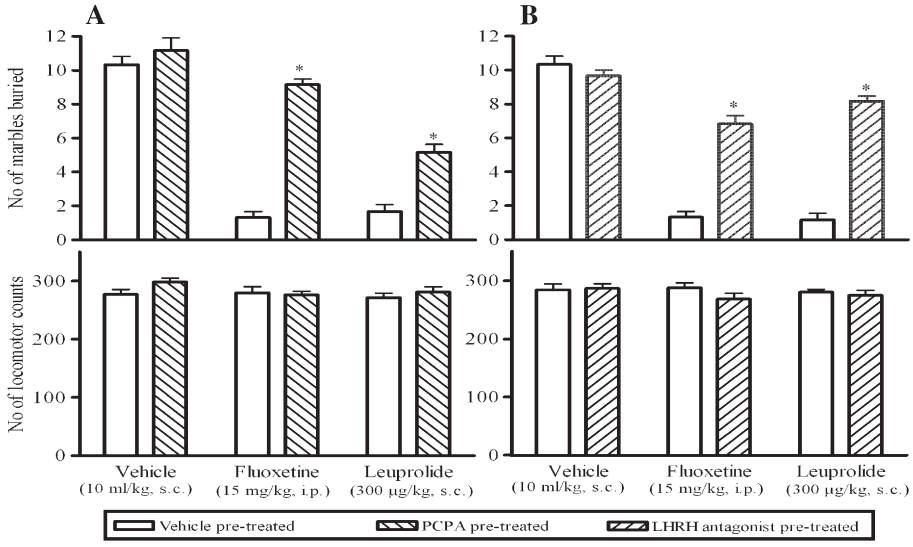

Fig. 2. Effect of PCPA and LHRH antagonist pre-treatments on the anti-compulsive effect of fluoxetine and leuprolide in mice; (A) pre-treated with parachlorophenylalanine (PCPA), and (B) pre-treated with LHRH antagonist. Separate groups of mice were injected with PCPA [300 mg/kg, i.p. (×3 days)] or vehicle [10 ml/kg, i.p. (×3 days], and 24 h after last dose, vehicle (10 ml/kg, s.c.) or fluoxetine (15 mg/kg, i.p.) or leuprolide (300 μg/kg, s.c.) were administered, and 30 min thereafter, individual mouse was tested for marble-burying behavior and locomotor activity. Separate groups of mice were injected with vehicle (10 ml/kg, i.p.) or LHRH antagonist (2.5 μg/mouse, s.c.) 30 min prior to vehicle (10 ml/kg, s.c.) or fluoxetine (15 mg/kg, i.p.) or leuprolide (300 μg/kg, s.c.), and 30 min thereafter, individual mouse was tested for marble-burying behavior and locomotor activity. Marble-burying behavior and locomotor activity was tested in separate groups of mice. Each bar represents mean±S.E.M. of data from 6 mice. *$P<0.001$ vs. respective fluoxetine and leuprolide control. (Two-way ANOVA followed by Bonferroni post hoc test).

significant LHRH antagonist pre-treatment-drug treatment interaction [F (2, 30)=52.13, $P<0.0001$], LHRH antagonist pre-treatment effect [F (1, 30)=147.4, $P<0.0001$] and drug treatment effect [F (2, 30)=134.3, $P<0.0001$]. All these treatments had no significant effect on the motor activity; LHRH antagonist pre-treatment-drug treatment interaction [F (2, 30)=0.8691, $P=0.4296$], LHRH antagonist pre-treatment effect [F (1, 30)=1.089, $P=0.3051$] and drug treatment effect [F (2, 30)=0.5421, $P=0.5871$] (Fig. 2B).

4. Discussion

This is the first report to show that leuprolide — a LHRH agonist, dose dependently attenuated marble-burying behavior in mice, and the effect was comparable to that shown by fluoxetine. The observed anti-compulsive effect of fluoxetine is well in accordance with earlier report (Takeuchi et al., 2002), and has been attributed to selective inhibition of serotonin reuptake.

Marble-burying behavior of mice is a well-accepted paradigm to screen anti-compulsive activity. It has been suggested that this model does not differentiate between anti-compulsive and anxiolytic drugs (Joel, 2006). Hence, the observed effects of fluoxetine and leuprolide can be either anti-compulsive or anxiolytic. However, it is to be noted that the task of marble-burying behavior is only inhibited by those doses of anxiolytics that disrupt the motor activity (Li et al., 2006; Pelleymounter et al., 2002). Fluoxetine and leuprolide did not affect the motor activity in the present investigation.

Leuprolide, being a LHRH agonist is likely to influence the estrogen levels by activating the release of luteinizing hormone and follicle stimulating hormone (Bhatia et al., 2002). The effect of estrogen is genomic in nature, and leuprolide produced its effect just after 30 min of administration. Therefore, it is difficult to relate the effect of leuprolide to its influence on sex hormones. However, estrogen-deficient male mice develop compulsive behavior (Hill et al., 2006) and obsessive–compulsive disorder exacerbates during premenstrual period and menopause (Vulink et al., 2006). Therefore, the involvement estrogen in the anti-compulsive effect of leuprolide cannot be also ruled out. In addition, LHRH has been reported to exhibit anti-dopaminergic-like activity (Kadar et al., 1992) and such agents augment the anti-compulsive effect of SSRI in refractory patients (Denys, 2006). Thus, the observed anti-compulsive effect of leuprolide may be subsequent to either of these effects of LHRH.

The studies further revealed a significant inhibition of marble-burying behavior when sub-effective dose of leuprolide (50 μg/kg) and fluoxetine (5 mg/kg) was administered together, which suggests a mutual relation between them. Serotonin is reported to induce the release of LHRH from median eminence (Vitale et al., 1986). Thus, it is possible that the sub-effective dose of fluoxetine might have increased LHRH secretion, which combined with the sub-effective dose of the leuprolide had an additive effect leading to a significant inhibition of marble-burying behavior.

In order to test the possible release of LHRH by fluoxetine, the anti-compulsive effect of fluoxetine was tested in mice pre-treated with LHRH antagonist. Pre-treatment with LHRH antagonist completely eliminated the anti-compulsive effect of leuprolide, whereas the effect of fluoxetine was partially but significantly attenuated. However, LHRH antagonist did not exhibit any *per se* effect. This suggests that fluoxetine also involves endogenous LHRH in its anti-compulsive effect.

In order to understand the interplay between LHRH and serotonin, the effect of leuprolide was tested in mice pre-treated with PCPA — an agent that depletes brain serotonin levels (Chiavegatto et al., 2001). It was noted that pre-treatment with PCPA significantly reduced the effect of leuprolide, which suggests the involvement of serotonin in its anti-compulsive effect, and as the reduction was partial, the involvement of other neurotransmitters is also possible. Incidentally, LHRH receptors are identified in the brain regions where serotonin dysfunction is believed to cause obsessive–compulsive disorder (Aouizerate et al., 2004) and, LHRH is reported to modulate the activity of several neurotransmitters, including serotonin (Pan et al., 1988). Thus, the modulation of serotonergic neurotransmission by leuprolide may be one of the reasons for its anti-compulsive effect. Such possibility supports the view that fluoxetine releases LHRH to probably enhance the effect of serotonin, and therefore, pre-treatment with LHRH antagonist partially attenuated the anti-compulsive effect of fluoxetine, whereas PCPA pre-treatment completely eliminated the same.

In conclusion, leuprolide — a LHRH agonist, exhibits anti-compulsive effect and fluoxetine partially involves LHRH in its effect. Further research is required to accurately assess the mutual relation between LHRH and serotonin in the control of obsessive–compulsive disorder.

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