Neuropsychopharmacology (2007) 32, 892–901

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Estrous Cycle-Dependent Changes in Basal and Ethanol-Induced Activity of Cortical Dopaminergic Neurons in the Rat

Laura Dazzi*[1,2,3], Emanuele Seu[1,2,3], Giulia Cherchi[1,2,3], Pier Paolo Barbieri[1,2,3], Alessandra Matzeu[1,2,3] and Giovanni Biggio[1,2,3]

[1]Department of Experimental Biology 'B. Loddo,' University of Cagliari, Cagliari, Italy; [2]Center of Excellence for Neurobiology of Dependence, University of Cagliari, Cagliari, Italy; [3]Unit of Neuropsychopharmacology, CNR Institute for Neuroscience, University of Cagliari, Cagliari, Italy

The influence of the estrous cycle on dopamine levels in the rat medial prefrontal cortex under basal and ethanol-stimulated conditions was evaluated by microdialysis. The basal dopamine concentration in the dialysate varied markedly during the estrous cycle, being highest in estrus and lowest in proestrus. Furthermore, a challenge intraperitoneal administration of ethanol (0.5 g/kg) induced a significant increase in dopaminergic output (+50%) during estrus but had no effect in diestrus or proestrus. Ovariectomy or pretreatment with either finasteride (a 5α-reductase inhibitor) or clomiphene (an estrogen receptor antagonist) prevented this ethanol-induced increase in dopamine concentration. The effect of ethanol was restored in ovariectomized rats by pretreatment with estrogen but not by that with progesterone. Our results thus show that the basal levels of dopamine in the prefrontal cortex are dependent on the phase of the estrous cycle. Furthermore, this dependence appears to be attributable to the effects of ovarian steroid hormones and results in a differential sensitivity of the dopaminergic neurons to ethanol. The hormone-induced changes in the activity of these neurons might contribute to the differences in drug sensitivity and mood state apparent among phases of the estrous cycle and between the sexes.
Neuropsychopharmacology (2007) **32,** 892–901. doi:10.1038/sj.npp.1301150; published online 12 July 2006

Keywords: ethanol; dopamine; estrogen; progesterone; estrous cycle; prefrontal cortex

INTRODUCTION

Clinical and experimental evidences have indicated that male and females differ in their behavioral and pharmacological responses to drugs of abuse, and in particular females appear to be more vulnerable than males to the reinforcing effects of psychostimulants, opiates, nicotine, and alcohol (Lynch *et al*, 2002; Roth *et al*, 2004; Carroll *et al*, 2004). Sex differences have been reported in animal models of all stages of drugs of abuse, including acquisition, maintenance, escalation, dependence, withdrawal, relapse, and treatment (Lynch *et al*, 2002; Roth *et al*, 2004; Carroll *et al*, 2004). Motivation to self-administer drugs of abuse in female rats varies as a function of the estrous cycle (Lynch *et al*, 2002), and ethanol consumption has been shown to be increased in women with premenstrual syndrome (Tobin *et al*, 1994; Chuong and Burgos, 1995; Allen, 1996). These observation, together with the evidence that no differences in ethanol pharmacokinetics have been associated with the different stages of the reproductive cycle both in rats (Robinson *et al*, 2002) and humans (Corrêa and Oga, 2004), suggest that sex differences in drug-seeking behavior as well as in drug sensivitity may be mediated by cyclic hormonal changes. Females are more susceptible than males to alcohol-related impairment of cognitive performance, especially in tasks involving delayed memory or divided attention functions (Savage *et al*, 2000), suggesting that mesocortical dopaminergic neurons might be involved in the modulation of cognitive impairments induced by this drug of abuse. In fact, dopaminergic neurons that project from the ventral tegmental area (VTA) to limbic and cortical regions of the forebrain play a major role in the physiological modulation of emotive and cognitive function (Goldman-Rakic, 1987; Jentsch *et al*, 1997; Sutton and Davidson, 1997). These neurons also contribute to the circuit responsible for drug reward (Pierce and Kalivas, 1997; Wolf, 1998; Childress *et al*, 1999; Tzschentke, 2001) as well as to the mediation of several effects of drugs of abuse including ethanol (Grace, 2000). Moreover, the activity of the mesocorticolimbic dopamine system shows sex differences (Becker, 1999) and is sensitive to modulation by the ovarian steroids estrogen and progesterone. Changes in the peripheral levels of these endogenous compounds are thus associated with those in the activity of nigrostriatal and

*Correspondence: Professor L Dazzi, Department of Experimental Biology 'B. Loddo,' Neuroscience Section, Cittadella Universitaria, Monserrato, SS. 554, Km 4.500, 09042 Cagliari, Italy, Tel: +39 070 675 4169, Fax: +39 070 675 4166, E-mail: dazzi@unica.it
Received 1 February 2006; revised 5 April 2006; accepted 22 May 2006
Online publication: 1 June 2006 at http://www.acnp.org/citations/Npp060106060065/default.pdf

mesolimbic dopaminergic systems (Becker, 1999). Estrogen also induces increases in dopamine turnover (Di Paolo *et al*, 1985, 1986) and amphetamine-stimulated release (Becker, 1990, 1999), decreases the number and sensitivity of pre- and postsynaptic D_2 dopamine receptors (Bazzett and Becker, 1994; Thompson *et al*, 2001; Zhou *et al*, 2002; Febo *et al*, 2003), and increases the density of the dopamine reuptake protein (Di Paolo *et al*, 1988; Morissette and Di Paolo, 1993). Progesterone, the precursor of the neuroactive steroid 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, or 3α,5α-THPROG), which is one of the most potent positive modulators of $GABA_A$ receptor function (Majewska *et al*, 1986; Majewska, 1992), decreases both basal and stimulated mesocortical dopaminergic transmission (Motzo *et al*, 1996; Dazzi *et al*, 2002b), as do other GABAergic drugs.

Both estrogen (McEwen and Parsons, 1982; Becker and Cha, 1989; Xiao and Becker, 1994; Dluzen, 2005) and ethanol (Phillips and Shen, 1996; Samson *et al*, 1997; Brodie and Appel, 1998) increase the activity of striatal dopaminergic neurons. The increase in the concentration of the progesterone metabolite 3α,5α-THPROG in rat brain elicited by systemic administration of ethanol correlates with the hypnotic, anticonvulsant, and sedative effects of ethanol (VanDoren *et al*, 2000). The local application of ethanol also increases the concentration of 3α,5α-THPROG as well as the amplitude of $GABA_A$ receptor-mediated inhibitory potentials recorded from pyramidal neurons in isolated hippocampal slices (Sanna *et al*, 2004). Furthermore, 3α,5α-THPROG is able to substitute for ethanol in a discriminative stimulus paradigm in both primates and rats (Bowen *et al*, 1999; Engel and Grant, 2001), to protect against seizures induced by ethanol withdrawal in rats (Finn *et al*, 2004; Devaud *et al*, 1995), and to potentiate the effect of an acute administration of ethanol on cortical dopaminergic neurons (Dazzi *et al*, 2002a).

Given the roles of estrogen and progesterone in modulation of the basal and ethanol-stimulated activity of dopaminergic neurons, the marked fluctuations in the plasma and cerebral concentrations of these ovarian hormones might be expected to contribute substantially to the changes in mood, cognitive performance, and drug sensitivity associated with progression through the menstrual cycle. The subunit composition of $GABA_A$ receptors in the brain has recently been shown to change markedly during the estrous cycle (Griffiths and Lovick, 2005; Lovick *et al*, 2005; Maguire *et al*, 2005), with these changes possibly having an impact on the threshold of excitability of various neuronal populations. These observations, together with the ability of both estrogen and progesterone to alter the function of GABAergic and dopaminergic systems, both of which are implicated in the behavioral and physiological effects of ethanol (Phillips and Shen, 1996; Koob *et al*, 1998), further suggest that these hormones may differentially influence the subjective, behavioral, and physiological effects of ethanol at different phases of the reproductive cycle.

To evaluate whether changes in estrogen and progesterone levels would affect the response of mesocortical dopaminergic neurons to ethanol, we have measured the basal levels of dopamine in dialysis samples collected from the prefrontal cortex of intact cycling female rats as well as the response of mesocortical dopaminergic neurons to an acute administration of ethanol at the different phases of the estrous cycle and after ovariectomy.

MATERIALS AND METHODS

Animals

Female Sprague–Dawley CD rats (Charles River, Como, Italy), with initial body masses of 200 to 220 g, were maintained under an artificial 12-h-light, 12-h-dark cycle (light on 0800–2000 hours) at a temperature of $22 \pm 2^{\circ}C$ and 65% humidity and with free access to standard food (Stefano Morini, San Polo D'Enza, Reggio Emilia, Italy) and water. The rats were acclimated to the animal facility for at least 8 days before experiments. Animal care and handling throughout the experimental procedures were in accordance with the statement revised and approved by the Society for Neuroscience in January 1995 and with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocols were also approved by the Animal Ethics Committee of the University of Cagliari.

Drugs

Ethanol (20%, w/v) was administered intraperitoneally (i.p.) at a dose of 0.5 g/kg of body mass. Progesterone was dissolved in olive oil by sonication for 4 h and was administered i.p. at a dose of 5 mg/kg in a volume of 2 ml/kg. Ethynylestradiol (a synthetic estrogen) was dissolved in distilled water with one drop of Tween 80 per 5 ml of solution and was injected subcutaneously (s.c.) at a dose of 30 μg per rat in 1 ml. Finasteride (a specific 5α reductase inhibitor) was obtained as Prostide tablets (Sigma Tau, Milan, Italy) containing 25 mg of the drug (Trapani *et al*, 2002); it was dissolved in 20% (w/v) 2-hydroxypropyl-β-cyclodestrin by sonication for 2 h and administered s.c. at a dose of 25 mg/kg in a volume of 3 ml/kg. Progesterone, ethynylestradiol, and finasteride were administered 25 and 1 h before the onset of microdialysis experiments (at 0800 hours on the day of surgery and at 0800 hours on the day of the experiment). The time points for treatment with each of the steroids or drugs have been selected on the basis of previous results from our lab in which both 3α,5α THPROG levels and dopamine activity have been measured after administration of these compounds (Dazzi *et al*, 2002a, b). Clomiphene (an estrogen receptor antagonist) was dissolved in distilled water with one drop of Tween 80 per 5 ml and was administered i.p. at a dose of 5 mg/3 ml/kg. Control rats received an equal volume of the respective vehicle.

Drug Treatment Protocols and Ovariectomy

The estrous cycle in the rat comprises four phases (estrus, diestrus 1, diestrus 2, proestrus) and has an average duration of 4 days. We determined the different phases of the estrous cycle from vaginal smears obtained daily at 0900 hours for about 2 weeks (two to four cycles) before experiments. Each phase is characterized by a specific number and morphology of cells in the vaginal fluid as revealed by light microscopy (Jablonka-Shariff *et al*, 1999) (Figure 1). Only animals with a regular estrous cycle (stages

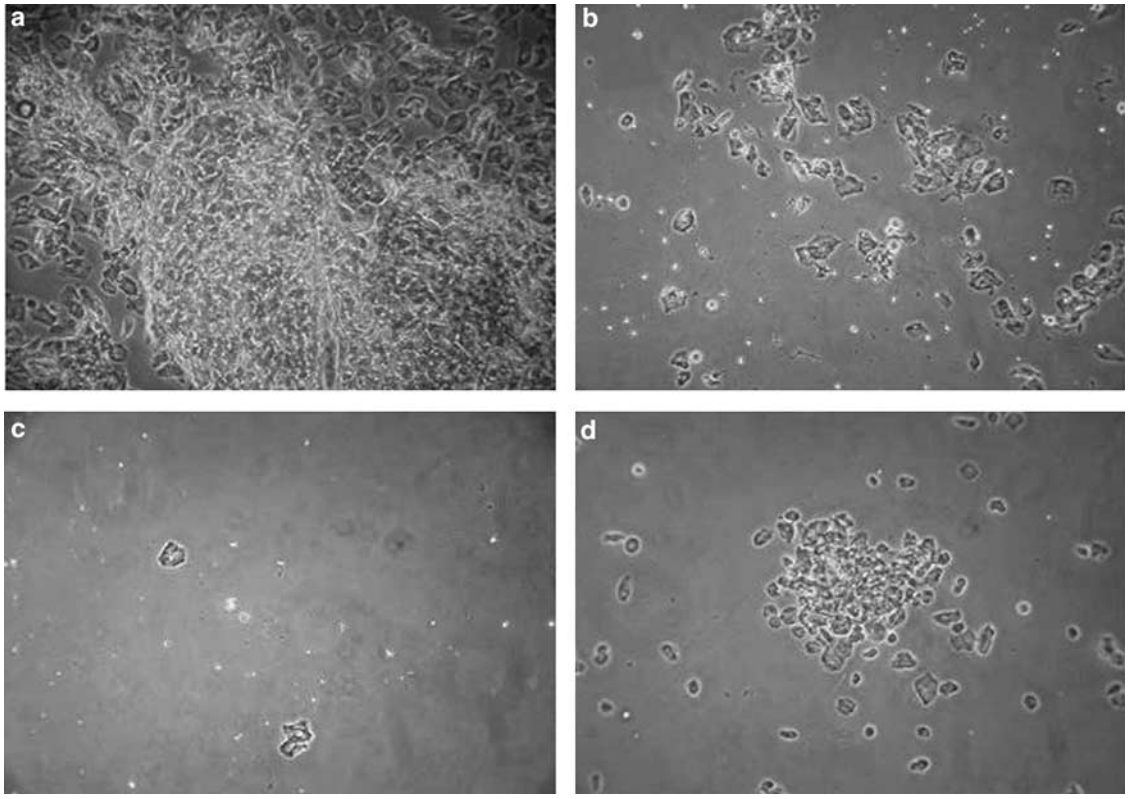

Figure 1 Microscopic identification of the different phases of the estrous cycle. Estrus (a), diestrus 1 (b), diestrus 2 (c), and proestrus (d) are each characterized by the number and morphology of the cells in vaginal fluid. Magnification × 100.

are in sequence and the entire cycle lasts 4–5 days) were used for experiments. Intact female rats in different phases of the estrous cycle were subjected to surgery for the insertion of a microdialysis probe. Microdialysis was initiated 24 h after probe implantation; when the concentration of dopamine had stabilized, ethanol (0.5 g/kg, i.p.) was administered. Other intact female rats were pretreated with either the estrogen receptor antagonist clomiphene (5 mg/kg, i.p.) or the 5α-reductase inhibitor finasteride (25 mg/kg, s.c.) before the acute administration of ethanol.

Female rats at least 2 months old, with body masses of 220–250 g, were anesthetized with Equithesin solution (162 ml of nembutal, 42.5 g of chloral hydrate, 21.26 g of $MgSO_4$, 396 ml of propylene glycol, and 100 ml of ethanol, adjusted to a volume of 1 l with distilled water) at a dose of 3 ml/kg (i.p.). The ovaries of rats were removed through two lateral cuts at the abdominal level. Control rats were anesthetized and subjected to sham surgery, without removal of the ovaries. The animals were then housed for 2 weeks in groups of four per cage to allow full recovery from the surgery. Beginning 1 week after surgery, vaginal smears were collected daily to confirm the loss of ovarian function; all ovariectomized (OVX) rats presented a smear consisting almost exclusively of a few leukocytes, similar to that observed in diestrus 2 for intact females. At 2 or 4 weeks after ovariectomy, the rats were anesthetized to allow insertion of a microdialysis probe and were divided into groups that were pretreated with various drugs before the acute administration of ethanol.

Implantation of the Microdialysis Probe and Experimental Procedures

Rats were anesthetized with chloral hydrate (0.4 g/kg, i.p.) and a concentric dialysis probe was inserted at the level of the medial prefrontal cortex (A +3.2, ML +0.8, V −5.3 relative to the bregma) according to the Paxinos atlas (Paxinos and Watson, 1982). The active length of the dialysis membrane (Hospal Dasco, Bologna, Italy) was restricted to 2 mm.

Experiments were performed 24 h after probe implantation, beginning at 0900 hours. Ringer's solution containing 125 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 23 mM $NaHCO_3$, and 1.5 mM potassium phosphate (pH 7.3) was pumped through the dialysis probe at a constant rate of 2 μl/min. Samples of dialysate were collected every 20 min and immediately analyzed for dopamine by high-performance liquid chromatography with electrochemical detection. The system consisted of an isocratic pump, an electrochemical detector (LC4B; BAS Bioanalytical System, West Lafayette, IN) operating at +650 mV relative to an Ag/AgCl reference electrode, and an LC18DB column (750 by 4.6 mm; Supelco 3 μm). The mobile phase consisted of 73.4 mM potassium phosphate buffer (pH 3.0), 3 mM octane-2-sulfonic acid, 0.1 mM EDTA, and 10% (v/v) methanol, and was pumped at a flow rate of 1 ml/min. Absolute dopamine concentrations were determined with the use of external standards; the detection limit for dopamine was 2 fmol per injection. The average neurotransmitter concentration in the last three samples before treatment was taken as 100%, and

post-treatment values were expressed as a percentage of the basal value. The mean *in vitro* recovery of the probes was 24 ± 3%. All probes were tested before implantation, and those with a recovery value outside of this range were not used.

At the end of each experiment, rats were transcardially perfused with 100 ml of physiological saline followed by 500 ml of a solution containing 4% formaldehyde, 1% calcium acetate, and 100 mM NaCl. The probe was removed, the brain was cut with the use of a Vibratome into serial coronal sections, and the placement of the probe was verified histologically. All rats in which the probe was located outside of the prefrontal cortex were excluded from the analysis. The locations of the probes were reconstructed and referred to the atlas of Paxinos and Watson (1982) (Figure 2).

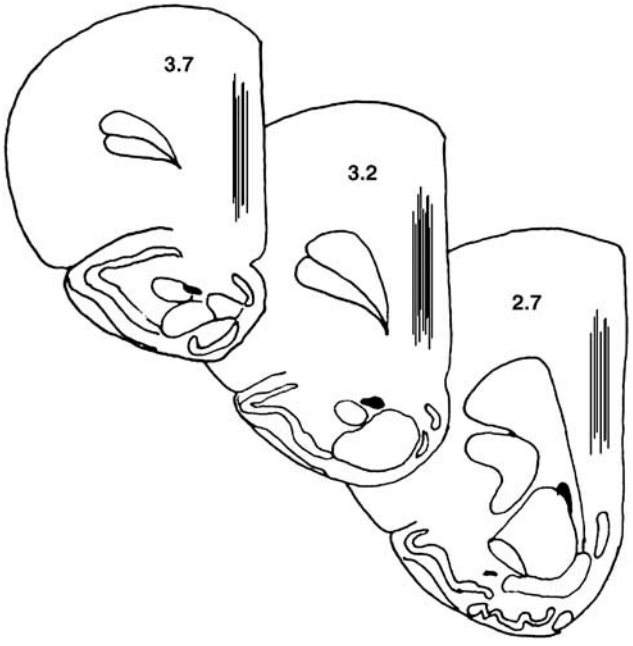

Figure 2 Localization of the dialysis probes within the prefrontal cortex (according to Paxinos and Watson, 1982). Numbers in the sections represent anteriority.

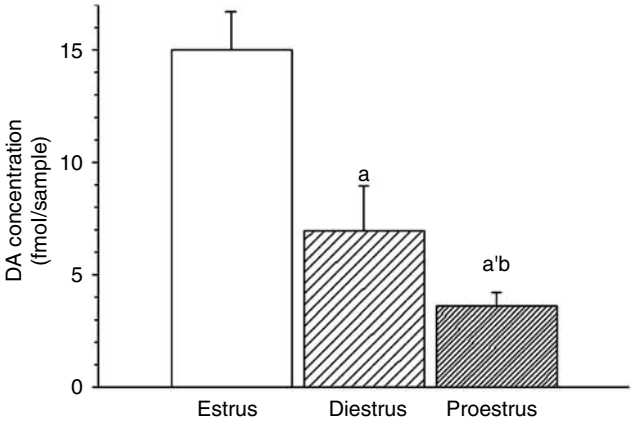

Figure 3 Changes in the basal concentration of dopamine in dialysate sample from the prefrontal cortex during the estrous cycle. Data are means ± SEM for at least 10 rats per group and are expressed as femtomoles of dopamine (DA) per 40-µl sample. $^{a}P<0.05$, $^{a}P<0.01$ *vs* basal value in estrus; $^{b}P<0.01$ *vs* basal value in diestrus.

Statistical Analysis

Data are presented as means ± SEM. Comparisons among groups were performed by one-way analysis of variance (ANOVA) for the data in Figure 3 or by two-way ANOVA for repeated measures, with factors being cycle phase and time points, for the data in Figure 4 and pretreatment and treatment for the data in Figures 5–7. The raw baseline values of dopamine concentration were used for statistical

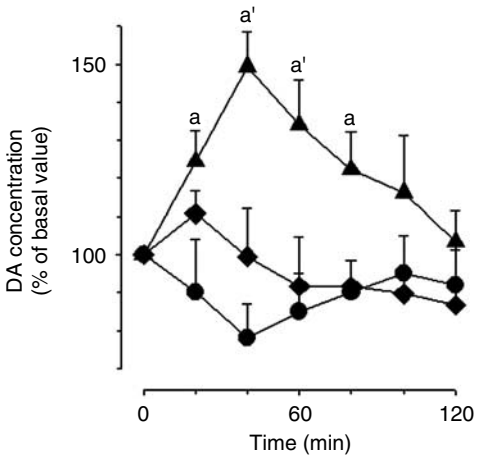

Figure 4 Effects of acute administration of ethanol (0.5 g/kg, i.p.) on the basal concentration of dopamine in dialysate sample from the prefrontal cortex during estrus (triangles), diestrus (diamonds), or proestrus (circles). Data are means ± SEM for at least five rats per group and are expressed as a percentage of the corresponding basal value. $^{a}P<0.05$, $^{a'}P<0.01$ *vs* basal value.

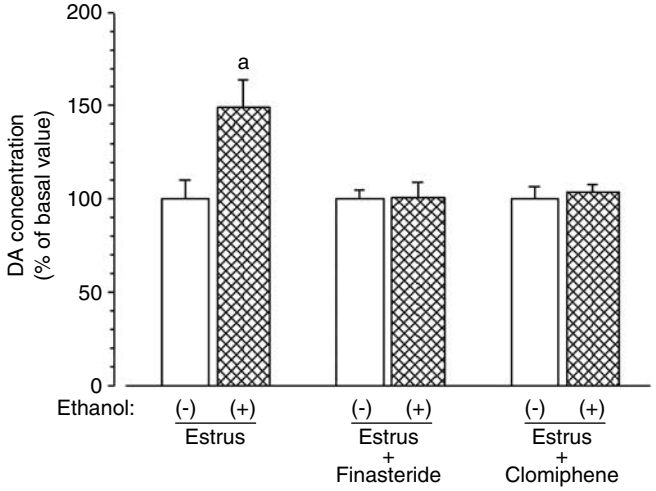

Figure 5 Effects of pretreatment with the estrogen receptor antagonist clomiphene (5 mg/kg, i.p.) or the 5α-reductase inhibitor finasteride (25 mg/kg, s.c.) on the ethanol-induced increase in the dopamine output in the prefrontal cortex during estrus. Finasteride was administered 25 and 1 h, and clomiphene was administered 1 h, before the onset of microdialysis. Data are expressed as means ± SEM for at least five rats per group, are expressed as a percentage of the corresponding basal value, and were obtained 1 h after the administration of ethanol (0.5 g/kg, i.p.). $^{a}P<0.01$ *vs* the basal value.

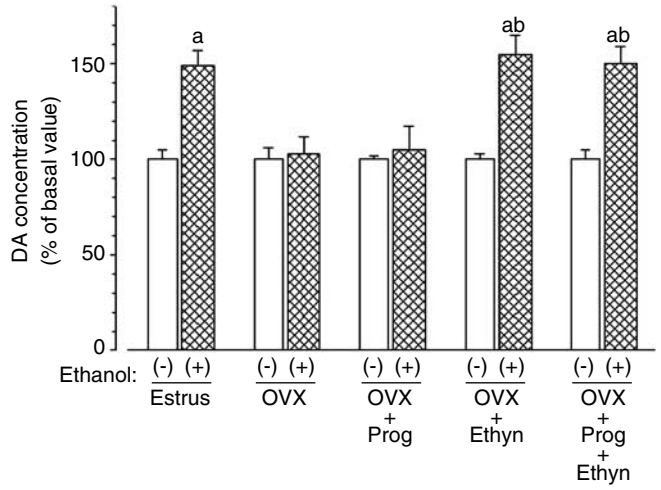

Figure 6 Effects of ovariectomy and of pretreatment with ethynylestradiol or progesterone in OVX rats on the ethanol-induced increase in cortical dopamine output. Female rats were subjected to ovariectomy 2 weeks before experiments. They were pretreated with vehicle, ethynylestradiol (30 μg, s.c.), progesterone (5 mg/kg, i.p.), or the combination of ethynylestradiol and progesterone both 25 and 1 h before the onset of microdialysis. They were then subjected to an intraperitoneal challenge with ethanol (0.5 g/kg), and the cortical concentration of dopamine was determined 60 min later. The effect of ethanol was also determined for sham-operated rats in estrus. Data are expressed as a percentage of the corresponding basal value and are means ± SEM for at least five rats per group. $^{a}P<0.01$ *vs* basal value; $^{b}P<0.01$ *vs* the corresponding value for control animals (OVX rats pretreated with vehicle).

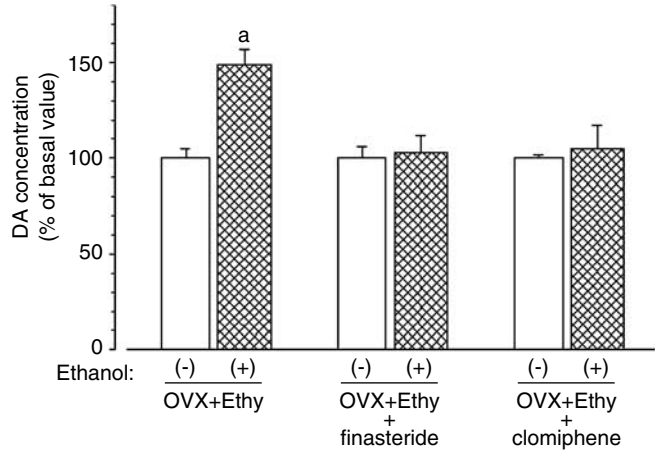

Figure 7 Effects of clomiphene or finasteride on the ethanol-induced increase in dopamine output in OVX rats pretreated with ethynylestradiol. Female rats were subjected to ovariectomy 2 weeks before experiments. Ethynylestradiol (30 μg, s.c.) and finasteride (25 mg/kg, s.c.) were administered 25 and 1 h, and clomiphene (5 mg/kg, i.p.) was administered 1 h, before the onset of microdialysis. Rats were then subjected to an intraperitoneal challenge with ethanol (0.5 g/kg). Data are expressed as a percentage of the corresponding basal value, are means ± SEM for at least five rats per group, and represent the maximal value obtained 60 min after ethanol administration. $^{a}P<0.01$ *vs* basal value.

analysis. Absolute basal dopamine concentrations are reported in the Results section. *Post hoc* comparisons were performed by Neuman–Keuls test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Changes in the Basal Levels of Dopamine in the Dialysates from Rat Prefrontal Cortex during the Estrous Cycle

We first measured the basal levels of dopamine in the prefrontal cortex of female rats during the estrous cycle (Figure 3). Given that the basal dopamine concentration did not differ between diestrus 1 and diestrus 2 ($P=2.056$), the values for these two phases were combined and are subsequently referred to as the diestrus value. The basal dopamine concentration was maximal during estrus (15 ± 1.7 fmol per 40-μl sample), was markedly decreased during diestrus (6.9 ± 2.0 fmol), and was lowest during proestrus (3.6 ± 0.6 fmol). ANOVA revealed a significant effect of cycle phase on basal dopamine concentration ($F(2,31)=14.5477$, $P<0.001$).

Differential Effects of Ethanol on Dopamine Output in the Prefrontal Cortex during the Estrous Cycle

Similar to our previous observations with male rats (Dazzi *et al*, 2002a), i.p. administration of ethanol (0.5 g/kg) induced a marked increase in cortical dopamine output in female rats during estrus (Figure 4). This increase was maximal (+50%) 40 min after ethanol administration and was no longer apparent at 100 min. The same dose of ethanol administered to different groups of female rats in diestrus or proestrus failed to significantly affect the basal concentration of dopamine. ANOVA revealed a significant main effect of cycle phase ($F(2,144)=2.5689$, $P<0.001$), a significant main effect of repeated measures ($F(6,144)=4.7983$, $P<0.001$), and a significant interaction between factors ($F(12,144)=8.4625$, $P<0.001$).

Inhibition of the Ethanol-Induced Increase in Cortical Dopamine Output during Estrus by Clomiphene, Finasteride, or Ovariectomy

The acute administration of the estrogen receptor antagonist clomiphene (5 mg/kg, i.p.) prevented the ethanol-induced increase in dopamine concentration observed during estrus (Figure 5). ANOVA revealed a significant main effect of pretreatment ($F(1,10)=2.8309$, $P<0.001$), a significant main effect of treatment ($F(1,10)=2.7285$, $P<0.001$), and a significant interaction between factors ($F(1,10)=0.9259$; $P<0.001$). Administration of the 5α-reductase inhibitor finasteride (25 mg/kg, s.c., once a day for 2 days) also completely inhibited the ethanol-induced increase in cortical dopamine output during estrus. ANOVA again revealed a significant main effect of pretreatment ($F(1,12)=4.0394$, $P<0.001$), a significant main effect of treatment ($F(1,12)=2.1819$, $P<0.001$), and a significant interaction between factors ($F(1,12)=1.0394$, $P<0.001$).

To clarify further the roles of progesterone and estrogen in the effect of ethanol on cortical dopaminergic neurons, we evaluated the effect of this drug on dopamine concentration in OVX rats. The basal dopamine concentration in dialysate sample from the prefrontal cortex of OVX rats was 5.9 ± 1.4 fmol per 40-μl sample, a value similar to that for intact female rats during diestrus (Figure 3). The administration of ethanol to OVX rats, however, failed

to increase dopamine output in the prefrontal cortex (Figure 6). ANOVA revealed a significant main effect of ovariectomy ($F(1,9) = 3.3717$, $P < 0.001$), a significant main effect of treatment ($F(1,9) = 3.0221$, $P < 0.001$), and a significant interaction between factors ($F(1,9) = 0.063$, $P < 0.001$).

Effects of Progesterone and Ethynylestradiol on the Sensitivity of Mesocortical Dopaminergic Neurons to Ethanol in OVX Rats

To better understand the relative roles of estrogen and progesterone in the effect of ethanol on mesocortical dopaminergic neurons, we examined whether the ethanol-induced increase in dopamine output could be restored in OVX rats by prior administration of progesterone (5 mg/kg, i.p., once a day for 2 days) or the synthetic estrogen ethynylestradiol (30 μg, s.c., once a day for 2 days) (Figure 6). The acute administration of ethanol failed to significantly affect dopamine concentration in the prefrontal cortex of OVX rats pretreated with progesterone ($F(1,11) = 0.0245$, $P = 0.6497$). In contrast, ethanol induced a significant increase (+ 55%) in dopamine output in OVX rats pretreated with ethynylestradiol ($F(1,12) = 2.9746$, $P < 0.001$); the magnitude of this effect of ethanol was similar to that apparent in sham-operated female rats using estrus. The same dose of ethynylestradiol administered 30 days (rather than 2 weeks) after ovariectomy failed to restore the ethanol-induced increase in dopamine concentration ($F(1,11) = 0.0152$, $P = 0.8624$) (data not shown). The combined administration of progesterone and ethynylestradiol in OVX rats did not modify further the effect of ethanol (+ 50% increase) on cortical dopamine output relative to administration of ethynylestradiol alone ($F(1,12) = 0.1163$, $P = 0.4354$).

The ethanol-induced increase in the extracellular dopamine concentration in OVX rats pretreated with ethynylestradiol was prevented by clomiphene ($F(1,10) = 2.6598$, $P < 0.001$) (Figure 7). Finasteride also completely inhibited the ability of ethanol to increase mesocortical dopaminergic output in OVX rats pretreated with ethynylestradiol ($F(1,10) = 2.2658$, $P < 0.001$) (Figure 7).

DISCUSSION

We have shown that there are marked differences in the concentration of dopamine in dialysate sample from the prefrontal cortex of female rats in the different phases of the estrous cycle, with the highest concentration apparent during estrus and the lowest during proestrus. Consistent with our data, previous studies have shown that the basal concentration of dopamine in the striatum and nucleus accumbens of rats is dependent on the circulating levels of estrogen and progesterone (Becker, 1999). Moreover, we have also now shown that the mesocortical dopaminergic pathway is sensitive to the acute administration of ethanol (0.5 g/kg, i.p.) during estrus but not during diestrus or proestrus. Furthermore, the ethanol-induced increase in dopamine concentration was prevented by ovariectomy and restored in OVX rats by pretreatment with a synthetic estrogen but not by that with progesterone, suggesting that estrogen plays a more important role than does progesterone in modulation of this effect of ethanol in female rats. In contrast, we previously showed that an increase in the brain content of 3α,5α-THPROG, induced by administration of its precursor progesterone, enhanced the ethanol-induced biphasic effect on cortical dopamine output in male rats (Dazzi *et al*, 2002a) and that this effect was abolished by the 5α-reductase inhibitor finasteride, which blocks the formation of 3α,5α-THPROG from progesterone.

Estrogen exerts a wide range of actions in the mammalian brain that extend far beyond its classical role as regulator of the hypothalamo-pituitary-gonadal axis. These actions include neurotrophic effects, such as promotion of cell survival (Sawada and Shimohama, 2003), modulation of synaptogenesis (Naftolin *et al*, 1996; Stein, 2001) and axonal and dendritic sprouting (Garcia-Segura *et al*, 2001; Leranth *et al*, 2000), and enhancement of neurogenesis (Beyer, 1999; Tanapat *et al*, 1999). A functional relation between the ability of estrogen to increase the synthesis of brain-derived neurotrophic factor and an increase in the excitability of specific neuronal populations in the rat hippocampus has also been demonstrated (Scharfman *et al*, 2003). In addition, estrogen modulates certain brain functions by affecting neurotransmitter levels within distinct neuronal populations as well as the expression of receptors and second messengers (Becker, 1990, 1999). Indeed, estrogen affects the concentration of dopamine in specific brain areas and induces rapid changes in the response of striatal neurons to D_1 and D_2 dopamine receptor agonists (Febo *et al*, 2003), possibly by producing an uncoupling of the D_2 receptor–G protein complex (Dluzen, 2005). Prolonged estrogen administration induces downregulation of presynaptic dopamine activity (Di Paolo *et al*, 1982; Morissette and Di Paolo, 1993) and produces a dopamine receptor supersensitivity (Hruska and Silbergeld, 1980; Di Paolo *et al*, 1981, 1982; Hruska, 1986) that results in a release from the inhibitory action of these receptors and enhancement of stimulated dopamine release. Moreover, estrogen increases the activity of the dopamine transporter in a manner dependent on phase of the estrous cycle, with higher activity during proestrus and lower activity in estrus (Morissette and Di Paolo, 1993; Thompson and Moss, 1997). Together, these various observations indicate that estrogen may play a major role in modulation of basal and ethanol-stimulated activity of cortical dopaminergic neurons. Consistent with this notion, we have now shown that blockade of estrogen receptors with clomiphene prevented both the effect of ethanol on the cortical concentration of dopamine in intact female rats during estrus as well as the ability of ethynylestradiol to restore this effect of ethanol in OVX rats. Estrogen may thus increase the activity of cortical dopaminergic neurons through a combination of mechanisms, including a reduction in inhibitory control exerted by autoreceptors (Hruska and Silbergeld, 1980; Di Paolo *et al*, 1981, 1982; Hruska, 1986), an increase in the activity of the dopamine transporter (Morissette and Di Paolo, 1993; Thompson and Moss, 1997), inhibition of Ca^{2+} influx (Mermelstein *et al*, 1996), uncoupling of the D_2 receptor–G protein complex (Dluzen, 2005), and modulation of neuronal plasticity (Naftolin *et al*, 1996; Beyer, 1999; Tanapat *et al*, 1999; Garcia-Segura *et al*, 2001; Leranth *et al*, 2000; Stein, 2001; Sawada and Shimohama, 2003; Scharfman *et al*, 2003).

Estrogen depletion induced by ovariectomy results in a marked decrease in dopaminergic cell density in the brain of nonhuman primates (Leranth *et al*, 2000), an effect that can be reversed by estrogen administration 10 days, but not 30 days, after ovariectomy. These results suggest that estrogen is necessary to maintain dopamine cells trophic and active and that a more prolonged estrogen depletion, as observed 30 days after ovariectomy, may result in the death of dopamine neurons. Our finding that estrogen administration 30 days after ovariectomy failed to restore the effect of ethanol on cortical dopaminergic neurons is consistent with the notion that estrogen is necessary to ensure an optimal level of dopaminergic neurotransmission. Thus, estrogen administration 14 days after ovariectomy would still be able to increase the activity of dopaminergic neurons, whereas it looses this capability after 30 days of estrogen depletion when at least part of the mesocortical dopamine neurons might be inactive or dead.

Similar to the effect of clomiphene and ovariectomy, the selective 5α-reductase blocker finasteride, which inhibits the conversion of progesterone to 3α,5α-THPROG, prevented the increase in dopamine output elicited by ethanol during estrus, indicating that both estrogen and progesterone might be necessary for the effect of ethanol on mesocortical dopaminergic neurons in female rats. Ethanol increases both peripheral (Van Doren *et al*, 2000) and central (Sanna *et al*, 2004) synthesis of 3α,5α-THPROG and modulates expression of the genes for various $GABA_A$ receptor subunits (Cagetti *et al*, 2003; Follesa *et al*, 2003; Sanna *et al*, 2003). The presence of progesterone as a source of 3α,5α-THPROG, which by acting at $GABA_A$ receptors mediates most of the behavioral, neurochemical, and molecular effects of ethanol (Morrow *et al*, 2001; Van Doren *et al*, 2000; Dazzi *et al*, 2002a; Sanna *et al*, 2003, 2004), seems to be required for many of the actions of this addictive substance. The possibility, however, that clomiphene and/or finasteride may antagonize the ethanol-induced increase in cortical dopamine output by acting at sites distal from where the ethynyl estradiol is acting, or by alternate mechanisms than the primary effect of these drugs, cannot be excluded.

The observation that 14 days after ovariectomy, ethynyl-estradiol pretreatment alone was able to restore the effect of ethanol on cortical dopamine output appears to contradict this hypothesis. However, although ovariectomy greatly reduces the amount of progesterone both in plasma and the brain, it does not completely eliminate it (Morissette and Di Paolo, 1993; Follesa *et al*, 2002), suggesting that the amount of this steroid remaining in the brain of OVX rats might be sufficient to potentiate the effect of estrogen. Our recent observation that ethanol increases brain steroidogenesis by a local action (Sanna *et al*, 2004), together with the suggestion that the antiseizure effect of estrogen might be mediated by formation of 3α,5α-THPROG (Frye and Rhodes, 2005), support the idea that the brain level of this neuroactive steroid might be increased by ethynylestradiol and ethanol administration to an extent sufficient for it to be able to potentiate estrogen action. This conclusion is further supported by our observation that finasteride abolished the ethanol-induced increase in the cortical extracellular concentration of dopamine in OVX rats pretreated with ethynylestradiol. Synergistic interactions between estrogen and 3α,5α-THPROG have also been described previously (Canonaco *et al*, 1989; Landgren and Selstam, 1995; Frye and Duncan, 1996; Wihlback *et al*, 2005).

Short-term exposure to progesterone affects the expression of $GABA_A$ receptor subunits and consequently the pharmacological properties of the receptors. In particular, such exposure to progesterone or to its metabolite 3α,5α-THPROG increases the expression of the α4 subunit (Gulinello and Smith, 2003), the presence of which renders $GABA_A$ receptors insensitive to benzodiazepines and confers to flumazenil the ability to act as a positive modulator (Wafford *et al*, 1996). The subunit composition of $GABA_A$ receptors is also thought to determine receptor sensitivity to ethanol. However, whereas low concentrations of ethanol were shown to selectively increase GABA-gated currents at receptors containing both α4 and δ subunits (Sundstrom-Poromaa *et al*, 2002; Wallner *et al*, 2004), a more recent study failed to confirm this finding (Borghese *et al*, 2005). Expression of the α4 subunit was also increased *in vitro* in cerebellar and hippocampal cell cultures (Follesa *et al*, 2000) as well as *in vivo* in the brain of rats (Biggio *et al*, 2003; Follesa *et al*, 2004) by chronic exposure to and subsequent withdrawal of progesterone, a paradigm that has been proposed to replicate the hormonal facets of premenstrual syndrome (Sundstrom-Poromaa *et al*, 2002). Another possible explanation for the difference in the effect of ethanol on dopaminergic neurons during the different phases of the estrous cycle is thus that physiological fluctuations in the concentration of progesterone during the estrous cycle lead to changes in the expression of $GABA_A$ receptor subunits that, in turn, result in the synthesis of $GABA_A$ receptors with different sensitivities to ethanol. Indeed, the subunit composition of $GABA_A$ receptors in various regions of the brain of mice and rats has been shown to change during progression of the estrous cycle (Griffiths and Lovick, 2005; Lovick *et al*, 2005; Maguire *et al*, 2005). The stimulatory effect of ethanol on cortical dopaminergic neurons was evident only during estrus, in which there is a fall in progesterone levels following the high values apparent in the preceding phase (Morissette and Di Paolo, 1993). Our previous data showing that administration of progesterone to male rats for 2 days potentiates the effect of ethanol on cortical dopaminergic neurons (Dazzi *et al*, 2002a) are in agreement with this hypothesis. Such treatment with progesterone increases expression of the δ subunit of the $GABA_A$ receptor and its association with the α4 subunit (Shen *et al*, 2005), thus possibly increasing the ability of ethanol to increase GABA-mediated currents at lower doses.

In conclusion, our data suggest the existence of a modulatory effect of both ovarian steroids on the activity of mesocortical dopaminergic neurons in female rats. Estrogen and progesterone thus appear to have a synergistic effect on ethanol-stimulated dopamine output in the prefrontal cortex during the estrous cycle. Estrogen likely has a stimulatory effect on the activity of this neuronal system, playing a permissive role in the effect of ethanol. Progesterone, through its metabolite 3α,5α-THPROG, may be necessary to modulate the subunit composition of $GABA_A$ receptors. Although it is not possible to assign specific roles to estrogen and progesterone in the regulation

of mesocortical dopaminergic activity, the observation that dopaminergic neurons are sensitive to a synergistic action of circulating steroid levels provides a clue to understanding how fluctuations in ovarian hormone production may amplify or ameliorate the symptomatology of psychiatric disorders characterized by altered mood and emotional states as well as the estrous cycle- and gender-related differences in drug sensitivity.

ACKNOWLEDGEMENTS

This work was supported by National institute on Alcohol Abuse and Alcoholism Grant U01AA13641, by the Sardinian Health Ministry (no 363, 12.04.05, c. 12076/00), as well as by GIO.I.A. Foundation (Pisa, Italy).

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