How hormonal contraceptives shape brain and behavior:

A review of preclinical studies

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Abstract

Steroid hormones influence different aspects of brain function, including development, neurogenesis, neuronal excitability, and plasticity, thus affecting emotional states, cognition, sociality, and reward. In women, their levels fluctuate across the lifespan and through the reproductive stages but are also altered by exogenous administration of hormonal contraceptives (HC). HC are widely used by women throughout their fertile life both for contraceptive and therapeutic benefits. However, awareness of their effects on brain function and behavior is still poorly appreciated, despite the emerging evidence of their action at the level of the central nervous system. Here, we summarize results obtained in preclinical studies, mostly conducted in intact female rodents, aimed at investigating the neurobiological effects of HC. HC can alter neuroactive hormones, neurotransmitters, neuropeptides, as well as emotional states, cognition, social and sexual behaviors. Animal studies provide insights into the neurobiological effects of HC with the aim to improve women's health and well-being.

Keywords: hormonal contraceptives; neuroactive steroids; anxiety; depression; social behavior; sexual behavior; learning and memory; female rats

Highlights

- Hormonal contraceptives (HC) affect brain function and behavior.
- We review preclinical studies on HC effects on neurobehavior.
- Animal studies provide insights on HC neurobiological effects.
- Understanding the neurobiological mechanisms of HC actions will improve women's well-being.

1. Introduction

Steroid hormones are crucial for normal brain function as they modulate brain development, neurogenesis, neuronal excitability, and neuronal plasticity, thus affecting emotional states, cognition, sociality, and reward. They exert these actions by binding to intracellular steroid receptors that promote gene transcription; in addition, some steroids can modulate ligand-gated ion channels located on the cell membrane thus exerting rapid non-genomic actions (McEwen and Milner, 2017; Porcu et al., 2016). Concentrations of endogenous steroid hormones fluctuate under several physiological conditions including development, puberty, the ovarian cycle, menopause, aging, as well as following several pathological conditions including neuropsychiatric diseases, neurodegenerative, inflammatory diseases. In addition, several pharmacological treatments against these diseases can restore altered levels of steroids. Steroid levels, particularly sex steroids also vary in response to hormonal contraceptives (HC) use (Del Río et al., 2018; Porcu et al., 2016; Porcu et al., 2019).

HC are a combination of synthetic estrogens and progestins, available as pills (combined oral contraceptives), transdermal patches or vaginal rings. Ethinyl estradiol (EE) is still the most used synthetic estrogen, even though some preparations using estradiol valerate or estetrol have the same contraceptive action with fewer side effects than EE (Apter et al., 2017; Nappi et al., 2014). Estrogens are paired to different types of progestins, including androgenic nortestosterone derivatives (levonorgestrel (LNG), norethindrone, desogestrel, gestodene, norgestimate), anti-androgenic progesterone derivatives (cyproterone acetate, chlormadinone acetate, nomegestrol acetate), and the spironolactone derivative drospirenone (Mitchell and Welling, 2020; Sitruk-Ware and Nath, 2010). Progestin-only contraceptives are also available as pills, injections, subdermal implants, and intrauterine devices. Emergency contraception also consists of a single administration of a higher dose of progestins, mostly LNG 1.5 mg or ulipristal acetate 30 mg (Regidor, 2018).

HC are used by 43% of women of reproductive age worldwide, mostly to prevent unintended pregnancies (United Nations, 2019). Since the first oral contraceptive developed in the 1960s, “the pill”, several forms of hormonal contraception are now available, including injectable formulations, subcutaneous implants, skin patches, intrauterine devices, and vaginal rings. Oral contraceptives remain one of the most used forms of hormonal contraception, especially in western countries (United Nations, 2019), mostly due to their high efficacy and rapid reversibility; in addition, they are prescribed as treatment for some gynecological diseases, such as endometriosis, polycystic ovary syndrome, dysmenorrhea, acne, and hirsutism.

Given that HC are mostly used by young, healthy women, much attention has been paid to their side effects. For instance, cardiovascular adverse effects have been reported, especially in women with other predisposing factors (i.e. smoking), but the lower HC formulations markedly reduce or abolish this risk (Levin et al., 2018; Williams and MacDonald, 2021), and evidence of improved cardiovascular outcomes following HC use has also been reported (Horvath et al., 2018). Likewise, potential risks for cervical and breast cancer are low and may depend on other variables related to duration of HC intake, reproductive history, increased risk due to genotype or to papilloma virus infection (Barańska et al., 2021; Brabaharan et al., 2022; Gierisch et al., 2013; Levin et al., 2018). On the other hand, HC use decreases the incidence of endometrial, ovarian and colorectal cancers, as well as benign breast disease (Brabaharan et al., 2022; Horvath et al., 2018; Levin et al., 2018). In addition, HC use in some women is also associated with headache, weight gain, nausea, changes in menstrual flow and decreased sexual desire, suggestive of an effect at the level of the central nervous system. Furthermore, emotional lability, irritability, and episodes of affective disorders such as depression or mood changes are also common reasons given by women for discontinuing effective HC use, often within the first three months, suggesting psychological effects of these drugs (Kulkarni, 2007; Kurshan and Epperson, 2006). Nonetheless, despite the large number of studies, there is still a lack of agreement on the effect of HC

on mood disturbances, with evidence for mood deterioration as well as mood improvement or stabilization (Bitzer et al., 2018; Böttcher et al., 2012; Cheslack-Postava et al., 2015; Fruzzetti and Fidecicchi, 2020; Hall et al., 2012; Keyes et al., 2013; Lewis et al., 2019; Lundin et al., 2017; Montoya and Bos, 2017; Rapkin, Biggio, et al., 2006; Rapkin, Morgan, et al., 2006; Robakis et al., 2019; Skovlund et al., 2016; Sundstrom Poromaa and Segebladh, 2012; Toffol et al., 2012). Indeed, brain imaging studies showed increased mean diffusivity in the fornix of HC users compared to non-users, suggestive of microstructural changes in white matter (De Bondt et al., 2013), as well as modified volumes in specific brain regions, along with altered functional connectivity in the frontal nodes of the executive network between HC users and non-users, with important consequences for emotional regulation (Engman et al., 2018; Gingnell et al., 2013; Lisofsky et al., 2016; Petersen et al., 2021; Petersen et al., 2014; Petersen et al., 2015; Pletzer et al., 2016; Pletzer et al., 2019; Pletzer et al., 2010; Taylor et al., 2021). Some of these effects differ between adolescent and adult HC users (Sharma, Fang, et al., 2020; Sharma, Smith, et al., 2020), may depend on duration of HC use and type of progestin, and may not be completely reversible (Pletzer et al., 2015).

Although it is now accepted that HC affect brain function, the mechanisms underlying such effects are not completely understood. Animal models may aid in investigating the neurobiological mechanisms behind HC actions on brain function, but insofar very few studies have examined the effects of HC in animals' brains. Here, we describe the current literature on preclinical studies in animals focused on the effects of HC on the central nervous system. Animal models to study effects of HC on brain and behavior have mostly used female rats or mice (see Table 1 for summary and references) and consist of administration of an estrogen-progestin combination, usually EE and LNG, or administration of a single synthetic steroid. Among progestins, LNG is the most studied; it is a second generation progestin that binds with high affinity to progesterone, androgen and mineralocorticoid receptors, but not estrogen receptors (Schindler et al., 2008). LNG is widely used in currently prescribed HC formulations and,

besides “the pill”, it is also the primary component of most emergency contraceptive pills, intra-uterine devices and subdermal implants (Regidor, 2018). Some older studies have tested mestranol in combination with other progestins such as norethindrone, norethisterone acetate, medroxyprogesterone acetate, or lynestrenol; however, studies examining the new generation of progestins with anti-androgenic effects (i.e. drospirenone, dienogest, trimegestone) are still lacking. Drugs are administered in a wide range of doses meant to mimic the HC formulations used by women. The route of administration is mostly subcutaneous or oral; the subcutaneous administration allows a slow rate of absorbance compared to other routes of delivery, while the oral route better mimics the one used by women; oral gavage via orogastric feeding needle is preferred to other oral delivery methods (i.e. voluntary intake; feeding syringe) to ensure each animal takes the same amount of drug. Implantable minipumps could also provide a suitable HC delivery system that allows constant delivery of the drug and could mimic subdermal implants used by women; however, this route of delivery is less common in preclinical research. Duration of treatment ranges from 1 day (rare) to 2 months, but the majority of the studies reported chronic treatments of 28-30 days, thus encompassing approximately 7 estrous cycles. Lifespan differs substantially between rats/mice and women; thus it is not simple to mimic duration of treatment. Studies using a short-term treatment, which encompasses approximately 1-3 estrous cycles, can provide useful information on the neurobiological effects of HC, but might be less relevant from a translational point of view, given that women usually take HC for longer periods of time. The majority of preclinical studies have examined HC effects during chronic exposure or the day after the last administration; studies examining putative long-term effects that may persist after weeks from drug discontinuation are very few and more research is needed. Overall, these studies showed that HC can alter neuroactive hormones, neurotransmitters, neuropeptides, as well as emotional states, cognition, social and sexual behaviors, as detailed in the next sections.

2. Hormonal contraceptives alter levels of neuroactive steroid hormones

HC prevent ovulation by inhibiting the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, and estradiol and progesterone from the ovary, thus preventing the fluctuations in circulating levels of such hormones that typically occur throughout the menstrual cycle (Lobo and Stanczyk, 1994). Furthermore, HC prevent the increase in serum levels of pregnenolone, testosterone and the progesterone derivative, allopregnanolone ((3α,5α)-3-hydroxypregnan-20-one or 3α,5α-THP), typically observed in the luteal phase of the menstrual cycle in women (Follesa et al., 2002; Paoletti et al., 2004; Rapkin, Morgan, et al., 2006). The neuroactive steroid allopregnanolone regulates mood, emotional states, the stress response, and its levels are altered in several neuropsychiatric diseases, including anxiety, depression, premenstrual dysphoric disorder, post-traumatic stress disorder, or alcohol use disorders (Bäckström et al., 2014; Girdler et al., 2012; Porcu and Morrow, 2014; Rasmusson et al., 2017). Interestingly, its synthetic form known as brexanolone has gained approval from the USA Food and Drug Administration for treatment of post-partum depression (Meltzer-Brody et al., 2018), and allopregnanolone has been proposed as a therapeutic approach for alcohol use disorders and mood disorders (Boero et al., 2020; Morrow et al., 2020; Paul et al., 2020).

Similar to the effects in women, HC also disrupt the rat estrous cycle, inhibiting ovulation and decreasing levels of neuroactive steroid hormones (Table 1). Long-term administration (subcutaneous injection, s.c., up to 4 weeks) of EE alone or in combination with different progestins decreased basal and GnRH-stimulated serum LH levels in intact female rats, and this latest effect normalized two weeks after drug discontinuation (Kuhl et al., 1984). In agreement, we reported that long-term administration (s.c. or oral gavage, up to 6 weeks) of EE (30 μg), LNG (125 μg), and their combination (EE-LNG) markedly decreased plasma, cerebrocortical and hippocampal levels of pregnenolone, progesterone and allopregnanolone, and that levels of such hormones returned to normal two weeks after HC

discontinuation (Follesa et al., 2002; Porcu et al., 2012; Santoru et al., 2014; Sassoè-Pognetto et al., 2007). Long-term EE-LNG treatment (oral gavage, 4 weeks) also decreased cerebrocortical concentrations of testosterone in intact female rats (Santoru et al., 2014), matching the lower serum testosterone levels observed in women using HC (Graham et al., 2007; Paoletti et al., 2004).

Circulating estradiol levels are also altered by HC in female Sprague-Dawley rats. LNG (125 μg) administered s.c. for five days decreased serum estradiol levels, compared to controls in proestrus (naturally high estradiol), but not in metestrus (naturally low estradiol), and such alteration normalized within five days from drug suspension, or following treatment with estrogen receptor (ERα and ERβ) agonists (Graham and Milad, 2013). Serum estradiol levels were also reduced following 3 weeks s.c. administration of EE (30 μg), LNG (60 μg), or their combination, compared to control rats in the early diestrus/metestrus phase of the ovarian cycle, when estradiol levels are naturally low compared to the proestrus phase; however, lower doses of EE (10 μg), LNG (20 μg), or their combination did not alter estradiol levels, suggesting that higher doses of EE and LNG may be required to inhibit the hypothalamic-pituitary-gonadal (HPG) axis in rodents (Simone et al., 2015).

The molecular mechanisms by which HC decrease neuroactive steroid levels may depend on inhibition of the HPG axis but may also include direct effects of the synthetic estrogen and progestins on enzymatic expression and activity. Indeed, LNG inhibits basal and LH-stimulated progesterone synthesis, as well as the conversion of pregnenolone to progesterone in cultured rat luteal cells, suggesting a potential direct action on 3β-hydroxysteroid dehydrogenase (Tellería et al., 1994). Similarly, LNG inhibits 5α-reductase activity in skin (Rabe et al., 2000). Whether HC alter the expression and activity of neurosteroidogenic enzymes in the brain is still undetermined; however, our observation that long-term EE-LNG treatment (s.c., 6 weeks) also reduces cerebrocortical concentrations of pregnenolone, progesterone and allopregnanolone in ovariectomized rats (Follesa et al., 2002), lead us to speculate that HC may have a direct effect on brain neurosteroidogenesis, independent from peripheral synthesis.

In summary, the HC-induced decrease in estradiol, progesterone and allopregnanolone levels may affect normal brain function and might contribute to some of the side effects of HC at the central nervous system level, given that these neuroactive hormones exert numerous beneficial effects in the brain, including mood enhancing effects, neuroprotection, and modulation of neuronal plasticity and cognition.

2.1. Hormonal contraceptives and stress-related hormone levels

In addition to the above-mentioned changes in estradiol, progesterone and allopregnanolone, HC also target the hypothalamic-pituitary-adrenal (HPA) axis and increase basal circulating cortisol in women (Kirschbaum et al., 1999; Paoletti et al., 2004; Wiegratz et al., 2003). However, their effects on free salivary and plasma cortisol are contradictory and inconclusive (Boisseau et al., 2013; Kirschbaum et al., 1999; Reynolds et al., 2013). HC users also show a blunted cortisol response to psychosocial or physical stress and to HPA pharmacological challenges with naltrexone, or adrenocorticotropic hormone (ACTH) (Kirschbaum et al., 1999; Kirschbaum et al., 1995; Roche et al., 2013), as well as blunted plasma ACTH content in response to corticotropin releasing hormone challenge (Jacobs et al., 1989), suggestive of reduced sensitivity to stress. Interestingly, similar responses were also observed in adult female cynomolgus monkeys treated with Triphasil® (a clinically prescribed tablet containing EE and LNG) that have a reproductive physiology comparable to women's (Henderson and Shively, 2004). Likewise, female rats chronically treated with EE-LNG (oral gavage, 4 weeks) also showed increased basal corticosterone levels and a blunted corticosterone response to acute restraint stress (Porcu et al., 2019).

Given that EE, but not progestins, induces a dose-dependent increase in cortisol binding globulin (CBG) levels (Wiegratz et al., 2003), this mechanism may likely account for HC effects on basal and stress-induced cortisol levels. Indeed, HC users with higher CBG content have blunted ACTH and cortisol responses to psychosocial stress, compared to users with lower CBG content (Kumsta et al., 2007).

Moreover, women using a LNG-releasing intrauterine device show an increased salivary cortisol response to stress (Aleknaviciute et al., 2017), further supporting the hypothesis that effects of HC on cortisol responses may depend on the estrogenic component and its ability to alter CBG levels.

On the other hand, HPA homeostasis is also regulated by the neuroactive steroid allopregnanolone acting on hypothalamic GABAergic neurons (Biggio et al., 2014; Gunn et al., 2015), and we hypothesized that the decrease in brain and plasma allopregnanolone levels, induced by long-term EE-LNG treatment (s.c. or oral gavage, 4-6 weeks) in female rats, might contribute to the altered basal and stress-stimulated corticosterone responses, thus leading to reduced sensitivity to effects of stress. However, acute restraint stress increased plasma allopregnanolone levels in controls but not EE-LNG-treated rats, suggesting that EE-LNG treatment prevents the allopregnanolone response to stress necessary to restore HPA axis homeostasis (Porcu et al., 2019). Thus, long-term treatment with HC may increase vulnerability to affective disorders via reduced sensitivity of the HPA axis to the effects of stress, a condition also observed in depression (Baumeister et al., 2014).

All the above-mentioned studies have mainly examined basal or acute stress effects of HC on the HPA axis. However, studying the effects of HC in conditions of chronic stress will be of utmost importance, especially in light of challenges currently imposed worldwide by the COVID-19 pandemic, in addition to other long-term stressful events that many women may experience in their life.

3. Hormonal contraceptives alter neurotransmitters, neuropeptides, and circadian rhythms

Regulation of the HPG axis, the main target of HC, is under the control of different neurotransmitters, including γ-aminobutyric acid (GABA), glutamate, monoamines, and acetylcholine; furthermore, the hypothalamus is also the brain area where circadian clocks controlling biological rhythms are located. Likewise, steroid hormones influence the synthesis of specific neuropeptides, such

as brain derived neurotrophic factor (BDNF). However, very few preclinical studies have examined HC effects on neurotransmitter systems and neuropeptides (Table 1).

3.1. Neurotransmitters

HC increased GABA levels, decreased glutamate levels, and altered glutamate decarboxylase and aminobutyrate activities in rat brain, depending on the hormonal combination (Table 1) (Daabees et al., 1981; Ghazal et al., 1976). Furthermore, we showed that long-term treatment (s.c., 4-6 weeks) with EE-LNG alters $GABA_A$ receptor subunit expression in the rat cerebral cortex and hippocampus (Table 1) (Follesa et al., 2002; Porcu et al., 2012). $GABA_A$ receptors belong to the ligand gated ion channel family and are formed by five subunits, typically 2 αs, 2 βs and a γ or δ subunit; 20 genes encoding $GABA_A$ receptor subunits have been identified (α1-6, β1-4, γ1-3, δ, ε, π, τ, ρ1-3), thus giving rise to a wide variety of receptor subtypes with different pharmacological properties, function and neuronal localization. The majority of brain $GABA_A$ receptors contains the γ2 subunit, which is required for proper synaptic anchoring of the receptor; thus receptors containing $\alpha1\beta\gamma2$, $\alpha2\beta\gamma2$ and $\alpha3\beta\gamma2$ subunits are located at the synapse, mediate phasic inhibition and are sensitive to benzodiazepines. In contrast, receptors containing the $\alpha4\beta\gamma2$, $\alpha5\beta\gamma2$ and $\alpha6\beta\gamma2$ combinations can also be located extrasynaptically, thus mediating both phasic and tonic inhibition, while the $\alpha4/6\beta\delta$ receptor subtype is exclusively located extrasynaptically, mediates tonic inhibition and is benzodiazepine insensitive. In addition to the binding site for GABA, all these receptors are positively modulated by endogenous neuroactive steroids such as allopregnanolone (Sieghart and Savić, 2018).

Long-term EE-LNG treatment (30-125 μg/rat, respectively, s.c., 6 weeks) increased mRNA expression for the γ2L and γ2S isoforms of the $GABA_A$ receptor in the rat cerebral cortex and such alteration normalized in two weeks from drug discontinuation (Follesa et al., 2002). Likewise, the

abundance of the γ2 protein increased in both cerebral cortex and hippocampus of EE-LNG-treated rats, compared to vehicle-treated rats. However, cerebrocortical and hippocampal mRNA expression and proteins for α1, α3, α4, α5, β1, β2, and β3 subunits were not affected (Follesa et al., 2002; Porcu et al., 2012), thus suggesting a selective effect of EE-LNG on the γ2 subunit. Further, the scaffold protein gephyrin, which colocalizes with synaptic $GABA_A$ receptors, was also not affected by EE-LNG treatment (30-125 μg/rat, respectively, s.c., 4 weeks) (Sassoè-Pognetto et al., 2007).

$GABA_A$ receptor subunit expression is influenced by fluctuations in allopregnanolone content that occur in physiological conditions like puberty (Shen et al., 2007), ovarian cycle (Lovick et al., 2005; Maguire et al., 2005), pregnancy and lactation (Concas et al., 1998; Maguire and Mody, 2008; Sanna et al., 2009), or following pharmacological treatments (Calza et al., 2010; Follesa et al., 2001; Locci et al., 2017; Modol et al., 2014; Shen et al., 2005; Smith et al., 1998). Thus, the marked decrease in cerebrocortical and hippocampal levels of allopregnanolone, induced by long-term EE-LNG treatment (s.c., 4-6 weeks) might contribute to the observed changes in $GABA_A$ receptor γ2 subunit expression (Follesa et al., 2002; Porcu et al., 2012; Sassoè-Pognetto et al., 2007). Intriguingly, γ2 subunit expression can change in response to other conditions associated to fluctuations in allopregnanolone content. For instance, during pregnancy elevated allopregnanolone levels are paralleled by a decrease in γ2 subunit mRNA and protein in rat cerebral cortex and hippocampus, a condition prevented by treatment with the 5α-reductase inhibitor finasteride that decreases brain allopregnanolone levels (Concas et al., 1998). Likewise, γ2 subunit mRNA and protein increase immediately before parturition when the brain concentrations of allopregnanolone abruptly decline (Concas et al., 1998); similarly, the abundance of γ2 subunit mRNA in the posterior paraventricular nucleus remains elevated during lactation, when brain concentrations of neuroactive steroids in rats continue to be low (Fénelon and Herbison, 1996). Thus, the increased expression of the $GABA_A$ receptor γ2 subunit, induced by long-term EE-LNG treatment (s.c., 4-6 weeks), might be related to the decrease in brain allopregnanolone levels. However, this

assumption is not supported by data showing that only long-term treatment (s.c., 4 weeks) with the progestin LNG alone alters $GABA_A$ receptor subunit expression, while EE alone does not, despite both treatments are equally effective in reducing brain allopregnanolone levels (Porcu et al., 2012). Hence, the HC-induced changes in γ2 subunit expression might be attributed to LNG, rather than to the low allopregnanolone content. LNG displays a potent progesterone-like activity, but it also binds to androgen receptors (Cabeza et al., 1995; Lemus et al., 1992) and to intracellular mineralocorticoid receptors (Sitruk-Ware and Nath, 2010); moreover, its A-ring reduced derivatives, 3α,5α- and 3α,5β-LNG, show enhanced binding affinity for androgenic receptors compared to LNG (Lemus et al., 1992). Thus, LNG or its metabolites might alter γ2 subunit expression through androgen receptors; indeed, chronic exposure to androgenic steroids also regulates $GABA_A$ receptor expression (McIntyre et al., 2002). Nonetheless, LNG might alter $GABA_A$ receptor subunit expression through its 3α,5α-and 3α,5β-reduced metabolites directly interacting with this receptor system, although a direct action of these LNG metabolites on the $GABA_A$ receptor has not been proven yet. To summarize, alterations in $GABA_A$ receptor expression induced by HC might contribute to some adverse effects sometimes experienced by women, given that the GABAergic transmission is involved in several psychophysiological states, such as anxiety, depression, sleep, cognitive function, seizures, and sexual behavior.

Very few studies, mostly conducted a while ago and thus using older formulations, have examined the effects of HC on other neurotransmitters' systems (Table 1). Acetylcholine and choline brain levels, as well as choline acetyltransferase activity did not change in both mice and rats following chronic oral HC treatment for 12 or 30 days (Daabees et al., 1981; Ladinsky et al., 1976). By contrast, HC, orally administered in different steroid and doses combinations, decreased dopamine levels in the striatum of rats and mice (Jori and Dolfini, 1976). Moreover, a decrease in tyrosine hydroxylase expression was also observed in the locus coeruleus following chronic s.c. administration of low EE-LNG doses (10-20 μg), while higher doses (30-60 μg) increased tyrosine hydroxylase mRNA but not protein levels (Simone et

al., 2015). Likewise, monoamine oxidase activity was reduced by oral treatment with estradiol in combination with norgestrel (Marchi and Cugurra, 1974). Finally, serotonin and its precursor tryptophan were increased in rat brain following acute administration of medroxyprogesterone acetate, as well as chronic treatment with EE in combination with different progestins (Baker et al., 1977; Daabees et al., 1981); however, administration of norethisterone acetate alone failed to alter these neurotransmitters in mice (Baker et al., 1977).

3.2. Neuropeptides

Chronic treatment with EE-LNG (s.c., 3 weeks) decreased hippocampal BDNF mRNA and protein in female rats (Simone et al., 2015), an effect most likely associated to the HC-induced reduction in brain and plasma levels of neuroactive steroid hormones. In fact, BDNF expression is strongly influenced by estradiol through a direct control of an estrogen response element present in the BDNF gene (Sohrabji et al., 1995). Furthermore, both progesterone and allopregnanolone increase BDNF expression in various brain regions (Almeida et al., 2019; Naert et al., 2007). Indeed, hippocampal BDNF expression changes across the rat estrus cycle (Scharfman et al., 2003), and serum BDNF levels in women fluctuate over the menstrual cycle, being highest during the luteal phase, although such fluctuations were absent in women taking HC (Pluchino et al., 2009).

Low EE doses (10 μg), alone or in combination with LNG, administered s.c. for 3 weeks, increased galanin content in the rat locus coeruleus, while higher EE doses (30 μg) were ineffective, despite increasing galanin mRNA (Simone et al., 2015). Galanin is a neuropeptide whose expression is regulated by estrogen; it is widely distributed in the nervous system where it modulates several physiologic functions including neurotransmitter release, hormone secretion, pain, metabolic homeostasis, sleep/wake homeostasis, cognition, innate immunity and inflammation (Lang et al., 2015). Galanin expression is altered in neurological and neuropsychiatric diseases, including depression and

anxiety/stress-related disorders (Lang et al., 2015), and given that it is a stress-inducible neuropeptide and cotransmitter in serotonergic and noradrenergic neurons, authors hypothesized that the EE-induced increase in galanin might contribute to changes in anxiety and cognition through inhibition of the noradrenergic transmission (Simone et al., 2015).

3.3. Biological rhythms

Biological rhythms regulate several physiological and behavioral functions and are controlled by the suprachiasmatic nucleus of the hypothalamus (Neumann et al., 2019). Short-term (8 days) s.c. administration of EE-LNG (30-30 μg/day) to late adolescent female Wistar rats, from postnatal day 50 to 58, prevented the peak fecal estradiol level that occurs during the estrus cycle, and reduced circadian rhythm power; furthermore, such 8 days HC treatment disrupted the 4-day temperature rhythms and reduced core body temperature (Table 1). Importantly, all these effects were observed during HC exposure and persisted after drug discontinuation and into early adulthood (Grant et al., 2021). Despite the artificial laboratory conditions, this study is the first to show an effect of HC on biological rhythms that may have long-term consequences even after treatment cessation.

4. Psychobehavioral effects of hormonal contraceptives

4.1. Emotional state

Emotional disorders such as depression and anxiety are more frequent in women than men, and the hormonal fluctuations associated with the ovarian cycle have been suggested to contribute to the increased vulnerability to mood disorders and to the greater risk of developing a depressive episode following puberty (Hantsoo and Epperson, 2017; Kuehner, 2017). Studies examining the effects of HC on mental health in women have yielded mixed results, with improvement (Keyes et al., 2013; Toffol et al.,

2012), no change (Cheslack-Postava et al., 2015; Lundin et al., 2017; Morssinkhof et al., 2021; Rapkin, Morgan, et al., 2006; Schaffir et al., 2016; Scheuringer et al., 2020), or worsening (Deci et al., 1992; Oinonen and Mazmanian, 2002; Skovlund et al., 2016; Sundstrom Poromaa and Segebladh, 2012) in symptoms of depression and anxiety. Young age is one of the factors that may account for these discrepancies. Increasing evidence from several recent studies reports that adolescents using HC are more likely to experience depressive symptoms and a first diagnosis of depression, to use psychotropic drugs and to have a higher relative risk for suicide (Anderl et al., 2022; Anderl et al., 2020; de Wit et al., 2020; Edwards et al., 2020; Gregory et al., 2018; Skovlund et al., 2018; Skovlund et al., 2016; Zettermark et al., 2018). Within HC users, adolescents show a greater blunted cortisol response to the Trier social stress test compared to adults, which has been hypothesized to contribute to their increased vulnerability to depression (Sharma, Smith, et al., 2020). Further, a genetic predisposition to mood disorders may also contribute to HC effects on mental health. HC users who carry the mineralocorticoid receptor haplotype 1 or 3 showed a higher rate of depressive symptoms, while those carrying the haplotype 2 appear to be protected against the adverse effects on mood (Hamstra et al., 2015), suggesting that HC may negatively influence emotional states in those women who may be genetically predisposed to mood disorders (Hamstra et al., 2017; Hamstra et al., 2016; Hamstra et al., 2015; Rapkin, Morgan, et al., 2006; Segebladh et al., 2009).

Endogenous steroid hormones modulate emotional state in humans. While, the anxiolytic effects of estradiol are controversial (Nouri et al., 2022), allopregnanolone concentrations are decreased in plasma and cerebrospinal fluid of depressed patients and can be normalized by antidepressant treatment (Uzunova et al., 1998), strongly suggesting that allopregnanolone may be involved in the etiology of depression (Boero et al., 2020; Bäckström et al., 2014). Endogenous steroid hormones also modulate emotional behavior in animal models (Hwang et al., 2020; Porcu et al., 2016) and we thus hypothesized that the decrease in the concentrations of estradiol, progesterone, and its neuroactive

metabolite allopregnanolone induced by long-term administration of HC would affect anxiety- and depressive-like behavior in female rats.

4.1.1. Anxiety-like behavior

The effects of HC on anxiety-like behavior have been examined in female rats (Table 1). Long-term treatment with EE-LNG (30-125 μg/rat, respectively, s.c., 6 weeks) induced an anxiety-like behavior in the elevated plus maze test (Follesa et al., 2002), and we hypothesized that such effect might be the consequence of decreased brain allopregnanolone content induced by HC, given that this neuroactive steroid exerts anxiolytic properties (Bitran et al., 1991). However, long-term treatment (s.c., 4 weeks) with EE alone failed to alter anxiety-like behavior, while LNG alone increased such behavior despite both treatments were equally effective in reducing brain allopregnanolone levels (Porcu et al., 2012). We thus hypothesized that the EE-LNG-induced anxiety-like behavior was likely due to a neuroactive action of LNG or its metabolites. LNG binds to progesterone receptors, but it also has a potent activity via androgen receptors. Moreover, it can be A-ring reduced to form 5α-LNG and 3α,5α-LNG, and to a minor level 3β,5α-LNG (Lemus et al., 1992). Indeed, acute administration of 3β,5α-LNG and 3α,5α-norethisterone exerted an anxiolytic effect in ovariectomized Wistar rats, while acute administration of LNG or norethisterone did not alter anxiety-like behavior in the burying behavior test (Picazo et al., 1998). Likewise, long-term LNG administration induced anxiety, mood disorders, and sleep problems in otherwise healthy women without a prior record of these events (Slattery et al., 2018; Wagner and Berenson, 1994).

By contrast, chronic administration of low EE and LNG doses (10 and 20 μg, respectively, s.c., 3 weeks) exerted an anxiolytic effect in the elevated plus maze test and the shock-probe defensive burying test; however, higher EE-LNG doses (30-60 μg) did not alter anxiety-like behavior in the elevated plus maze test and EE alone at the higher dose (30 μg) increased freezing in the shock-probe defensive

burying test (Simone et al., 2015). The EE-LNG-induced changes in anxiety-like behavior correlated with tyrosine hydroxylase expression in the locus coeruleus, suggesting an involvement of the noradrenergic transmission in the HC-mediated anxiety-like behavior in rats (Simone et al., 2015). Thus, HC may modulate anxiety-like behavior through multiple different mechanisms; future studies in rodents are necessary to elucidate these mechanisms to better target potential side effects of these drugs.

4.1.2. Depression-like behaviors

We further hypothesized that the reduction in brain allopregnanolone content induced by long-term EE-LNG treatment might affect depressive-like behavior in female rats (Table 1). However, we found that such treatment (EE-LNG 30-125 μg/rat, respectively, oral gavage, 4 weeks) did not alter sucrose intake in the sucrose preference test (Santoru et al., 2014) that assesses anhedonia, a core symptom of depression (Willner, 1997). We also performed the forced swim test, and we observed a reduction in immobility time in EE-LNG-treated rats compared to vehicle-treated rats (Santoru et al., 2014), indicative of an antidepressant effect of EE-LNG, according to the original interpretation (Porsolt et al., 1977). However, this behavioral paradigm may not be appropriate to detect antidepressant-like behavior in the presence of stress and anxiety, and it has been suggested that immobility in the forced swim test may represent a switch from active to passive behavior in order to cope with an acute stressor (Estanislau et al., 2011; Molendijk and de Kloet, 2015). Thus, given that HC blunt the HPA axis response to acute stress in both women (Kirschbaum et al., 1999; Kirschbaum et al., 1995) and female rats (Porcu et al., 2019), the reduction in immobility induced by long-term EE-LNG-treatment might be due to an inability to cope with the acute swim stress, rather than to a change in depressive-like behavior.

4.1.3. Fear extinction

Fear conditioning and extinction is a valuable experimental paradigm to study the mechanisms underlying emotional learning; this behavioral model is modulated by hormone levels and can be used in different species (Maeng and Milad, 2015). Women using HC have impaired fear extinction, measured as an increase in recovery of fear, compared to free-cycling women in the high estrogen phase, suggesting that fear responses persist for a longer time in HC users (Graham and Milad, 2013). Similarly, impaired extinction recall was observed in female rats following LNG administration (125 μg, s.c., 5 days; Table 1). In both women and female rats, HC did not alter acquisition of fear and extinction (Graham and Milad, 2013). The decrease in estradiol levels induced by HC or by LNG alone might contribute to the impairment in fear extinction. In fact, ERα or ERβ agonist administered to LNG-treated rats reduced freezing during extinction recall. Moreover, estradiol enhances the consolidation of extinction in naturally cycling women, as its administration avoided recovery of fear during extinction recall (Graham and Milad, 2013). In addition, the reduction in progesterone and allopregnanolone levels induced by long-term HC exposure (s.c. or oral gavage, 4-6 weeks; Follesa et al., 2002; Porcu et al., 2012; Santoru et al., 2014) may also contribute to the impaired extinction recall, given that these neuroactive hormones also contribute to fear extinction (Milad et al., 2009; Pibiri et al., 2008).

4.2. Social behavior

Rodents, like humans, are social species and a detailed observation of their behavior in a social context can provide information on their social abilities and social status in terms of being dominant or submissive towards a conspecific. We evaluated social interaction in the resident-intruder test, where agonistic behaviors (follow the intruder, dominant postures, and attacks) displayed by the resident rat towards an intruder are scored to estimate dominance and aggressive social interaction. Long-term EE-LNG treatment (30-125 μg/rat, respectively, oral gavage, 4 weeks) alters social interaction in adult

female rats (Table 1); in fact, such treatment decreased both frequency and duration of dominant and agonistic behaviors suggesting that EE-LNG-treated rats were less dominant compared to vehicle-treated rats (Santoru et al., 2014). Similarly, in cynomolgus monkeys HC (Triphasil®, a EE-LNG tablet) increased frequency of contact aggressions received and relaxed vigilance (Henderson and Shively, 2004). Social and aggressive interactions are mediated by steroid hormones in both animals and humans (Soma et al., 2008). While testosterone mediates aggressive behavior and attacks in males (Koolhaas et al., 1980), estradiol and progesterone fluctuations during the ovarian cycle influence aggression in female rats, a behavior blunted by ovariectomy (Ho et al., 2001). Thus, multiple neuroactive steroids may be influencing agonistic behavior in EE-LNG-treated female rats. In fact, long-term EE-LNG treatment decreases brain and plasma progesterone levels (Follesa et al., 2002; Porcu et al., 2012), as well as cerebrocortical testosterone levels (Santoru et al., 2014), which might contribute to the decrease in social dominance observed in female rats, given that testosterone levels are associated with aggressive behavior and social dominance in both animals and humans (Archer, 2006; Van de Poll et al., 1988). Allopregnanolone also increases aggressive behaviors in male mice subjected to the resident-intruder test (Fish et al., 2001); thus, the decrease in dominant and agonistic behaviors induced by long-term EE-LNG treatment in female rats might be related to the blunted allopregnanolone content following such treatment (Follesa et al., 2002; Porcu et al., 2012; Santoru et al., 2014). However, allopregnanolone is less likely to be involved; in fact, a different outcome in the resident-intruder test has been reported in other experimental conditions of reduced brain and plasma allopregnanolone levels. For instance, a single exposure to estradiol on the day of birth that induces defeminization in adult female rodents and persistently decreases brain and plasma allopregnanolone levels (Calza et al., 2010; Locci et al., 2017), increased agonistic behavior in adult female rats (Berretti et al., 2014). Likewise, male rats and mice subjected to juvenile social isolation display an aggressive behavior along with marked reductions in brain allopregnanolone content (Pinna et al., 2003; Serra et al., 2007).

Long-term EE-LNG treatment (30-125 μg/rat, respectively, oral gavage, 4 weeks) further decreased social investigation in the resident-intruder test (Table 1). In particular, body and anogenital investigation were reduced in EE-LNG-treated female rats, compared to vehicle-treated female rats (Santoru et al., 2014). While the reduced anogenital investigation might be related to a decrease in sexual interest toward the intruder, as this is an appetitive behavior aimed to establish, maintain and promote sexual interaction (Everitt, 1990), the significance of the reduced social investigation is unclear. Interestingly, HC use negatively influences affective empathy towards strangers (Kimmig et al., 2021), and lower levels of prosocial behavior and emotional empathy were also reported in women using HC, compared to non-HC users, and such effect appeared to be associated with the lower progesterone levels in HC users (Strojny et al., 2021). Moreover, both social and sexual stimuli are influenced by oxytocin. To the best of our knowledge, the effects of HC on the oxytocinergic system have not yet been investigated in animal models. In women, oxytocin levels fluctuate across the menstrual cycle, being lower during the luteal phase, while no such fluctuations were observed in women taking HC (Salonia et al., 2005), although other studies reported an increase in plasma oxytocin in women under HC treatment (Silber et al., 1987; Stock et al., 1994). Moreover, HC alter oxytocin-mediated reward sensitivity to social and sexual stimuli in women (Scheele et al., 2016), suggesting that they may also influence social and sexual behavior through the oxytocinergic system.

4.3. Sexual behavior

HC have been reported to influence women's sexual well-being although evidence remains controversial (Both et al., 2019; Caruso et al., 2022) and may depend on the form of HC used (Guida et al., 2017). An improvement, likely due to the increased feeling of security and self-confidence (Buggio et al., 2022; Guida et al., 2005; Strufaldi et al., 2010), no change (Pastor et al., 2013), and a decrease in libido, sexual arousal, and frequency of sexual activities (Battaglia et al., 2012; Caruso et al., 2004;

Graham et al., 2007; Graham et al., 1995; Malmborg et al., 2020; Smith et al., 2014; Wallwiener et al., 2010) have been reported; indeed adverse changes in sexuality and mood were the strongest predictors for HC termination in the first six months of use (Sanders et al., 2001).

Estrogen and progesterone synergistically promote sexual behavior in animals (Gilman and Hitt, 1978); likewise, allopregnanolone also controls this behavior by increasing proceptive and receptive behaviors in female rats (Frye et al., 1998). Therefore, the decrease in neuroactive steroids induced by HC may contribute to sexual well-being in both women and animals. In line with this hypothesis, we reported that long-term EE-LNG treatment (30-125 μg/rat, respectively, oral gavage, 4 weeks) significantly decreased the frequency of proceptive behaviors (ear wiggling, hops and darts) displayed by receptive females in order to attract and facilitate the male mounts; receptive behaviors (lordosis) were also reduced, though not significantly (Table 1) (Santoru et al., 2014). Likewise, in non-human primates HC induce a reduction in the ability to attract mates and in the cyclic increase in female anogenital swelling, indices of decreased proceptive behaviors (Guy et al., 2008; Nadler et al., 1992). These preclinical findings support evidence from a recent meta-analysis of 12 clinical studies involving 9427 participants reporting that sexual desire can be significantly compromised by HC use, although other measures of sexual dysfunction in women are not affected (Huang et al., 2020).

Animal models allow us to test the involvement of each neuroactive steroid in regulation of sexual behavior. We thus investigated whether administration of estradiol and/or progesterone could reinstate sexual behavior in EE-LNG-treated rats. Estradiol administration *per se* did not alter lordosis and proceptive behaviors; by contrast, progesterone, alone or in combination with estradiol, restored such behaviors, suggesting that the pronounced decrease in brain and peripheral levels of progesterone, induced by long-term EE-LNG treatment in female rats, contributes to the reduced sexual behavior (Santoru et al., 2014). Moreover, the effects of progesterone on sexual behavior appear to be mediated by its neuroactive metabolite allopregnanolone. Indeed, brain allopregnanolone content is increased

when rats are maximally receptive or following mating, and systemic administration or intracerebral infusion of this neuroactive steroid promotes mating in ovariectomized rats (Frye, 2001; Frye and Rhodes, 2006; Glaser et al., 1985; Henderson, 2007). On the contrary, inhibition of allopregnanolone biosynthetic enzymes diminishes sexual behavior in female rats (Frye and Vongher, 2001). In agreement with these results, we observed that administration of finasteride (a 5α-reductase inhibitor that blocks the conversion of progesterone to allopregnanolone), which *per se* did not alter receptive and proceptive behaviors, prevented reinstatement of proceptive behaviors following progesterone administration to EE-LNG-treated rats, suggesting that the decrease in proceptive behaviors induced by HC treatment in female rats is related to the decrease in the brain allopregnanolone levels (Santoru et al., 2014).

Sexual behavior is also influenced by testosterone in both animals and humans (Frye, 2001; Giraldi et al., 2004; Stuckey, 2008), and HC decrease serum testosterone levels in women (Graham et al., 2007; Paoletti et al., 2004), as well as its cerebrocortical concentrations in female rats (Santoru et al., 2014). Nevertheless, administration of progesterone, finasteride or their combination did not alter cerebrocortical testosterone levels in EE-LNG-treated rats, suggesting that the decrease in proceptive behaviors induced by long-term EE-LNG treatment is not related to testosterone concentrations (Santoru et al., 2014). Overall, these results further support the involvement of allopregnanolone in regulation of sexual behavior in rodents and in the HC-induced alterations, suggesting that allopregnanolone might contribute to negative changes in sexual well-being experienced by some women using HC.

4.4. Learning and memory

It is well accepted that neuroactive steroid hormones regulate learning and memory. Several studies have examined the effects of HC on cognitive performance in women leading to mixed results.

Specifically, HC can cause amelioration (Gurvich et al., 2020; Plamberger et al., 2021; Rosenberg and Park, 2002), no difference (Gogos, 2013; Gravelsins et al., 2021; Islam et al., 2008; Mihalik et al., 2009; Mordecai et al., 2008; Rosenberg and Park, 2002), or worsening (Bradshaw et al., 2020; Griksiene et al., 2018; Griksiene and Ruksenas, 2011) in cognitive abilities, compared to naturally cycling women. Reasons for such discrepancies may include different cognitive domains analyzed (verbal fluency, visuospatial abilities, mental rotation task, etc.), as well as different duration of treatment and doses and type of progestin administered. Few examples include different outcomes in mental rotation performance (i.e. the spatial cognitive ability to mentally detect objects with different spatial orientation) depending on the type of progestin (Griksiene et al., 2018; Griksiene and Ruksenas, 2011; Wharton et al., 2008), or the different dose of EE (Beltz et al., 2015) used. Moreover, HC can induce either improvement (Gogos et al., 2014; Mordecai et al., 2008), or no difference (Gordon and Lee, 1993; Islam et al., 2008; Wharton et al., 2008) in verbal memory, a traditionally female-favoring domain. By contrast, visuospatial ability, a male-favoring domain, did not change between HC users and non-users (Gogos et al., 2014; Gordon and Lee, 1993; Islam et al., 2008; Mordecai et al., 2008), except for one study that reported opposite effects depending on the kind of progestin (androgenic or anti-androgenic) used (Wharton et al., 2008).

Very few studies have examined learning and memory in animals following HC exposure. Long-term treatment with EE-LNG (30-125 μg, respectively, oral gavage, 4 weeks) did not alter spatial learning and memory in female Sprague-Dawley rats that underwent the Morris water maze test (Santoru et al., 2014). The same treatment also did not alter behavioral flexibility in the Morris water maze test (Boi et al., submitted). Likewise, in the same rat strain chronic EE-LNG treatment (30-60 μg, respectively, s.c., 3 weeks) did not alter visuospatial memory in the novel object recognition and novel context recognition tests (Simone et al., 2015). However, lower doses of EE (10 μg), LNG (20 μg), and their combination impaired recognition memory in the novel object recognition test, while the higher EE dose (30 μg)

improved preference for the novel object compared to vehicle-treated rats (Simone et al., 2015). By contrast, in the novel context recognition test none of the EE and LNG treatments or their combinations affected memory, except for administration of LNG (20 μg) that improved recognition memory compared to vehicle-treated rats (Simone et al., 2015).

Given that HC treatment blunts levels of estradiol, progesterone and allopregnanolone that are known to affect learning and memory performance (Chesler and Juraska, 2000; Johansson et al., 2002), these results were unexpected. However, they may depend upon the experimental conditions and the specific memory domains examined. We cannot rule out the possibility that HC may affect cognition in different experimental settings (species/strain, doses, duration of treatment, behavioral test, etc.) used. For instance, chronic EE administration (0.125 or 0.3 μg/rat, s.c. osmotic pump, 18 days) to adult ovariectomized rats decreased working memory in the water radial arm maze, with no change in spatial reference memory in the Morris water maze (Mennenga et al., 2015). However, we believe these results, albeit relevant to the understanding of estrogen effects on cognitive performance, lack translational value because women taking HC for contraceptive purposes have an intact HPG axis.

By contrast, another preclinical study examined the effects of 4-months exposure to HC in a rodent model of menopause in which adult intact Fisher-344 female rats received a weekly injection of medroxyprogesterone acetate (3.5 mg/rat) from months 4 to 8, were ovariectomized on month 10, and tested on month 12, following an additional treatment with either vehicle or medroxyprogesterone acetate (to mimic hormone replacement therapy). Such treatment impaired working memory in the water radial arm maze but did not change spatial reference memory in the Morris water maze (Braden et al., 2011). This finding may be relevant for those women currently undergoing hormone replacement therapy in menopause, who are more likely to have used HC for contraceptive purposes, compared to older generations of women for which HC were not yet available.

The molecular mechanisms beyond the HC-induced changes in cognitive performance are still unknown. Simone and collaborators showed that impaired memory in the novel object recognition test was associated to reduced hippocampal BDNF mRNA expression, the neurotrophin implicated in learning and memory. Further, such decrease was also positively correlated with tyrosine hydroxylase mRNA expression in the locus coeruleus, suggesting the involvement of noradrenergic transmission on BDNF expression and possibly on HC-induced cognitive performance (Simone et al., 2015).

4.5 Reward

Steroid hormones affect reward in both animals and humans. Administration of estradiol or progesterone modulates reward in rodents (Anker and Carroll, 2010; Yoest et al., 2014); likewise, allopregnanolone has rewarding properties (Finn et al., 1997; Fish et al., 2014; Sinnott et al., 2002), influencing alcohol and cocaine intake (Anker and Carroll, 2010; Morrow et al., 2006). Reward reactivity in women is affected by hormonal fluctuations across the menstrual cycle (Bayer et al., 2013; Dreher et al., 2007), and by hormone therapy in perimenopausal women (Thomas et al., 2014). Thus, HC likely affect reward, although only few studies have examined this aspect. HC users showed increased sensitivity to monetary reward (Bonenberger et al., 2013), but also a reduction in the oxytocin-induced reward associated to the partner's attractiveness (Scheele et al., 2016). Further, HC use differentially modulated smoking satisfaction across the menstrual cycle, as well as affect after smoking abstinence (Hinderaker et al., 2015). EE (0.3 μg/rat, daily intravenous injection for 56 days) was recently shown to modulate nicotine self-administration in ovariectomized Long-Evans rats (Maher et al., 2021); however, this model lacks translational value to study the effects of HC, given that these drugs are taken by young premenopausal women. Thus, future studies in animal models of reward-related behavior are needed to examine putative effects of long-term HC exposure on reward.

5. Conclusions and future directions

We reported evidence that HC affect brain function through many effects on neuroactive steroid hormones, neurotransmission, neuropeptides, and related psychobehavioral impact (Table 1). Preclinical studies using animal models to investigate HC effects on brain function and behavior are limited and we must consider several methodological differences, including species, strains, age, housing environment, doses, route of administration and duration of treatment used, when interpreting and translating current results to the clinic. Studies examining the effects of newer HC formulations currently used by women or bi-or tri-phasic formulations are lacking; likewise, time-course studies to examine effects of long-term exposure are needed, as some women may be exposed to HC for several years starting from adolescence to menopause. Nonetheless, animal models remain a valuable tool to investigate the cellular and molecular mechanisms involved in HC action on brain function, given that they allow for the direct measurement of several neuromodulators in brain tissue. In fact, other aspects remain to be explored.

An important one pertains to HC effects on the adolescent brain, given that these drugs are frequently used by adolescents for therapeutic and contraceptive purposes. The adolescent brain is not fully mature yet, and its maturation is influenced by the surge in steroid hormones levels that occurs at puberty. Therefore, it is quite relevant to understand the effects of HC during adolescence especially whether the depletion in steroid hormones induced by use of these drugs can have long-term consequences on the developing brain. Indeed, the adolescent brain may respond differently than the adult brain to the psychobehavioral effects of HC (Cahill, 2018), and clinical studies suggest that HC use during adolescence blunts the response to stress, alters brain working memory processing for negative stimuli and resting state functional connectivity (Sharma, Fang, et al., 2020; Sharma, Smith, et al., 2020), conditions that may contribute to the increased vulnerability to mood disorders (Anderl et al., 2022; Anderl et al., 2020; de Wit et al., 2020; Gregory et al., 2018; Skovlund et al., 2018; Skovlund et al., 2016;

Zettermark et al., 2018). One challenging aspect in studying the effects of HC in adolescence using animal models is that adolescence in rodents has a short timeframe (approximately two weeks from postnatal day 28 to 42). Estradiol and progesterone levels start to rise at postnatal day 40 in female rats, but reach stable levels comparable to the adult ones only at postnatal day 48; likewise, the estrus cycle stabilizes by postnatal day 75 (Vetter-O'Hagen and Spear, 2012). Therefore, treatment with HC before that timeframe is likely to disrupt the normal development of the HPG axis in adolescent rodents, with long-term consequences for brain development. In addition, approximately ¼ of sexually active adolescents reported using emergency contraception, which consists of a single higher dose of a progestin, usually levonorgestrel (Williams et al., 2021). The consequences of such intervention for brain function are still understudied and might be particularly critical during adolescence when the brain is still under development and highly responsive to steroid hormones.

Another important aspect is the reversibility of HC effects on neurotransmission and behavior after treatment discontinuation. We showed that neuroactive steroid levels and expression of the $\gamma 2$ subunit of the $GABA_A$ receptor normalize two weeks after drug discontinuation (Follesa et al., 2002), but not all the behavioral effects induced by long-term HC treatment are related to the decrease in the above-mentioned parameters. Furthermore, we have preliminary evidence of epigenetic changes that persist up to two weeks after EE-LNG treatment discontinuation in female rats (Boi et al., submitted). Thus, whether HC may induce any persistent effects on behavior or other molecular markers remains to be clarified.

Emerging evidence in women using HC points to the importance of genotype in the susceptibility to certain neuropsychiatric symptoms (i.e., mineralocorticoid receptor haplotypes, catechol-o-methyltransferase, (Gravelsins et al., 2021; Hamstra et al., 2017; Hamstra et al., 2016; Hamstra et al., 2015)). Animal models allow for manipulation of targeted genes to better understand the genetic component involved in certain HC-mediated behavioral phenotypes. Furthermore, clinical advances

driven by progress in genetics and by neuroimaging will facilitate the development of new useful animal models that can combine small-animal imaging techniques, optogenetics and measurement of high-throughput central and peripheral biomarkers, to investigate HC effects on brain function.

Most preclinical studies suggest that some of the behavioral effects induced by HC administration can be attributed to their action on HPG axis suppression and subsequent decrease in brain levels of endogenous GABAergic neuroactive steroids. However, we must keep in mind that the synthetic estrogen and progestins in HC formulations can be neuroactive themselves, as they bind to steroid receptors throughout the brain. Indeed, it has been shown that some of their metabolites, 3β,5α-LNG and 3α,5α-norethisterone, can have anxiolytic properties, similar to other 3α,5α-reduced metabolites of progesterone (Picazo et al., 1998). Moreover, LNG inhibits 5α-reductase activity in skin (Rabe et al., 2000), and basal and LH-stimulated progesterone synthesis, as well as the conversion of pregnenolone to progesterone (Tellería et al., 1994), suggesting a direct action of this progestin on enzymatic activity. Therefore the actions of HC on brain function and behavior can be mediated by both mechanisms, the reduction in endogenous neuroactive steroids and a direct effect of exogenous hormones, and future studies should consider this aspect when examining the neurobiological mechanisms of HC.

Animal models are indispensable tools for research on the neurobiological mechanisms underlying the effects of HC on brain function and behavior that may be helpful to shed light on some of the side-effects of HC on the central nervous system that affect women's well-being.

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Table 1. Preclinical studies on the neurobiological effects of hormonal contraceptives.

	Effect	Species	Steroid formulation	Route of administration	Schedule	References
Neuroactive hormones						
Pregnenolone	Decreased levels in cerebral cortex, hippocampus and plasma	Sprague-Dawley rat	EE-LNG 30-125 μg, EE 30 μg, LNG 125 μg	s.c. s.c. s.c.	Once daily for 6 weeks Once daily for 4 weeks	Follesa et al., 2002; Sassoè-Pognetto et al., 2007; Porcu et al., 2012
Progesterone	Decreased levels in cerebral cortex, hippocampus and plasma	Sprague-Dawley rat	EE-LNG 30-125 μg, EE 30 μg, LNG 125 μg	s.c. s.c. s.c. Oral gavage	Once daily for 6 weeks Once daily for 4 weeks	Follesa et al., 2002; Sassoè-Pognetto et al., 2007; Porcu et al., 2012, Santoru et al., 2014
	Abolished cyclicity	Chimpazee	EE 50, 100 and 400 μg + Norethindrone 500 μg	Oral consumption	Once daily for 27 days	Nadler et al., 1992
Allopregnanolone	Decreased levels in cerebral cortex, hippocampus and plasma	Sprague-Dawley rat	EE-LNG 30-125 μg, EE 30 μg, LNG 125 μg	s.c. s.c. s.c. Oral gavage	Once daily for 6 weeks Once daily for 4 weeks	Follesa et al., 2002; Sassoè-Pognetto et al., 2007; Porcu et al., 2012, Santoru et al., 2014
Estradiol	Decreased levels in serum	Sprague-Dawley rat	EE-LNG 30-60 μg, EE 30 μg, LNG 60 μg	s.c.	Once daily for 3 weeks	Simone et al., 2015
	No effect in serum levels		EE-LNG 10-20 μg, EE 10 μg, LNG 20 μg			
	Decreased serum levels	Chimpazee	EE 50, 100 and 400 μg + Norethindrone 500 μg	Oral consumption	Once daily for 27 days	Nadler et al., 1992
Testosterone	Decreased levels in cerebral cortex	Sprague-Dawley rat	EE-LNG 30-125 μg	Oral gavage	Once daily for 4 weeks	Santoru et al., 2014
	Abolished cyclicity	Chimpazee	EE 50, 100 and 400 μg + Norethindrone 500 μg	Oral consumption	Once daily for 27 days	Nadler et al., 1992
Corticosterone	Increased levels in plasma	Sprague-Dawley rat	EE-LNG 20-60 μg	Oral gavage	Once daily for 4 weeks	Porcu et al., 2019
Cortisol	Increased levels in plasma	Cynomolgus monkey	EE-LNG (Triphasil), EE 0-6.8 μg and LNG 0-21.3 μg range/month	Oral tablet	Once daily for 24 months	Henderson and Shively, 2004
Luteinizing hormone	Decreased serum levels	SIV rat	EE 30 and 50 μg + LNG 125, 250 or 500 μg, Desogestrel	s.c.	Once daily for 4 weeks	Kuhl et al., 1984

			125 µg, Norethisterone 2 mg, Chlorimadinone acetate 2 mg, or Cyproterone acetate 2 mg			
	Abolished cyclicity	Chimpazee	EE 50, 100 and 400 µg + Norethindrone 500 µg	Oral consumption	Once daily for 27 days	Nadler et al., 1992
Neurotransmitters						
GABA	Increased levels in brain	Albino rat	Mestranol, Mestranol + Lynestrenol	Oral	Once daily for 12 days	Ghazal et al., 1976
			EE, Norethisterone acetate, EE + Norethisterone acetate	Oral	Once daily for 12 days	Daabees et al., 1981
	No effect	Albino rat	Lynestrenol	Oral	Once daily for 12 days	Ghazal et al., 1976
			Medroxyprogesterone acetate	i.m.	Single administration	Daabees et al., 1981
Glutamate	Decreased levels in brain	Albino rat	Lynestrenol	Oral	Once daily for 12 days	Ghazal et al., 1976
			Norethisterone acetate	Oral	Once daily for 12 days	Daabees et al., 1981
	No effect	Albino rat	Mestranol, Mestranol + Lynestrenol	Oral	Once daily for 12 days	Ghazal et al., 1976
			Medroxyprogesterone acetate	i.m.	Single administration	Daabees et al., 1981
			EE, EE + Norethisterone acetate	Oral	Once daily for 12 days	
Acetylcholine	No effect	Mouse, Rat	Mestranol + Lynestrenol, + Norethindrone, or + Norethisterone	Oral	Chronic, 30 days	Ladinsky et al., 1976
		Albino rat	Medroxyprogesterone acetate	i.m.	Single administration	Daabees et al., 1981
			EE, Norethisterone acetate, EE + Norethisterone acetate	Oral	Once daily for 12 days	
Dopamine	Reduced levels in striatum	Mouse, rat	Mestranol + Lynestrenol, + Norethindrone, or + Norethynodrel	Oral	Once daily for 4 or 30 days	Jori and Dolfini, 1976
Serotonin	No effect	Mouse	Norethisterone acetate	Injection (unspecified)	Once daily for 42 days	Baker et al., 1977
	Increased brain levels	Mouse	EE + Norethisterone acetate	Injection (unspecified)	Once daily for 42 days	Baker et al., 1977
	Increased brain levels	Albino rat	Medroxyprogesterone acetate	i.m.	Single administration	Daabees et al., 1981

			EE, Norethisterone acetate, EE + Norethisterone acetate	Oral	Once daily for 12 days	
Tryptophan	No effect	Mouse	Norethisterone acetate	Injection (unspecified)	Once daily for 42 days	Baker et al., 1977
			EE + Norethisterone acetate			
	Increased brain levels	Albino rat	Medroxyprogesterone acetate	i.m.	Single administration	Daabees et al., 1981
			Norethisterone acetate, EE + Norethisterone acetate	Oral	Once daily for 12 days	
Enzymes						
Glutamate decarboxylase	Increased activity	Albino rat	Mestranol	Oral	Once daily for 12 days	Ghazal et al., 1976
	No effect	Albino rat	Lynestrenol, Mestranol + Lynestrenol	Oral	Once daily for 12 days	Ghazal et al., 1976
Aminobutyrate aminotransferase	Decreased activity	Albino rat	Mestranol, Lynestrenol	Oral	Once daily for 12 days	Ghazal et al., 1976
	No effect	Albino rat	Mestranol + Lynestrenol	Oral	Once daily for 12 days	Ghazal et al., 1976
Choline acetyltransferase	No effect on activity	Mouse, Rat	Mestranol + Lynestrenol, Norethindrone, or Norethisterone	Oral	Chronic, 30 days	Ladinsky et al., 1976
Tyrosine hydroxylase	Increased mRNA in locus coeruleus	Sprague-Dawley rat	EE-LNG 30-60 µg	s.c.	Once daily for 3 weeks	Simone et al., 2015
	Decreased mRNA in locus coeruleus		EE-LNG 10-20 µg, EE 10 µg, LNG 20 µg			
	Decreased protein in locus coeruleus		EE-LNG 10-20 µg, EE 10 µg			
Monoamine oxydase	Decreased activity	Rat	EE + Norgestrel	Oral	Once daily for 1 or 2 months	Marchi and Cugurra, 1974
Receptors						
$GABA_A$	Increased γ2 subunit in cerebral cortex and hippocampus	Sprague-Dawley rat	EE-LNG 30-125 µg, LNG 125 µg	s.c. s.c. s.c.	Once daily for 6 weeks Once daily for 4 weeks	Follesa et al., 2002; Sassoè-Pognetto et al., 2007; Porcu et al., 2012
	No effect on α1, α3, α4, α5, β1, β2, and β3 subunits in cerebral cortex and hippocampus	Sprague-Dawley rat	EE-LNG 30-125 µg, LNG 125 µg	s.c. s.c.	Once daily for 6 weeks Once daily for 4 weeks	Follesa et al., 2002; Porcu et al., 2012
Gephyrin	No effect on mRNA and protein in cerebral cortex and hippocampus	Sprague-Dawley rat	EE-LNG 30-125 µg	s.c.	Once daily for 4 weeks	Sassoè-Pognetto et al., 2007
Neuropeptides						

BDNF	Decreased mRNA expression in the hippocampus	Sprague-Dawley rat	EE-LNG 10-20 µg, EE 10 µg	s.c.	Once daily for 3 weeks	Simone et al., 2015
Galanin	Increased mRNA expression in the locus coeruleus	Sprague-Dawley rat	EE-LNG 10-20 and 30-60 µg, EE 30 µg, LNG 60 µg	s.c.	Once daily for 3 weeks	Simone et al., 2015
	Increased protein in the locus coeruleus		EE-LNG 10-20 µg, EE 10 µg			
Biological rhythms						
Circadian rhythm power	Reduced	Wistar rat	EE-LNG 30-30 µg	s.c.	Once daily for 8 days	Grant et al., 2021
Temperature rhythms	Disrupted	Wistar rat	EE-LNG 30-30 µg	s.c.	Once daily for 8 days	Grant et al., 2021
Core body temperature	Reduced	Wistar rat	EE-LNG 30-30 µg	s.c.	Once daily for 8 days	Grant et al., 2021
Behavioral tests						
Locomotor activity	Reduced activity	Mouse	Norethisterone acetate EE+ Norethisterone acetate	Injection (unspecified)	Once daily for 42 days	Baker et al., 1977
	No effect	Sprague-Dawley rat	EE-LNG 10-20 and 30-60 µg, EE 10 and 30 µg, LNG 20 and 60 µg	s.c.	Once daily for 3 weeks	Simone et al., 2015
Elevated plus maze	Anxiety-like behavior	Sprague-Dawley rat	EE-LNG 30-125 µg, LNG 125 µg	s.c. s.c.	Once daily for 6 weeks Once daily for 4 weeks	Follesa et al., 2002; Porcu et al., 2012
	Anxiolytic-like behavior	Sprague-Dawley rat	EE-LNG 10-20 µg, EE 10 µg, LNG 20 µg	s.c.	Once daily for 3 weeks	Simone et al., 2015
Shock-probe defensive burying	Anxiolytic-like behavior	Sprague-Dawley rat	EE-LNG 10-20 µg, EE 10 µg	s.c.	Once daily for 3 weeks	Simone et al., 2015
	Anxiety-like		EE 30 µg			
Sucrose preference	No depressive-like behavior	Sprague-Dawley rat	EE-LNG 30-125 µg	Oral gavage	Once daily for 4 weeks	Santoru et al., 2014
Forced swim	No depressive-like behavior	Sprague-Dawley rat	EE-LNG 30-125 µg	Oral gavage	Once daily for 4 weeks	Santoru et al., 2014
Fear conditioning	Impaired extinction recall	Sprague-Dawley rat	LNG 100 and 500 µg/kg	s.c.	Once daily for 4 days	Graham and Milad, 2013
Morris water maze	No effect on spatial learning and memory	Sprague-Dawley rat	EE-LNG 30-125 µg	Oral gavage	Once daily for 4 weeks	Santoru et al., 2014
Novel object recognition	Impaired learning	Sprague-Dawley rat	EE-LNG 10-20 µg, EE 10 µg, LNG 20 µg	s.c.	Once daily for 3 weeks	Simone et al., 2015
	Improved learning		EE 30 µg			
Novel context recognition	No effect on learning and memory	Sprague-Dawley rat	EE-LNG 10-20 and 30-60 µg, EE 10 and 30 µg, LNG 60 µg	s.c.	Once daily for 3 weeks	Simone et al., 2015

	Improved learning		LNG 20 µg			
Resident-intruder	Decreased dominant behavior Decreased social investigation	Sprague-Dawley rat	EE-LNG 30-125 µg	Oral gavage	Once daily for 4 weeks	Santoru et al., 2014
Sexual activity	Decreased proceptive behavior No effect on lordosis	Sprague-Dawley rat	EE-LNG 30-125 µg	Oral gavage	Once daily for 4 weeks	Santoru et al., 2014
Sexual activity	Abolished anogenital swelling cyclicity	Chimpazee	EE 50, 100 and 400 µg + Norethindrone 500 µg	Oral consumption	Once daily for 27 days	Nadler et al., 1992
Sexual activity	Decreased mounting frequency No effect on copulatory frequency	Hamadryas baboon	Medroxyprogesterone acetate 3.5 mg/kg	i.m.	Single injection	Guy et al., 2008

Only studies conducted in intact female animals are reported. Abbreviations: EE, ethynyl estradiol; LNG, levonorgestrel; i.m., intramuscular; s.c., subcutaneous.