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Effects of hormonal contraceptives on the brain

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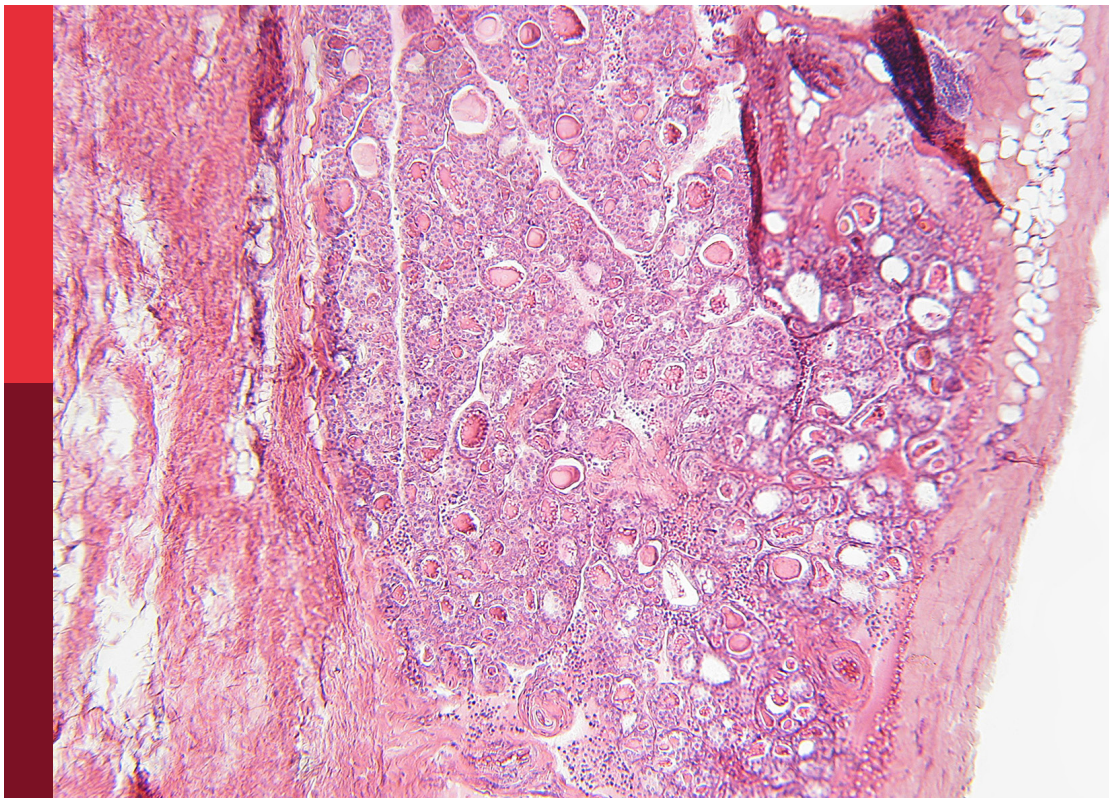

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Effects of Hormonal Contraceptives on the Brain

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Editorial: Effects of hormonal contraceptives on the brain

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Editorial on the Research Topic
Effects of hormonal contraceptives on the brain

Hormonal contraceptives just celebrated their 60th anniversary and are used by 150 million women worldwide (1). While the brain, as a neuroendocrine organ, is in fact the primary target of hormonal contraceptives, we know relatively little about their effects on the brain beyond the suppression of the hypothalamic-pituitary-gonadal axis (2). Combined oral contraceptives (COCs), as the most commonly used form of hormonal contraceptives, downregulate endogenous hormone production, abolish the cyclical fluctuations of endogenous hormones and replace them by constant levels of potent synthetic estrogens and progestins (3). These mechanisms may affect brain areas with a high sensitivity to estrogenic and progestagenic actions (for reviews see 4, 5), as has been demonstrated for the hippocampus (6, 7), amygdala (8–10) and prefrontal cortex (8, 11, 12) among others. These areas are involved in cognitive and emotional processing, and there is increasing awareness of potential alterations in these processes due to hormonal contraceptive use (13).

In fact, first evidence indicating an association between the use of COC and depressive symptoms dates back to the 1960s (14). Nowadays, several studies have shown that oral contraceptive use may result in improved and stabilized mood in some women (15), but mood worsening and depressive symptoms in others (16). Some studies see a blunted cortisol response as partly responsible for the altered vulnerability of COC users to mood disorders (17), an effect that was also highlighted in the current Research Topic (Hogsted et al.). Yet, it is still unclear, which factors determine how a woman will react to COC treatment. Understanding the mechanisms underlying this dissociation of two groups of women with differential affective responsivity to COC treatment is critical, from a medical as well as a methodological viewpoint. Adverse mood effects are among the most common reasons for COC discontinuation (as also pointed out in this topic by Pletzer et al.) thus introducing substantial sampling bias in studies on long-term COC users. It has been suggested that adolescent onset of COC use, as well as previous depressive episodes may represent risk factors for developing adverse mood symptoms during COC treatment (18). In contrast, it has been discussed, whether particularly women with premenstrual dysphoric disorder (PMDD) profit from the mood-stabilizing effects of COCs. Indeed, some COCs with anti-androgen activity have been approved for the treatment of premenstrual dysphoric disorder (19), though recent meta-analyses suggest that in comparison with placebo women with

PMDD show no significant improvement in terms of depressive symptoms upon treatment with COCs (20).

In the current Research Topic, seven articles address the complex interactions between COC use and emotional processing in different female samples (Beinhölzl et al.; Doornweerd et al.; Larsen et al.; Lewis et al.; Pletzer et al.; Robertson et al.; Schmidt et al.).

Focusing on the risk factor of adolescent onset, Doornweerd et al. followed depression and anxiety trajectories of adolescent girls between the age of 13 until 18. Their results demonstrate that girls who never used COCs during adolescence showed increased levels of depressive and anxiety symptoms in late adolescence compared to girls who started using COCs at some point during the study. These results are in line with the several studies indicating potential mood-stabilizing effects of OC intake, particularly in women who do not develop adverse mood symptoms during the first months of treatment and thus become long-term COC users (21), but are in contrast to studies suggesting that adolescent onset of COC use is associated with higher risk for mood disorders (18, 22). However, COC users showed higher risky behavior and also differed in several aspects from never users. This study indicates that COC use needs to be considered in the development of internalizing symptoms from adolescence into adulthood, nicely points out that relevant confounders need to be considered, and adds to research showing that COC use is associated with mental health.

Given that a history of depression represents a risk factor for developing depressive symptoms during COC use (18), depressed patients are of particular interest with regards to their neuro-behavioral responses to COC use. In the current Research Topic, two manuscripts focused on a population with depression and evaluated the efficacy of antidepressant treatment in COC users at the behavioural (Beinhölzl et al.) and neurotransmitter level (Larsen et al.). They found no strong evidence for a difference in treatment response between COC users and non-users diagnosed with depression (Larsen et al.), but also no evidence for changes in emotional attention during a one-week anti-depressant treatment in healthy COC users (Beinhölzl et al.).

Focusing on women diagnosed with PMDD, Robertson et al. investigated the feasibility of a modern COC pill containing 17-beta estradiol rather than ethinylestradiol as an estrogenic component and the new anti-androgenic progestin nomegestrol acetate. The results demonstrated that 20% of women reported emotional side effects and discontinued COC treatment, but the majority of women (75%) reported positive mood changes and reduced depression, anxiety and stress scores during COC treatment.

While the majority of previous studies on emotional processing in COC users have focused on negative emotionality using aversive stimulus material, Schmidt et al. investigated the neural correlates of positive stimulus processing in COC users. However, results revealed no significant group or phase differences in either subjective stimulus evaluations or neural reactivity towards positive stimuli. Null findings were also obtained with regards to value-based decision making during COC use in comparison to naturally cycling females (Lewis et al.) and associations between personality factors and contraceptive choice (Pletzer et al.). The systematic reporting of null findings supported by Bayesian analyses is vital in avoiding publication bias and providing a balanced overview of the aspects of human emotion and cognition affected by hormonal contraceptive use.

Regarding cognitive changes during hormonal contraceptive use, previous studies have yielded mostly inconsistent results due to mostly small sample sizes and a lack of control for the contraceptive formulation used (23). A systematic review arrived at the conclusion that the most consistent finding is a moderate increase in memory tasks, while results regarding spatial performance are inconclusive, but may depend on the hormonal contraceptive formulation used (24). In this Research Topic, three articles have addressed the issue of spatial abilities during COC use including well-powered samples and strictly controlling for both, the progestin type and estrogen dose contained in the COC (Hampson et al.; Koebele et al.; Noachtar et al.).

While previous studies suggested that mostly the androgenicity of the progestin determined the directionality of COC dependent effects on spatial abilities, Hampson et al. observed only moderate effects of androgenicity, while estrogenic potency had a more substantial impact. Supporting the idea of a role for ethinylestradiol in diminishing spatial performance, Koebele et al. observed beneficial effects of the anti-androgenic progestin drospirenone on spatial working memory in rats, which were reversed by ethinylestradiol. Furthermore, ethinylestradiol increased the expression of glutamate decarboxylase (GAD), hinting at inhibitory effects, in the perirhinal cortex. Also, in the study by Noachtar et al., deactivation of the caudate and postcentral gyrus during a spatial navigation task was more prominently related to the duration of COC use in users of levonorgestrel containing pills. Connectivity patterns however were related to the duration of COC use irrespective of androgenicity. Thus, both human studies suggest only moderate roles of androgenicity in the modulation of spatial abilities by COC at the behavioral (Hampson et al.) and brain levels (Noachtar et al.). The studies do however report contrary findings regarding verbal fluency performance: While Hampson et al. observed moderate increases in the number of words produced during the active intake phase, Noachtar et al. found a negative association between verbal fluency performance and longer duration of COC use. The results suggest potentially differential effects of short- and long-term contraceptive use on verbal memory.

Importantly, all three studies go well beyond the traditional cross-sectional design, comparing COC users to naturally cycling women, but rather rely on longitudinal and correlational designs focusing on short term hormone administration, withdrawal or the duration of COC use. An even more stringent longitudinal approach was chosen by Jensen et al., who followed a single subject's resting brain connectivity over two whole cycles – one with and one without COC treatment. Modularity, system segregation and characteristic path length were significantly higher across the natural compared to the contraceptive cycle, hinting at alterations in the hierarchical organization of resting brain networks during COC use. Together with a previous study using the same approach (25), these network analyses particularly highlight the effects of an absent cyclicity of hormonal fluctuations in COC users, rather than focusing on the reduction in endogenous hormone levels or the potency of the synthetic hormones administered. However, in the majority of studies, it cannot be determined, which of the neuroendocrine mechanisms described above are responsible for the changes observed during COC treatment. In that respect, levonorgestrel containing intra-uterine devices (IUDs) may represent a new frontier for the neuroscience of hormonal contraception given that hormonal IUDs do not abolish endogenous hormonal fluctuations (for review see 26). First neuroimaging data from IUD users are presented within this Research Topic by Beltz et al.. IUD users provide a

promising natural experiment for the interplay between exogenous and endogenous sex hormones, and they are likely qualitatively different from OC users with whom they are often grouped in hormonal contraceptive research.

Finally, with respect to delineating the mechanisms underlying hormonal contraceptive-dependent changes in the brain, this Research Topic particularly benefits from the integration of human neuroimaging studies with animal research shedding light on the mechanisms at work (Koebele et al.; Huang et al.). The benefits of this inter-disciplinary integration are manifold. While human neuroimaging studies may identify regions of interest for microstructural analyses in animals, animal studies are for example able to delineate the effects of the estrogenic and progestagenic components of COCs, as outlined in the work by Koebele et al. within this topic. These approaches may in turn lay the groundwork for future neuroimaging studies in humans.

In summary, 14 manuscripts addressing a variety of topics in the realm of hormonal contraception are enclosed in this special issue. We are particularly proud to point out that the majority of first and senior authors on these articles are female. This collection of papers offers a wide range of approaches/perspectives to help our understanding of synthetic hormones' effects and to open new paths in this field of research. Particularly, these studies increase our understanding of temporal relationships through longitudinal studies, as well as hormonal contraceptive type-specific effects on brain, behaviour and mental health. With neuroendocrinological research thriving in the past decades, it now seems within our grasp to deepen our understanding of the complex relationship between synthetic hormones, cognition and emotional well-being. After 60 years of hormonal contraceptive use, it seems like high time to do so.

Author contributions

BP wrote the first draft of the manuscript. EC, EH-L, AL and BD provided revisions, which were incorporated by BD and BP. All authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Pilot Data on the Feasibility And Clinical Outcomes of a Nomegestrol Acetate Oral Contraceptive Pill in Women With Premenstrual Dysphoric Disorder

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Background: Up to 80% of reproductive-aged women experience premenstrual symptoms. Premenstrual Dysphoric Disorder (PMDD) is a severe form, affecting 2-5% of women. Combined oral contraceptive pills (COCPs) are used in the treatment of PMDD. Clinical practice suggests that a newer COCP containing nomegestrol acetate (2.5mg) and 17-beta estradiol (1.5mg), may be a suitable treatment for mood symptoms in PMDD.

Materials and Methods: This was a clinical follow-up feasibility study of women who had attended the Monash Alfred Psychiatry research centre, Women's Mental Health Clinic, with a diagnosis of PMDD. 67% of the sample also had concurrent cPTSD, 29% co-morbid anxiety, and 20% depression. They were recommended treatment with nomegestrol acetate/17-beta estradiol. Eligible women were contacted by telephone to answer a questionnaire to assess women's subjective response to nomegestrol acetate/17-beta estradiol, acceptability and the Depression, Anxiety and Stress Scale-21 (DASS-21) after being recommended nomegestrol acetate/17-beta estradiol. The paired-sample t-test was used to determine if there were any statistically significant differences in the DASS-21 scores over the study observation period (before and after taking nomegestrol acetate/17-beta estradiol).

Results: 35 (74.5%) women reported a subjective positive mood response to nomegestrol acetate/17-beta estradiol, 31 (63.3%) adhered to the medication, and only 10 (20.4%) women reported side effects as the main reason for discontinuing nomegestrol acetate/17-beta estradiol. There were statistically significant reductions ($p<0.05$) in the overall DASS-21 scores from before women commenced nomegestrol acetate/17-beta estradiol and after commencement of treatment.

Conclusions: This preliminary study supports the acceptability and effectiveness of nomegestrol acetate/17-beta estradiol as a treatment for mood symptoms in PMDD. Further research, particularly a randomized controlled trial, is required to elucidate the effect of nomegestrol acetate/17-beta estradiol treatment on mood in PMDD.

Keywords: nomegestrol acetate/17-beta estradiol, mood, premenstrual dysphoric disorder, depression, contraception

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INTRODUCTION

The prevalence of premenstrual symptoms in women of reproductive age is reported to be up to 80% (1). At the severe end of the spectrum is premenstrual dysphoric disorder (PMDD), affecting 2-5% of women (2). The mood symptoms associated with PMDD cause clinically significant distress, interfering with the ability to work, quality of home life and interpersonal relationships and contributing to a high degree of morbidity in this population (3).

The gonadal hormones (estrogen, progesterone and testosterone) have been shown to influence neurochemistry, brain function and the activity of neurotransmitters gamma-aminobutyric acid (GABA), serotonin and dopamine *via* genomic and non-genomic activity at their respective receptors (4). A recent, comprehensive systematic literature review has summarized the neuroimaging findings demonstrating structural, neurochemical and functional markers of PMDD. The functional brain alterations reported in PMDD involve activation of the amygdala, insular cortex and involvement of the cerebellum, and prefrontal areas. Both hyperactivity of the amygdala and hypoactivity of the PFC are associated with PMDD symptoms severity during the late luteal phase of the menstrual cycle (5).

There is also evidence that estrogen is neuroprotective, in many different brain regions, including the brain stem, with concomitant improvements in mood symptoms (6).

Progesterone, however, can contribute to worsening of mood symptoms in susceptible women, evidenced by worsening of mood symptoms in women who use progesterone-only forms of contraception such as the progesterone-only pill and the levonorgestrel intrauterine device (7). Depressive symptoms in women with PMDD are correlated with these hormonal fluctuations; that is, depression worsens in the premenstrual phase, when estrogen is at its lowest and symptoms improve with the onset of menses (2), suggesting that women with PMDD have an abnormal sensitivity to the hormonal fluctuations that occur across the menstrual cycle. In particular, it is believed that women with PMDD have an abnormal GABA response to changes in allopregnanolone levels (a metabolite of progesterone) across the menstrual cycle, contributing to negative mood symptoms (8).

Management options for PMDD include non-pharmacological treatments, hormonal treatments such as the combined oral contraceptive pill (COCP), estrogen patches and micronized progesterone, as well as antidepressants either intermittently or continuously, or a combination of treatments. If these pharmacological treatments are unsuccessful, gonadotropin-releasing hormone (GnRH) analogues and surgery (hysterectomy and bilateral oophorectomy) with menopausal hormone therapy may be required (4).

Given that fluctuations in hormones clearly contribute to PMDD symptom on and off-set, management with hormonal treatment such as the COCP to stabilize the gonadal hormones is an appropriate option. COCPs have mixed evidence in the management of mood symptoms. While studies suggest some COCPs worsen mood symptoms (7, 9), others suggest there may be no impact on mood in most women (10–13), and others report improvement in mood symptoms with hormonal treatment (14–16). The discrepancies that exist between studies to date could be due to the population studied, the COCP preparation, including the type of progesterone and if the COCP is taken continuously (skipping the inactive pills and taking the active tablets only continuously) or intermittently.

Studies of newer generation COCPs containing antimineralocorticoid and antiandrogenic progestins such as drospirenone are promising (4, 14, 17). COCPs containing antiandrogenic progestins are believed to have less adverse mood effects as they block androgenic hormone properties associated with irritability, a key feature of PMDD (18, 19). An initial study by Freeman et al (20) showed that women with PMDD treated with drospirenone (3mg) and ethinylestradiol (30mcg) (Yasmin), had a reduction in PMDD symptoms compared to placebo. Similar findings were identified by Pearlstein et al (21) and Yonkers et al (22). A subsequent Cochrane review article by Lopez, Kaptein and Helmerhorst (23) concluded that drospirenone containing COCPs may help in the management of PMDD, but that there is also a large placebo effect.

Nomegestrol acetate (2.5mg) and 17-beta estradiol (1.5mg) (Zoely, Merck Sharp & Dohme) is a newer COCP that has been successful in clinical practice for the off-label treatment of mood symptoms in PMDD. Nomegestrol acetate/17-beta estradiol is a monophasic preparation with an extended regimen of 24 active pills followed by four placebo pills (24). It contains a synthetic estrogen (17-beta estradiol) that is structurally identical to endogenous estrogen, whereas most other COCPs contain ethinylestradiol. 17-beta estradiol can cross the blood brain barrier, interact with serotonin receptors and regulate cerebral blood flow to the amygdala and dorsolateral prefrontal cortex, and many other areas of the brain, including important mood-control brainstem centers, all involved in depression (25).

Nomegestrol is structurally similar to progesterone and has strong antigonadotrophic and moderate antiandrogenic activity and no effect on estrogenic, glucocorticoid or mineralocorticoid activity (26). A pooled analysis by Witjes et al (27)of the randomized, open-label, multicenter studies by Mansour et al (28) and Westhoff et al (29) compared the effect of nomegestrol acetate/17-beta estradiol versus drospirenone/ethinylestradiol on premenstrual and menstrual symptoms in healthy women, using the Moos Menstrual Distress Questionnaire Form C (MDQ-C). Women taking nomegestrol acetate/17-beta estradiol experienced a significant improvement in the pain, water retention, negative affect, impaired concentration and behavior change domain scores in the menstrual phase compared with drospirenone/ethinylestradiol users. Arousal scores worsened with nomegestrol acetate/17-beta estradiol but not with drospirenone/ethinylestradiol. The authors concluded that nomegestrol acetate/17-beta estradiol is significantly associated with improvements in many of the MDQ-C domain scores compared with drospirenone/ethinylestradiol (27).

Currently, drospirenone containing COCPs are the recommended COCPs for the treatment of PMDD (4).

To date, there have not yet been any studies investigating the impact of nomegestrol acetate/17-beta estradiol treatment on mood in women with PMDD.

This study was undertaken to determine the acceptability and the subjective opinion of nomegestrol acetate/17-beta estradiol treatment on mood in women with PMDD and to determine the changes in the DASS-21 scores before and after nomegestrol acetate/17-beta estradiol treatment in women with PMDD.

MATERIALS AND METHODS

Study Design

This study was a single centre clinical follow-up study that included a questionnaire formulated by the authors (ER and JK) to assess the acceptability and women's subjective response to nomegestrol acetate/17-beta estradiol and the clinical outcomes using self-report questionnaire the DASS-21. Project 285/17 WMHC Database was approved by the Alfred Hospital Ethics Committee on July 3rd, 2020.

Study Participants

Study participants were identified from the Monash Alfred Psychiatry research centre (MAPrc) Women's Mental Health Clinic (WMHC) database. The WMHC provides second opinion consults by expert psychiatrists and psychologists for women with a range of psychiatric disorders. The clinic carefully considers the impact of hormonal changes in the management of mental illness. Women who had attended the WMHC at MAPrc from January 2018 to June 2020, had a clinic diagnosis of PMDD, and had been recommended nomegestrol acetate/17-beta estradiol were included. The diagnosis of PMDD was based on a modified clinical assessment using the central 4 items of the Caroline Premenstrual Assessment Scoring System (C-PASS) (30) - the severity of depressive symptoms, duration of symptoms in the premenstrual phase, relative symptom change from pre to post menstrual phase and absolute clearance of symptoms in the post menstrual phase. Hence a semi-structured C-PASS was used to diagnose PMDD.

Only women who had previously completed the WMHC database Participant Consent Form were eligible for inclusion. Women were excluded if they had never taken the recommenced treatment of nomegestrol acetate/17-beta estradiol or taken nomegestrol acetate/17-beta estradiol for less than one month. If women had previous self-harming behavior or a suicide attempt soon after taking nomegestrol acetate/17-beta estradiol they were excluded for safety reasons. Confidentiality was ensured and verbal consent gained from the participants at the start of the follow-up phone call.

Data (demographics, early life trauma, and DASS-21) was collected as part of the participants' clinical appointment at the WMHC. Menopausal status (reproductive or perimenopausal) was determined by the treating clinician at the clinic appointment based on a comprehensive assessment, including questions about the menstrual status and menopausal symptoms. Follow-up phone calls were made to all eligible participants (those patients with PMDD who have been recommended nomegestrol acetate/17-beta estradiol), and were approximately 20 minutes in length. During this follow-up phone call, the participants completed the DASS-21 scale and were asked questions about their use of nomegestrol acetate/17-beta estradiol, if they take it continuously, side effects of nomegestrol acetate/17-beta estradiol, their subjective opinion of the effect of nomegestrol acetate/17-beta estradiol on their mood and other treatments that they were previously or currently taking.

The Depression, Anxiety, and Stress Scale-21

The DASS-21 is a validated and reliable self-report questionnaire to measure the negative emotional states of depression, anxiety and stress (31, 32). In a clinical population, the DASS-21 has been shown to accurately represent clinical status and changes after treatment (33).The scale has a total of 21 self-report items with responses rated on a 4-point Likert scale ranging from 0 (never) to 3 (almost always). Higher scores suggest a higher level of depression, anxiety and stress. Given the small sample size of this study, only the overall score of DASS-21 was considered for data analysis purposes.

Statistical Analysis

The analysis was performed using the IBM statistics program, SPSS (version 26; IBM Corp., Armonk, NY). To determine if continuous data (DASS-21 pre-post overall scores) were normally distributed, Shapiro-Wilk test for normality was used and histograms were generated. The skewness and kurtosis were within the acceptable range and the overall normality results showed no major violation of normality ($p>0.01$). To determine if there were any statistically significant differences in DASS-21 scores between the first visit and the follow-up call, the paired-t test was used. Given the sample size was relatively small and collected from a more heterogeneous patient population, and to reduce any potential bias in the data, we used bootstrapping technique to reduce its impact by generating a bias-corrected (BCa) confidence interval. Thus, using bootstrapping, 1000 random samples were generated to determine the BCa confidence interval to further support the robustness of the results.

Additional sensitivity analyses were carried out by running the same analysis using the non-parametric Wilcoxon signed-rank to check for the robustness of the assumptions. Also, sensitivity analyses were conducted one by one to check if the findings would change with specific subgroups of participants and/or for the key baseline and medical cofounding variables; The variables included age, history of posttraumatic stress disorder (PTSD), smoking, alcohol use, concurrent anxiety, depression, and complex post traumatic stress disorder (cPTSD), taking a selective serotonin reuptake inhibitor (SSRI), antidepressant, antipsychotic, benzodiazepine, anticonvulsant, current use of estrogen patch, and menopausal status (reproductive or peri-menopausal).

RESULTS

The mean age of the 49 women included in this study was 36 years (sd=8.07). The number of clinic visits during the study

period ranged from one to four with a median of two clinic visits (**Table 1**).

The clinical characteristics of the participants are summarized in **Table 2**. The majority of women were at the reproductive stage (81.6%), with the remaining women being perimenopausal. The majority of women (89.8%) had a history of trauma, including physical, emotional, sexual, peer and neglect. All women had a diagnosis of PMDD, with 33 (67.3%) women having a concurrent diagnosis of complex posttraumatic stress disorder (cPTSD), 28.6% of women had co-morbid anxiety and 20.4% had co-morbid depression, 18 (46.2%) women were taking an SSRI, 10 (25.6%) women were taking another antidepressant, 8 (20.0%) were taking an antipsychotic, 6 (15.4%) were taking a benzodiazepine and 13 (32.5%) were taking an anticonvulsant.

The current and past hormonal treatment and the patients' opinion on its effectiveness on mood were reported in **Table 3**. Around 85.7% of women had previously used a different COCP, including ethinylestradiol and levonorgestrel or drospirenone combinations. Use of a progesterone-only treatment in the past was reported by 19 (38.8%) women. 38.1% of the women who had previously taken a different COCP reported a negative mood response; while 68.4% of women taking a progesterone only treatment reported a negative mood response (**Table 3**).

All women had taken nomegestrol acetate/17-beta estradiol at some point after their first clinical appointment at WMHC. High adherence was reported, with 31 (63.3%) women still currently taking nomegestrol acetate/17-beta estradiol. Nearly half (46.9%) of the women had taken nomegestrol acetate/17-beta estradiol for more than one year with most women (71.4%) taking it continuously. Out of 18 (36.7%) women who had discontinued nomegestrol acetate/17-beta estradiol, side effects were the most commonly provided reason (55.6%), 4 (22.2%) women reported that nomegestrol acetate/17-beta estradiol did not help with their mood, 5.6% of women stated medical reasons for stopping treatment and 5.6% of women were attempting to conceive or pregnant.The most common side effects reported were breakthrough bleeding (40.8%) and decreased libido (24.5%). Thirty five (74.5%) women reported a subjective positive effect of nomegestrol acetate/17-beta estradiol on their mood, 6 (12.8%) reported a neutral effect and 6 (12.8%) reported a negative effect (**Table 4**).

To evaluate the secondary outcome of the study; the clinical effectiveness of nomegestrol acetate/17-beta estradiol, a paired-sample t-test was used to assess the mean differences of the DASS-21 overall scores. The mean, SD and SE of DASS-21 scores from the clinic visit and phone call are displayed in**. Table 5**. After checking for normality assumption and outliers the paired-sample t-test was used to assess mean differences. On average,

TABLE 1 | Participant characteristics.

Characteristic	n (%)*
Age (mean, sd)	36.1 (8.07)
Location	
Metropolitan	35 (71.4%)
Regional/rural	14 (28.6%)
Employment	
Employed	34 (70.8%)
Unemployed	9 (18.8%)
Student	5 (10.4%)
Education	
Up to 12	6 (12.5%)
Vocational training	12 (25.0%)
Bachelor's degree	20 (41.7%)
Post-graduate degree	10 (20.8%)
Living situation	
With others	36 (87.8%)
Alone	5 (12.2%)
Relationship status	
Partnered	31 (64.6%)
Single	17 (35.4%)
Smoking	
No	39 (84.8%)
Yes	7 (15.2%)
Alcohol	
No	21 (44.7%)
Yes	26 (55.3%)
Recreational drugs	
No	41 (89.1%)
Yes	5 (10.9%)
Menopausal status	
Reproductive	40 (81.6%)
Peri-menopausal	9 (18.4%)
Menstrual cycles	
Regular	29 (70.7%)
Irregular	12 (29.3%)
History of trauma	
No	5 (10.2%)
Yes	44 (89.8%)
Number of clinic visits since 2018-2020 (median, range)	2 (1-4)

**n varies from 41-49 due to some missing data for demographic characteristics.*

TABLE 2 | Participant clinical characteristics.

Characteristic	n	n (%)*
Comorbidity of cPTSD	**49**	
No		16 (32.7%)
Yes		33 (67.3%)
Depression	49	
No		39 (79.6%)
Yes		10 (20.4%)
Anxiety	49	
No		35 (71.4%)
Yes		14 (28.6%)
Current use of SSRI	39	
No		21 (53.8%)
Yes		18 (46.2%)
Other Antidepressants	39	
No		29 (74.4%)
Yes		10 (25.6%)
Antipsychotics	40	
No		32 (80.0%)
Yes		8(20.0%)
Benzodiazepine	39	
No		33 (84.6%)
Yes		6 (15.4%)
Anticonvulsant	40	
No		27 (67.5%)
Yes		13 (32.5%)

**n varies from 41-49 due to some missing data for demographic characteristics.*

TABLE 3 | Other hormonal treatment and subjective opinions on their mood response compared to Nomegestrol acetate/17-beta estradiol.

Treatment	
Past combined oral contraceptive pill (COCP)	n=49
Never	7
Yes to any	(14.3%)
(Ethinylestradiol/levonorgestrel, Ethinylestradiol/norethisterone, Ethinylestradiol/drospirenone, Ethinylestradiol/cyproterone)	42 (85.7%)
Progesterone only treatment[b]	
Previous	19
Current	(38.8%)
	1
	(2.0%)
Estrogen patch	
Previous	14
Current	(28.6%)
	13
	(26.5%)
Subjective mood response to previous COCP[a]	n=42
Positive	6
Neutral	(14.3%)
Negative	14
Missing info	(33.3%)
	16
	(38.1%)
	6
	(14.3%)
Subjective mood response to previous progesterone only treatment[c]	n=19
	0
Positive	(0.0%)
Neutral	4
Negative	(21.1%)
Missing info	13
	(68.4%)
	2
	(10.5%)
Subjective opinion of Nomegestrol acetate/17-beta estradiol on mood	n=47
	35
Positive	(74.5)
Neutral	6 (12.8)
Negative	6 (12.8)

[a]*n=42.*
[b]*Progesterone only treatment includes minipill, Mirena, Implanon, prometrium.*
[c]*n=19.*

patients reported improved mood change from the clinic visit (M= 27.27, SE=2.00), to follow-up phone call (i.e. before and after taking nomegestrol acetate/17-beta estradiol) (M = 17.03, SE = 1.65) using the self-reported overall score of DASS-21. The difference, 10.23, BCa 95% CI [646, 14.26], was significant t(29) = 4.72, p = 0.001 and represented a large effect size, d =0.86. The post-hoc power anlaysis showed achieved power of 0.99 for this study (**Table 5**). Additional sensitivity analyses were carried out to check the robustness of the findings. A similar significant result found when a non-parametric Wilcoxon Signed Rank test was used to compare the median instead of mean for DASS-21 overall scores ($p<0.05$). In addition, series of sensitivity analyses were conducted on specific subgroups of participants for some key baseline characteristics or potential cofounders one by one. The sensitivity analyses showed similar significant mean differences for groups with no alcohol use, no smoking, no concurrent depression, anxiety or cPTSD, no current use of an estrogen patch, no use of an SSRI, other antidepressant, antipsychotic, benzodiazepine, and anticonvulsant users. Similar significant findings on overall DASS-21 scores were found for women at reproductive stage, with comorbidity of cPTSD, younger than 39 years old and those who used nomegestrol acetate/17-beta estradiol continuously during the study period.

TABLE 4 | Adherence to nomegestrol acetate/17-beta estradiol and potential side effects for non-adherence at time 2 of interview.

Variables	n (%)
Discontinued nomegestrol acetate/17-beta estradiol:	n=49
No	31 (63.3%)
Yes	18 (36.7%)
Reasons for discontinuing nomegestrol acetate/17-beta estradiol:	*n=18*
Side effects	10 (55.6%)
Want pregnancy	1 (5.6%)
Did not help mood	4 (22.2%)
Didn't need anymore	2 (11.1%)
Medical reasons	1 (5.6%)

TABLE 5 | Paired-sample t-test for comparing pre-post overall DASS-21 scores (n=30)[a].

DASS-21 scores	Mean (SD)	SE	T (*p*)	BCa 95%
Clinic visit			4.72 (0.001)	6.46 - 14.26
Overall score	27.27 (10.96)	2.00		
Phone call				
Overall score	17.03 (9.08)	1.65		

[a]*Only women with scores from both clinic visit and phone call included.*

DISCUSSION

In our pilot study, we evaluated the feasibility and clinical effectiveness of nomegestrol acetate/17-beta estradiol in women with PMDD. Over two-thirds of women adhered to nomegestrol acetate/17-beta estradiol during the study period, while around 20% of them reported discounting of the medication due to side effects. The number of women reporting a positive mood response to nomegestrol acetate/17-beta estradiol was much higher than for previously used COCPs and there was a significant reduction in their self-reported overall DASS-21 score.

The individual differences in response to a COCP could be due to the type of COCP and if the COCP was taken continuously or intermittently. The most common previously used COCP in our group was ethinylestradiol and levonorgestrel. Levonorgestrel is an older synthetic form of progesterone with androgenic properties and therefore may worsen mood symptoms in women with PMDD (34). The improved response to nomegestrol acetate/17-beta estradiol compared to other COCPs in our study could be due to both the different progesterone and estrogen in nomegestrol acetate/17-beta estradiol, a topic to be further explored in future studies.

Eighteen women in our study discontinued nomegestrol acetate/17-beta estradiol, with side effects being the most

commonly provided reason. The rate of reported side effects from nomegestrol acetate/17-beta estradiol in our study appears higher than what has been previously reported by Mansour et al. (28) and Westhoff et al (29). In our study, breakthrough bleeding was the most common side effect in 40.8% of women compared to 11.7% and 9.1% reported in the two above studies, respectively. This discrepancy could be due to our small sample size but could also be due to our categorization of breakthrough bleeding. Of note, in the above studies, the incidence of breakthrough bleeding with nomegestrol acetate/17-beta estradiol was different to the number of women who identified breakthrough bleeding as a side effect. In both studies, approximately 30% of women had breakthrough bleeding in the first cycle and this decreased to approximately 12-25% across the remaining 12 cycles. We did not ask women when their breakthrough bleeding had occurred or how often it had occurred. It is known that breakthrough bleeding is common in women when initially taking a COCP. Nomegestrol acetate/17-beta estradiol is a low dose estrogen pill (estradiol alone is more rapidly metabolized than ethinylestradiol, which after liver metabolism persists as other estrogen metabolites), and this may be why women initially experience higher rates of breakthrough bleeding with nomegestrol acetate/17-beta estradiol compared with other COCPs. Nomegestrol has a longer half-life than other progestogens studied, which is consistent with its reduction in the frequency of bleeding days over time (28).

More women in our study reported weight gain (18.4%) compared to the 7.9% reported by Mansour et al (28) and the 9.5% reported by Westhoff et al (29). These two studies measured participants weight throughout the study period, whereas we asked for the women's subjective opinion of weight changes since taking nomegestrol acetate/17-beta estradiol.

The secondary outcome in this study focused on clinical outcomes. This follow-up study suggests that nomegestrol acetate/17-beta estradiol may be an effective management option for mood symptoms in women with PMDD. The statistically significant improvements in the overall DASS-21 scores before and after treatment with nomegestrol acetate/17-beta estradiol, support our findings, that the majority of women reported a subjective positive mood response to nomegestrol acetate/17-beta estradiol.

Of note, there is a high prevalence of trauma in our population. 89.8% of women in our study had a history of trauma with cPTSD being a concurrent clinic diagnosis in 67.3% of women.

cPTSD occurs in individuals who have experienced prolonged traumatization and is characterised by emotional dysregulation, dissociation, somatization and poor self-esteem (35). It is understood that trauma and posttraumatic stress disorder (PTSD) are independently associated with PMDD (36). Our research suggests that stress can lead to numerous neurobiological changes, including neuroendocrine disruption of the hypothalamic-pituitary-adrenal axis (HPA) and hypothalamic-pituitary-gonadal axis (HPG). Feedback links between the HPA axis and the HPG axis may explain the connection between cPTSD and PMDD. Nomegestrol acetate/17-beta estradiol could be an effective adjunct treatment in women with PMDD and cPTSD to stabilize hormonal fluctuations that may contribute to emotional dysregulation.

Limitations of our study include our small sample size and the study design; in that it was an observational study with no comparison group. Given the retrospective nature of this study, there is a risk of recall bias in the women reporting their subjective mood response to nomegestrol acetate/17-beta estradiol and side effects, particularly more so for the women who were no longer taking nomegestrol acetate/17-beta estradiol. The DASS-21 score is not specific to PMDD and we did not specify the phase of the menstrual cycle women were at when they first completed the DASS-21 at the clinic, prior to commencing nomegestrol acetate/17-beta estradiol. We excluded women who had self-harming or suicidal behavior soon after taking nomegestrol acetate/17-beta estradiol for safety reasons which could have resulted in an overestimation of the positive effect of nomegestrol acetate/17-beta estradiol given that we excluded women who had a negative mood response. We also excluded women who used nomegestrol acetate/17-beta estradiol for less than one month, as it usually takes at least 1 month to see treatment effects. This may have introduced selection bias as there is a possibility the women who did not initially tolerate nomegestrol acetate/17-beta estradiol stopped taking the treatment after a few weeks. Furthermore, we did not take other factors into consideration that may influence mood and thus may change response to nomegestrol acetate/17-beta estradiol.

CONCLUSIONS

This is the first study to investigate the feasibility and clinical effectiveness of nomegestrol acetate/17-beta estradiol treatment in the management of PMDD mood symptoms. Our findings support what we have seen in clinical practice and suggest that nomegestrol acetate/17-beta estradiol could be well tolerated by women suffering from PMDD and could be an effective first line treatment option for women with PMDD. Future research is required, particularly a randomized controlled trial of a large sample comparing nomegestrol acetate/17-beta estradiol to other recommended first line treatments for PMDD, either a drospirenone containing COCP or an SSRI.

AUTHOR'S NOTE

The manuscript has been submitted solely to this journal and is not published in the press or submitted elsewhere.

DATA AVAILABILITY STATEMENT

The data analyzed in this study was obtained from routinely collected information as part of standard treatment in the Women's Mental Health Clinic, Alfred HREC project 285-17],

the following restrictions apply: 1) That the aim/ purpose for the data access is clearly stated and must be for a genuine research purpose, 2) That the name and affiliation of the person/s requesting data access must be clearly stated 3) That the requesting individual/s must declare all and any conflict of interest - including past & current employment and any commercial involvement. 4) That any patient identifying details are kept confidentialRequests to access these datasets should be directed to the corresponding author: Professor Jayashri Kulkarni AM, jayashri.kulkarni@monash.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Alfred Ethics Committee. The patients/ participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

This clinical follow-up study was conceived by JK and CT. JK, consultant psychiatrist, had ultimate patient care responsibility. CT, endocrinologist, provided endocrine specialist care for patients. The initial questionnaire, phone calls, data collection, and data entry were completed by ER. NT assisted ER with the implementation of the objective DASS-21 scale and data analysis. In the revision and resubmission, LK, biostatistician, contributed enormously to the reanalyses and other work as per the reviewers' suggestions. All authors contributed to the article and approved the submitted version.

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Stress-Hormone Dynamics and Working Memory in Healthy Women Who Use Oral Contraceptives *Versus* Non-Users

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Background: Women who use oral contraceptives (OCs) may have a higher risk of developing a depression, which is associated with both vulnerability to stress and cognitive dysfunction. OCs disrupt the hypothalamic-pituitary-gonadal (HPG) axis by suppressing endogenous sex steroid production including estradiol. The HPG axis and the hypothalamic-pituitary-adrenal (HPA) axis are known to interact, possibly through modulations driven by estradiol. OCs may affect HPA regulation capacity, i.e., disturb cortisol dynamics such as the cortisol awakening response (CAR), and influence cognition such as working memory (WM). We hypothesize that OC use is associated with blunted cortisol dynamics and impaired WM performance relative to non-users.

Methods: Data from 78 healthy women in the reproductive age were available from the CIMBI database. We evaluated if CAR and WM differed between OC users (n=25) and non-users (n=53) and if the level of estradiol modulated the OC use effect on CAR or WM in generalized least square models.

Results: We found that OC users had a blunted CAR (p= 0.006) corresponding to a 61% reduction relative to non-users; however, no estradiol-BY-OC use interaction effect was observed on CAR. Also, OC users had higher cortisol levels at awakening compared to non-users (p = 0.03). We observed no effect of OC use or an estradiol-BY-OC use interaction effect on WM. Also, within the OC user group, neither CAR nor WM was associated with suppressed estradiol. CAR was not associated with WM.

Conclusion: Healthy women who use OCs have blunted cortisol dynamics relative to non-users. However, we could not detect OC use effects on working memory in our sample size. We speculate that disrupted cortisol dynamics may be important for the emergence of depressive symptoms in OC users.

Keywords: cortisol, oral contraceptives, hormonal contraceptives, working memory, estradiol, depression, cortisol awakening response, HPA-axis

INTRODUCTION

Combined synthetic estrogen and progestogen OCs are widely used by women in the reproductive age. More than 50% of Scandinavian women begin using hormonal contraceptives before the age of 17 (1), and 42% of Danish women of fertile age use OCs (2). Women who start treatment with oral contraceptives (OCs) are at a higher risk for developing a depressive episode relative to non-users (3). This phenomenon is particularly pronounced in adolescence (3), as also supported by recent independent findings showing that adolescents who use OCs report more depressive symptoms (4) and are more likely to start psychotropic drugs (5) compared to their unmedicated peers. Also, adolescent girls who use OCs have long-lasting vulnerability for depression into adulthood even after discontinuing OCs (6). Depressive episodes are associated with both vulnerability to stress and cognitive dysfunction (7).

The hypothalamic-pituitary-adrenal (HPA) axis dysfunction is a key feature of MDD and several other psychiatric diseases (8). The HPA axis generates diurnal cortisol rhythms and responses to stress or other stimuli. A dynamic cortisol response is considered critical for a healthy adaptation to stress and therefore support resilience (9). Regulation of the HPA axis has suggested to be affected by OCs (10) as blunted or absent cortisol responses to psychosocial stress tests have been observed in healthy women using OCs (10–13). Thus, OCs may, at least in some women, add to the risk of developing psychiatric disorders by increasing vulnerability to psychosocial stress. OCs disrupt the Hypothalamic-Pituitary-Gonadal (HPG) axis and consequently suppress endogenous ovarian hormone production, including estradiol (14). The HPG and HPA axes are intimately related, and sex steroid receptors are pervasively expressed in key parts of the neural circuitry controlling the HPA axis (15). Overall, estradiol plays a significant role in HPG and HPA axis interactions (15). Thus, OC-induced suppression of the HPG axis including estradiol-suppression may well affect the HPA axis and thereby cortisol dynamics, which is putatively critical to mental health. So far, the leading explanation for the observed changes in cortisol dynamics in OC users has been that cortisol binding globulin (CBG) increases in response to OCs and thus less free cortisol is available (13, 16, 17). However, as suggested by Kirschbaum et al. (17), HPA and HPG relations might also play a role.

One way to characterize the HPA axis is by examining the cortisol awakening response (CAR), which is a superimposition on the circadian rhythm of cortisol release that occurs in response to awakening (18). CAR can be easily assessed by home-sampling of saliva, allowing the observation of HPA dynamics in a natural setting. In contrast to more extreme stress test responses, CAR reflects the HPA axis output in a basic everyday condition that may be particularly relevant to mental health risk and resilience mechanism associated with OC use. CAR is known to be influenced by various state and trait factors (19). So far, cortisol responses in OC users have mostly been examined in relation to high intensity HPA axis stimuli such as psychosocial stress or pain (11–13, 20–22), whereas data on OC effects on diurnal features of the HPA axis such as CAR, e.g., responses to an every-day HPA axis stimuli such as awakening, are sparse and inconclusive. Also, early studies did not include factors later known to affect CAR (17–19, 23), and one study only examined adolescent OC users without providing full CAR characterization (13). In this study we include several factors known to affect CAR as suggested by Stalder et al. (2010) (18) and provide a full CAR characterization.

We further want to examine the relationship between WM and OC use as sex steroids, especially estradiol, appear to affect cognitive functions and in particular working memory (WM) (24), which notably is also impaired in MDD (25). WM is the ability to temporarily store and manipulate information that is required to carry out cognitive tasks such as comprehension, thinking and reasoning. In healthy naturally cycling women, WM seems to be impaired during menstrual bleeding when estradiol is low, and in pregnant women WM is improved in late pregnancy when estradiol is high (26, 27). Overall some studies demonstrated better performance by OC users on some cognitive domains by such as emotional memory, susceptibility to false memories and especially verbal memory, while others found no difference in cognition examining visuo-spatial memory, verbal fluency, and attention (28, 29).

We hypothesize that women who use OC relative to non-users have a blunted CAR, which may be associated with low endogenous levels of estradiol. We further explore if OC use is associated with worse performance on cognitive tests of WM, possibly in a manner dependent on estradiol.

Abbreviations: OCs, oral contraceptives; HPG, hypothalamic-pituitary-gonadal; HPA, hypothalamic-pituitary-adrenal; CAR, cortisol awakening response; WM, working memory; IUD, intrauterine devices; AUCi, area under the curve with respect to increase from baseline at awakening; LNS, letter number sequencing; SDMT, symbol digit modalities test.

MATERIALS AND METHODS

Participants

Data were available from the Center for Integrated Molecular Brain Imaging (CIMBI) database (30). We included data from

healthy women within the reproductive age between 18 and 50 years of age with no psychiatric history. All the women had concurrent information on contraceptive use, cortisol dynamics (CAR), and cognitive functioning (WM). Overview on study population selection from the CIMBI database is shown in **Figure 1**. The CIMBI database contained data from 110 healthy women that met the inclusion criteria. Our exclusion criteria were (1) Women above 45 with early menopause or unknown menopausal status measured with follicle stimulating hormone, (2) incorrect collection of home cortisol samples, and (3) hormonal contraceptives other than OCs or hormonal intrauterine devices (IUDs). Eighteen women were excluded from the study: two women >45 years of age had missing follicle stimulating hormone data, thus menopausal status was unknown, and seven women were excluded due to non-compliance when collecting home cortisol saliva samples. Further, we excluded a user of progestin-only pills, a nuva-ring user, and one woman with unspecified type of IUD. Cortisol saliva samples were analyzed in larger batches to minimize experimental noise. One small (n= 6) batch of saliva samples could not be efficiently compared to later batches as they significantly differed from internal standard quality control samples, which are run routinely in each batch; consequently, we excluded six women with incomparable saliva samples. Thus, data from 78 women were available for analysis. Fifty-three women did not use hormonal contraception (non-users): 44 women reported no use of contraceptives and nine women had copper IUDs (see **Figure 1**). Fourteen women using hormonal IUDs were included for additional analysis but were not included in the OC user group or non-user group (see **Figure 1** for overview on study population selection). All OC users used combined ethinylestradiol and progesterone contraception, however with slightly different types of gestagene and small variations in estradiol dose. None of the participants had a history of psychiatric or current severe somatic illness. Participants from the database had been recruited for different neuroimaging projects between 2007 and 2018, and all women provided written consent for inclusion in the CIMBI database. All projects were approved by the Ethics Committee of Copenhagen and Frederiksberg or of Region, Denmark [(KF) 01-274821, (KF)01-2006-20, H-15004506, H-1-2010-085, H-4-2012-105, H-6-2014-057, H-15017713].

Clinical and Genetic Measures

All participants were screened with physical and neurological examinations and clinical blood samples. Information on the serotonin transporter genotype (5-HTTLPR) was obtained in terms of 5-HTTLPR high-expressing (LA/LA) or low-expressing (LG or S carrier) variants (31). This enabled us to test if those variants were associated with CAR, as earlier studies have indicated associations with cortisol responses to a standardized psychosocial stress exposure (31). Information on smoking and daily number of cigarettes was further collected. Plasma estradiol samples were obtained maximum 3 days after the collection of

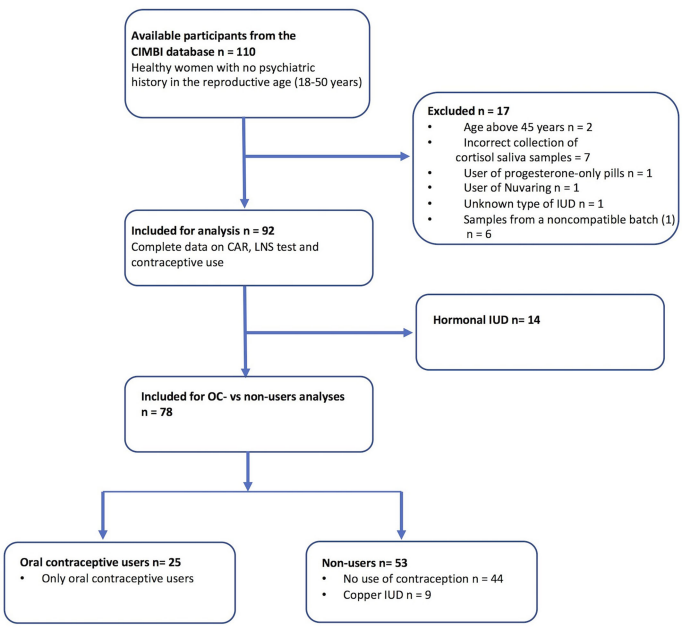

FIGURE 1 | Overview on study population selection from the CIMBI database. Participants recruited from the Center for Integrated Molecular Brain Imaging (CIMBI) database. Inclusion criteria were healthy women with no psychiatric history in the reproductive age <50 years. A complete dataset was required with concurrent information on cortisol awakening response (CAR), Letter-Number-Sequence (LNS) test, and contraceptive use. Exclusion criteria were (1) women above 45 years of age with early menopause or unknown menopausal status measured with follicle stimulation hormone (FSH), (2) incorrect collection of home cortisol samples, and (3) hormonal contraceptives other than OCs or intrauterine devices (IUDs). Further, six women had incomparable cortisol data due to batch variations of the cortisol saliva samples.

the saliva cortisol samples. Twenty-four participants had their estradiol blood samples collected later than 3 days from saliva collection or WM test. These women were excluded from analyses. Thus, estradiol data were available for analysis on 49 women for CAR analysis (OC users: 15; non-users: 34) and 52 for WM analysis (OC users: 14; Non-users: 38) out of the 78 women (see **Figure 1** in supplementary for selection process). The menstrual phase for the non-user women was unknown, except for 26 out of 53 non-user women who had their blood samples collected during the follicular phase as defined by the design of the study they participated in. Hospital analysis method of plasma sex steroid levels was based on antibody reagents from Estradiol II and Elecsys® Estradiol III, Roche. Comparability of estradiol analysis is treated in further details in Larsen et al., 2020. Seventeen out of 73 estradiol samples were below the lower detection limit (0.04 and 0.09 nmol/L, respectively).

Cortisol Measurements

Saliva samples were collected in Salivette® tubes (Sarstedt, Neubringen, Germany). We measured the cortisol awakening response (CAR), i.e., the dynamic increase in cortisol levels that occurs within the first hour upon morning awakening. For the assessment of CAR, participants were instructed to collect saliva sample immediately after awakening and after 15, 30, 45, and 60 min by chewing a swab until it was fully saturated with saliva. Participants were told to avoid food, drinking, brushing teeth, and smoking during the first hour after waking up. Further, the participants were instructed to take a saliva sample at bedtime the same day as collection of CAR. The women noted whether the CAR samples were collected on a work, study, or rest day and at which time the samples were collected including time at awakening. Our inclusion criteria assured that when saliva samples were collected more than 10 min after awakening, the datasets were not included in our analyses. The saliva samples collected at home were stored in the refrigerator and returned to the laboratory the day after completion of sampling, and if collected during the weekend, maximum 3 days after collection. The CAR measurements were computed as the area under the curve with respect to increase from baseline at awakening (AUCi). The AUCi captures change over multiple time points and is thereby an index of change (32). The saliva samples were analyzed in different batches across the collection period (2007 to 2018) to minimize experimental noise. We present data from six different batches. Participant training, instructions, home-sampling procedures, storing, and cortisol analyses were carried out as described in Frokjaer et al., 2013. Salivary cortisol concentrations were determined by an electrochemiluminescence immunoassay (ECLIA) method on Modular Analytics E170 equipment (Roche, Mannheim, Germany) and for two of the most recent batches by a chemiluminescence immunoassay (CLIA) method on the IDS-iSYS automatic analyzer (IDS PLC, Boldon, UK). The two methods were equally distributed in the two groups. The intra- and inter-assay variation of both methods was <15%.

Cognitive Tests

All cognitive tests were performed by or supervised by a trained neuropsychologist. The single tests in the program are described in further details in Dam et al., 2020. The main task used to index WM function was the Letter Number Sequencing (LNS) test from WAIS-III. The LNS is a verbal WM task where the test subject is asked to listen to a jumbled sequence of letters and numbers before mentally sorting and reciting them back starting with the numbers in numerical order followed by the letters in alphabetical order. Number of completed trials is the main outcome of the test with scores ranging from 0 to 23. As a second test we used the Symbol Digit Modalities Test (SDMT), which provides a measure of psychomotor speed and WM capacity. The SDMT involves visual processing and is a paper-and-pencil test of 90 seconds, in which the test subject has to translate abstract shapes to numbers based on a translation key that the test subject must learn and hold in working memory in order to increase speed. Number of correctly translated symbols is the main outcome of the test. Data on SDMT were missing for two participants. For descriptive purposes, intelligence factor (IQ) was tested with Reynolds Intellectual Screening Test (RIST), which provides a proxy measure of the general, age-adjusted IQ. The time interval between cortisol saliva samples and WM tests was 0 to 112 days with a mean of 3 days. Seventy-one out of 78 participants had less than 2 weeks between WM test and cortisol saliva sample collection.

Questionnaires

The participants' mental well-being was assessed with Cohen's Perceived Stress test (Cohen's PSS), Major Depression Inventory (MDI), and Profile of Mood States (POMS). PSS was placed at the day of home collection of the cortisol saliva samples. MDI indexes symptoms of depression according to the ICD-10 diagnostic system and can be used as a screening tool for MDD. The individual subscales of the POMS can be summarized in the Total Mood Disturbances (TMD). Additionally, information on education and sleep quality was collected with questionnaires. Education level was scored on a five-point Likert scale: One corresponded to having no vocational degree and five if they had more than four years of higher academic education. Data on education level were missing for three participants. Information on sleep quality was obtained with a global score from Pittsburgh Sleep Quality Index (PSQI) with higher scores indicating worse sleep. PSQI was completed 0–5 days before cortisol sample collection. PSQI questionnaire data were available for 66 women (OC users: 21; Non-user: 45).

Statistics

We used a Welch's t-test for continuous measures and a Fisher's test for categorical parameters to determine if there were any group differences in demographics, cognition, psychometrics, and hormone levels. Our main analysis was the association between AUCi CAR and OC use. As secondary cortisol analyses, we examined (1) differences in cortisol at wake-up (wake-up cortisol) and evening cortisol values between OC users and non-users. (2) In a subgroup of 49 women (OC user: 15; non-user: 34), we investigated if there was an estradiol-BY-OC use interaction effect on CAR. (3) We further tested an association between CAR and hormonal IUD (IUD-users: n=14; non-users: n=53). All CAR and cortisol analyses were tested in generalized least squares models and

adjusted for age, BMI (which differed between OC users and non-users), and work day status (work/study day *vs.* rest day) as, in the earlier studies, anticipation of a working day has been shown to be associated with an enhanced CAR (18).

Follow-up sensitivity analyses were performed to evaluate the robustness of identified significant associations. Alternative models were tested by adjusting for relevant covariates that could potentially affect CAR or the HPA axis according to guidelines (18). We specifically considered smoking and 5-HTTLPR genotype (LA/LA *versus* not-LA/LA) as some studies suggest that they may affect the HPA axis (31, 33, 34). We further examined state covariates proposed by consensus guidelines for the assessment of CAR (18): season (summer *vs.* winter), BMI, TMD, PSQI global sleep quality index, and work day status (work/study day *vs.* rest day) (see **Table 2**). Even though we already tested the effect of work day *vs.* rest day on CAR, we further tested if time at awakening could influence our main model by replacing work up status with awakening time (reported in **Supplementary Material** only). To test if absolute cortisol values at wake-up drove a group difference in AUCi CAR, we also evaluated the effect of adjusting our main analysis for cortisol awakening values. As a second sensitivity analysis, we used the generalized least squares test to account for possible batch effects. We thus accounted for potential random effects in each batch due to variations in technical analyses.

In our main working memory analyses, a generalized least squares test was performed to test for an effect of OC use on working memory [LNS (n=75)/SDMT (n=73)] (see **Figure 2** in supplementary for selection of population for WM analysis). As secondary working memory analyses, we investigated (1) if there was an estradiol-BY-OC use interaction effect on WM (SDMT/LNS) in a subgroup of 52 women (OC user: 15; non-user: 38) (see **Figure 1** in supplementary for selection of population for estradiol analysis). (2) Within the OC user group we examined if plasma estradiol was associated with CAR (n = 15) or WM (n=14). (3) Finally, we tested if a relationship between WM and CAR existed in a generalized least squares test in the manuscript adjusted for age. The analyses were constrained to data points that were collected maximum 14 days apart. All WM models were adjusted for age and education score except the analysis within the OC user group as they all had identical education scores. Since WM is a component of IQ scoring, we chose to adjust for education score rather than IQ (35). See **Supplementary Material** for regression report on WM and OC use association adjusted for parameters differing between the OC user and non-user group. We did not use outlier detection or incorporate outlier exclusion in our analyses. All statistical tests and graphical presentations were performed in R Statistics version 1.2.5001 (R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL (https://www.R-project.org/).

RESULTS

Participants

Demographic, psychometric, cognitive, and endogenous hormonal data of the study population are presented in **Table 1**. The two groups (OC users *vs.* non-users) were similar in mean age, IQ, education score, depressive symptoms (MDI), stress (PSS), and distribution of 5-HTTLPR-genotypes. The groups differed on several parameters even though they all were within the normal range. On sleep quality (PSQI global score), the OC users displayed worse sleep within the last month compared to non-users with a mean above the cutoff score of 5, indicating impaired sleep (p-value=0.09). OC users had worse scores on mood (TMD) compared to non-users (p-value = 0.05). Both groups were clearly within the mentally healthy spectrum as also supported by very low MDI scores. OC users scored higher on education level (p-value= 0.02) and in the SDMT-test (p-value=0.06). As expected, compared to the non-users, OC users had suppressed endogenous estradiol and progesterone levels (estradiol p-value=0.07, progesterone p-value=0.01). OC users had progesterone levels below 5 nmol/L, suggesting anovulation and good compliance to oral contraceptives. Data on estradiol levels were missing for five women, and data on progesterone levels were missing for six women. Most women were within the normal range of BMI (weight in kg/height in m^2), except three who were underweight (BMI <18,5), 11 who were overweight (25–30), and one who was obese (BMI >30). BMI was significantly higher in non-users. The frequencies of work day status (p-value=0.01) and BMI (p-value=0.09) were distributed differently, but the frequencies of 5HTLLPR genotype and smoking were not (p-value>0.39). Most participants reported no use of other medications, two women used mild non-steroid allergy medication in terms of antihistamines, and one of these also had a nasal steroid spray prescribed although she did not use it.

Association Between CAR and OC Use

OC users displayed a significantly diminished AUCi CAR by β= −203 nmol/L*minutes (CI: [−343; −63], p-value= 0.006) relative to non-users, corresponding to a 61% reduction (see **Figure 2** and **Table 2**: model B) in a model adjusted for age, work day status, and BMI. This indicates a blunted CAR in OC users, as illustrated in **Figure 2**. In our secondary analyses, (1) we observed no estradiol-BY-OC use-effect on CAR (β= −564, CI 95% [−3,075;1,946], p-value= 0.66). (2) Within the OC user group, estradiol was not associated with CAR (β= −32, CI 95% [−100;35], p-value= 0.34). Also, OC users had higher cortisol levels at awakening compared to non-users (β= 3.43, CI 95% [0.41;6.46], p-value= 0.03), but absolute evening cortisol did not differ (β= −0.22, CI 95% [−1.34;9.14], p-value= 0.25). See **Figure 3** for unadjusted mean values for each CAR time point in the two groups. (3) Effects from hormonal IUD relative to naturally cycling non-users were not evident on CAR (β= 122, CI 95% [−83;328], p-value= 0.25). When including the hormonal IUD group in the non-user group, the results were largely similar to the results above (when constraining our analyses to compare OC users with natural cycling women) (see **Supplementary Material**).

Follow-up sensitivity analysis showed that BMI contributed to the CAR model (**Table 2**). We further evaluated age, season (winter *vs.* summer), Cohen's PSS, TMD, smoking, sleep quality, and 5-HTTLPR genotype as potential significant covariates for a CAR and OC use association. Those variables did not contribute

TABLE 1 | Demographic, cognitive, psychometric, and hormonal data.

Clinical parameters	OC-user (n = 25)	Non-user (n = 53)	Range	p-values	n
Age	23.6 (2.42)	25.1 (5.21)	18–39	0.09	78
BMI	21.5 (1.7)	23.2 (2.8)	17–32	0.002	78
IQ	109 (6.8)	110 (7.26)	96–126	0.53	78
Education score	4.7 (0.8)	4.1 (1.5)	1–5	0.03	75
LNS	12.5 (2.6)	12.6 (2.6)	6–19	0.94	78
SDMT	68.2 (9.8)	68.2 (10)	44–87	0.06	76
Cohen's PSS	7.0 (5.5)	8.2 (6.4)	0–23	0.39	78
MDI	5.1 (2.9)	5.1 (3.4)	0–15	0.93	76
TMD	-4.2 (11.8)	2.4 (16.1)	–21–58	0.05	76
Sleep quality	5.1 (2.9)	3.8 (2.1)	1–10	0.09	66
P-Estradiol nmol/L	0.12 (0.2)	0.48 (1.4)	0.04–10	0.07	74
P-Progesterone nmol/L	0.99 (0.43)	3.93 (7.74)	0.4–41	0.01	71
Categorical variables	**OC-user (n = 25)**	**Non-user (n = 53)**		**p-values**	
Smoking					
- Non-smokers	96% (n = 24)	83% (n = 44)		0.39	
- Light smokers	4% (n = 1)	7% (n = 4)			
- Intermediate smokers	0% (n = 0)	4% (n = 2)			
- Missing value	0% (n = 0)	6% (n = 3)			
5-HTLLPR genotype					
- LA/LA	28% (n = 7)	23% (n = 12)		0.78	
- Other genotypes	72% (n = 18)	77% (n = 41)			
Day of cortisol saliva samples					
- Work/study day	36% (n = 9)	68% (n = 36)		0.01	
- Rest day	64% (n = 16)	32% (n = 17)			
BMI					
- Underweight (<18)	4% (n = 1)	4% (n = 2)		0.09	
- Normal weight (18–25)	92% (n = 23)	75% (n = 40)			
- Overweight	4% (n = 1)	19% (n = 10)			
- Obese	0% (n = 0)	2% (n = 1)			

*Mean, standard deviation, and range are shown for clinical parameters in each group. The categorical variables are presented showing the distribution of smoking, 5-HTLLPR genotype, whether the cortisol saliva samples were collected on a work, study, or rest day, and BMI. For clinical parameters, statistical differences were calculated with Welch's t-test, and for the categorial variables, differences were calculated with Fisher's test. Sleep quality was assessed with Pittsburgh Sleep Quality Index (PSQI). PSQI global score ranges overall sleep quality from 0 to 21 with higher scores indicating worse sleep. Total mood disturbance (TMD) ranging from 0 to 200 with higher scores indicating mood disturbances. Body mass index (BMI), Letter-Number-Sequence test (LNS), Cohen's Perceived Stress test (Cohen's PSS), Major Depression Inventory (MDI). Light smoker = max 5 cigarettes per day, intermediate smoker = 5–15 cigarettes per day. *For calculation of Fischer's test, we pooled BMI under 25 versus BMI above 25.*

to the reported results (**Table 2**), nor were they correlated with the CAR (p-value> 0.35). As expected, CAR was moderately intercorrelated with absolute cortisol levels at awakening (Pearson correlation = −0.45). Nevertheless, when adjusting our main model for awakening cortisol levels, CAR remained to be associated with OC use at a borderline significant level (β:−122, CI 95% [−245;−2.25], p-value = 0.058).

Cortisol data from six different batches were pooled in the present study (see **Figure 4**). We evaluated possible batch effects by adding to the main CAR model an additive batch effect on the mean and a multiplicative batch effect on the variance. The resulting model was fitted using generalized least square. The difference in batch effect size compared to effects in the main CAR analysis was 16% (Batch analysis β: −260 and CAR analysis β: −203) with similar p-values (p-value=0.007 and p-value=0.006). We saw no evidence for substantial batch effects. Therefore, pooling of the six batches did not drive the outcome of our main analysis.

OC Use and Working Memory

In our main analyses we observed a trend towards better SDMT performance in OC users (β= 3.88, CI 95%: [−1.2; 8.96], p= 0.14).

TABLE 2 | The effect of OC-use on CAR evaluated in generalized least square analyses in alternative models with increasing complexity.

Covariates for adjustment	Effect (nmol/L*minutes)	95% CI	p-value
A. No adjustment	−228	[−354; −103]	<0.001
B. Age, work day status, BMI	−203	[−343; −63]	0.006
C. Age, BMI, Cohen's PSS, 5HTTLPR genotype, TMD, smoking, work day status, season, sleep quality*	−238	[−418; −57]	0.013

*Model B was chosen as our main model. Work day status describes whether cortisol saliva samples were collected on a work/study or rest day. 5-HTTLPR genotype status is defined as LA/LA versus not-LA/LA. Sleep quality was assessed with global score of the Pittsburgh Sleep Quality Index (PSQI). Season, summer versus winter. Cohen's PSS, Cohen's Perceived stress scale; TMD, total mood disturbance. * Model C only includes 63 participants due to missing PSQI data (n=12) and missing TMD data (n=2).*

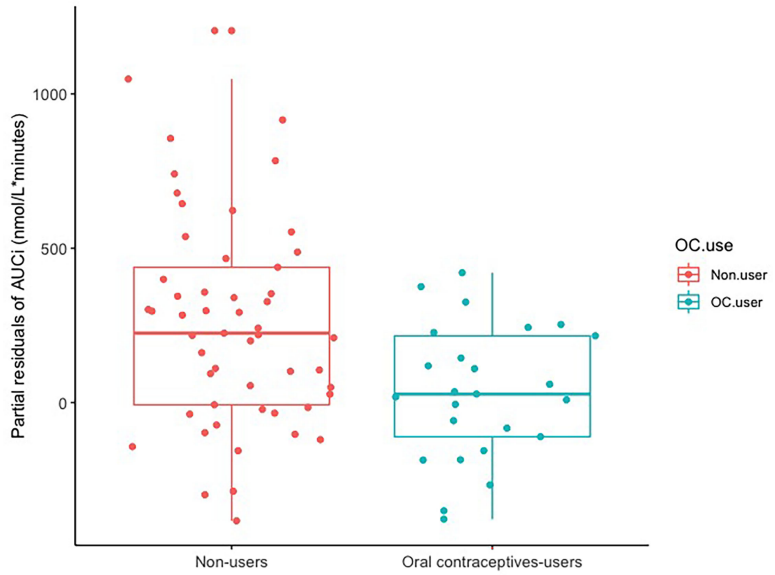

FIGURE 2 | CAR in oral contraceptive users *versus* non-users. Boxplot showing partial residuals of CAR AUCi (nmol/L*minutes) to remove the effect of age, BMI and work day status (work/study day *vs.* rest day). Oral contraceptive users display a significantly reduced AUCi CAR compared to non-users (p-value= 0.006). CAR, cortisol awakening response; AUCi, area under the curve with respects to increase.

In contrast, we found no OC use effect on LNS (β = 0.09, CI 95% [−1.48; 1.3], p-value= 0.9) (see **Figures 5A, B**). In our secondary analyses, (1) we saw no estradiol-BY-OC use-effect on LNS or SDMT. (2) Likewise, in an analysis constrained to the OC user group only, estradiol was not associated with LNS or SDMT. (3) Finally, we found no evidence for an association between CAR and LNS or SDMT. All results from secondary analysis are presented in **Table 3**.

DISCUSSION

We here report the effect of OC use on CAR and WM in a group of healthy premenopausal women. We observed that healthy women who use OCs have blunted HPA axis dynamics in terms of CAR with a 61% reduction, compared to non-users, and at the same time higher absolute levels of cortisol at awakening. Meanwhile, we observed no statistically significant association

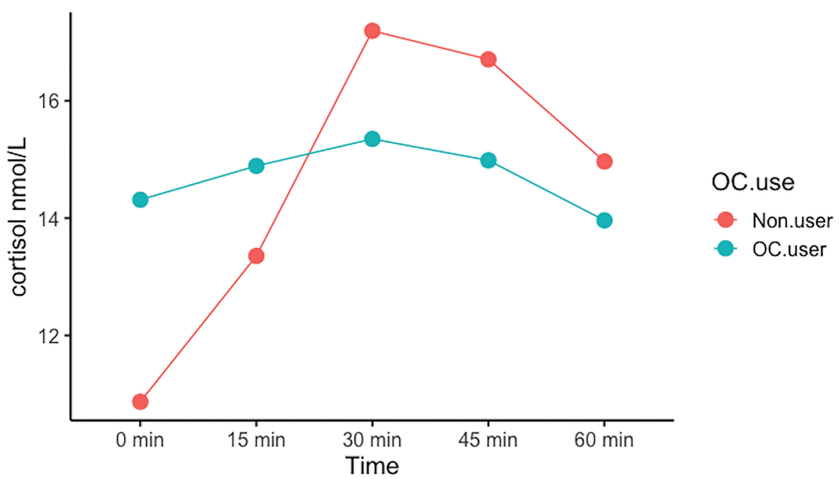

FIGURE 3 | The cortisol awakening response in OC-users and non-users. Mean cortisol values at each time point during the first hour after wake up (0 min) depicting the cortisol awakening response. The 95% confident intervals are presented as the shadowed area surrounding the line.

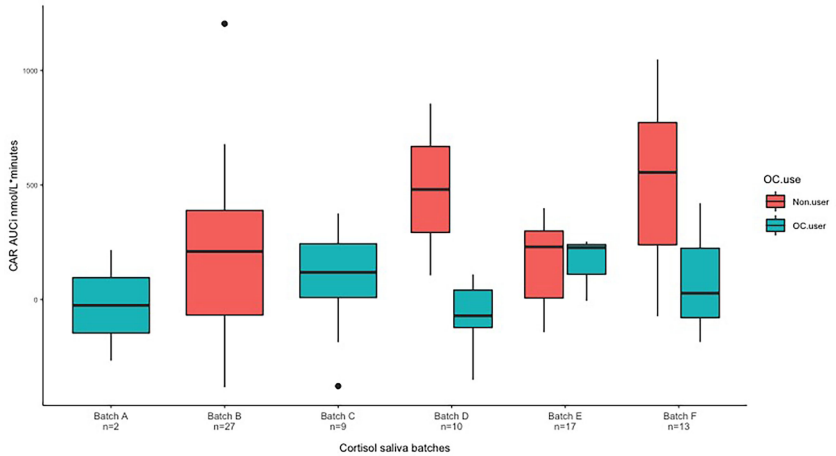

FIGURE 4 | Variance seen by boxplot based on unadjusted observations. We here present data from six different batches. Batch D: non-users n = 2, OC-users n = 8. Batch E: Non-users n = 14, OC-users n = 3. Batch F: non-users n = 10, OC-users n = 3. CAR, cortisol awakening response; AUCi, area under the curve with respect to increase.

between OC use and WM, except for a trend towards better visual WM performance in OC users. Furthermore, no group-dependent effects were observed in the association between estradiol and CAR or WM.

OC Use and HPA Dynamics

As hypothesized, we found that OC users displayed a significantly diminished CAR with a 61% reduction relative to non-users (**Figure 2** and **Table 2**: model B) in a model adjusted for age, work day status, and BMI, consistent with a blunted CAR in OC users, a finding that was robust also when tested in alternative models. Our findings align with prior studies examining CAR and cortisol response to a social stress test in OC users *versus* non-users, which have reported attenuated cortisol responses in OC users (11–13, 36). Taken together with previous studies, our finding indicates that OC users display blunted HPA axis dynamics. Earlier attempts to address CAR differences between OC users and non-users have been inconclusive as they did not adjust for factors later known to clearly affect CAR, such as work day *vs.* rest day, smoking, and sleep quality (10, 17, 18, 23). Another study examining cortisol and state anxiety induced by exam-like stress showed attenuated cortisol response in OC users, but this did not reach statistical significance (37). There might be longer lasting consequences of initiating OCs in early life, i.e., in adolescence when the brain is not fully matured. A recent study found no overall difference between OC users and non-users when exposed to a social stress test, but notably found a reduced response in OC users who had initiated OC prior to puberty relative to in adulthood (20). Also, Bouma et al., 2009, only found a slightly blunted morning cortisol in adolescent females who presumably had used OCs for a short period. Altogether most studies suggest a blunted

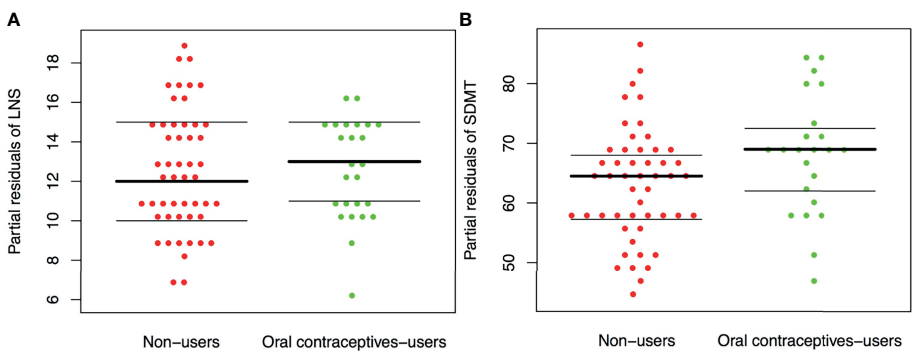

FIGURE 5 | **(A, B)** Working memory in oral contraceptive users *versus* non-users. Boxplot showing partial residuals of working memory test to remove the effect of age and education based on a generalized least square model. Working memory was tested with the Letter-Number-Sequencing (LNS) **(A)** test and the Symbol-Digit-Modalities-test (SDMT) **(B)** in oral contraceptive-users (OC-users) and women using no oral contraceptives (non-users). There was no difference between OC-users and non-users on the LNS (p = 0.9), but a trend towards better performance in OC-users was observed on the SDMT (p = 0.14). WM, working memory; LNS, Letter-number-sequence test; SDMT, Symbol-Digit-Modalities test; OC, oral contraceptive; CAR, cortisol awakening response.

TABLE 3 | Results from secondary working memory analyses.

Secondary WM analysis		Effect	95% CI	P-value	n
(1) Estradiol-BY-OC-use interactions					
	LNS	−4.61	[−27; 18]	0.68	51
	SDMT	0.32	[−78; 78]	0.99	51
(2) Estradiol-WM associations within OC-users					
	LNS	−4.7	[−29; 19]	0.70	15
	SDMT	1.6	[−79; 76]	0.97	15
(3) CAR-WM associations					
	LNS	0.0002	[−0.002; 0.002]	0.88	66
	SDMT	0.0002	[−0.007; 0.007]	0.96	66

WM, working memory; LNS, Letter-number-sequence test; SDMT, Symbol-Digit-Modalities-test; OC, oral contraceptive; CAR, cortisol awakening response.

cortisol response in OC users, which aligns with our finding. This supports that OC use compromises cortisol responsiveness across a range of stimuli of the HPA-axis.

Our secondary analyses were less conclusive: (1) We did not find an estradiol-BY-OC user group effect on CAR and plasma estradiol, and plasma estradiol was not associated with CAR within the OC user group. We speculate this finding may reflect that long-term suppression of sex steroids in OC users is affecting HPA dynamics, in contrast to natural dynamics of estradiol in non-users, independent on the actual levels of estradiol. The estradiol levels in the non-user group were lower than might be expected for natural cycling women, which is most likely because many were recruited in the early follicular phase (26 out of 53 non-user women), which was a requirement in certain studies some of the participants were recruited for. Kirschbaum et al. (1999) only found a CAR difference between oral contraceptive women and normal cycling women in luteal phase but not the follicular phase (17). Thus, this points to a difference across all phases, and one might speculate if our results had been even more evident if we had only included women from the luteal phase. However, as the estradiol sample size was small, these results should be interpreted with caution. (2) As an index for absolute cortisol levels, we examined wake-up cortisol values, which were significantly higher in OC users, and evening cortisol, which did not differ between OC users and non-users. However, there is currently no consensus in terms of absolute levels as both amplified, reduced, and unaltered levels have all been reported (11–13, 36, 38). Interestingly, even after adjusting our main analysis for cortisol awakening values, we continued to see a trend (p=0.058) towards a blunted CAR in OC users. Awakening time (see **Supplementary**) and sleep quality did not affect the association between CAR and OC use. Thus, several properties (circadian and superimposition) of the HPA-axis may well be affected by OC use. As the HPA axis contributes with generating diurnal rhythm, a high wake-up value may also reflect disturbed HPA axis dynamics. Also, it is presumably difficult to generate a forceful CAR if the wake-up value is already high. Absolute evening values did not differ between OC users and non-users. We believe this supports that OC use disturbs the dynamics rather than the absolute cortisol levels per se.

Central mechanism may be involved in the association between OC use and CAR as sex steroid receptors are pervasively expressed in key parts of the neural circuitry controlling the HPA axis, amongst these hippocampus, allowing sex steroids to modify the neuroendocrine response to stress (15). Other factors may also play a role such as changes in the levels of cortisol binding globulin in OC users (13, 16), which ostensibly would decrease the free fraction of cortisol that pass to saliva. However, this does not align with our observation of increased cortisol levels at awakening in OC users.

CAR has been suggested to be a potential marker of hippocampal function where the magnitude of CAR is positively related to hippocampal volume (39), as a well-functioning hippocampus may be necessary for CAR to occur (39). Further, the hippocampus expresses high levels of estrogen receptors and shows great plasticity, which covaries with estrogen (40). Thus, the OC-induced attenuation of CAR could potentially be mediated by a hippocampal pathway. Along this line, Clow et al. (2010) (41) suggested that the hippocampus may play a role in the regulation of CAR prior to awakening. During sleep, the hippocampus inhibits cortisol secretion, and it is particularly active during REM sleep, which is dominant in later stages of sleeping and immediately pre-awakening. At awakening, hippocampal activation switches off (41). This might add to explain the increased awakening cortisol levels and impaired quality of sleep we observed in OC users. The hippocampus also appears to play an important role in the development of depression (42), and hippocampal volume has been linked to OC use (43). Future studies may illuminate if hippocampus volumes are related to OC use in a manner coupled to HPA-axis outputs and dynamics or cognitive performance.

In summary, our findings are in line with the notion that OC-induced suppression of endogenous sex steroids can compromise HPA axis dynamics as demonstrated by a blunted CAR and a change in diurnal features, i.e., cortisol levels at awakening. The effect of initiating OCs at different brain maturation states, especially in adolescence, and the reversibility after cessation of OCs should be investigated in future studies.

OC Use, Estradiol, and Working Memory

Existing literature consistently demonstrates that sex steroids, in particular estradiol, affect WM in natural cycling, pregnant, and menopausal women (24, 26, 27). Contrary to our expectation, we did not find a significant association between OC use and WM. Also, we did not find an estradiol-by-OC use interaction effect on WM, and the plasma estradiol concentration was not associated with WM within the OC user group. However, a non-significant trend towards better SDMT performance in OC users was

observed, but this might be a spurious finding. Notably, previous studies reporting an association between sex steroids and WM function used visuo-spatial WM tasks including spatial WM, paragraph recall, and mental rotation (24, 26, 27). This could help explain why we observed a trend-like improvement in performance for OC users on the visual SDMT task but not the verbal LNS task. It should also be noted that the SDMT task does not exclusively assess WM functioning, but it is also commonly used to index processing speed (44); the observed trend could therefore reflect changes in processing speed instead of WM function. In addition, the LNS task design makes it particularly challenging and mentally taxing for the participant. Decreased perseverance in hormonal contraceptive users has been linked to worse performance on both simple and challenging tasks (45), and we speculate if this may be related to a less sensitive HPA axis, which would lower the capacity for OC users to mobilize extra cognitive resources. We also speculate if the combination of reduced perseverance and the more challenging auditive nature of the LNS task may explain why we did not see a similar trend with LNS as with the less challenging SDMT. In summary, we find no evidence for impaired WM in OC users, and the trend-like effect observed might be spurious. However, our sample size was modest with regard to detecting moderate to smaller effects on cognition, and we cannot exclude that such effects could be detected in larger sample sizes. Furthermore, effects on different cognitive domains need to be disentangled.

OC Use and Risk for Depression

Our results suggest that OC users are not able to mobilize an efficient HPA axis response to a daily stimulus as the transition from sleep to awake. This may also imply that OC users have a limited biological capacity to handle stress, which can add as a risk factor for mood disorders. Interestingly, we also observed that OC users scored higher on mood (TMD) and displayed worse sleep relative to non-users, perhaps suggesting subclinical effects correlate to a less dynamic HPA axis function in OC users. Frokjaer et al. (2015) demonstrated that gonadotropin-releasing hormone-agonist (GnRHa)-induced suppression of endogenous sex steroids in healthy women triggers subclinical depressive symptoms (46). This points to possible subclinical effects from ovarian sex steroid suppression in otherwise healthy individuals. Several large epidemiological studies have suggested an association between OCs and depression, especially among adolescents (3, 4, 6). It is also important to emphasize that the majority of women using OCs tolerate them well and do not experience adverse effects. However, a particular subgroup of vulnerable or hormone-sensitive individuals could be at risk of developing depressive episodes when exposed to hormonal transitions. Potential estrogen-sensitive transcripts predicting hormone-induced mood changes have been identified; however, it is unknown if these markers of estrogen sensitivity translates to OC use or other, naturally occurring, hormonal transitions (47). Future work should consider intervention studies to illuminate potential causal links between use of hormonal contraceptives, changes in HPA axis dynamics and potential emergence of depressive symptoms.

Methodological Considerations and Limitations

Our study has important strengths and limitations that should be considered when interpreting our findings. A main strength is that we have detailed CAR measures in a larger sample compared to earlier studies (Kirschbaum et al., 1995: n=59; and Kirschbaum et al., 1999: n=61) and adjusted for relevant covariates. Furthermore, our sample comprised healthy women exposed to an everyday HPA axis stimulus, i.e., the natural transition from sleep to awakening. This allowed us to investigate HPA-axis dynamics in the absence of psychosocial stress. As a limitation, first, a possible "healthy user" bias might exist in our sample since (a) the women included in our study did not develop depression after starting OCs, and (b) they did not experience any mood deteriorations extensive enough to terminate the use of OCs. Therefore, we cannot exclude that the association between OC use and CAR might be stronger in high-risk or patient groups. Second, the cortisol measures in our study were determined by two different methods due to changes in the hospital laboratory across the data collection period. Although we observed no batch effects and the frequency of data points determined with the two methods were equally distributed between OC user and no-user groups, this may have added larger variation in our data, which in terms may have reduced our power to detect a difference between groups. Third, there were some limitations regarding menstrual cycle phase data and estradiol: (a) Information on menstrual phase were not available for all naturally cycling women in the non-user group, which limits our ability to control for menstrual phase effects. (b) Ideally, plasma estradiol should have been collected the same day as the cortisol saliva samples and not up to 3 days later. (c) The analyses with estradiol had small sample sizes. (d) Time of day for the estradiol measure was not standardized. Since subtle diurnal fluctuations cannot be excluded, one may speculate if it has added variation to our data.

Fourth, we did not have data on which kind of oral contraceptive was used by the women nor if the women collected their cortisol samples on an active or an inactive pill day. Finally, causality cannot be inferred because of the cross-sectional nature of the study.

Consistent with our hypothesis, we observed that women who use oral contraceptives have blunted cortisol dynamics relative to non-users. OC use did not appear to be coupled to WM performance relative to non-users; however, a trend towards better WM performance on the SDMT was observed in OC users. We speculate that the observed effects on the HPA axis may lead to an inadequate response to stress that in an adverse environment may contribute as a risk factor for depression in sensitive individuals.

DATA AVAILABILITY STATEMENT

Due to the General Data Protection Regulation, the data that support the findings of this study are not readily available. Data in the Cimbi database can be accessed by application (http://www.cimbi.dk/db).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Capital Region of Denmark, Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EH: Data curation, formal analysis, investigation, methodology, project administration, software, visualization, validation, writing—original draft. CB: Validation, writing—review and editing. VD: writing—review and editing. AN: Resources, writing—review and editing; NJ: Resources, writing—review and editing; BO: Formal analysis, software, data curation, supervision, writing—review and editing. DS: Supervision, writing—review and editing. VF: Conceptualization, funding acquisition, resources, investigation, methodology, validation, writing—original draft, supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.731994/full#supplementary-material

Supplementary Figure 1 | Selection of participants for estradiol interaction analysis.

Supplementary Figure 2 | Selection of participants for working memory analysis.

Supplementary Table 1 | Main analysis including hormonal IUD users in non-user group. When including hormonal IUD users in the non-user group, we found similar results as in our main analysis.

Supplementary Table 2 | Main analysis adjusting for awakening time instead of work day status. Replacing work day status with awakening time did not change our results as awakening time did not contribute to the model (p-value = 0.79).

Supplementary Table 3 | CAR regression report for (A) Main model, (B) Model including all covariates in CAR analysis.

Supplementary Table 4 | Regression report for main WM analyses including potential covariates: **(A)** LNS, **(B)** SDMT. **(A)** Analysis includes data from 64 women as information on PSQI was not available on all women. **(B)** Analysis includes data from 62 women as information on PSQI was not available on all women.

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Event-Related Potentials in Women on the Pill: Neural Correlates of Positive and Erotic Stimulus Processing in Oral Contraceptive Users

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Background/Aims: Exposure toward positive emotional cues with – and without – reproductive significance plays a crucial role in daily life and regarding well-being as well as mental health. While possible adverse effects of oral contraceptive (OC) use on female mental and sexual health are widely discussed, neural processing of positive emotional stimuli has not been systematically investigated in association with OC use. Considering reported effects on mood, well-being and sexual function, and proposed associations with depression, it was hypothesized that OC users showed reduced neural reactivity toward positive and erotic emotional stimuli during early as well as later stages of emotional processing and also rated these stimuli as less pleasant and less arousing compared to naturally cycling (NC) women.

Method: Sixty-two female subjects (29 NC and 33 OC) were assessed at three time points across the natural menstrual cycle and corresponding time points of the OC regimen. Early (early posterior negativity, EPN) and late (late positive potential, LPP) event-related potentials in reaction to positive, erotic and neutral stimuli were collected during an Emotional Picture Stroop Paradigm (EPSP). At each appointment, subjects provided saliva samples for analysis of gonadal steroid concentration. Valence and arousal ratings were collected at the last appointment.

Results: Oral contraceptive users had significantly lower endogenous estradiol and progesterone concentrations compared to NC women. No significant group differences in either subjective stimulus evaluations or neural reactivity toward positive and erotic emotional stimuli were observed. For the OC group, LPP amplitudes in reaction to erotic vs. neutral pictures differed significantly between measurement times across the OC regimen.

Discussion: In this study, no evidence regarding alterations of neural reactivity toward positive and erotic stimuli in OC users compared to NC was found. Possible confounding factors and lines for future research are elaborated and discussed.

Keywords: oral contraceptives (OCs), event-related potentials (ERP), neural reactivity, emotional processing, early posterior negativity (EPN), late positive potential (LPP), gonadal steroids, subjective stimulus evaluations

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INTRODUCTION

The majority of previous research regarding effects of oral contraception (OC) has been focusing on its physiological aspects (Frye, 2006) and side effects like cancer risk (Gierisch et al., 2013), or thromboembolic events (Oedingen et al., 2018). Psychophysiological aspects of OC use have been studied less systematically (Pletzer and Kerschbaum, 2014). OCs might, however, be shaping mind and behavior via their influence on the endocrine as well as the central nervous system. So called combined OCs (COCs) are the most commonly used ones and contain 20–35 μg ethinyl-estradiol (EE) and varying progestin components (Dhont, 2010; Liang et al., 2012). These synthetic steroids unfold negative feedback effects on the hypothalamus-pituitary-gonadal (HPG-) axis. During intake they consequently reduce serum levels and cyclical fluctuations of neurosteroid precursors (i.e., progesterone, pregnenolone, EE, and testosterone) as well as neurosteroids (i.e., allopregnanolone and allotetrahydrodeoxycorticosterone) (Rapkin et al., 2006; Zimmerman et al., 2014). Levels of endogenous gonadal steroids also stay suppressed during the 7 days OC break, even though some residual ovarian activity can occur (van Heusden and Fauser, 1999). Neurosteroids impact activity as well as organization of the nervous system due to their influence on synaptic transmission, myelination, apoptosis, and dendritic spine plasticity via genomic and non-genomic pathways (Del Río et al., 2018). Accordingly, neuroanatomical alterations regarding gray and white matter density and/or volume have been reported in OC users – typically in areas of the limbic system (Brønnick et al., 2020; Taylor et al., 2021) which is known for its key role in emotional processing (Frühholz et al., 2014). Using structural magnetic resonance imaging (MRI) data, Hertel et al. (2017) observed lower hippocampal volume and hypothalamic-pituitary-adrenal (HPA-) axis alterations (i.e., elevated baseline cortisol levels) consistent with chronic stress in OC users. Lisofsky et al. (2016) reported reduced gray matter volume in OC users' left amygdala and anterior parahippocampal gyrus. In contrast, Sharma et al. (2020b) reported greater white matter volume in OC users' right amygdala, left parahippocampal gyrus and left hippocampus while not observing any gray matter differences in these regions. Regarding brain connectivity, Petersen et al. (2014, 2015) reported reduced cortical thickness in parts of the default and salience network in OC users and altered resting state functional connectivity in the default and executive control network, which are involved in reward processing and evaluation of internal and external stimuli. Correspondingly, differences between OC users and naturally cycling (NC) women have been reported in a variety of psychophysiological functions, including stress responsivity, fear conditioning, cognition and socio-emotional behaviors such as emotional processing (Warren et al., 2014; Montoya and Bos, 2017; Lewis et al., 2019).

Due to previously reported associations of OC use and depression (Skovlund et al., 2017; Wit et al., 2020) as well as a reduction of general well-being and its subdomains positive well-being, self-control and vitality (Zethraeus et al., 2017), most of the existing neuropsychological OC research has been investigating processing of negative stimuli (Brønnick et al., 2020), or adverse mood effects (Schaffir et al., 2016). Using functional magnetic resonance imaging (fMRI), Petersen and Cahill (2015) reported reduced bilateral amygdala reactivity toward negative visual stimuli in OC users. Gingnell et al. (2013) additionally collected daily symptom ratings and observed lower emotional reactivity toward negative facial expressions in OC users in the left insula, left middle frontal gyrus and the bilateral inferior frontal gyri along adverse mood effects such as depressed mood, mood swings and fatigue after one cycle of OC treatment in a placebo controlled, double-blind randomized controlled trial (RCT) (Gingnell et al., 2013). While the authors studied women who had previously experienced negative mood symptoms during OC intake (Gingnell et al., 2013), results on general mood effects of OC use are still inconclusive (Schaffir et al., 2016). Some studies report mood worsening – especially during the hormone-free phase (Lundin et al., 2017). Others indicate beneficial effects including mood improvement, higher mood stability and a decrease in negative premenstrual affect (Oinonen and Mazmanian, 2002; Jarva and Oinonen, 2007; Hamstra et al., 2017). In contrast, positive emotional states and processing of positive stimuli under OC use are examined only very rarely. These factors are, however, highly relevant for well-being and mental health (Alexander et al., 2021). Regarding depression, numerous studies reported a reduction of positive affect (Gençöz, 2002; Wichers et al., 2007; Horner et al., 2014) and stressed the importance of targeting low positive affect specifically in anti-depressive treatments (Nutt et al., 2007; Werner-Seidler et al., 2013; Jonge et al., 2017). Depressive subjects also show reduced neural reactivity toward positive emotional stimuli which might explain the occurrence of depressive symptoms, such as loss of interest and anhedonia (Shestyuk et al., 2005; Epstein et al., 2006). In one of the few studies on this topic regarding OC use, Jarva and Oinonen (2007) observed blunted self-reported emotional reactivity following positive mood inductions while emotional reactivity following negative mood inductions was unchanged. The authors argued that OC's mood stabilizing effect, accompanied by reduced positive emotional reactivity, might resemble depressive states reported by some users. Further research in this field is highly necessary, especially since perceived adverse influences of OCs on mental health are a common reason for discontinuation of their use (Sanders et al., 2001). The same is true for deteriorations in *female sexual function* (Sanders et al., 2001) which refers to the entirety of psychological and physiological aspects of the female sexual response (Basson, 2008). Some authors argue that OCs should have positive effects on female sexuality as their conception-suppressing effect ensures greater sexual freedom and reduces pregnancy-related anxieties during sexual intercourse (Burrows et al., 2012). Others have reported lower female sexual function scores in users of oral vs. other forms of contraception (Wallwiener et al., 2010, 2015), or reduced scores in its subdomains including arousal, pleasure, orgasm and lubrication (Smith et al., 2014). Results are so far inconclusive and differ greatly with respect to study design (Davis and Castaño, 2004; Pastor et al., 2013). Still, RCTs and meta-analytic reviews suggest that the female sexual function subdomain *desire* might be affected by OC use (Zethraeus et al., 2016; Huang et al., 2020). Altered processing of erotic stimuli

has been reported in females suffering from hyposexual desire disorder (Bianchi-Demicheli et al., 2011; Woodard et al., 2013). Regarding OC use, Abler et al. (2013) compared different stages of erotic stimulus processing in 12 OC users and 12 NC women. They reported reduced brain activity during anticipation of erotic stimuli. During viewing of erotic pictures and videos, no consistent significant differences were observed between both groups. OC and NC subjects did also not differ in arousal ratings of erotic stimuli. While this is – to be best of our knowledge – the only study that explicitly compared erotic stimulus processing in NC and OC women, erotic stimulus processing has more often been examined in association with the natural menstrual cycle (MC) using fMRI (Gizewski et al., 2006; Zhu et al., 2010) as well as event-related potential (ERP) techniques (Krug et al., 2000; Munk et al., 2018, 2020).

The use of ERPs has important advantages because their high temporal resolution allows to assess immediate vs. prolonged neural reactions associated with attending to and processing of motivationally salient stimuli (Brown et al., 2013). Compared to commonly conducted (f)MRI investigations, ERP studies also have fewer exclusion criteria (e.g., dental braces, retainers, bone plates and screws, implants) (McLoughlin et al., 2014) allowing greater and more integrative samples. In neuroendocrine research, which is already characterized by numerous exclusion criteria (i.e., absence of any physical or psychological illness, no medication intake, regular menstrual cycles), this is a huge benefit regarding representativeness and generalizability of results. Most ERP studies regarding MC effects focused on the late positive potential (LPP) – an EPR component most prominent on centro-parietal electrode sites. It starts around 400 ms after stimulus onset and can last for several hundred ms. The LPP is sensitive to emotional content with typically higher amplitudes toward emotional vs. neutral stimuli (Schupp et al., 2004, 2006; Hajcak and Olvet, 2008). It is therefore suggested to reflect prolonged and facilitated attention toward motivationally salient emotional stimuli and their processing (Foti et al., 2009; Hajcak et al., 2012). Regarding the MC, Krug et al. (2000) observed higher LPP amplitudes toward erotic pictures around ovulation when these stimuli are highly relevant for fertility and reproduction. Similarly, Munk et al. (2018, 2020) reported that LPP amplitudes toward erotic vs. neutral stimuli were associated with estradiol concentration which typically peaks during ovulation. As OCs suppress ovulation and inhibit conception, they might also reduce motivational salience of erotic stimuli. Furthermore, the LPP has been associated with activity of the dopaminergic reward system (Cuthbert et al., 2000; Munk et al., 2016). Activity of this system is modulated by estradiol and testosterone, both of which are downregulated by OC intake (Rapkin et al., 2006; Zimmerman et al., 2014; Montoya and Bos, 2017). Recently, another ERP component has been suggested to be especially sensitive to erotic content– the early posterior negativity (EPN) (Frank and Sabatinelli, 2019; Farkas et al., 2020). The EPN is defined as a relative negativity at temporo-occipital electrode sites that is also sensitive to emotional content but occurs earlier than the LPP at approximately 150–300 ms after stimulus onset (Schupp et al., 2004, 2006; Hajcak et al., 2012). It is therefore associated with earlier and broader stimulus distinction compared to the LPP (Weinberg and Hajcak, 2010; Farkas et al., 2020). To the best of our knowledge, no study so far assessed the EPN in association with MC or OC effects. However, gender differences have been observed with reduced EPN modulation in women vs. men (Weinberg and Hajcak, 2010), indicating some involvement of sex steroids. Furthermore, Farkas et al. (2020) observed greater EPN negativity in reaction to erotic and nudist scenes compared to other pleasant and unpleasant visual stimuli and interpreted this as a marker of heightened attention for sexual opportunities – a process that might be modulated by OC use.

To differentiate between earlier vs. later stages of emotional processing might be relevant in association with OC use, as a recent ERP study provided initial evidence for differential effects of OC use at different processing stages (Monciunskaite et al., 2019). In this only ERP study on the effects of OCs to date, the authors used a passive viewing task to examine processing of (highly) unpleasant and pleasant (i.e., erotic) as well as neutral social visual stimuli derived from the International Affective Picture System (IAPS). Monciunskaite et al. (2019) reported significantly lower LPP amplitudes and global field power (GFP) in OC users compared to NC subjects in reaction to all picture categories. When comparing emotional-neutral difference scores in the LPP, however, the only significant finding was an attenuated unpleasant-neutral difference in OC users. Furthermore, OC users showed higher GFP (trend level) and significantly higher parieto-occipital activity compared to NC women in an earlier time window (<350 ms), pointing toward differential effects of OC use on early vs. later stages of emotional processing (Monciunskaite et al., 2019). An effect of emotion regulation strategies such as reappraisal on neural reactivity was discussed, yet, no subjective stimulus evaluations were assessed to support this hypothesis (Monciunskaite et al., 2019). Previous results on subjective stimulus evaluations in OC users have revealed inconsistent results. Whereas some studies reported no differences regarding positive, neutral, negative (Armbruster et al., 2017) nor erotic (Abler et al., 2013) stimuli, others reported higher valence ratings in reaction to emotional- and lower arousal ratings in reaction to neutral stimuli in users of different forms of hormonal contraceptives (HCs) (Spalek et al., 2019).

Another important limitation of Monciunskaite et al.'s (2019) – and also other studies on OC use – is that OC users were assessed only once, namely during the active period of the OC regimen. Yet, most adverse effects of OC use are being reported during the 7 days break (Sulak, 2000; Kelly et al., 2010). Studies that included measurements during the OC free week reported differences in brain connectivity and emotional processing between active and OC free periods. Nasseri et al. (2020) noticed higher amygdala-ventromedial prefrontal cortex (vmPFC) coupling in the left hemisphere after stress exposure during the active vs. OC free period and interpreted this in terms of better emotion regulation abilities during OC intake. Similarly, Radke and Derntl (2016) reported higher accuracy in an affective responsiveness task during the active vs. OC free period of the regimen. As Herrera et al. (2020) point out, consideration of OC regimen is also important to disentangle effects associated with either high exogenous hormone intake or reduced endogenous hormone production.

Therefore, the current study examined early vs. prolonged stages of positive and erotic stimulus processing in OC users across different time points of the OC regimen. A within-subjects repeated-measurements design was chosen in order to overcome limitations of frequently used between-subjects designs, in which groups of women in different phases of the OC regimen/MC have been compared. The most important aim was to elucidate overall differences between OC using and NC women as well as OC regimen – rather than MC related – effects on emotional processing.

Regarding subjective stimulus evaluations as well as neural reactivity toward emotional stimuli, following hypotheses were investigated:

(1) Higher valence and arousal ratings as well as EPN modulation and LPP amplitudes in reaction to emotional vs. neutral stimuli (Manipulation Check).
(2) Lower valence and arousal ratings as well as EPN modulation and LPP amplitudes in OC users.
(3) A modulation of ERPs by OC regimen period (active vs. OC free).

MATERIALS AND METHODS

Participants

Women were recruited at the University of Giessen via circular emails and flyers and screened for fulfillment of inclusion criteria using a telephone-based interview, which was conducted after subjects were found eligible using a self-designed online screening questionnaire. Inclusion criteria were: age between 18 and 35 years, nulliparous, absence of any physical or psychological illness, no intake of medication or drugs (incl. tobacco) influencing the central nervous- or endocrine system, right-handedness, body-mass index (BMI) $\geq 18 \leq 26$ kg/m^2 and normal or corrected-to-normal vision. Parous women were excluded as several studies indicate endocrine differences between nulliparous and parous women (e.g., Bernstein et al., 1985; Hill et al., 1986; Musey et al., 1987; Barrett et al., 2014). Left-handed subjects were excluded to avoid lateralization effects (Sainburg, 2014). 87 native German-speaking females started participation in the study. Testing could not be completed with 21 women (N = 15: contact restrictions during the coronavirus pandemic; N = 3: no detectable ovulation, N = 3: difficulties with scheduling), leaving a sample of 66 (30 NC and 36 OC) women. NC women reported a regular MC duration of 26–30 days (mean ± SD: 27.97 ± 1.22). 22 (73%) stated to have previously used hormonal contraceptives. While a hormone-free interval of at least 6 months was required for participation, mean time since discontinuation was 49.14 months (SD = 30.99, range: 7–177 months). The OC group consisted of women using combined monophasic OCs with an EE-dosage <50 μg for at least 6 months. An OC regimen of 21/7 (21 days OCs and 7 days OC free) was required for participation. Mean duration of OC intake was 58.18 months (SD = 31.12). 25 subjects used OCs that contained androgenic progestins; eleven subjects used OCs containing anti-androgenic progestins. Androgenicity of progestin components was classified according to Wiegratz and Thaler (2011). One subject in the OC group was excluded from all further analyses as she reported – after completion of the study – that she was using an OC ("Zoely") which contains estradiol instead of EE and is taken in a 24/4 regimen. An overview of EE-dosages and progestin components can be found in the **Supplementary Material**.

Subjects participated in exchange for a monetary compensation of 10€/h or research participation credit. Written informed consent was obtained. The study was conducted in accordance with the declaration of Helsinki and was approved by the local ethics committee of the University of Giessen, Department of Psychology (application number: 2018-0022).

Study Design

This study was conducted as a counter-balanced repeated-measurements design. Each woman attended three testing sessions across one or two consecutive MCs or OC regimen. Females in the OC group were tested on days 7–9 (mean ± SD: 7.97 ± 0.82) and days 14–16 (mean ± SD: 15.03 ± 0.79) of their OC blister (measurement times AP1 and AP2, respectively) as well as during their OC free week (measurement time IP), i.e., days 24–26 after starting a new OC blister (mean ± SD: 24.91 ± 0.70). Measurements during the OC free week corresponded to the first 2 days of the withdrawal bleeding to ensure that women were in a hormone-withdrawal state. Testing dates of the NC group were adapted to the women's average MC length. Measurements during follicular phase (FO) were conducted on days 7–9 (mean ± SD: 7.78 ± 0.63). Measurements during luteal phase (LU) were terminated according to the following formula: average MC length – 2/3 days. Consequently, they were conducted on days 23–28 (mean ± SD: 25.57 ± 1.25). For a correct termination of measurements during ovulation (OV), NC women were provided with LH-tests (Femometer LH Ovulation Rapid Test Strip; sensitivity 25 miu/ml; Hangzhou Clongene Biotech Co., Ltd.; Hong Kong) that they were supposed to use for seven consecutive days around their predicted ovulation. After announcing a positive LH-test result, subjects were assessed within 24 h (mean ± SD: 8.79 ± 7.03). OV measurements were conducted between days 10–20 (mean ± SD: 14.90 ± 2.44). To avoid sequence effects, phase of first measurement was counter-balanced. 11/16 women started during FO/AP1, 7/14 subjects during OV/AP2 and 12/5 subjects during LU/IP. It was aimed to schedule all three testing appointments at approximately the same time of the day (morning, noon, and afternoon) to account for the circadian rhythmicity in gonadal steroid secretion. To ensure similar between-session intervals for the NC and OC group, OC users were tested twice during their active OC period with the second time point corresponding to ovulation in NC women. Testing dates for both groups are illustrated in **Figure 1**.

Emotional Picture Stroop Paradigm

To assess neural reactivity toward emotional stimuli, an Emotional Picture Stroop Paradigm (EPSP) was conducted. EPSPs were previously successfully used as an implicit measurement tool regarding sexual interest

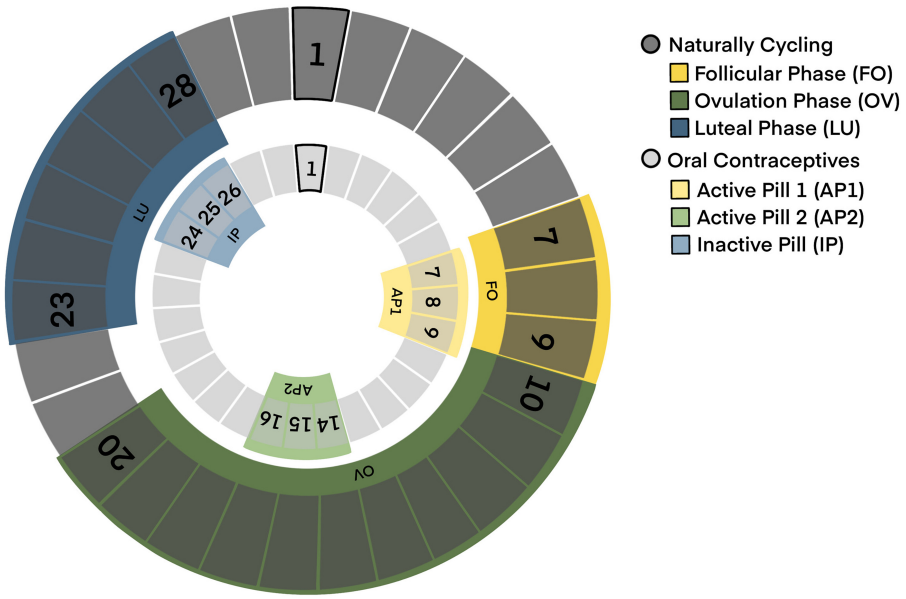

FIGURE 1 | Testing dates for naturally cycling and oral contraceptive using women.

(Ciardha and Gormley, 2009; Munk et al., 2020). The combination of EPSPs and ERP collection is, moreover, advantageous in assessing selective attention and has proven as a valid instrument in emotion research (Thomas et al., 2007; Bertsch et al., 2009; Franken et al., 2009). Based on previous research (Munk et al., 2020), three main categories were included: erotic stimuli included couples in underwear and erotic poses (couple erotic) as well as lightly dressed males (male erotic) and females (female erotic). Positive stimuli included single smiling individuals of both sexes; neutral stimuli consisted of couples (couple neutral) and single individuals (person neutral) in neutral poses and with neutral facial expressions, and trees as a non-social neutral stimulus category. In order to validate picture selection regarding valence and arousal, an online pilot-study in n = 134 women (mean age ± SD: 25.57 ± 8.99) had been conducted before. Women rated 84 preselected pictures (12 per subcategory) on valence and arousal. Pictures were retrieved from www.shutterstock.com and consecutively gray-scaled. Eight pictures per subcategory were chosen for the main study based on the following criteria: High valence as well as arousal ratings for each of the erotic subcategories, high valence and medium arousal ratings for the positive category and medium valence along low arousal ratings for the neutral categories. Higher arousal ratings were obtained for the erotic vs. positive and both emotional vs. the neutral category. Valence ratings were higher for the positive vs. erotic and both emotional vs. the neutral category.

During the EPSP, gray-scaled pictures were presented in a size of 640 pixels × 480 pixels on a black background. Subjects were seated in a comfortable chair in a dimly lit room at a 60 cm distance to the screen of 24″. They were instructed to indicate the picture's frame color (blue, green, red, and yellow) by pressing the respective button as fast and as accurately as possible on a response pad (MilliKey^{TM} MH-5; Lab Hackers Research Equipment, Halifax, Canada). Picture presentation lasted until subjects executed a response. An inter-trial interval (white fixation cross) with a random duration of 1,000–1,500 ms (mean 1,250) was presented between trials (**Figure 2**). Each picture was presented twice in each frame color resulting in 64 trials per subcategory and a total of 448 trials divided into four blocks of 112 trials each. Order of trials was randomized. Between blocks, a 30 s break was included. To familiarize participants with color-button arrangements and thereby reducing eye-movements during the task, subjects were run on 40 practice trials with colored squares before block one and on an additional 12 practice trials before block two to four, respectively. Stimulus presentation and response recording were controlled by Presentation Software 21.1 (Neurobehavioral Systems Inc., Albany, CA, United States) run on a Pentium (Intel Corp., Santa Clara, CA, United States)-based personal computer. Depending on the subjects' speed, the EPSP took approximately 20 min (including breaks and practice trials).

Event-Related Potentials Recording and Quantification

Throughout the EPSP, EEG was continuously recorded from 64 active (Ag/AgCl) electrodes mounted in an elastic cap (actiCap snap, Easycap GmbH, Herrsching, Germany). Electrode sites were re-referenced online to FCz. Scalp impedances were kept below 20 kΩ. Signals were recorded using Brain Vision Recording Software (Version 1.22.0101) and a BrainAmp DC amplifier (both Brain Products GmbH, Gilching, Germany) with a sampling rate of 500 Hz and a band-pass filter between 0.1 and 80 Hz. Brain Vision Analyzer software 2.2.0 (Brain Products GmbH, Gilching, Germany) was used for offline

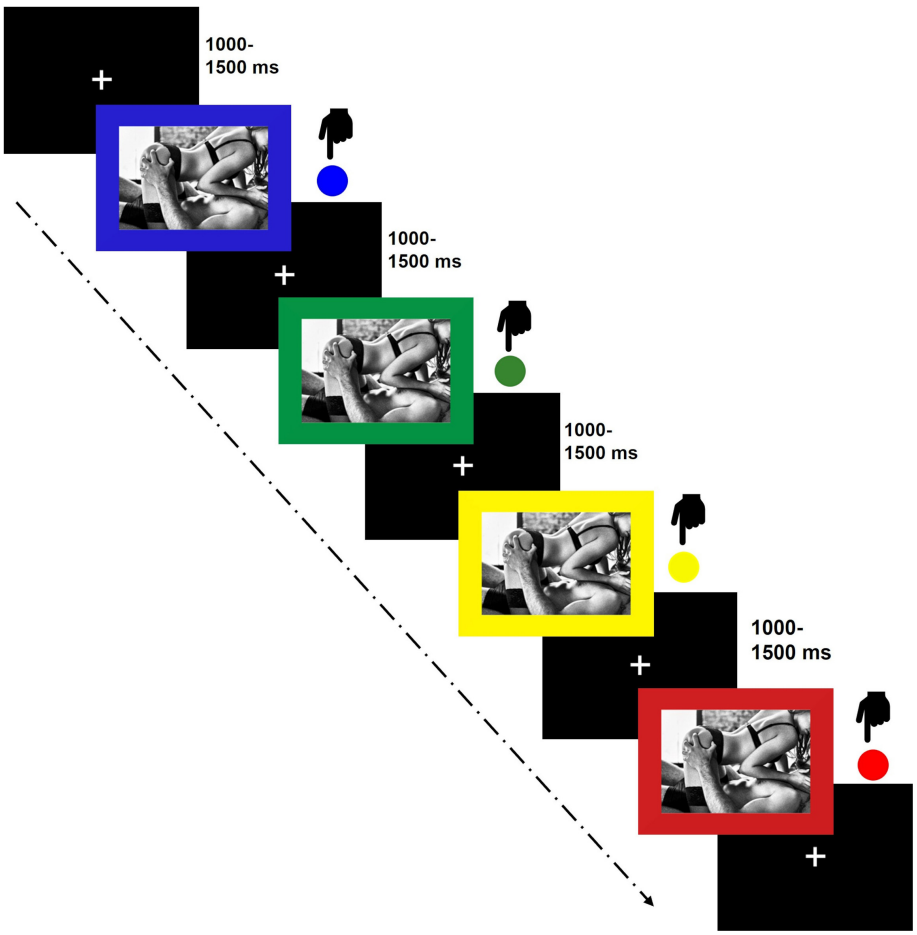

FIGURE 2 | Emotional Picture Stroop Paradigm (EPSP).

TABLE 1 | Participant characteristics of naturally cycling (NC) women and oral contraceptive (OC) users.

	Entire sample	NC	OC	Test-statistics
Age (mean ± *SD*)	23.24 ± 2.83	23.72 ± 2.56	22.82 ± 3.03	$t_{(60)} = 1.26$, $p = 0.212$
BMI (mean ± *SD*)	21.26 ± 1.87	21.37 ± 2.21	21.17 ± 1.55	$t_{(60)} = 0.42$, $p = 0.676$
Age at menarche (mean ± *SD*)	12.98 ± 1.75	12.62 ± 1.24	13.30 ± 2.07	$t_{(60)} = 1.55$, $p = 0.127$
Relationship status (% in a relationship)	75.80	72.40	78.80	$\chi^2_{(1)} = 0.34$, $p = 0.559$

processing. Raw EEG data was filtered with a 0.5 Hz (12 dB/oct per order) high-pass Butterworth IIR. Data was then visually inspected for non-ocular artifacts, and these were subsequently excluded. Blink- and eye- movement artifacts were removed using an Independent Component Analysis (ICA-) algorithm as implemented in Brain Vision Analyzer. Afterward, data was filtered using a 30 Hz (12 dB/oct per order) low-pass Butterworth IIR and a 50 Hz Notch filter, re-referenced to an average reference and segmented (stimulus locked; −200 ms to 1,000 ms). For each stimulus category, grand average waveforms were computed and baseline-corrected using the pre-stimulus interval (−200 ms to 0 ms). Three subjects (2 OC and 1 NC) had to be excluded from further analyses due to extensive EEG artifacts caused by movements, muscle tension or teeth grinding. For the remaining subjects, an average of 59.75 (*SD* = 3.25) trials per stimulus subcategory were included in the analysis. Symmetrical clusters were chosen for analysis of both ERP components. In accordance with its typical temporo-occipital topography, mean amplitudes of electrodes PO7, PO8, O1, and O2 in the time window between 150 and 250 ms were extracted to quantify the EPN component. For analyses of the LPP, mean amplitudes in the temporal window between 400 and 800 ms were extracted at centro-parietal electrodes CP1, CP2, P1, and P2. For both ERP components, choice of time window and electrodes was based on visual inspection of grand average waveforms, significant electrode inter-correlations and results of previous research (Farkas et al., 2020; Schindler et al., 2020).

TABLE 2 | Mean (M), standard deviation (SD) and range of estradiol concentration in pg/ml across follicular (FO), ovulation (OV) and luteal phase (LU) in naturally cycling (NC) women and across active OC pill phase one (AP1), active OC pill phase two (AP2), and the inactive OC pill phase (IP) in oral contraceptive (OC) users.

NC				
	FO	**OV**	**LU**	**Mean**
M	4.15	5.06	4.84	4.68
SD	0.94	1.28	1.37	0.91
Range	2.94–6.53	2.69–7.96	2.70–9.15	2.86–7.25
OC				
	AP1	**AP2**	**IP**	**Mean**
M	3.98	3.99	4.47	4.15
SD	1.08	1.40	1.62	1.15
Range	2.38–6.05	2.15–7.91	2.54–11.41	2.37–6.72

TABLE 3 | Mean (M), standard deviation (SD) and range of progesterone concentration in pg/ml across follicular (FO), ovulation (OV) and luteal phase (LU) in naturally cycling (NC) women and across active OC pill phase one (AP1), active OC pill phase two (AP2) and the inactive OC pill phase (IP) in oral contraceptive (OC) users.

NC				
	FO	**OV**	**LU**	**Mean**
M	43.22	68.82	106.79	72.94
SD	25.76	101.92	55.16	45.31
Range	22.08–119.21	12.21–578.60	38.69–239.08	28.64–278.48
OC				
	AP1	**AP2**	**IP**	**Mean**
M	35.99	35.74	41.29	37.67
SD	12.70	18.62	37.61	17.96
Range	19.03–75.33	18.75–102.55	14.49–190.26	19.31–104.37

Endocrine Analyses

Prior to EEG recording, saliva samples for analysis of progesterone (P), 17β-estradiol (E2) and testosterone (T) were collected using Salicaps (IBL, Hamburg, Germany). These were stored in a freezer at −20°C before being analyzed using enzyme-linked-immunosorbent assay (ELISA-) kits (IBL Hamburg, Germany) in a fully automated manner (BEP2000, Siemens Healthineers, Eschborn, Germany). Only P and E2 were analyzed for the current study. All analyses were run in duplicates. Intra-assay variability coefficients were 7.5 and 7.8% for E2 and P, respectively. Inter-assay variability coefficients were 6.3% for E2 and 1.3% for P. The standard curves were of expected slope and shape and according to the manufacturer, all ELISA-kits were validated by LC-MS. Subjects were instructed to neither eat nor drink (except water) for 2 h prior to testing to avoid possible contamination of samples. Outliers were replaced using a 90% winsorization for the NC and OC groups, respectively. Consequently, values below the 5th or above the 95th percentile were replaced with values corresponding to the 5th or 95th percentile, respectively. Winsorization has proven as a reliable method for outlier correction (Kennedy et al., 1992; Rivest, 1994).

Subjective Stimulus Evaluations

During their last appointment, subjects rated valence and arousal of the EPSP pictures on a 9-point rating scale (arousal: 1 calm and relaxed, 9 very aroused; valence: 1 very unpleasant, and 9 very pleasant) using Self-Assessment Manikins (SAM) (Bradley and Lang, 1994). Subjects were simultaneously presented with a printed version of all pictures belonging to the same subcategory (erotic: couple, male, female; neutral: couple, person, tree; positive). Presentation order of categories was randomized in order to avoid sequence effects. Stimulus evaluations were collected after the last EEG-recording to reduce the influence of conscious stimulus evaluations on neural processing.

Statistical Analyses

Mean concentration of ovarian steroids was compared between groups using independent sample *t*-tests for (a) estradiol and (b) progesterone concentration, respectively. Differences between MC/OC phases were analyzed using rmANOVAs separately for each group. For analysis of the subjective stimulus evaluations, arousal and valence ratings were entered into two separate rmANOVAs with the within-subjects factor stimulus category (three steps) and the between-subjects factor group (two steps). Since OC/MC phase of stimulus evaluation was not balanced, this was also added as a between-subjects factor.

To compare EPN and LPP amplitudes between groups, ERP amplitudes were averaged across measurement times. They were then entered into two rmANOVAs with the within-subjects factors electrode (four steps) and stimulus category (three steps) and the between-subjects factor group (two steps). To assess OC-regimen related effects, ERP amplitudes were then compared between measurement times across the OC regimen using rmANOVAs with the within-subjects factors electrode (four steps), measurement time (three steps) and stimulus category (three steps). Statistical analyses were conducted using IBM SPSS Statistics version 27 (IBM Corp., Somers, NY, United States) with an α-level set to 0.05. For all rmANOVAs Greenhouse–Geisser correction was used in case of violated sphericity assumption. Bonferroni correction was applied to control for multiple testing in *post hoc* analyses.

RESULTS

Participants

The final sample consisted of 62 subjects (29 NC and 33 OC). Groups did not differ in age, BMI, age at menarche or relationship status (**Table 1**). Age and duration of OC use were unrelated, $r = 0.266$, $p = 0.134$, but age was positively correlated with duration of the hormone free interval in previous OC users, $r = 0.723$, $p < 0.001$.

Gonadal Steroid Concentration

Mean estradiol (**Table 2**), $t_{(60)} = 2.02$, $p = 0.048$, $d = 0.514$, as well as progesterone concentration (**Table 3**), $t_{(60)} = 4.12$, $p < 0.001$, $d = 1.049$, differed significantly between groups, with, respectively, higher concentrations in the NC compared

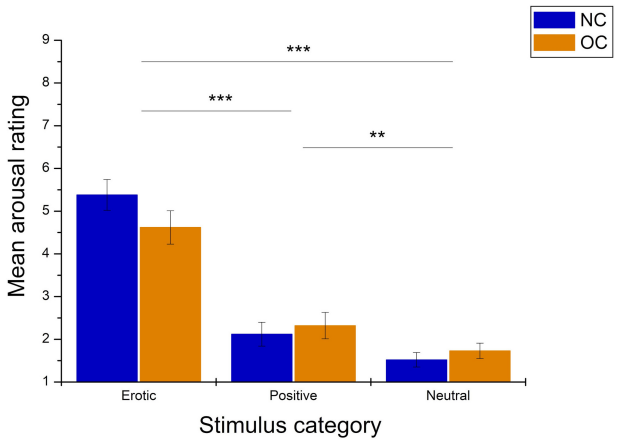

FIGURE 3 | Mean arousal ratings in reaction to erotic, positive and neutral stimuli in naturally cycling (NC) women and oral contraceptive (OC) users. Error bars reflect standard error of the mean (SEM). ***$p < 0.001$, **$p < 0.01$.

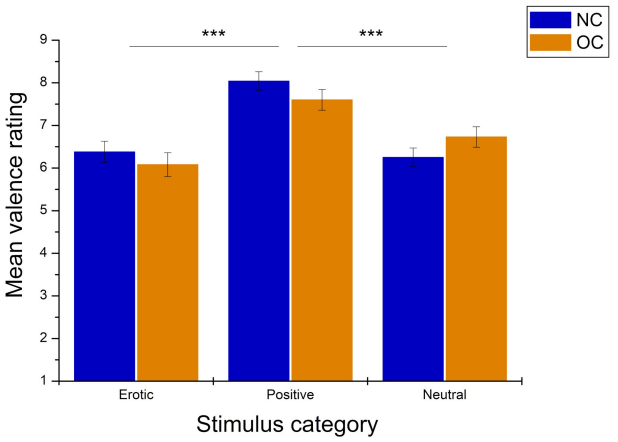

FIGURE 4 | Mean valence ratings in reaction to erotic, positive and neutral stimuli in NC women and OC users. Error bars reflect SEM. ***$p < 0.001$.

to the OC group. For the NC group, estradiol concentration (**Table 2**) differed significantly between MC phases, $F_{(2,56)} = 6.85$, $p = 0.002$, $\eta_p^2 = 0.197$. Pairwise comparison revealed that estradiol levels were significantly lower during FO compared to OV, $p = 0.010$, and LU, $p = 0.015$. No difference was observed between OV and LU, $p = 1.00$.

Progesterone concentration also differed significantly between MC phases, $F_{(1.58,44.36)} = 7.48$, $p = 0.003$, $\eta_p^2 = 0.211$, with significantly higher concentrations during LU compared to FO, $p < 0.001$, and no significant differences in FO and LU compared to OV (all $p \geq 0.119$) as illustrated in **Table 3**.

As expected, no phase differences in gonadal steroid concentration were observed for the OC group, estradiol (**Table 2**): $F_{(1.39,44.53)} = 2.85$, $p = 0.086$, $\eta_p^2 = 0.082$; progesterone (**Table 3**): $F_{(1.52,48.68)} = 0.68$, $p = 0.473$, $\eta_p^2 = 0.021$.

Subjective Stimulus Evaluations

Arousal Ratings

Arousal ratings differed significantly between stimulus categories, $F_{(1.66,93.04)} = 126.72$, $p < 0.001$, $\eta_p^2 = 0.694$ and were not affected by group, $F_{(1,56)} = 0.13$, $p = 0.720$, $\eta_p^2 = 0.002$. The stimulus category × group interaction was also not significant, $F_{(1.66,93.04)} = 3.04$, $p = 0.062$, $\eta_p^2 = 0.052$. Pairwise comparison revealed significantly higher arousal ratings in reaction to erotic vs. neutral and positive stimuli (all $p < 0.001$). Positive stimuli were rated as more arousing compared to neutral stimuli, $p = 0.004$. See **Figure 3** for illustration.

Valence Ratings

As shown in **Figure 4**, valence ratings also differed significantly between stimulus categories, $F_{(1.78,99.45)} = 29.08$, $p < 0.001$, $\eta_p^2 = 0.342$. Pairwise comparison revealed that positive stimuli were rated as significantly more pleasant compared to erotic and neutral stimuli (all $p < 0.001$), while no differences were observed between the latter two categories, $p = 0.750$. Valence ratings did not differ between groups, $F_{(1,56)} = 0.15$, $p = 0.704$, $\eta_p^2 = 0.003$, nor was there a significant stimulus category × group interaction, $F_{(1.78,99.45)} = 2.50$, $p = 0.094$, $\eta_p^2 = 0.043$.

Neural Reactivity

Early Posterior Negativity

Regarding the EPN, analyses revealed a main effect of stimulus category, $F_{(1.37,82.23)} = 70.79$, $p < 0.001$, $\eta_p^2 = 0.541$. Pairwise comparison showed that erotic stimuli evoked greater EPN negativity compared to neutral and positive stimuli (all $p < 0.001$). No differences were observed between positive and neutral stimuli, $p = 0.061$. Mean amplitude was 3.31 μV (*SEM* = 0.41) in reaction to erotic, 4.89 μV (*SEM* = 0.38) in reaction to positive and 4.69 μV (*SEM* = 0.38) in reaction to neutral stimuli (**Figure 5**).

Groups did neither differ significantly in EPN amplitudes, $F_{(1,60)} = 1.05$, $p = 0.311$, $\eta_p^2 = 0.017$, nor was there a significant group × stimulus category interaction, $F_{(1.37,82.23)} = 0.14$, $p = 0.869$, $\eta_p^2 = 0.002$. Results are illustrated in **Figure 6**. EPN amplitudes did not differ in dependence of OC regimen, $F_{(2,64)} = 1.27$, $p = 0.288$, $\eta_p^2 = 0.038$ and there was no significant OC regimen × stimulus category interaction, $F_{(4,128)} = 1.48$, $p = 0.213$, $\eta_p^2 = 0.044$.

Late Positive Potential

Late positive potential amplitudes differed significantly in dependency of stimulus category, $F_{(1.69,101.36)} = 67.87$, $p < 0.001$, $\eta_p^2 = 0.531$. Erotic stimuli elicited greater LPP amplitudes compared to neutral and positive ones (all $p < 0.001$). LPP amplitudes toward positive vs. neutral stimuli did not differ significantly, $p = 1.00$ (**Figure 7**).

There was no significant main effect of group, $F_{(1,60)} = 1.04$, $p = 0.311$, $\eta_p^2 = 0.017$, and no significant group × stimulus interaction, $F_{(1.69,101.36)} = 0.53$, $p = 0.560$, $\eta_p^2 = 0.531$, $\eta_p^2 = 0.009$. Results are illustrated in **Figure 8**.

For the OC group, LPP amplitudes differed across the OC regimen in dependency of stimulus category, as indicated by a significant stimulus × OC regimen interaction,

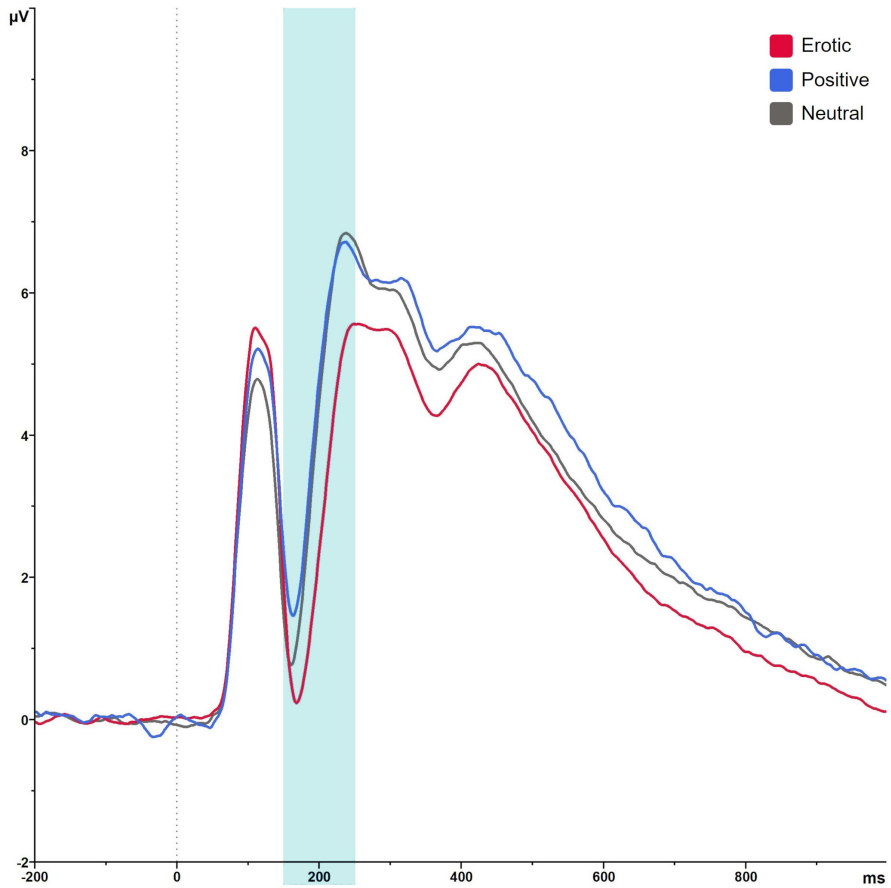

FIGURE 5 | Grand average on electrode PO7 in reaction to erotic, positive and neutral stimuli in the time window 150–250 ms.

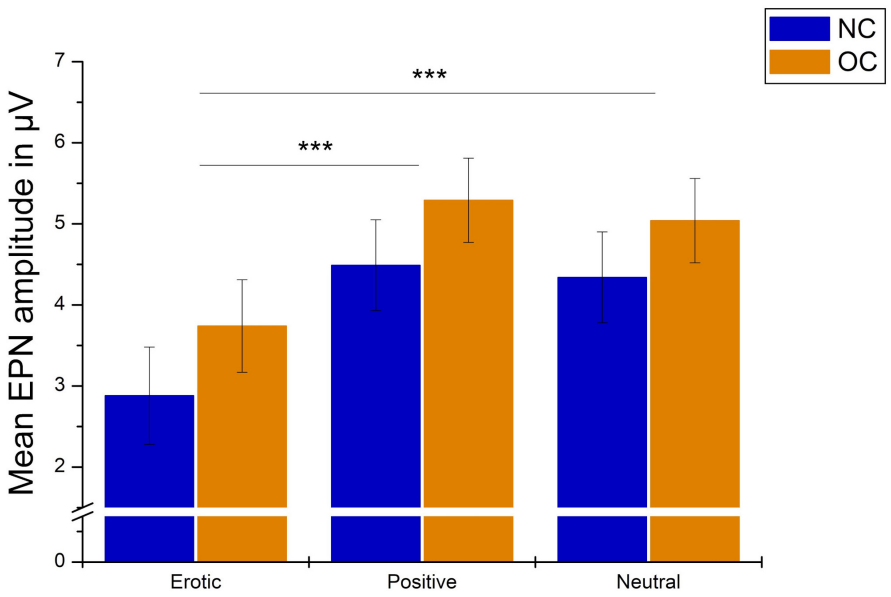

FIGURE 6 | Mean EPN amplitudes in µV averaged over electrodes PO7, PO8, O1, and O2 in reaction to erotic, positive and neutral stimuli in NC women and OC users. Error bars reflect SEM. ***$p < 0.001$.

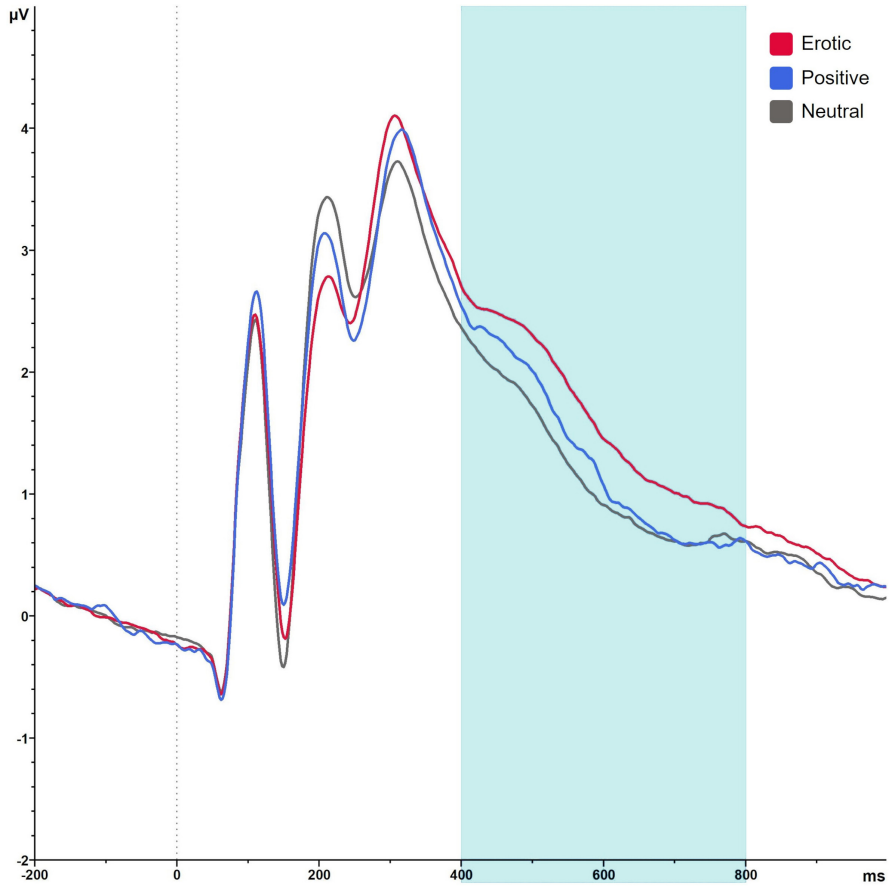

FIGURE 7 | Grand average on electrode P2 in reaction to erotic, positive and neutral stimuli in the time window 400–800 ms.

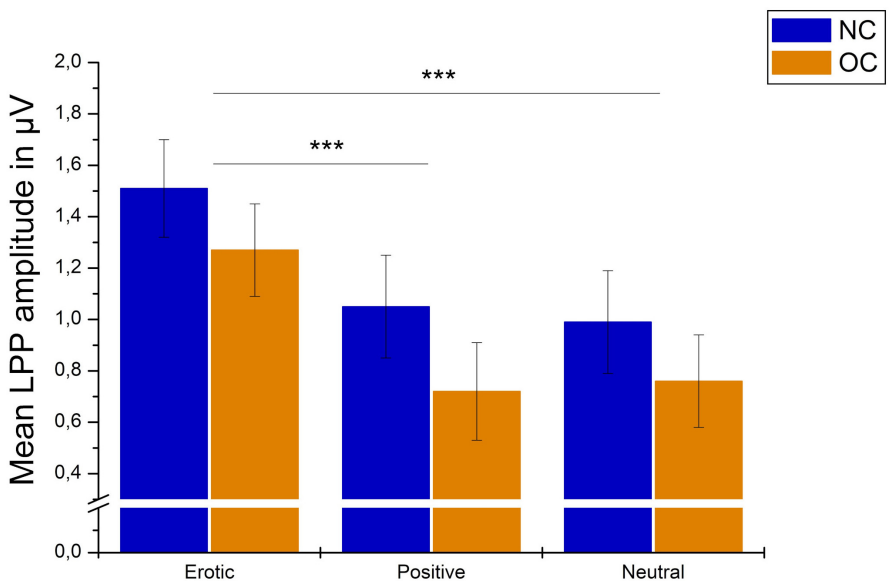

FIGURE 8 | Mean LPP amplitudes in µV averaged over electrodes CP1, CP2, P1, and P2 in reaction to erotic, positive and neutral stimuli in NC women and OC users. Error bars reflect SEM. ***$p < 0.001$.

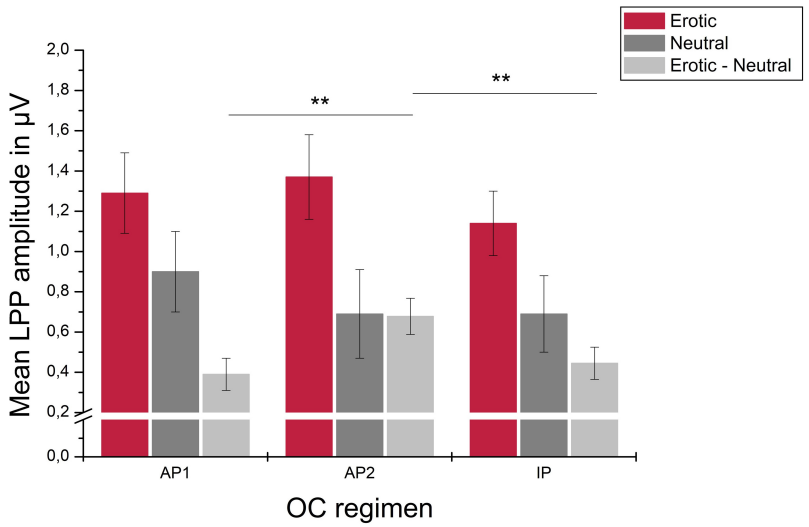

FIGURE 9 | Mean LPP amplitudes in µV averaged over electrodes CP1, CP2, P1, and P2 in reaction to erotic and neutral stimuli as well as the erotic – neutral difference in OC users across active OC pill phase one (AP1), active OC pill phase two (AP2) and the inactive OC pill phase (IP). Error bars reflect SEM. $^{**}p < 0.01$.

$F_{(3{\cdot}07,98{\cdot}07)} = 3.85$, $p = 0.011$, $\eta_p^2 = 0.107$. *Post hoc* analyses revealed no main effect of measurement time for neither stimulus category, erotic: $F_{(2,64)} = 1.38$, $p = 0.259$; neutral: $F_{(2,64)} = 1.59$, $p = 0.212$; positive: $F_{(2,64)} = 0.14$, $p = 0.872$. The amplitude difference between erotic and neutral stimuli, however, differed significantly across OC regimen measurement times, $F_{(2,64)} = 9.58$, $p < 0.001$, $\eta_p^2 = 0.230$, and was higher during AP2 compared to AP1 and IP, $p = 0.001$, respectively. Descriptively, this effect was due to lower reactivity toward neutral stimuli (AP1 vs. AP2) and higher neural reactivity toward erotic stimuli (AP1 vs. AP2 and AP2 vs. IP). Results are illustrated in **Figure 9**.

DISCUSSION

The current study examined neural correlates of early and later stages of emotional processing in NC and OC women using an ERP approach. NC women and OC users did not differ significantly in neural reactivity toward positive or erotic visual stimuli, nor did they rate valence and arousal of these stimuli differently. In OC using women, LPP but not EPN amplitude differences between erotic and neutral stimuli varied significantly between measurement times across the OC regimen with significantly higher differences during AP2 compared to AP1 and IP – that is, during the second week of OC intake compared to the first and the OC free week.

Gonadal Steroid Concentration

Endogenous estradiol as well as progesterone concentrations were lower in the OC than in the NC group. These results are in line with most previous studies (Rapkin et al., 2006; Petersen and Cahill, 2015; Zethraeus et al., 2017) and are caused by OCs downregulating effect on the HPG-axis. Yet, not all studies did report differences in estradiol levels between NC and OC subjects (Monciunskaite et al., 2019), possibly due to the use of between- vs. within-subjects designs. In the current study, reliability of endocrine analyses was increased by strict termination of measurements in three distinct cycle phases and the assessment of these distinct phases in the same women, which helped to reduce between-subject variance. If distinct phases are being assessed in different women, high-between subject variance can hinder detection of phase and/or group differences. Within-subject designs are, therefore, advantageous in order to disentangle these effects. In the NC group, endocrine concentrations showed expected variations across the menstrual cycle. Estradiol concentration was highest during ovulation and remained elevated during luteal compared to follicular phase. This corresponds to the second estrogen peak observed during mid-luteal phase (Gandara et al., 2007). Progesterone levels peaked during luteal phase with a significant increase compared to follicular phase and intermediate concentrations during ovulation.

Subjective Stimulus Evaluations

Regarding stimulus arousal ratings, both emotional categories were rated as significantly more arousing compared to neutral stimuli and erotic stimuli were also rated as more arousing compared to positive stimuli. This is in line with previous research (Jacob et al., 2011; Maffei et al., 2015). With regard to valence ratings, positive stimuli were rated as significantly more pleasant than neutral and erotic stimuli. In contrast to our expectation, erotic stimuli were not rated as more pleasant than neutral stimuli. Higher pleasantness ratings for erotic vs. neutral stimuli have previously been reported (Jacob et al., 2011), but might depend on the explicity of erotic stimuli, perceived embarrassment while watching such stimuli, or specific features of included neutral stimuli (Maffei et al., 2015). Specific setting characteristics (online vs. on-site) could also affect the results with anonymous online ratings possibly being more honest. In

the current study, no significant differences between the NC and OC group in valence or arousal ratings regarding any stimulus category were observed. Accordingly, Armbruster et al. (2017) reported no differences in subjective stimulus evaluations between NC and OC women in reaction to positive, negative and neutral stimuli. Subtle differences in valence and arousal ratings in reaction to positive, negative and neutral stimuli have previously been reported by Spalek et al. (2019). However, the authors did not control for measurement time during MC/OC regimen and their sample included various types of HCs (COCs, patches, hormonal IUDs, and progestin-only pills).

Regarding erotic stimuli, Abler et al. (2013) did not observe group differences in arousal ratings. The current results, while remaining on a trend level ($p = 0.062$ for the stimulus × group interaction), indicate slightly lower arousal ratings in OC users compared to NC women. This finding is relevant in the light of adverse sexual effects reported by some OC users (Wallwiener et al., 2010, 2015; Smith et al., 2014; Zethraeus et al., 2016; Huang et al., 2020) and should be more closely examined in future studies, possibly in combination with measures of sexual function and/or self-reported adverse OC effects.

Results suggest that conscious processing of emotional stimuli might not be influenced very strongly by OC intake. Previous results regarding modulation of subjective stimulus evaluations by MC phase were inconsistent with some studies reporting no (Zhang et al., 2013) and others domain-specific effects (Lusk et al., 2015; Mačiukaitė et al., 2017). To the best of our knowledge, no study so far assessed OC regimen related effects in stimulus evaluations. As MC/OC regimen time points of stimulus evaluation were not balanced between subjects in the current sample, no conclusion can be drawn in this respect.

Neural Reactivity in Naturally Cycling vs. Oral Contraceptive Using Women

No significant differences were observed in neural reactivity between NC and OC women neither for early (EPN) nor for later (LPP) stages of emotional processing. These differences were expected due to reported adverse side effects of OC intake, including depression (Skovlund et al., 2017; Wit et al., 2020), a reduction of general well-being (Zethraeus et al., 2017) or sexual function (Wallwiener et al., 2010, 2015; Smith et al., 2014; Zethraeus et al., 2016), and previous research indicating reduced reactivity to negative emotional stimuli under OC treatment (Gingnell et al., 2013; Petersen and Cahill, 2015; Monciunskaite et al., 2019). However, Abler et al. (2013) also reported no significant differences in neural activity during viewing of erotic videos and pictures between OC using and NC women. They did observe subtle differences in activity of the precentral gyrus during expectation of erotic stimuli but only when comparing follicular NC women to OC users that had previously taken OC pills for up to 2 months without any OC break, which limits generalizability of reported results.

Generally, an important factor that needs to be considered in OC research and interpretation of its results is the duration of OC use. In case of the Gingnell et al. (2013) study, emotional processing was assessed after one cycle of OC treatment and only women who had previously experienced OC related side effects were included. Mean duration of OC intake in the current sample was approximately 4 years. Consequently, prolonged rather than initial effects of OC use were assessed. Initial effects of OC treatment – as assessed after one treatment cycle – might diminish over time leading to non-significant findings in cross-sectional studies. Correspondingly, Jarva and Oinonen (2007) reported greater differences between NC and OC women in emotional reactivity if those took OCs for less than 2 years. Exploratory analyses in the underlying sample revealed no significant association between duration of OC use and neural reactivity, neither in the EPN nor in the LPP. However, no subject in the current sample had used OCs for less than 12 months and only four had used them for less than 2 years. Consequently, no conclusions could be drawn on initial OC treatment effects.

Nevertheless, some effects of OCs on brain structure and neurochemistry might last for months or years after OC discontinuation (Pletzer et al., 2019) and such enduring effects could also mask group differences if NC women have previously used HCs. Importantly, 22 out of 29 subjects in the current NC sample reported previous HC use. Therefore, reported results might not be generalizable to HC naïve women. Adverse side effects are the main reason for terminating (oral) hormonal contraception (Sanders et al., 2001; Huber et al., 2006). The relatively long mean duration of OC use in the underlying OC group suggests an absence of major side effects. This phenomenon is called self-selection bias or survivor-effect and is highly relevant in cross-sectional OC research (Brønnick et al., 2020). Consequently, OC users in the current sample might not be representative of OC users in general, but of those without major side effects. These women might also experience only subtle alterations of psychophysiological processes. However, 42% of OC users in the current sample reported to experience some sort of OC related side effects with mood swings and depressive mood being most frequently reported. Practical reasons, partnership characteristics or a positive cost-benefit ratio (cycle control, beneficial effects on acne or menstrual pain) might result in continuation despite side effects (Frost and Darroch, 2008; Merkh et al., 2009; Egarter et al., 2013). To differentiate effects of initial, prolonged or previous OC use, future studies should include higher proportions of OC naïve women. As recent studies suggest higher vulnerability to OC related neurophysiological alterations and side effects during puberty (Anderl et al., 2020; Sharma et al., 2020a; Wit et al., 2020), time of OC initiation should also be considered. Furthermore, information about side effects and reasons for initiation (i.e., contraception vs. health-related reasons) should also be collected more thoroughly.

Current research suggests that adverse effects following OC use occur only in a subgroup of women (Schaffir, 2006; Burrows et al., 2012; Schaffir et al., 2016). Accordingly, Scheuringer et al. (2020) conducted a placebo-controlled RCT and assessed attentional biases and depressive symptoms after 3 months of OC intake. While they did not observe effects of OCs on depressive symptoms, or interference in a verbal emotional Stroop task, previous OC use and high trait anxiety significantly predicted depressive symptoms at the end of the trial. Genetic

factors could also contribute to such susceptibility and might be similar to those predisposing women to experience other reproductive affective disorders like premenstrual dysphoric disorder, postpartum or perimenopausal depression (McEvoy et al., 2017; Bromberger and Epperson, 2018). Regarding sexual side effects, polymorphisms associated with female sexual function such as the estrogen-receptor α polymorphism (rs2234693) (Armeni et al., 2017) could be relevant as well. Such factors should be considered in future research to prospectively improve contraception counseling.

In a previous ERP study on OC use, Monciunskaite et al. (2019) reported significantly lower LPP amplitudes toward emotional as well as neutral stimuli in OC users. As the study design of this study was relatively similar (ERP approach, student sample, comparable sample size, and long OC duration), previously outlined factors are unlikely to account for diverging results. However, the sample in the Monciunskaite et al. (2019) study was restricted to users of anti-androgenic progestins. In the current sample, a majority of women used androgenic progestins. While most studies do not control for different OC formulations, some recent results indicate that androgenic vs. anti-androgenic progestins differentially affect cognitive and socio-emotional processes. For instance, Gurvich et al. (2020) reported lower accuracy in an emotional face discrimination paradigm in anti-androgenic vs. androgenic OC users. Pletzer et al. (2015) observed higher accuracy in a neutral face recognition paradigm and higher gray matter volumes in the fusiform gyrus, the fusiform face area and the parahippocampal place area as well as the cerebellum in users of anti-androgenic progestins. Face recognition accuracy did not differ significantly between NC women and users of androgenic progestins. In a later study, Pletzer et al. (2016) reported that observed differences between NC and OC women in resting-state-connectivity were mostly attributable to the group of anti-androgenic OC users. Affected regions belonged to limbic as well as occipital networks involved in processing of visual emotional stimuli. Exploratory analysis in the current sample revealed a trend (p = 0.052 for the main effect of group) toward lower LPP amplitudes in anti-androgenic OC users compared to NC women and users of androgenic OCs. EPN amplitudes did not differ significantly between groups. However, subjective stimulus evaluation differed significantly between groups. Irrespective of stimulus category, users of anti-androgenic OCs rated stimuli as significantly less pleasant compared to androgenic OC users, who did not differ from the NC group. Regarding stimulus arousal, erotic stimuli were rated as significantly less arousing in the anti-androgenic- compared to the androgenic OC as well as the NC group. Test statistics regarding these results are provided in the **Supplementary Material**. These results should be treated with caution due to the small sub-sample size (23 subjects used androgenic OCs and 10 subjects anti-androgenic OCs). Taken together with results described above, they nevertheless suggest that pooling different OC formulations might prevent detection of group differences between NC and OC women and differential effects of progestin types should be considered in future research. Research regarding effects of synthetic progestins on the CNS is still very sparse (Gruber and Huber, 2003) and most studies also did not include/differentiate newer progestins (Rapkin et al., 2006; Africander et al., 2011; Porcu et al., 2019). Therefore, as a first step, more basic neuroscientific research, examining differential effects of OC preparations on i.e., neurosteroid concentration and/or neuroanatomy, is needed.

Other possible explanations for the diverging results between the current and the Monciunskaite et al. (2019) sample are design and paradigm-related differences. In the current study, each subject was assessed three times across the MC/OC regimen [compared to once in Monciunskaite et al.'s (2019) sample]. Measurements during ovulation in the NC and during the OC free week in the OC group were included. Furthermore, Monciunskaite et al. (2019) used a passive viewing paradigm whereas an active task requiring subjects to react to presented stimuli was used in the current study. Previous research indicated higher mind wandering tendencies in OC users (Raymond et al., 2019) – a confound that should be more relevant in passive vs. active tasks and might explain reduced LPP amplitudes in the Monciunskaite et al. (2019) sample. Further research, possibly including eye-tracking measures, is necessary, especially since NC and OC women might differ in their attention to contextual features of presented (erotic) stimuli (Rupp and Wallen, 2007).

Regarding erotic picture processing, another factor should be considered in future research: Prause et al. (2015) observed lower LPP amplitudes in subjects reporting less than two sexual intercourse partners during the last 12 months compared to subjects reporting two or more partners. Importantly, this difference was more pronounced in less explicit sexual images. As erotic pictures in the current study included males and females in underwear (i.e., no naked genitals were depicted) and couples during "petting" rather than penetrative intercourse, they can be classified as rather less explicit. Hence, differences in sexual activity between both groups could also have confounded current results. Subjects did not report the number of current and/or previous sexual intercourse partners. Relationship status – as the most similar measure in the underlying study – was, however, not significantly associated with early or late neural reactivity.

While no significant differences occurred between the NC and OC group, the erotic-neutral difference in LPP amplitudes was modulated by measurement time during the OC regimen with significantly higher differences during the second week of active OC intake. Importantly, this effect remained significant when controlling for phase of first measurement. Most previous studies on OC use did not examine different time points across the OC regimen at all, or if they did, compared one time point during the active period and one time point during the OC free week in a between-subjects design. Within-subjects designs – as used in the current study - help to reduce variance in order to better disentangle OC regimen effects. The higher erotic-neutral difference at AP2 resulted from lower neural reactivity to neutral stimuli (AP1 vs. AP2) and higher neural reactivity to erotic stimuli (AP1 vs. AP2 and AP2 vs. IP). Mean amplitude in reaction to erotic stimuli was highest at AP2, although this effect was not statistically significant. Previous research reported higher coupling of brain regions relevant for emotion regulation during the active vs. inactive period of

the OC regimen (Nasseri et al., 2020) and Radke and Derntl (2016) reported higher accuracy during the active OC treatment in an affective responsiveness task. Taken together, reported results suggest that emotional processes are modulated by the OC regimen, with possibly higher emotional abilities (higher emotional reactivity to erotic vs. neutral stimuli, better emotion regulation and affective state evaluation) during active vs. inactive periods of the OC regimen.

After initiation of a new OC blister, ovarian suppression is usually reached after seven consecutive days of OC intake (Curtis et al., 2006). Most manufacturers recommend additional contraception if a pill is forgotten during the first 7 days after the break, but not if it is forgotten on days 8–14. Missing pills adjacent to the OC free interval is associated with greater risk of pregnancy (Curtis et al., 2006). The AP1 measurement in the current study corresponded to days 7–9 after starting a new OC blister and the AP2 measurement was conducted on days 14–16. Therefore, AP2 might correspond to the time point of maximal ovarian suppression and most stable endocrine status. Correspondingly, higher reactivity to erotic vs. neutral stimuli was noted at this point. This could be interpreted in terms of positive mood effects during steady hormonal states in OC users (Ott et al., 2008). Lower reactivity to erotic vs. neutral stimuli during the OC free week is in line with reported adverse effects during the hormone absent interval of the OC regimen (Sulak, 2000; Kelly et al., 2010). In the current study, it was aimed to assess NC and OC women on similar days of the MC/OC regimen with equal between measurement intervals. To further elucidate OC regimen related differences in emotional processing, future studies should terminate measurements in the OC group in consideration of results regarding ovarian function at different time points of the OC regimen (Curtis et al., 2006).

Limitations

Several limitations need to be considered in the interpretation of the presented results. Most subjects were students and mean age was comparably young. Strict inclusion/exclusion criteria, furthermore, reduces representativeness of the sample.

Regarding included stimulus categories, no significant differences were observed in neural reactivity to positive vs. neutral stimuli. This contradicts previous research (Schupp et al., 2000; Bublatzky et al., 2014; Munk et al., 2016). However, neural reactivity has proven to be arousal- rather than valence dependent (Schupp et al., 2000; Leite et al., 2012). Comparing arousal ratings between subcategories revealed that positive stimuli were rated as more arousing compared to trees but did not differ significantly from neutral persons and couples which could explain the observed similarity in neural reactivity.

Conclusion

Sixty years after introduction of "the pill," the question *how* OCs affect brain structure and functions remains unclear and understudied (Porcu et al., 2019). Hence, in a first step, more basic neuroscientific research is needed.

In the current study, no significant differences were observed either in subjective stimulus evaluations, or in neural reactivity toward positive or erotic emotional stimuli. Neural reactivity toward erotic vs. neutral stimuli was modulated by time point of the OC regimen with greater differences during the second week of the OC regimen. This could result from steady endocrine states and should be more closely examined in future studies. Future studies should control for duration of current and/or previous OC use and other possible confounding factors such as the number of sexual intercourse partners. Furthermore, it seems to be highly relevant to distinctively examine different OC formulations, as different progestins could have differential effects on neural and socio-emotional processes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Local Ethic Commission of the Faculty of Psychology and Sport Science at Justus-Liebig-University Gießen. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NS, JH, and AM: conceptualization and study design. NS: data acquisition and investigation, formal analysis, visualization, and writing – original draft. NS and AM: writing – review and editing. AM: project administration. JH and AM: supervision and resources. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.798823/full#supplementary-material

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The Impact of Hormonal Contraceptive Use on Serotonergic Neurotransmission and Antidepressant Treatment Response: Results From the NeuroPharm 1 Study

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Background: Hormonal contraceptive (HC) use has been associated with an increased risk of developing a depressive episode. This might be related to HC's effect on the serotonergic brain system as suggested by recent cross-sectional data from our group, which show that healthy oral contraceptive (OC) users relative to non-users have lower cerebral serotonin 4 receptor (5-HT4R) levels. Here, we determine if cerebral 5-HT4R binding differs between HC non-users, OC users, and hormonal intrauterine device (HIUD) users among women with an untreated depressive episode. Also, we test if antidepressant drug treatment response and its association with pre-treatment 5-HT4R binding depends on HC status.

Methods: [^{11}C]-SB207145 Positron Emission Tomography imaging data from the NeuroPharm-NP1 Study (NCT02869035) were available from 59 depressed premenopausal women, of which 26 used OCs and 10 used HIUDs. The participants were treated with escitalopram. Treatment response was measured as the relative change in the Hamilton Depression Rating Scale 6 items ($r\Delta HAMD_6$) from baseline to week eight. Latent variable models were used to evaluate the association between global 5-HT4R binding and OC and HIUD use as well as $r\Delta HAMD_6$.

Results: We found no evidence of a difference in global 5-HT4R binding between depressed HC users and non-users ($p \geq 0.51$). A significant crossover interaction ($p=0.02$) was observed between non-users and OC users in the association between baseline global 5-HT4R binding and week eight $r\Delta HAMD_6$; OC users had 3-4% lower binding compared to non-users for every 10% percent less improvement in $HAMD_6$. Within the groups, we observed a trend towards a positive association in non-users

(p_{adj}=0.10) and a negative association in OC users (p_{adj}=0.07). We found no strong evidence of a difference in treatment response between the groups (p=0.13).

Conclusions: We found no difference in 5-HT4R binding between HC users *vs*. non-users in depressed women, however, it seemed that 5-HT4R settings differed qualitatively in their relation to antidepressant drug treatment response between OC users and non-users. From this we speculate that depressed OC users constitutes a special serotonin subtype of depression, which might have implications for antidepressant drug treatment response.

Keywords: hormonal contraception, oral contraception, hormonal intrauterine device, [11C]SB207145, serotonin, major depressive disorder, serotonin 4 receptor, sex steroid hormones

INTRODUCTION

Hormonal contraception (HC) is used by millions of women worldwide to avoid pregnancies as well as for other indications such as dysmenorrhea, menorrhagia, and acne (1, 2). In spite of HC being used for more than 60 years it is still debated whether HC use causes mood deterioration and development of depressive episodes (3). Recent findings from large epidemiological studies have suggested that starting on HC is associated with an increased risk of being diagnosed with Major Depressive Disorder (MDD), being prescribed antidepressants (4, 5), and even attempting or completing suicide (6–8). Notably, the risk is higher when HC is initiated in adolescence (9, 10) and moreover, HC use in adolescence is associated with lasting vulnerability for MDD in adulthood (11), which may relate to HC being introduced in a critical stage of brain development (12). Even though HCs are widely used, we still have limited understanding on how HC affects the brain and which implications it may have for mental health (13).

HCs exist in different types in terms of hormonal content and route of administration such as oral contraceptives (OCs), hormonal intrauterine devices (HIUDs), vaginal rings, injections and subdermal implants (14). OCs and HIUDs are the types most widely used (1). HCs contain synthetic female sex hormones either in form of a progestin alone (HIUDs and progesterone-only pills) or in a combination with an estrogen (combined OCs), most frequently ethinylestradiol (14). The synthetic steroids in combined OCs and in high-dose progesterone-only pills act by suppressing the hypothalamic-pituitary-gonadal hormonal axis resulting in suppression of the endogenous hormone production, disrupted follicular maturation, and inhibition of ovulation (15). HIUDs and low-dose progesterone-only pills on the other hand only inhibit ovulation in 60-85% of the time and even as low as 15% of the time after one year of HIUD use. Instead, their primary mechanism of action is induction of local inflammation in the cervical mucous to prevent access of sperm and by thinning of the uterus lining to prevent implantation of a fertilized egg (16, 17). Further, the synthetic steroids in HC induce an increase in the sex hormone binding globulin level further lowering the bioavailable fraction of sex hormones (18). These profound changes in the sex hormone milieu may shape brain biology both in terms of brain structure (19) and function (20). Recently, we have shown that OC use appears to affect the brain's serotonin signaling system (21), which is a key system for maintaining mental health and is involved in the pathophysiology of MDD and the treatment hereof (22). We used the molecular imaging technique ([^{11}C]SB207145-radioligand positron emission tomography (PET)) to quantify the postsynaptic serotonin 4 receptors (5-HT4R). The 5-HT4Rs are sensitive to chronic synaptic serotonin manipulation such that they are inversely correlated to serotonergic tonus (23–25) making it an interesting tool to study MDD pathophysiology. In a population of healthy women, we found that women using OCs had 9-12% lower 5-HT4R level globally in the brain compared to non-users (21). In comparison, our group also found 7-8% lower 5-HT4R global binding in unmedicated depressed individuals compared to healthy controls, and intriguingly, this gap was only evident in those who remitted after eight weeks of antidepressant treatment with a selective reuptake inhibitor (SSRI), indicating that those responding to the treatment may have a serotonergic subtype of MDD (26). The SSRI works by targeting the serotonin system, which induces an additional downregulation of 5-HT4R levels in neostriatum, a phenomenon confirmed in both a depressed and healthy cohort (23, 26). From this, we speculate whether the mechanisms causing the lower 5-HT4R binding in OC users and depressed individuals are similar and if it has any implications for the SSRI treatment response in women with a depressive episode.

We sought to investigate this by determining 1) if the 5-HT4R binding differs between depressed women who do not use HC *vs*. those using OCs and HIUDs, 2) if antidepressant drug treatment response is affected by HC use, 3) if an association between pre-treatment 5-HT4R brain binding and antidepressant drug treatment response depends on HC status, and 4) if neostriatal 5-HT4R levels are sensitive to SSRI treatment in HC users. We hypothesize that if the effects from OC and MDD are not additive, we will not be able to detect a difference in baseline 5-HT4R binding. However, when we account for treatment response, and thus may account for the serotonergic subtype of MDD, we will be able to detect a negative main effect of OC use on 5-HT4R binding. In line with that, we hypothesize that an association between baseline binding and treatment response will depend on OC use, such that an association is found in non-users but not in OC users, as they may constitute a more homogenous group in terms of 5-HT4R downregulation. We further

hypothesize that the effect of HIUD use on 5-HT4R binding will be in the same direction as of OC use, but with smaller effect size, due to the smaller degree of synthetic steroid exposure and larger degree of preserved ovulations (i.e., preserved hormonal cycle).

METHODS

We included patient data from the NeuroPharm-NP1 clinical trial (27), which aimed to predict MDD treatment outcome with the use of potential biomarkers including 5-HT4R PET brain scans. The study was approved by the Committees on Health Research Ethics in the Capital Region of Denmark (H-15017713), the Danish Data Protection Agency (04711/RH-2016-163), and the Danish Medicines Agency (NeuroPharm-NP1, EudraCT-number 2016-001626-34) and was pre-registered on clinicaltrials.gov (NCT02869035). A detailed description of the study design and the study elements can be found in the clinical trial protocol (27). The present study used information on HC use and baseline 5-HT4R PET imaging data together with clinical outcome measures after eight weeks of antidepressant drug treatment. It represents initially unplanned analyses motivated by the observed difference in 5-HT4R binding in healthy women who use OC ***vs***. those who do not use OC (21). The treatment started at a daily dose of 10 mg of the SSRI, escitalopram, and was increased to a daily dose of maximally 20 mg, depending on treatment response and adverse reactions. In case of non-response at week four, patients were switched to duloxetine, a serotonin-norepinephrine reuptake inhibitor (SNRI). Compliance was confirmed by serum drug levels after eight weeks of treatment. A subset of the study population was rescanned after eight weeks of treatment to map the change in 5-HT4R level. Allocation to rescan happened continuously until allotted rescans were completed.

Study Population

The study population consists of all the depressed premenopausal women (defined as < 50 years of age) from the NeuroPharm-NP1 study with available baseline 5-HT4R PET data and information on HC use. One patient was excluded from the analyses on 5-HT4R imaging data due to an interrupted PET scan (n=59 for the first analysis). Eight patients dropped out during follow-up, six OC users and two non-users (n=52 for the second and n=51 for the third analysis). Twenty-six of the remaining women were rescanned at follow-up; 15 were non-users, six were OC users and five were HIUD users (n=26 for the fourth analysis). The women were suffering from an unmedicated moderate to severe (> 17 points on the Hamilton Depression Rating Scale 17 items ($HAMD_{17}$)) depressive episode confirmed by a psychiatrist at inclusion. The current depressive episode was single or recurrent and did not involve acute severe suicidal ideation or psychosis. They had no prior or present history of other major psychiatric disorders confirmed by use of the diagnostic tool, the Mini International Neuropsychiatric Interview version 6.0 (28), or any other severe somatic illness confirmed by a basic somatic screening. Level of education was calculated as the number of completed school years (7-12 years) summed with a Likert score indexing highest completed or commenced degree ranging from one (no vocational degree) to five (>four years of higher learning at university level). Information about relationship status was acquired *via* interview. Blood tests to assess plasma estradiol and progesterone levels were taken at the baseline PET scan as part of standard biochemical screening.

Hormonal Contraception

Information about HC use was acquired *via* face-to-face interview at the time of the PET scan and *via* a written questionnaire which included a question on the specific HC type. The HC type was divided into HIUD and OC. OCs included different generations of combined OCs (n=20) and progestogen-only pills (n=6), as specified in **Table S1**.

Clinical Outcome Measure

The clinical outcome measure was the relative change in Hamilton Depression Rating Scale 6 items ($r\Delta HAMD_6$) in percentage from baseline to week eight. In the larger NeuroPharm-NP1 study, the categorical treatment response categories, remitters *vs*. non-responders, were used as the primary clinical outcome and $r\Delta HAMD_6$ was included as the secondary outcome (27). For this study, which only includes the women, we only applied $r\Delta HAMD_6$ to avoid underpowered statistics.

Imaging

PET acquisition and quantification is detailed in the trial protocol (27) and briefly summarized here: A high-resolution research tomography Siemens PET scanner (CTI/Siemens, Knoxville, TN, USA) (256 × 256 × 207 voxels; 1.22 × 1.22 × 1.22 mm) was used for acquiring PET scans. Each scan was obtained from a 120 min dynamic PET acquisition immediately after a 20 second intravenous bolus injection of the [^{11}C] SB207145 tracer ligand. Motion correction was performed using the AIR 5.2.5 software (29). High-resolution structural T1-weighted magnetic resonance images were acquired on a Siemens 3-Tesla Prisma scanner with a 64-channel head coil. The images were segmented into cerebrospinal fluid, white- and gray matter and were co-registered with PET images using the Statistical Parametric Mapping software (SPM8, The Wellcome Centre for Human Neuroimaging, UCL, London, UK). Regions were automatically delineated from the MR image *via* the user-independent algorithm in the Pvelab software (30). Regions of interest (ROIs) were neostriatum, hippocampus and neocortex, as these were lower in 5-HT4R binding in the depressed cohort in the NeuroPharm-NP1 Study (26), and as these regions represent low, intermediate, and high expression levels of brain 5-HT4Rs (31) and lastly, as these regions have previously shown lower 5-HT4R levels in OC users (21). As previous studies only found an effect of SSRI treatment on 5-HT4R binding in neostriatum, we only used this region to test whether this was also true in the HC users. Regional non-displaceable binding potentials (BP_{ND}) were quantified using the simplified reference tissue model with cerebellum as reference tissue (32).

Statistics

For the descriptive statistics, we compared between the HC non-users, the OC users, and the HIUD users, and p-values were computed with Fisher's exact test for the categorical outcomes, Kruskal-Wallis Rank Sum Test for the continuous and the discrete variables and when relevant, Dunn's test was used for *post hoc* analyses where multiple comparisons were corrected for with the Bonferroni-Holm method. For plasma progesterone and estradiol, we used Gehan test (33) due to censored values (18 estradiol- and 35 progesterone samples were below the detection limit of 0.09 nM and 0.6 nM, respectively).

To evaluate if HC use was associated with global 5-HT4R brain binding (aim 1), we used a linear latent variable model (LVM) where the effects of HIUD- and OC use were mediated through a shared latent variable (hereafter phrased as the global LV) across brain regions of low (neocortex), intermediate (hippocampus) and high (neostriatum) 5-HT4R binding (31). By introducing the global LV, we were able to account for the large inter-correlation in 5-HT4R binding between brain regions. The binding in each region was adjusted independently, i.e., not through the global LV, for age, BMI, 5-HTTLPR genotype (LALA or non-LALA), and injected [11C]SB207145 mass per kg bodyweight as these are considered to influence 5-HT4R PET measurements (34–36). 5-HT4R BP_{ND} values were log-transformed prior to modeling and the regional estimates are expressed as a percent difference in 5-HT4R binding in the OC users *vs.* the non-users and in the HIUD users *vs.* the non-users. To test if the treatment response differed between the groups (aim 2), we used the Kruskal-Wallis Rank Sum Test. For reporting the effect size, we used the Mann-Whitney parameter (37), which gives the probability that a randomly selected individual from one group had a worse treatment response compared to one from another group. An estimate of 0.5 indicates no difference in treatment response. To evaluate if an association between baseline 5-HT4R BP_{ND} and week eight $r\Delta HAMD_6$ depends on OC/HIUD use (aim 3), we extended our LVM by including an interaction term between $r\Delta HAMD_6$ and the HC group to be mediated through the global LV. The estimates related to $r\Delta HAMD_6$ are expressed as the percent change in 5-HT4R BP_{ND} per 10% change in $HAMD_6$ ($r_{10}\Delta HAMD_6$). To evaluate the effect of eight weeks of SSRI/SNRI treatment on neostriatal 5-HT4R binding across groups (aim 4), we used linear regression adjusting for the difference in injected [11C]SB207145 mass per kg bodyweight between baseline and week eight scans. Due to the smaller re-test samples within the OC and the HIUD group, the p-values were also computed with a permutation test with 10,000 permutations.

Diagnostic tools were used to assess the adequacy of the models' assumptions. We used chi-squared tests to evaluate specification of the covariance structure for each LVM with chi-square < 0.05 indicating suboptimal specification [section 6.2.4 in (38)]. Missing data in the analyses regarding treatment response were handled using complete case analysis. Two-sided statistical tests were used and $p < 0.05$ was considered statistically significant. Confidence intervals and p-values computed for the regional effects were adjusted for three comparisons using the single-step Dunnett's procedure (39). Statistical analyses were performed in R (40) and LVMs were estimated with the lava package (38).

RESULTS

Study Population Profile

The clinical profile and PET parameters of the study population at baseline are shown in **Table 1**. The non-users, the HIUD users, and the OC users were similar in terms of proportion suffering from first depressive episode and in clinician rated depressive symptoms ($HAMD_{6/17}$) at baseline. The depressive symptoms scored within a moderate-to-severe depressive episode as restricted by the inclusion criteria. The educational level tended to differ between the groups (p=0.06) such that the HC users tended to have lower education. As expected, the OC users had lower plasma estradiol levels (median [Q1, Q3]: 0.09 nM [0.09, 0.18]) compared to the non-users (median [Q1, Q3]: 0.30 nM [0.16, 0.64]), p_{adj}=0.0002, and the HIUD users (median [Q1, Q3]: 0.23 [0.17, 0.26]), p_{adj}=0.01. The OC users had lower plasma progesterone levels but only compared to the non-users (p_{adj}=0.04). The HIUD users did not seem to differ from the non-users in plasma hormone levels.

Hormonal Contraception and Serotonin 4 Receptor Binding

In support of the LVM structure, we found significant region-specific bindings loading onto the global LV for all regions (p<0.001). From the chi-squared test we found no evidence for a lack of fit (p_{adj}>0.79), so no additional covariance was added to the model. There was no evidence for an association between 5-HT4R binding and OC use (p=0.51) or HIUD use (p=0.73) (**Figure 1**). The corresponding non-significant regional estimates for OC use varied between -2.8% and -1.9%, and for HIUD use between -2.1% and -1.4%. No gross deviation from the normality assumption was observed.

Antidepressant Treatment Response

Week eight depression profiles are shown in **Table 2**. We found no evidence of a difference in proportion of patients who switched to duloxetine after week four (p=0.34) or in week eight treatment response ($r\Delta HAMD_6$) between the groups (p=0.13).The estimated probability of finding a smaller reduction in $HAMD_6$ in an OC and an HIUD user was 66% (95% CI: [0.48; 0.80], p=0.08) and 68% (95% CI: [0.46; 0.83], p=0.11), respectively, compared to in a non-user (**Figure 2**), and notably, this was at a non-significant level as the confidence intervals included 0.5 (p-values were not corrected for multiple comparisons). The OC users seemed to require higher doses of escitalopram compared to the non-users (p_{adj}=0.04) and the HIUD users (p_{adj}=0.01); 77.8% of the OC users were treated with the highest recommended daily dose of 20 mg compared to 35.0% and 22.2% of the non-users and the HIUD users, respectively. Correspondingly, the OC users also had a higher plasma concentration of escitalopram at week eight compared to the HIUD users (p_{adj}=0.03), and at a trend level compared to the non-users (p_{adj}=0.06). The dropouts' depression profiles, in

TABLE 1 | Clinical profile and PET parameters at baseline.

	Non-users (n=23)	HIUD users (n=11)	OC users (n=26)		
	n (%)	**n (%)**	**n (%)**	**p-value$^{\alpha}$**	**n**
First MDD episode	10 (43.5%)	5 (45.5%)	8 (30.8%)	0.61	60
5-HTTLPR L_AL_A genotype	8 (34.8%)	1 (9.1%)	7 (26.9%)	0.32	60
In relationship	7 (30.4%)	6 (54.5%)	9 (34.6%)	0.47	60
	Median [Q1, Q3]	**Median [Q1, Q3]**	**Median [Q1, Q3]**	**p-value$^{\beta}$**	**n**
Age	26.2 [22.9, 30.3]	23.7 [22.5, 24.4]	23.3 [21.6, 25.7]	0.25	60
BMI [kg/m^2]	23.9 [20.5, 31.2]	22.1 [19.2, 23.9]	21.8 [20.0, 24.7]	0.31	60
Educational level	16.0 [16.0, 17.0]	15.0 [12.5, 16.5]	14.5 [13.0, 16.0]	0.06	54
$HAMD_6$	12.0 [11.5, 13.5]	13.0 [12.0, 13.5]	12.5 [12.0, 13.0]	0.87	60
$HAMD_{17}$	22.0 [21.0, 24.0]	25.0 [21.0, 27.0]	23.0 [20.2, 25.0]	0.58	60
P-estradiol [nM]	0.30 [0.16, 0.64]	0.23 [0.17, 0.26]	0.09 [0.09, 0.18]	0.20$^{\gamma}$ 0.0002$^{\delta}$ 0.01$^{\epsilon}$	60
P-progesterone [nM]	0.90 [0.60, 15.00]	0.60 [0.60, 3.85]	0.60 [0.60, 0.67]	0.59$^{\gamma}$ 0.04$^{\delta}$ 0.59$^{\epsilon}$	60
Injected dose [MBq]	600.2 [573.6, 604.2]	591.3 [526.8, 602.4]	602.5 [589.8, 605.2]	0.21	59
Injected tracer mass/kg [µg/kg]	$8.6x10^{-3}$ [$6.5x10^{-3}$, $1.3x10^{-2}$]	$8.6x10^{-3}$ [$6.0x10^{-3}$, $1.7x10^{-2}$]	$9.2x10^{-3}$ [$6.4x10^{-3}$, $1.2x10^{-2}$]	0.91	59
Cerebellum, area under curve [kBq/ml]	10380.8 [9105.6, 12660.3]	10755.5 [9055.6, 11657.2]	11118.2 [9058.6, 13413.4]	0.47	59

HIUD, hormonal intrauterine device; OC, oral contraceptive; MDD, Major Depressive Disorder; BMI, Body Mass Index; $HAMD_{6/17}$, Hamilton Depression Rating Scale 17 or 6 items; MDI, Major Depressive Inventory; $^{\alpha}$p-values are computed with Fischer's exact test. $^{\beta}$p-values are computed with Kruskal-Wallis Rank Sum Test except for P-estradiol and P-progesterone, for which Gehan test was used due to censored values and they were corrected for three comparisons with the Bonferroni-Holm method. $^{\gamma}$Non-users vs. HIUD users. $^{\delta}$Non-users vs. OC users. $^{\epsilon}$HIUD users vs. OC users.

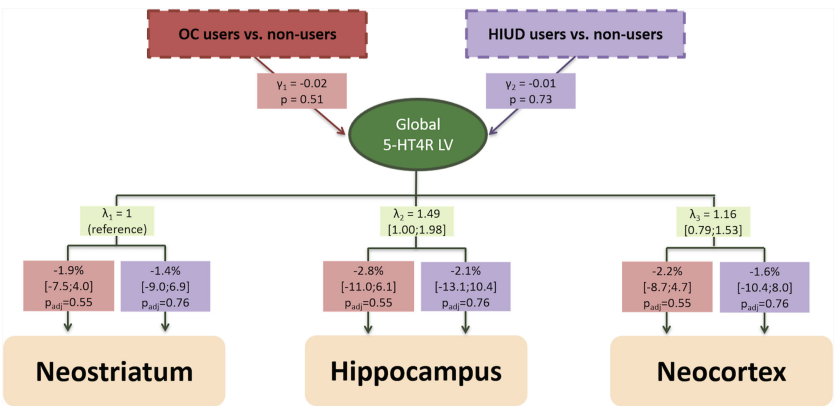

FIGURE 1 | The estimated latent variable model for the effect of oral contraceptive (OC) and hormonal intrauterine device (HIUD) use on baseline 5-HT4R BP_{ND} in women with an untreated depressive episode. γ is the effect on the global latent variable interpreted as global (log-transformed) 5-HT4R BP_{ND} effects. λ is the loading on each region. The boxes beneath the loadings indicate the percentage difference in 5-HT4R binding for each brain region in OC- and HIUD users compared to non-users. Regional bindings were adjusted for Age, BMI, 5-HTTLPR genotype and injected tracer mass per kg bodyweight (not shown). P-values and confidence intervals are adjusted for 3 comparisons by use of the Dunnett's test.

terms of baseline $HAMD_{6/17}$ as well as relative change in $HAMD_6$ in percentage from baseline to week one, two, four and eight and reason for dropout, are presented in **Table S2**.

Pre-treatment Serotonin 4 Receptor Binding and Antidepressant Treatment Response

Again, the LVM was supported by the region-specific bindings loading onto the global LV ($p<0.001$). The chi-squared test showed no evidence of misspecification of the covariance structure ($p_{adj}>0.41$). The estimated associations between baseline global 5-HT4R binding (represented as the global LV) and treatment responses across each group are shown in **Figure 3** with a trend towards a positive slope in the non-users ($1.33x10^{-2}$ (p=0.10)), a trend towards a negative slope in the OC users ($-1.27x10^{-2}$ (p=0.07)), and a non-significant negative slope in the HIUD users ($-3.34x10^{-4}$ (p=0.75)). The corresponding correlation coefficients are 0.39 for the non-users, -0.46 for the OC users, and -0.12 for the HIUD users. In the OC users, the slope differed by $-2.59x10^{-2}$ (p=0.02) from the non-users. In **Figure 4**, the LVM is summarized in three layers (A, B and C). The first (A) shows the trend towards a positive association

TABLE 2 | Clinical depression profile at week eight.

	Non-users (n=21)	HIUD users (n=11)	OC users (n=20)		
	n (%)	**n (%)**	**n (%)**	**p-value$^{\alpha}$**	**n**
Switchers to duloxetine	1 (4.8%)	2 (18.2)	2 (10)	0.34	52
	Median [Q1, Q3]	**Median [Q1, Q3]**	**Median [Q1, Q3]**	**p-value$^{\beta}$**	**n**
rΔHAMD$_6$ [%]	-63.6 [-78.6, -46.2]	-50.0 [-64.1, -23.7]	-45.3 [-64.9, -15.1]	0.13	52
P-escitalopram [nM]	68.1 [43.3, 102.8]	42.5 [36.0, 108.5]	105.7 [74.1, 139.8]	0.40$^{\gamma}$	47
				0.06$^{\delta}$	
				0.03$^{\epsilon}$	
	n (%)	**n (%)**	**n (%)**	**p-value$^{\alpha}$**	**n**
Escitalopram dose:					
5 mg	0 (0.0)	1 (11.1)	0 (0.0)	0.26$^{\gamma}$	47
10 mg	2 (10.0)	2 (22.2)	1 (5.6)	0.04$^{\delta}$	
15 mg	11 (55.0)	4 (44.4)	3 (16.7)	0.01$^{\epsilon}$	
20 mg	7 (35.0)	2 (22.2)	14 (77.8)		

HIUD, hormonal intrauterine device; OC, oral contraceptive; rΔHAMD$_6$, relative change in Hamilton Depression Rating Scale 6 items from baseline. $^{\alpha}$p-value is computed with Fischer's exact test. $^{\beta}$p-values are computed with Kruskal-Wallis Rank Sum Test and, for post hoc analyses, with Dunn's test corrected for three comparisons with the Bonferroni-Holm method. $^{\gamma}$Non-users vs. HIUD users. $^{\delta}$Non-users vs. OC users. $^{\epsilon}$HIUD users vs. OC users.

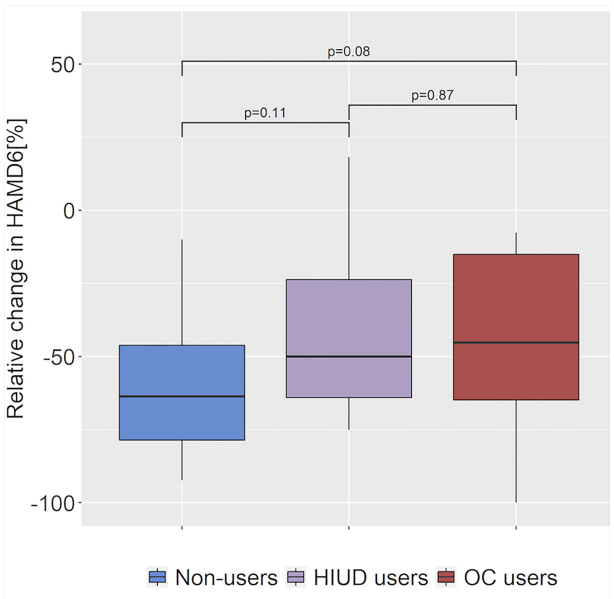

FIGURE 2 | Antidepressant drug treatment response at week eight across hormonal contraceptive user status. Difference in relative change in Hamilton Depression Rating Scale 6 items (HAMD6) from baseline between the HC non-users, the hormonal intrauterine device (HIUD) users and the oral contraceptive (OC) users. A larger negative change corresponds to a better improvement of depressive symptoms. P-values are computed with Mann-Whitney tests with no correction for multiple comparisons.

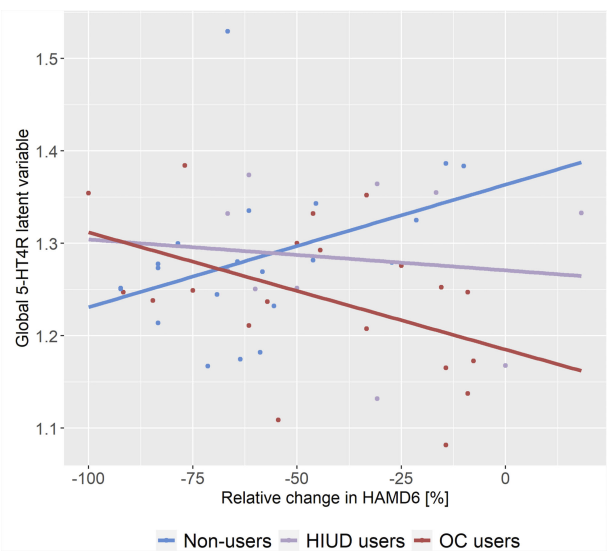

FIGURE 3 | Estimated global 5-HT4R latent variable summarizing the association between baseline global 5-HT4R brain binding and week eight antidepressant drug treatment response across the groups. The slopes are the association between baseline (un-medicated) 5-HT4R binding and change in Hamilton Depression Rating Scale 6 items (HAMD$_6$) in each group, respectively. A negative change in HAMD$_6$ mirrors an improvement of depressive symptoms. The slopes are 1.33×10^{-2} (p_{adj}=0.10) for the non-users, -3.34×10^{-3} (p_{adj}=0.75) for the hormonal intrauterine device (HIUD) users, and -1.27×10^{-2} (p_{adj}=0.07) for the oral contraceptive (OC) users.

between the 5-HT4R binding and $r_{10}\Delta HAMD_6$ mediated through the global LV in the non-users (p=0.10). The second layer (B) shows how this association differs in the HC users from the non-users. The regional estimates show that per 10% less improvement in HAMD$_6$, the OC users had 2.6-4.0% ($p_{adj} \leq 0.03$) lower binding at baseline compared to the non-users. The third layer (C) shows the estimated effect of HC use on 5-HT4R binding if no treatment response was achieved at week eight, i.e., the estimated HC effect when accounting for the confounding factor of a suggested serotonergic subtype of MDD. However, as we have only few observations at this part of the response scale, these estimates are calculated based on the model and less on actual observed differences. At the regional level, the percent effect of OC use varied between -24.2% and -16.3% ($p_{adj} \leq 0.01$). No gross deviation from the normality assumption was observed for any of the LVMs.

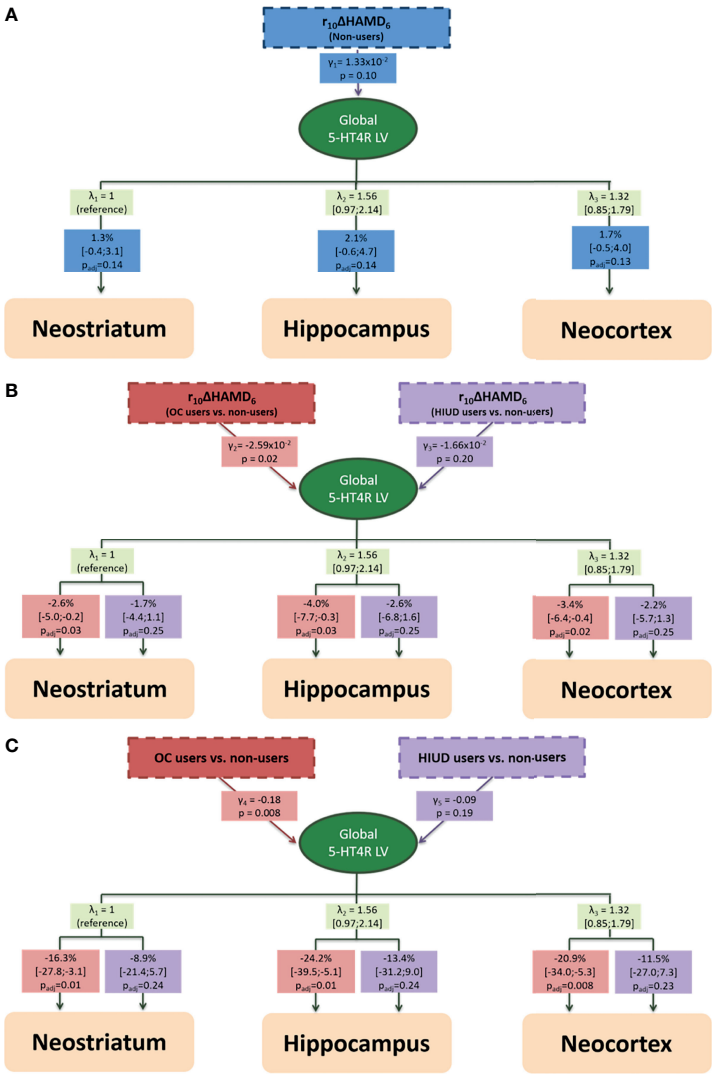

FIGURE 4 | The estimated latent variable model for the association between baseline 5-HT4R binding and week eight antidepressant treatment response as a function of hormonal contraceptive status shown in three layers **(A–C)**. **(A)** The association between baseline 5-HT4R binding and treatment response in non-users. **(B)** The difference in the association between baseline 5-HT4R binding and treatment response between the non-users and the oral contraceptive (OC) and the hormonal intrauterine device (HIUD) users, respectively. **(C)** The estimated effect of OC and HIUD use on 5-HT4R binding when no change in Hamilton Depression Rating Scale 6 items (HAMD6). γ is the estimates of the association with the global latent variable interpreted as global (log-transformed) 5-HT4R BP_{ND} estimates. λ is the loading on each region. The boxes beneath the loadings indicate the percentage difference in 5-HT4R binding for each brain region. Regional bindings were adjusted for Age, BMI, 5-HTTLPR genotype and injected tracer mass per kg bodyweight (not shown). P-values and confidence intervals are adjusted for 3 comparisons. r_{10}ΔHAMD$_6$: 10% relative change in Hamilton Depression Rating Scale 6 items.

Antidepressants' Effect on Serotonin 4 Receptor Binding

In **Figure 5**, we show the effects of eight weeks of SSRI/SNRI treatment on the 5-HT4R BP_{ND} across the groups. A reduction in 5-HT4R BP_{ND} appeared to be present in all groups with an estimated reduction in the non-users of 7.0% (95% CI: [-12.1; -1.6], p=0.02). Due to the small sample sizes in the HIUD- and the OC group, the effect sizes are less reliable, and the p-values are also computed after 10,000 permutations. It revealed that, if the null hypothesis is true (i.e., no change in 5-HT4R BP_{ND} after eight weeks of SSRI treatment), the observed lower 5-HT4R BP_{ND} would be found by chance in 9-10% of the cases, hence, a reduction in 5-HT4R binding is only found at a trend level in the HIUD and the OC users.

DISCUSSION

In this study, we found no evidence of a difference in 5-HT4R binding in unmedicated depressed women who use OCs or

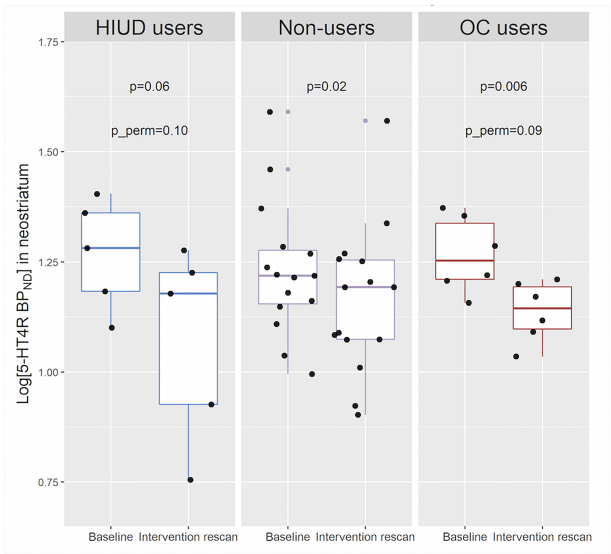

FIGURE 5 | The effect of eight weeks of SSRI/SNRI treatment on neostriatal 5-HT4R binding across the groups. p_perm: p-values based on 10,000 permutations to account for the small sample sizes in the oral contractive (OC)- and the hormonal intrauterine device (HIUD) user groups.

HIUDs relative to non-users. We found a trend towards an association between baseline global 5-HT4R BP_{ND} and treatment response in the non-users and the OC users and these were in opposite directions with a significant crossover interaction, such that the OC users had 3-4% lower binding compared to the non-users for every 10% percent less improvement in $HAMD_6$ at week eight. Based on our model, the main effect of OC use on regional 5-HT4R binding at zero improvement in depressive symptoms was estimated to be between -16 and -24%. In addition, we observed no strong evidence of a difference in treatment response between the groups, although, the estimated probability of finding a smaller reduction in $HAMD_6$ in an OC and an HIUD user was numerically higher compared to in a non-user. Last, we found that as in the non-users, both the HIUD users and the OC users seemed to react to SSRI treatment in terms of neostriatal 5-HT4R downregulation, however, only at a trend level.

Hormonal Contraceptive Use and Brain Serotonin

In a healthy population we found OC use to be associated with 9-12% lower global 5-HT4R binding (21), which was not seen in this depressed cohort, however, the depressed cohort is also 7-8% lower in binding compared to a healthy population (26). Hence, the OC effect on 5-HT4R binding may be obscured by the effects of the MDD as expected, and thus we see no indication of the effects of OC and MDD being additive. Comparable to OC use, we found a numerically lower, but still statistically non-significant effect of HIUD use. In the previous study on the healthy population (21), we were underpowered to investigate an effect of HIUD use. Thus, brain signatures of HIUD use remain to be studied since HIUD use is also associated with an increased risk of developing MDD (4, 5).

Whereas we found no difference in 5-HT4R binding level at baseline between the groups, we observed a crossover interaction between the OC users and the non-users in the association between baseline 5-HT4R BP_{ND} and treatment response, but notably, the association within each group was only borderline significant, which may be due to power issues. As we were not expecting to see a negative trend in the association in the OC users, this highlights the question whether the 5-HT4R setting may differ qualitatively, i.e., whether the mechanisms behind a lower 5-HT4R level differ. As shown in a mixed male-female cohort of MDD patients, only those responding to treatment had a lower baseline (unmedicated) 5-HT4R level compared to healthy controls, so it has been speculated whether they have a serotonergic subtype of MDD, in which the lower binding is due to a compensatory upregulation of the serotonergic tonus as an attempt to maintain euthymia (26). In contrast, we speculate whether all depressed OC users belong to a special serotonergic subgroup with varying degree of serotonergic involvement. We suggest that the mechanism affecting the 5-HT4R level in OC users may be driven by a hormone-dependent decrease in gene expression levels due to a suppressed hormone state in OC users, which align with previous observations in healthy OC users (21). This also aligns with preclinical research showing that estradiol supports serotonergic signaling in terms of increased capacity of serotonin synthesis and reuptake (41, 42), reduced capacity of serotonin degradation (41), increased neural firing (43) and increased serotonin receptor availability (44), which mainly happen *via* gene expression through estrogen receptor alpha and -beta (45). Also notably, estradiol increases 5-HT4R mRNA expression in the anterior pituitary cells in rats (46). We speculate that the more the 5-HT4R expression level as well as other parts of the serotonin signaling are compromised by the suppressed hormone state in OC users, the worse the treatment response, and in contrast, only the non-users with a serotonergic component show a better response to an SSRI targeting this system. If this theory is applied on our results and we compare the 5-HT4R levels at $r\Delta HAMD_6$ equal to 0% (i.e., the results from the third layer of the LVM), we compare the effect of OC use with a population with a non-serotonergic subtype of MDD, which reveals an OC effect equal to about -20%. However, since this result is based on our model with rather few observations in this range of the treatment response, it should be interpreted with some caution.

The association between baseline binding and treatment response did not differ between the HIUD users and the non-users, however, the estimate was in the same direction as in the OC users. It is possible that, if replicated in larger sample sizes, this reflects an effect of HIUD use on serotonin brain signaling.

Hormonal Contraceptive Use and Antidepressant Treatment Response

The estimated probability of finding a smaller reduction in $HAMD_6$ in an OC and an HIUD user compared to a non-user was 66% and 68%, respectively, but the confidence intervals included 50%, so we have insufficient evidence to conclude that

differences in the treatment response exist. This might be a question of power as the confidence intervals were wide, e.g., the OC users *vs*. the non-users 95% confidence spanned from 48% to 80%. Larger samples would help narrow the confidence intervals to help us discriminate between differences in treatment response *vs*. no or very small differences. The median $r\Delta HAMD_6$ was, at the non-significant level, about 18 percent point and 14 percent point lower in the OC and the HIUD users, respectively, relative to the non-users. This is equivalent to about two points based on median $HAMD_6$ scores at baseline. Treatment response could be affected by educational level (47) as the non-users tended to have higher educational level, however, this could also be related to the HC users not having started their final degree, yet, as the median age in the HC users was about 23 *vs*. 26 in non-users. Adjusting for educational level did not have any notable impact on the result (not shown). Limited and insufficient clinical evidence exists addressing whether HC use affects SSRI treatment response. The STAR*D study reported a trend towards better remission rates to citalopram treatment in 226 HC users compared to 670 HC non-users, however, this effect was not robust to adjustment for potential confounders (48). Another study, based on 17 double-blind, placebo-controlled clinical trials with 1698 women, found no difference in treatment response between the OC users and the non-users measured by change in $HAMD_{17}$ (49). It has been highlighted that the studies are limited by lacking information on the types of HCs used, for not including or missing response rates across the trials, and for including studies with variable SSRI dosages (50). The latter could be relevant as higher dosage was required in the OC users, which could make up for less response in OC users relative to non-users. Correspondingly, serum levels of escitalopram seemed to be higher in the OC users, which might also be a result of an OC-induced inhibition of the cytochrome P450 (CYP) hepatic enzymes, CYP2C19 and CYP3A4 (51), which makes up about 70% of the escitalopram metabolism (52).

Preclinical evidence points towards a modulatory role of estradiol on antidepressant drug effects in rats (53–56). One study found that exogenous administration of 17 beta-estradiol as well as ethinylestradiol in ovariectomized female rats facilitates the antidepressant-like effects of fluoxetine and desipramine in a forced swim test (53). In support of that, other studies found that deficiency of brain estrogen levels attenuated sertraline- (54) and duloxetine (55)-induced antidepressive behaviors in mice and it correlated with serotonin turnover in hippocampus and prefrontal cortex (54, 55). In contrast, another study found that acute administration of estrogen with progesterone blocked the antidepressant-like effect of desipramine, but only after acute, not chronic administration (56).

Suppressed levels of endogenous estradiol thus might affect SSRI treatment response and possibly this involves the 5-HT4R. A difference in the 5-HT4R setting could potentially affect the treatment effects as it plays a key role in serotonergic neuronal firing from the dorsal raphe nuclei with projections to most parts of the brain (57).Thus, a lower 5-HT4R agonism capacity could make the serotonergic brain function less adaptable to environmental demands. This aligns with rodent data showing that 5-HT4R partial agonists reduce stress-induced antidepressive behavior (58), have fast acting antidepressant-like effects (59), and that the antidepressant-like effects of SSRIs depends on 5-HT4R activation (60). Whereas our data highlight that treatment response depends on the initial 5-HT4R setting in an OC-dependent manner, we also see a trend towards the expected SSRI-effect on the neostriatal 5-HT4R level after eight weeks of treatment, supporting that the 5-HT4R setting in HC users is sensitive to SSRIs.

CONCLUSION AND PERSPECTIVES

In a depressed cohort, we found no evidence of a difference in the 5-HT4R level between the non-users and the HC users, but the associations between 5-HT4R binding and treatment response were in opposite directions in the OC users and the non-users; the lower the global 5-HT4R binding before treatment start the worse the treatment response in the OC users in contrast to better treatment response in the non-users. We found no strong evidence of a difference in treatment response between the groups. From this study, we speculate that depressed OC users constitutes a special serotonin subtype of MDD which might have implications for SSRI treatment. We will in a planned longitudinal study address the causal relationship between OC use and lower 5-HT4R binding and future and properly-designed studies must address if OC use affects treatment response in depressed women and whether they perhaps could benefit from tapering off OC use. Also, the effects of HIUD use on brain serotonin signatures remains to be investigated in future studies.

Methodological Considerations

When interpreting the results from this study, some limitations should be considered; 1) the sample size is small, so interpretation should be made with caution, especially with regard to HIUD use and when comparing treatment responses, 2) the OC group was pooled from users of different types of OCs (combined OCs and progesterone-only pills) with different hormone content, which may blur the results, 3) we lacked information on previous HC use among the non-users and information on starting day and pill-free days in the HC users, which could have helped us understand the potential role of HC use in their depressive episode, 4) we had 8 dropouts during follow-up which were mainly OC users. However, based on their last response rate (**Table S2**), they seem more or less equally distributed between those showing some degree of response and those who do not, so it appears not to introduce a selection bias.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: For accessing the dataset, a Cimbi

database application is required. Requests to access these datasets should be directed to the corresponding author (vibe.frokjaer@nru.dk) or access can be requested through the procedures outlined here: https://cimbi.dk/index.php/documents/category/3-cimbi-database.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Committees on Health Research Ethics in the Capital Region of Denmark (H-15017713). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SL and VF contributed to study conceptualization, data collection, analysis and interpretation of results, participated in drafting the manuscript, and approved the final version prior to submission. BO contributed to analysis and interpretation of data, revised the manuscript critically for important intellectual content, and approved the final version prior to submission. KK-F, AP, VD, and CS contributed to data collection and the acquisition of data, revised the manuscript critically for important intellectual content, and approved the final version prior to submission. GK and MJ contributed to study conceptualization and interpretation of data, revised the manuscript critically for important intellectual content, and approved the final version prior to submission. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.799675/full#supplementary-material

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Prenatal Progestin Exposure-Mediated Oxytocin Suppression Contributes to Social Deficits in Mouse Offspring

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Epidemiological studies have shown that maternal hormone exposure is associated with autism spectrum disorders (ASD). The hormone oxytocin (OXT) is a central nervous neuropeptide that plays an important role in social behaviors as well as ASD etiology, although the detailed mechanism remains largely unknown. In this study, we aim to investigate the potential role and contribution of OXT to prenatal progestin exposure-mediated mouse offspring. Our *in vitro* study in the hypothalamic neurons that isolated from paraventricular nuclei area of mice showed that transient progestin exposure causes persistent epigenetic changes on the OXT promoter, resulting in dissociation of estrogen receptor β (ERβ) and retinoic acid-related orphan receptor α (RORA) from the OXT promoter with subsequent persistent OXT suppression. Our *in vivo* study showed that prenatal exposure of medroxyprogesterone acetate (MPA) triggers social deficits in mouse offspring; prenatal OXT deficiency in OXT knockdown mouse partly mimics, while postnatal ERβ expression or postnatal OXT peptide injection partly ameliorates, prenatal MPA exposure-mediated social deficits, which include impaired social interaction and social abilities. On the other hand, OXT had no effect on prenatal MPA exposure-mediated anxiety-like behaviors. We conclude that prenatal MPA exposure-mediated oxytocin suppression contributes to social deficits in mouse offspring.

Keywords: autism spectrum disorders, oxytocin, oxidative stress, progestin, social deficits

Abbreviations: ASD, autism spectrum disorders; ChIP, chromatin immunoprecipitation; EPM, elevated plus maze; ERE, estrogen response element; ERβ, estrogen receptor β; MBT, marble-burying test; MPA, medroxyprogesterone acetate; O2, superoxide anions; OXT, oxytocin; OXTR, oxytocin receptor; PVN, paraventricular nuclei; RORA, retinoic acid-related orphan receptor alpha; ROS, reactive oxygen species; SI, social interaction; SOD2, superoxide dismutase 2; USV, ultrasonic vocalization.

INTRODUCTION

Autism spectrum disorders (ASD) are a series of neurodevelopmental disorders characterized by symptoms including social deficits and restricted or repetitive behaviors (1, 2). While the potential mechanism for ASD remains unclear, many factors, including environmental exposure, sex, and epigenetic modifications, are reported to be associated with ASD development (1, 3, 4). It has been reported that ASD patients have increased steroidogenic activity and that abnormal steroid levels may be involved in ASD development (5, 6). We have previously reported that maternal exposure to either progestin (7, 8) or androgens (9) contribute to autism-like behaviors in offspring; and the epidemiological study shows that maternal hormonal exposure may be associated with autism development (10).

Oral contraceptive hormones, primarily including estrogens and progestins, were originally used starting around 60 years ago for birth control by preventing ovulation; this time period has been reported to coincide with the dramatic increase in ASD prevalence (8, 10). Our epidemiological study has shown that the following 3 risk factors are highly associated with ASD: 1) Use of progestin to prevent threatened abortion, 2) Use of progestin contraceptives at the time of conception, and 3) prenatal consumption of progestin-contaminated food (10). We then hypothesize that maternal exposure to oral contraceptive hormones, especially progestin, may be associated with autism development.

Oxytocin (OXT) is a neuropeptide primarily secreted by hypothalamic neurons that located in either the paraventricular nuclei (PVN) or supraoptic nuclei (SON) (11). OXT, in conjunction with oxytocin receptor (OXTR) (12), has been reported to play an important role in regulation of social recognition and anxiety-like behaviors (13–16) as well as many other kinds of pathophysiological processes (17). OXT/OXTR signaling abnormalities have been associated with ASD (18, 19). We have previously reported that maternal diabetes-mediated OXTR suppression contributes to social deficits in mouse offspring (20), while the detailed mechanism for the role of OXT in ASD development remains largely unknown (21).

Estrogen receptor β (ERβ) is widely expressed in a variety of brain regions and has been reported to be associated with anxiety-like behaviors and ASD development (8, 22–24). We have previously reported that ERβ expression is reduced in the amygdala, contributing to prenatal progestin exposure-mediated autism-like behaviors in rat offspring (7, 8). Additionally, ERβ regulates the expression of superoxide dismutase 2 (SOD2), modulating cellular oxidative stress (25). Interestingly, both ERβ and SOD2 are suppressed in maternal diabetes-mediated autism-like mouse offspring (26). ERβ is highly expressed and co-localized in OXT neurons in the hypothalamic region, and OXT may be regulated directly or indirectly by ERβ, while the possible mechanism remains largely unknown (12, 27, 28).

In this study, we aim to investigate the role and mechanisms for maternal progestin exposure-mediated OXT suppression and its contribution to social behaviors in offspring. Our *in vitro* study in mouse hypothalamic neurons showed that transient treatment by 10μM of medroxyprogesterone acetate (MPA) for 3 days triggers persistent OXT suppression through epigenetic modifications and subsequent dissociation of ERβ and retinoic acid-related orphan receptor α (RORA) (29) from the OXT promoter, indicating that ERβ and RORA may play a role in progestin-mediated OXT suppression. We then conducted the *in vivo* mouse study, and we found that prenatal exposure to MPA triggers OXT suppression as well as autism- and anxiety-like behaviors in offspring. Prenatal OXT deficiency had no effect on prenatal MPA exposure-induced anxiety-like behavior, but it partly mimicked prenatal MPA exposure-mediated social deficits in offspring. We next conducted postnatal gene manipulation of ERβ and RORA targeting to hypothalamic OXT neuron-located PVN area, and we found that postnatal ERβ expression partly ameliorated prenatal MPA exposure-induced social deficits, while postnatal RORA expression had no effect. Furthermore, postnatal OXT peptide injection to the third ventricle partly ameliorated prenatal MPA exposure-induced social deficits in offspring as well. We conclude that prenatal MPA exposure-mediated oxytocin suppression contributes to social deficits in mouse offspring.

MATERIALS AND METHODS

An expanded section for Materials and Methods is available in **Supplementary Information** (see **Data S1**), and the details for used primers are available in **Table S1**.

Reagents and Materials

The primary hypothalamus neurons were isolated from the paraventricular nucleus (PVN) area of experimental mice. The antibodies for β-actin (sc-47778), p53 (sc-126), RORA (sc-518081), RXRα (sc-515929) and SOD2 (sc-30080) were purchased from Santa Cruz Biotechnology. The oxytocin (OXT) from tissue, culture medium, serum and cerebrospinal fluid (CSF) was determined using the Oxytocin ELISA Kit (ab133050) according to manufacturers' instructions.

Generation of OXT Reporter Construct

The genomic DNA was purified from primary mouse hypothalamic neurons, and the mouse OXT promoter (2kb upstream + first exon) was identified from Ensembl gene ID: OXT-201 ENSMUST00000028764.6, and amplified by PCR, then subcloned into the pGL3-basic vector (# E1751, Promega) using the following primers with underlined restriction sites: OXT forward: 5'-gcgc-acgcgt- cta acc taa agc cca aag ctg -3' (Mlu I) and OXT reverse: 5'- gtac- aagctt- ctt gcg cat atc cag gtc cag -3' (Hind III). To map the progestin-responsive element on the OXT promoter, the related OXT deletion reporter constructs were generated using PCR techniques and subcloned into pGL3-basic vector (30).

Generation of Expression Lentivirus

The mouse ERβ expression lentivirus was prepared previously in our lab (20). The cDNA for mouse RORA was purchased from

Open Biosystems and then amplified using the following primers with underlined restriction sites: RORA forward primer: 5'- gtac - gggccc- atg gag tca gct ccg gca gcc -3' (ApaI) and RORA reverse primer: 5'- gtac - tctaga- tta ccc atc gat ttg cat ggc -3' (Xba1), and then subcloned into the pLVX-Puro vector (from Clontech). The lentivirus for ERβ, RORA, and empty control were expressed using Lenti-X^{TM} Lentiviral Expression Systems (from Clontech) and concentrated according to manufacturers' instructions (26).

DNA Methylation Analysis

The DNA methylation on the OXT promoter was evaluated using a methylation-specific PCR-based method as described previously with minor modifications (31–33). The mouse genomic DNA was extracted and purified from primary hypothalamic neurons, and then treated by bisulfite modification through EpiJET Bisulfite Conversion Kit (#K1461, from Fisher). The treated DNA was then amplified using the following primers: Methylated primer: forward 5'- tga aaa ata gtt ttt ggt tag ggc -3' and reverse 5'- ctc tta aat caa att att cca cgc t -3'; Unmethylated primer: forward 5'- gaa aaa tag ttt ttg gtt agg gtg t -3' and reverse 5'- ctc tta aat caa att att cca cac t -3'. Product size: 198bp (methylated) & 197bp (unmethylated); CpG island size: 227bp; Tm: 68.4°C. The final DNA methylation results were normalized by DNA unmethylated results as input.

In Vivo Mouse Experiments

Generation of neuron-specific OXT knockout mice. The OXT$^{fl/fl}$ mouse, which has loxP flanking sites targeting exon 3 of the OXT gene, was generated by *in vitro* fertilization and was obtained for this study as a generous gift from Dr. Haimou Zhang (Hubei University). The Oxytocin-Ires Cre mice (Oxt^{Cre}, #024234), which expresses Cre recombinase under the control of the oxytocin promoter, was obtained from Jackson Laboratories. To generate neuron-specific OXT$^{-/-}$ null mice (Oxt^{Cre}-OXT$^{fl/fl}$), OXT$^{fl/fl}$ mice were cross-bred with Oxt^{Cre} mice for over 4 generations on the C57BL/6J background. Positive offspring were confirmed by genotyping through PCR using specific primers (see **Table S1**) for the presence of both loxP sites within OXT alleles and Cre recombinase (34, 35). The experimental animals were either OXT wild type (WT) or OXT null (OXT$^{-/-}$) mice with C57BL/6J genetic background as described above.

Mouse Protocol 1: Prenatal treatment by progestin MPA or OXT deficiency. Female mice (3-month old) were mated with males, and the pregnant dams were verified, then received either MPA treatment (20 mg/kg body weight, which is similar or equal to high-dose of women exposure) or control (CTL) group that received vehicle only, which containing 1% ethanol in organic sesame oil, and 0.1 ml of drugs were given every 2 days by peritoneal injection from day 1 until offspring delivery for ~21 days in total. The above treated dams were then randomly assigned to the below 4 groups: Group 1: OXT WT background dams receiving CTL injection (CTL/WT); Group 2: OXT WT background dams receiving MPA injection (MPA/WT); Group 3: OXT null background dams receiving CTL injection (CTL/OXT$^{-/-}$); Group 4: OXT null background dams receiving MPA injection (MPA/OXT$^{-/-}$). 10 dams were assigned for each group, and one representative offspring was selected randomly from each dam for experiments and analysis. Nine representative offspring were selected from the 10 in total in order to account for potential death of an experimental animal during the process. Hypothalamic neurons from PVN area were isolated on embryonic day 18 (E18), and the offspring were then fed by normal chow until 7-8 weeks old, after which they were given behavior tests. The offspring were then sacrificed; the serum and CSF were collected for OXT analysis and various brain tissues, including the amygdala, hypothalamus (PVN area) and hippocampus, were isolated for further biological assays, including gene expression and oxidative stress.

Mouse Protocol 2: Postnatal manipulation of ERβ/RORA lentivirus-carried expression. At 6-week of age, offspring of OXT wild type background that received either the CTL or MPA treatment as described in Mouse Protocol 1 were anesthetized by a mixture of ketamine (90 mg/kg) and xylazine (2.7 mg/kg) and implanted with a guide cannula targeting the PVN area by the direction of an ultra-precise stereotax (Kopf Instruments) using the coordinates of 0.85 mm posterior to the bregma, 0.15 mm lateral to the midline, and 4.8 mm below the skull surface (36). The lentivirus for expression of ERβ (↑ERβ), RORA (↑RORA), or empty (EMP) was infused immediately by a flow rate of 0.5 μl/h after placement of the cannula and minipump, and in total, 0.5μl of (2×10^3 cfu) lentivirus was infused in 1 hour, and the lentivirus was dissolved in artificial cerebrospinal fluid (aCSF), which containing 140 mM NaCl, 3 mM KCl, 1.2 mM Na2HPO4, 1 mM MgCl2, 0.27 mM NaH2PO4, 1.2 mMCaCl2, and 7.2 mM dextrose in pH 7.4. The experimental animals were randomly separated into the following 4 groups (10 mice each group). Group 1: CTL treated offspring received vehicle lentivirus infusion (CTL/P-EMP); Group 2: MPA treated offspring received vehicle lentivirus infusion (MPA/P-EMP); Group 3: MPA treated offspring received ERβ lentivirus infusion (MPA/P-↑ERβ); Group 4: MPA treated offspring received RORA lentivirus infusion (MPA/P-↑RORA). To confirm a successful lentivirus injection into PVN area, the cannula placement was checked histologically postmortem by injection of 0.5μl India ink. Animals whose dye injections were not located in the PVN area were excluded from the final analysis, and the offspring were used for behavior tests after two-week of lentivirus infusion followed with biological assays as indicated in Mouse Protocol 1 (37).

Mouse Protocol 3: Postnatal administration of OXT peptides. The offspring (6-week old) from Mouse Protocol 1 were anesthetized and implanted with a guide cannula targeting the third ventricle at the midline coordinates of 1.8 mm posterior to the bregma and 5.0 mm below the skull surface (36). Two weeks were allowed for mice to recover from surgery, and each mouse then received injection with either aCSF as vehicle (VEH) control or oxytocin peptide (OXT, dissolved in aCSF) *via* pre-implanted cannula (36, 38). The experimental animals were then randomly separated into the following 4 groups (10 mice each group). Group 1: CTL treated offspring received vehicle injection (CTL/P-VEH); Group 2: MPA treated offspring received vehicle injection (MPA/P-VEH); Group 3: CTL treated offspring

received OXT peptide injection (MPA/P-OXT); Group 4: MPA treated offspring received OXT peptide injection (MPA/P-OXT). The oxytocin (0.1 mM, diluted in aCSF, 1 μg/20μl aCSF) or vehicle was locally administered *via* the installed catheter (39). 20 min (including a period for 5 min-adaptation in the test cage) after the injection, the offspring were used for behavior tests followed by biological assays, as indicated in Mouse Protocol 1 (37).

Animal Behavior Tests

The animal behavior tests were evaluated at ages of 7-8 weeks old from offspring unless otherwise mentioned. Anxiety-like behavior was determined by the marble-burying test (MBT) and the elevated plus maze (EPM) tests (7). Autism-like behavior was determined by ultrasonic vocalization (USV), social interaction (SI) test and a three-chambered social test (40–42), and the details for these tests are described in **Supplementary Information**.

Isolation of Brain Tissues

The brain tissues were isolated from experimental offspring for further biological assays. The experimental mouse was deeply anesthetized through free breathing of isoflurane vapor (> 5%). The whole blood was then withdrawn by heart puncture for PBMC isolation and the mouse was perfused transcardially by 20 ml cold perfusion solution for 5 min. The skull was cut using a pair of small surgical scissors and the brain was carefully freed from the skull before being transferred to a petri dish (60 mm×15 mm) that was filled with ice-cold DPBS solution. The targeted brain regions, including the amygdala, hypothalamus (PVN area) and hippocampus, were dissected under the surgical microscope under the referred location from the atlas outlined in *The Mouse Brain in Stereotaxic Coordinates (3rd Edition)*. A separate petri dish was prepared for each of the target regions. The whole dissection process was carried out in the span of no more than one hour. The dissected tissues were then frozen at -80°C for either immediate use or later biological assays (43, 44).

Collection of Cerebrospinal Fluid

The procedure for CSF collection is based on a previously established protocol with minor modifications. In brief, the mouse was anesthetized and the shaved head was clamped in place for dissection under a dissecting microscope. The layers of muscles were carefully dissected away using forceps and the dura over the cisterna magna was exposed. This area has large blood vessels running through, which is optimal for capillary insertion and CSF collection. The angle of the glass capillary was carefully adjusted and the sharpened tip of glass capillary was aligned and eventually tapped through the dura to collect CSF using a micromanipulator control. Approximately 20 μl of CSF was automatically drawn into the capillary tube once the opening was punctured. The glass capillary was gently removed from the mouse by micromanipulator control and the CSF was then mixed with 1 μl of 20x protease inhibitor in a 1.5 ml centrifuge tube for a quick centrifugation (pulse spin for 5 seconds at maximal speed), and the CSF samples were aliquoted for either immediate analysis or stored at -80°C (45).

In Vitro Primary Culture of Hypothalamic Neurons

The isolation of hypothalamic neurons was carried out following a previously described procedure with minor modifications. Three to five hypothalami from PVN area of mice on embryonic day 18 (E18 rats) were isolated, pooled, and then dissociated into single cell suspension by trituration. They were then transferred to a culture dish, which containing primary DMEM culture medium, 10% FBS, 10% heat-inactivated horse serum, 20mM D-glucose and combined antibiotics (from Invitrogen). The osmolarity of medium was then adjusted to 320-325 mOsm using glucose. The subsequent cell suspension was then split into tissue culture flasks that coated with 100μg/ml of poly-L-lysine (Sigma). 24 hours of incubation were allowed for cells to attach to the flask at 37°C with 5% CO2, the medium was then refreshed for cells to growth until confluent for further biological assays (46). The isolated primary hypothalamic neurons were used for *in vitro* cell culture study until passage 3. For mapping of progestin-responsive element on the OXT promoter, the cells were immortalized by an hTERT lentivirus vector for a longer life span (up to passage 12) to achieve better transfection efficiency and higher experimental stability as described previously (47, 48).

RESULTS

Transient Progestin Treatment Causes Persistent OXT Suppression and Oxidative Stress; ERβ Expression Completely, While RORA Expression Partly, Reverses This Effect

We first determined the possible effect of MPA treatment on OXT expression. Mouse hypothalamic neurons were treated by MPA for 3 days and then cultured for another 3 days in the absence of MPA, but with the infection of either ERβ (↑ERβ) or RORA lentivirus (↑RORA) for biological assays. Our results showed that 3-day MPA treatment significantly suppressed OXT mRNA levels and that OXT mRNA remained low after removal of MPA. Infection of ERβ lentivirus completely, while RORA expression partly, reversed this effect (see **Figures 1A, B**). We also measured mRNA expression of these genes at the end of the treatment on day 6, and the results showed that lentivirus infection of either ERβ or RORA was successful. Transient MPA treatment significantly suppressed expression of ERβ, SOD2 and RORA, and the expression remained low during subsequent MPA absence (see **Figure 1B**). We then evaluated protein levels of these genes by either western blotting (see **Figures 1C, D**, **S1A**) or ELISA for OXT (see **Figure 1E**), and the expression pattern was similar to that of mRNA levels. In addition, we conduct immunostaining of OXT for the hypothalamic neurons that isolated from PVN area of mice, and the results showed that almost all the neurons had OXT expression (see **Figure S2**), indicating a successful OXT neuron preparation. We also evaluated the potential effect of MPA on OXTR expression and the results showed that MPA had no effect, while ERβ expression

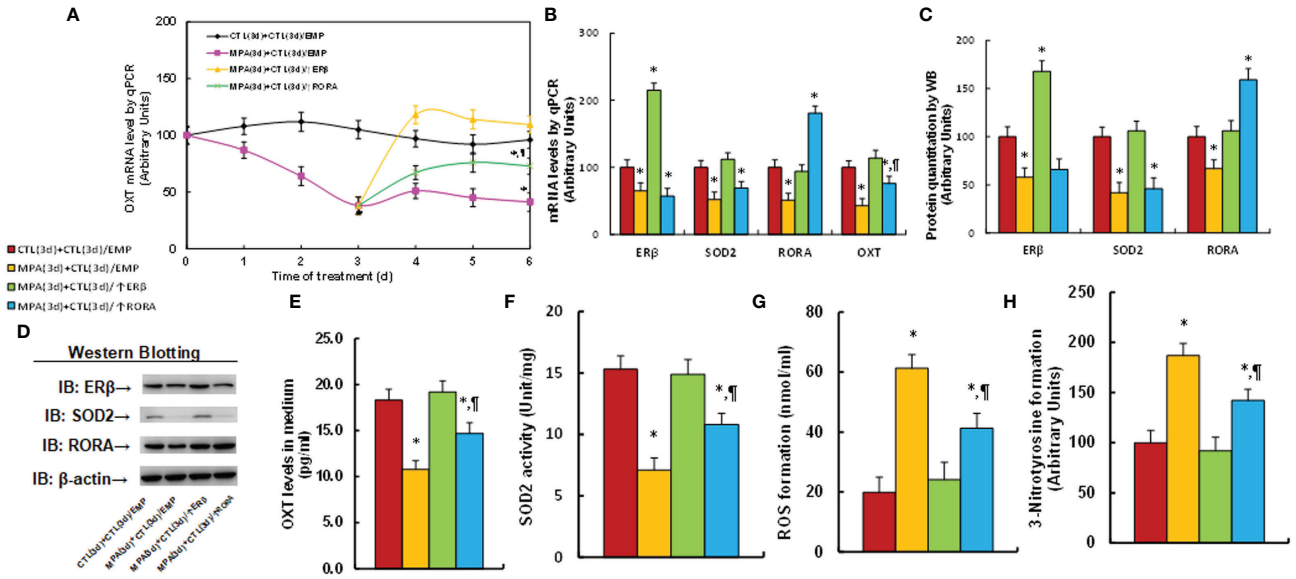

FIGURE 1 | Transient MPA treatment causes persistent OXT suppression and oxidative stress; ERβ expression completely, while RORA expression partly, reverses this effect. Mouse hypothalamic neurons were treated with either 10 μM MPA or vehicle control (CTL) for 3 days. The cells were infected by empty (CTL), ERβ (↑ERβ), or RORA (↑RORA) lentivirus on day 3 and the cells were then cultured without MPA for another 3 days in the presence of 1% FBS before being harvested for biological assays. **(A)** OXT mRNA levels on different time points, n=4, $^{*}P < 0.05$, vs. day 0 group; $^{¶}P < 0.05$, vs. day 3 group. (B–H) Biological assays on day 6. **(B)** mRNA levels, n=4. **(C)** Protein quantitation, n=5. **(D)** Representative western blots for **(C)**. **(E)** OXT levels in culture medium, n=5. **(F)** SOD2 activity, n=5. **(G)** ROS generation, n=5. **(H)** 3-nitrotyrosine generation, n=5. $^{*}P < 0.05$, vs. CTL(3d)+CTL(3d)/EMP group; $^{¶}P < 0.05$, vs. MPA(3d)+CTL(3d)/EMP group. Data were expressed as mean ± SD.

significantly increased OXTR mRNA levels (see **Figure S3**). We then measured the effect of MPA on oxidative stress, and the results showed that MPA treatment significantly decreased SOD2 activity (see **Figure 1F**) and increased ROS formation (see **Figure 1G**) and 3-nitrotyrosine formation (see **Figure 1H**). Again, ERβ expression completely, while RORA expression partly, reversed this effect. Furthermore, we determined the potential effect of other progestins on OXT expression and epigenetic changes. The results showed that estrogen (E2), progesterone (P2) and NGM had no significant effect, while almost all transient treatments of progestin, including LNG, NES, NET, NETA, NEN and OHPC, induced persistent OXT suppression and increased H3K27me2 modification on the OXT promoter (see **Table 1**). We conclude that transient progestin treatment causes persistent OXT suppression and oxidative stress in hypothalamic neurons.

MPA Induces OXT Suppression by Epigenetic Modifications and Subsequent Dissociation of ERβ and RORA From the OXT Promoter

We evaluated the potential molecular mechanism for MPA-induced OXT suppression. The conditionally immortalized hypothalamic neurons from PVN area were transfected by

TABLE 1 | Transient progestin exposure causes persistent epigenetic changes on the OXT promoter and the subsequent OXT suppression.

Transient Progestin Exposure	OXT mRNA level by Qpcr (% control)	H3K27me2 modification by ChIP (% control)
E2	106 ± 11	91 ± 12
P4	92 ± 10	108 ± 11
LNG	76 ± 9*	163 ± 8*
MPA	43 ± 10*	215 ± 11*
NES	69 ± 12*	121 ± 10
NET	58 ± 11*	147 ± 10*
NETA	71 ± 8*	168 ± 12*
NEN	62 ± 10*	177 ± 9*
NGM	87 ± 11	119 ± 13
OHPC	64 ± 12*	188 ± 12*

*Primary mouse hypothalamic neurons were treated with either 10 μM of progestin (dissolved in 0.1% DMSO) or vehicle control for 3 days before then being cultured for another 3 days in the absence of progestin in the presence of 1% FBS during 6-day treatment. The cells were harvested for mRNA analysis and ChIP analysis on the OXT promoter. E2, 17β-estradiol; P4, progesterone; LNG, levonorgestrel; MPA, medroxyprogesterone acetate; NES, nestorone; NET, norethindrone; NETA, norethindrone acetate; NEN, norethynodrel; NGM, norgestimate; OHPC, hydroxyprogesterone caproate; *, P<0.05, vs control group. n=4, results were expressed as mean ± SD.*

either OXT full length (pOXT-2000) or deletion reporter constructs and then treated by MPA for luciferase reporter assay. Our results showed that MPA-induced OXT suppression had no significant changes in the constructs of -2000, -1600, -1200, -800, -600, -400 and -200, while the suppression was significantly diminished in deletion constructs of -100 and -0, indicating that the MPA-responsive element is located in the range of -200~-100 on the OXT promoter (see **Figure 2A**). We then searched all the potential binding motifs in the range of -200~-100 on the OXT promoter and found that there were two RXRα motifs at -188 and -105, two estrogen response element (ERE) motifs at -182 (marked in red) and -169, one motif for RORA at -163 (marked in red) and one for p53 at -135, respectively (see **Figure 2B**). We then mutated these potential binding motifs respectively in the OXT full length reporter constructs and transfected them for reporter assay. The results showed that single mutants (marked in green, see **Figure 2B**) of ERE at -162 (M-182/ERE) and RORA at -163 (M-163/RORA) significantly diminished MPA-induced OXT suppression, while other single mutants had no effect (see **Figure 2C**). We then transfected either single or double mutants of M-182/ERE and M163/RORA to investigate the effect of MPA, and the result showed that single mutant of either M-182/ERE or M-163/RORA partly, while double mutant M-182/ERE/163/RORA

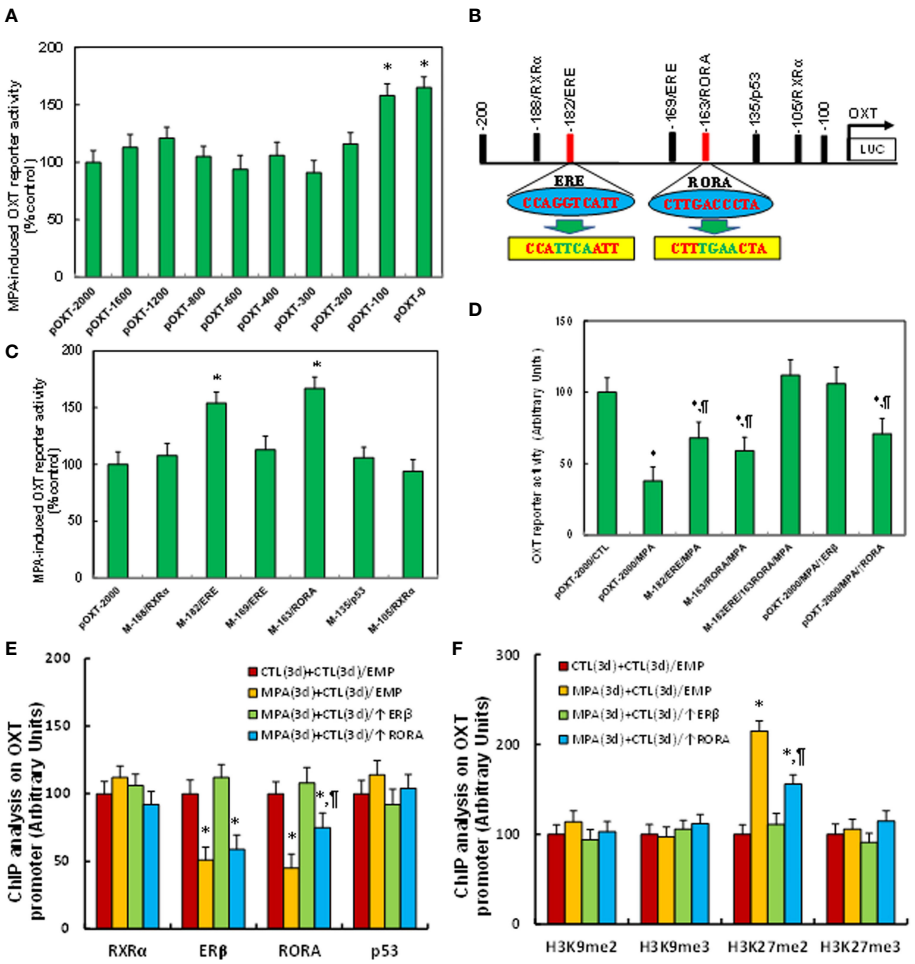

FIGURE 2 | MPA induces OXT suppression by epigenetic modifications and the subsequent dissociation of ERβ and RORA from the OXT promoter. **(A)** The immortalized mouse hypothalamic neurons from PVN area were transfected by either OXT full length (pOXT-2000) or deletion reporter constructs. After 24 hours, cells were then treated by either control or 10μM MPA for 3 days and the OXT reporter activities were then calculated, n=5. *$P < 0.05$, vs. pOXT-2000 group. **(B)** Schematic model for the possible transcriptional binding element on the OXT promoter with one of ERE and RORA binding site (in red) as well as related mutation sites (in green). **(C)** The cells were transfected with either wild type OXT reporter construct (pOXT-2000) or single point mutation construct as shown in **(B)** and then treated by either control or MPA for 3 days, and the OXT reporter activities were then determined, n=5. *$P < 0.05$, vs. pOXT-2000 group. **(D)** The cells were transfected by either OXT full length (pOXT-2000), single mutant, double mutants as indicated, or infected by ERβ lentivirus (↑ERβ), and then treated by either control or MPA for 3 days, and the OXT reporter activities were then determined, n=5. *$P < 0.05$, vs. pOXT-2000/CTL group; ¶$P < 0.05$, vs. pOXT-2000/MPA group. **(E)** ChIP analysis for transcription factor binding ability assay, n=4. **(F)** ChIP analysis for histone 3 methylation, n=4. *$P < 0.05$, vs. CTL(3d)+CTL(3d)/EMP group; ¶$P < 0.05$, vs. MPA(3d)+CTL(3d)/EMP group. Data were expressed as mean ± SD.

completely, reversed MPA-induced suppression. ERβ expression completely, but RORA expression partly, reversed MPA-induced suppression (see **Figure 2D**). We also evaluated the binding ability of these motifs by ChIP techniques, and the results showed that MPA treatment significantly decreased the binding abilities of ERβ and RORA on the OXT promoter. Again, ERβ expression completely, but RORA expression partly, reversed MPA-induced suppression (see **Figure 2E**). We finally evaluated MPA-mediated epigenetic changes on the OXT promoter by ChIP techniques. The results showed that MPA treatment significantly increased H3K27me2 modifications on the OXT promoter, but had no effect on H3K9me2, H3K9me3 or H3K27me3. ERβ expression completely, while RORA expression partly, reversed this effect (see **Figure 2F**). In addition, we found that MPA treatment had no effect on the OXT promoter for DNA methylation (see **Figure S4**), histone 4 methylation (see **Figure S5A**) and histone 3 acetylation (see **Figure S5B**). We conclude that MPA induces OXT suppression by epigenetic modifications and the subsequent dissociation of ERβ and RORA from the OXT promoter.

Prenatal OXT Deficiency Mimics Prenatal MPA Exposure-Mediated OXT Suppression and Oxidative Stress

We determined the effect of prenatal OXT deficiency on prenatal MPA exposure-mediated OXT suppression and oxidative stress. The OXT wild type (WT) or OXT null ($OXT^{-/-}$) background dams were exposed to either control (CTL) or MPA and the hypothalamic neurons or tissues from PVN area of offspring were isolated for analysis. We first evaluated gene expression in hypothalamic tissues, and found that MPA exposure significantly decreased mRNA levels of ERβ, SOD2, RORA and OXT in hypothalamic tissues. Prenatal OXT deficiency showed no further effect, although it decreased OXT mRNA levels in the control (CTL) group (CTL/OXT-/-), indicating that OXT knockdown in these animals was successful (see **Figure 3A**). We also measured protein levels for the genes through either western blotting (see **Figures 3B, C, S1B**) or ELISA for OXT (see **Figure 3D**), and the expression pattern was similar to that of mRNA levels. In addition, we measured gene expression in tissues of both the amygdala (see **Figure S6A**) and

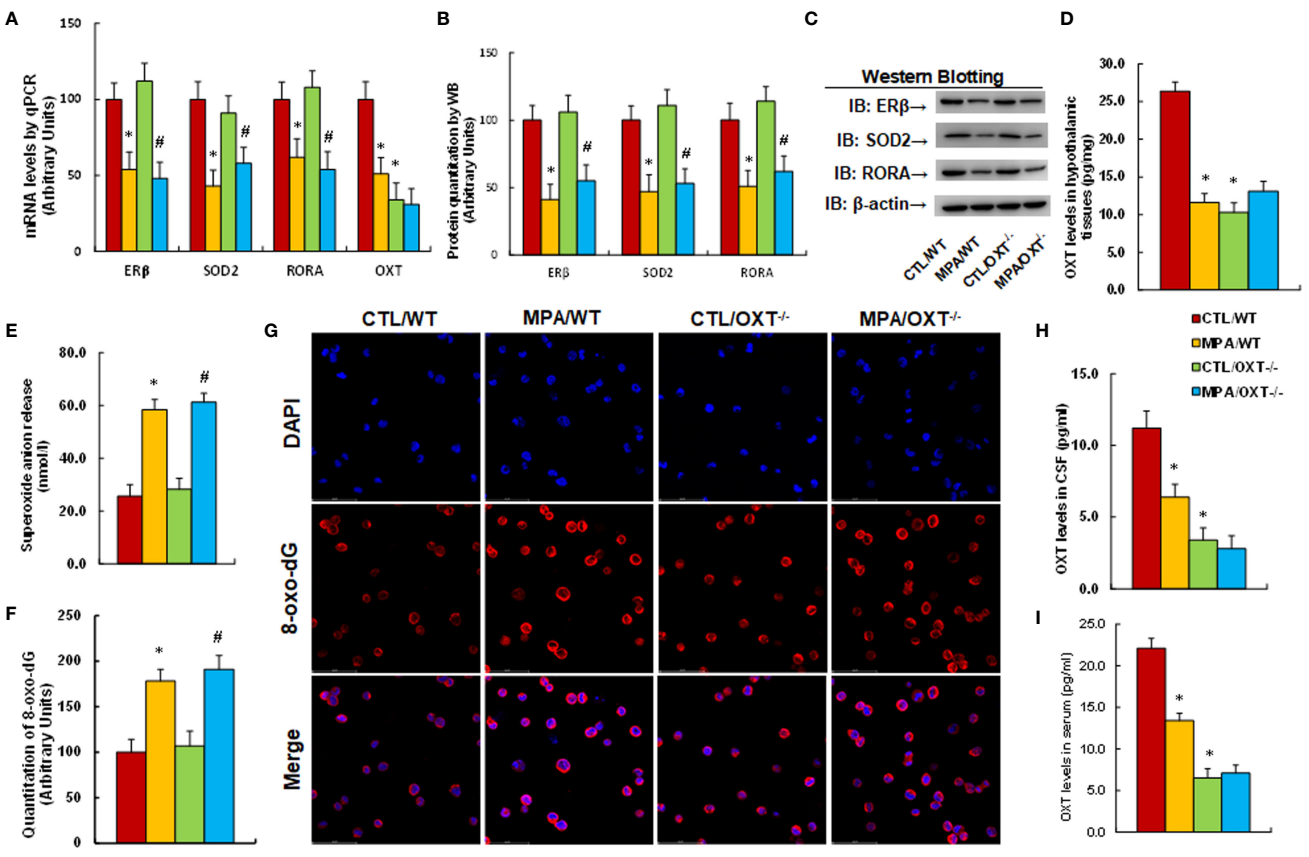

FIGURE 3 | Prenatal OXT deficiency mimics prenatal MPA exposure-mediated OXT suppression and oxidative stress. The OXT wild type (WT) or OXT null ($OXT^{-/-}$) background dams were treated by either control (CTL) or MPA and the hypothalamic neurons or tissues from PVN area of offspring were isolated for biological assays. **(A-D)** The hypothalamic tissues were isolated for analysis. **(A)** mRNA levels, n=4. **(B)** Protein quantitation, n=5. **(C)** Representative western blots for **(B)**. **(D)** OXT levels in hypothalamic tissues, n=5. **(E)** Superoxide anion release in hypothalamic tissues, n=5. **(F, G)** Immunostaining of hypothalamic neurons. **(F)** 8-oxo-dG staining quantitation, n=5. **(G)** Representative 8-oxo-dG staining (red) and DAPI staining (blue). **(H)** OXT levels in CSF, n=5. **(I)** OXT levels in serum, n=5. $^{*}P < 0.05$, vs. CTL/WT group; $^{\#}P < 0.05$, vs. CTL/OXT-/- group. Data were expressed as mean ± SD.

hippocampus (see **Figure S6B**), and the results showed that MPA exposure decreased mRNA levels of ERβ, SOD2 and RORA in the amygdala but had no effect in the hippocampus. OXT knockdown showed no further effect. We also evaluated the effect of MPA and OXT deficiency on oxidative stress in hypothalamic tissues, and the results showed that prenatal MPA exposure significantly increased superoxide anion release (see **Figure 3E**) and 8-oxo-dG formation (see **Figures 3F, G**), while prenatal OXT deficiency showed no effect. We then evaluated OXT peptide levels in both the CSF (see **Figure 3H**) and serum (see **Figure 3I**), and found that prenatal MPA exposure significantly decreased OXT levels, and prenatal OXT deficiency achieved a further decrease. We conclude that prenatal OXT deficiency mimics prenatal MPA exposure-mediated OXT suppression and oxidative stress.

Prenatal OXT Deficiency Partly Mimics Prenatal MPA Exposure-Mediated Social Deficits in Mouse Offspring

We determined the potential effect of prenatal MPA exposure and OXT deficiency on animal behaviors. We first evaluated anxiety-like behaviors, and our results showed that offspring in the prenatal MPA exposure (MPA/WT) group buried less marbles in the marble-burying test (MBT) test (see **Figure 4A**) and spent less time in the Open Arm and more time in Closed Arm during the elevated plus maze (EPM) test (see **Figure 4B**) compared to the control (CTL/WT) group. We then evaluated autism-like behaviors, and the results showed that mice in the MPA/WT group had fewer ultrasonic vocalizations in the USV tests (see **Figure 4C**) and spent significantly less time sniffing, mounting and interacting in total during the social interaction (SI) tests (see **Figure 4D**). They spent less time sniffing in the Stranger 1 side and more time in the Empty side for sociability (see **Figure 4E**); additionally, they spent more time in the Stranger 1 side and less time in the Stranger 2 side for social novelty (see **Figure 4F**) during the three-chambered social test compared to the CTL/WT group. OXT deficiency had no effect on the MBT, EPM or USV tests, while it slightly decreased sniffing and total interaction time in the SI test and slightly decreased social ability and social novelty in the three-chambered social tests. We conclude that prenatal OXT deficiency partly mimics prenatal MPA exposure-mediated social deficits in mouse offspring.

Postnatal ERβ Expression Completely, While Postnatal RORA Expression Partly, Reverses Prenatal MPA Exposure-Mediated OXT Suppression and Oxidative Stress in Offspring

Pregnant dams were given either control (CTL) or MPA treatment, and the subsequent offspring received either empty (EMP), ERβ (↑ERβ) or RORA (↑RORA) lentivirus in the PVN area before then being sacrificed for analysis. We first determined gene expression in the hypothalamic tissues that isolated from

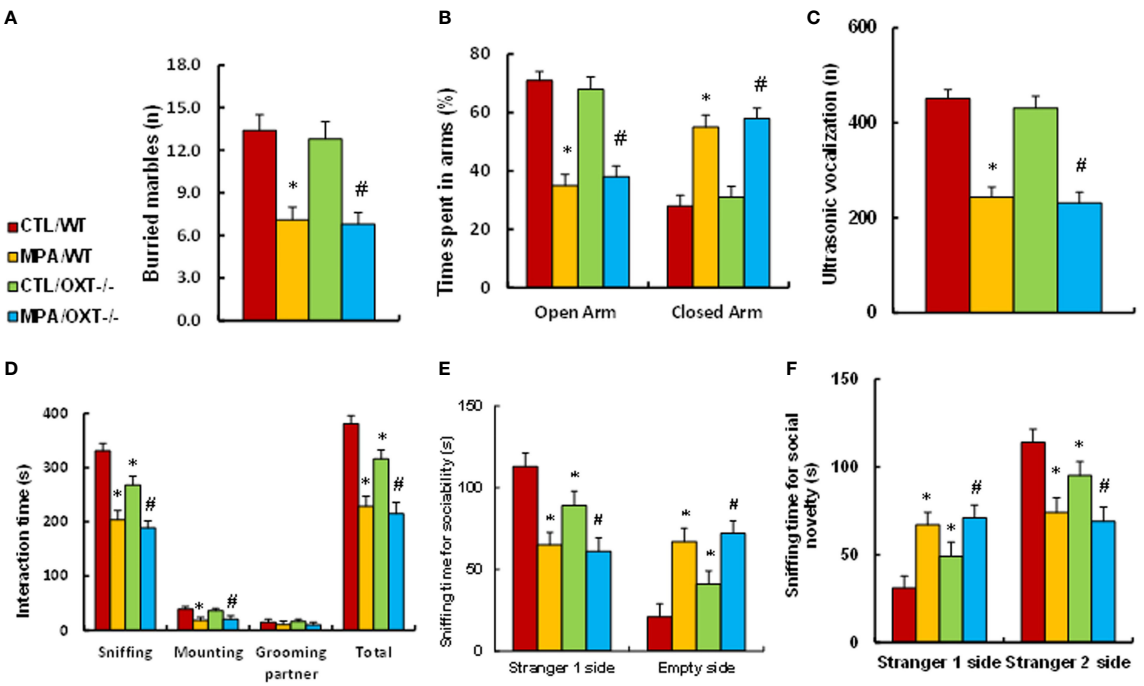

FIGURE 4 | Prenatal OXT deficiency partly mimics prenatal MPA exposure-mediated social deficits in mouse offspring. The OXT wild type (WT) or OXT null ($OXT^{-/-}$) background dams were treated by either control (CTL) or MPA, and the subsequent offspring were used for animal behavior tests. **(A)** MBT test, n=9. **(B)** EPM test, n=9. **(C)** Ultrasonic vocalization, n=9. **(D)** Social interaction (SI) test, n=9. **(E, F)** Three-chambered social tests for sociability **(E)** and social novelty **(F)**, n=9. $^{*}P < 0.05$, vs. CTL/WT group; $^{\#}P < 0.05$, vs. CTL/OXT-/- group. Data were expressed as mean ± SD.

PVN area of offspring, and found that infection of either ERβ or RORA lentivirus significantly increased mRNA levels, respectively, indicating a successful gene manipulation. Additionally, ERβ expression (MPA/P-↑ERβ) completely reversed MPA exposure-mediated gene suppression of ERβ, SOD2, RORA and OXT. RORA expression (MPA/P-↑RORA) showed no effect on ERβ and SOD2, while it partly reversed MPA exposure-mediated OXT suppression (see **Figure 5A**). We also measured protein levels for the genes using either western blotting (see **Figures 5B, C**, **S1C**) or ELISA for OXT (see **Figure 5D**), and the expression pattern was similar to that of mRNA levels. Moreover, we measured gene expression in the other brain regions, and the results showed that ERβ expression completely reversed MPA exposure-mediated gene suppression of ERβ, SOD2 and RORA in the amygdala, while RORA expression showed no effect (see **Figure S7A**). Neither prenatal MPA exposure nor postnatal gene manipulation showed any effect on gene expression in the hippocampus (see **Figure S7B**). We also evaluated the effect of MPA exposure and postnatal gene manipulation on oxidative stress in hypothalamic tissues, and the results showed that postnatal ERβ expression completely, while RORA expression partly, reversed prenatal MPA exposure-mediated increased superoxide anion release (see **Figure 5E**) and 8-OHdG formation (see **Figure 5F**). We then evaluated OXT peptide levels in both the CSF (see **Figure 5G**) and serum (see **Figure 5H**), and the results showed that postnatal ERβ expression completely, while RORA expression partly, reversed prenatal MPA exposure-mediated OXT suppression. We conclude that postnatal ERβ expression completely, while postnatal RORA expression partly, reverses prenatal MPA exposure-mediated OXT suppression and oxidative stress in offspring.

Postnatal ERβ Expression Partly Ameliorates Prenatal MPA Exposure-Mediated Social Deficits in Mouse Offspring, While Postnatal RORA Expression Has no Effect

We evaluated animal behaviors of offspring with prenatal MPA exposure and postnatal gene manipulation. Our results showed that postnatal expression of either ERβ or RORA showed no effect on MPA exposure-mediated anxiety-like behaviors, as measured using the marble-burying test (MBT) test (see **Figure 6A**) and elevated plus maze (EPM) test (see **Figure 6B**). We also evaluated autism-like behaviors, and the results showed that postnatal expression of either ERβ or RORA showed no effect on MPA exposure-mediated decreased ultrasonic vocalization in USV tests (see **Figure 5C**). On the other hand, postnatal ERβ expression partly ameliorated MPA exposure-mediated impaired social interaction, including sniffing and total interaction time, as measured in the social interaction (SI) tests (see **Figure 6D**). Additionally, it partly ameliorated MPA exposure-mediated impaired sociability (see **Figure 6E**) but not social novelty (see **Figure 6F**) during the

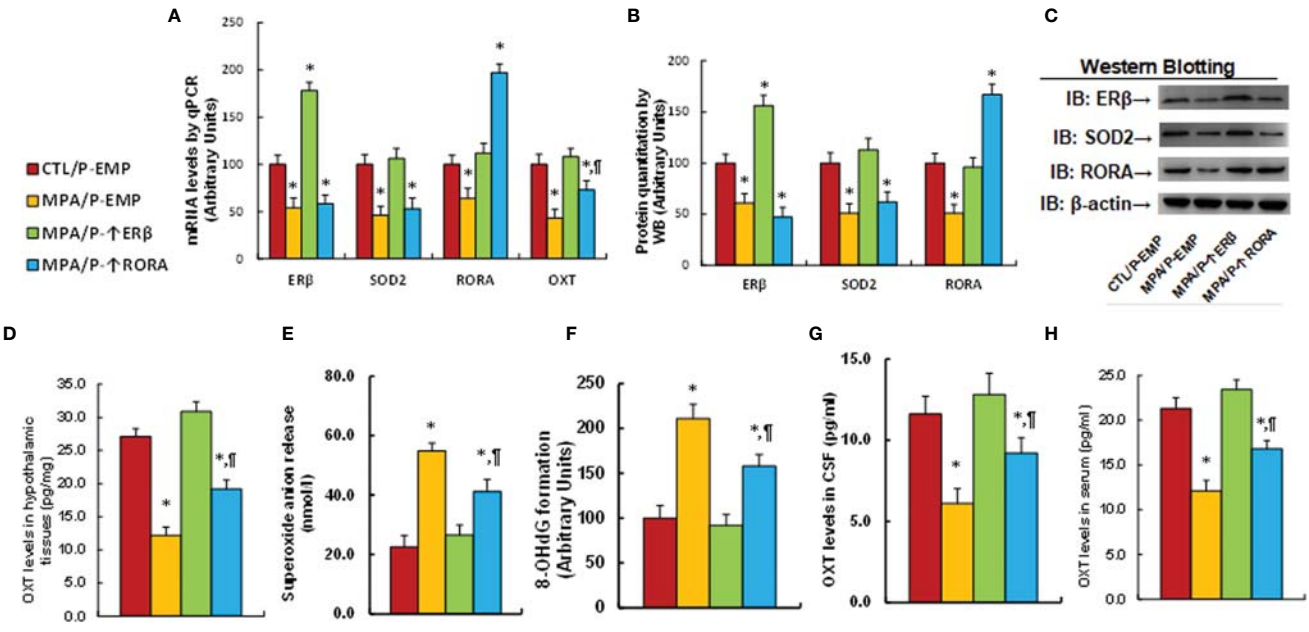

FIGURE 5 | Postnatal ERβ expression completely, while postnatal RORA expression partly, reverses prenatal MPA exposure-mediated OXT suppression and oxidative stress in offspring. The pregnant dams were treated with either control (CTL) or MPA, and the subsequent offspring received either empty (EMP), ERβ (↑ERβ) or RORA (↑RORA) lentivirus, and the offspring were then sacrificed for biological assays. (A–F) The hypothalamic tissues from PVN area were isolated for biological assays: **(A)** mRNA levels, n=4. **(B)** Protein quantitation, n=5. **(C)** Representative western blots for **(B)**. **(D)** OXT levels in hypothalamic tissues, n=5. **(E)** Superoxide anion release, n=5. **(F)** 8-OHdG generation, n=5. **(G)** OXT levels in CSF, n=5. **(H)** OXT levels in serum, n=5. *$P < 0.05$, vs. CTL/P-EMP group; ¶$P < 0.05$, vs. MPA/P-EMP group. Data were expressed as mean ± SD.

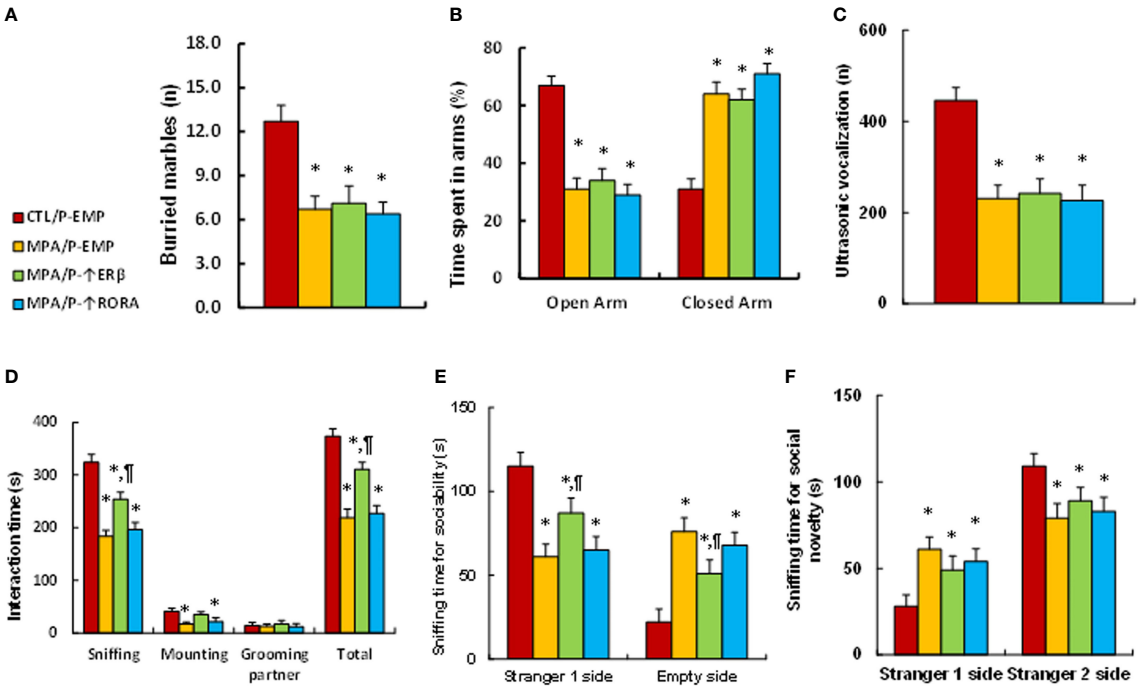

FIGURE 6 | Postnatal ERβ expression partly ameliorates prenatal MPA exposure-mediated social deficits in mouse offspring, while postnatal RORA expression has no effect. The pregnant dams were treated with either control (CTL) or MPA, and the subsequent offspring received empty (EMP), ERβ (↑ERβ) or RORA (↑RORA) lentivirus before then being used for animal behavior tests. **(A)** MBT tests, n=9. **(B)** EPM tests, n=9. **(C)** Ultrasonic vocalization, n=9. **(D)** Social interaction (SI) test, n=9. **(E, F)** Three-chambered social tests for sociability **(E)** and social novelty **(F)**, n=9. $^{*}P < 0.05$, vs. CTL/WT group; $^{\#}P < 0.05$, vs. CTL/OXT-/- group. Data were expressed as mean ± SD.

three-chambered social test. Postnatal RORA expression showed no effect on MPA exposure-mediated behaviors in offspring (see **Figures 6C–F**). We conclude that postnatal ERβ expression partly ameliorates prenatal MPA exposure-mediated social deficits in mouse offspring.

Postnatal Injection of OXT Peptide Partly Reverses Prenatal MPA Exposure-Mediated Social Deficits in Mouse Offspring

Pregnant dams were treated with either control (CTL) or MPA, and the subsequent offspring received either vehicle (VEH) or OXT peptide injection through the third ventricle for biological assays. We first determined gene expression in hypothalamic tissues that isolated from PVN area, and found that OXT peptide showed no effect on MPA exposure-mediated gene suppression of ERβ, SOD2, RORA or OXT (see **Figure 7A**). We also measured OXT peptide levels after OXT injection, and found that postnatal OXT injection significantly increased OXT levels in the CSF compared to the control (CTL/P-VEH) group (see **Figure 7B**) and also partly reversed MPA exposure-mediated decreased OXT serum levels (see **Figure 7C**). We evaluated animal behaviors in the offspring, and our results showed that postnatal OXT injection showed no effect on MPA exposure-mediated anxiety-like behaviors, as measured through the marble-burying test (MBT) test (see **Figure 7D**) and elevated plus maze (EPM) test (see **Figure 7E**). We also evaluated autism-like behaviors, and the results showed that postnatal OXT injection showed no effect on MPA exposure-mediated decreased ultrasonic vocalization in USV tests (see **Figure 7F**). On the other hand, postnatal OXT injection partly ameliorated MPA exposure-mediated impaired social interaction, as indicated through sniffing and total interaction time during the social interaction (SI) tests (see **Figure 7G**). Additionally, it partly ameliorated MPA exposure-mediated impaired sociability (see **Figure 7H**) but not social novelty (see **Figure 7I**) during the three-chambered social test. We conclude that postnatal OXT injection partly ameliorates prenatal MPA exposure-mediated social deficits in mouse offspring.

DISCUSSION

In this study, we found that transient progestin treatment triggers persistent epigenetic changes and OXT suppression in hypothalamic neurons. Prenatal MPA exposure induces OXT suppression, oxidative stress and social deficits in offspring. OXT knockdown mice partly mimics, while postnatal ERβ expression or postnatal OXT peptide injection partly ameliorates, prenatal MPA exposure-mediated social deficits in mouse offspring.

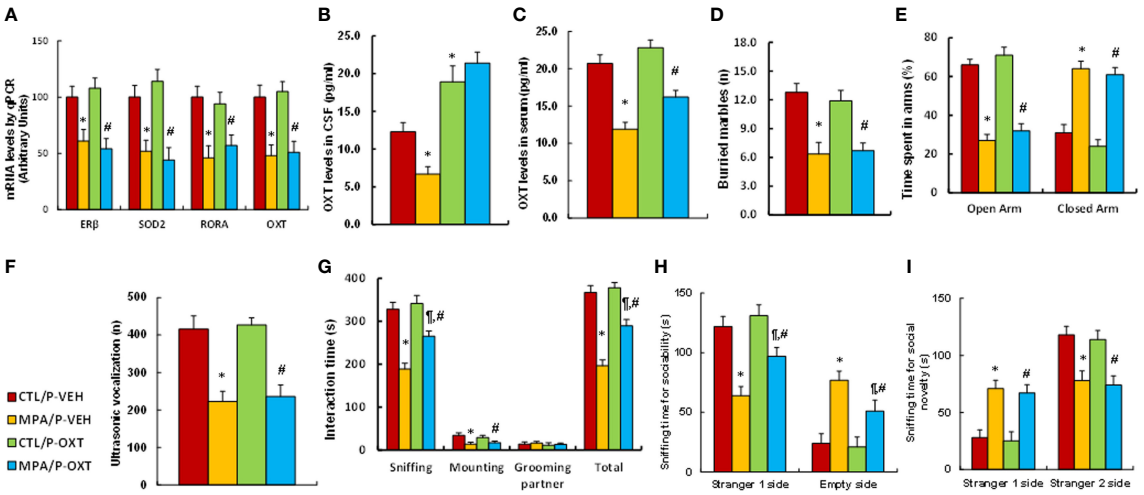

FIGURE 7 | Postnatal injection of OXT partly ameliorates prenatal MPA exposure-mediated social deficits in mouse offspring. The pregnant dams were treated with either control (CTL) or MPA, and the subsequent offspring received either vehicle (VEH) or OXT peptide injection through the third ventricle. The animals were then used for analysis. **(A)** The hypothalamic tissues were used for mRNA analysis, n=4. **(B)** OXT levels in CSF, n=5. **(C)** OXT levels in serum, n=5. **(D–I)** Offspring were used for animal behavior tests. **(D)** MBT tests, n=9. **(E)** EPM tests, n=9. **(F)** Ultrasonic vocalization, n=9. **(G)** Social interaction (SI) test, n=9. **(H, I)** Three-chambered social tests for sociability **(H)** and social novelty **(I)**, n=9. $^{*}P < 0.05$, vs. CTL/P-VEH group; $^{¶}P < 0.05$, vs. MPA/P-VEH group; $^{\#}P < 0.05$, vs. CTL/P-OXT group. Data were expressed as mean ± SD.

Effect of Prenatal Progestin Exposure

Our *in vitro* study in hypothalamic neurons showed that transient progestin treatment induces persistent epigenetic modifications even after removal of progestin and subsequently dissociates both ERβ and RORA from the OXT promoter, triggering OXT suppression. The *in vivo* study in mouse models showed that prenatal MPA exposure induces OXT suppression, partly contributing to social deficits in mouse models. In addition, the regular MPA dose for treatment of women contraception is reported as 150mg (49), and the high MPA dose for tumor suppression is in the range of 400-2000mg daily (50), and those doses can be calculated as 2.5-33.3mg/kg body weight if the average weight of women is considered as 60kg. Given the fact that the practical human exposure time can be 3 months (first trimester is most sensitive for ASD development) or more during the pregnancy (10, 51), while the exposure time of pregnant dams is much less, can only reach to 21 days in maximum, we finally chose MPA dose of 20mg/kg body weight for prenatal treatment of pregnant dams to mimic the possible high dose of MPA for human exposure. Furthermore, our *in vitro* and *in vivo* study showed that progestin exposure induces suppression of ERβ and RORA in addition to OXT suppression, which is consistent with our previous finding in rat models (7, 8), indicating that ERβ may play an important role in prenatal progestin exposure-mediated social deficits in mouse offspring. In addition, our results showed that prenatal progestin exposure triggers social deficits in rodents, which is consistent with previous reports that maternal hormone exposure is a potential risk factor for ASD (52, 53), modulating a neurogenic response and social recognition during development (54, 55).

Role of ERβ and RORA in OXT Expression

Our *in vitro* study showed that the OXT promoter has the potential binding sites of ERβ and RORA, which are responsible for progestin treatment-mediated OXT suppression. This indicates that RORA may also play a role in OXT expression that adds to the significant effect of ERβ, which is consistent with previous findings that RORA plays a critical role in embryo development (35) and is associated with autism development (56). Interestingly, our *in vitro* study found that ERβ expression completely, while RORA expression partly, reverses progestin treatment-mediated OXT suppression. Furthermore, *in vivo* mouse study showed that postnatal ERβ expression completely, while RORA expression partly, reverses prenatal MPA-mediated OXT suppression in hypothalamic neurons. Postnatal ERβ expression can partly ameliorate prenatal MPA exposure-mediated social deficits in mouse offspring, while postnatal RORA expression has no effect. The results indicate that the effect of ERβ expression overcomes the effect of RORA expression, which can be explained with the hypothesis that ERβ expression-mediated SOD2 up-regulation (25) diminishes progestin exposure-mediated oxidative stress and epigenetic modifications (26), subsequently restoring the binding ability of both ERβ and RORA on the OXT promoter. In addition, it has been previously reported that OXT expression is regulated by estrogen and ERβ (57, 58). Sharma et al. has shown that ERβ forms a functional complex with cAMP response element-binding protein (CBP) and steroid receptor coactivator-1 (SRC1) in the presence of ERβ ligand, and subsequently regulating the OXT expression through ERE binding site on the OXT promoter (57). On the other hand, our results show that MPA treatment induces histone modification on the OXT promoter, resulting in ERβ

dissociation from ERE binding motif on the OXT promoter, triggering OXT suppression. Furthermore, the progestin-responsive ERE binding motif identified in this work is different with previous study (57), and the progestin exposure-mediated OXT suppression is epigenetic modification-based persistent suppression. In this study, a novel mechanism for progestin-mediated OXT suppression through ERβ and RORA is reported.

Role of OXT and Social Deficits

OXTR is expressed in a variety of human tissues and is highly expressed in limbic regions such as the amygdala (12, 20). It has been reported that the OXT/OXTR signaling pathway plays a role in regulation of a variety of social behaviors (11, 16) as well as ASD etiology (18, 19, 59) and is involved with anxiety-like behaviors (13, 14). Our results showed that OXTR expression does not change in response to progestin treatment, while OXT expression is reduced persistently. Furthermore, prenatal OXT deficiency in OXT knockdown mice partly mimics prenatal MPA exposure-mediated social deficits, including impaired social interaction and social ability, but showed no effect on anxiety-like behaviors, as measured in MBT and EPM tests. Furthermore, postnatal expression of ERβ in the PVN area or through postnatal OXT peptide injection in the third ventricle partly ameliorates prenatal MPA-exposure-mediated social deficits; again, there is no effect on anxiety-like behaviors. This can be partly explained through the hypothesis that postnatal OXT manipulation is only effective in certain OXT-responsive areas, but cannot mimic the whole endogenous OXT-responsive area (60). However, it is clear that OXT peptides do have some effect on modulating social behaviors in mouse offspring. On the other hand, recent placebo-controlled trial using intranasal OXT therapy showed no significant effect on ASD children and adolescents, which can be explained because intranasal OXT administration may not reach sufficient OXT concentrations in OXT-responsive areas of the central nervous system (61).

CONCLUSIONS

Transient progestin treatment induces epigenetic changes, triggering persistent OXT suppression. Postnatal ERβ expression in hypothalamic regions or postnatal OXT peptide injection partly ameliorates postnatal MPA exposure-mediated impaired social interaction and social abilities in mouse offspring. We conclude that maternal progestin exposure-mediated oxytocin suppression contributes to social deficits in mouse offspring.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee from Foshan Maternity & Child Healthcare Hospital at Southern Medical University.

AUTHOR CONTRIBUTIONS

PY wrote the paper. PY and XS designed, analyzed the data and interpreted the experiments. RS and HY performed part of the gene analysis. HZ performed part of the mouse experiments. SH and JZ performed the remaining experiments. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.840398/full#supplementary-material

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No Differences in Value-Based Decision-Making Due to Use of Oral Contraceptives

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Fluctuating ovarian hormones have been shown to affect decision-making processes in women. While emerging evidence suggests effects of endogenous ovarian hormones such as estradiol and progesterone on value-based decision-making in women, the impact of exogenous synthetic hormones, as in most oral contraceptives, is not clear. In a between-subjects design, we assessed measures of value-based decision-making in three groups of women aged 18 to 29 years, during (1) active oral contraceptive intake (N = 22), (2) the early follicular phase of the natural menstrual cycle (N = 20), and (3) the periovulatory phase of the natural menstrual cycle (N = 20). Estradiol, progesterone, testosterone, and sex-hormone binding globulin levels were assessed in all groups *via* blood samples. We used a test battery which measured different facets of value-based decision-making: delay discounting, risk-aversion, risk-seeking, and loss aversion. While hormonal levels did show the expected patterns for the three groups, there were no differences in value-based decision-making parameters. Consequently, Bayes factors showed conclusive evidence in support of the null hypothesis. We conclude that women on oral contraceptives show no differences in value-based decision-making compared to the early follicular and periovulatory natural menstrual cycle phases.

Keywords: oral contraceptives, ovarian hormones, value-based decision-making, impulsive choice, delay discounting, probability discounting, risk, loss

INTRODUCTION

Our everyday life is determined by the decisions and choices which we have made or did not make – no matter how big or small. To make these decisions, we often draw on the cognitive process of value-based decision-making. In this complex cognitive process, potential rewards are balanced against their potential costs, i.e., a certain delay or probability of obtaining or losing something. Value-based decision-making comprises different facets, in which the dimensions amount, delay, or

probability differ (1). We speak of *delay discounting* if a person is faced with the decision between a smaller, sooner reward and a larger, later reward. Risk-aversion/seeking and loss aversion are captured in *probability discounting* which is a decision between a sooner, small certain reward (or loss) and a later, less certain but larger reward (or loss). Choice behavior is considered more impulsive if a person tends to choose smaller, sooner rewards over larger, later rewards.

Women and men differ in some aspects of value-based decision-making [for review, see Ambrase et al. (2)]. For example, women show bias towards frequent but smaller rewards, while men tend to maximize rewards even if their strategy is not optimal. Women also tend to regret suboptimal changes in their decision-making strategy and thus are more sensitive to information about previous rewards (3–5). Emerging evidence suggests that ovarian hormones, such as estradiol and progesterone, affect value-based decision-making in women (2). Ovarian hormones fluctuate across the menstrual cycle [~ 28 days; Bull et al. (6)]: In the *follicular* phase, both estradiol and progesterone levels are low in the beginning, with estradiol slowly rising and surging before *ovulation* (periovulatory phase, ~ day 14). Following ovulation, estradiol and progesterone rise again in the *luteal* phase, peaking bluntly. It has been shown that women made more impulsive choices in the early follicular phase, i.e., when both estradiol and progesterone were low, while at the same time women were less likely to wait for a higher reward, compared with the periovulatory phase (7). Similarly, women were also less sensitive for immediate rewards with rising estradiol levels, but this effect was mainly driven by women with lower frontal dopamine levels (8). Hence, decision-making processes may be affected by the interaction between ovarian hormones and neurotransmitter systems involved in decision-making – especially the dopaminergic system (9).

While most studies focused on menstrual cycle related effects on decision-making as the menstrual cycle provides a natural experimental model for investigating influences of endogenous ovarian hormones in women, we know only little about possible effects of exogenous ovarian hormones, such as in oral contraceptives (OCs). More than 100 million women worldwide use OCs (10), as OC-use provides an effective option for contraception as well as for managing cycle-related physiological symptoms. While the physiological side effects of OC-use are relatively well understood (e.g., cardiovascular risk), only little research has been dedicated to the effects of OCs on behavior, brain function or their association with psychopathology [but see (11–14)]. Steep delay discounting, risk-seeking and insensitivity to loss characterize mental disorders such as attention-deficit hyperactivity disorder (15), bipolar disorders (16), or substance use disorders (17). To give but one example, substance use disorders are two times more prevalent in men than in women (18), but women show more severe illness courses [for review see Becker (19)]. In women, drug use escalates more quickly and shows patterns of bingeing more often; moreover, women have poorer outcomes regarding quitting and treatment (20, 21). Evidence from rodent and human studies suggests that effects of ovarian hormones on underlying mechanisms of decision-making contribute to these differences (as reviewed by 2): Women have higher ratings of craving and show greater subjective responses to drug stimuli in the follicular phase compared with the luteal phase. However, a recent review of the relationship between OC-use and smoking-related symptoms found only mixed results, e.g., for craving, and could not report about any published data on OC-use and smoking cessation outcomes (22). Given the fact that one out of four smokers use OCs and that OC-use is related to increased nicotine metabolism (22), further research is needed to explore hormonal treatment developments and, more specifically, to investigate potential benefit/harm and secondary effects of OC-intake.

The most widely prescribed OCs contain a synthetic estrogen (ethinyl estradiol) and a synthetic progesterone (progestin) (10). These combined formulations prevent pregnancies by inhibiting ovulation because endogenous estradiol and progesterone fluctuations are suppressed. While endogenous estradiol and progesterone levels are constantly low in OC-users (23, 24), exogenous hormone levels are on a steadily high level (25). This substitution with higher-affinity, synthetic hormones has been shown to lead to structural brain differences in OC-users compared with naturally cycling women: e.g., OC-users had smaller right putamen volumes (26) as well as lower thickness of the lateral orbitofrontal cortex and the posterior cingulate cortex (27). Especially the lateral orbitofrontal cortex region is essential for the cognitive control of behavior, including response inhibition to stimuli with changing reward value (28). Besides its impact on brain structure, OC-use has also been found to increase resting state functional connectivity in the salience network, central executive network, reward network, as well as in the subcortical limbic network (26), which provides a mechanistic insight for putatively altered value-based decision-making in OC-users.

Overall, results from studies investigating the impact of OCs on value-based decision-making are mixed [for review, see Lewis et al. (29)]. OC-users were more sensitive to monetary rewards and had enhanced blood-oxygen level dependent (BOLD) responses during reward expectation in the anterior insula and inferior prefrontal cortex compared with naturally cycling women (30). Another study found greater neural activation in the amygdala, putamen, and executive frontal areas to food stimuli in OC-users compared with naturally cycling women in the follicular phase, but no differences between OC-users and naturally cycling women in the luteal phase (31). However, these studies were limited by their small sample size [N = 24; (30)] or lack of behavioral outcome measures (31). Two other studies found blunted reward responses in OC-users compared with naturally cycling women: Scheele et al. (32) reported enhanced attractiveness ratings of the partner's face together with increased BOLD responses in nucleus accumbens and ventral tegmental area in naturally cycling women after oxytocin administration, but not in OC-users. Jakob et al. (33) found that only naturally cycling women showed a significant effect of polymorphisms of the dopamine transporter (DAT1-genotype) on reinforcement learning, while OC-using women did not show any such behavioral variations according to DAT1-genotype differences. Especially the latter study provides a first hypothesis about how decision-making processes may be affected by the interaction

between ovarian hormones and neurotransmitter systems involved in decision-making, namely the dopaminergic system. Based on these previous studies, we expect OC-users to show differences in value-based decision-making compared with naturally cycling women. However, we cannot hypothesize the direction of this difference, i.e., if OC-users show more or less impulsive decision-making compared with naturally cycling women.

To this end, we investigated value-based decision-making in women using OCs and compared this group with two other groups of naturally cycling women with different hormonal profiles. In this study, three groups of women underwent a value-based decision-making test battery (34), which measured different facets of value-based decision-making: delay discounting, risk-seeking for gains/losses, and loss aversion. The three hormonal profile groups comprised (1) women using OCs (active pill intake, OC group), (2) women in the early follicular phase (days 2-5 of their cycle, fNC group), and (3) women during the periovulatory phase (± 3 days around ovulation, oNC group). Based on the literature reported earlier, we hypothesized (a) less impulsive choices in the fNC group compared with the oNC group, and (b) differences in value-based decision-making between the OC-group and both naturally cycling groups; the direction of this difference, however, remained exploratory.

MATERIALS AND METHODS

Sample Description

A total of 67 healthy female students were recruited from the University of Tübingen and participated in the study. We excluded five participants: three women did not show a luteinizing hormone (LH) surge in the predefined time frame, two women used progestogen-only contraception or recently switched the OC brand. The remaining 62 participants formed three hormonal profile groups, (1) the OC group (n = 22, mean age = 22 ± 2), (2) the fNC group (n = 20, mean age = 22 ± 3), and (3) the oNC group (n = 20, mean age = 24 ± 4). Inclusion criteria were 18-35 years of age, no history of any neurological or mental disorders and no (other) hormonal treatment within the past three months. For the OC group, we included women using monophasic OCs (containing a synthetic estrogen and a synthetic progesterone; an overview of the oral contraceptives and their compounds used by the study participants can be found in **Supplementary Table 2**) for at least six months (mean duration: 3.3 years ± 1.7 years) and measured them during their active pill intake phase (days 2-21). Inclusion criteria for the fNC and oNC groups were an average cycle length of 21-35 days and no hormonal contraception for the past six months. We tested women in the oNC group during their fertile period, i.e., ± 3 days around the detection of the LH peak (predicting ovulation within 2 days, using NADAL hLH ovulation strips, nal von minden GmbH, Moers/Germany). Women in the oNC group reported the first day of bleeding after the measurement to confirm the test results. We measured women in the fNC group on days 2-5 of their menstruation. All women were comparable in age, verbal intelligence, and executive functioning. **Table 1** shows all sociodemographic and neuropsychological characteristics as well as the serum hormone profiles.

Experimental Procedure

After we received written informed consent from participants, we checked all inclusion and exclusion criteria and asked for menstrual cycle features, OC intake history, as well as gynecological characteristics (e.g., premenstrual syndrome, pregnancies, endometriosis, polycystic ovary syndrome etc.). The German version of the Structured Clinical Interview

TABLE 1 | Sample description (mean and standard deviation) and hormone profiles per hormonal profile group.

Demographic information and questionnaires	OC	fNC	oNC	*p*-value
N	22	20	20	
Age (years)	22 (2)	22 (3)	24 (4)	.208
Impulsiveness (BIS-15)	29.0 (6.4)	28.7 (5.1)	33.0 (7.0)	**.058**†
State anxiety (STAI)	34.9 (9.6)	33.2 (4.8)	33.1 (7.0)	.99
Positive mood (PANAS)	31.9 (6.0)	31.2 (6.2)	30.3 (7.2)	.72
Negative mood (PANAS)	13.8 (4.2)	12.9 (4.0)	12.7 (4.6)	.44
Verbal intelligence (WST)	31.8 (3.2)	32.5 (2.4)	31.7 (3.6)	.69
Executive functioning (TMTB-A in sec)	15.5 (9.5)	15.1 (13.5)	16.2 (12.5)	.96
Hormone profiles	**OC**	**fNC**	**oNC**	***p*-value**
Estradiol (pmol/l)	67.0 (30.1)	165.9 (45.8)	516.7 (352.2)	**<.001**, OC = fNC < oNC
Progesterone (nmol/l)	1.3 (0.7)	2.1 (0.9)	6.6 (8.0)	**<.001**, OC = fNC < oNC
Testosterone (nmol/l)	0.8 (0.2)	1.1 (0.3)	1.2 (0.3)	**<.001**, OC < fNC = oNC
SHBG (nmol/l)	182.0 (107.1)	65.1 (33.0)	53.9 (23.2)	**<.001**, OC > fNC = oNC

OC, women using oral contraceptives; fNC, naturally cycling women in the early follicular phase; oNC, naturally cycling women during periovulatory phase; BIS-15, German short version of the Barrat Impulsiveness Scale; STAI, State-Trait Anxiety Inventory; PANAS, Positive and Negative Affect Scale; WST, Wortschatztest; TMTB-A, Trail Making Test; SHBG, sex hormone binding globulin; bold values indicate statistically significant differences; †*Marginally significant.*

[SCID; Wittchen et al. (35)] was used to exclude any history of mental disorder. Neuropsychological tests comprised verbal intelligence [Wortschatztest WST; Schmidt and Metzler (36)] and executive functioning [trail making test TMT; Reitan (37)]. Affective functioning was assessed with the Positive and Negative Affect Scale [PANAS; Watson et al. (38)], state anxiety with the State-Trait Anxiety Inventory [STAI; Laux et al. (39)]. Impulsiveness was assessed with the German short version of the Barrat Impulsiveness Scale [BIS-15; Meule et al. (40)]. Thereafter, participants underwent the value-based decision-making battery (34), as well as two other behavioral tasks (Tübinger Empathy Test and a sexual approach avoidance task; reported in (41). The Ethics committee of the Medical Faculty of Tübingen approved the study.

Value-Based Decision-Making Battery

The value-based decision-making battery measured different facets of impulsive choice, which were implemented in four tasks: delay discounting, probability discounting for gains, probability discounting for losses, and mixed gambles (34).

Participants repeatedly had to decide for one of two offers, which were presented simultaneously on a computer screen for 5 seconds. Offers were randomly assigned to the left or to the right of the screen and participants had to decide by pressing the respective button. For each trial, the participant's choice was indicated with a frame before presenting the next offer. The test battery took about 20 minutes. All task choices were hypothetical and participants were not informed about outcomes. Since hypothetical monetary rewards have been shown to produce similar results as real monetary rewards [e.g., (42, 43)], participants were paid a fixed amount of money for compensation after completing the test battery.

The delay discounting (DD) task consisted of 50 trials in which participants had to choose between a smaller, immediate amount of money and a larger, later amount (3-50 €; delays of 3 days, 1 week, 2 weeks, 1 month, 2 months, 6 months or 1 year). This task measured the extent to which individuals discount rewards as a function of delay, where stronger discounting is described by higher k values.

In the probability discounting for gains (PDG) and probability discounting for losses (PDL) tasks, participants had to decide between a small, but sure gain or loss of money and a larger amount of money with changing probabilities (3-50 €, probabilities of 2/3, 1/2, 1/3, 1/4, 1/5; 50 trials respectively). The PDG task measured risk-aversion, described by the preference for sure over probabilistic amounts, which is indicated by higher k values. Higher k values in the PDL task describe a preference for the probabilistic offer over the certain one and therefore captured risk-seeking.

In the mixed gambles (MG) task, participants had to gamble for winning (1-40 €) or losing (5-20 €) money or to reject to gamble over the course of 50 trials. This task measured loss aversion. Higher λ values resulted from participants who tended to reject gambles and therefore weighed losses relatively higher.

The tasks used a trial-by-trial adaptive Bayesian approach, that allows an efficient and precise estimation of the impulsive choice parameters k or λ (34). After each trial, the individual indifference point is estimated based on previous choices and informs the options in the next trial. Additionally, a consistency parameter β was computed for each task. Large values of β describe consistent choices, i.e., a higher probability of choosing the option with a higher value; small values of β represent inconsistent choices. The mathematical modeling and parameter estimation for the four tasks can be found in the **Supplementary Material**, together with the posterior distributions of the estimated parameters k and λ.

The value-based decision-making battery, including instructions, binary choices, outcomes, and the parameter estimation algorithm was implemented using MATLAB, Release 2010a (The MathWorks, Inc., Natick, MA) and Psychotoolbox 3.0.10, based on the Psychophysics Toolbox extensions (44, 45).

Hormone Sampling and Analysis

Blood levels of estradiol, progesterone, testosterone, and sex hormone binding globulin (SHBG) were assessed to confirm cycle phase and inter-individual differences in sex steroid concentrations. Samples were analyzed using chemiluminescence immunoassays (CLIA; Centaur, Siemens). Measurement units were nmol/l for progesterone, testosterone and SHBG, and pmol/l for estradiol. The analytical sensitivity of the assays is 27.2 pmol/l for estradiol, 0.67 nmol/l for progesterone, 0.09 nmol/l for testosterone, and 1.6 nmol/l for SHBG. For the intra-assay accuracy, the maximum coefficient of variation is 11.1% for estradiol, 12.4% for progesterone, 8.5% for testosterone, and 3.8% for SHBG. The reported overall variation of the assays is 13.3% for estradiol, 12.7% for progesterone, 12.6% for testosterone, and 6.5% for SHBG.

Data Analysis

Analyses were conducted using R version 3.6.2 (46), using parametric statistical methods with two-tailed significance at $p < .05$. We used log transformations of k, λ, and β to fulfill the assumptions of parametric testing; BIS-15 total scores were centered to the mean. Mean differences between groups in age, questionnaire data, and hormonal profiles were analyzed using univariate ANOVAs. Each task of the battery (DD, PDG, PDL, and MG) was analyzed in separate univariate ANOVAs, with *group* (OC, fNC, and oNC) as between-subjects factor and η_p^2 as a measure of effect size. In an exploratory analysis, we also included BIS-15 scores as covariate. We used Pearson's r to characterize the correlations between k/λ and β. We conducted a sensitivity power analysis using G*Power version 3.1.9.4 (47) to calculate the critical population effect size with 80% power. Our remaining sample ($N = 62$) was sufficiently powered to detect a small to medium effect ($f^2 = 0.21$).

RESULTS

Demographics and Hormone Concentrations

The hormonal profile groups did not differ in age, mood and anxiety scores, verbal intelligence, and executive functioning (**Table 1**). Impulsiveness differed marginally between groups, as

measured with the BIS-15 questionnaire, $F(2,59) = 2.99$, $p = .058$. Hormone concentrations varied as expected across the hormonal phases which were examined (**Table 1** and **Figure 1**): estradiol, $F(2,59) = 28.08$, $p < .001$, progesterone, $F(2,59) = 7.95$, $p < .001$, testosterone, $F(2,59) = 15.95$, $p < .001$, and SHBG, $F(2,59) = 23.28$, $p < .001$ (**Supplementary Table 3** contains single serum hormone profiles for all participants).

Delay Discounting

Running a one-way ANOVA with *group* as between-subjects factor, we found no significant group effect for the DD task, $F(2,59) = .59$, $p = .560$, $\eta_p^2 = .019$ (**Figure 2**). Adding BIS-15 as a covariate did not show any association with parameter k (delay discounting), $F(3,58) = .42$, $p = .738$.

To substantiate the null effect observed in the DD task, a Bayesian analysis approach using the Bayesian information criterion BIC (as described by 48) was applied to allow for the evaluation of the probability of the null hypothesis being true (i.e., that there is no difference between the groups). We provide a detailed description of the approach in the **Supplementary Material**. Bayesian analyses revealed that the probability of the null hypothesis was $p_{BIC} = .97$. According to criteria suggested by Masson [see also (49)], this reflects strong evidence for the null hypothesis (.50-.75 weak,.75-.95 positive,.95-.99 strong, >.99 very strong).

Correlation analyses showed significant correlations of k and β for the fNC ($r = -.48$, $p = .033$) and oNC ($r = -.51$, $p = .021$) groups, but not for the OC group ($r = -.02$, $p = .919$; **Figure 3**). In other words, stronger discounting correlated with more inconsistent choices for the naturally cycling groups (fNC and oNC), but not for the OC group. However, the correlation coefficients between groups did not differ significantly (fNC vs. OC, $z = 1.51$, $p = .13$; fNC vs. oNC, $z = 0.12$, $p = .91$; oNC vs. OC, $z = 1.63$, $p = .1$; Bonferroni-corrected at $\alpha = .017$).

Probability Discounting of Gains

We found no significant differences between groups for the PDG task, using a one-way ANOVA with *group* as between-subjects factor, $F(2,59) = .55$, $p = .560$, $\eta_p^2 = .019$ (**Figure 2**). Adding BIS-15 as a covariate did not show any association with parameter k (risk-aversion), $F(3,58) = 1.17$, $p = .330$.

For the PDG task, Bayesian analyses revealed that the probability of the null hypothesis was $p_{BIC} = .97$. This reflects strong evidence for the null hypothesis.

Correlation analyses showed no significant correlations of k and β for any of the groups (OC $r = -.13$, fNC $r = .05$, oNC $r = -.33$; all $p > .05$).

Probability Discounting of Losses

Running a one-way ANOVA with *group* as between-subjects factor, we found no significant group effect for the PDL task, $F(2,59) = .67$, $p = .517$, $\eta_p^2 = .022$ (**Figure 2**). Adding BIS-15 as a covariate did not show any association with parameter k (risk-seeking), $F(3,58) = .44$, $p = .722$.

For the PDL task, Bayesian analyses revealed that the probability of the null hypothesis was $p_{BIC} = .97$. This reflects strong evidence for the null hypothesis.

Correlation analyses showed no significant correlations of k and β for any of the groups, (OC $r = .15$, fNC $r = -.10$, oNC $r = .14$; all $p > .05$).

Mixed Gambles

We found no significant differences between groups for the MG task, running a one-way ANOVA with *group* as between-subjects factor, $F(2,59) = 1.83$, $p = .169$, $\eta_p^2 = .058$ (**Figure 2**). BIS-15 as a covariate was significantly associated with parameter λ (loss aversion) for all three groups, $F(3,58) = 2.87$, $p = .044$, $\eta_p^2 = .071$ (**Figure 4**). This means that less impulsive participants tended to reject gambles and therefore weighed losses higher, regardless in which group they were in.

For the MG task, Bayesian analyses revealed that the probability of the null hypothesis was $p_{BIC} = .91$. This reflects positive evidence for the null hypothesis.

Correlation analyses showed no significant correlations of λ and β for any of the groups, (OC $r = -.33$, fNC $22r = -.09$, oNC $r = -.12$; all $p > .05$).

DISCUSSION

In the present study, we investigated value-based decision-making in women with different hormonal profiles. We

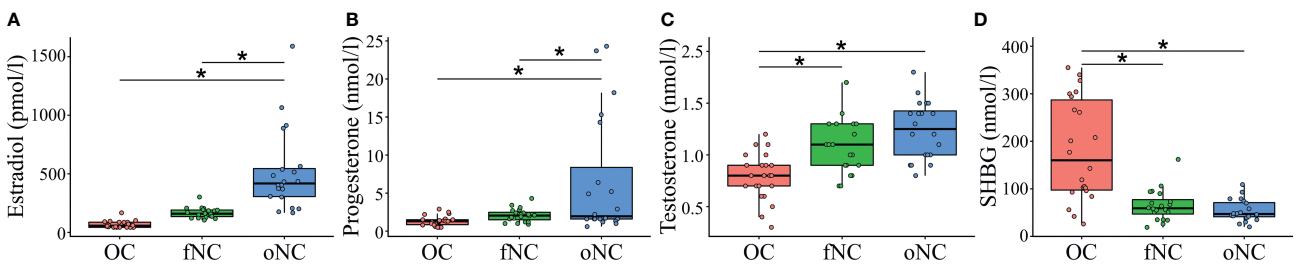

FIGURE 1 | Serum hormone profiles for each hormone profile group, showing **(A)** estradiol in pmol/l and **(B)** progesterone, **(C)** testosterone, and **(D)** sex hormone binding globulin (SHBG) in nmol/l. OC, women using OCs; fNC, women in the early follicular phase; oNC, women during periovulatory phase. Whiskers indicate variability outside the upper and lower quartiles, '*' denote significance levels at $p < .05$.

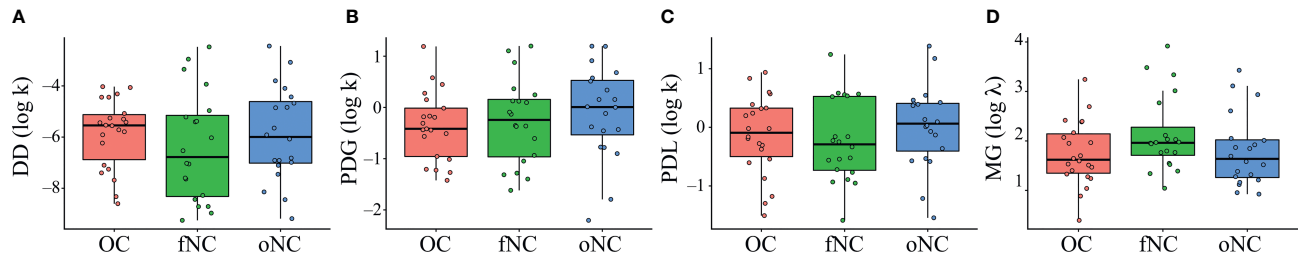

FIGURE 2 | Boxplots showing outcomes for the parameter k of the **(A)** delay discounting (DD), **(B)** probability discounting for gains (PDG), and **(C)** probability discounting for losses (PDL) tasks, and λ of the **(D)** mixed gambles (MG) task. Each dot represents an individual subject. OC, women using OCs; fNC, women in the early follicular phase; oNC, women during periovulatory phase. Whiskers indicate variability outside the upper and lower quartiles.

measured the value-based decision-making constructs delay discounting (DD), risk-seeking for gains (PDG) and losses (PDL), and loss aversion (MG). The three groups did not differ in the main outcome parameters k for the DD, PDG, and PDL tasks, and λ for the MG task. We substantiated these null effects using a Bayesian analysis approach, which reflected positive to strong evidence for the null hypothesis, i.e., that there are no differences between groups. The BIS-15 total score as a covariate was not associated with the k parameters of the DD, PDG, and PDL tasks, only with parameter λ (loss aversion) of the MG task. Here, more impulsive participants in all groups tended to reject gambles, which means that they weighed uncertain losses higher than uncertain gains. In a more exploratory fashion, we also ran correlation analyses between k and β to learn more about decision behavior. For the DD task, k and β significantly correlated for the fNC and the oNC groups, but not for the OC group. This means that in naturally cycling women, steeper discounting correlated with more inconsistent choice behavior – but not in women using OCs. Inconsistent choices describe a lower probability of choosing the option with a higher value.

Based on the current literature, we hypothesized (a) less impulsive choices in the fNC group compared with the oNC group, and (b) differences in value-based decision-making between the OC-group and both naturally cycling groups; however, the direction of this difference remained exploratory. Our results did not confirm these hypotheses. One explanation might be the relative scarcity of studies investigating value-based decision-making in different hormonal profile groups. Therefore, formulating straightforward hypotheses might have been premature. Most results so far came from small samples [e.g., Bonenberger et al. (30)], using different tasks, characterizing hormonal profile groups differently [for review, see (29)], and, in general, replication studies are missing [but see Diekhof et al. (50)]. Diekhof et al. (50) replicated results from a within-subjects design in a between-subjects design and showed that avoidance learning capacity is reduced when women were in the high estradiol state of the late follicular phase as compared to the mid luteal phase with more progesterone influence. Although this probabilistic feedback learning task differed in some aspects to the task battery used in our study, the similarity between these tasks lies in maximizing reward by choosing a certain option and, thus, falls within the concept of value-based decision-making. The study by Diekhof et al. (50) not only supports that choice behavior is influenced by hormonal fluctuations, but also confirms the use of between-subjects designs in studies investigating different hormonal states.

Still, in the present study we did not find an effect of different hormonal states on value-based decision-making, especially no difference between the naturally cycling groups and the OC group. One explanation might be a possible hormone-genotype interaction. Jakob et al. (33) investigated how estradiol levels and polymorphisms of the dopamine transporter (DAT1) interact: only naturally cycling women showed a significant effect of DAT1-genotype on reinforcement learning, i.e., a decrease in the ability to avoid punishment with rising estradiol levels in 9RP carriers, while OC-using women did not show any such behavioral variations according to DAT1-genotype differences. This hints at a first hypothesis about how decision-making processes may be affected by the interaction between ovarian hormones and neurotransmitter systems involved in decision-making, namely the dopaminergic system. In the same vein, Jacobs and D'Esposito (51) showed how the interaction between baseline dopamine and estradiol can shape prefrontal cortex dependent working memory performance across the cycle. Here, the effect of estradiol was beneficial or detrimental, depending on the catechol-O-methyltransferase (COMT) genotype, which is involved in metabolizing released dopamine. However, this coupling seems to work differently in women using OCs, leading to no observable variation in behavior (33). The hypothesis of more general differences between naturally cycling women and women using OCs is still uptrend and has been confirmed for several cognitive and behavioral processes [emotion recognition: (13, 52–54); memory performance: (55); fear conditioning and extinction: (56)], however, based on our results, it might not hold true for value-based decision-making.

Evidence increasingly points to considerable effects on brain circuitry and structure following administration of metabolic hormones in form of OC-use [e.g., (26, 27, 57)], however, we do not fully understand the action of OCs on brain and behavior.

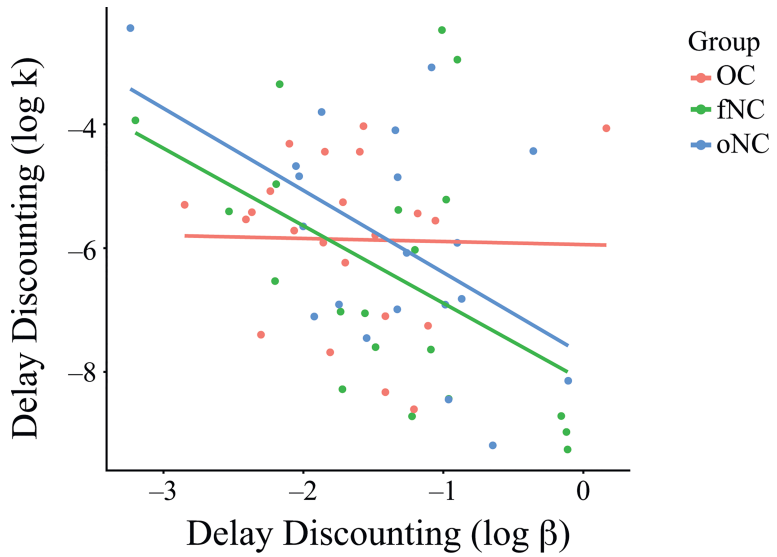

FIGURE 3 | Relationship between the parameters k and β of the delay discounting task per group. Correlation analyses showed significant correlations of k and β for the fNC (r = -.48, p = .033) and oNC (r = -.51, p = .021) groups, but not for the OC group (r = -.02, p = .919; **Figure 3**). However, the correlation coefficients between groups did not differ significantly (fNC vs. OC, z = 1.51, p = .13; fNC vs. oNC, z = 0.12, p = .91; oNC vs. OC, z = 1.63, p = .1; Bonferroni-corrected at α = .017). Each dot represents an individual subject. OC, women using OCs; fNC, women in the early follicular phase; oNC, women during periovulatory phase.

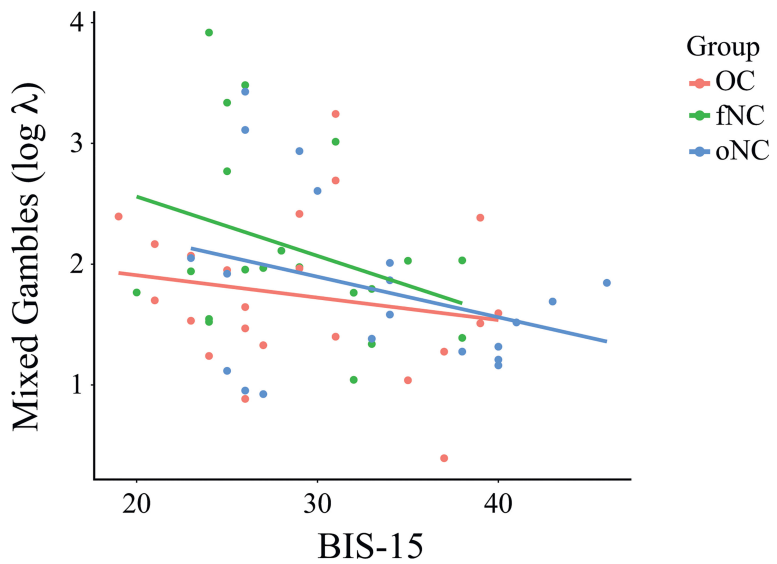

FIGURE 4 | Relationship between the parameter λ (loss aversion) of the mixed gambles task and BIS-15 total score per group. BIS-15 as a covariate was significantly associated with loss aversion for all three groups, $F(3,58)$ = 2.87, p = .044, η_p^2 = .071 Each dot represents an individual subject. OC, women using OCs; fNC, women in the early follicular phase; oNC, women during periovulatory phase.

The present null finding extends the scarce literature on OC-effects on value-based decision-making, especially on the behavioral level. Here, we found no differences between naturally cycling women and women using OCs in making value-based decisions. This result is important for understanding female-specific development, maintenance, and treatment trajectories in mental disorders which are characterized by steep delay discounting, risk-seeking, and

insensitivity to loss, as e.g., reported in patients with substance use disorders. It is just as important to know if and how OC-use impacts behavior related to mental health as well as to highlight which behavior is potentially not affected. Still, further research is needed to investigate potential benefit/harm as well as secondary effects of OC-intake on female behavior.

Limitations

Some limitations have to be noted for the present study. We only used hypothetical monetary rewards. Although hypothetical monetary rewards have been shown to produce similar results as real monetary rewards [e.g., (42, 43)], it would have also been interesting to use real monetary rewards as well as food stimuli. Moreover, we used a relatively new task approach, which has only been used by few studies so far [e.g., (58)]. However, this new approach for adaptive parameter estimation and offer presentation is quick, reliable, and outperforms the most widely used classical approaches.

Also, OC-users in our study had a quite varying mean intake duration of 3.3 years ± 1.7 years. We ruled out a possible impact of these varying intake durations on the results of our study by correlating duration of OC-use with task performance (DD r = -.16, PDG r = -.12, PDL r = .42, MG r = -.33; all p >.05).

Another limitation is that we only tested young female university students, a group with a presumably very good ability to wait for rewards in the first place. They probably did not differ much in the tested value-based decision-making facets at baseline. One solution would be to use a within-subjects design. However, Diekhof et al. (50) could replicate their results of a within-subjects design in a between-subjects design on avoidance learning capacity and therefore provide first evidence for using between-subjects designs in studies investigating influences of different hormonal states.

Another limitation concerning the study design is that we compared OC-users only with the early follicular and periovulatory phases of naturally cycling women, and not with the luteal phase. Firstly, we aimed at contrasting naturally cycling women, i.e., women with a fluctuating hormonal milieu, with women which do not have hormonal fluctuations, at least over certain period, i.e., during active pill intake. Secondly, we further divided the naturally cycling women in a group with overall low endogenous hormone levels, here the fNC group, and a group with high endogenous estradiol levels, here the oNC group, as we had specific hypotheses based on prior knowledge about the impact of endogenous estradiol on value-based decision-making [e.g., (7, 8)]. The luteal phase of the menstrual cycle shows elevated levels of both estradiol and progesterone, which makes it difficult to disentangle specific effects of either one. To this end, we decided to measure a group with overall low endogenous hormone levels (fNC group) and a group with high endogenous estradiol levels only (oNC group), and compare these groups with women with overall low endogenous hormone levels and high exogenous hormone levels (OC group). Therefore, we could ground our hypotheses about the naturally cycling groups on existing literature on estradiol effects on decision-making and focus on the rather exploratory hypotheses about OC effects in this domain. To substantiate the null findings in our study, we encourage to use larger sample sizes and measure women in a longitudinal design, e.g., a naturally cycling group measured at several time-points during the menstrual cycle in comparison with OC-users measured across a similar time-scale.

Conclusion

We investigated the impact of different hormonal profiles on the value-based decision-making constructs delay discounting, risk-aversion, risk-seeking, and loss aversion in women. The three groups – early follicular, periovulatory, and OC-using women – did not differ in the main outcome parameters. We underpinned these null effects using a Bayesian analysis approach, i.e., that there are no differences between groups. While more general differences between naturally cycling women and women using OCs have been confirmed for several cognitive and behavioral processes, it might not be the case for value-based decision-making. Understanding the influence of endogenous and exogenous hormones is important in the context of mental disorders with a focus on decision-making deficits and a known sexual dimorphism.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the Medical Faculty of Tübingen. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CL, NK, MS, JS, and BD designed the study. SP designed the task. CL and A-CK coordinated the study and acquired the data, which CL analyzed. NK, SP, and MS critically revised the analysis. CL wrote the article, which all authors reviewed. All authors approved the final version to be published and can certify that no other individuals not listed as authors have made substantial contributions to the paper.

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Stable Anxiety and Depression Trajectories in Late Adolescence for Oral Contraceptive Users

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Background: The use of oral contraceptives (OCs) has been associated with increased incidences of anxiety and depression, for which adolescents seem to be particularly vulnerable. Rather than looking at singular outcomes, we examined whether OC use is associated with depressive and anxiety symptom trajectories from early adolescence into early adulthood.

Materials and Methods: Data from 178 girls were drawn from the Research on Adolescent Development and Relationships (RADAR-Y) younger cohort study. We used assessments on 9 waves from age 13 until 24. Developmental trajectories of ratings on the Reynolds Adolescent Depression Scale (RADS-2) and the Screen for Child Anxiety Related Emotional Disorders (SCARED) were compared between never and ever users of OCs.

Results: Never users showed increases in depressive and anxiety symptoms in late adolescence, whereas OC users showed a stable level of symptoms throughout adolescence. This effect remained after adjusting for baseline differences between groups in romantic relationships, sexual debut, educational level, smoking, drinking, and drug use. Age of OC use onset did not significantly predict symptom development.

Conclusions: OC use in adolescence was related to an altered developmental trajectory of internalizing symptoms, in which OC users did not show an increase in depressive and anxiety symptoms in late adolescence, whereas never users did. The question remains whether this altered symptom trajectory can be considered a protective effect of OC use on psychopathology. Additional research is needed to improve our understanding of the long-term consequences of OC use on mental health.

Keywords: development, adolescence, anxiety, depression, oral contraceptives (OCs)

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INTRODUCTION

The oral contraceptive pill (OC; "the pill") has been on the market for half a century, is used by about 100 million women world-wide, and is one of the most extensively studied pills in the history of medicine (1, 2). In countries such as the Netherlands, about one in five adolescent girls use OCs to prevent pregnancy, alleviate dysmenorrhea, and/or treat acne (3, 4). Whereas, the physical

side effects of OCs have been subject to a considerable amount of research, a surprising lack of knowledge exists about its effects on affect, including effects on affect-related brain function (5–7). Some studies suggest that women taking OCs will not be subjected to adverse emotional changes (8, 9), or might even experience a protective effect on their mental health (10–14). However, in recent large observational studies OC use has been associated with higher depressive symptoms (15–17), use of antidepressants (17, 18), and an increased risk of suicide (19). Evidence regarding OCs and anxiety has been limited, with some studies showing that OC use is associated with the onset of anxiety symptoms (20, 21), and lower efficacy of exposure-based therapy (22, 23).

The mixed findings have raised doubts about whether the association of OC use with mood deterioration is due to hormonal disruption. The OC pill contains synthetic versions of the hormones estradiol (E) and progesterone (P) and suppresses natural gonadal hormone production (2). Since natural fluctuations of E and P have been associated with emotion regulatory functioning and mood, OC use may result in emotion dysregulation and internalizing symptoms (6). On the other hand, the different outcomes could merely result from existing and unmeasured group differences between OC users and non-users that make OC users inherently more at risk of psychopathology beyond any influence of OC hormones (24). For instance, behavioral and social factors, such as earlier sexual debut, age at menarche, and smoking, have been identified as confounders for both OC use and depression (8, 25, 26).

Additionally, the conflicting evidence may be especially prominent in studies of only adult OC users, as studies including information on adolescent OC use find adolescents to be particularly vulnerable to developing mood symptoms and disorders after OC use (15–17, 27). This finding may be explained by the absence of the survivor bias effect in first time adolescent OC users compared to adult women with possible previous OC experiences. Then again, adolescent OC users may also be more susceptible to the change in hormonal milieu after starting OCs. Adolescence is considered to be a vulnerable period for the development of depression and anxiety disorders (28–30), with girls being twice as likely to experience depressive and anxiety symptoms compared to boys (31, 32). During this developmental period, the gonadal hormones are at the forefront of brain development (33), and hormonal disruptions are therefore likely to affect brain development and mental health in the long run (34). Given that adolescent girls use OCs at an increasingly younger age and for purposes beyond contraception (3), it is important to gain more insight into the effects of OC's on mental health in adolescence.

Whereas depression in adolescents taking OCs has been the focus in some previous longitudinal studies, the effect of adolescent OC use was mostly assessed retrospectively. Therefore, the present study aimed to examine OC's effect on both depression and anxiety symptoms throughout adolescence. Our study also goes beyond prior research by modeling OC effects on the depressive and anxiety symptom trajectories from early adolescence into early adulthood. These symptom trajectories may be more sensitive to OCs effect than assessing clinical diagnoses and differences in singular outcomes. This allows examining the effect of OC use on symptom development over time while taking the naturally fluctuating development of internalizing symptoms into account. Data were used from a longitudinal data set in the Netherlands on adolescent development. Whereas, baseline information before OC onset is often unavailable in prior work, the current dataset allowed us to include measurements on mental health and adolescent developmental markers over the course of several years before and after OC onset. We used this information to a) examine the relationship between OC use and depression and anxiety symptom development throughout adolescence and into early adulthood, b) explore the effect of age of OC onset on these symptom trajectories, and c) identify possible confounders and risk factors in adolescent development of an effect of OC use on internalizing symptoms.

MATERIALS AND METHODS

Participants and Procedure

Participants were drawn from the ongoing Research on Adolescent Development and Relationships (RADAR-Y) young population cohort study (35). They were recruited in schools in the province of Utrecht and four cities the Netherlands in 2006. After the first assessment, participants we used in the present study were followed up for nine measurement waves up to 2017. At the first assessment, 214 adolescent girls were included with a mean age of 13.01 years (SD = 0.44). Until age 18, measurements were done annually; afterwards (after wave 6) they were done biannually. From this sample only participants were included who provided information on oral contraceptive use. Thirty-five participants had missing data on contraceptive use. Four participants used other forms of hormonal contraceptives (e.g., injection or intrauterine device), of which 3 girls had used OCs prior to the current alternative contraceptive. The final sample consisted of 178 native Dutch girls, with a mean age of 12.99 (SD = 0.43) at Wave 1 and 23.83 (SD = 0.43) at Wave 9. Most participants had a medium or high socioeconomic status (SES) (85.4%).

Measures

Oral Contraceptive Use

Current and past use of OC was determined via self-report at Wave 6 with the question: "which (hormonal) contraceptive do you currently use?" Participants could indicate when the first intake of their current contraceptive occurred [in months and years, which previous (other) contraceptive they had used, and which dates they started and stopped (in months and years)]. No information was available on OC type. Girls who reported no previous or current OC use were defined as never users of OCs. Ever users of OCs were defined as girls who reported to use OCs currently or previously. Follow up analyses used age of OC onset ($n = 137$), which was determined by age, date of assessment, and date of onset OC use. Early OC users were defined as users with first intake of OCs before the age of 15 and late OC users were defined as starting OC use at age 15 or older. The age of 15 was selected as a cutoff to separate early and middle/late adolescence

into groups comparable in size ($n = 33$ for early users, $n = 44$ for late users).

Depression

Depressive symptoms were assessed by the Dutch adjusted version of the Reynolds Adolescent Depression Scale, 2nd ed. (RADS-2) (36). The RADS-2 is a self-report measure developed to measure cognitive, motor, somatic and interpersonal depressive symptoms in adolescents. The scale consists of 23 items (the anhedonia subscale was not administered on Waves 2-6) on a 4-point Likert scale ranging from 1 (almost never) to 4 (most of the time), with a minimum score of 23 and maximum score of 92. Items are part of the subscales dysphoric mood, negative self-evaluation, and somatic complaints. In this study, the scale showed good internal consistency across all waves, with a Cronbach's alpha of 0.93. A previous study has shown good psychometric properties for the RADS-2 in adolescents (37).

Anxiety

Anxiety symptoms were assessed with the Screen for Child Anxiety Related Emotional Disorders (SCARED) (38). The SCARED is a self-report instrument designed to measure anxiety symptoms in children and adolescents. The SCARED consists of 38 items scored on a 3-point Likert scale ranging from 1 (almost never) to 3 (often), with a minimum score of 38 and maximum score of 114. The scale assesses the subscales somatic/panic, general anxiety, separation anxiety, social phobia, and school phobia. The internal consistency for the SCARED was good across all waves, with a Cronbach's alpha of 0.93. Multiple previous studies have shown the SCARED has good psychometric properties (38–40).

Covariates

First, covariates were selected if they had previously been associated with OC use and/or internalizing symptoms: age at menarche (25), (age at) sexual debut [yes/no] (25), romantic relationships [yes, no] (41), smoking history [no, yes] (26), religion [not religious, religious] (42), alcohol use [0-5, no alcohol this past month to alcohol every day this month] (43), hard drug use [no, yes] (44), childhood trauma [Childhood Trauma Questionnaire (CTQ)] (45), neuroticism [BIG5] (46), educational level [wave 3] (47), and SES [low, middle/high] (48). Next, the variables that were significantly different between never and ever OC users (or never, early, and late users) were included in the analyses (**Table 1**). These were sexual debut ($p < 0.001$), romantic relationships ($p < 0.001$), education ($p = 0.006$), religion ($p = 0.045$), alcohol use ($p = 0.049$), smoking history ($p = 0.033$) and drug use ($p = 0.082$ for never/ever, $p = 0.040$ for never/early/late).

Analyses

Descriptive statistics were computed for all OC groups (never users, ever users, early users and late users). Growth curve models were used to describe the effect of OC use on the developmental trajectories of both depressive and anxiety symptoms (49). First, a best-fit model was determined that best captured the observed symptom changes in different developmental periods for both depressive and anxiety symptoms. This was done by comparing the chi-square difference ($\Delta\chi^2$) in log likelihood (-2LL) of a one-slope model, two slope model or three slope model with age centered by subtracting 16 from the original values (to compare slopes for early and late adolescence). The one-slope model assumed a linear relationship between age and anxiety or depressive symptoms, where the two-slope model fitted two slopes before and after age 16. The three-slope model plotted one slope for early adolescence (< 14 years), one slope for middle to late adolescence (14–18 years), and one slope for young adulthood (> 18 years). The best fitting model used two age slopes to model the development of symptoms from age 13–24.

The final growth curve model assessed within-subject variation using a random intercept and the two age slopes. In the crude models, between-subject variation was modeled by fixed effects for OC use [never/ever]. The age slopes and two-way interaction effects of OC use and both slopes were included to estimate whether the course of depressive and anxiety symptoms differed according to OC use. The adjusted models included the covariates romantic relationships, sexual debut, educational level, religion, alcohol use, drug use, and smoking history as fixed effects. This resulted in two crude and two adjusted models for OC use effects on depressive and anxiety symptoms, respectively. All growth curve analyses were performed using PROC MIXED in SAS 9.4 (SAS Inc, Cary, NC).

Follow-up analyses grouped OC use as never, early (< 15 years), or late (≥ 15 years) users to examine the effect of age of OC onset on mood development. Additionally, sensitivity analyses were conducted by restructuring the data using age of OC onset in a 2-slope model, setting the slopes to before and after OC onset (slopes before and after the mean age of OC onset for never users). Modeling the effect of OC use based on age of OC onset rather than centered age resulted in similar main results (see **Supplementary Table 1** and **Supplementary Figure 1**). The main growth curve models that used age to model depressive and anxiety symptoms had more power than models using OC onset, because 41 participants had missing data for date of OC onset.

RESULTS

Of the total sample of 178 girls, 60 reported they have never used hormonal contraceptives before and 118 reported they have used OCs. Of the OC users, 6 girls were not currently using OCs but had used them in the past, and the other 112 were currently using OCs. For 77 girls, the age of OC use onset could be determined; the other 41 girls had missing data for date of OC onset. The mean age of OC onset was 14.9 (SD = 1.6). Of the OC users, 33 were considered early users and 44 considered late users. **Table 1** displays the descriptive statistics for never and ever users of OC and for early and late users. OC users were more likely to have had romantic relationships and their sexual debut. Additionally, OC users had a lower level of education, and were more likely to have smoked, have drunk alcohol and be non-religious compared to never users. In **Supplementary Table 2** the correlations between

TABLE 1 | Study sample characteristics depending on history of oral contraceptive use.

Characteristic	Never users ($n = 60$)	Ever users ($n = 118$)	*P*	Early users ($n = 33$)	Late users ($n = 44$)	*P*
Age, mean (SD)	12.9 (0.4)	13.0 (0.5)	0.086	13.0 (0.4)	13.0 (0.5)	0.975
Age at menarche, mean (SD)	12.5 (1.4)	12.5 (1.4)	0.187	12.1 (1.5)	12.7 (1.5)	0.142
Age at sexual debut, mean (SD)	14.7 (4.4)	15.1 (2.1)	0.537	15.0 (1.2)	15.8 (3.0)	0.186
Sexual debut, N(%)	12/60 (20)	93/115 (80.9)	<0.001	28/33 (84.8)	33/41 (80.5)	0.624
Romantic Relationships, N(%)	32/56 (57.7)	93/102 (91.2)	<0.001	24/27 (88.9)	35/40 (87.5)	0.863
Education, mean (SD)	6.4 (2.2)	5.3 (2.5)	0.006	5.2 (2.7)	5.9 (2.4)	0.297
Low Family SES, N(%)	7/59 (11.7)	17/117 (14.4)	0.627	3/33 (9.1)	4/44 (57.1)	1.00
Religious, N(%)	37/60 (61.7)	54/118 (45.8)	0.045	11/33 (33.3)	24/44 (54.5)	0.064
Smoking history, N(%)	18/60 (30.0)	55/118 (46.6)	0.033	17/33 (51.5)	14/44 (31.8)	0.081
Alcohol Use, mean (SD)	1.1 (0.9)	1.4 (0.8)	0.049	1.4 (1.0)	1.3 (0.7)	0.613
Drug Use, N(%)	11/60 (18.3)	36/118 (30.5)	0.082	14/33 (42.4)	14/44 (31.8)	0.338
Childhood Trauma, mean (SD)	1.3 (0.3)	1.3 (0.3)	0.985	1.3 (0.3)	1.3 (0.3)	0.913
Neuroticism[a], mean (SD)	4.3 (1.17)	4.3 (1.22)	0.928	4.4 (1.1)	4.4 (1.2)	0.987

[a] *Scores reflect emotional stability with lower scores meaning higher levels of neuroticism.*

the descriptive characteristics and internalizing symptoms are listed. **Table 2** describes the means and standard deviations of depressive and anxiety symptoms of the final sample at each wave. **Supplementary Table 3** lists the correlations between anxiety and depression for each wave.

Anxiety and Depressive Symptom Trajectories

The best fit model that was used to determine the effect of OC use on symptom development throughout adolescence modeled symptom trajectories over two slopes before and after 16 years for both depression [$\chi^2(6) = 875.9$, AIC = 9997.2, BIC = 10020.7] and anxiety [$\chi^2(6) = 846.9$, AIC = 9010.2, BIC = 9033.7]. For the depressive symptoms, there was no significant main effect of slope 1 (ß = 0.02, $p = 0.0.947$). For the whole sample, depressive symptoms significantly increased in late adolescence (ß = 0.38, $p = 0.027$).

The development of anxiety symptoms in adolescence did not show significant main effects of slopes. Anxiety symptoms remained stable in early (ß = −0.43, $p = 0.147$) and late adolescence (ß = 0.08, $p = 0.613$).

OC Use and Anxiety and Depressive Symptoms

The trajectories over time for depression and anxiety are shown in **Figure 1**. **Table 3** shows the model fit, standardized regression coefficients and standard errors of the models. Depressive symptoms showed an overall increase in late adolescence with a significant main effect of slope 2 ($p = 0.003$). There was a significant interaction effect of slope 2 and OC use ($p < 0.001$): never users of OC showed an increase in depressive symptoms in late adolescence, whereas OC users showed a stable trajectory of depressive symptoms throughout adolescence and young adulthood. The model adjusted for covariates showed similar results, with a significant main effect of age slope 2 ($p = 0.001$) and a significant interaction effect of age slope 2 and OC use ($p < 0.001$), indicating a general increase in depressive symptoms in late adolescence for girls and women who have never used OCs.

In the crude model for anxiety symptoms, only the interaction effect between slope 2 and OC use was significant ($p = 0.023$). Again, an increase toward the end of adolescence and young adulthood was seen only for never users of OCs (**Table 2**). OC users showed a slight decrease of anxiety symptoms in late adolescence. Adjusting for the covariates showed similar results, with a significant interaction effect of age slope 2 and OC use ($p = 0.020$).

Age of OC Use and Anxiety and Depressive Symptoms

Grouping OC use into early and late users resulted in the developmental courses depicted in **Figure 2** (see **Supplementary Table 4** for model fit, standardized regression coefficients and standard errors of the models). For depressive symptoms, results only showed a significant interaction between age slope 2 and OC use ($p < 0.001$). An increase in depressive symptoms was shown for never users of OCs in late adolescence (> 16 years). Late pill users showed a stable trajectory throughout adolescence and early pill users showed a decrease in depressive symptoms after the age of 16. This effect remained after full adjustment for all covariates ($p < 0.001$).

The growth model for anxiety did not show significant effects of age or OC use, the interaction effect of age slope 2 and OC use had a *p*-value of 0.112. Adjusting for covariates resulted in the same non-significant interaction effect of age slope 2 and OC use ($p = 0.099$).

Sensitivity Analyses

The sensitivity analysis using a 2-slope model before and after OC onset showed similar effects as the main models comparing never and ever users, see **Supplementary Table 1** and **Supplementary Figure 1**. A significant increase in symptoms for

TABLE 2 | Depressive and anxiety symptoms per study wave.

	Depressive symptoms M(SD)			Anxiety symptoms M(SD)		
Wave	**All**	**Never users**	**Ever users**	**All**	**Never users**	**Ever users**
Age 13 (W1)	39.5 (11.9)	38 (11.7)	40.3 (12)	53.6 (10.4)	54.2 (11.7)	53.3 (9.5)
Age 14 (W2)	38.2 (13.1)	36.8 (14.2)	39 (12.5)	52.2 (12.4)	53.9 (14.5)	51.2 (11)
Age 15 (W3)	40.3 (14.6)	37.9 (14)	41.6 (14.8)	53.5 (13.4)	52.4 (13.7)	54.1 (13.3)
Age 16 (W4)	40.3 (14.3)	38.9 (14.9)	41.1 (14)	52.5 (13.5)	52.7 (14.7)	52.5 (12.9)
Age 17 (W5)	39.1 (13.9)	38.4 (13.6)	39.6 (14)	51.6 (11.8)	51.1 (11.9)	51.9 (11.7)
Age 18 (W6)	39.9 (13.9)	40.3 (14.4)	39.7 (13.7)	52.2 (12.6)	52.5 (13.9)	52 (11.9)
Age 20 (W7)	40.0 (11.9)	41.6 (13.2)	38.7 (10.7)	52.9 (10.7)	54.4 (12.5)	51.7 (9)
Age 22 (W8)	41.4 (13.4)	44.9 (15.7)	38.4 (10.4)	53.2 (11.4)	55 (13.5)	51.5 (9)
Age 24 (W9)	42.6 (13.2)	47.5 (15.1)	38.7 (10.1)	53.1 (12.3)	56.9 (15.3)	50.2 (8.2)

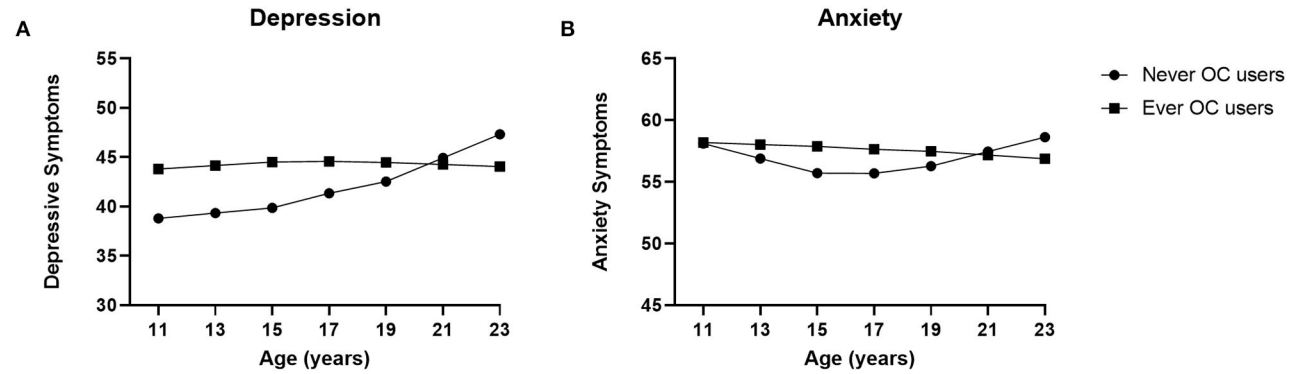

FIGURE 1 | Oral Contraceptive (OC) use and anxiety and depression modeled symptom trajectories. Growth curve models for the depressive and anxiety symptoms for never users of oral contraceptives (OCs) and ever users of OCs. **(A)** In late adolescence depression scores increased significantly for never users, whereas OC users showed stable depression scores throughout adolescence. **(B)** An increase in anxiety symptoms toward late adolescence was significant only for never users of OCs, with stable levels of anxiety symptoms for OC users. Means are adjusted for romantic relationships, sexual debut, education level, religion, smoking history, alcohol use, and drug use.

never users in late adolescence was seen for both depressive ($p = 0.002$) and anxiety ($p = 0.028$) symptoms. When considering age of OC onset, the interaction effects were similar ($p = 0.006$ for depression, $p = 0.086$ for anxiety) compared to the models using age to model the slopes. However, in these models the slopes for early and late OC use did not differ from each other ($p > 0.05$) but only from never users ($p < 0.05$).

DISCUSSION

This study found an altered symptom trajectory of anxiety and depressive symptoms for OC users in late adolescence. Specifically, OC users showed a stable course of depressive and anxiety symptoms throughout adolescence whereas never users showed an increase of internalizing symptoms in late adolescence. When adjusting the developmental trajectories for baseline differences between groups (romantic relationships, sexual debut, educational level, smoking, drinking, and drug use) the effects of OC use remained significant. Furthermore, comparing development before and after OC use resulted in the same effects. That is, no change in symptom development between groups was found before start of OC use, whereas OC users showed a more stable course after OC onset without the increase in symptoms seen on never OC users. Earlier start of OC use even showed a decrease in depressive symptom development after the age of 16, but this effect disappeared when taking the development before and after OC onset into account.

This apparent "protective" role of OC use on internalizing symptoms has been found previously (8, 10–14, 50). In these studies, women using OCs showed lower prevalence of depressive symptoms (12, 13), Major Depressive Disorder (MDD), Generalized Anxiety Disorder (GAD) and Panic Disorder (PD) (10), and even lower risk of past-year suicide attempts (12) compared to non-users. This could be due to a stabilizing effect of OC use on hormonal fluctuations of E and P throughout the menstrual cycle and its co-occurring stabilization of mood variability (51).

The importance of considering relevant confounders when studying OC side effects is shown by these aforementioned studies. They showed that controlling for confounders (including age, BMI, physical activity, chronic disease, number of sexual partners) rendered results non-significant for the protective effects of OCs on psychopathology (8, 10), most likely because

TABLE 3 | Model fit, standardized regression coefficients and standard errors of fixed and random effects as predictors of development of depressive and anxiety symptoms with never and ever users of oral contraceptives.

Parameters	Estimate	SE	χ^2	df	AIC	BIC
Depression - crude						
Intercept	38.11	1.76	815.64	6	8868.6	8890.9
Age 1	0.22	0.46				
Age 2	1.11*	0.25				
OC use	2.41	2.41				
OC use*age 1	−0.11	0.58				
OC use*age 2	−1.25**	0.33				
Depression - adjusted						
Intercept	39.09	3.68	700.41	6	8067.0	8088.2
Age 1	0.27	0.49				
Age 2	1.20*	0.26				
OC use	4.54	2.56				
OC use*age 1	−0.09	0.61				
OC use*age 2	−1.30**	0.34				
Romantic relationships	−4.23	2.53				
Sexual debut	−0.06	2.32				
Educational level	0.51	0.34				
Religion	−1.48	1.67				
Alcohol use	−3.06*	1.05				
Smoking	4.20*	1.85				
Drug use	5.18*	2.05				
Anxiety - crude						
Intercept	51.85	1.65	785.84	6	8098.4	8120.7
Age 1	−0.64	0.51				
Age 2	0.47	0.25				
OC use	0.89	2.04				
OC use*age 1	0.56	0.64				
OC use*age 2	−0.74	0.33				
Anxiety - adjusted						
Intercept	55.11	3.42	692.46	6	7339.8	7361.1
Age 1	−0.60	0.53				
Age 2	0.59	0.24				
OC use	2.69	2.32				
OC use*age 1	0.52	0.68				
OC use*age 2	−0.74*	0.32				
Romantic relationships	−4.66	2.38				
Sexual debut	−0.05	2.20				
Educational level	0.21	0.32				
Religion	−0.72	1.58				
Alcohol use	−3.10*	0.99				
Smoking	2.39	1.75				
Drug use	5.93*	1.94				

*$p < 0.05$, **$p < 0.001$; Adjusted models are corrected for educational level, religion, smoking history, alcohol use and drug use; SE, standard error; df, degrees of freedom. AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion.*

OC use in these studies was associated with increased health-promoting behavior (12). In our study, the stabilizing effect of OC use on internalizing symptoms remained after adjustment for relevant risk factors. However, OC use in our study was associated with increased risk behavior for (mental) health problems. Namely, OC users had a lower educational level, were more likely to have had romantic relationships, their sexual debut, a history of smoking, and were heavier drinkers. These are behaviors that are generally associated with an increased risk of internalizing disorders (26, 43, 44). Our results may

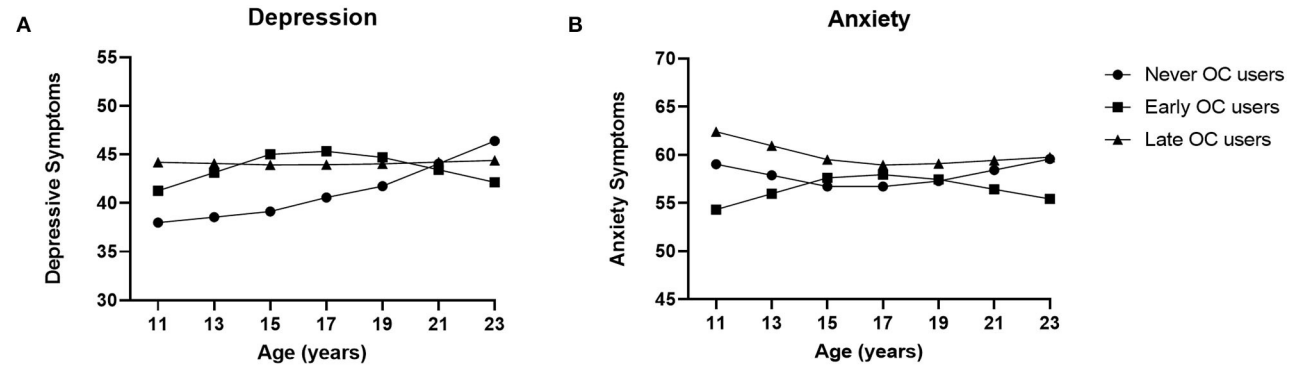

FIGURE 2 | Oral Contraceptive (OC) use, age of onset, and anxiety and depression modeled symptom trajectories. Growth curve models for the depressive and anxiety symptoms for never, early (<15 years), and late (≥15 years) users of oral contraceptives (OCs). **(A)** Never users increased significantly in depressive symptoms in late adolescence, whereas late pill users had a stable trajectory throughout adolescence and early pill users decreased in symptoms after age 16. **(B)** Never, early and late OC users did not show significantly different anxiety symptom trajectories in adolescence. Means are adjusted for sexual debut, romantic relationships, education level, religion, smoking history, alcohol use, and drug use.

therefore better fit with the buffer perspective on risky behavior, which suggests that experimenting with alcohol and drugs may not be detrimental *per se* but can be part of developmentally normative activities (52–54). Beyond the covariates regarding risky behavior that were included in this study, OC use may function as an overall indicator of a more outgoing and social lifestyle with OC users representing the typically developing teenagers. As interpersonal difficulties are considered correlates and risk factors for internalizing disorders such as depression (55), the increased socialization of OC users could buffer the increased depression and anxiety symptoms in late adolescence seen in non-users. However, if these groups are to be considered inherently and substantially different, with OC users reflecting the more normative developing adolescents, it could be expected that the groups showed a significant difference in development before OC onset. This was, however, not the case.

Both our raw and modeled data show an overall increase for depressive symptoms in late adolescence, which is in line with previous studies using the same dataset that have shown an increase in internalizing symptoms in late adolescence for boys and girls (56–58). In the study by Nelemans et al. (57), the results for girls showed a larger intercept and less steep incline in depressive symptoms in late adolescence compared to boys. This would imply a more stable course of symptoms over time in girls, which is possibly an effect of higher baseline levels for girls in general or due to the contribution of OC users. The first inclination would be to interpret the finding of a decrease in internalizing symptoms for OC users in late adolescence as protective. Conversely, the increase in symptoms at the end of adolescence could be considered beneficial and part of developmentally appropriate behavior (59), especially because this increase is seen in both boys and girls (57). Adolescence is a developmental period in which internal changes and external challenges result in higher turmoil than in either childhood or adulthood (60). This significant transition period requires adolescents to adapt to the developmental tasks of that period and provides an opportunity to attain psychological autonomy in adulthood (61). As such, OC use may prevent adolescents from learning to express a range of emotions and developing adequate coping strategies.

Our finding that earlier age of OC onset in adolescence strengthens the effect of OCs on depressive symptoms builds on previous research showing that considering age of OC onset is essential when studying mood related side effects of OC use (15–17, 27). However, this effect disappeared when group differences were assessed after OC onset rather than according to age. The previous effect may then be explained by duration of use, as earlier OC users automatically became longer OC users. The effect of age of OC onset may also not be of added value in this study as only adolescent OC users were included, but remains an important factor to be considered when studying adult OC users.

Strengths, Limitations and Future Directions

In our study we were able to analyze symptom trajectories as a function of OC use over time, which may give more information about effects on mental health than comparing outcomes at any one given time point. The current dataset entailed extensive information regarding adolescent development, which made it possible to include and correct for relevant characteristics predictive of mental health in those years. The methodological challenges when examining OC use on mental health symptoms include problems such as survivor bias and confounding. Our study included information on previous OC use, so we were able to include ever users rather than just current users. Additionally, we were able to correct for most known confounders for both internalizing symptoms and OC use after which the effects remained significant.

Nevertheless, methodological limitations of epidemiological studies like this one are that confounding by unmeasured variables remains a possibility and causality cannot be inferred. For example, our data did not have information on premenstrual symptoms (PMS) or non-contraceptive reasons for using OCs such as acne reduction. The potential protective effect of OCs

on mental health may be due to the reduction of hormonal fluctuations, so the positive effects of OC use may be reserved for women who suffer from the hormonal fluctuations of the menstrual cycle and experience a high degree of premenstrual symptoms and related internalizing symptoms that are alleviated by the stabilizing effects of OCs (11). In addition, future research should consider social functioning as a possible buffer in OC users for future psychopathology. Lastly, the contribution of sexual orientation to the increase in internalizing symptoms in never users should be explored. Non-heterosexual girls will be less likely to start OCs and are at increased risk of developing internalizing symptoms in adolescence (62, 63).

It should also be noted that current and past OC use was based on self-report, and was not confirmed by medical reports. Age of onset of OC use could therefore be subject to inaccuracies. Additionally, information on OC use was only assessed at wave 6 when participants were, on average, 18 years old. Participants starting OC use at a later age may have been wrongly classified as "never" users. However, the vast majority of OC users were probably classified correctly at wave 6, because Dutch girls start OC use on average at age 16 (4). Moreover, a wrongful classification would only mean an underestimation of the stabilizing effect seen in OC users. Removing the wrongfully classified OC users from the never user group would also remove the dampening effect seen in OC users from the already increased symptom trajectories seen in never users. In a sensitivity analysis shown in **Supplementary Table 5**, analyzing only waves 1 through 6 (without 7–9), we found similar results for depression symptoms with significant group differences in late adolescence. Therefore, despite increased differences between groups in standard deviations, the pill effect on depressive symptoms remains apparent in late adolescence. However, the increase in anxiety symptoms in late adolescence for never users rendered insignificant when analyzing only waves 1 through 6. This further showed that the effect of OC use on anxiety symptoms was inconsistent in our sample and depends on the method of analysis (never/ever use, never/early/late use, crude/adjusted, number of waves). Another limitation was the lack of information on type and dose of OC. Considering the different androgenic properties of possible progestins in OCs, different effects on mood are possible and important to consider (7).

After controlling for the aforementioned variables, an effect of OC use on mental health could possibly still be explained by residual confounding by unknown confounders that we could not account for. For example, the confounders could reflect a bigger social buffer effect as mentioned above. At the same time, previous epidemiological studies on OC use and depression have also shown multiple group differences in factors including SES, sexual activity, ethnicity, educational level, BMI, age at menarche, and smoking (8, 15, 16). These recurring group differences point to our sample being representative of the general population.

Our results show the importance of considering age of OC onset when researching mood related effects. However, OC onset data were not available for 41 participants, resulting in more missing data in these analyses compared to the never/ever analyses. Therefore, the results of this analysis have to be interpreted with caution due to lower power and could potentially explain the lack of significant findings with respect to age of OC use onset and anxiety symptom development.

CONCLUSION

In conclusion, this study underlines the importance of considering OC use in the development of internalizing symptoms from adolescence into adulthood and adds to research showing that OC affects mental health. Our results suggest that OC users show a stable trajectory of anxiety and depressive symptoms throughout adolescence whereas girls who do not use OCs show an increase in internalizing symptoms in late adolescence, even after adjusting for confounders. The stabilizing trajectory in OC users is surprising given that OC users reported more health risk behaviors, but it may reflect a group of typically developing girls. The question is whether this altered symptom trajectory in OC users can be considered as a protective effect of OC use on psychopathology and which developmental pattern can be seen as the normative development of internalizing symptoms in adolescent girls. The answer may lie in future studies predicting adaptive outcomes in later life using the separate developmental trajectories of depressive and anxiety symptoms based on OC use. Given the high number of adolescent girls that start OCs in adolescence (3, 4), additional research is needed to improve our understanding of the long-term consequences of OC use on mental health.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: https://doi.org/10.17026/dans-zrb-v5wp (For Waves 1-7, data used for this study are available on request to the corresponding author).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Committees of the Utrecht Medical Center and VU University Medical Center, Netherlands, and the Ethical Committee of the Faculty of Social Science of the Utrecht University, Netherlands. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AD: formal analysis, writing—original draft, and visualization. SB, SN, EM, IE, JB, and WM: conceptualization and writing—review and editing. LG: formal analysis, conceptualization, writing—original draft, and supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2022.799470/full#supplementary-material

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Evaluating the Cognitive Impacts of Drospirenone, a Spironolactone-Derived Progestin, Independently and in Combination With Ethinyl Estradiol in Ovariectomized Adult Rats

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Oral contraceptives and hormone therapies require a progestogen component to prevent ovulation, curtail uterine hyperplasia, and reduce gynecological cancer risk. Diverse classes of synthetic progestogens, called progestins, are used as natural progesterone alternatives due to progesterone's low oral bioavailability. Progesterone and several synthetic analogs can negatively impact cognition and reverse some neuroprotective estrogen effects. Here, we investigate drospirenone, a spironolactone-derived progestin, which has unique pharmacological properties compared to other clinically-available progestins and natural progesterone, for its impact on spatial memory, anxiety-like behavior, and brain regions crucial to these cognitive tasks. Experiment 1 assessed three drospirenone doses in young adult, ovariectomized rats, and found that a moderate drospirenone dose benefited spatial memory. Experiment 2 investigated this moderate drospirenone dose with and without concomitant ethinyl estradiol (EE) treatment, the most common synthetic estrogen in oral contraceptives. Results demonstrate that the addition of EE to drospirenone administration reversed the beneficial working memory effects of drospirenone. The hippocampus, entorhinal cortex, and perirhinal cortex were then probed for proteins known to elicit estrogen- and progestin- mediated effects on learning and memory, including glutamate decarboxylase (GAD)65, GAD67, and insulin-like growth factor receptor protein expression, using western blot. EE increased GAD expression in the perirhinal cortex. Taken together, results underscore the necessity to consider the distinct cognitive and neural impacts of clinically-available synthetic estrogen and progesterone analogs, and why they produce unique cognitive profiles when administered together compared to those observed when each hormone is administered separately.

Keywords: drospirenone (DRSP), ethinyl estradiol (EE), cognition, rat model, contraceptive

INTRODUCTION

Most individuals use some form of contraception during their reproductive years (Daniels and Abma, 2018). In the past several decades, there has been a rise in the popularity of exogenous hormone-containing methods, including oral contraceptives, intrauterine devices, vaginal rings, and subcutaneous implants due to their high reliability not only in preventing unintended pregnancy, but also for their value in treating a range of other health-related indications such as endometriosis, acne, and premenstrual dysphoric disorder (PMDD; Dayal and Barnhart, 2001). It is currently estimated that 79.3% of women have used the oral contraceptive pill at some point during their life; 13.9% of United States women ages 15–44 report current use of the pill between 2015 and 2017; this percentage increases to 19.5% for users in the 20–29 year old age range (Centers for Disease Control and Prevention, 2019). Likewise, some women undergoing the menopause transition opt to take hormone therapy to alleviate unwanted symptoms including hot flashes, dyspareunia, and vaginal dryness (Pinkerton et al., 2017). Furthermore, oral contraceptives are often prescribed for pregnancy prevention during the menopause transition when fertility is variable (Ikhena and Johnson, 2012; Liu, 2021). Thus, a significant number of people will have had exposure to hormone-containing therapies at some point in the reproductive lifespan, and it is imperative to understand the long-term health effects of hormone-containing contraceptives and menopausal hormone therapies.

Progestins act by inhibiting ovulation and altering the uterine and cervical environment for pregnancy prevention (Rivera et al., 1999). If an estrogen-containing formulation is used and the uterus is intact, the progestin component also facilitates prevention of endometrial hyperplasia, making combined oral contraceptives the most popular form of oral contraceptive use (Hall and Trussell, 2012). Due to the low oral bioavailability of natural 17β-estradiol (E2) and progesterone, synthetic forms of estrogen and progesterone are most often utilized. Ethinyl estradiol (EE) is the synthetic estrogen used in nearly all combined oral contraceptive formulations. However, a wide range of progesterone synthetics exist, collectively called progestins. Progestins are derived from a variety of parent molecules structurally related to either testosterone or progesterone. This results in different pharmacological and pharmacokinetic profiles, including variable affinities to the steroid hormone receptors for progesterone, androgens, estrogens, glucocorticoids, and mineralocorticoids (Schindler et al., 2003; Kuhl, 2005; Sitruk-Ware and Nath, 2010). Although progestins have similar progesterone-like effects peripherally, research suggests that many progestins have a negative impact on cognition and reverse neuroprotective estrogen effects (Chesler and Juraska, 2000; Bimonte-Nelson et al., 2004b, 2006; Rosario et al., 2006; Harburger et al., 2007, 2009; Braden et al., 2010, 2011, 2015; Lowry et al., 2010), while others have neutral or even beneficial effects when administered independently (Braden et al., 2017; Prakapenka et al., 2018). Given the prevalence and diversity of progestins used in contraceptives and hormone therapies, it is of critical importance to better understand how synthetic hormones impact the brain and behavior beyond their prescribed uses.

While most United States Food and Drug Administration (FDA)-approved progestins are structurally similar to testosterone and progesterone, the progestin drospirenone is derived from a novel source: spironolactone, an anti-androgenic aldosterone antagonist (Krattenmacher, 2000; Archer et al., 2015). This makes the molecular structure and function of drospirenone unique. Aldosterone is an adrenal-derived hormone that regulates water retention and blood pressure; thus, beyond drospirenone's capacity to bind to the progesterone receptor with high affinity and its structural similarity to progesterone compared to other clinically-available progestins (Muhn et al., 1995; Fuhrmann et al., 1996), it may also modulate fluid retention that naturally occurs during the menstrual cycle (Foidart, 2005; Fenton et al., 2007; Bitzer and Paoletti, 2009). Drospirenone possesses spironolactone-derived anti-androgenic and anti-mineralocorticoid receptor properties without concomitant glucocorticoid receptor activity (Schindler et al., 2003). As such, drospirenone-containing contraceptives are FDA-approved to treat acne vulgaris and PMDD, a mood disorder different from premenstrual syndrome affecting approximately 5% of women (Fenton et al., 2007; Hofmeister and Bodden, 2016). Although drospirenone was reported to increase deep vein thrombosis when it was first popularized, this finding has since been refuted, and its safety profile is considered consistent with other clinically-used progestins (Larivée et al., 2016). It remains a popular progestin component in both oral contraceptives (e.g., YAZ®, Yazmin®, Ocella™, Slynd®, and Nextsellis®) and menopausal hormone therapies (e.g., Angeliq®) at the time of this writing.

Though drospirenone and EE have been reported to improve anxiety, PMDD symptoms, and psychosexual wellbeing in reproductive age women (Paoletti et al., 2004; Pearlstein et al., 2005; Yonkers et al., 2005; Nappi et al., 2009) and drospirenone plus E2 was shown to not impact cognition in menopausal women (Davison et al., 2013), little attention has been dedicated to methodically understanding drospirenone's impact on memory and anxiety-like behaviors from a preclinical perspective. Given drospirenone's unique pharmacological properties and potential for alleviating cognitive symptoms associated with PMDD, it is of significant interest to evaluate its effects on cognition alone and in combination with the synthetic estrogen EE. Our laboratory has demonstrated unique cognitive effects of progestins and estrogens depending on whether the drugs are administered alone or in combination with each other, and which animal model is used to evaluate these effects. For example, the progestin levonorgestrel has null or beneficial effects on spatial working memory when given alone (Braden et al., 2017; Prakapenka et al., 2018), but its beneficial effect is attenuated when combined with E2 in middle-aged ovariectomized (Ovx; surgical ovary removal) rats (Prakapenka et al., 2018). Yet, when E2 and levonorgestrel were co-administered to middle-aged rats with intact but follicle-depleted ovaries, this combined treatment regimen benefited spatial memory, anxiety-like, and depressive-like behaviors (Koebele et al., 2021a). Our laboratory has also reported spatial

memory impairments associated with the progestins segesterone acetate (Woner et al., 2019) and medroxyprogesterone acetate (Braden et al., 2010, 2011).

Estrogens, progesterone, and their synthetic analogs, impact multiple neural systems to influence learning and memory processes. For example, both E2 (Nakamura et al., 2004; Joh et al., 2006) and the progestin medroxyprogesterone acetate (Pazol et al., 2009; Braden et al., 2010) act on the λ-aminobutyric acid (GABA)ergic system, which is the primary inhibitory neurotransmitter system in the brain and a critical modulator of normal learning and memory. Indeed, E2 is known to modulate hippocampal GABAergic activity (Murphy et al., 1998; Nakamura et al., 2004; Moura and Petersen, 2010; Wójtowicz and Mozrzymas, 2010) and synaptic plasticity (Woolley and McEwen, 1993, 1994; Woolley et al., 1997; Miranda et al., 1999; Barha and Galea, 2010; Frankfurt and Luine, 2015; Smith et al., 2016). Although the hippocampus is the most well-characterized structure in terms of E2's impact on cognition, newer studies implicate ovarian hormones in functional changes in the perirhinal and entorhinal cortices, including E2-induced decreases in perirhinal cortex dendritic spine density (Gervais et al., 2015). Recently, it was shown that membrane-bound estrogen receptors enhanced synaptic excitation in the entorhinal cortex, while progesterone and allopregnanolone did not alter entorhinal synaptic responses (Batallán Burrowes et al., 2021). Systemic and intracranial E2 infusions into the perirhinal and entorhinal cortices enhanced novelty preference in Ovx rats, but impaired delayed-non-match to sample task performance (Petrulis and Eichenbaum, 2003; Gervais et al., 2013, 2016), demonstrating hormone-mediated cognitive-behavioral implications. We have previously reported altered glutamate decarboxylase (GAD)65+GAD67 expression in the hippocampus and surrounding cortical areas of Ovx rats treated with medroxyprogesterone acetate (Braden et al., 2010), which points to a putative mechanism for the detrimental cognitive effects of progestogens. Natural progesterone can reverse estrogen-induced growth factor increases in the entorhinal cortex in aged female rats (Bimonte-Nelson et al., 2004a), while the progestin segesterone acetate increased expression of insulin-like growth factor-1 receptor (IGF1-R), which is important for neurogenesis, in the frontal cortex of mice (Chen et al., 2018). Estrogen receptors also frequently colocalize with IGF1-R receptors on neuronal and glial cells, which may promote estrogen-induced neuroprotective effects (Garcia-Segura et al., 2007). Indeed, even short-term E2 administration has been shown to produce long-lasting increases in IGF1-R expression and benefit cognition (Witty et al., 2013), while IGF1-R blockade alters E2-induced hippocampal plasticity changes and spatial memory enhancements (Nelson et al., 2014). While evaluations of neurobiological mechanisms of the synthetic analog EE are in their infancy, our laboratory reported dose-dependent effects of EE on memory, wherein a higher dose impaired memory (Mennenga et al., 2015a). This underscores the need to investigate neural actions of EE, as well as whether drospirenone has similar neurobiological actions as other progestogen-mediated effects reported in the literature.

Elucidating whether drospirenone differentially impacts cognition in contrast to progestins from traditional derivatives is an important step toward refining and discovering novel pharmacotherapies that can provide long-term health benefits, including potential neuroprotection. In order to assess the impact of drospirenone on cognition, two experiments were performed. Using young adult, Ovx Fischer-344-CDF (F344-CDF) rats, Experiment 1 evaluated a range of doses of drospirenone to determine an optimal dosing regimen that impacted cognition. Experiment 2 incorporated the optimal dose determined from Experiment 1 and combined this dose with EE to investigate cumulative effects of the drugs on cognitive performance. Brains from both experiments were analyzed for GAD65, GAD67, and IGF1-R protein expression.

MATERIALS AND METHODS

Experimental procedures and statistical analyses were identical for Experiment 1 and Experiment 2.

Subjects

One hundred sexually inexperienced 3-month-old female F344-CDF rats were obtained from Charles-River Laboratories (Raleigh, NC, United States). Forty subjects were included in Experiment 1 and 60 subjects were included in Experiment 2. Upon arrival to the animal facility, all rats were pair-housed, provided with free access to food and water for the duration of the experiment, and were maintained on a 12-h light/dark cycle for the entirety of the experiment. Procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Ovariectomy

After 9 ± 1 day of acclimation to the vivarium, all rats underwent Ovx in order to initiate a "blank ovarian hormone slate," which permits the evaluation of specific treatment effects without interactions with endogenously circulating hormones. Rats were anesthetized via inhaled isoflurane anesthesia. Five mg/kg/mL of the NSAID carprofen (Rimadyl®; Pfizer Pharmaceutical, Hospira Inc., Lake Forest, IL, United States) was administered to prevent post-surgical discomfort. Following sterilization of the surgical area, all rats received bilateral dorsolateral incisions through the skin and muscle. Ovaries and tips of the uterine horns were ligated and excised on each side. Muscle was sutured with dissolvable Vicryl suture and a local anesthetic, bupivacaine (Marcaine®; Pfizer Pharmaceutical, Hospira Inc., Lake Forest, IL, United States), was applied topically to the incision site. Skin was sutured with dissolvable Vicryl suture. All rats received two mL of sterile saline subcutaneously to prevent post-surgical dehydration.

Hormone Treatment: Experiment 1

Drospirenone is abbreviated as DRSP in reference to treatment groups. Rats were randomly assigned to one of the following treatment groups (n = 10/group): Vehicle (sesame oil, control),

DRSP-Low (12.5 μg/day), DRSP-Medium (30 μg/day), or DRSP-High (300 μg/day). Two ± one days after Ovx surgery, daily subcutaneous treatment injections began. Each treatment was administered in 0.1 mL of sesame oil in the scruff of the neck, and continued throughout the entirety of the experiment until euthanasia. The low DRSP dose was based on the most common dose prescribed to women in a combined oral contraceptive (3 mg/day), adjusted for an average weight (250 *g*) Ovx rat. Clinically, the ratio of DRSP to EE in a typical combined oral contraceptive is 100:1, so the medium DRSP dose reflected this ratio when considering the range of EE doses used in Experiment 2. The high DRSP dose was a replication of the dose used in a prior study from another laboratory (Muhn et al., 1995) that resulted in ovulation inhibition in ovary-intact adult rats.

Hormone Treatment: Experiment 2

Rats were randomly assigned to one of the following treatment groups (n = 10/group): Vehicle (sesame oil, control); DRSP (30 μg DRSP/day); EE-Low (0.125 μg EE/day), EE-High (0.3 μg EE/day), DRSP + EE-Low (30 μg DRSP/day + 0.125 μg EE), or DRSP + EE-High (30 μg DRSP/day + 0.3 μg EE). All treatments were administered in the same fashion as Experiment 1, in 0.1 mL of sesame oil in the scruff of the neck beginning 2 ± 1 days after Ovx and continued daily throughout the experiment until euthanasia. The EE doses utilized were based on prior research from our laboratory; the Low-EE dose represented a typical dose of EE in a modern-day oral contraceptive (30–35 μg/day), and the High-EE dose represented the higher doses of EE prescribed in earlier generations of oral contraceptives (75–80 μg/day), each adjusted for rat body weight (Mennenga et al., 2015a). The DRSP-Medium dose (30 μg/day) was chosen for use in Experiment 2 based on Experiment 1 results.

Vaginal Cytology

Eighteen days after the first hormone injection, vaginal smears were performed for three consecutive days to confirm successful Ovx in Vehicle-treated rats and evaluate how DRSP and/or EE treatment impacted vaginal epithelial cells after Ovx. Cytology was characterized based on specifications in Goldman et al. (2007) and Koebele and Bimonte-Nelson (2016), whereby: diestrus smears contained leukocytes with or without the presence of cornified cells; proestrus smears contained round, nucleated epithelial cells and cornified cells present in clusters; estrus was defined by the presence of cornified cells; and metestrus contained a combination of cornified cells, leukocytes, round cells, and keratinized, needle-like cells (Goldman et al., 2007; Koebele and Bimonte-Nelson, 2016).

Body Weights

Beginning at Ovx surgery (baseline), weekly weights (grams) were recorded for all rats until the end of each experiment.

Behavioral Battery

One month after daily hormone treatment initiation, rats were assessed on the water radial-arm maze (WRAM) and Morris water maze (MM) to evaluate spatial working and reference memory (RM). Following water maze tasks, rats were tested on the open field task (OFT) to assess locomotor activity and anxiety-like behavior.

Water Radial-Arm Maze

The WRAM was an eight arm apparatus used to test spatial working and RM in rodents, as previously described (Bimonte and Denenberg, 1999; Bimonte et al., 2000; Bimonte-Nelson et al., 2015). Working memory required updating within a session. RM remained constant through the entirety of the task across days. Each arm was identical in size (29.7 cm long × 12.7 cm wide) and evenly spaced, radiating out from the circular center of the maze. Black non-toxic powdered paint was used to make the water (18–20°C) opaque. Four out of the eight arms contained hidden platforms placed 2 cm below the water's surface at the beginning of each daily testing session. The specific locations of the platforms were constant within a rat for all testing days, but platform location combinations varied among rats and combinations were counterbalanced across treatment groups. Salient spatial cues were present on the walls around the maze to assist with spatial navigation.

Rats underwent baseline WRAM testing for 12 consecutive days, with four trials administered per daily testing session (one trial per hidden platform). The trial began when the experimenter placed the rat in the non-platformed start arm. Rats had 3 min per trial to locate a hidden platform. If the rat did not locate a platform in the maximum allotted time of 3 min, the experimenter led the rat to the nearest hidden platform. Once a platform was located, the rat was permitted to stay on it for 15 s, and then the experimenter removed the rat from the maze and placed it back into a heated testing cage for a 30 s inter-trial-interval. During those 30 s, the experimenter removed the just-found platform from the maze for the remainder of the daily testing session and gently stirred the water with a net to obscure potential olfactory cues and remove any debris from the water. The rat was placed back into the maze for the remaining three trials in an identical manner. Following 12 days of baseline testing, on day 13, a 6-h delay was implemented between trials two and three to test delayed working memory retention. Cognitive performance on the WRAM was quantified by the number of non-platformed arm entries—called errors—committed prior to locating a platform on each trial. An arm entry was quantified when the rat's nose passed a designation mark 11 cm into the arm that was visible to the experimenter but not visible to the rat. Errors were defined in one of three categories: working memory correct (WMC) errors were entries into a previously platformed arm (which may occur on trials 2–4), RM errors were entries into a never-platformed arm for the first time within a daily testing session (capped at four errors), and working memory incorrect (WMI) errors were defined as repeat entries into never-platformed arms within a daily testing session.

Morris Water Maze

The MM was a large round tub (diameter = 188 cm) filled with 18–20°C black-painted water used to assess spatial RM (Morris et al., 1982; Bimonte-Nelson et al., 2015; Morris, 2015). One

hidden platform was submerged within the northeast quadrant of the maze, where it remained for all days and trials. Spatial cues were placed on the walls in the testing room to aid in spatial navigation. Each rat received four trials per day for five days. On each trial, a rat was dropped off from a cardinal direction (north, east, south, or west). The order of drop-off locations was the same for all rats within a day, but changed across days. The maximum trial time was 60 s. If a rat did not locate the hidden platform within the maximum allotted time, the experimenter led the rat to the platform. Once the rat found the platform, it remained on the platform for 15 s prior to being returned to its heated testing cage for an inter-trial-interval of approximately 15 min. The rats' swim paths were recorded using Ethovision software (Noldus Instruments, Wageningen, Netherlands). On the fifth testing day, an additional trial was implemented following the four baseline trials. During this trial, called the probe trial, the platform was removed from the maze and the rats were allowed to swim freely for 60 s to assess spatial localization to the platform.

Open Field Task

The OFT measured locomotor activity and anxiety-like behavior. This task has been shown to be sensitive to the presence and absence of ovarian hormones (Hiroi and Neumaier, 2006). The OFT was a 100 cm × 100 cm × 30 cm black plexiglass arena. Although some paradigms use a bright light in the center of the maze, this assay was completed in red light (i.e., darkness for rats), as we have previously published (Koebele et al., 2021a). This is because rats with significant anxiety-like phenotypes tend not to move at all if the center of the arena is lit. One day prior to the OFT, the arena was cleaned with an enzyme cleaner to remove any odors in and on the box. Rats were acclimated to the anteroom of the testing area for at least 30 min. Each rat was placed in the arena along the north wall. The experimenter quietly exited the room while the rat was allowed to explore the arena freely for a 10-min trial. The rat was then placed back in its testing cage and removed from the room. The experimenter counted and removed any fecal boli from the arena, cleaned the arena with water, and dried it with paper towel prior to the next subject's trial. Dependent variables assessed in the OFT were total distance moved, as well as distance moved and time spent in the arena center (inner nine squares), small center (inner-most square), and arena corners. Twenty-five evenly spaced squares were digitally overlaid on the OFT tracks, and distance moved and time spent were recorded using Ethovision tracking software.

Euthanasia

One day following OFT completion, rats were deeply anesthetized with inhaled isoflurane anesthesia. Brains were removed and the dorsal hippocampus, entorhinal cortex, and perirhinal cortex of the right hemisphere were rapidly raw dissected, weighed, and frozen at −70°C until western blot analysis. Ovx status was verified at necropsy and the uterine horns were removed from the body cavity, trimmed of visible fat, and wet weight was recorded.

Western Blot Protein Analysis

Right hemisphere dorsal hippocampus, entorhinal cortex, and perirhinal cortex from each experiment were analyzed for GAD65 expression, GAD67 expression, and IGF1-R expression via western blots. Frozen raw tissue samples were suspended in a 1:25 weight-to-volume RIPA buffer solution [150 mM NaCl, 1%Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50-mM Tris–HCl, protease inhibitor (Millipore-Sigma, CAT#5892791001)], and phosphatase inhibitor (Millipore-Sigma, CAT#524625). Tissues were kept on ice at all times and homogenized using a probe sonicator (Ultrasonic Processor, Cole Parmer, IL, United States), and then centrifuged at 10,000 rpm for 10 min at 4°C. Cleared supernatants were collected, aliquoted, and frozen at −70°C until analysis. Bicinchoninic acid protein assays (Thermo-Fisher Scientific, Pittsburgh, PA, United States) were used to determine sample protein concentrations.

Within an experiment, treatment groups were counterbalanced and equally represented on each gel run. The NuPAGE PowerEase electrophoresis system was utilized for tissue processing. Tissue samples for each region were loaded at an equal protein concentration and were run on a 4–12% NuPAGE Bis-Tris gel in an XCell SureLock Mini-Cell with MOPS running buffer (Invitrogen, Carlsbad, CA, United States) and transferred to an Immobilon polyvinylidene difluoride membrane. The membrane was washed in 1× Tris-buffered saline with 0.1% Tween (TBST) and blocked in 5% non-fat milk for 1 h at room temperature. Following blocking, the membrane was washed in 1× TBST and incubated overnight on a shaker at 4°C with anti-GAD65 (1:5000; Abcam, ab26113; 65 kDa), anti-GAD67 (1:10,000; Abcam, ab 26116; 67 kDa), anti-IGF1-R (1:1000; Cell Signaling, #9750S; 95 kDa), and loading control anti-beta-actin (1:20,000; Cell Signaling, #4970S; 45 kDa) in 5% non-fat milk. The following day, the membrane was washed in 1× TBST and incubated with secondary antibodies anti-mouse horseradish peroxidase (HRP; 1:2000; Cell Signaling #7076S) for GAD65 and GAD67, and anti-rabbit HRP (1:2000; Cell Signaling #7074) for IGF1-R and beta-actin for 1 h at room temperature in 5% non-fat milk. The membrane was washed in 1× TBST, and developed using chemiluminescence (Lumiglo and peroxide, Cell Signaling #7003S) in a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). Films were scanned to the computer as JPEG files at 600 dpi. Densitometry analyses were completed using ImageJ software (Gallo-Oller et al., 2018). GAD65, GAD67, and IGF1-R bands were normalized to corresponding beta-actin for each blot.

Statistical Analyses

A priori two-group comparisons between each DRSP group and Vehicle were completed using repeated measures analysis of variance (ANOVA) for Experiment 1. For Experiment 2, each DRSP, EE, and DRSP + EE group was compared to Vehicle using two-group comparisons. Additionally, we compared EE-Low and EE-High groups to each other to assess dose-dependent effects, as well as compared EE-Low, EE-High, and DRSP groups to the combinations of DRSP + EE to assess effects of the hormones alone vs in combination with one another to evaluate differential

cognitive effects when hormone treatments are co-administered (Prakapenka et al., 2018; Koebele et al., 2021a). Alpha level was set to 0.05 for all analyses. Generalized eta squared (η_G^2) was calculated for repeated measures ANOVA as a measure of effect size (Olejnik and Algina, 2003; Bakeman, 2005). For all non-repeated measures ANOVA, eta squared (η^2) was reported. Standard effect size guidelines were applied to interpretations of η_G^2 and η^2, with 0.02 as a small effect, 0.13 as a medium effect, and 0.26 as a large effect (Olejnik and Algina, 2003; Bakeman, 2005).

Water radial-arm maze data were separated into three phases based on error-making patterns in the learning curve, as we have previously published (Mennenga et al., 2015b; Braden et al., 2017; Prakapenka et al., 2018; Koebele et al., 2019, 2021a). Day 1 was considered training and was excluded from the analysis. Days 2–5 were the Early Acquisition Phase when rats are exploring the maze and learning the rules of the task. Days 6–9 were the Late Acquisition Phase, when error scores begin to decrease but there is still variability in performance as rats consolidate the win-shift rules, such that they have to shift spatial locations to be rewarded (i.e., removed from the maze) on each trial. Days 10–12 were the Asymptotic Phase, when rats are reaching peak performance and approaching asymptotic error scores. WMC, RM, and WMI errors were the dependent measure analyzed separately for the Early Acquisition Phase, Late Acquisition Phase, and Asymptotic Phase. Trials (three trials for WMC, four trials for RM and WMI) were nested within days as repeated measures. Treatment was the independent variable. Based on prior findings indicating working memory load-dependent hormone effects, we also analyzed the moderate (Trial 3) and maximum (Trial 4) working memory load trials separately, as previously done (Braden et al., 2010; Mennenga et al., 2015a; Koebele et al., 2017, 2019, 2021b; Prakapenka et al., 2018).

Morris water maze data were analyzed using repeated measures ANOVA, with Treatment as the independent variable and Swim Distance to Platform (cm) as the dependent variable, with four trials nested within the five days as repeated measures for each two-group comparison. Groups were evaluated separately for probe trial performance [percent of total swim distance in the northeast (previously platformed) Quadrant vs the southwest (opposite) Quadrant].

Open field task data were analyzed using ANOVA, with Treatment as the independent variable, and Total Distance Moved (cm), Center Distance, Center Time, Small Center Distance, Small Center Time, Corner Distance, and Corner Time as dependent variables for each two-group comparison.

Western blot protein analyses were completed using ANOVA, with Treatment as the independent variable and GAD65, GAD67, and IGF1-R expression normalized to beta-actin (arbitrary units; AU) in the dorsal hippocampus, entorhinal cortex, and perirhinal cortex as the dependent variable for each two-group comparison. Body weight and uterine weight were also analyzed using ANOVA, with Treatment as the independent variable and Weight (*g*) as a dependent variable for each two-group comparison.

Pearson's r correlations were completed between IGF1-R expression, GAD65 expression, and GAD67 expression in the dorsal hippocampus, entorhinal cortex, and perirhinal cortex with WMC and WMI errors on the WRAM for each phase across all trials, as well as for Trial 3 only and Trial 4 only. The False Discovery Rate (FDR) correction using the Benjamini-Hochberg procedure was applied with an FDR = 0.25 (Benjamini and Hochberg, 1995; McDonald, 2014).

RESULTS

Experiment 1

Water Radial-Arm Maze

Early Acquisition Phase

Across all days and trials of the Early Acquisition Phase, there was a main effect of Treatment for the Vehicle vs DRSP-Low comparison [$F_{(1,18)} = 5.71$, $p < 0.05$, $\eta_G^2 = 0.02$] where rats treated with DRSP-Low made fewer WMC errors on Trials 2–4 compared to Ovx rats without hormone treatment. All DRSP-treated groups made fewer errors than Vehicle-treated rats on the moderate working memory load trial (Vehicle vs DRSP-Low: [$F_{(1,18)} = 5.69$, $p < 0.05$, $\eta_G^2 = 0.06$]; Vehicle vs DRSP-Medium: [$F_{(1,18)} = 10.50$, $p < 0.01$, $\eta_G^2 = 0.10$]; and Vehicle vs DRSP-High: [$F_{(1,18)} = 7.57$, $p < 0.05$, $\eta_G^2 = 0.07$]; **Figure 1A**). A Treatment main effect was also present for WMI errors on the moderate memory load trial for the Vehicle group vs the DRSP-Low group [$F_{(1,18)} = 7.83$, $p < 0.05$, $\eta_G^2 = 0.08$] and vs the DRSP-Medium group [$F_{(1,18)} = 5.13$, $p < 0.05$, $\eta_G^2 = 0.05$], where each DRSP-treated group made fewer WMI errors compared to Vehicle-treated rats (**Figure 1B**). There were no differences in working memory performance on the maximum working memory load trial during early acquisition, nor were there RM effects in the Early Acquisition Phase.

Late Acquisition Phase

There were no main effects of Treatment during the Late Acquisition Phase for WMC, WMI, or RM errors for any two-group comparison.

Asymptotic Phase

There was a main effect of Treatment for WMI errors for the Vehicle vs DRSP-Medium comparison [$F_{(1,18)} = 4.47$, $p < 0.05$, $\eta_G^2 = 0.02$] and a Trial × Treatment interaction for this comparison [$F_{(3,54)} = 4.47$, $p < 0.01$, $\eta_G^2 = 0.06$] across all trials and days of the Asymptotic Phase. On the maximum working memory load trial, DRSP-Medium rats made fewer WMI errors compared to Vehicle treated rats ([$F_{(1,18)} = 4.47$, $p < 0.05$, $\eta_G^2 = 0.07$], **Figure 1C**). There were no significant effects for WMC or RM errors in the Asymptotic Phase.

Delayed Memory Retention

Working memory correct errors on Trial 3 from the final day of baseline testing (day 12) were compared to WMC errors on Trial 3 after the 6-h delay (the first post-delay trial on day 13) for each treatment group. Post-delay errors on Trial 3 were increased for Vehicle rats [$F_{(9,1)} = 5.44$, $p < 0.05$, $\eta_G^2 = 0.52$] and DRSP-Low rats [$F_{(9,1)} = 10.79$, $p < 0.01$, $\eta_G^2 = 0.33$] compared to the previous day's performance in each group (**Figures 1D,E**). DRSP-Medium and DRSP-High groups did not show a delay-induced impairment (**Figures 1F,G**).

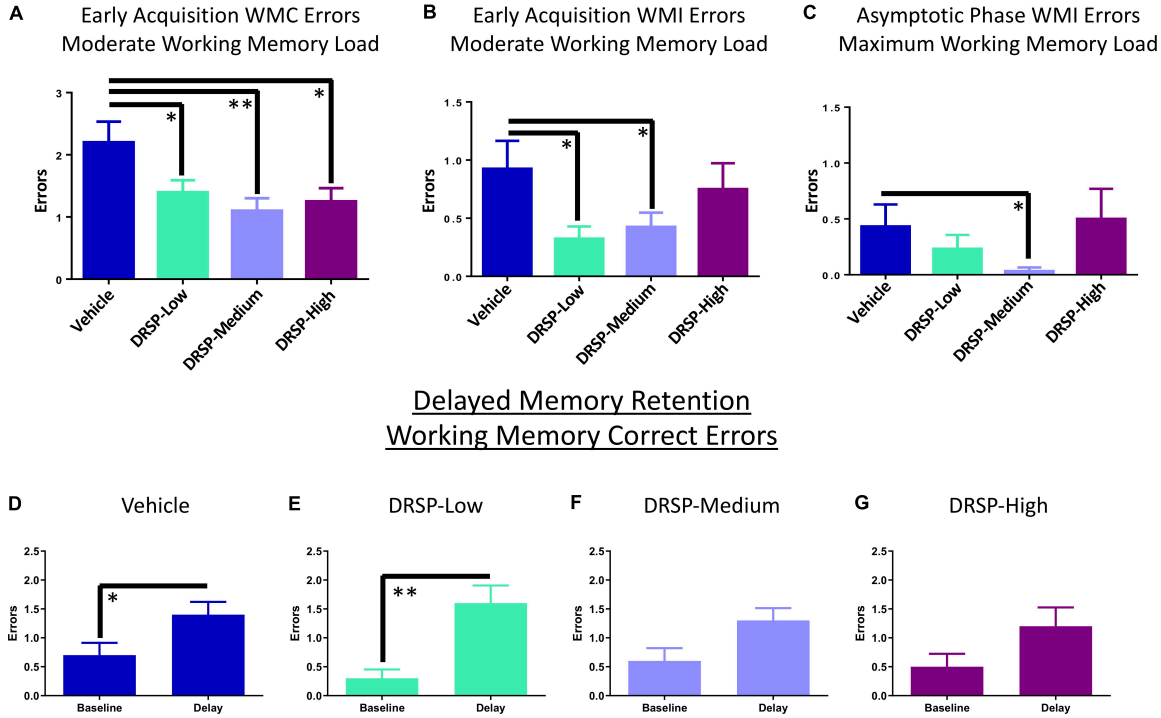

FIGURE 1 | Experiment 1 Water radial-arm maze. **(A)** During Early Acquisition, all groups treated with drospirenone had improved WMC performance compared to rats without hormone treatment when working memory load was moderately taxed. **(B)** This working memory benefit was extended to WMI errors on the moderate working memory load trial for DRSP-Low and DRSP-Medium groups compared to rats without hormone treatment. **(C)** During the Asymptotic Phase, DRSP-Medium continued to enhance WMI performance compared to rats without hormone treatment on Trial 4, the maximum working memory load trial. Rats treated with Vehicle **(D)** or DRSP-Low **(E)** showed impaired delayed memory retention on Trial 3 following a 6-h delay compared to the previous day's baseline performance. Rats treated with **(F)** DRSP-Medium and **(G)** DRSP-High treatment did not exhibit a statistically significant delay-related impairment. $^{*}p < 0.05$ and $^{**}p < 0.01$. Vehicle $n = 10$, DRSP-Low $n = 10$, DRSP-Medium $n = 10$, and DRSP-High $n = 10$.

Morris Water Maze

Across all days and trials, there was a main effect of Treatment for the Vehicle vs DRSP-Medium comparison [$F_{(1,18)} = 7.37$, $p < 0.05$, $\eta_G^2 = 0.02$], and for the Vehicle vs DRSP-High comparison [$F_{(1,18)} = 7.21$, $p < 0.05$, $\eta_G^2 = 0.03$] where rats treated with the medium or high dose of drospirenone swam less distance to the platform compared to Vehicle-treated rats (**Figures 2A–D**). Each group was analyzed separately on the probe trial. There was a Quadrant main effect for each group (Vehicle: [$F_{(9,1)} = 60.17$, $p < 0.0001$, $\eta_G^2 = 0.23$]; DRSP-Low: [$F_{(9,1)} = 174.65$, $p < 0.0001$, $\eta_G^2 = 0.27$]; DRSP-Medium: [$F_{(9,1)} = 78.85$, $p < 0.0001$, $\eta_G^2 = 0.18$]; and DRSP-High: [$F_{(9,1)} = 56.32$, $p < 0.0001$, $\eta_G^2 = 0.15$]). This indicated that all rats, regardless of treatment, swam a greater percent of total distance in the target, compared to the opposite, quadrant (**Figure 2E**).

Open Field Task

DRSP administration at any dose had no impact on overall locomotor activity or measures of anxiety-like behavior in Ovx rats as measured in the OFT.

Peripheral Markers of Hormone Stimulation

Vaginal Smears

All groups displayed diestrus-like or blank cytology, indicating that Ovx was successful and that daily drospirenone treatment at all doses evaluated did not stimulate the vaginal epithelium following Ovx.

Body Weight and Uterine Weights

There were no main effects of Treatment on body or uterine weights at euthanasia for any two-group comparison, indicating that drospirenone administration at any assessed dose did not influence body or uterine weight after Ovx.

Western Blot Protein Analysis

Representative blots for each protein within all assessed brain regions are pictured in **Figure 3A**. There were no effects of Treatment for GAD65 expression, GAD67 expression, or IGF1-R expression in the dorsal hippocampus, entorhinal cortex, or perirhinal cortex for any two-group comparison (**Figures 3B–D**), indicating the drospirenone treatment after Ovx did not impact

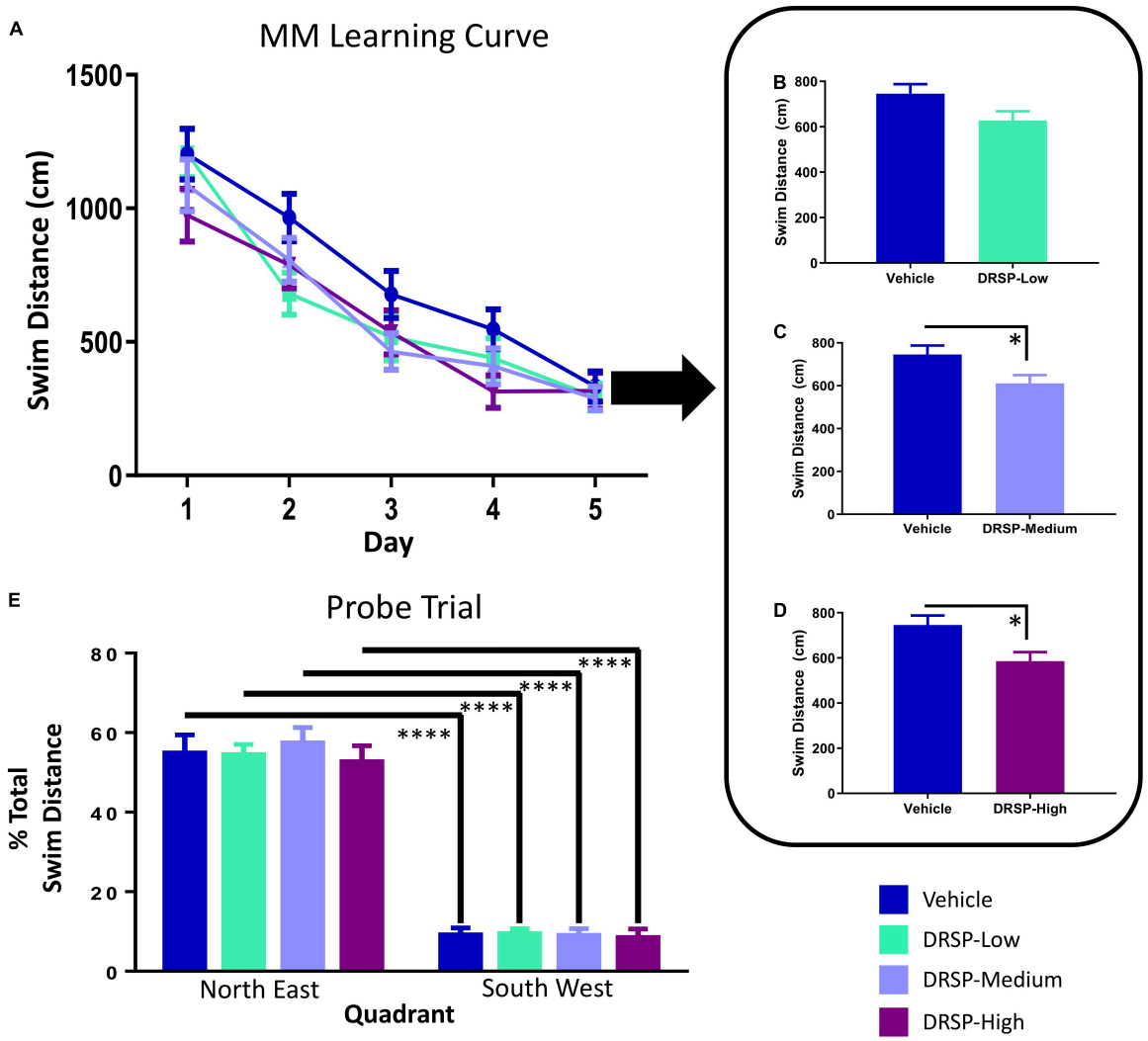

FIGURE 2 | Experiment 1 Morris water maze. Across all days of testing **(A)**, rats treated with the DRSP-Medium dose **(C)** and the DRSP-High dose **(D)** swam less distance to reach the platform compared to rats without hormone treatment, while the DRSP-Low group **(B)** performed similarly to the Vehicle group. **(E)** On the probe trial, all groups localized to the previously platformed target quadrant. *$p < 0.05$ and ****$p < 0.0001$. Vehicle $n = 10$, DRSP-Low $n = 10$, DRSP-Medium $n = 10$, and DRSP-High $n = 10$.

GAD or IGF1-R protein expression in brain regions important for spatial learning and memory.

Correlations

After correcting for multiple comparisons using the FDR method, there were no significant correlations between western blot results and WRAM performance in Experiment 1.

Experiment 2

Water Radial-Arm Maze

Early Acquisition Phase

There was a Trial × Treatment interaction for WMI errors for the Vehicle vs EE-Low group [$F_{(3,54)} = 3.03$, $p < 0.05$, $\eta_G^2 = 0.01$]. Rats treated with DRSP + EE-Low made fewer RM errors during early acquisition compared to Vehicle-treated rats [$F_{(1,18)} = 5.17$, $p < 0.05$, $\eta_G^2 = 0.01$]. DRSP-treated rats made fewer WMI errors than Vehicle-treated counterparts on the moderate working memory load trial alone [$F_{(1,18)} = 7.03$, $p < 0.05$, $\eta_G^2 = 0.08$], replicating findings from Experiment 1 (**Figure 4A**). Furthermore, rats treated with DRSP alone made fewer WMI errors on the moderate working memory load trial compared to DRSP + EE-Low rats [$F_{(1,18)} = 7.79$, $p < 0.05$, $\eta_G^2 = 0.07$] and DRSP + EE-High rats [$F_{(1,18)} = 10.23$, $p < 0.01$, $\eta_G^2 = 0.09$], indicating that the addition of EE at either dose impaired working memory compared to drospirenone alone during the Early Acquisition Phase (**Figure 4A**).

Late Acquisition Phase

Across all days and trials in the Late Acquisition Phase, the EE-Low vs DRSP + EE-Low comparison revealed a main effect of Treatment [$F_{(1,18)} = 5.09$, $p < 0.05$, $\eta_G^2 = 0.04$] as well

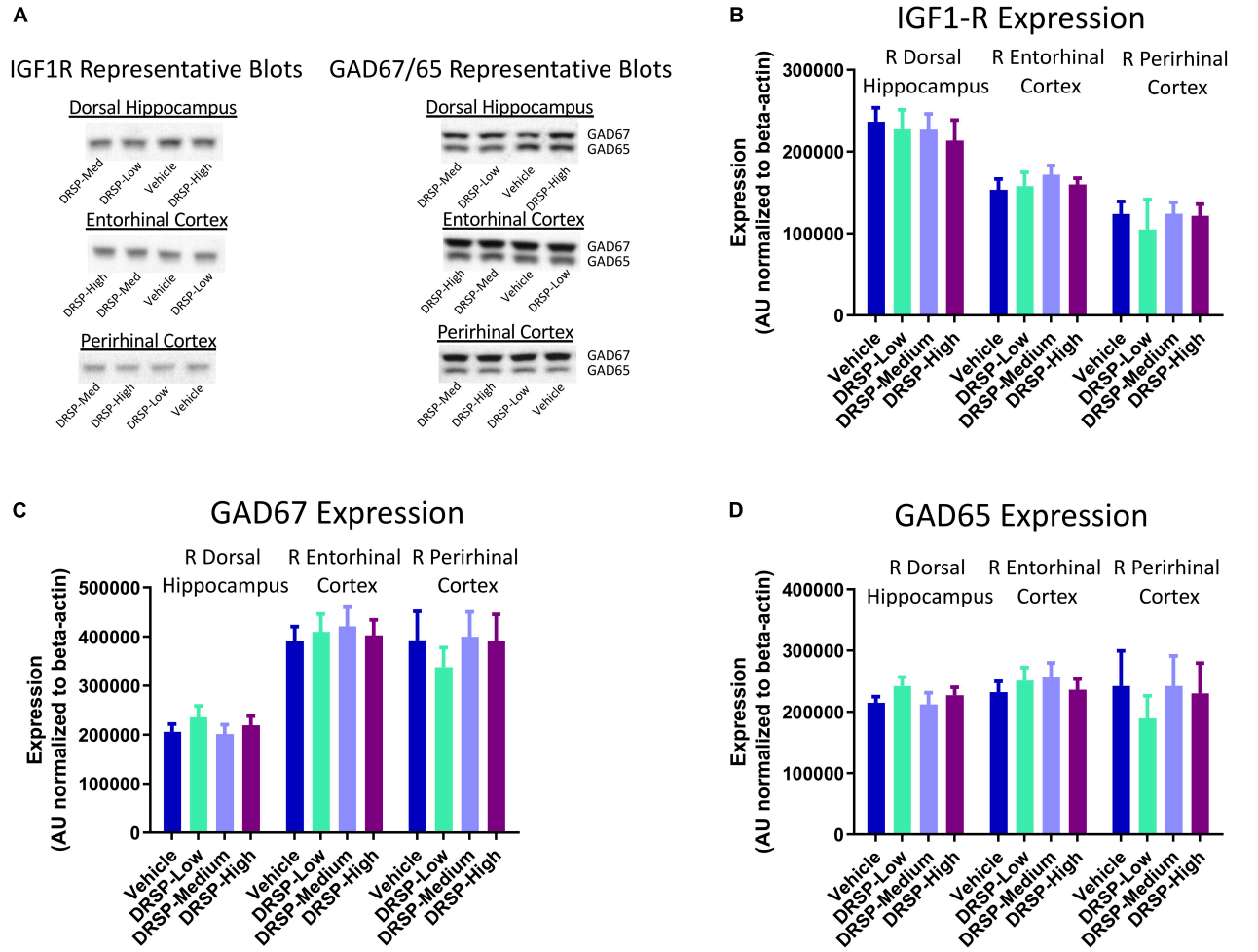

FIGURE 3 | Experiment 1 Western blot protein analysis. **(A)** Representative images of IGF1-R, GAD65, and GAD67 western blot analysis within the dorsal hippocampus, entorhinal cortex, and perirhinal cortex. There were no Treatment differences in IGF1-R expression **(B)**, GAD67 expression **(C)**, or GAD65 expression **(D)** for any brain region evaluated. Vehicle n = 10, DRSP-Low n = 10, DRSP-Medium n = 10, and DRSP-High n = 10.

as a Trial × Treatment interaction [$F_{(2,36)} = 3.79$, $p < 0.05$, $\eta_G^2 = 0.03$] for WMC errors. A Trial × Treatment interaction for WMI errors was present for the Vehicle vs EE-High comparison [$F_{(3,54)} = 4.54$, $p < 0.01$, $\eta_G^2 = 0.05$] and for the EE-High vs DRSP + EE-High comparison [$F_{(3,54)} = 4.28$, $p < 0.01$, $\eta_G^2 = 0.07$]. The DRSP + EE-High group made fewer WMI errors on the moderate working memory load trial compared to EE-High alone [$F_{(1,18)} = 5.65$, $p < 0.05$, $\eta_G^2 = 0.09$], suggesting that after initial learning takes place, the addition of drospirenone to a high dose of EE may prevent working memory impairments compared to a high dose of EE alone when working memory is moderately taxed (**Figure 4B**). On the maximum working memory load trial, the DRSP + EE-Low-treated group made more WMC errors compared to the EE-Low alone group [$F_{(1,18)} = 5.17$, $p < 0.05$, $\eta_G^2 = 0.07$; **Figure 4C**].

Asymptotic Phase

Across all days and trials on the Asymptotic Phase, there was a Trial × Treatment interaction for RM errors for the EE-Low vs EE-High comparison [$F_{(3,54)} = 3.17$, $p < 0.05$, $\eta_G^2 = 0.05$]. When the moderate and maximum working memory load trials were evaluated separately, no statistically significant effects were revealed for working memory during the Asymptotic Phase for any planned comparison.

Delayed Memory Retention

Working memory correct errors on Trial 3 from the last day of regular WRAM testing (day 12) were compared to Trial 3 after the 6-h delay (first post-delay trial) for each treatment group. Post-delay errors on Trial 3 were increased for the EE-Low group [$F_{(9,1)} = 19.29$, $p < 0.01$, $\eta_G^2 = 0.44$; **Figure 4E**], the EE-High group [$F_{(9,1)} = 16.20$, $p < 0.01$, $\eta_G^2 = 0.36$; **Figure 4F**], and the DRSP + EE-Low group [$F_{(9,1)} = 12.52$, $p < 0.01$, $\eta_G^2 = 0.50$; **Figure 4H**] compared to the previous day performance of each respective group. Rats treated with Vehicle, DRSP, or DRSP + EE-High did not exhibit a significant delay-induced impairment (**Figures 4D,G,I**).

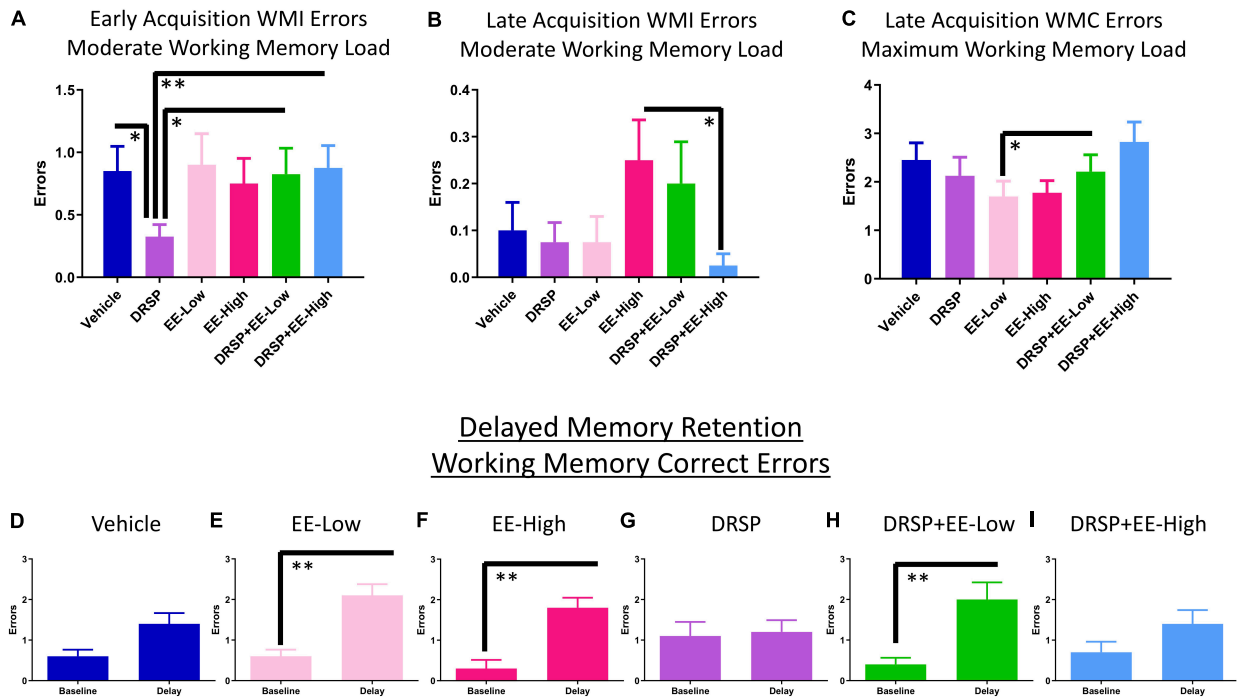

FIGURE 4 | Experiment 2 Water radial-arm maze. **(A)** During early acquisition, rats treated with drospirenone only showed enhanced WMI performance on the moderate working memory load trial compared to rats without hormone treatment, or rats given a combination of drospirenone plus a low or high dose of EE. **(B)** During the Late Acquisition Phase, rats treated with a combination of drospirenone and high EE had enhanced WMI performance on the moderate working memory load trial compared to rats treated with a high dose of EE only. **(C)** Rats treated with a combination of drospirenone and low EE showed impaired performance on the maximum working memory load trial compared to a low dose of EE alone during Late Acquisition. **(D)** Rats without hormone treatment did not exhibit a delay-induced memory impairment, **(E,F,H)** while rats treated with EE-Low, EE-High, and DRSP + EE-Low were significantly impaired following a 6-h delay. **(G,I)** The DRSP group and the DRSP + EE-High group were not significantly impaired following a 6-h delay. $^{*}p < 0.05$ and $^{**}p < 0.01$. Vehicle $n = 10$, DRSP $n = 10$, EE-Low $n = 10$, EE-High $n = 10$, DRSP + EE-Low $n = 10$, and DRSP + EE-High $n = 10$.

Morris Water Maze

There were no Treatment effects for any two-group comparisons, nor any Day × Treatment interactions (**Figure 5A**). There was a main effect of Quadrant for each group (Vehicle: $[F_{(9,1)} = 63.43$, $p < 0.0001$, $\eta_G^2 = 0.13]$; DRSP: $[F_{(9,1)} = 40.74$, $p < 0.0001$, $\eta_G^2 = 0.21]$; EE-Low: $[F_{(9,1)} = 191.53$, $p < 0.0001$, $\eta_G^2 = 0.50]$; EE-High: $[F_{(9,1)} = 76.74$, $p < 0.0001$, $\eta_G^2 = 0.32]$; DRSP + EE-Low: $[F_{(9,1)} = 92.29$, $p < 0.0001$, $\eta_G^2 = 0.17]$; and DRSP + EE-High: $[F_{(9,1)} = 63.36$, $p < 0.0001$, $\eta_G^2 = 0.14]$), with each treatment group swimming a greater percent of total distance in the previously platformed quadrant compared to the opposite quadrant (**Figure 5B**).

Open Field Task

Total Distance Moved (cm) in the arena was a marker of locomotor activity (**Figure 6A**); a main effect of Treatment was observed for the EE-Low vs EE-High comparison $[F_{(1,18)} = 10.77$, $p < 0.01$, $\eta^2 = 0.60]$, with EE-High rats having a greater distance covered in the 10 min trial. Additionally, DRSP + EE-High rats covered a greater distance compared to the DRSP only group $[F_{(1,18)} = 4.96$, $p < 0.05$, $\eta^2 = 0.28]$ and compared to the DRSP + EE-Low group $[F_{(1,18)} = 8.41$, $p < 0.01$, $\eta^2 = 0.47]$. EE-High rats covered more distance in the center of the arena, an indicator of anxiolytic behavior, compared to Ovx rats without hormone treatment $[F_{(1,18)} = 5.92$, $p < 0.05$, $\eta^2 = 0.33]$ and Ovx rats treated with a lower dose of EE $[F_{(1,18)} = 9.14$, $p < 0.01$, $\eta^2 = 0.51]$ (**Figure 6B**). The DRSP + EE-High group moved more than the DRSP + EE-Low group in the center of the arena $[F_{(1,18)} = 11.28$, $p < 0.01$, $\eta^2 = 0.63]$. Small Center Distance (cm) was the distance traveled in the immediate center of the arena, and is an additional marker of anxiolytic behavior (**Figure 6C**). EE-High treated rats traveled more distance in the Small Center compared to EE-Low rats $[F_{(1,18)} = 4.71$, $p < 0.05$, $\eta^2 = 0.26]$. DRSP + EE-High-treated rats traveled more distance in the Small Center compared to DRSP + EE-Low rats $[F_{(1,18)} = 4.56$, $p < 0.05$, $\eta^2 = 0.25]$. Corner Distance analyses, a measure of anxiety-like behavior (**Figure 6D**), revealed the Vehicle group traveled more distance in the corners compared to the EE-Low group $[F_{(1,18)} = 5.41$, $p < 0.05$, $\eta^2 = 0.30]$, as well as the DRSP + EE-Low group $[F_{(1,18)} = 6.99$, $p < 0.05$, $\eta^2 = 0.39]$. The DRSP + EE-High group moved more distance in the Corners compared

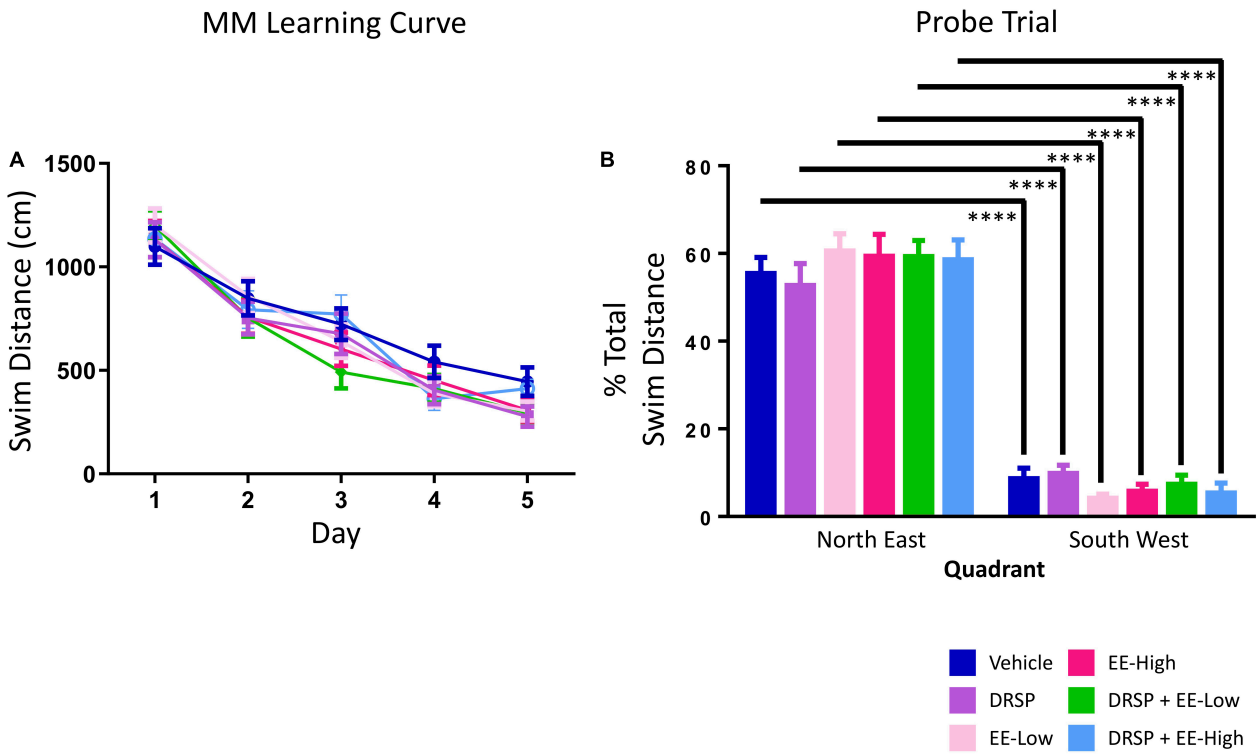

FIGURE 5 | Experiment 2 Morris water maze. **(A)** There were no Treatment differences across MM Days 1–5. **(B)** All groups localized to the target quadrant during the probe trial. ****$p < 0.0001$. Vehicle $n = 10$, DRSP $n = 10$, EE-Low $n = 10$, EE-High $n = 10$, DRSP + EE-Low $n = 10$, and DRSP + EE-High $n = 10$.

to the DRSP + EE-Low group [$F_{(1,18)} = 8.04$, $p < 0.05$, $\eta^2 = 0.45$].

For Time analyses in the OFT (**Figure 6E**), the EE-High group spent more time in the Center compared to the Vehicle group [$F_{(1,18)} = 7.02$, $p < 0.05$, $\eta^2 = 0.39$], the EE-Low group [$F_{(1,18)} = 8.40$, $p < 0.01$, $\eta^2 = 0.47$], and the DRSP + EE-High group [$F_{(1,18)} = 4.69$, $p < 0.05$, $\eta^2 = 0.26$], indicating that EE-High treatment decreased anxiety-like behavior. Small Center time did not differ for any comparison (**Figure 6F**). For Corner Time (**Figure 6G**), Ovx rats without hormone treatment spent more time in the corners compared to the EE-High group [$F_{(1,18)} = 9.05$, $p < 0.01$, $\eta^2 = 0.50$] and compared to the DRSP + EE-Low group [$F_{(1,18)} = 10.42$, $p < 0.01$, $\eta^2 = 0.58$]. EE-Low rats spent more time in the corners than rats receiving the combination of DRSP + EE-Low treatment [$F_{(1,18)} = 5.21$, $p < 0.05$, $\eta^2 = 0.29$]. Overall, Vehicle treatment was associated with increased anxiety-like behaviors, and EE-High treatment was associated with decreased anxiety-like behaviors.

Peripheral Markers of Hormone Stimulation

Vaginal Smears

The Vehicle group and the DRSP group exhibited blank or diestrus-like smears for all three days evaluated, indicating successful Ovx and a lack of stimulation from daily drospirenone treatment alone, replicating results from Experiment 1. Rats treated with EE-Low, EE-High, DRSP + EE-Low, and DRSP + EE-High exhibited cornified cells across all days, indicating EE-induced stimulation of the vaginal epithelium that was not qualitatively altered by concomitant drospirenone administration.

Body Weights and Uterine Weights

Two-group comparisons were completed for body weight at the end of the experiment (**Figure 7A**). The Vehicle group weighed more than the EE-Low group [$F_{(1,18)} = 36.26$, $p < 0.0001$, $\eta^2 = 2.01$], EE-High group [$F_{(1,18)} = 26.24$, $p < 0.0001$, $\eta^2 = 1.46$], DRSP + EE-Low group [$F_{(1,18)} = 40.02$, $p < 0.0001$, $\eta^2 = 2.22$], and DRSP + EE-High group [$F_{(1,18)} = 36.61$, $p < 0.0001$, $\eta^2 = 2.03$]. The DRSP group weighed more than the DRSP + EE-Low group [$F_{(1,18)} = 53.94$, $p < 0.0001$, $\eta^2 = 3.00$] and the DRSP + EE-High group [$F_{(1,18)} = 51.22$, $p < 0.0001$, $\eta^2 = 2.85$]. Collectively, EE-treated groups, with and without concomitant drospirenone administration, did not differ from one another, suggesting that EE administration prevents weight gain in Ovx rats, and drospirenone treatment does not further alter body weight when combined with EE, at least with the current experimental parameters.

Wet uterine weight (g) was lower in the Vehicle group compared to the EE-Low group [$F_{(1,18)} = 246.14$, $p < 0.0001$, $\eta^2 = 13.56$], EE-High group [$F_{(1,18)} = 153.73$, $p < 0.0001$, $\eta^2 = 8.62$], DRSP + EE-Low group [$F_{(1,18)} = 351.73$, $p < 0.0001$, $\eta^2 = 19.4$], and DRSP + EE-High group [$F_{(1,18)} = 114.6$, $p < 0.0001$, $\eta^2 = 6.45$] (**Figure 7B**). Uterine weights in the Vehicle group and DRSP group did not differ from one another,

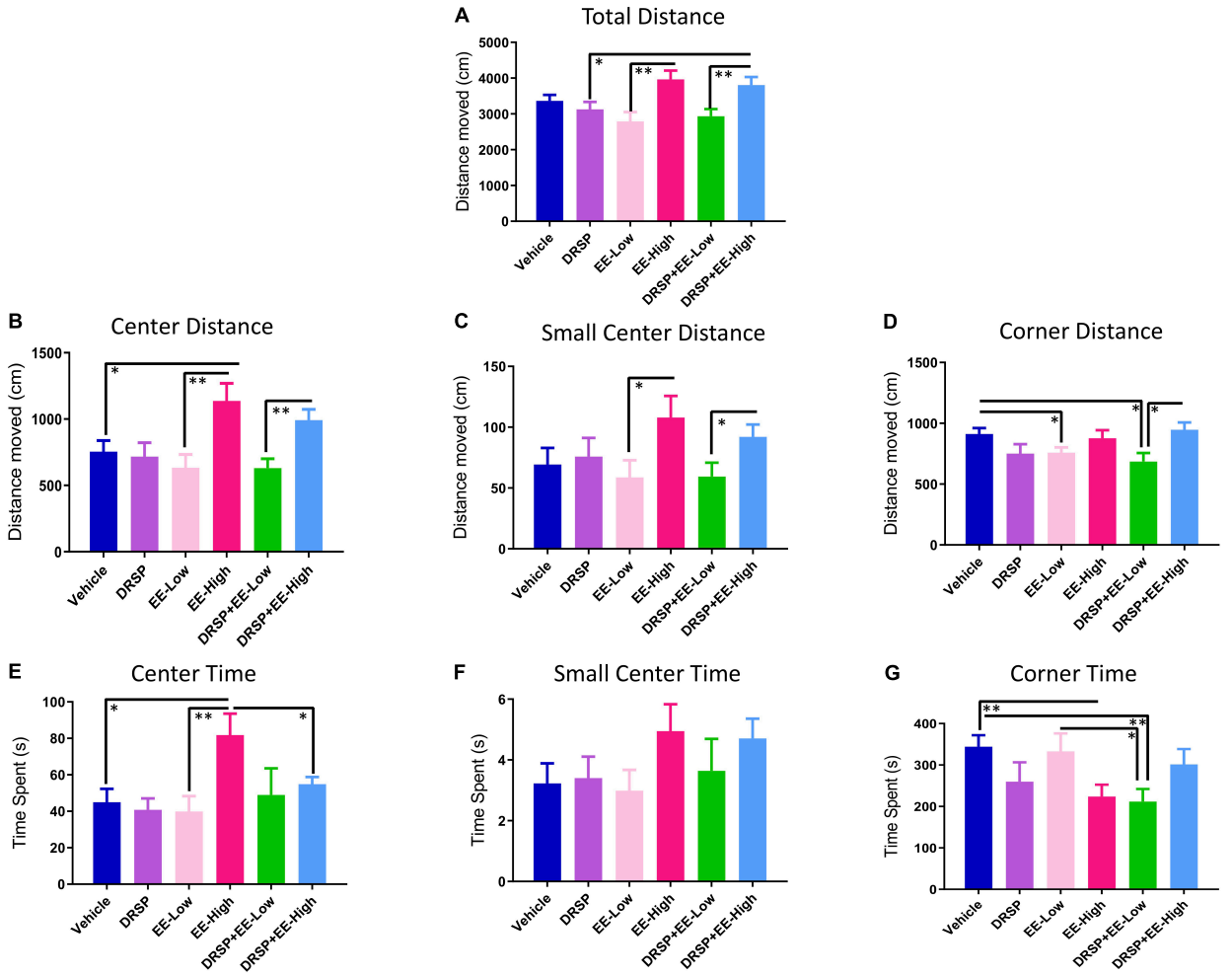

FIGURE 6 | Experiment 2 Open field task performance. Performance varied across: **(A)** Total Distance Moved, **(B)** Center Distance, **(C)** Small Center Distance, **(D)** Corner Distance, **(E)** Center Time, **(F)** Small Center Time, and **(G)** Corner Time, in the OFT. In general, the EE-High group exhibited decreased anxiety-like behavior in the Open Field. *$p < 0.05$ and **$p < 0.01$. Vehicle $n = 10$, DRSP $n = 10$, EE-Low $n = 10$, EE-High $n = 10$, DRSP + EE-Low $n = 10$, and DRSP + EE-High $n = 10$.

replicating findings from Experiment 1. The DRSP group also had significantly lower uterine weights compared to the DRSP + EE-Low group [$F_{(1,18)} = 386.84$, $p < 0.0001$, $\eta^2 = 22.25$] and DRSP + EE-High group [$F_{(1,18)} = 118.16$, $p < 0.0001$, $\eta^2 = 6.53$]. Uteri from the EE-High group weighed more than the EE-Low group [$F_{(1,18)} = 7.60$, $p < 0.01$, $\eta^2 = 0.43$]. Overall, the data indicate that unopposed EE-High treatment significantly increases uterine weights, while drospirenone treatment at the administered dose does not have a significant influence on uterine weights when given alone or combined with EE.

Western Blot Protein Analysis

Representative blots for each protein within all brain regions analyzed are pictured in **Figure 8A**. There were no differences in IGF1-R expression, GAD65 expression, or GAD67 expression for any comparison in the right dorsal hippocampus or right entorhinal cortex (**Figures 8B–D**). EE-Low treated-rats had increased GAD67 expression [$F_{(1,18)} = 10.62$, $p < 0.01$, $\eta^2 = 0.59$; **Figure 8C**] and GAD65 expression [$F_{(1,18)} = 5.96$, $p < 0.05$, $\eta^2 = 0.33$; **Figure 8D**] in the right perirhinal cortex compared to Vehicle-treated rats. DRSP + EE-High treated rats [$F_{(1,18)} = 5.26$, $p < 0.05$, $\eta^2 = 0.29$] also exhibited increased GAD67 expression in the right perirhinal cortex compared to Vehicle-treated rats (**Figure 8C**). There were no differences in IGF-1R expression in the right perirhinal cortex (**Figure 8B**).

Correlations

After correction for multiple comparisons using the FDR method, results revealed a negative correlation for GAD65 expression in the right entorhinal cortex and WMC errors committed on Trial 3 during the Asymptotic Phase for the EE-High group, such that greater GAD65 expression was associated with fewer WMC errors on the moderate working memory load trial ($R^2 = 0.78$, $p < 0.001$, Benjamini-Hochberg p value = 0.243, **Figure 9A**). Within the perirhinal cortex, the DRSP + EE-Low

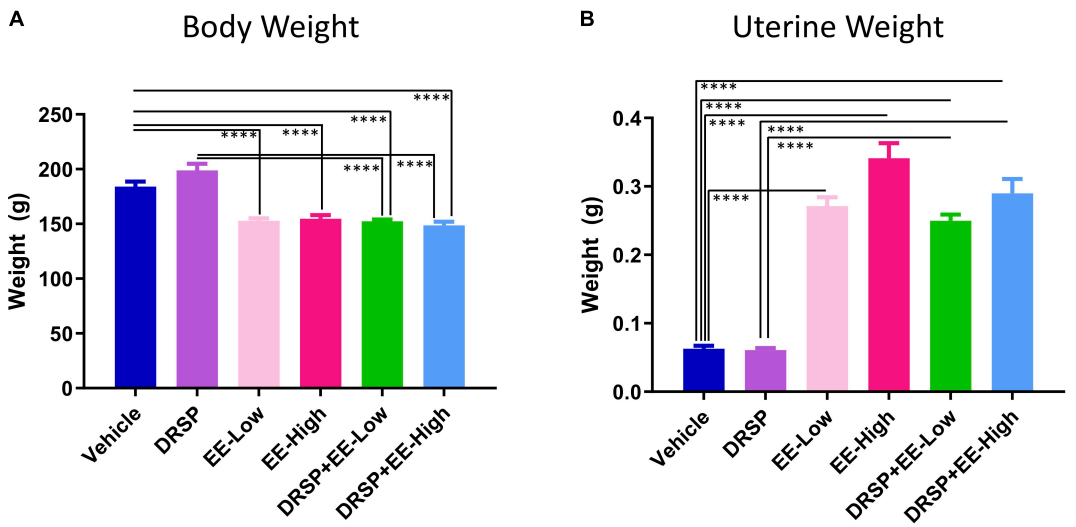

FIGURE 7 | Experiment 2 Body weight and uterine weight. All subjects were ovariectomized. **(A)** Rats treated with EE alone and in combination with drospirenone weighed significantly less than rats without hormone treatment. Rats treated with drospirenone weighed more than those that received drospirenone plus EE at a low or high dose. **(B)** Uterine weights from rats treated with EE weighed significantly more at euthanasia compared to rats without hormone treatment. Rats treated with drospirenone had lower uterine weights compared to rats treated with a combination of drospirenone and EE, but did not differ from rats without hormone treatment. ****$p < 0.0001$. Vehicle $n = 10$, DRSP $n = 10$, EE-Low $n = 10$, EE-High $n = 10$, DRSP + EE-Low $n = 10$, and DRSP + EE-High $n = 10$.

group showed a positive correlation between IGF1-R expression and WMC errors on Trial 3 during the Early Acquisition Phase, where greater IGF1-R expression was associated with more WMC errors ($R^2 = 0.73$, $p < 0.001$, Benjamini-Hochberg p value = 0.243, **Figure 9B**). Furthermore, within the perirhinal cortex, the DRSP + EE High group had a positive correlation between GAD67 expression and WMC errors on Trial 4 during the Early Acquisition Phase ($R^2 = 0.72$, $p < 0.001$, Benjamini-Hochberg p value = 0.243, **Figure 9C**), whereby higher perirhinal cortex GAD67 expression was associated with more WMC errors on the maximum working memory load trial. Interestingly, the DRSP + EE-High group had a negative correlation between GAD65 expression and WMI errors on Trial 3 during the Early Acquisition Phase, with greater GAD65 expression levels in the perirhinal cortex were associated with fewer WMI errors on the moderate working memory load trial ($R^2 = 0.72$, $p < 0.001$, Benjamini-Hochberg p value = 0.243, **Figure 9D**).

DISCUSSION

Drospirenone's unique pharmacological properties (Fuhrmann et al., 1996; Schindler et al., 2003; Kuhl, 2005; Bitzer and Paoletti, 2009) and its continued popularity in the clinic (The Medical Letter on Drugs Therapeutics, 2020) merited investigation into its impact on the brain and behavior. Collectively, we showed dose-dependent cognitive benefits of drospirenone in young Ovx rats, which were modified by the addition of the synthetic estrogen EE. Brain assessments indicated that while drospirenone alone did not impact GAD65, GAD67, or IGF1-R expression within the parameters tested, EE modified GAD65 and GAD67 expression, providing a putative mechanism through which this synthetic estrogen impacts cognition, in a similar fashion to the interplay between endogenous E2 and GABAergic activity (Moura and Petersen, 2010). Relationships between protein expression and working memory performance were evident, although they were dependent upon WRAM phase, brain region, and hormone treatment.

Water Radial-Arm Maze: Spatial Working Memory

In Experiment 1, the Medium dose of drospirenone, which most closely models the typical ratio of drospirenone to EE used in combined oral contraceptive formulations, had beneficial effects on spatial working memory when memory load was taxed compared to Ovx-Vehicle-treated rats. The Low and High doses of drospirenone showed working memory benefits during the Early Acquisition Phase, but only the Medium drospirenone dose continued to elicit benefits for spatial working memory when working memory was maximally taxed in the Asymptotic Phase. A 6-h delay on the WRAM significantly impaired working memory performance for Vehicle and DRSP-Low treated groups, but not the DRSP-Medium and DRSP-High groups, on the post-delay trial, suggesting the potential of a dose-dependent effect for delayed memory retention. Drospirenone's dose-dependent effects may follow a U-shaped pattern, where too little or too high of a dose negatively impacted performance, while the medium

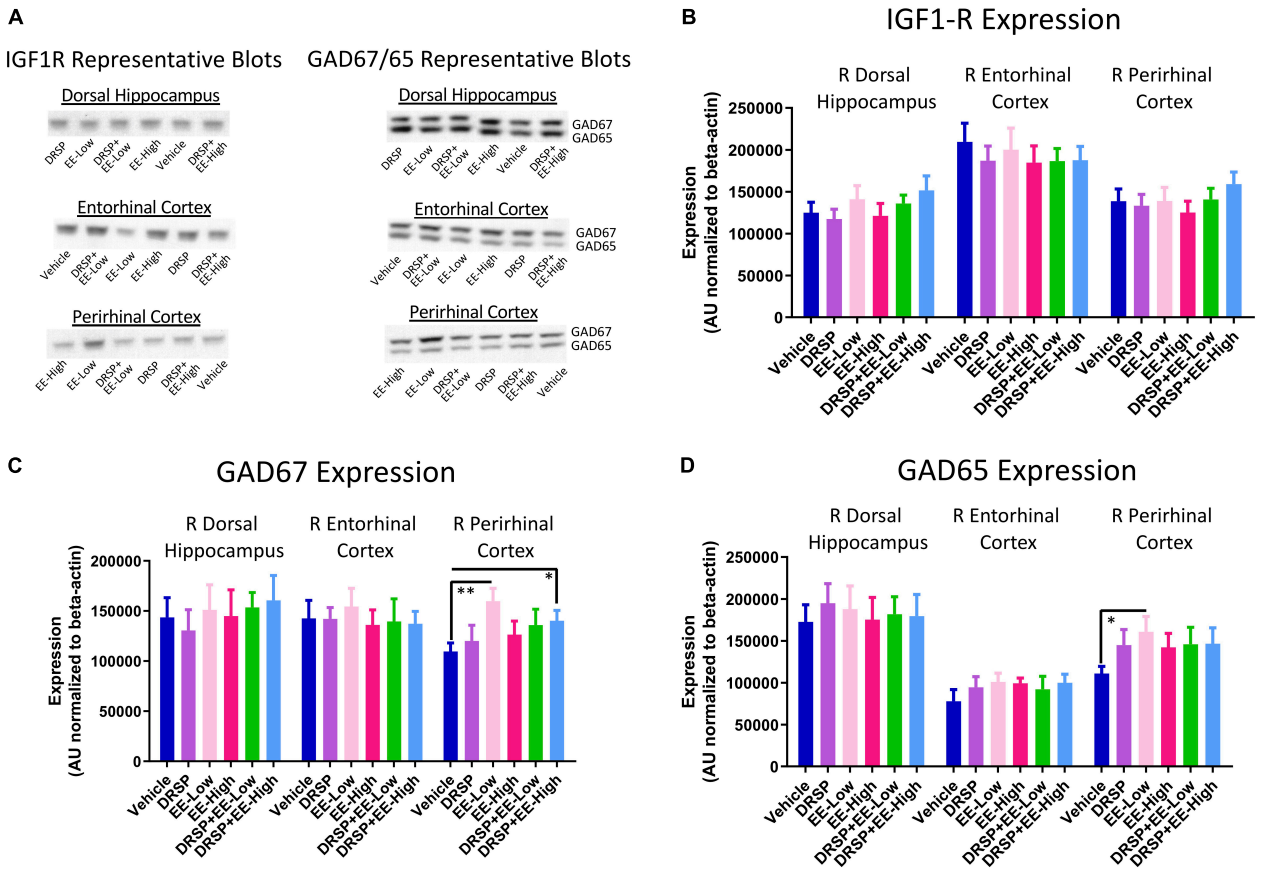

FIGURE 8 | Experiment 2 Western blot protein analysis. **(A)** Representative images of IGF1-R, GAD65, and GAD67 western blot analysis within the dorsal hippocampus, entorhinal cortex, and perirhinal cortex. **(B)** There were no differences in IGF1-R expression between groups in any brain region evaluated. **(C)** Rats treated with a low dose of EE or drospirenone plus a high dose of EE showed increased GAD67 expression in the perirhinal cortex compared to rats without hormone treatment. No differences in GAD67 were detected between groups in the dorsal hippocampus or entorhinal cortex. **(D)** Rats treated with a low dose of EE had increased GAD65 expression in the perirhinal cortex compared to rats without hormone treatment. No differences in GAD65 were detected between groups in the dorsal hippocampus or entorhinal cortex. $^{*}p < 0.05$ and $^{**}p < 0.01$. Vehicle $n = 10$, DRSP $n = 10$, EE-Low $n = 10$, EE-High $n = 10$, DRSP + EE-Low $n = 10$, and DRSP + EE-High $n = 10$.

dose provided an optimal range to exert meaningful positive effects on memory performance. Prior research in women has demonstrated similar GABA-mediated *U*-shaped effects of allopregnanolone on mood (Andréen et al., 2009; Bäckström et al., 2015); animal models have also shown *U*-shape effects of reproductive hormones on cognition, including rapid effects mediated by membrane-bound receptors (Acosta et al., 2009; Foster, 2012).

In Experiment 2, drospirenone was administered alone and in combination with two doses of EE that reflected commonly prescribed doses in combined oral contraceptives. We replicated the finding from Experiment 1 that drospirenone alone enhanced spatial working memory when memory load was taxed compared to Ovx rats without hormone treatment during the Early Acquisition Phase. Drospirenone-treated rats also had enhanced performance compared to both combinations of DRSP + EE in the Early Acquisition Phase, suggesting that concomitant EE administration attenuated the beneficial effects of drospirenone alone. These results support and extend prior findings from our laboratory with levonorgestrel, wherein this progestin was beneficial when administered alone, but resulted in spatial working memory impairments when administered in combination with E2 in middle-aged Ovx rats (Prakapenka et al., 2018). During Late Acquisition, EE-High treatment impaired working memory compared to DRSP + EE-High treatment, indicating a potential mnemonic benefit of combined hormone treatment in relation to EE-only treatment, at least at a high dose. However, Low-EE treated rats made fewer working memory errors than combined DRSP + Low-EE-treated rats during Late Acquisition, suggesting that working memory outcomes could be dependent on both dose and combination of hormones in this young adult Ovx model.

During the delayed memory retention evaluation, EE-Low, EE-High, and DRSP + EE-Low groups were impaired, but the Ovx-Vehicle group did not show a significant delay-induced impairment. Similar to the first experiment, both the DRSP group and the DRSP + EE-High group did not exhibit poorer performance following the delay. These findings point to a

potential protective effect of drospirenone on delayed memory retention alone and in combination with EE-High treatment.

Morris Water Maze: Spatial Reference Memory

In Experiment 1, the DRSP-Medium and DRSP-High doses showed benefits for RM performance compared to Ovx Vehicle-treated rats across all days of MM. This effect of the Medium drospirenone dose was not found in Experiment 2. In fact, there were no differences in performance for any comparison on the MM task in Experiment 2; thus, drospirenone may have a more consistent beneficial effect in the working memory domain. In both experiments, all groups spatially localized to the platform location by the end of testing.

Open Field Task: Locomotor and Anxiety-Like Behavior

In Experiment 1, drospirenone administration alone at any dose did not impact locomotor or anxiety-like behavior. In Experiment 2, rats treated with a high dose of EE alone and in combination with drospirenone exhibited increased locomotor activity. Rats treated with a high dose of EE also had increased center distance and time, indicative of decreased anxiety-like behavior. These group differences may have been due, in part, to an overall change in locomotor activity induced by the high EE dose; as such, these findings should be interpreted with caution in the context of being solely related to anxiety-like alleviation by the high dose of EE alone and in combination with drospirenone, specifically. E2 levels are known to impact locomotor and anxiety-like behavior, and thus this finding is concordant with prior research in surgical and transitional menopause models (Blizard et al., 1975; Lund et al., 2005; Hiroi et al., 2006; Hiroi and Neumaier, 2006; McLaughlin et al., 2008; Koebele et al., 2021a). The investigation of effects of EE on anxiety assessments are currently limited, although dose-dependent beneficial effects of EE have been reported in ovary-intact rats (Simone et al., 2015). While drospirenone has beneficial impacts on PMDD symptoms and mood in the clinical setting (Paoletti et al., 2004; Foidart, 2005; Pearlstein et al., 2005; Yonkers et al., 2005; Fenton et al., 2007; Nappi et al., 2009; Archer et al., 2015; Hofmeister and Bodden, 2016), it is possible that the detrimental effect of Ovx on anxiety-like behavior (Blizard et al., 1975; Hiroi and Neumaier, 2006; Diz-Chaves et al., 2012; Hiroi et al., 2016) overrode any potential benefit of drospirenone alone in this assessment. Future studies should evaluate ovary-intact animals as well as implement additional tasks that measure more nuanced aspects of anxiety-like behavior to further investigate the role of drospirenone on specific symptoms of anxiety, mood, depression, and affect in a preclinical model.

Peripheral Measures of Hormone Treatment

Drospirenone alone did not alter body weight, uterine weight, or induce vaginal cytology changes compared to vehicle treatment in Ovx rats in Experiment 1. In Experiment 2, all rats treated with EE, alone or in combination with drospirenone, weighed less than Vehicle-treated rats. In addition, rats treated with drospirenone weighed more than rats treated with a combination of drospirenone and EE at both doses. This was somewhat surprising, given the anti-mineralocorticoid receptor properties of drospirenone as it pertains to water retention and metabolic effects (Muhn et al., 1995; Fuhrmann et al., 1996; Sitruk-Ware, 2004, 2006; Foidart, 2005). However, it is important to note that all rats were surgically menopausal and did not have ovaries; therefore, it is possible that the detrimental metabolic effects of Ovx impacted body weight over and above any potential effect that drospirenone could have had on body weight. To this end, an evaluation of drospirenone administration on weight maintenance or gain in ovary-intact rats would be informative in the future. All rats treated with EE, alone and in combination with drospirenone, exhibited vaginal cytology indicative of vaginal epithelium stimulation. Furthermore, uterine weight was increased for all EE-treated rats at the end of the experiment, supporting the idea that EE stimulates uterine tissue growth, a finding we have previously reported (Mennenga et al., 2015a). The dose of drospirenone given in combination with EE treatments did not attenuate uterine weight at the end of the experiment, suggesting the dose given was insufficient to counter EE-induced uterine stimulation, an effect previously observed in intact rats, but not widely studied (Adeyanju and Olatunji, 2019). Different ratios of drospirenone to EE will be important to investigate in future studies.

Brain Assessment and Correlations With Behavior

Drospirenone alone did not alter proteins of interest associated with spatial learning and memory, including GAD65, GAD67, and IGF1-R in the dorsal hippocampus, entorhinal cortex, or perirhinal cortex. The observed beneficial cognitive impact of drospirenone may be regulated by different neural systems, or there may be region-dependent effects of drospirenone in brain areas that we did not assess herein. Alternatively, the chronic nature of Ovx and daily hormone administration could have led to reorganizational processes in the systems of interest by the time brains were evaluated. Another possibility is that drospirenone-induced alterations are not evident at the level of protein expression for the selected markers, at least at the time point and hormone modulation experimental parameters assessed.

In Experiment 2, the EE-Low group had increased GAD65 and GAD67 protein expression in the perirhinal cortex compared to Ovx rats without hormone treatment. Although the perirhinal cortex is traditionally associated with visual recognition memory, Schulz-Klaus and colleagues reported a reduction in anxiety-like behaviors by temporarily inactivating the perirhinal cortex through intracranial infusions of the $GABA_A$ receptor agonist muscimol (Schulz-Klaus et al., 2005). Furthermore, infusion of E2 into the perirhinal and entorhinal cortices, as well as systemic E2 administration, impaired performance on a delayed non-match-to-sample object recognition test, but enhanced novelty preference (Gervais et al., 2013, 2016), suggesting a broader role for the perirhinal and entorhinal cortices in complex cognitive tasks. Interestingly, the EE-Low group had enhanced

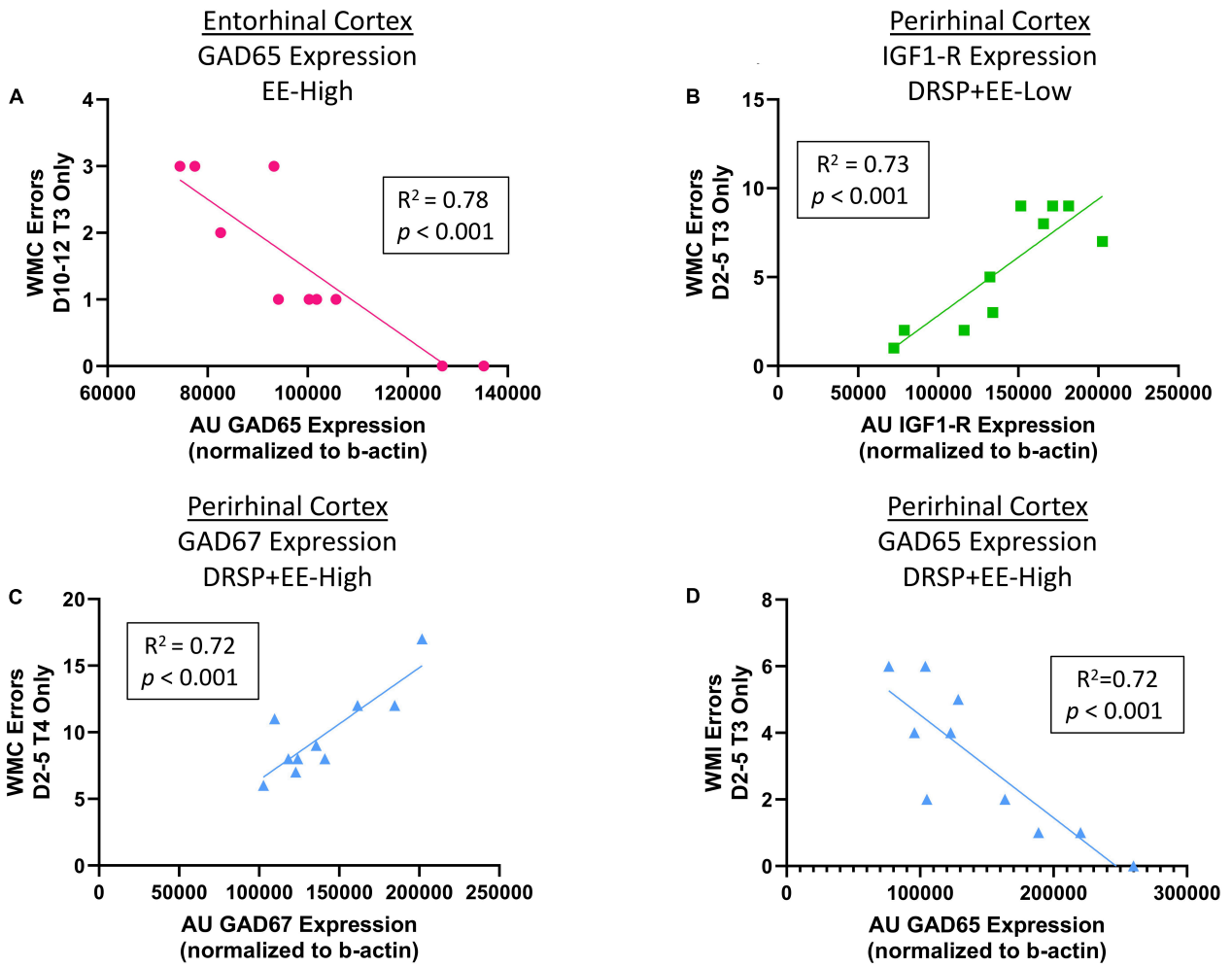

FIGURE 9 | Experiment 2 Correlations between proteins of interest and WRAM performance. **(A)** Within the EE-High group, greater GAD65 expression in the entorhinal cortex was associated with fewer WMC errors when working memory was moderately taxed during the Early Acquisition Phase of WRAM. **(B)** Within the DRSP + EE-Low group, greater IGF1-R expression in the perirhinal cortex was associated with more WMC errors when working memory was moderately taxed during the Early Acquisition Phase of WRAM. **(C)** Within the DRSP + EE-High group, greater GAD67 expression in the perirhinal cortex was associated with increased WMC errors on the maximum working memory load trial during Early Acquisition. **(D)** Within the DRSP + EE-High group, greater GAD65 expression in the perirhinal cortex was associated with decreased WMI errors on the moderate working memory load trial during Early Acquisition. Vehicle $n = 10$, DRSP $n = 10$, EE-Low $n = 10$, EE-High $n = 10$, DRSP + EE-Low $n = 10$, and DRSP + EE-High $n = 10$.

working memory compared to the DRSP + EE-Low group, yet exhibited greater anxiety-like behaviors compared to the EE-High group in the OFT. Thus, the outcomes associated with changes in perirhinal cortex GAD expression following synthetic EE administration may be dose- and task- dependent, and should be further explored.

Within the perirhinal cortex, greater GAD67 and IGF1-R expression was correlated with increased WMC errors during the Early Acquisition Phase of the WRAM in DRSP + EE-High and DRSP + EE-Low groups, respectively. Although endogenous E2 has been associated with increased IGF1-R expression and long-term cognitive benefits (Witty et al., 2013), poorer performance associated with higher levels of IGF1-R suggests that either the synthetic E2 analog EE plays a unique role in IGF1-R expression, or that the addition of the synthetic progestin drospirenone alters the relationship between IGF1-R and enhanced cognition. Within the DRSP + EE-High group, greater GAD65 expression was associated with fewer WMI errors, indicating that the perirhinal cortex has a more complex role in spatial working memory than previously noted. In the entorhinal cortex, which is a gateway for information transfer between the hippocampus and other cortical regions in the context of learning (Kitamura et al., 2015), greater GAD65 expression was associated with fewer errors in the EE-High group. Just as endogenous E2 is a known regulator of GABAergic activity in the hippocampal complex (Wójtowicz and Mozrzymas, 2010), synthetic EE may also modulate aspects of the GABAergic system in the context of learning and memory in a similar fashion.

Preclinical research on synthetic ovarian hormones in the context of cognition and neurobiological correlates is generally

limited; yet, findings from work on endogenous ovarian hormones have helped our understanding. For example, as EE impacted memory and GAD expression herein, E2 has also been shown to increase GAD mRNA levels in the hippocampus, regulate GABAergic activity, initiate IGF1-R signaling (Weiland, 1992; Murphy et al., 1998; Nakamura et al., 2004; Garcia-Segura et al., 2007), increase hippocampal IGF1-R expression (Witty et al., 2013), improve working memory, and decrease anxiety-like behavior following Ovx (Bimonte and Denenberg, 1999; Hiroi et al., 2006, 2016; Hiroi and Neumaier, 2006). Concomitant natural progesterone treatment has been shown to reverse the effect of E2 on hippocampal GAD mRNA expression (Weiland, 1992) and on neurotrophin expression (Bimonte-Nelson et al., 2004a), as well as attenuate E2's cognitive benefits in surgical and transitional menopause models (Bimonte-Nelson et al., 2004b, 2006; Koebele et al., 2021a). Yet, progesterone has acute beneficial neuroprotective effects following ischemic injury (Singh and Su, 2013), an outcome which has recently been extended to synthetic progestins including drospirenone acting through a GABA-mediated mechanism (El Amki et al., 2019). The timing of hormone treatment after surgical menopause may also be key to interpreting findings related to neurobiological correlates. For example, Nakamura and colleagues demonstrated that GAD65 immunoreactive cells in the hippocampus decreased 10 days after Ovx compared to three days after Ovx, and E2 increased GAD65 immunoreactive cells 10 days, but not three days, after Ovx (Nakamura et al., 2004). Treatment in the current experiment began 48 h after surgery; thus, GAD-mediated effects may not be evident in all groups as a result of immediate hormone treatment. In the current report, the synthetic estrogen EE modulated memory performance, anxiety-like behaviors, and GAD expression in unique ways compared to reports of endogenous analogs. Thus, while EE and drospirenone are molecularly similar to natural E2 (Kuhl, 2005; Sitruk-Ware and Nath, 2011) and progesterone (Muhn et al., 1995; Fuhrmann et al., 1996; Schindler et al., 2003; Kuhl, 2005), respectively, careful consideration must be given to the dose and treatment regimen if the goal is to mimic or improve the effects of endogenous hormones. In the future, it will be important to further investigate additional parameters to parse the distinct effects of synthetic vs natural hormones on outcomes of interest. It is important to note that all major neurotransmitter systems are impacted by ovarian hormone loss and treatment (Barth et al., 2015); thus, other proteins and cellular signaling cascades, as well as a broad range of brain regions involved in spatial working memory and attention (e.g., frontal cortex), should be investigated to establish a mechanism for the cognitive effects observed with drospirenone.

CONCLUSION

A growing body of research supports the tenet that oral contraceptives impact cognition and alter brain function in task- and composition- dependent manners (e.g., Gogos, 2013; Egan and Gleason, 2012; Beltz et al., 2015, 2022; Porcu et al., 2019; Taylor et al., 2020; Ycaza Herrera et al., 2020; Gravelsins et al., 2021; Lewis et al., 2022; Menting-Henry et al., 2022). Overall, the current series of experiments found that the spironolactone-derived progestin drospirenone has beneficial effects for spatial working memory performance in a young adult Ovx rat model, and that the synthetic estrogen EE has variable effects on behavior that depend on dose and combination with drospirenone. Future investigations using an ovary-intact rat model with these combination therapies would be beneficial to understand how clinically-prescribed treatments impact cognitive function in a normally-cycling reproductive system, as the majority of women prescribed combined oral contraceptives have an intact uterus and ovaries. The primary endpoint for behavior in this series of experiments focused on cognitive domains involving working memory and anxiety-like behaviors, as these domains are known to be impacted by changes in endogenous sex steroid hormones. Future studies would benefit from an expansion of cognitive domains and related brain areas evaluated, particularly given the changes observed with EE in the perirhinal cortex herein, which has a crucial role in recognition memory that is modulated by E2 (Petrulis and Eichenbaum, 2003; Gervais et al., 2013, 2016). Moreover, further investigations into the mechanistic mediators of how different synthetic estrogens and progestins affect brain functions, including probing a greater breadth of protein markers, dendritic spine density, and cellular signaling, all of which are known to be impacted by E2, will likely yield new breakthroughs and pathways of insight for future clinical treatments (Nelson et al., 2014; Gervais et al., 2015; Smith et al., 2016; Batallán Burrowes et al., 2021). While drospirenone has promise for beneficial cognitive effects, the search continues for an estrogen-progestin combination therapy that results in beneficial, or even null, cognitive outcomes in addition to its contraceptive and non-contraceptive peripheral benefits.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Arizona State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SK: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing—original draft, writing—reviewing and editing, visualization, supervision, and project administration. MP: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing—original

draft, writing—reviewing and editing, and project administration. JP, CB-L, SN-S, VP, IS, HB, SP, and CC: investigation, validation, and writing—review and editing. HB-N: conceptualization, methodology, validation, formal analysis, funding acquisition, resources, writing—original draft, writing—reviewing and editing, visualization, supervision, and project administration. All authors contributed to the article and approved the submitted version.

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Effects of an Oral Contraceptive on Dynamic Brain States and Network Modularity in a Serial Single-Subject Study

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Hormonal contraceptive drugs are used by adolescent and adult women worldwide. Increasing evidence from human neuroimaging research indicates that oral contraceptives can alter regional functional brain connectivity and brain chemistry. However, questions remain regarding static whole-brain and dynamic network-wise functional connectivity changes. A healthy woman (23 years old) was scanned every day over 30 consecutive days during a naturally occurring menstrual cycle and again a year later while using a combined hormonal contraceptive. Here we calculated graph theory-derived, whole-brain, network-level measures (modularity and system segregation) and global brain connectivity (characteristic path length) as well as dynamic functional brain connectivity using Leading Eigenvector Dynamic Analysis and diametrical clustering. These metrics were calculated for each scan session during the serial sampling periods to compare metrics between the subject's natural and contraceptive cycles. Modularity, system segregation, and characteristic path length were statistically significantly higher across the natural compared to contraceptive cycle scans. We also observed a shift in the prevalence of two discrete brain states when using the contraceptive. Our results suggest a more network-structured brain connectivity architecture during the natural cycle, whereas oral contraceptive use is associated with a generally increased connectivity structure evidenced by lower characteristic path length. The results of this repeated, single-subject analysis allude to the possible effects of oral contraceptives on brain-wide connectivity, which should be evaluated in a cohort to resolve the extent to which these effects generalize across the population and the possible impact of a year-long period between conditions.

Keywords: oral contraceptive (OC), functional connectivity (FC), functional magnetic resonance imaging (fMRI), menstrual cycle, steroid hormones, dynamic functional connectivity (dFC), hormonal contraceptive, brain modularity

INTRODUCTION

Naturally cycling women undergo menstrual cycles for approximately a third of their lifespan, involving profound sex steroids level fluctuations across 24- to 36-day cycles, frequently with coinciding fluctuations in mood, impulsivity, and irritability (Pletzer et al., 2017; Lewis et al., 2019). Such hormonal rhythms are significantly altered by oral contraceptive (OC) medication, which (Iversen et al., 2020) are used by more than 100 million women globally (Brynhildsen, 2014) and in Denmark 42% of women in the reproductive age use OCs, while 80% have used them at some point in their lives (Skovlund et al., 2016). The most common OCs combine an estrogen and a progestin to downregulate endogenous ovarian sex steroid hormone levels, resulting in inhibited follicular growth, egg maturation, and ovulation, thus preventing pregnancy. There has been a significant focus on somatic side effects of OC use, such as the increased risk of thromboembolic disease (Amoozegar et al., 2015; Roach et al., 2015; Keenan et al., 2018). However, women also report adverse impacts on psychological wellbeing, e.g., mood instability, irritability, sadness, symptoms of depression and anxiety, and a decrease or lack of libido (Guen et al., 2021) which has recently received increased attention. Epidemiological studies show an association between starting an OC and the emergence of depressive episodes, especially among adolescents (Skovlund et al., 2016; Zettermark et al., 2018; Anderl et al., 2020, 2021).

Sex-steroid milieu changes have been found to alter brain biology, including hippocampal plasticity (Barth et al., 2016; Taylor et al., 2020) and serotonergic neurotransmission (Barth et al., 2015), both crucial to maintaining mental health (Frokjaer, 2020). In addition, mental disorders, such as anxiety and mood disorders, can be exacerbated during certain menstrual cycle phases, including premenstrual symptom worsening (Pinkerton et al., 2010; Green and Graham, 2022; Kuehner and Nayman, 2021). Hormonal fluctuations across the menstrual cycle putatively trigger severe depressive symptoms in some women, i.e., premenstrual dysphoric disorder (PMDD), which can be treated with specific OCs but worsened by others (Rapkin et al., 2019).

Although OCs are beneficial for reproductive health and well tolerated by some women, it is necessary that we examine their effect on brain function and how this may affect mental health. Previous neuroimaging studies of the relationship between hormonal dynamics during the menstrual cycle, OC, and brain function have predominately used task-based functional magnetic resonance imaging (fMRI) during a few selected time points, i.e., during the follicular and luteal phases (Dubol et al., 2020). Most studies on OCs use a between-subject design that can be subject to individual-based confounding factors, e.g., duration and onset of OC use (Montoya and Bos, 2017) and self-selection bias (Brønnick et al., 2020). Test-retest reliability of task-based fMRI measures is overall poor and is further impacted by repeated measurements, i.e., habituation effects (Elliott et al., 2020). Thus, studies have increasingly investigated resting-state fMRI (rs-fMRI) by applying dense sampling across multiple time points in individual subjects (Arélin et al., 2015; Pritschet et al., 2021b).

Recently, the "28andMe" project (Pritschet et al., 2020) acquired serial measures of rs-fMRI of one woman once per day during one natural menstrual cycle. A year later, the study was repeated while on a combined OC with estrogen and an androgenic progestin. The initial report on this data found that 17β-oestradiol appeared to facilitate tighter coherence within static functional brain-networks, while progesterone had the opposite effect (Pritschet et al., 2020). Additionally, network reorganization occurred in several networks across the menstrual cycle, most strikingly in a default mode subnetwork localized to prefrontal cortex regions during the ovulatory hormone peaks (Mueller et al., 2021). This reorganization was not present while on OC, despite a similar mid-cycle oestradiol peak on OC, suggesting that this OC constrained or blunted default mode network (DMN) connectivity during estrogen fluctuations.

These studies did not evaluate graph-theoretical estimates of overall brain connectivity either on a network-wise or global level. Modularity and system segregation are static estimates of whole-brain connectivity related to relative within- and between-network connectivity strength (Cohen and D'Esposito, 2016; Sporns and Betzel, 2016). In contrast, characteristic path length is a network-independent measure of "connectedness" between brain regions, representing an approximation of capacity for information flow throughout the brain (Rubinov and Sporns, 2011). In contrast to "static" functional connectivity measures, which evaluate average connectivity across the entire scan session, thus presuming signal stationarity, dynamical functional connectivity (dFC) provides a framework for estimating signal fluctuations within a resting-state scan session (Chang and Glover, 2009; Preti et al., 2016; Cabral et al., 2017). Using dFC, we may characterize discrete time-varying brain connectivity patterns, denoted brain states, whose expression may be related to the use of OC medication.

Here we leverage the repeated rs-fMRI acquisition framework of the "28andMe" project, where we evaluate whether the above graph-theoretical and dynamical whole-brain connectivity measures differed throughout the natural and OC cycles measured one year apart. This explorative approach characterizes how these measures may vary during this subject's natural cycle and when on OC medication. Evaluating differences in these measures during the natural and OC cycle offers a novel perspective into how OC use may affect brain function. The nature of the dataset does not allow us to discriminate with certainty between effects of OC or effects of time between the two series of measurements, which should be kept in mind when interpreting the results, but offers a fundament for hypotheses to be tested in other study designs.

MATERIALS AND METHODS

The 28andMe dataset for this study was obtained from the OpenNeuro database (Accession Number: ds002674, version 1.0.5, doi: 10.18112/openneuro.ds002674.v1.0.5) (Pritschet et al., 2021a) and is available under the CC0 license. The subject,

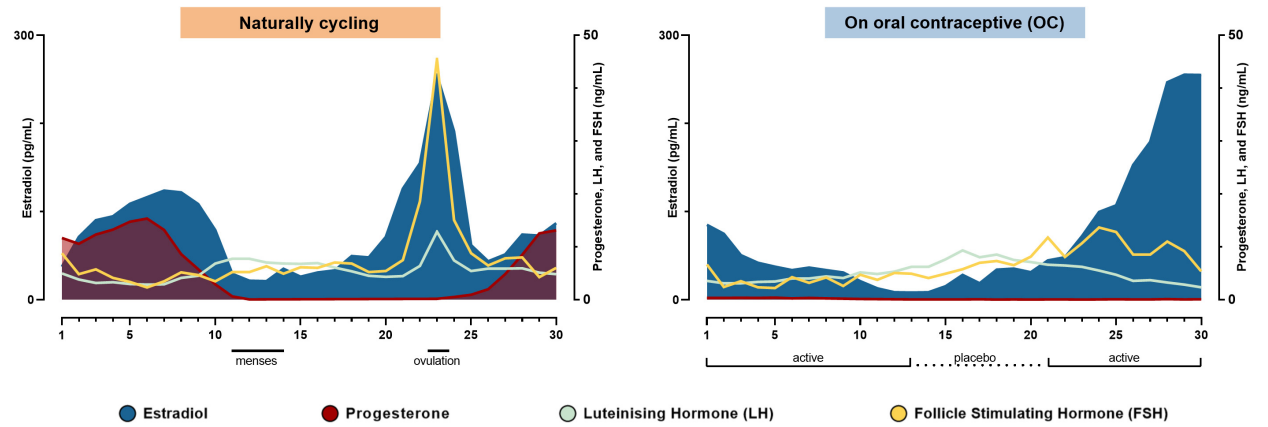

FIGURE 1 | Hormonal timelines in each of the two conditions. The subject's hormone levels during the natural cycle and while taking the oral contraceptive in the order of experiment days. Modified from Taylor et al. (2020).

the study design, and magnetic resonance imaging (MRI) acquisition are described previously (Pritschet et al., 2020, 2021b; Taylor et al., 2020) and summarized here. MRI acquisition and fMRI preprocessing steps are described in the **Supplementary Material**.

The Subject and Study Design

The subject was a right-handed 23-year-old Caucasian female graduate student with no neuropsychiatric or endocrine disorders or prior head trauma history. She had a history of regular menstrual cycles (no missed periods, cycle length 26–28 days) and had not taken hormone-based medication in the 12 months before study onset.

The subject underwent rs-fMRI daily at 11 a.m. for 30 consecutive days. 12 months later the subject repeated the 30-day fMRI protocol while on a monophasic hormonal contraceptive regime of 21 active days (20 μg ethinylestradiol and 100 μg levonorgestrel, Aubra, Afaxys Pharmaceuticals) and 7 placebo days, which she began 10 months before the second data collection. The subject began each test session with daily behavioral assessments and blood measurements, see Taylor et al. (2020).

Hormone levels were not attached in the OpenNeuro data set and were extracted from **Figure 1** from Taylor et al. (2020) using WebPlotDigitizer 4.5 (Rohatgi, 2021). The study days were transformed into cycle days as in Taylor et al. (2020) and plotted in **Figure 1**.

Graph-Theory Measures

To evaluate graph-theory measures, we considered a graphical representation of the brain, where brain regions are described as nodes and edges between those nodes that represent connection strength. The set of connections (i.e., connectivity strengths) can be represented as a connectivity matrix, a diagonally symmetrical $n \times n$ matrix containing continuous values, where n is the number of nodes (Schaefer et al., 2018) and each matrix element represents the magnitude of estimated static connectivity between that node pair, expressed either as a Pearson's correlation coefficient or Fisher's transformed r-to-z (r2z) score.

We summarize the graphical representation of static brain connectivity using several established metrics (Rubinov and Sporns, 2009) including modularity (Cohen and D'Esposito, 2016) and system segregation (Chan et al., 2014) which relate to network connectivity through the lens of modular brain networks, and characteristic path length (CPL) (Rubinov and Sporns, 2009), which evaluates connectivity of the brain considered as a whole. See **Supplementary Figure 1A** for a simplified diagram. Brain regions were allocated based on the Schaefer-400 atlas (Schaefer et al., 2018). This matrix can be expressed as a graph that can be made sparse by applying a threshold and retaining only edges above that threshold. Depending on the metric, edge strength can be weighted, reflecting observed connectivity strength (a value between 0 and 1), or binarized (0 or 1) based on the defined threshold. In the absence of an *a priori* optimal threshold, binarization was performed over a range of thresholds. For modularity and system segregation analyses, regions were allocated to seven canonical resting-state networks: the visual, somatomotor, dorsal attention, ventral attention (salience), limbic, default mode, and executive control networks from a commonly used parcelation (Yeo et al., 2011). Further elaboration on the precise graph theory metrics utilized in this analysis are available in the **Supplementary Material**.

Dynamic Functional Brain Connectivity

We estimate dynamic connectivity structures using Leading Eigenvector Dynamics Analysis (LEiDA) (Cabral et al., 2017), followed by diametrical clustering as in Olsen et al. (2021; see **Supplementary Figures 1B–E**). The brain was parcelated into cortical regions from the Schaefer-100 atlas (Schaefer et al., 2018). LEiDA assesses dynamic functional connectivity by estimating regional instantaneous phases through the Hilbert transform, constructing a $P \times P$ phase coherence map for every timepoint t, and extracting its leading eigenvector (see **Supplementary Section 2** and **Supplementary Figure 1**

for more details). The leading eigenvector represents the dominant instantaneous connectivity pattern for every functional volume acquired. Clusters in the pool of leading eigenvectors (assumed independent) are informative of general states of brain connectivity. Diametrical clustering is a *k*-means type clustering algorithm which acknowledges the unit norm and sign invariance of eigenvectors and which, for a specified number of states *k* produces dynamic brain connectivity states and a labeling for all acquired volumes (Dhillon et al., 2003). We evaluated *k* in the range 2–20 and, for each scan session and *k*, computed the fractional occurrence of all states.

Statistical Analysis

We evaluated differences in brain imaging measures between the natural cycle and OC condition using paired *t*-tests, our statistical significance threshold was set at $\alpha < 0.05$. As our estimates across both 30-day periods are effectively time series, we considered evidence for autocorrelation. See **Supplementary Figure 2** for correlograms of the paired difference for all metrics. Based on visual inspection we observed potential autoregressive effects for bCPL and modularity. For these, we modeled the autocorrelation with lag 1 using a generalized least squares regression of the paired differences using the *nlme* package version 3.1 for R and reported the associated *p*-value (Pinheiro et al., 2021). One ROI (122/400 from Schaefer 400, temporal pole) was removed from bCPL analyses as it produced infinite path lengths in some scans; this had a negligible impact on other bCPL values.

All confidence intervals (*CI*) presented are 95% confidence intervals. For tests of brain state fractional occurrence, we employ within-*k,* Bonferroni-corrected statistical significance thresholds to control the family-wise error rate (FWER).

Visualizations and Code

MATLAB (The MathWorks Inc.) and R 4.1[1] were used to generate the results presented here; the corresponding code has been made publicly available at https://github.com/anders-s-olsen/28andme. BrainNet Viewer 1.7[2] was used to generate connectivity visualizations (Xia et al., 2013). Some plots were constructed using *ggplot2* in R (Wickham, 2016). The BrainConnectivity toolbox was used to estimate CPLs, and bCPL was calculated with the *distance_bin* function (Rubinov and Sporns, 2009).

RESULTS

Weighted Graph-Theory Measures

System Segregation

System segregation across the natural cycle (0.98 ± 0.05) was statistically significantly greater than across the OC cycle (0.92 ± 0.06; difference: 0.052, 95% *CI*: 0.023:0.081, $p = 9.19 \times 10^{-4}$; **Figure 2A**) with a large effect size (Cohen's $d = 0.97$, 95% *CI*: 0.33–1.61).

Weighted Characteristic Path Length

Weighted characteristic path length across the natural cycle (0.86 ± 0.01) was statistically significantly greater than across the OC cycle (0.85 ± 0.01; difference: 0.0071, 95% *CI*: 0.002–0.012, $p = 0.0078$; **Figure 2B**) with a medium effect size ($d = 0.62$, 95% *CI*: 0.15–1.11).

Binarized Graph-Theory Measures

Modularity

Estimates of modularity were statistically significantly higher during the natural cycle (0.01 at threshold 0.1, 0.23 at threshold 0.25) compared to the OC cycle (−0.02 at threshold 0.1, 0.20 at threshold 0.25) across the range of connectivity thresholds (range of mean difference in modularity 0.028–0.036; all $p \leq 1.2 \times 10^{-5}$; **Figure 2C**) with a large to medium effect size across the range of connectivity thresholds ($d = 1.00$, 95% *CI*: 0.46–1.55 at threshold 0.1 to $d = 0.69$, 95% *CI*: 0.26–1.11 at threshold 0.25). See **Supplementary Figure 3** for plots of estimates and *p*-values across threshold values.

Binarized Characteristic Path Length

Estimates of bCPL were statistically significantly longer in the natural cycle (1.64 at threshold 0.1, 2.02 at threshold 0.25) compared to the OC cycle (1.62 at threshold 0.1, 1.98 at threshold 0.25) across the range of connectivity thresholds (range of mean difference in bCPL 0.021–0.049; all $p \leq 3.1 \times 10^{-5}$; **Figure 2D**) with a large effect size across the range of connectivity thresholds ($d = 0.93$, 95% *CI*: 0.29–1.56 at threshold 0.1 to $d = 1.02$, 95% *CI*: 0.33–1.70 at threshold 0.25). See **Supplementary Figure 3** for plots of estimates and *p*-values across threshold values.

Dynamic Functional Brain Connectivity

The evaluation of differences between natural and OC cycle dynamic functional connectivity identified two brain states for which the fractional occurrence differed statistically significantly in 8 and 10 of 19 models, respectively. In total, 209 statistical tests were performed. Summary Bonferroni-corrected *p*-values are presented in **Figure 3C** across the range of $k \in \{2, \ldots, 20\}$.

For $k \geq 3$, we observed one brain state ("State 1," green triangle in **Figure 3C**) for which the fractional occurrence was significantly higher in the OC state ($k = 7$: estimate = 0.0455; *CI* = 0.0209, 0.0701; $p < 0.001$; $p_{\text{FWER}} = 0.005$, Cohen's $d = 1.00$; **Figure 3A**). The difference in fractional occurrence of this state between the natural cycle and OC condition was statistically significant over the interval $k \in \{3, \ldots, 10\}$. State 1 is mainly characterized by functional coherence between regions related to the dorsal attention network and, to some extent, limbic, and control networks (see **Supplementary Figure 4** for centroid loadings). These are in turn antisynchronous with regions related to the visual and DMNs and partly to the salience/ventral attention network (**Figure 3A**). Brain state 1 was structurally similar across *k* (**Supplementary Figure 4**).

In contrast, for $k \geq 4$, we observed a second brain state ("State 2," purple triangle in **Figure 3C**) for which the fractional occurrence was statistically significantly higher during the natural cycle ($k = 7$: estimate = −0.0502; 95% *CI* = −0.0789, −0.0213; $p = 0.001$; $p_{\text{FWER}} = 0.009$, $d = 0.92$, **Figure 3B**). This effect

[1] https://cran.r-project.org/

[2] https://www.nitrc.org/projects/bnv/

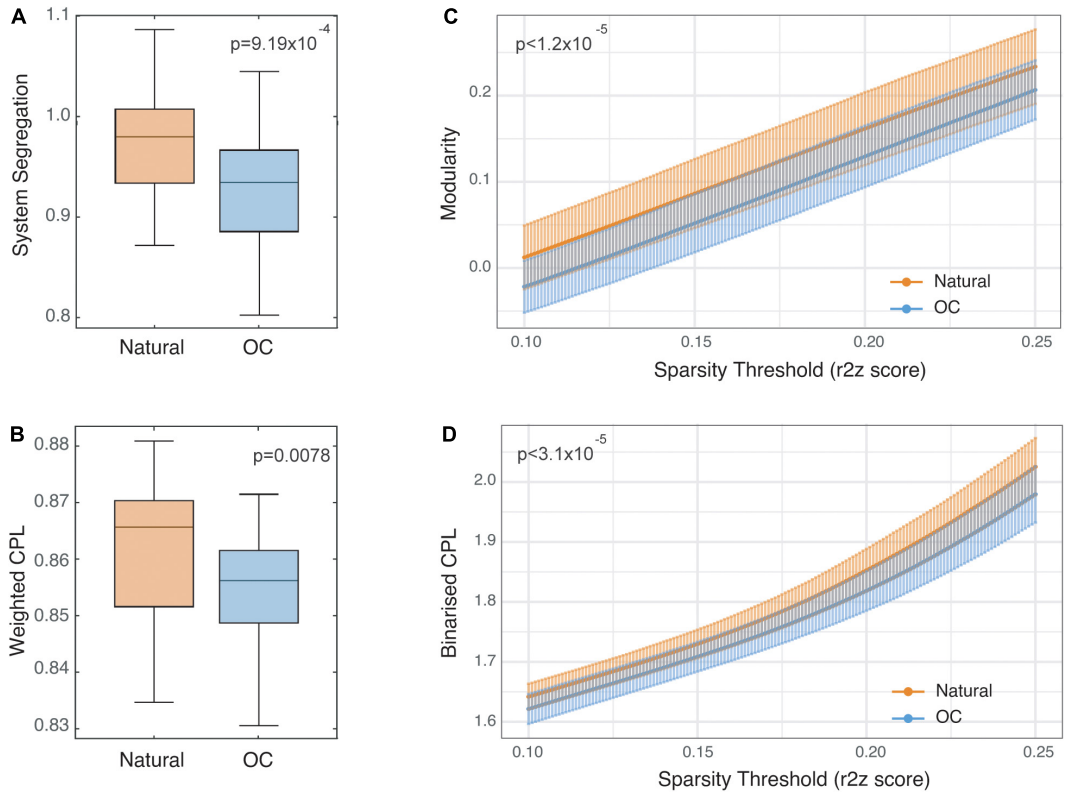

FIGURE 2 | Static functional connectivity comparisons between naturally cycling and oral contraceptive (OC) condition scans. Panels **(A,B)** show Tukey's boxplots representing system segregation and weighted characteristic path length values (CPL), respectively. The horizontal lines represent medians, and the vertical limits of the colored area represent the first and third quartiles. Notches represent 95% confidence intervals for comparing medians, calculated as $\frac{1.58 \times IQR}{\sqrt{n}}$. Panels **(C,D)** show modularity and binarized CPL values reported for each condition at a range of sparsity threshold values between 0.1 and 0.25 and are plotted as mean ± SD.

was statistically significant for $k \in \{4, 5, 7, 9, 12, \ldots, 18\}$. We note that the highlighted brain states for $k \in \{4, 5, 9\}$ are structurally somewhat different than for the rest of the range, particularly regarding the representation of the visual network (see **Supplementary Figure 4**). Brain state 2 is characterized by functional coherence between the dorsal attention and salience networks, and, to some extent, the somatomotor network. These are in turn antisynchronous with regions related to the visual, default-mode, control, and limbic networks (**Figure 3** and **Supplementary Figure 4**).

DISCUSSION

Here we investigated static and dynamic functional connectivity during two 30-day periods a year apart during which a single healthy woman completed daily rs-fMRI scan sessions. Contrasting the 30-day period during which the subject had her natural menstrual cycle with the data from the OC condition, we observed higher modularity, system segregation, and characteristic path length during her natural cycle relative to OC (**Table 1**). These findings suggest that OC may alter brain network organization and point to a whole-brain connectivity architecture that is less strongly partitioned into resting-state networks during OC use. Dynamic functional connectivity analysis identified two discrete brain states for which the fractional occurrence was significantly altered between the natural cycle and OC condition. Together, these results suggest an association between OC use and changes in brain network segregation, including connectivity dynamics in this single subject.

Brain Connectivity Organization Associated With Oral Contraceptive Use

We show that the subject's static brain connectivity during rs-fMRI in the OC cycle relative to the natural cycle was less modular and less segregated into independent systems, i.e., had greater between vs. within network connectivity. Also, on average, shorter paths were required for information transfer between brain regions in the OC cycle.

A recent meta-analysis of 12 studies showed that low brain modularity was associated with major depressive disorder (MDD) with a small to medium effect size (Hedge's $g = -0.33$) (Xu et al., 2021). CPL is a measure of information transfer efficiency (Bullmore and Sporns, 2012). In contrast to modularity, system segregation and CPL were not associated with MDD (Xu et al., 2021), and current data investigating the relation between CPL and MDD and anxiety disorders and the effects of previous pregnancy and PMDD are conflicting (Dan et al., 2020;

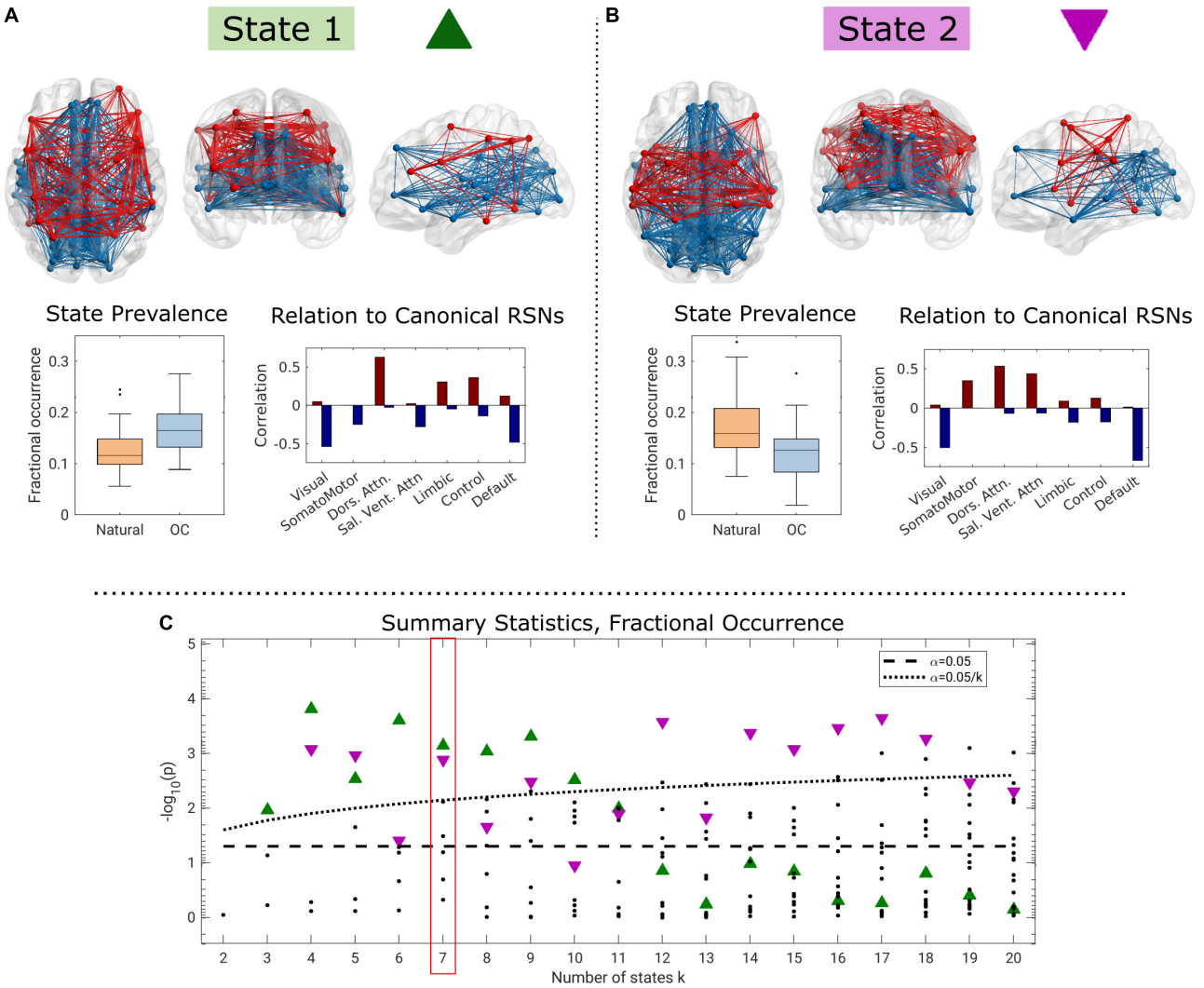

FIGURE 3 | Two brain states for $k = 7$ identified as having significantly elevated fractional occurrence when oral contraceptive medication is used compared to a natural menstrual cycle **(A)**, and opposite **(B)**, and summary p-values across the range of k **(C)**. In the connectivity visualizations, nodes are only shown if the corresponding centroid element has a strength of at least 50% of the maximum absolute loading. In panel **(C)**, green triangles correspond to state 1, purple triangles to state 2, and the dotted black line corresponds to the within-k Bonferroni-corrected threshold for statistical significance.

TABLE 1 | Graph theory measures and state prevalence during oral contraceptive (OC) and naturally cycling state 1 and 2 are both states involving the frontoparietal network.

	Modular graph theory		Global graph theory		Leading Eigenvector Dynamics Analysis (LEiDA)	
	Modularity	System segregation	Weighted characteristic path length (CPL)	Binarized CPL	Fractional occurrence	
OC vs natural	↓Modularity	↓Segregation	↓Path length	↓Path length	↑State 1▲	↓State 2▼

Chu et al., 2021; Guo et al., 2021; Zhang et al., 2021). Additionally, across MDD patients, these measures appear to vary by the age of disease onset, further complicating interpretation of these metrics (Yun and Kim, 2021).

Transient reorganization of functional brain networks during the NC has been observed in the 28andme dataset by Mueller et al. (2021); the most striking reorganization occurred in a DMN subnetwork localized to regions of the prefrontal cortex, during peaks in oestradiol and gonadotropins, which was not observed during OC despite a similar oestradiol peak. This suggests that the OC-induced suppression of gonadotropins reduces network flexibility and the brain's ability to reorganize at the mesoscale level in response to oestradiol (Mueller et al., 2021). Similarly, using a novel metric of brain macroscale information processing

known as brain turbulence, higher turbulence levels at lower scales (i.e., long distances in the brain) and higher information transmission across scales were observed in the luteal versus the follicular phase (Filippi et al., 2021). In contrast, during OC-use, there were no shifts in information processing across the OC-cycle. Taken together, these findings could be interpreted as increased stability or blunted dynamics by the OC-induced hypothalamic-pituitary-gonadal (HPG) axis suppression. This may be related to the decreased modularity, system segregation and CPL that we observed during OC when compared to NC.

A previous analysis of the 28andMe data showed oestradiol's ability to modulate information transfer efficiency within the DMN was present in both NC and OC (Pritschet et al., 2020). However, the subject has a retained oestradiol peak during OC, which is uncommon (Río et al., 2018), and may be specific to that subject or that cycle. We cannot exclude that, women on OCs with a more profoundly suppressed HPG axis would display stronger effects of OC use compared to natural cycling, an effect that should be considered in future population studies evaluating similar effects.

Oral Contraceptive Associated With Changes in Brain Dynamics

The highlighted dFC brain states 1 and 2 were characterized by loadings (**Figure 3** and **Supplementary Figure 4**), that to a large degree, mapped on to functional networks (Yeo et al., 2011). State 1, and the OC condition, is visually characterized by reduced within-network connectivity for the DMN, and slightly increased within-network connectivity for the dorsal attention network. Although this is numerically consistent with previous studies reporting, reduced DMN-connectivity in depressed individuals, it is relevant to note that this effect is of small size and variable across study populations (Yan et al., 2019; Tozzi et al., 2021). Although the DMN and visual networks appear together with the antisynchronous dorsal attention network in both states, it appears that the somatomotor, salience, limbic, and executive control networks shift their associations (**Figure 3**). However, for both the executive control and limbic networks the effects appear to be confined to single regions rather than the network. Expressed crudely, OC use is associated with a shift in dorsal attention and DMN connectivity with the limbic and executive control networks to the somatomotor and salience networks in the evaluated subject. Functional hyperconnectivity has previously been reported in relation to depression. A similar study in the same dataset employed edge time series (Esfahlani et al., 2020) to detect communities of distinct high-amplitude fluctuations (Greenwell et al., 2021). The study focused on two communities, of which one displayed opposed fluctuation between regions in the DMN with the dorsal, salience, and sensorimotor networks, similar to state 2 presented here. Likewise, the second community was characterized by opposed co-fluctuations in the control and dorsal attention networks with the DMN, similar to state 1 presented here. Although edge time series is fundamentally different to LEiDA and diametrical clustering, it is encouraging that the two studies show converging results. Taken together, our findings suggest hormonal contraceptive effects on the occurrence of brain states and corresponding network-specific connectivity in a single subject.

Methodological Considerations

The major limitation of this study is that all analyses were performed on a single individual, across single cycles and in relation to one single type of OC, and that data acquisition for the two conditions was performed 1 year apart. Future studies in a cohort of women, ideally across multiple cycles are required to establish population-level effects of OC use on distributed brain connectivity estimates. Additionally, it is unclear whether the observed differences in brain connectivity are due to OC use (or not) or other factors that may have changed between these two scan periods. Nevertheless, it is a strength of these data that this individual was scanned 30 times, limiting sensitivity to spurious differences that may have emerged were data collected across fewer days. This represents an intriguing dataset with which we have generated observations that support novel hypotheses regarding OC effects on brain connectivity that can be evaluated in larger cohorts.

The reported estimates of functional connectivity based on binarized connectivity matrices are limited due to the undirected selection of a sparsity threshold. We demonstrate that reporting results over a range of thresholds that preserve a balance between randomness and regularity of network connectivity (Bassett and Bullmore, 2016) can provide results that are less constrained by *a priori* threshold selection, though further work must work to refine the optimal threshold range for the human functional connectome. Additionally, we show convergence between binarized and weighted analysis frameworks for both global and network-based analyses.

By using LEiDA to delineate dFC structures, we impose little prior knowledge on the optimal number of brain states and instead evaluate a range of brain state partitions. Inevitably, there will be discrepancies between estimated brain states, dependent on model order k. For both highlighted brain states, slight alterations in state loadings occur in the transitions from $k = 5$ to $k = 6$, and again from $k = 11$ to $k = 12$ (**Supplementary Figure 4B**). This indicates that the significant changes in brain state dynamics observed in our statistical tests probably arise from only a subset of the regions in the identified brain states.

Combined OCs with the antiandrogenic progestins, i.e., Drospirenone and Desogestrel compared to androgenic, appear to have different effects on functional brain connectivity, cognition and mood (Poromaa and Segebladh, 2012; Pletzer et al., 2015, 2016). The OC used by the individual in this study was a combined OC with the androgenic progestin Levonorgestrel. Thus, the effects observed in this individual may be particular to this type of OC.

Perspectives on Future Research

Several epidemiological studies consistently suggest an association between starting an OC and the emergence of

depressive episodes, especially among adolescents (Skovlund et al., 2016; Zettermark et al., 2018; Anderl et al., 2020, 2021). Of concern, adolescents are initiating OC use at increasingly younger ages, often shortly after the onset of puberty (Parkes et al., 2009), when the brain is undergoing organizational changes and maturation related to the surge of sex hormones (Levitt, 2003), which may affect brain architecture (Cahill, 2018; Sharma et al., 2020). Furthermore, many countries have approved and are increasing over-the-counter access to OCs without age restrictions (ACOG, 2019; MHRA, 2021). Considering this and evidence from our analysis and other studies that OC use is associated with changes in brain functional connectivity, it is relevant for future studies to evaluate whether effects on brain functional connectivity is a mediator of OC use on mental health in adolescents.

Also, OC-related brain changes beyond brain network organization or mechanisms underlying such changes should be determined in future populations. Such changes may include key features of the serotonin signaling system (Larsen et al., 2020), since serotonin (5-HT) is a neurotransmitter implicated in modulating functional brain activity and neuropsychiatric pathophysiology (Roseman et al., 2014; Schaefer et al., 2014; Beliveau et al., 2015; Arnone et al., 2018) and estrogen and progesterone target the serotonin system (Barth et al., 2015).

Summary

Our analyses have shown significant alterations in static and dynamic functional connectivity associated with OC use in an open access dataset of an individual scanned daily over 30 days two times 1-year apart. We show that the OC state had statistically significantly lower network modularity and characteristic path length in static estimates of network connectivity and changes in the dynamic connectivity. Due to the limitations of this study, we cannot conclude whether these phenomena generalize or a related to OC use *per se*. However, if the observed findings are related to OC use, this would point to OC-related effects on network dynamics in cognitive and emotional regulation networks. While it might stabilize cognitive and emotional function in some individuals, it might blunt it in others, contributing to an increased vulnerability toward psychiatric disorders. Although these findings are premature for clinical decision-making, they offer hypotheses to be tested in future cohorts designs to determine how the brain-network organization varies across the natural menstrual cycle and how it may be altered in the context of different OCs in different age groups. We propose that these analyses prompt further investigation into the effects of OCs on brain function, especially given the widespread use of these drugs in both adolescent and adult healthy individuals and those with psychiatric disturbances.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://openneuro.org/datasets/ds002674/versions/1.0.5.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of California, Santa Barbara Human Subjects Committee. The participant provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KJ, DM, and AO wrote the initial draft of the manuscript. KJ, DM, AO, SL, and VF contributed to the conception and design of the study. DM, AO, and SB performed the statistical analyses. All authors contributed to the interpretation of the analyses, the manuscript revision, and have approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.855582/full#supplementary-material

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Intrauterine Device Use: A New Frontier for Behavioral Neuroendocrinology

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Intrauterine devices (IUDs) are the most-used reversible contraceptive method for women in the world, but little is known about their potential modulation of brain function, cognition, and behavior. This is disconcerting because research on other hormonal contraceptives, especially oral contraceptives (OCs), increasingly shows that exogenous sex hormones have behavioral neuroendocrine consequences, especially for gendered cognition, including spatial skills. Effects are small and nuanced, however, partially reflecting heterogeneity. The goal of this paper is to introduce IUD use as a new frontier for basic and applied research, and to offer key considerations for studying it, emphasizing the importance of multimodal investigations and person-specific analyses. The feasibility and utility of studying IUD users is illustrated by: scanning women who completed a functional magnetic resonance imaging mental rotations task; taking an individualized approach to mapping functional connectivity during the task using network analyses containing connections common across participants and unique to individual women, focusing on brain regions in putative mental rotations and default mode networks; and linking metrics of brain connectivity from the individualized networks to both mental rotations task performance and circulating hormone levels. IUD users provide a promising natural experiment for the interplay between exogenous and endogenous sex hormones, and they are likely qualitatively different from OC users with whom they are often grouped in hormonal contraceptive research. This paper underscores how future research on IUD users can advance basic neuroendocrinological knowledge and women's health.

Keywords: brain function, connectivity, fMRI, gender, intrauterine device, networks, oral contraceptive, spatial skills

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INTRODUCTION

There has been a recent uptick in the biopsychological study of hormonal contraceptives, partially reflecting women's increased scientific participation and funding emphases (1–4). Indeed, hormonal contraceptives are not only important for their contraceptive and medical benefits, but also as a natural experiment for exogenous sex hormone influences on the brain, cognition, and

behavior, which are severely under-studied domains of women's health. Hormonal contraceptives come in many forms, with intrauterine devices (IUDs) being the most-used worldwide (159 million users; 5). Oral contraceptives (OCs), however, are the most widely-studied form, likely owing to their prevalence in North America and Europe (5). Thus, there are perplexing knowledge gaps regarding neuroendocrine links to cognition and behavior in IUD users. This paper presents vital considerations for filling these gaps and illustratively showcases how multimodal study designs and person-specific methods have potential to accurately reflect the heterogeneity present–but often erroneously ignored–among all women, particularly in relation to ovarian hormone influences (e.g., 6).

Most empirical research on hormonal contraceptives considers users to be homogenous, and thus, combines women using different forms (e.g., IUDs, OCs, and implants; 2–4, 7). This is problematic because hormonal contraceptives have varying exogeneous hormone constituents and doses, and thus, have varying influences on endogenous hormone levels. For instance, combined OCs contain a synthetic estrogen (usually ethinyl estradiol) and a progestin varying in androgenicity, from anti-androgenic to highly androgenic. In many monophasic formulations, women receive stable doses of both hormones for 21 days followed by a placebo for 7 days (although schedules vary). In many triphasic formulations, women receive consistent doses of ethinyl estradiol for 21 days with progestin doses increasing slightly every 7 days for 3 weeks, followed by a placebo for 7 days. The pills alter endogenous ovarian hormone secretion through negative feedback mechanisms and prevent pregnancy by inhibiting ovulation. Most IUDs, however, release a relatively constant dose of the progestin levonorgestrel, which is moderately-to-highly androgenic, for up to three or five years (8). They prevent pregnancy by instigating local changes to reproductive biology (e.g., in tissue within the endometrial cavity), and their systemic impacts on endogenous ovarian hormone levels (especially because they do not contain estradiol), and on brain function and behavior, are unclear. Their reported side effects, however, include acne, headaches, and breast tenderness (8), and women using IUDs have shown risks for depression similar to OC users (9), suggesting that effects may be systemic.

Thus, there is significant heterogeneity among hormonal contraceptives. This heterogeneity is exacerbated by the established heterogeneity in women's neuroendocrine function, including in receptor sensitivity and in lifestyle factors that affect hormone function (10, 11). It is, therefore, not surprising that research on the neural, cognitive, and behavioral consequences of hormonal contraceptive use offers only a few consistent results. One of them concerns depression, as noted above (9). Another concerns OCs and spatial skills. OC progestin androgenicity has been positively associated with three-dimensional (3D) mental rotations performance (12–15), which shows a large gender difference in which men–on average–outperform women (see 1). There is also indication that OC ethinyl estradiol dose is inversely related to mental rotations performance (12). These findings broadly align with reviews and recent empirical work suggesting that high androgens (and perhaps progestogens) as well as low estradiol may facilitate mental rotations performance in women (1, 2, 4, 16, 17). There are, surprisingly and unfortunately, no studies that focus on mental rotations performance (or any aspect of cognition) in IUD users as a homogenous group; when they are studied, IUD users are combined with other hormonal contraceptive users, increasing heterogeneity and limiting inferences (e.g., 13, 18).

The neural substrates underlying hormonal contraceptives and mental rotations performance are also not well-understood (see 2). Generally, functional magnetic resonance imaging (fMRI) studies show that mental rotation tasks engage occipital and parietal regions and some temporal and frontal regions, especially in the right hemisphere, and these regions are linked to gender differences in task performance (1, 19–21). Men typically recruit visual and parietal regions more strongly than women, and women tend to engage frontal regions, such as the inferior frontal gyrus, more than men. These differences are thought to be related to gender differences in strategy use (22, 23). They may also be linked to testosterone and progesterone, but especially to estrogen, as the hormones have been shown to modulate brain activity underlying spatial task performance across the natural menstrual cycle (24–27).

It is necessary to emphasize, however, that this extant literature overwhelmingly relies on traditional neuroscience methods; studies come from a functional localization perspective and focus on task-related brain regions identified through cognitive subtraction (28). For instance, focus might be on parietal activation during mental rotations versus passive viewing, determined by averaging brain activity across trials and participants, often regardless of their hormone milieus. Although they have led to important findings, these methods can also result in null or inaccurate findings because the brain operates as a network (e.g., different parietal regions communicate with several different frontal regions during rotation; 29), hormone milieus vary within and between individuals (7, 8), and people are heterogeneous in their cognition and behavior (30). A person-specific neural network perspective could overcome these limitations. For instance, although the default mode network, which includes midline and lateral parietal regions as well as the medial prefrontal cortex (31), is more active during rest than tasks, it contributes to cognitive function and task performance (32, 33). Women also appear to have greater connectivity (i.e., synchrony) of default mode regions during rest than do men (32, 34). Interestingly, no work has examined the interplay between the default mode network and a set of regions constituting a putative mental rotations network, especially in relation to sex hormones.

FEASIBILITY AND UTILITY OF STUDYING IUD USE

There is a pressing need for future research to examine the neuroendocrine underpinnings of links to behavior and cognition, such as mental rotations performance, in IUD users.

In doing so, it is vital to conduct multimodal investigations that assess links among hormone levels (e.g., circulating, inferred from hormonal contraceptive dosing, or otherwise marked by hormone activity levels), brain function, and behavior (e.g., mental health reports or cognitive task performance), and to consider heterogeneity among women in those links.

Multimodal Data Collection

To illustrate the feasibility and utility of a multimodal person-specific approach, data from 11 IUD users is briefly presented (M_{age}=28.37, SD_{age}=5.40; 55% White, 27% Asian, 18% Black; 73% non-Hispanic). Participants are from an ongoing fMRI study that was conducted with approval from the University of Michigan Institutional Review Board; all participants provided informed consent. All participants were using slow-release IUDs containing the androgenic progestin levonorgestrel (nine were using Mirena®, one Kyleena®, and one Skyla®). They had been using the IUDs for at least the past three months and had no reproductive health issues (e.g., polycystic ovary syndrome) or previous pregnancies. They were also not using medications containing sex hormones.

Among other study procedures, participants completed a 60-minute online monitored survey and received a 60-minute MRI scan. The morning of the scan, they provided approximately 2mL of saliva, which was collected *via* passive drool within 30 minutes of waking. Saliva samples were assayed using high sensitivity estradiol, progesterone, and testosterone enzyme-linked immunosorbent assay kits according to manufacturer instructions (35) by the Core Assay Facility at the University of Michigan. They were assayed in duplicate and averaged for analyses. See **Supplementary Materials** for details, including assay sensitivities and intra-assay coefficients of variation. The top third of **Table 1** shows means and standard deviations (in pg/mL) for all three hormones. These hormone levels do not appear to be suppressed, as are hormone levels in OC users (e.g., 36); in fact, progesterone in IUD users may be elevated compared to both naturally cycling women and OC users (e.g., 37). Thus, these data are consistent with insinuations that IUDs have systemic effects.

TABLE 1 | Multimodal data for IUD users (n=11): Endogenous hormone levels, mental rotations task performance, and person-specific network densities.

Hormone Assessments (in pg/mL)	IUD Users	
	M	*SD*
Estradiol	1.33	.71
Progesterone	239.01	124.99
Testosterone	132.82	64.88
In-Scanner Behavior		
Mental Rotations Performance (% correct)	75.00	8.39
Neural Network Densities		
Total network complexity	35.45	4.53
Within-MRN density (proportion of total)	.34	.03
Within DMN density (proportion of total)	.18	.03
Between-network density (proportion of total)	.19	.05

IUD, intrauterine device; M, mean; SD, standard deviation.

During each scan, participants completed two unique runs of a slow event mental rotations task (27). Each run contained 16 trials during which participants determined whether a pair of 2D or 3D objects formed from small blocks were accurate rotations of each other. The 3D condition was based on the traditional Shepard and Metzler task (38), and the 2D condition controlled for basic visual processing, decision-making, and rotation. Task timing is shown in **Supplemental Figure S1**. Each run lasted 4 min 24s, and correct responses were recorded. Behavior is vital to the interpretation of brain function, and the middle third of **Table 1** shows that IUD users correctly identified whether the rotated 2D or 3D objects were the same in 75% of trials, on average.

Neuroimaging data were acquired using a GE Discovery MR750 3.0 Tesla scanner with a standard coil (Milwaukee, WI). Structural data consisted of 208 slices from a T1 SPGR PROMO sequence (TI=1060ms, TE=Min Full, flip angle=8°, FOV=25.6 cm, slice thickness=1mm, 256x256 matrix, interleaved). Before the task, a fieldmap was acquired using a spin-echo EPI sequence (TR=7400ms, TE=80ms, FOV=22.0cm, 64x64 matrix, interleaved). Functional data consisted of 40 interleaved slices collected during an EPI sequence (TR=2000ms, TE=25 ms, flip angle=90°, FOV=22.0 cm, slice thickness=3mm, 64x64 matrix, 134 volumes). Standard preprocessing was conducted, as described in **Supplementary Materials**. Blood oxygen level-dependent (BOLD) time series were then extracted from ten regions of interest (ROIs) with 10mm diameters, four that constituted the default mode network (DMN) and six that constituted a putative mental rotations network (MRN; see **Supplemental Table S1** for central coordinates), following past work (2, 27, 39). Individual differences in anatomical structure were addressed by intersecting ROIs with participants' binarized grey matter masks (generated using FSL's FAST; 40). Time series from the two runs were concatenated after processing.

Person-Specific Functional Connectivity

Person-specific connectivity analyses were conducted on the mental rotations task-related fMRI data in order to reveal potential individual differences in the neuroendocrinology of IUD use. Specifically, the BOLD time series for each participant was submitted to group iterative multiple model estimation (GIMME), which has been validated in extensive largescale simulations (e.g., 41). Details can be found in tutorials (42, 43) and empirical applications (e.g., 6, 44, 45). Briefly, GIMME uses a data-driven approach based on Lagrange Multiplier tests to add directed contemporaneous (same-volume) or lagged (from one volume to the next) connections to participants' null networks (with no connections). In this application, GIMME added group-level connections (reflecting systematic effects of IUDs) that were significant for at least 75% of the sample to the networks of all women in the sample, followed by individual-level connections (reflecting heterogeneity) for each woman until the model fit well according to standard indices. All connections (i.e., whether at the group- or individual-level) were fit uniquely to each woman's data, and thus, have individualized weights. Each participant's network was then characterized by its overall complexity (i.e.,

number of connections) as well as its subnetwork densities (i.e., number of network connections divided by complexity): within the MRN, within the DMN, and between the MRN and DMN.

The 11 person-specific neural networks generated by GIMME fit the data well, as indicated by average fit indices: $\chi^2(109.55) = 554.17$, $p<.001$, RMSEA=.121, SRMR=.036, CFI=.957, NNFI=.926. **Figure 1A** presents the network for one individual IUD user. Black nodes represent MRN ROIs, and blue nodes represent DMN ROIs. The network reflects homogeneity, as it prioritized contemporaneous (solid lines) and lagged (dashed lines) group-level connections consistent across all IUD users, which are shown as thick black lines. Notice that most group-level connections are between contralateral ROIs (e.g., left and right parietal, lateral parietal, and superior parietal) or ROIs in the same network; only a few are between ROIs in different networks (e.g., from the posterior cingulate cortex to the left inferior frontal gyrus). Heterogeneity was also reflected in the contemporaneous and lagged individual-level connections unique to this participant, which are shown as thin gray lines in **Figure 1A**. For this woman, complexity was 33, and the MRN and DMN densities were 33 and 15, respectively, with a 21 between-network density. The bottom third of **Table 1** shows average complexity and network densities across all IUD users, and **Figure 1B** also graphically shows the average densities. As expected for these task-related fMRI data, the density of connections within the MRN was greater than within the DMN or between the two networks.

Finally, **Figure 1C** shows how neural network densities were related to multimodal study data, including in-scanner mental rotations task performance and endogenous hormone levels. Task performance was positively related to overall neural network complexity, especially to the density of the MRN, as well as to progesterone and even testosterone levels, which were correlated with each other, consistent with the androgenic pharmacokinetic properties of the progestin-based IUDs being used by this sample. The density of the DMN, however, was inversely correlated with all hormones.

DISCUSSION

IUD users provide a novel and promising natural experiment for neuroendocrinological research and are prevalent worldwide (5), but they remain understudied. Research on IUD users–as an independent group not combined with other hormonal contraceptive users–is necessary and feasible. It is necessary because IUDs have functional properties that inherently differ from those of other hormonal contraceptives, such as OCs, which suppress endogenous hormones levels and inhibit ovulation (36). In fact, the illustrative data presented here indicate that circulating progesterone may be enhanced in IUD users. More work is sorely needed to determine the extent to which salivary assays of endogenous hormones reflect or are modulated by intrauterine administrations of synthetic

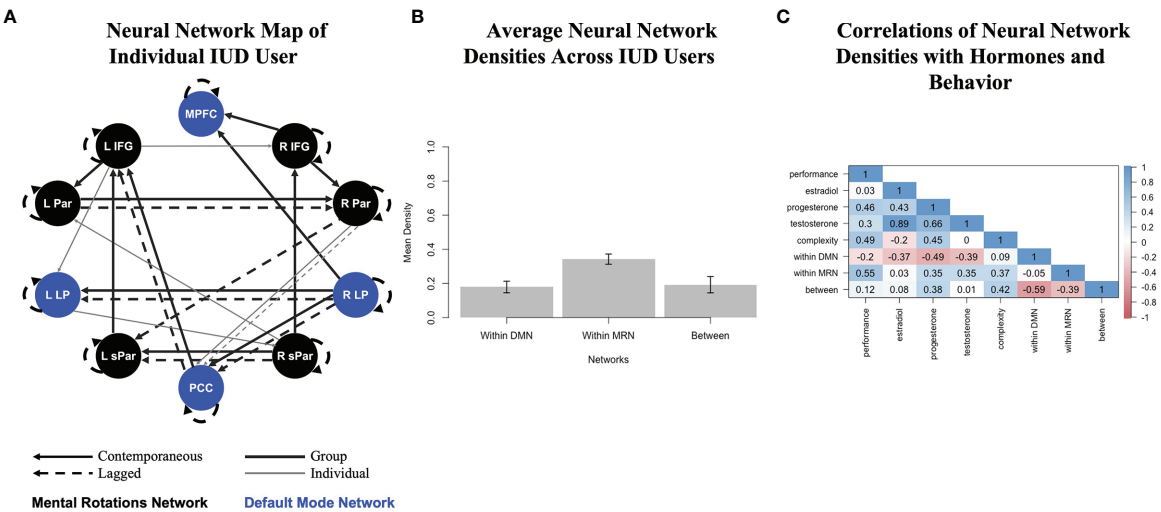

FIGURE 1 | Analysis pipeline linking multimodal data in IUD users who completed a mental rotations fMRI task and provided saliva for hormone assays. **(A)** A person-specific neural network generated by group iterative multiple model estimation (GIMME) for one IUD user. Black nodes show putative mental rotations network regions, and blue nodes show default mode network regions. Solid lines are contemporaneous (same-volume) connections, and dashed lines are lagged (next-volume) connections. Thick black lines are group-level connections significant for at least 75% of the sample, but estimated for all IUD users, and thin gray lines are individual-level connections unique to this IUD user; all participants had corresponding estimated networks (though not depicted here). All connections also have a direction (positive or negative) and beta weight associated with them (also not depicted here). This woman's network fit her functional data well ($\chi^2(112)=652.60$, $p<.001$, RMSEA=.135, SRMR=.039, CFI=.955, NNFI=.924). R, right; L, left; IFG, inferior frontal gyrus; Par, parietal; LP, lateral parietal; sPar, superior parietal; MPFC, medial prefrontal cortex; PCC, posterior cingulate cortex. **(B)** Average neural network densities extracted from the person-specific GIMME networks of all IUD users (and divided by overall network complexity), with error bars showing standard deviations. **(C)** Correlations among multimodal data, including mental rotations task performance in the scanner, endogenous hormone levels, and neural network features, including overall complexity and network densities. Color-coded correlations are shown in the matrix, with dark red reflecting strong inverse relations through dark blue reflecting strong positive relations.

hormones, and this work must consider different data collection methods (e.g., saliva versus serum) and analysis approaches (e.g., ELISA versus mass spectrometry; 46).

Moreover, research with IUD users is arguably more feasible than research on ovarian hormones *via* menstrual cycle phase comparisons in naturally cycling women or even *via* active versus placebo pill comparisons in OC users, as it does not require repeated assessments or phase monitoring, which is not only difficult, but often inaccurate (7).

When studying the neural consequences of the interplay between exogenous and endogenous hormones in IUD users, behavioral assessments and heterogeneity are vital to consider. Regarding behavior, it is prudent to examine behaviors that have already been linked to hormonal contraceptives outside of the scanner, such as mental rotations performance, in order to reveal underlying neural mechanisms (1, 2, 4). Utilizing tasks that maximize power is also important for detecting robust and reliable effects (47, 48). The mental rotations task used in this feasibility demonstration was statistically powerful because it contained 3D (experimental) and 2D (control) conditions instead of a control condition that did not require rotation (see 27).

Regarding neural heterogeneity, multivariate connectivity analyses that incorporate individual differences (see 48), or better yet, person-specific effects, are well-suited to capturing multimodal associations in IUD users; in this way, GIMME has particular utility (41–43). As seen in the illustrative analysis within this paper, GIMME mapped connections among ROIs in the MRN and DMN in a data-driven way, such that only the most meaningful ROI connections were added to participants' individualized networks. Specifically, if model parameters indicated that certain connections were statistically informative for most IUD users, then those connections were estimated uniquely in all women's networks based on their own time series. Thus, GIMME provided group-level inferences without averaging! This has incredible utility for future studies of IUD users–and of other heterogenous samples–as human neuroendocrine processes are unique due to individual differences in biology (e.g., hormone receptor sensitivity; 10), psychology (e.g., emotion; 49), and context (e.g., modulation by stress; 11). Averaging across these heterogeneous samples can falsely exaggerate findings, cancel out effects, or distort inferences (30). Person-specific networks, though time-intensive and complex, are more likely to accurately reflect neuroendocrine nuances.

Conclusions

The goal of this paper was to highlight the value of IUD users as a natural experiment for studying both exogenous and endogenous sex hormone links to gendered neurocognition (namely, mental rotations), by utilizing multimodal research designs and person-specific approaches to the analysis of fMRI data. Future investigations should focus on IUD users as an independent group; it may rarely be appropriate to combine IUD users with OC users to create a general "hormonal contraceptive" group. Future investigations should also triangulate hormonal, neural, and behavioral data, and analyze these data in ways that accurately reflect heterogeneity within IUD users, who have unique neuroendocrine milieus. Indeed, effects of IUD use are likely to be both systemic within women, and unique to individual women. This means that future investigations are important for both revealing ovarian hormone influences on the brain and behavior, and for advancing multimodal and person-specific methods within behavioral neuroendocrinology.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because data use agreements need to be established. Requests to access the datasets should be directed to Adriene Beltz, abeltz@umich.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Michigan IRB (Health Sciences and Behavioral Sciences). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AB conceptualized and directed the study with critical input from KK and JJ; MD helped collect the data; AB and MD analyzed the data with critical input from NC; AB, MD and NC drafted the manuscript; all authors provided critical revisions and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.853714/full#supplementary-material

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Effects of oral contraceptives on spatial cognition depend on pharmacological properties and phase of the contraceptive cycle

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The central nervous system effects of oral contraceptives (OCs) are not well-documented. In a set of 3 studies, we investigated a specific cognitive function, mental rotation, in healthy women currently using OCs for contraceptive purposes (n = 201) and in medication-free controls not using OCs (n = 44). Mental rotation was measured using a well-standardized and extensively validated psychometric test, the Vandenberg Mental Rotations Test (MRT). In an initial study (Study 1), current OC users (n = 63) were tested during the active or inactive phases of the contraceptive cycle in a parallel-groups design. Studies 2 and 3 were based on an archival dataset (n = 201 current OC users) that consisted of data on the MRT collected in real-time over a 30-year period and compiled for purposes of the present work. The OCs were combined formulations containing ethinyl estradiol (10-35 ug/day) plus a synthetic progestin. All 4 families of synthetic progestins historically used in OCs were represented in the dataset. Cognitive performance was evaluated during either active OC use ('active phase') or during the washout week of the contraceptive cycle ('inactive phase') when OC steroids are not used. The results showed a significant phase-of-cycle (POC) effect. Accuracy on the MRT was mildly diminished during the active phase of OC use, while scores on verbal fluency and speeded motor tasks were modestly improved. The POC effect was most evident in women using OCs that contained first- or second-generation progestins (the estrane family of progestins or OCs containing levonorgestrel), but not in women using OCs containing recently developed progestins and lower doses of ethinyl estradiol. Using independently established ratings of the estrogenic, androgenic, and progestogenic intensities of the different OC formulations, each brand of OC was classified according to its distinct endocrine profile. Multiple regression revealed that the effects of OC use on the MRT could be predicted based on the estrogenic strength of the contraceptives used. Estrogenic potency, not androgenic or

anti-androgenic effects of the OC pill, may underlie the effects of OC usage on spatial cognition.

KEYWORDS

oral contraceptive, hormonal contraceptive, menstrual cycle, estrogen, androgen, ethinyl estradiol, mental rotation, visuospatial

1 Introduction

Oral contraceptives (OCs) have been available since the 1960s as a trusted method of contraception and are used by millions of women world-wide. Most contemporary OCs are 'combined' formulations that consist of an orally administered estrogen (typically ethinyl estradiol, EE2) in combination with a progestin. At present, more than 20 different synthetic progestins are available for contraceptive use. Different brands of OCs are differentiated by which progestins they contain, their pharmacological properties, and the dosages of estrogen and progestin that are used. Despite widespread adoption by women, scientific knowledge of OC actions in the central nervous system (CNS) is still rudimentary. In recent years, there have been multiple calls for increased study of the CNS effects of OCs (1, 2).

OCs disrupt the endocrine environment of the female menstrual cycle. Through feedback inhibition of the hypothalamic-pituitary-gonadal axis, OCs inhibit gonadotropin secretion and the rising concentrations of endogenous 17β-estradiol that trigger ovulation (3). Because ovulation is prevented, the luteal phase increase in progesterone that normally follows ovulation does not occur. Circulating testosterone is reduced by 40-60%, as revealed by assays of free testosterone in the serum or saliva of OC users (e.g., 4, 5). While endogenous production of sex steroids is inhibited, OCs serve as an *exogenous* source of estrogens and progestins, whose resulting levels in the bloodstream vary depending on the brand of OC used and individual differences in metabolism and clearance (6, 7; but see 8). While standard commercial immunoassays of serum or saliva confirm the lower levels of endogenous hormones, they are typically insensitive to the exogenous hormones contained in OC pills, rendering the exogenous hormone 'invisible' in standard laboratory assays. Importantly, however, the OC steroids can interact physiologically with hormone receptors located in the CNS (and elsewhere in the body) and thus possess a potential to influence CNS activity and function (e.g., 9, 10). Indeed, recent studies employing advanced neuroimaging techniques such as functional MRI, while subject to methodological limitations, suggest OC use may be associated with subtle changes in brain structure and resting-state and/or task-driven neuronal activity (see 11 for review). Changes have been identified in several different brain regions, including hippocampal and neocortical sites known to participate in higher-order cognitive processes (11, 12).

Although empirical data are still sparse, OCs might plausibly be expected to influence certain spheres of cognitive function. Furthermore, the effects are likely to be selective. Over the past 3 decades, the naturally-occurring forms of estradiol and progesterone, which vary over the menstrual cycle in healthy *non*-OC users, have been discovered to modify certain cognitive functions, notably those that are sex-differentiated (i.e., display sex-related differences in performance). These effects are mediated by the binding of ovarian steroids available in the bloodstream to estrogen-, androgen-, or progesterone receptors in the CNS (13, 14). Local alterations in neurochemistry or synaptic function are then initiated *via* modulatory effects of the steroids on gene transcription or *via* rapid non-genomic signaling mechanisms (14, 15). While evidence is not entirely consistent, high levels of 17β-estradiol, the major estrogen present in naturally-cycling women of reproductive age, have been associated with modest increases in verbal fluency, perceptual speed and accuracy, and possibly verbal memory (e.g., 16–18), but also diminished performance on tests of visuospatial ability including mental rotation tests (e.g., 16, 19–21). One question, therefore, is what effect does the use of *oral contraceptives* have on women's cognition? Secondly, are the effects of OCs attributable to their suppression of the endogenous steroids? Or to the exogenous hormones supplied by the OCs themselves? Based on precedent established by studies investigating natural forms of the hormones, visuospatial cognition is one possible candidate to exhibit an OC-linked effect.

To date, studies of cognition and perception in women using OCs are few in number. Possible effects of OCs on a range of outcomes have been suggested, from olfactory and inner ear processes (4, 22, 23), to motor execution or planning (24), memory processes (25–28) and controversially, vulnerability to mood disorders (e.g., 29). Empirical support for such effects, however, is limited and unsystematic. Within the realm of cognitive function, a handful of studies has focused on visuospatial cognition, defined as the capacity to envision in the 'mind's eye' the visual appearance, positions, or movements of objects (30). One common example of a visuospatial ability is mental rotation, i.e. imagining the movement of an object

around its axis or envisioning the orientation of a rotated object when viewed from different vantage points (see Figure 1).

Existing studies of OCs and mental rotation have produced inconsistent findings, are typically limited to comparisons of naturally-cycling women (NC) and mixed groups of OC users (often without controlling for the phase of the menstrual cycle where cognitive testing is performed), and are subject to a range of methodological issues. These include small sample sizes that render it difficult to draw conclusions, particularly given the diversity of OC formulations available. On average, cognitive studies have included a mean sample size of only 24 OC users (32). The earliest work on mental rotation suggested no differences exist between OC users and non-users, with both groups of women however showing improvement in accuracy if tested during the menstrual phase of their menstrual cycle (33, 34). Other studies, to the contrary, have suggested that active use of OCs might improve mental rotation slightly compared with non-OC users (e.g., 17, 35, 36), or that either improved or diminished performance can be found depending on the 'androgenicity' (or 'anti-androgenicity') of the progestin constituent of OCs, with superior visuospatial ability observed under conditions where an OC contains a progestin having a greater capacity to bind to androgen receptors (37). Of note, some but not all of the progestins used in OCs are derived from 19-nortestosterone and exhibit residual binding affinity for androgen receptors. On the other hand, our own past work has suggested that visuospatial performance may be linked to the estrogenic, not androgenic, effects of the OC pill (35, 38).

Despite early publication barriers, our laboratory has systematically collected data over many years to investigate the effects of natural and synthetic reproductive steroids on cognitive function, perceptual processes, and motivational variables in both OC users and non-users. Relevant to the present report, we have collected data over the past 3 decades using the Vandenberg test of mental rotation (MRT; 31) to study visuospatial function. Vandenberg's test is a modification of the Shepard-Metzler (39) figures and is a 'paradigmatic' index of visuospatial ability. We first began collecting data on the MRT in 1991 as part of our wider body of work on the modulation of visuospatial abilities by endogenous estradiol in women. The resulting data from non-OC users has been published (e.g., 21, 40), but much of our OC data (often collected either as control groups or to assure that cognitive testing was conducted blind to women's endocrine status) has not. In the present article we report previously unpublished findings from a historical dataset of OC users, compiled *via* a series of real-time studies carried out in our laboratory between 1991 and 2015. These data were collected by former students in the laboratory and together comprise an unreported dataset of significant informational value because the number of OC users is unusually large (N = 201), it consists of healthy young adults using OCs strictly for contraceptive purposes (absent of pre-existing medical conditions) who were tested on a well-validated, widely-used, cognitive test (the MRT; 31) collected in real time over multiple 'generations' of the synthetic progestins used in standard OCs. Data throughout this 30-year period were collected by several different experimenters but used identical test stimuli, identical test administration procedures (the 24-item version of the MRT, administered using a 4-minute time limit for each half of the test), and identical scoring criteria (31; see details below). All assessments were carried out under well-controlled, in-person, one-on-one test sessions in our laboratory. Internet testing was avoided. To our knowledge, it constitutes the largest dataset ever collected on mental rotation in women who use OCs and every 'generation' of contraceptive progestin, from the first to most recent, is represented. As such, the dataset is a rare, possibly unique, resource to test the general hypothesis that OCs influence cognitive function, and the specific hypothesis that the use of OCs is associated with hormonally-driven effects on mental rotation.

The purpose of the present report is to explore whether OCs influence higher-order cognitive processes, using mental rotation as a target function. We aimed to test whether any OC effect could be identified; to explore whether the effect differs across different families of OCs; and to investigate if a phase-of-cycle effect is associated with OC use, as is demonstrated across

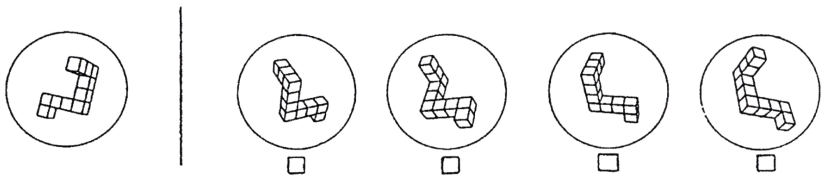

FIGURE 1
An example item from a task that requires mental rotation, the ability to 'rotate' an object in one's mind. This item is from the Vandenberg Mental Rotations Test (MRT), a psychometric tool developed and standardized by Vandenberg and Kuse (31). Each of the 24 items on the test depicts a target object (left) that can be rotated to match only two of the multiple-choice options shown on the right. Which two are correct? Mental rotation is an elemental process involved in visuospatial cognition. [Reprinted from *Hormones and Behavior*, Vol. 65, Hampson E, Levy-Cooperman N, Korman JM, Estradiol and mental rotation: Relation to dimensionality, difficulty, or angular disparity? pp. 238-248, 2014, with permission from Elsevier].

the natural menstrual cycle in naturally-cycling women (18–21). A phase-of-cycle effect has not been established in OC users to date. This question, however, is important theoretically because it addresses the unresolved issue of whether cognitive effects of OCs are caused by the exogenous steroids present in OC pills or by their suppression of endogenous 17β-estradiol or the naturally-occurring progestogen, progesterone. To address these questions, we first report (in Study 1) the results of a medium-sized investigation of OC users and non-user controls who were tested on a set of sex-differentiated cognitive tasks while actively taking their OCs or during the 'off' week of the contraceptive cycle (when no hormone is used and therefore concentrations of exogenous hormones are minimized). In Study 2, we then examine the hypothesis of phase-of-cycle effects across all 4 families of synthetic progestins (explained further below) using our full dataset of 200 OC users. Finally, in Study 3, we end by investigating whether observed effects of OCs on mental rotation are related to the estrogenic or androgenic actions of OC pills, and which hormonal constituent is most important in driving the mental rotation effect. This question is explored in Study 3 *via* multiple regression methods.

2 Study 1: the 'on-off' study

2.1 Background and hypothesis

In naturally-cycling (NC) women who do not use OCs, accuracy on mental rotation tests shows small but systematic variations across the menstrual cycle (for a recent review see 41). Scores on the MRT tend to improve during menses, the phase of the menstrual cycle when circulating levels of ovarian steroids are lowest, and are modestly diminished at phases where 17β-estradiol concentrations are at their peak (e.g., 18–21, 33, 42). Conversely, high estradiol is associated with slight improvements in performance on certain motor planning/execution tasks and in verbal fluency (16, 18). (Verbal production is assessed in the laboratory by having participants generate words, phrases, or sentences that meet experimenter-defined semantic, lexical, or phonetic criteria). At lower levels of estradiol increase, menstrual cycle effects are harder to detect (e.g., 43), consistent with the relatively small effect sizes of these hormonal influences.

In women using OCs, two early studies (33, 34) raised the possibility that a similar phase-of-cycle (POC) effect might be detectable among OC users. However, these early findings were largely dismissed because of methodological concerns (e.g., failing to analyze OC users separately from non-users in statistical analyses; inconsistent findings). As a result, POC effects among OC users remain unconfirmed. A few later studies that did try to address the POC question were complicated by practice effects that rendered the findings inconclusive (e.g., 26, 44, 45). While generally recognized as more statistically powerful, within-subject designs are not recommended in situations where differential carryover effects can be anticipated (46). The MRT is known to elicit a substantial practice effect (47) and to have decreased validity with multiple test exposures. To avoid the complications caused by repeat testing, therefore, we used a between-subjects design to test the hypothesis of a POC effect.

Based on past investigations of NC women, we hypothesized that a POC effect (operationalized here as a difference in performance between the active and inactive phases of the contraceptive cycle) would likewise be found in OC users if the exogenous hormones contained in OCs are responsible, because tissue concentrations of OC steroids are higher during the active phase of the OC cycle (when hormones are ingested on a daily basis) than during the inactive phase (when no hormone is used, resulting in washout of exogenous hormones and onset of menses). Conversely, the suppression of endogenous hormone production caused by OC use endures during the inactive phase of the contraceptive cycle (e.g., 26, 44) and may even last for weeks in some women. In particular, 17β-estradiol and progesterone levels either remain stable during the inactive phase or show a minute rise only, approximating early follicular values (e.g., 26, 48, 49). Consequently, no differences in MRT performance were anticipated between the active and inactive phases if suppression of endogenous hormones is the mechanism responsible for cognitive differences between OC users and non-users. Importantly, following past observations in NC women, we predicted that any cognitive effects seen in OC users would be directionally-selective, and that the direction of effect would depend on the precise cognitive processing requirements demanded by the tasks performed.

2.2 Methods

2.2.1 Participants

OC users ($N = 63$) were recruited *via* poster advertisements at a Canadian university and reimbursed for their participation (includes two participants with partial data only). All participants were right-handed and had been using the same brand of OC for a minimum of 4 mo prior to participating in the study. Only women using 'low-dose' OCs were included (30-35 ug EE2 per day). As the dataset is historic, all participants were taking first or second generation progestins (see Supplementary Materials Table S1, for a full list of the OC brands used). Volunteers were pre-screened by telephone interview to assure that the inclusionary and exclusionary criteria were met. Participants were considered ineligible if they had been using OCs for less than 4 mo, were left-handed, had any history of psychiatric, endocrine, or neurological conditions, or if they used any prescription medication other than OCs.

Data from a demographically-matched group of NC women ($N = 44$), recruited in the same manner and tested on the MRT,

were also included. The NC group was recruited from the same source and were matched on educational level and within 1.5 years of age. The NC women were tested at either menses, when ovarian output is negligible (corresponding to the 'inactive' phase), or at higher estrogen ('active') phases of their menstrual cycle, as confirmed by salivary radioimmunoassays of 17β-estradiol and progesterone collected at the study visit (see 21 for full details). The purpose of the NC group was to provide a context for the MRT scores observed in the OC users, by illustrating a typical level of performance in demographically-matched NC controls, who had been tested at 2 phases of the natural ovarian cycle found in past work to differ in their average level of performance on the MRT (e.g., 18, 20). Mean age was 22.17 ± 3.25 years (SD).

2.2.2 Procedure

The study employed a between-subjects design to avoid potential confounding introduced by repeated exposure to the cognitive test materials. Each participant reported individually to our lab for a 60-min study visit where a brief set of verbal and spatial cognitive tasks and simple motor tasks were administered by a trained examiner. Participants also filled out a standardized mood scale (Profile of Mood States, 50), a handedness inventory (51), and an auditory test (not relevant to the present report).

Based on information collected during telephone pre-screening, half the women were scheduled to be tested during the inactive phase of the OC cycle (Days 5, 6, or 7 of the 7-day inactive phase) when no hormone is taken, and half were tested during the 21-day active phase when OC pills containing active EE2 plus a progestin are taken. Assignment to phase was counterbalanced. Because it can take several days for exogenous hormone concentrations to stabilize at the beginning of a new OC package (6, 7, 52), women at the active phase were tested after at least 5 days of active pill use and, if using a multiphasic brand of OC (with varying hormone levels) were tested after taking at least 2 pills containing the highest hormone content. The average number of active pills remaining in the present package on the day that cognitive testing was performed was 7.45 pills (*SD* = 4.14). Participants were required to bring their current package of OC pills to the study visit to allow the researchers to objectively verify the name and type of OC used, the prescription information, and to verify the exact number of pills remaining on the date of cognitive testing.

2.2.3 Cognitive tests

Five cognitive tests were administered. Detailed descriptions can be found in Supplementary Table S2. These tasks were selected because they, or highly similar tests, have exhibited sensitivity to circulating levels of reproductive hormones in past studies of the natural menstrual cycle. Visuospatial tests included the Mental Rotations Test (MRT; 31) and the Paper Folding Test (53). The MRT is widely used in laboratory settings to assess proficiency of mental rotation. The 24-item version of the standard MRT was used with a four-minute limit on each half of the test. On each item participants had to identify which two of four forced-choice alternatives were rotated versions of a target object. The only difference between the target object and the two correct objects was their angular orientation in space. The test was scored using the standard scoring criteria recommended by the original test developers (31) and the final score was corrected for guessing (max correct = 48). The second test, Paper Folding (53), assessed a different form of spatial ability. Each of the 20 items requires predicting where punched holes would be located in a folded piece of paper once the paper is unfolded again. The correct answer is identified from among 5 alternatives. Six minutes were allowed. The number correct summed over all 20 items was calculated, and the total score was corrected for random guessing (53).

As seen in Supplementary Table S2, the remaining cognitive tasks were non-spatial. They consisted of two verbal fluency tasks, and a simple motor learning task (Manual Sequence Box; 54) that required learning and then executing a sequence of 3 hand postures until a criterion of 10 consecutive sequences without error was reached. In a prior study using a repeated-measures design, we previously reported that this form of motor learning is facilitated during the active phase of the OC cycle, compared with the inactive phase (24). Over the natural menstrual cycle, several independent labs have reported superior verbal fluency during high-estrogen phases compared with low (16, 18), and a correlation between verbal fluency scores and circulating levels of 17β-estradiol has been seen in NC women (18, 55–57).

2.2.4 Statistical analysis

Scores on the MRT were analyzed using a univariate ANOVA with Contraceptive Status (OC or NC) and Phase-of-Cycle (active, inactive) as independent variables. Because the NC women were originally recruited for a separate study, they overlapped with the OC group only for the MRT, but received different verbal fluency tasks and therefore could not be included in the statistical analyses of the non-spatial measures. Accordingly, the other measures were analyzed for the OC users alone using separate tests (one-tailed *t*-tests) that did not include the NC women.

2.3 Results

Results for the MRT are shown in Figure 2. A robust phase-of-cycle (POC) effect was found. The POC effect was evident in both the NC and OC groups, $F(1,101) = 24.72$, $p < .001$. The interaction between POC and contraceptive status (OC user or non-user) was not significant, $F(1,101) = 0.38$, $p = .538$. The magnitude of the effect, expressed as Cohen's $\underline{d}$ (58) was $\underline{d} = 0.84$

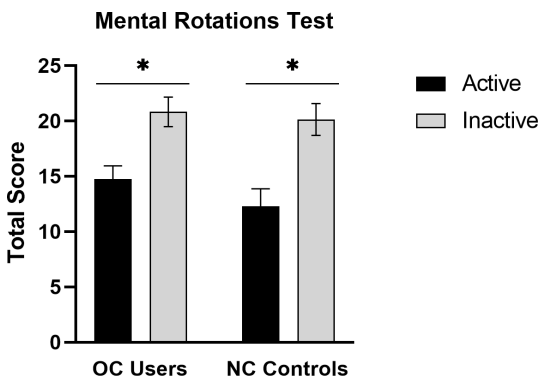

FIGURE 2
Mean accuracy on the MRT at the active and inactive phases of the menstrual cycle in oral contraceptive users (OC users, n = 61) and naturally-cycling controls (NC controls, n = 44). A robust phase-of-cycle effect was found in both groups. Among OC users, mental rotation performance was superior during the inactive phase of the contraceptive cycle (grey bars), when exogenous hormone intake is absent. *$p < .01$.

for OC users and d = 1.14 for the NC controls (see Figure 2). As expected, accuracy on the MRT was significantly higher in OC users who were tested during the inactive (menstrual) phase of the OC cycle, compared with OC users tested under 'active' hormone conditions. In other words, performance was poorer under active OC use.

The other visuospatial task, Paper Folding, showed the same pattern of means as the MRT (see Table 1), and approached significance, $t(59) = 1.57$, $p = .061$. In the present sample, average accuracy on the Paper Folding test was higher during the inactive phase, d = 0.41. The smaller effect size is commensurate with the smaller sex difference that is usually reported for Paper Folding, relative to the MRT task (59).

A significant but reversed direction of effects was found for the Manual Sequence Box. A significant POC effect was seen, Acquisition: $t(38.6) = 2.30$, $p = .013$; Execution: $t(47.4) = 2.06$, $p = .022$. As expected, OC users displayed better motor performance during the active than inactive phase (Table 1). This confirms results reported for the same manual sequencing task by Szekely et al. (24) in an earlier, independent, sample of OC users. Both of the verbal fluency tests likewise showed a significant or near significant POC effect, $p = .029$ for Controlled Associations; $p = .055$ for the Oral Fluency test (Table 1). On both fluency tests, word retrieval was superior during the active phase of the OC cycle, when exogenous hormones are used on a daily basis. Thus the direction of the POC effect for the MRT task (superior performance at the *inactive* phase) was reversed on the verbal measures, which depend on separate brain regions and do not recruit visuospatial processes. This suggests the visuospatial effect is selective.

Because the OC group contained women taking both monophasic ($n = 24$) and multiphasic ($n = 39$) OC formulations, a secondary ANOVA was run to test if the MRT results might be attributable to this difference. The results, however, showed no significant difference in accuracy between the women using mono- ($M = 18.32$) or multiphasic OCs ($M = 17.99$), $F(1,57) = 24.72$, $p = .866$. The MRT scores were nearly identical in both subgroups.

2.4 Discussion

Study 1 confirmed a significant POC effect among OC users. The direction of the effect under active OC use differed for the MRT versus the verbal tasks, demonstrating selectivity based on function. For all tasks, the direction of effect conformed to the POC effects reported to occur in naturally-cycling women, in association with naturally-occurring variations in ovarian hormones across the menstrual cycle (e.g., 16, 18, 42). In particular, lower accuracy on visuospatial tasks and improved verbal performance has been found in NC women when elevated concentrations of ovarian hormones are available to the CNS.

Study 1 confirmed early reports by Moody (33) or Silverman and Phillips (34) that imply a potential POC effect might exist in OC users (see also 26 for parallel findings on a verbal memory task). Those early studies were inconclusive, reflecting methodological issues that left the findings open to question. Consistent with Moody (33) and Silverman and Phillips (34), all participants in our ON-OFF study used OCs containing 1st or 2nd generation progestins. Thus, while Study 1 demonstrates a

TABLE 1 Mean performance (SD) on the MRT and other cognitive tasks at the active and inactive phases of the contraceptive cycle.

	Active Phase	Inactive Phase	Cohen's d	*p*-value
Mental Rotations Test (# correct)	14.72 (5.67)	20.82 (8.50)	d = 0.84	$p = .001$
Paper Folding (# correct)	11.97 (3.67)	13.36 (3.06)	d = 0.41	0.061
Oral Fluency (# words generated)	12.44 (3.53)	10.75 (4.66)	d = -0.41	0.055
Controlled Associations (# words)	30.45 (7.70)	26.82 (7.01)	d = -0.49	0.029
Box Task – time to acquisition (sec)	12.02 (2.42)	14.46 (5.23)	d = -0.60	0.013
Box Task – speeded execution (sec)	14.30 (3.83)	16.95 (5.88)	d = -0.53	0.022

The Manual Sequence Box is a timed measure, therefore a lower score indicates better performance.
The symbol # stands for "number" as in "number correct".

POC effect it does not establish whether a POC effect can also be found in women using modern 3rd or 4th generation OCs that contain lower EE2 concentrations and/or progestins possessing different pharmacological properties.

Recent OC studies have overlooked the question of POC effects. This might reflect lack of awareness, or the exclusion of women not at the active phase of the OC cycle in certain studies (e.g., 60, 61), or small sample sizes inadequately powered to detect POC effects. In some studies data are simply collected at random points in the OC cycle and then combined, irrespective of the possibility of POC effects on outcomes (35). Many recent investigations have compared OC users as a whole with NC women (who may or may not be assessed at known points in the natural menstrual cycle) to discover how the performance of OC users might differ from NC controls (e.g., 62). Failure to understand or identify POC effects is potentially an important source of measurement error in OC studies. Claims of 'better' or 'worse' performance by OC users are not well-substantiated by studies that fail to control for POC effects. If not controlled, apparent differences in accuracy on the MRT between OC and NC groups could merely reflect chance differences in the proportions of women in the 2 groups who happened to be tested during menses. On the other hand, if POC effects are attributable to the exogenous hormones present in OCs, then POC effects conceivably might be less visible for current formulations because they are attenuated by the lowered EE2 doses used in many contemporary OCs or are linked only to specific subclasses of synthetic progestins.

3 Study 2: phase of cycle effects across different families of oral contraceptives

3.1 Background and hypothesis

The objective of Study 2 was to explore the generalizability of POC effects across the different classes of progestins used in OCs. We analyzed an archival dataset consisting of 201 OC users, to investigate if POC effects are evident when the family of OC pills being used is considered as an independent variable.

The term 'generation' is used in 2 different senses in the medical literature. Some researchers define generations based on the timing of an OC's introduction into the North American marketplace, while others use the term to denote the distinct families of progestins available for use in OCs based on differences in molecular structure (63). We adopt the latter convention here. The progestin families are distinguished by their pharmacological profiles and derivation from either 19-nortestosterone or 17-hydroxyprogesterone or, more recently, spironolactone (64). Endocrine differences between the families include their capacity to exert androgenic or anti-androgenic side-effects, but also their capacity to exert estrogenic or anti-estrogenic effects (or neither), their progestogenic intensity, presence of mineralocorticoid effects, effects on serum binding proteins (e.g., SHBG), and their relative ability to bind to classical steroid hormone receptors (65). The estrane- and early gonane-based OCs used in Study 1 have stronger androgenic effects *in vivo* (but often contain higher doses of EE2) than many OCs based on 3rd or 4th generation progestins (10, 66), some of which have *anti*-androgen effects at concentrations used therapeutically (e.g., drospirenone; 65). One widely-recognized classification of the progestin families, which is based on structural properties of the progestins, is shown in Table 2 (64, 67), and was used to classify individual OC products in the present study. Because of differences in their pharmacological properties, the class of progestin used in a given OC may be relevant to understanding its ability to influence cognitive performance. While all OCs contain a progestin, not all families may have an equal potential to modify scores on the MRT or other cognitive tests, and consequently to cause POC effects.

3.2 Methods

3.2.1 Participants and description of dataset

Our historical dataset consisted of 204 adult females using various brands of hormonal contraceptives available by prescription in Canada between 1991 and 2015 (n = 201 with oral route of administration; see Table 2 for a list of OC brands used). Our dataset is a compilation of all available OC users tested in studies conducted in our laboratory from 1991-2015, who were using low-dose OCs (35 ug/day EE2 or less). The OC brands present in the dataset are rich and widely varied and represent those commonly used by healthy undergraduates at our institution and how they evolved over 3 decades. Fewer 4th generation OCs are available in the dataset (n = 22) than earlier generations of OCs due to the relatively recent introduction of DRSP-based contraceptives, the lesser frequency of their use in North America versus Europe (see 2, 60), and more limited recruitment by our lab for studies involving the MRT over the last 10 years. A criterion for inclusion in the dataset was having performed the 24-item (not 20-item) MRT test of Vandenberg and Kuse (31) as part of an individually supervised study visit to our laboratory, using a time limit of 4-min (not 3-min or 5-min) for each part of the test. All participants performed other cognitive tests too, but because the specifics of those additional tests varied from study to study, depending on the original purpose of the work, only the MRT could feasibly be analyzed here.

Mean age of the participants was M = 20.56 (SD = 2.32), range = 17-35, with no significant differences across the 4 families of progestins (Table 2). Mean daily EE2 dose was matched across the progestin families within 1ug/day, except for the 2nd generation gonanes, which were slightly lower. Except

TABLE 2 The four families of contraceptive progestins and specific brand names represented in the dataset (*N* = 204).

Generation and Family Name	Specific Progestins Used in OCs	Brand Names Represented	Mean Age of Participants (Range)
Generation 1 (Estranes) *N* = 58	norethindrone, norethynodrel, norethindrone acetate, ethynodiol diacetate	Brevicon 1/35, Brevicon 0.5/35, Demulen 30, Demulen 1/35, Loestrin 1/20, Loestrin 1.5/30, Lolo, Micronor, Minestrin 1/20, Ortho 1/35, Ortho 0.5/35, Ortho 10/11, Ortho 7/7/7 Synphasic	*M* = 21.01 Range = 18-27
Generation 2 (Gonanes) *N* = 76	levonorgestrel, dl-norgestrel	Alesse, Aviane, Alysena, Minovral, Portia, Triphasil, Triquilar	*M* = 20.40 Range = 18-30
Generation 3 (Third Generation Gonanes) *N* = 48	desogestrel, gestodene, norgestimate, etonogestrel, norelgestromin	Freya, Linessa, Marvelon, Cyclen, Tri-Cyclen, Tricyclen-Lo, Tricira Lo, Evra*, NuvaRing*	*M* = 20.39 Range = 18-35
Generation 4 (Spironolactone Derivatives and C-21 Progestins) *N* = 22	drospirenone, cyproterone acetate†	Yasmin, Yaz, CyEstra, Ginette, Diane-35	*M* = 20.43 Range = 17-27

*Evra (n = 1) and NuvaRing (n = 2) contain third generation progestins but are not administered orally.
†Cyproterone acetate is an old progestin but is included in Gen 4 in the present report because, like drospirenone, it is a potent anti-androgen. It has anti-androgen activity approximately three-fold greater than drospirenone's (65).

for the ON-OFF study, all data were collected in a blinded fashion. Participants were tested at either the active or inactive phases of the OC cycle. Data acquisition for the 2 phases was always carried out in parallel. Blind testing meant that fewer data from the inactive phase were available, but within each of the contributing datasets the active and inactive phases were matched for the era of their data collection and were thus matched in the compiled dataset as a whole. All participants had an educational level of year 1 of university or higher. Exact details of OC brand names, number of tablets remaining, and any other OC details were collected for each participant during the laboratory visit where the cognitive testing was performed. Although the duration of time on the present OC was not always recorded, average time on OC was ≥4 months where data were available and consequently the pattern of cognitive differences was expected to be stable (pharmacokinetic changes may occur between the first and third cycles of OC use before pharmacologic stability is reached, see 68).

For purposes of the present analysis, data were examined with and without a set of 44 NC controls (non-OC users), described above, who performed the MRT under identical administration and scoring conditions as part of their participation in a study of the MRT and the natural menstrual cycle performed by our lab (21). For all NC controls, phase of the menstrual cycle on the date of cognitive testing was confirmed *via* high-sensitivity radioimmunoassays of 17β-estradiol and progesterone.

The final size of the entire dataset including all groups of participants was 245 individuals.

3.2.2 Statistical analysis

To investigate whether the POC effect replicates across all 4 families of OCs commonly recognized, we performed a factorial ANOVA, with Phase-of-Cycle (active, inactive) and pharmacological family ('generation' of progestin) as independent variables, using our archival dataset to test the hypothesis of a generalized POC effect that is associated with OC use and is found pan-generationally. Specifically, we compared accuracies achieved on the MRT during the active and inactive phases of the contraceptive cycle, with women grouped according to the family (class of progestin) to which their OC belonged. A generalized POC effect was predicted to be evident in the ANOVA as a main effect of phase-of-cycle.

Phase of cycle is not the only variable that may influence accuracies on the MRT test. Accuracy may vary across the individual generations of OCs based on differences in their androgenicity. The same ANOVA was also used, therefore, to evaluate a second hypothesis. Wharton et al. (37) was the first to posit that women using 4th generation progestins based on drospirenone (DRSP) might exhibit poorer visuospatial ability (e.g., on mental rotation tests) because of the anti-androgenic qualities of DRSP, which is a partial androgen receptor antagonist (65). In other words, *overall* accuracy on the MRT was predicted to differ systematically across the progestin

families as a result of differences in their ability to transactivate androgen receptors (37). Accordingly, our ANOVA was examined to reveal if the different families of OCs showed absolute differences in accuracy on the MRT (i.e., a main effect of progestin family). It should be noted that Wharton's original findings, which served as the basis for this proposition, were based on a total sample size of only $n = 7$ Yasmin users, raising questions about reproducibility. Two subsequent studies have produced mixed support for Wharton's hypothesis (44, 60; see also 69). In general, empirical tests have been limited, and the proposed androgen mechanism remains an open question. We analyzed differences between the progestin generations to begin to shed light on the question of mechanism.

3.3 Results

3.3.1 Is a POC effect seen uniformly across all families of OCs?

Factorial ANOVA with Phase-of-Cycle and OC Family as between-subjects factors was used to contrast accuracies on the MRT in women tested during the active and inactive phases of the cycle. Only users of *oral* contraceptives were included; transdermal formulations were excluded from the ANOVA. Three outliers scoring below chance on the MRT and one woman whose brand of OC was ambiguous also had to be excluded.

The magnitude of the POC effect for each family of contraceptives is displayed in Figure 3. Contrary to our hypothesis of a pan-generational effect, a POC effect was identified only for some, but not all, families of OCs. The ANOVA revealed a significant main effect of Phase, $F(1,228) = 9.77$, $p = .002$, whereby accuracy on the MRT was mildly lower in women tested during the active phase of OC use ($M = 17.55$ items correct) than the inactive phase ($M = 20.41$ correct; $\underline{d} = 0.36$, across all generations combined). However, the magnitude of the POC effect varied significantly depending on which progestin family was used, Phase x Generation interaction: $F(4,228) = 2.87$, $p = .024$. Tukey *post-hoc* tests revealed that a robust POC effect was identifiable among early generation OC users and among the NC women, but the POC effect decreased progressively in size for more recent families of OCs and was essentially absent among women who used 4th generation progestins (see Figure 3).

3.3.2 Effects of the progestin family on MRT scores

Wharton et al.'s hypothesis (2008) predicts that any effect of the progestin family ought to be clearest when exogenous hormones are used *actively*. In light of the Phase x Generation interaction, and to isolate any effect of the progestin families most effectively, a simple effects ANOVA was therefore performed, limiting the analysis to the active phase of the OC cycle only (Figure 4). The analysis was run with and without NC controls tested at the *in*active phase to represent a basal level of MRT performance. As shown in Figure 4, simple effects revealed a significant effect of the OC family on MRT scores if accuracy was evaluated under conditions of active hormone intake, $F(4,140) = 3.28$, $p = .013$. Tukey-Kramer *post-hoc* tests showed that 1st generation progestins (estranes), even though they possess a moderately high degree of androgenic activity (66), performed significantly worse during OC intake ($M = 13.84$ items correct, $n = 32$) than users of either 2nd ($M = 18.77$, $n = 43$) or 3rd generation progestins ($M = 19.79$, $n = 24$), $p = .050$ and $p = .036$ respectively. Women using 1st generation progestins did not differ significantly from those using 4th generation progestins[1] ($p = .321$), who likewise had slightly lower accuracies ($M = 17.86$, $n = 22$). No evidence of *superior* accuracy was found in 2nd generation pills containing levonorgestrel ($M = 18.77$, $n = 43$), even though 2nd generation pills are the most highly androgenic OCs of all in terms of their endocrine effects (10). In short, family-dependent differences were observed if OC users were evaluated during active intake, but the pattern of group differences did not support the hypothesis of an effect driven by androgenic properties of the progestins (37).

If the analysis was restricted to monophasic OCs only, or was limited only to OCs containing 30 ug/day of EE2 or higher, the *n*'s were reduced but the pattern of group differences remained unchanged.

3.4 Discussion

Contrary to our hypothesis, Study 2 failed to show a POC effect that generalized across all progestin families. Rather, a robust POC effect was found only for Gen1 OCs, replicating the effect found in Study 1 but in a sample twice as large ($n = 63$ versus 134 Gen1 and 2 users, respectively). We also found differences in accuracy among the progestin families, but the differences did not conform to the androgen hypothesis. Differences were visible during active OC use only. If tested during the inactive phase of the OC cycle, when exogenous hormone is not used, accuracy on the MRT was fairly uniform across all OC groups (grey bars, Figure 3). During the inactive phase, accuracies in OC users resembled NC women tested at menses, when circulating hormone concentrations are at a nadir. The fact that all groups performed so similarly at the inactive phase suggests that the effects seen for the MRT at the active

1 Because the 4th generation OCs showed no evidence of a phase-of-cycle effect, we ran an analysis with all 22 4th generation users included, to bolster the limited sample size available for the 4th generation group and maximize the likelihood that any group difference would be detected.

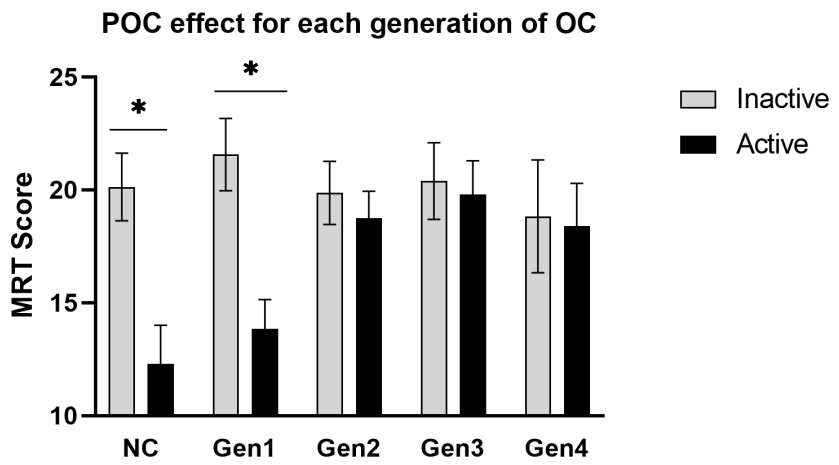

FIGURE 3
The POC effect for each pharmacological family of progestins. A robust phase-of-cycle (POC) effect was seen among users of early generation OCs (Gen1, n = 55) and naturally-cycling women (NC, n = 44), but to a lesser extent or not at all among the more recent families of progestins (Gen2, n = 74; Gen3, n = 44; Gen 4, n = 22). *$p < .01$.

phase are due to short-term, quickly reversible, effects of OC steroids in the CNS.

Wharton and colleagues (2008) argued that any effect of OCs on mental rotation might reflect their androgenic (or anti-androgenic) properties. Anticipated effects on MRT accuracy would be positive or negative, respectively. This prediction is plausible based on several studies of naturally-cycling (NC) women, which suggest that in non-OC users higher endogenous levels of circulating androgens (e.g., 19, 20) or the experimental administration of testosterone (e.g., 70, 71), is associated with greater accuracy on mental rotation tests. However, some large-sample studies have failed to support any connection between MRT performance and circulating androgen concentrations (e.g., 72). Studies involving OC users are rare and sample sizes are typically small. To our knowledge, Study 2 is the largest to date to test for androgen-related group differences and is the only study to include all 4 families of OC progestins. Our data suggest that androgen activity in the CNS is

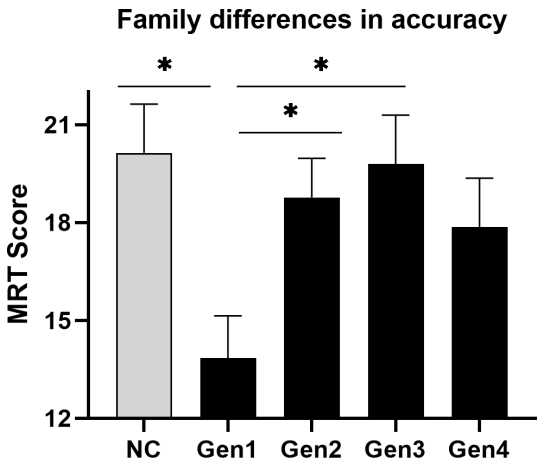

FIGURE 4
Differences in mean accuracy on the MRT among the progestin families when exogenous steroids were used. During the active phase of the contraceptive cycle (black bars), when OC steroids are taken on a regular daily basis, accuracy of performance on the mental rotation test (MRT) varied significantly across the different families of OCs. The grey bar represents the performance of naturally-cycling controls (NC) tested at menses (inactive phase), which is shown here as a neutral condition. Among OC users, family differences were minimal at the inactive phase (not shown in figure) where no active hormone is used. *$p < .05$.

unlikely to be the major driver of OC family ('generation') differences in MRT scores, although it may contribute in a minor capacity to mental rotation along with other variables. Independent of androgens, a separate body of work has suggested that high levels of *estrogens* are associated with lower scores on the MRT in naturally-cycling women (18–21). Because 1st and 2nd generation OCs tend to be higher in estrogenic activity but also higher in androgenic activity, it is unclear how these physiological effects might combine to influence cognitive performance. The question of androgenic versus estrogenic influences as the basis for group differences in the performance of OC users on the MRT was addressed in Study 3.

4 Study 3: estrogenic, androgenic, and progestogenic actions of the OC pill

4.1 Background and hypothesis

The endocrine effects of OCs vary along several different dimensions simultaneously. Although the OC brands within each progestin family share certain common features, they also exhibit differences that might be relevant to developing a full understanding of their CNS effects. Heterogeneity in the endocrine profile across OC brands is present, both within and between the various pill families. To test whether this heterogeneity is relevant to predicting the mental rotation effect, we followed up Study 2 by systematically coding the endocrine profiles of each of the individual OC brands represented in our dataset. This was done to capture differences that might be relevant to cognitive performance, and to allow further insights into exactly which endocrine characteristics are responsible for the MRT effect.

Individual brands vary in the intensities of their estrogenic, androgenic, and progestogenic actions *in vivo*, depending on dosage differences and which particular forms of estrogen and progestin they contain. Most 1st (and many 2nd) generation OCs contain 30-35 ug/day of ethinyl estradiol (EE2), while some OCs developed recently contain lower EE2 concentrations (20-25 ug/day). The estrogenic activity of OCs is not exclusively attributable to their estrogen constituent, however. Some progestins used in OCs (notably the 1st and 2nd generation families) have progestogenic effects, but also exert varying degrees of estrogenic (or sometimes anti-estrogenic) effects (3). Third generation gonanes have no intrinsic estrogen activity. They were developed to minimize androgenic side-effects associated with the 2nd generation progestins, which in turn are more highly androgenic than the moderate intensity estranes (1st generation progestins). Recently, 4th generation progestins, notably DRSP, have been developed and are purer progestogens, devoid of androgen activity (73). Some of the newer progestins are potent progestogens despite their absence of androgenic effects and possess several-fold greater progestogenic effectiveness than progesterone itself (65). The progestin doses actually used in OCs are reduced accordingly (3). Some progestins (e.g., cyproterone acetate, dienogest, drospirenone) demonstrate anti-androgenic activity at the concentrations used in OCs (65). Based on these complexities, many OCs simultaneously possess estrogenic, androgenic, and progestogenic actions in terms of their biological actions in tissue, and exert each of these effects to varying degrees depending on exactly which brand of OC and which family of progestin we consider. Any of these biological activities or some combination of them might explain the observed effects of OCs on MRT performance.

There have been at least 2 previous attempts to formally investigate the hormonal underpinnings of the visuospatial effect in women using OCs. In a group of 56 OC users, all of whom were using 1st or 2nd generation OCs and were tested during the active phase of the OC cycle, Hampson and Moffat (38) reported that the overall estrogenic potencies of the OCs used by women in their sample were a significant inverse predictor of performance on 2 visuospatial tests. Relative potencies had been established by bioassays or receptor studies (74; see 38) and reflected the combined estrogenic effect of each brand of OC attributable jointly to both its estrogen and progestin constituents. Using an alternative approach and a larger sample size, Beltz etal. (35) found that the nominal EE2 dose in a group of OC users significantly predicted scores on the MRT (β = -.26). Importantly, in the Beltz et al. study (2015), OC users were analyzed as a combined group without considering POC effects. Thus, OC users were included in the statistical analysis irrespective of whether they had been tested on the MRT at the active or inactive phase of the OC cycle. Accordingly, Beltz's study may underestimate the true magnitude of the estrogen effect. In the present analysis, we adopted the approach advocated by Hampson and Moffat (38). Thus, biological potency not face dosage was used as an index of hormone action.

4.2 Methods

4.2.1 Coding of OC brands

For each woman in our dataset, the specific brand of OC pill being used was independently coded for its estrogenic, androgenic, and progestogenic properties by using standard tables of the relative potencies of OCs available in Dickey and Seymour (67) or earlier editions of the same source (e.g. 74). Numerical values assigned by Dickey to each specific OC brand are data-driven and based on *in vivo* bioassays or receptor studies (e.g. 75) derived from humans or from pre-clinical investigations of laboratory animals. As such, they are an objective quantification of each brand's capacity to exert biological effects. The estrogenic activity of each brand of OC

is expressed relative to pure ethinyl estradiol, the androgenic activity of each brand is expressed relative to methyltestosterone, and progestogenic activity is expressed relative to norethindrone (67). Importantly, this method of coding considers the total bioactivity of each OC brand integrated over a 28-day window relative to its index compound (e.g., methyltestosterone) and, in contrast to classifications based on dosage alone, accounts for not only differences across brands in dose administered but also differences in the intrinsic biological strengths of the individual progestins and any incremental estrogenic or androgenic actions attributable to each OC's progestin component. It therefore affords a more refined and accurate picture of true differences in the endocrine effects of different OC formulations than dose considered alone. In effect, this endocrine coding method places all OC brands on a common measurement scale, allowing biological differences among OCs to be reflected irrespective of the identities of the specific progestins that comprise each brand. Relative to straight dosage-based comparisons, it offers greater precision, corresponds more closely to clinical observations (75), can encompass multiphasic as well as monophasic OC formulations, and avoids the need to entertain only small homogeneous groupings involving a single progestin when comparing different brands of OCs (*cf.* 35). Although some inexactness is still acknowledged (67, 75), part of which stems from individual variation in women's metabolism of the contraceptive steroids (7, but see 8), average differences between brands on the dimensions of estrogenicity, androgenicity, and progestogenicity are captured best by bioassay data making it a superior choice as an index of true differences between OCs in their capacity to exert endocrine effects *in vivo*.

Accordingly, each OC brand used by the 200 women in our dataset was assigned 3 independent ratings based on Dickey and Seymour (67) or related sources (74), reflecting its estimated estrogenic, progestogenic, and androgenic actions *in vivo*. Users of 2 OCs in our dataset, namely one 4th generation (Diane-35; alternatively CyEstra, n = 5) and one 1st generation OC (Demulen 30, n = 5) could not be coded, because these brands of OCs are not approved for use in the USA and thus no endocrine ratings for them were available in Dickey and Seymour (67).

4.2.2 Statistical analysis

Multiple linear regression with forced entry was used to investigate whether the estrogenic, androgenic, or progestogenic actions of OCs are significant predictors of accuracy on the MRT, and to evaluate their relative importance. The total MRT score of each participant was entered as a dependent variable, and the estrogenic, progestogenic, and androgenic ratings for each specific brand of OC from Dickey and Seymour (67) were used as predictor variables. In our sample, age was not significantly correlated with MRT scores (r = .03), likely reflecting the narrow age range of the present dataset and the fact that the MRT shows negligible age-related changes in young adulthood. Thus age was not entered as a predictor variable in the regressions. Progestogenic potency of each OC was included as a predictor for completeness, despite a lack of evidence based on previous literature to suggest a progestogen-based effect on MRT performance in either naturally-cycling women (e.g., 18–21) or in the only previous study of OCs to address this question (35). The OC ratings of progestogenic potency were skewed and were log-transformed prior to analysis to reduce skewness. Two outliers who had a total score ≤3 on the MRT (out of a possible maximum score of 48) were excluded when performing the regressions.

Only women evaluated at the active phase of the OC cycle were included in the regression analysis (n = 104). A matching regression performed for OC users evaluated on the MRT at the inactive phase can be found in the Supplementary Materials (Table S3).

4.3 Results

Forced-entry regression revealed that the endocrine profile of the OC pills significantly predicted scores achieved on the MRT, $F(3,99) = 3.28$, p = .024. The regression model is summarized in Table 3 (top). Individually, only an OC's estrogenic potency, but not its androgenic or progestogenic potency, was a significant predictor of accuracy (β = -.30, p = .004). OCs higher in biological estrogen activity were associated with lower scores on the MRT, irrespective of their nominal EE2 dosage. The relationship is shown in Figure 5 as a simple scatterplot with line of best fit. This outcome agrees with the results of Hampson and Moffat (38) who found that higher

TABLE 3 Results of the multiple regression analyses.

	R	R^2	F	Predictor	Beta	t, p-value
OC Users at Active Phase (n = 104)	.30	.09	3.28*	Estro	-.30**	-2.93, p = .004
				Andro	-.08	-0.77, p = .443
				Progest	-.08	-0.80, p = .423
Generations 1 and 2 Only (n = 70)	.39	.15	3.88*	Estro	-.40**	-3.30, p = .002
				Andro	-.13	-0.95, p = .346
				Progest	.10	0.74, p = .461

Dependent variable = Total MRT score (max = 48). Estro, estrogenic potency; Andro, androgenic potency; Progest, progestogenic potency (log-transformed).
*p < .05, **p < .01.

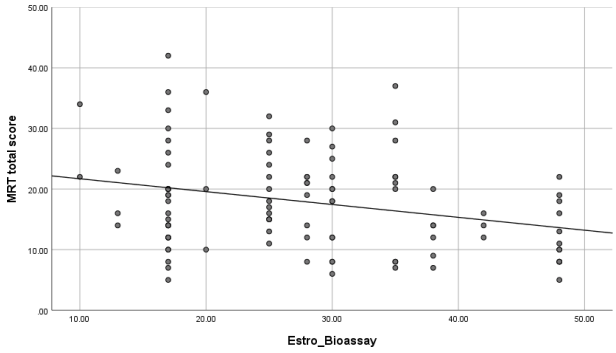

FIGURE 5
Scatterplot of the zero-order correlation (with best-fitting regression line) between overall accuracy on the Mental Rotation Test (MRT) and the estrogenic potencies of the oral contraceptives (OCs) used in our sample. All OC users were tested during the active pill ingestion phase of the contraceptive cycle (n = 104). OCs higher in estrogen potency were associated with poorer spatial accuracy on the MRT test. Estrogenic potencies for each contraceptive brand were obtained from tables in Dickey and Seymour (67), *Managing contraceptive pill patients and other hormonal contraceptives* (17th ed) or earlier editions of the same source, and are based on bioassays or receptor studies. Estro_Bioassay = relative estrogen activity of each OC brand.

estrogen potency of OC brands predicted poorer scores on a different but related visuospatial test, Space Relations from the Differential Aptitude Test (76). It also agrees with Beltz et al. (35) who identified a negative association between EE2 dose and accuracy on the MRT, based on differences in nominal OC dosage instead of overall biological activity as indicated by *in vivo* pharmacological studies (67). In support of these findings, high estradiol phases of the menstrual cycle are reportedly associated with lower accuracies on the MRT among naturally-cycling women (e.g., 18–21, 33, 42).

Current OCs that contain 3rd and 4th generation families of progestins possess limited or no androgenic activity, and often have only weak estrogen effects due to reduced doses of EE2 in a number of the 3rd and 4th generation formulations (e.g., Yaz = 20 ug/day). Re-running the multiple regression with only the 1st and 2nd generation OCs included (n = 70) produced an acceptable range in both the estrogenic (range = 10 to 48) and androgenic potencies (range = .17 to .80) and increased the magnitude of the predictive relationships observed (Table 3, bottom). Here, too, only differences in estrogen potency across OC brands were a significant predictor of accuracies on the MRT, β = -.40, p = .002 (Table 3).

5 General discussion

Mental rotation is a basic visuospatial ability used in science, technology, construction, and engineering disciplines that require the accurate visualization of spatial relationships among objects, parts of objects, or the visualization of movements in three-dimensional space. In the present work, we analyzed an archival dataset of OC users who were tested on a standardized test of mental rotation, the MRT (31). With an overall sample size of 201 OC users it is, to our knowledge, the largest sample to date to address the question of whether mental rotation is influenced by the use of OCs. We found a significant POC effect—a difference between average accuracies on the MRT during active pill usage compared with a baseline condition where no active hormone was being used. Accuracy was higher under the no-use ("inactive") conditions. The POC effect was seen prominently in women using early-generation OC pills, was attenuated in users of second-generation progestins, and was virtually absent among women taking current, third-generation, contraceptives. Changes in the hormonal constituents of OCs over time likely explain these generational effects. In particular, regression of MRT scores on the estrogenic, androgenic, and progestogenic biopotencies of a wide range of OC brands across 4 generations of OC pill formulations revealed that the capacity of an OC to produce estrogenic effects in tissue is one variable which contributes to individual differences in visuospatial performance.

Our findings reinforce cognitive differences seen over the menstrual cycle in naturally-cycling women, although those differences like the ones seen here are often subtle. During the past 3 decades, studies of NC women (not using OCs) have reported that performance on the MRT is modestly reduced at phases of the ovarian cycle characterized by high levels of circulating 17β-estradiol (see 41, for a recent review). A negative correlation between individuals' scores on the MRT and current estradiol concentrations in serum or saliva has been shown (e.g., 18–21). Using a different paradigm, oral contraceptive use, the present data converge on the notion that reproductive steroids have subtle effects on specific visuospatial functions and extend these observations to the synthetic steroids that are used in OCs.

Our data are among the first to suggest a POC effect in OC users. They extend early findings by Moody (33) and Silverman and Phillips (34). While those early studies had significant

methodological shortcomings that rendered them inconclusive, they helped to inform the POC hypothesis tested in the present report. Even earlier data from our own lab (77), using an independent dataset not included in the present report found that OC users assessed at the active phase of the OC cycle differed significantly from NC women assessed during menses on several other sex-differentiated cognitive tasks (that did not include the MRT) and concluded that 'functionally high levels' of hormones present during OC use might have implications for certain dimensions of cognitive performance.

Although mental rotation was our major focus, it was not the only cognitive function to show a POC effect in the present work. Study 1 also revealed a phase-of-cycle effect on rapid motor learning and execution and on word fluency. Unlike the MRT, which exhibits a well-established performance advantage in favor of males, fluency and motor learning exhibit sex differences in favour of *females* in the general population (78, 79). Relative to the MRT, these tasks showed an opposite, reversed, direction of POC effect in the present study, i.e. improvement during the *active* phase of the contraceptive cycle. This too is consistent with studies of naturally-cycling women showing similar functional selectivity in the cognitive effects seen under high 17β-estradiol conditions (e.g., 18). In OC users, studies investigating non-spatial facets of cognition are infrequent. Almost all have focused narrowly on declarative memory (but see 26, 69). This highlights the novelty of the present findings, which go beyond mental rotation.

Insofar as data are available, our results are compatible with POC effects identified in past studies of OC users on non-spatial tasks. Mordecai etal. (26) found improved verbal memory during the active pill phase in a study using a within-subjects design. Likewise, Hampson (25) found improved working memory during active usage. Verbal fluency and motor learning, however, have rarely been studied. In the present data, all effects seen under active OC use, including the MRT, are in directional agreement with effects on cognitive functions identified under high estradiol conditions in naturally-cycling women. The fact that similar effects are observed under active OC use suggests that exogenous hormone intake *via* the OC pill is the agent likely responsible for these cognitive effects. While OCs also inhibit the *endogenous* production of ovarian steroids, this inhibition is not quick to resolve. In most OC users, endogenous concentrations remain at very low early follicular values during the 7-day inactive phase, i.e. they closely resemble the menstrual phase concentrations of naturally-cycling women, where ovarian activity is negligible (e.g., 12, 26, 44). The suppression of endogenous production, because it spans across both the active and inactive phases, does not offer a satisfactory explanation for the POC effects we observed over the OC cycle, particularly their time course, which includes a rapid dissolution of the effects in the inactive phase followed by their re-appearance upon the return of exogenous steroid intake.

Several caveats must be noted. Though it is an implausible mechanism to explain the cognitive effects seen here, the inhibition of endogenous hormones by OCs might nevertheless contribute to other phenomena associated with long-term OC use such as, for example, incremental reductions in bone density that accrue under long-term exposure (80). Such a possibility is not ruled out by the present findings. Secondly, the cognitive functions we studied here were intentionally selected because they are known to exhibit differences in mean performance as a function of an individual's biological sex, and there is reason to believe they may be sensitive to circulating hormones (see 81). However, many if not most cognitive functions are not sexually differentiated. Accordingly, they might be expected to show no changes in response to OC use based on these same considerations. The present findings illustrate that OC-mediated effects on CNS functions are possible and offer 'proof of principle', but further research will be needed to define which cognitive functions more generally are influenced by OCs and which are not, and the present findings should not be overgeneralized. Revealing the breadth and limits of the POC phenomenon is an important objective for future research endeavors. Thirdly, the fact that we saw a perceptible difference in mental rotation performance when exogenous hormones were higher does not imply OCs produce 'supra-physiological' concentrations, as is sometimes claimed, or that they even reach the working concentrations attained during a natural menstrual cycle by their endogenous analogs. Indeed, direct assays of EE2 concentrations and their 24-hr time course following active pill ingestion (e.g., 82, 83), as well as the diminished magnitude of the POC effect we observed in users of contemporary OCs that contain lower doses of EE2, suggest contemporary OCs have physiological effects that are fairly modest.

The diminishing POC effect we saw over recent generations of OCs is a novel finding. It is unlikely to be explained by cohort effects, because all families of OCs in our dataset showed similar mean performance on the MRT if they were tested during the inactive phase of the OC cycle (Figure 3). Accuracies matched those of non-users tested at the same phase, when ovarian production of hormones is quiescent. Progestin family differences were seen only among women tested during the active phase of intake (which was always evaluated concurrently with the inactive phase during data collection). We propose instead that a diminished POC effect and generally higher levels of MRT performance during the active phase for more recent than older OCs reflects changes in the hormonal constituents of OCs over the past 3 decades. These include reductions in EE2 dose and associated changes in progestins, or a shortened inactive phase (e.g., Yaz, Loestrin 24) where exogenous washout normally occurs, or other changes that attenuate endocrine differences between the active and inactive phases of the OC cycle. If POC effects vary by generation it might explain inconsistencies in past literature regarding OC effects on cognition.

One popular theory speculates that effects of OCs on mental rotation are attributable to their progestin constituent, specifically the extent to which an OC exerts androgenic or anti-androgenic effects *in vivo* (37; or see also 84). The present dataset failed to support the androgen hypothesis, either in terms of the group

differences found across the different families or in our multiple regression analyses where androgen activity was taken into account on a brand-by-brand basis. Specifically, androgen potency was not a significant contributor to MRT scores in our regressions, and the slightly diminished MRT score seen among 4th generation users was so slight as to be non-significant.

Lack of support for the androgen hypothesis is not altogether surprising. Androgenic effects of OCs are very weak compared to testosterone itself, making it less likely that effects on cognition would be perceptible. This hypothesis is still largely speculative and there is a lack of empirical support for it more broadly. Wharton et al (37) result showing lower accuracy on the MRT in Yasmin users (n = 7) was not replicated by Griksiene and Ruksenas (44), who found no differences between 3rd (n = 10) and 4th generation OCs (n = 11) on a mental rotation test. A later study found that women taking anti-androgenic OCs (n = 35) were less accurate at mental rotation than naturally-cycling women, but other generations of progestins were not evaluated and thus no true generational effect was actually demonstrated (60). Recently, Gurvich et al. (69) found no significant POC effect on a different type of visuospatial task requiring recall of a learned route through a grid, but anti-androgenic OCs (n = 17) did perform more poorly than OCs containing levonorgestrel (n = 18), an androgenic progestin.

Our study is the first to evaluate androgenicity in a dataset where all 4 historic families of OC progestins were represented, enabling us to see a fuller picture. It is worth re-emphasizing that progestins in the present study were classified by their chemical structure not by the timing of their introduction into the marketplace. Thus our Gen1 and Gen2 families included OC formulations that are still available, albeit used infrequently by women today. For example, Alesse and Minovral were both considered 2nd generation OCs in the present work because they both contain levonorgestrel, but were introduced at very different times. Only 50% of the Gen1 brands listed in Table 3 are still marketed. For these reasons, our dataset is of scientific value but POC effects may be less applicable to many OC users at a clinical level today. Studies that include only the 2 most recent OC generations do not evaluate androgenicity across its entire range. While we believe that classification based on progestin identities is the most theoretically defensible, it is possible that different results would be obtained if brands of OCs were classified into generations based on market timing instead, which is an alternative basis for classifying progestins sometimes used in epidemiologic studies (63).

While POC effects may be less likely, current brands still vary considerably in their estrogen potency and our data suggest that pill estrogenicity may be the primary contributor to OC-related differences in performance on the MRT. It may also underlie the differentially large POC effect seen for the highly estrogenic Gen1 OCs. In our regressions, we used an empirically-derived coding scheme (67) based on published evidence from bioassay tests (e.g., actions in the rat ventral prostate relative to methyltestosterone; 85) to quantify the estrogenic, androgenic, and progestogenic effectiveness of each individual brand of OC pill. This method of quantifying bioactivity avoids the pitfalls of trying to make comparisons across OC brands based on dosage alone. A given brand may have higher or lower estrogen activity than its nominal EE2 dose implies, depending on which exact progestin the EE2 is paired with (e.g., see Ortho 0.5/35; 74). Progestogenic potency was also coded, but the progestogenic activity of the OCs in our dataset was found to be highly skewed. Although corrected by log-transformation, we consider our progestogen results to be only provisional and further investigation in future work is recommended. On the other hand, our regressions did support an estrogen-driven effect of OCs on women's MRT scores. OC brands having greater estrogenic effects were associated with lower accuracies on the MRT (Figure 5), irrespective of generation. This pattern was most pronounced among OCs possessing greater levels of estrogenicity, despite the higher androgenicity that is also associated with 1st and 2nd generation pills. Our study is the first to assess all 3 biological effects simultaneously, allowing their independent effects on spatial ability to be identified. Our findings demonstrate the relevance of estrogen and converge with similar conclusions by two previous studies (35, 38) that used smaller less diverse samples and implicated the estrogen activity of OCs in spatial cognition by utilizing alternative methods. In our dataset, Gen1 OCs were significantly higher in estrogenic activity than the other Gens as demonstrated by objective bioassay tests, which might explain the lower MRT accuracies seen for Gen1 OCs during the active phase of pill ingestion and the larger POC effect in Gen1 including significant 'bounceback' in MRT performance during the inactive phase.

An effect on the MRT that is mediated by estrogen is plausible given similar effects reported for 17β-estradiol in naturally-cycling women (e.g. 18, 20, 21). Little is known of ethinyl estradiol's sites of action in the CNS, but ERα and ERβ are expressed regionally in several regions of the CNS that are important in cognitive functioning (14, 86, 87) and EE2 is able to bind to intracellular ERs. In fact, EE2 displays an affinity for the ERα receptor even greater than the endogenous ligand, 17β-estradiol (88). Following oral ingestion of an active OC pill, serum concentrations of EE2 peak approximately 1-2 hrs later and may transiently reach mid-follicular values before declining to early follicular levels until the next pill is taken 24-hr later (82, 83). EE2 concentrations seen at peak vary depending on the EE2 content of the OC pill that is taken (52, 82) and on individual differences in absorption and metabolism (7; but see 8). Although the peak concentration is not sustained for long and typically drops to low basal levels within just a couple of hours, genomic effects initiated by the initial EE2 availability in the bloodstream might be longer-lasting. The time course of the effects initiated is not presently known. The natural ligand, 17β-estradiol, has been shown in laboratory animals to exert a range of effects on neurotransmitter synthesis, release, and metabolism (14) through binding to estrogen response elements at acceptor sites on the nuclear DNA and influencing the transcription of target genes. Estradiol also exerts rapid membrane-associated

effects in neurons, as well as effects on synaptic architecture in responsive brain regions (15). It remains to be confirmed if the synthetic estrogen EE2 has similar effects at the neuronal level. Recent functional imaging studies have reported differences in regional brain activity and connectivity in OC users compared with women not using OCs (11), suggesting a potential for cognitive/behavioral outcomes to be affected by OC use, but few studies have looked at outcomes in terms of overt behavioral changes that might be relevant to women's actual day-to-day functioning. Not all OCs may be equally capable of exerting CNS effects. Average estrogen doses have been lowered from 150ug in 1960 to as little as 10-15 ug/day today. It is possible that cognitive effects only occur in conjunction with OCs having high enough doses of EE2 to produce an impact on the brain circuitry that underlies cognitive function.

The present report has limitations, but also several strengths. We used prospective recruitment and assignment to conditions in Study 1. Studies 2 and 3 relied on retrospective data. As such, the present study cannot conclusively establish causation because our design is purely observational. Accordingly, a prospective placebo-controlled design would be desirable in a future study. A placebo control is difficult, however, where contraception is the topic of investigation due to ethical issues surrounding assignment of fertile women to a placebo condition. Although prone to practice effects, future work on cognitive function might also consider using a within-subjects design where cognition is evaluated in OC users before and after the onset of treatment. To capture changes in OC formulations over a 30-year time window, as in Study 2 or 3, a between-subjects design inevitably must be used. Strengths of the present work include the broad temporal scale it covers; use of a consistent, trusted and standardized spatial test; and data collection that was performed in parallel for both the active and inactive phases of the cycle in all 'eras' covered by our 30-year window. Although societal changes occurred over this interval, differences in average scores on the MRT for the progestin families were seen only at the active (not the inactive) phase of the OC cycle. Steady levels of accuracy at the inactive phase over the 30 years of data collection argues against societal changes in educational practices, gendered play, or unknown social changes as explanations for the family-wise differences we observed. The fact that we found better not worse performance on the MRT during the inactive (menstrual) phase of the cycle would similarly argue against negative stereotypes related to menstruation as the basis for a POC effect.

6 Conclusions

The present study reinforces and further validates studies of NC women reporting an effect of estradiol on spatial functions. Our data suggest generalizability to ethinyl estradiol, the synthetic form of estradiol used in nearly all current OCs. Lower estrogen potency was associated with better spatial performance. Effects tended to be modest in size and are not of clinical concern. These findings advance our emerging understanding of OC effects in the human CNS and at a broader level the associations between reproductive steroids more generally and female brain function. Knowledge of such effects can help promote more informed decision-making on the part of OC users and their healthcare providers. Our use of an endocrine coding scheme that reflects the biological effectiveness of OCs *in vivo* and not just face dosage can be applied in future research studies, in order to promote a deeper and more accurate analysis of OC effects in behavioral investigations.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Non-Medical Research Ethics Board, University of Western Ontario, London, Ontario, Canada. The patients/participants provided their written informed consent to participate.

Author contributions

EH was responsible for study conception, study design, and data interpretation. KE contributed to study design, data acquisition, and preliminary scoring. EM contributed to data acquisition and scoring. CF contributed to data compilation, data checking, and literature search. Statistical analysis was performed by EH, who wrote the first draft of the manuscript. All authors had an opportunity to contribute to the interpretation of analyses and manuscript revision, and have approved the final version. EH was responsible for funding acquisition and resources, and for student supervision. All authors contributed to the article and approved the submitted version.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.888510/full#supplementary-material

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The attention-emotion interaction in healthy female participants on oral contraceptives during 1-week escitalopram intake

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Previous findings in healthy humans suggest that selective serotonin reuptake inhibitors (SSRIs) modulate emotional processing *via* earlier changes in attention. However, many previous studies have provided inconsistent findings. One possible reason for such inconsistencies is that these studies did not control for the influence of either sex or sex hormone fluctuations. To address this inconsistency, we administered 20 mg escitalopram or placebo for seven consecutive days in a randomized, double-blind, placebo-controlled design to sixty healthy female participants with a minimum of 3 months oral contraceptive (OC) intake. Participants performed a modified version of an emotional flanker task before drug administration, after a single dose, after 1 week of SSRI intake, and after a 1-month wash-out period. Supported by Bayesian analyses, our results do not suggest a modulatory effect of escitalopram on behavioral measures of early attentional-emotional interaction in female individuals with regular OC use. While the specific conditions of our task may be a contributing factor, it is also possible that a practice effect in a healthy sample may mask the effects of escitalopram on the attentional-emotional interplay. Consequently, 1 week of escitalopram administration may not modulate attention toward negative emotional

distractors outside the focus of attention in healthy female participants taking OCs. While further research in naturally cycling females and patient samples is needed, our results represent a valuable contribution toward the preclinical investigation of antidepressant treatment.

KEYWORDS

selective serotonin reuptake inhibitors, attention-emotion interaction, oral hormonal contraceptives, female mental health, emotional flanker task

Introduction

Selective serotonin reuptake inhibitors (SSRIs) are the first line pharmacological treatment for major depressive and anxiety disorders (Cleare et al., 2015; Hieronymus et al., 2018). While the key molecular mechanisms of SSRI action are relatively well understood, with occupancy of the serotonin transporter (Artigas et al., 2002), resulting in an upregulation of serotonin neurotransmission, the specific mechanism by which SSRIs improve mood still remains unclear. Moreover, though upregulation of serotonin neurotransmission occurs within a relatively short timeframe, clinically relevant changes in mood often take up to several weeks to manifest (Frazer and Benmansour, 2002). Recent findings in healthy participants (Harmer et al., 2004, 2011) and depressed patients (Harmer et al., 2009b; Roiser et al., 2012) have shed some light on this apparent temporal inconsistency by suggesting that SSRIs may modulate implicit emotional and social processing via earlier changes in selective attention (Harmer et al., 2009a). Critically, these attentional changes are hypothesized to *precede* improvements in mood via modulated processing of emotional stimuli and other downstream neuroadaptive effects (Harmer et al., 2017). In particular, evidence suggests that antidepressant action may be attributed to early changes in biased orienting of attention to aversive stimuli, a process that rebalance negative affective bias by increasing the relative recognition of positive over negative stimuli. On this account, the proposed delay is largely mediated by translation of changes in emotional bias to improved mood. While this model is promising, findings are inconsistent and remains to be fully evaluated.

Several lines of evidence have provided support for this attentional-emotional interplay. For example, evidence from neuroimaging studies indicates an SSRI-induced alteration in limbic blood oxygenation level dependent (BOLD) activation in response to emotional stimuli in healthy participants, showing a decrease of activation toward aversive stimuli (Harmer et al., 2006; Murphy et al., 2009a; Sladky et al., 2015), and an increase in response to positive stimuli (Norbury et al., 2009; Outhred et al., 2014). More recent research has presented evidence that a single 20 mg dose of escitalopram can blunt neural responses to averse stimuli in health (Lewis et al., 2021). Moreover, attentional networks are modulated by SSRI-administration, specifically, by increasing the engagement of the medial and dorsolateral prefrontal cortices (Fales et al., 2009; Ma, 2015), regions known for facilitating selective attention (Ochsner and Gross, 2005). Additionally, attentional bias training shows changes in emotional processing, measured by reduced amygdala reactivity to aversive information and changes in amygdala-prefrontal cortex connectivity (Cohen et al., 2016). Behavioral findings, however, remain comparatively inconsistent. While some studies have shown altered processing of emotional stimuli following subacute SSRI intake (Harmer et al., 2003, 2004), others have reported contrary outcomes, and did not find an effect of SSRI-administration on processing of affective stimuli (Skandali et al., 2018) nor on inhibitory performance and response re-engagement (Drueke et al., 2010) in healthy humans. Browning et al. (2007b), for example, reported that acute administration of 20 mg citalopram may draw participants attention toward positive words, but also enhances recognition of anxiety-related stimuli in healthy volunteers. Additionally, further findings also show that 7 days of 20 mg citalopram induces a decrease in memory for negative information in health (Browning et al., 2011). Another study, however, support the reversing effect of SSRIs on cognitive biases, showing that 1 week of 20 mg citalopram reduces attentional orienting to threatening stimuli, in contrast to reboxetine, a selective noradrenergic reuptake inhibitor (Murphy et al., 2009b). Rose et al. (2006) reported that 7 days administration of 10 mg escitalopram did not have a significant influence on cognitive flexibility, auditory selective attention, verbal learning and recall. Consequently, findings in healthy subjects have been inconclusive, thus presenting a significant barrier toward translating these results to patient samples.

These mixed findings may be a result of varying study designs or other overlooked contributing factors, such as small sample sizes, statistical power, selection bias, and the various distinct methodological approaches between studies. One largely overlooked variable, however, for example is sex. In a systematic review of 51 placebo-controlled trials in healthy participants (Knorr et al., 2019), many studies exhibited unequal sex distribution (62% male and 38% female) and the reported findings were thus largely contradictory. This disparity in sex among participants may be a significant contributing factor, given that sex, sex hormones, and the menstrual cycle are

known to modulate both serotonergic signaling (Barth et al., 2015) and SSRI responsivity (LeGates et al., 2019). Moreover, menstrual cycle phase influences the recognition of emotional stimuli (Derntl et al., 2008; Gasbarri et al., 2008; Guapo et al., 2009), state of arousal (Goldstein et al., 2005), and attention (Pilarczyk et al., 2019). Therefore, it is necessary to control for these potential modulating factors, for example by choosing just female participants with regular use of oral contraceptives (OCs). Given that no study to date has taken this approach, it is not clear whether the hypothesis of an SSRI induced modulation of earlier attentional processing is reproducible in a well-powered, healthy female sample with regular OC intake.

The aim of the current study, therefore, is to test whether 7 days of SSRI administration modulates early attentional orienting during emotional processing in healthy female individuals using OCs. In so choosing a healthy sample, we aimed to refine applicability of this hypothesis of SSRI action (Harmer and Cowen, 2013) to an overlooked, yet substantial demographic of SSRI users, with a view toward enhancing applicability to patient samples. To assess the effects of SSRI intake on the interaction between attentional and emotional processing, we administered 20 mg of escitalopram or placebo for 1 week (with an additional assessment after a 1-month wash-out period). We chose 20 mg escitalopram due to its robust blockade of up to 80% of the serotonin transporter (Klein et al., 2006), relatively fast onset of action (Sanchez et al., 2014), higher clinical efficacy (Garnock-Jones and McCormack, 2010), and higher tolerability relative to other common SSRIs (Cipriani et al., 2009). Participants performed a modified version of the emotional flanker task (mEFT) at four time points: before drug administration, after a single dose, after 7 days of SSRI intake, and after a 1-month wash-out period. Unlike previously used paradigms (Harmer et al., 2003; Drueke et al., 2009), the mEFT simultaneously combines attentional and emotional information (Lichtenstein-Vidne et al., 2012). This combination of 1-week of escitalopram intake and our tailored task conditions, therefore, was specifically designed to investigate *early* effects of SSRI intake on the attentional-emotional interplay in an underrepresented sample, namely, female participants with regular OC use. We hypothesized that 1-week escitalopram intake would reduce focus on negative distractor stimuli during task performance, resulting in enhanced selective attention as indicated by disengaging from negative distractors and reduced RT in case of negative distractors, compared to placebo.

Materials and methods

Participants

Participants were recruited from the general public and the local database of the Max Planck Institute for Human Cognitive and Brain Sciences. Eighty-eight participants were rigorously screened, and exclusions were made based on tobacco use, other medication use, presence or history of neurological or psychological disorders, body-mass index (BMI) outside the range of 18.5–25 kg/m^2, alcohol abuse, or drug abuse. We screened for psychiatric and neurological health using the Structured Clinical Interview for DSM-IV Axis I Disorders (non-patient version) (SCID-I) (First, 2002), the Hamilton Depression Rating Scale (Hamilton, 1960), the Revised NEO Personality Inventory (McCrae et al., 2005) and the Mood Spectrum Self–Report Measure (Dell'Osso et al., 2002). Electrocardiogram (ECG) recordings were used to screen for abnormal QT times. All participants were female, taking estrogen- and progesterone-combined OCs to downregulate pulse frequency of gonadotropin releasing hormone, for at least 3 months prior to participation. By suppressing levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH), OC-use thus prevents follicular development and ovulation (Bastianelli et al., 2018) thereby controlling for sex hormone fluctuations during natural menstrual cycle (Mihm et al., 2011) that may modulate SSRI responsivity (LeGates et al., 2019). In addition, we strictly limited assessments to the pill-interval to control for potential hormonal effects during the pill-free interval. Participants were between 18 and 35 years of age. Seventy-one participants were enrolled, of whom 65 completed the assessment week. Six participants voluntarily discontinued participation during the assessment week. Additionally, two (placebo $n = 2$) participants did not complete a follow-up assessment. Of the 63 participants who completed the study, three were excluded due to QC concerns (**Figure 1**). All participants were monitored by a physician during the study, provided written informed consent prior to enrollment, and received financial compensation. We obtained ethical approval from the Ethics Committee of the Faculty of Medicine at Leipzig University (approval number 390/16-ek) and conducted all study procedures in accordance with the Declaration of Helsinki of 2013.

Study design

We administered 20 mg of escitalopram ($n = 29$) or placebo ($n = 31$) to healthy female participants for 7 days. During the administration week, we assessed behavioral responses to an emotional distractor task. Task performance was initially assessed at baseline, prior to escitalopram or placebo intake. Following the baseline measurement, participants were randomly assigned to receive either escitalopram or placebo using a 1:1 allocation method. Both the participant and the experimenter were blind to condition allocation. Behavioral performances were subsequently assessed after a single dose (Day 1), and again following the third assessment, which took place after 7 days (Day 7). Escitalopram and placebo intake

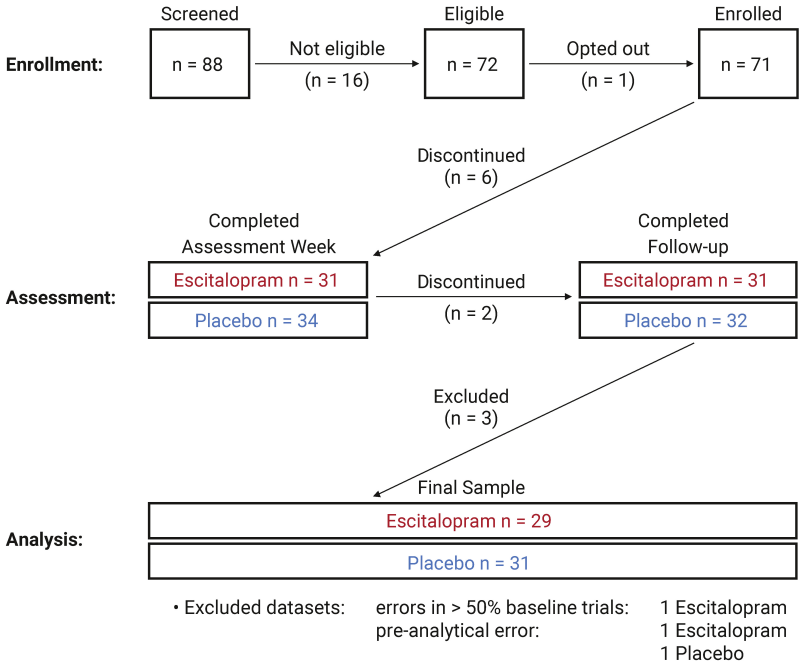

FIGURE 1
Inclusions and exclusions: The figure depicts the number of participants included in each step of enrollment, assessment, and analysis. Due to self-reported side effects, 6 participants voluntarily discontinued participation during the assessment week (placebo = 2, escitalopram = 4). Two participants did not return for the follow-up assessment. Three participants (placebo = 1, escitalopram = 2) were excluded following implementation of quality control measures.

occurred at fixed times each day. All participants returned for a follow-up assessment following a 4–6-week follow-up, in which no escitalopram or placebo was administered. ECG recordings were conducted at Days 1, 4, 7, and follow-up, to monitor QT intervals. Adverse reactions to escitalopram were recorded using the antidepressant side-effects checklist (ASEC) (Uher et al., 2009). Changes in mood and anxiety were recorded with different psychological inventories: the POMS (German language version of the profile of mood spectrum) (McNair et al., 1981), a validated (Gibson, 1997) self-report measure assessing various mood status, the DAS (Dysfunctional attitude scale) (Weissman and Beck, 1978), a validated (Weissman, 1979; de Graaf et al., 2009) questionnaire to assess negative attitudes and cognitive vulnerability and the STAI (State trait anxiety index) (Spielberger et al., 1983) validated (Guillén-Riquelme and Buela-Casal, 2014) measuring both state and trait anxiety, transient reactions in specific situations, and specific attributes of personality.

Task procedure and primary outcome measure

We assessed emotional and attentional responses using a mEFT (Lichtenstein-Vidne et al., 2012), implemented in EPrime 2.0 professional (Stahl, 2006) running on the Windows XP operating system. Visual task stimuli were obtained from the International Affective Picture System (IAPS; Bradley et al., 2001). The experiment contained 3 blocks of 32 trials each, resulting in 96 trials. Participants were instructed to indicate whether a target picture appeared above or below a fixation cross, via a keypress, while ignoring flanker images. In each trial, peripheral distracting pictures contained negative or neutral emotional valences while targets were either negative or positive. Participants were asked to respond as quickly and accurately as possible. The outcome measure was reaction time (RT), measured in milliseconds (ms), and calculated as the difference between the time of target onset and participant response. Three independent within-subject variables were manipulated: flanker location congruency with the target picture (congruent, incongruent), flanker valence (negative, neutral), and target valence (negative, positive) (**Figure 2**).

Data analysis

Demographics and mood assessment

Statistical analyses were conducted with the Statistical Package for the Social Sciences (SPSS, v24). Peripheral plasma escitalopram levels were quantified by high-performance chromatography using quality control (QC) sample (Teichert et al., 2020). Moreover, we measured plasma levels of FSH

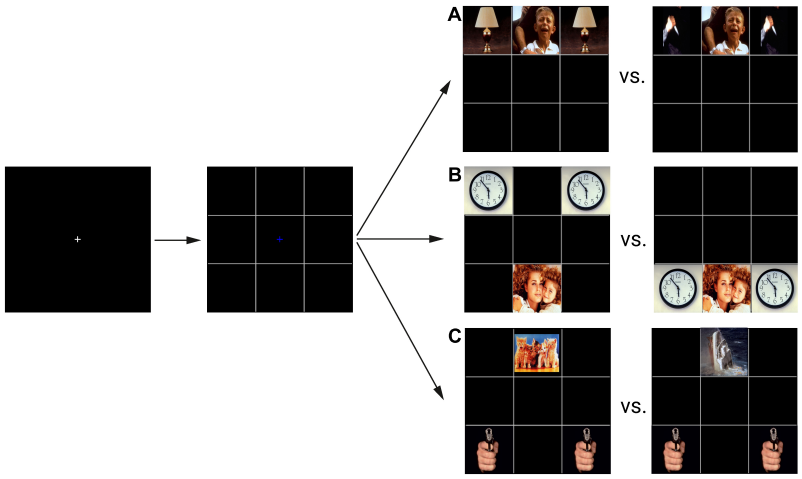

FIGURE 2
Visual stimulus of the emotional distractor task. Depicted here is an example of a typical trial. Participants view a fixation cross which is then replaced by a second fixation cross in a 9-panel grid. Both the target picture and the distractors subsequently appear, prompting participants to indicate the target picture location, while ignoring distracting flanker pictures. The different conditions of this panel are shown: flankers were neutral or negative **(A)** and incongruent or congruent to the target pictures **(B)**; targets were either positively or negatively valenced **(C)**. Target location was either above or below the previously shown fixation cross. Ms, milliseconds. Images are from The International Affective Picture System (IAPS).

and LH to assess regular OC-induced suppression of these hormones. We used independent sample *t*-tests to assess potential group differences in age, BMI, and downregulated hormonal profiles. ASEC scores at both single dose and steady state were also assessed with independent sample *t*-tests. Potential changes in mood and anxiety, as recorded by the DAS, STAI, and POMS were each assessed separately using a 2×3 analysis of variance (ANOVA) with *group* and *time* as factors. Results of mood and anxiety analyses were considered statistically significant at a Bonferroni-corrected α-level of $p < 0.016$ to account for multiple testing (0.05/3).

Data preprocessing and quality control

We preprocessed all data using EPrime (version 2.0). Each assessment contained 10 practice trials that were excluded during pre-processing. To identify outlier trials, we calculated the mean and standard deviation for each trial. Individual outlier trials were removed with SPSS, using a cutoff of $\pm$ 2.5 standard deviations from the mean (2% of all trials for all participants). Additionally, only trials in which a correct response was given were included (98% of all trials of all participants).

Validation of task performance

Prior to analyses comparing SSRI and placebo conditions, we performed a control analysis to confirm that participants understood task instructions. To this end, we assessed performance at baseline in all participants who completed this measurement ($n = 71$) and passed data QC, without reference to group allocation or to further exclusion. In line with previous studies with this task (Lichtenstein-Vidne et al., 2012), we specified an ANOVA to test for a main effect of congruency, a main effect of flanker valence, and for an interaction between congruency and target valence.

Analysis of attentional and valence-dependent task performance

To test our main hypothesis, we employed a five-way repeated measures mixed model analysis of variance (ANOVA) in SPSS. Here, we specified 5 independent variables; *group* (escitalopram, placebo), *time* (baseline, Day 1, Day 7, Follow-up), *target valence* (positive, negative), *flanker valence* (negative, neutral), and *congruency* (congruent, incongruent). We specified *group* as the between subjects factor, *time* as the within subjects factor and RT in each of the target, flanker valence, and congruency conditions as the dependent variable.

Correlation between peripheral plasma escitalopram levels and modified version of the emotional flanker task performance

We tested potential correlations between peripheral measures of plasma escitalopram and behavioral performance using a bivariate Pearson's correlation implemented in SPSS. Here we correlated peripheral plasma levels acquired at day 7 of escitalopram intake with the mean performance in task performance during each of the valence and congruency conditions in the escitalopram group only ($n = 29$). We employed 6 models to assess a potential association between (i) congruency (congruent and incongruent RT), (ii) flanker valence (Negative and Neutral RT) and (iii) target valence

conditions (Negative and Positive RT). Results were considered statistically significant at a Bonferroni corrected threshold of $p < 0.008$ (0.05/6) to account for multiple testing.

Bayesian analysis

Finally, we employed a Bayesian estimation to assess the likelihood of the null hypothesis in each task condition. To this end, we used JASP (v0.12.2—JASP Team, 2020) to implement a Bayesian repeated measures ANOVA for each condition in all cognitive and valence specific analyses [i.e., one Bayesian approach for each of the congruency (*congruent/incongruent)*, target valence (*negative/positive*), and flanker valence (*negative/neutral*) conditions], resulting in 6 Bayesian estimations in total.

Results

Demographics

Analyses of demographic variables yielded no significant group differences on any baseline control measures. Group comparisons of time between final escitalopram or placebo intake and follow-up measurement also indicated no significant differences. Escitalopram levels were consistent with previously reported data (Rao, 2007). Analyses of ASEC scores indicated a significant group difference in mean self-reported side effects at single dose ($t = -3.389$, $p = 0.001$) but not at steady state ($t = -0.675$, $p = 0.502$). Ovulation inhibition was confirmed as suppressed via gonadotropins measurement (Goldzieher et al., 1970; **Table 1**). Estrogen- and progesterone-combined hormonal contraceptives taken by the participants are listed in **Table 2**.

Mood and anxiety monitoring

We did not observe group differences on measures of either (i) state anxiety [time: $F(1, 55) = 1.939$, $p = 0.154$, partial eta$^2 = 0.066$; time by group: $F(1, 55) = 2.941$; $p = 0.061$; partial eta$^2 = 0.097$; group: $F(1, 1) = 0.665$, $p = 0.418$, partial eta$^2 = 0.012$], or (ii) mood [DAS: time: $F(1, 59) = 2.374$, $p = 0.102$, partial eta$^2 = 0.074$; time by group: $F(1, 59) = 1.024$, $p = 0.365$, partial eta$^2 = 0.034$; group: $F(1, 1) = 0.812$, $p = 0.371$; partial eta$^2 = 0.013$]; POMs [time: $F(1, 60) = 7.207$, $p = 0.002$, partial eta$^2 = 0.194$; time by group: $F(1, 60) = 1.145$, $p = 0.325$, partial eta$^2 = 0.037$; group: $F(1, 1) = 0.396$, $p = 0.531$, partial eta$^2 = 0.006$].

Baseline replication analysis

Analysis of baseline ($n = 71$) performance yielded an outcome comparable to previous studies, with a significant main effect for location congruency [$F(1, 70) = 42.306$; $p < 0.001$; partial eta$^2 = 0.377$], represented by faster RTs in congruent vs. incongruent trials. Additionally, results showed a congruency by flanker valence interaction [$F(1, 70) = 4.570$; $p = 0.036$; partial eta$^2 = 0.061$], with slower RTs in congruent trials during negative flankers as compared to the neutral condition. In contrast to previous findings, however, we did not observe a significant effect of distractor valence [$F(1, 70) = 1.758$; $p = 0.189$; partial eta$^2 = 0.024$] nor target valence [$F(1, 70) = 2.942$; $p = 0.091$; partial eta$^2 = 0.040$].

Task performance analysis over time

Five-way analyses of group performance over time

Our results show a significant effect of time [$F(1, 3) = 33.163$; $p < 0.001$; partial eta$^2 = 0.364$], with decreased RTs during performance of each target valence, flanker valence, and congruency condition over the course of the experiment. However, there was no significant group by time effect [$F(1, 3) = 0.128$; $p = 0.943$; partial eta$^2 = 0.002$]. Analysis of a congruency effect show significance [$F(1, 1) = 127.753$; $p < 0.001$; partial eta$^2 = 0.688$], but we did not observe a

TABLE 1 Demographic analysis overview: Baseline and demographic variables across both groups.

Demographics	Escitalopram ($M \pm SD$)	Placebo ($M \pm SD$)	*t*-value	*p*-value
Age (years)	24 ± 3	23 ± 4	0.99	0.33
BMI (kg/m^2)	22 ± 1.7	21 ± 1.7	1.08	0.28
Lutropin (μ/l)	2.0 ± 2.7	1.4 ± 2.0	0.92	0.34
Follitropin (μ/l)	2.9 ± 3.2	2.1 ± 3.0	0.99	0.33
Escitalopram single dose (ng/ml)	20 ± 5	n.d.	–	–
Escitalopram steady state (ng/ml)	46 ± 11	n.d.	–	–
Time to follow-up (days)	33 ± 5	35 ± 7	1.37	0.18

Results show no difference on any baseline or demographic measure, nor on the time in between the completion of the assessment week and the onset of the follow-up measurement. Values refer to mean and standard deviation.
kg/m^2, kilogram force per square meter; u/l, units per liter; ng/ml, nanograms/milliliters.

TABLE 2 Contraceptive usage.

Group	Number of participants	Compound (dose)
Placebo	12	Ethinylestradiol 0.03 mg (dienogest 2 mg)
	4	Ethinylestradiol 0.03 mg (chlormadinonacetat 2 mg)
	1	Ethinylestradiol 0.03 mg (desogestrel 0.15 mg)
	4	Ethinylestradiol 0.02 mg (levonorgestrel 0.1 mg)
	5	Ethinylestradiol 0.03 mg (levonorgestrel 0.15 mg)
	1	Ethinylestradiol 0.02 mg (desogestrel 0.15 mg)
	3	Ethinylestradiol 0.03 mg (levonorgestrel 0.125 mg)
	1	Ethinylestradiol 2.7 mg (etonogestrel 11.7 mg)
Escitalopram	2	Ethinylestradiol 0.02 mg (levonorgestrel 0.1 mg)
	2	Ethinylestradiol 0.03 mg (levonorgestrel 0.15 mg)
	1	Ethinylestradiol 0.02 mg (desogestrel 0.15 mg)
	3	Ethinylestradiol 0.03 mg (chlormadinonacetat 2 mg)
	17	Ethinylestradiol 0.03 mg (dienogest 2 mg)
	1	Ethinylestradiol 0.02 mg (levonorgestrel 0.1 mg)
	1	Ethinylestradiol 0.03 mg (levonorgestrel 0.125 mg)
	1	Ethinylestradiol 0.03 mg (desogestrel 0.15 mg)
	1	Ethinylestradiol 0.02 mg (drospirenon 3 mg)

Listed is an overview of taken hormonal contraceptives from the study participants. All participants used combined contraceptives to inhibit ovulation.

significant group by congruency interaction [$F(1, 1) = 0.071$; $p = 0.791$; partial eta$^2 = 0.001$]. Complimentary analysis of the flanker valence conditions (*negative, neutral*) shows no significant effect [$F(1, 1) = 1.176$; $p = 0.283$; partial eta$^2 = 0.020$] nor an interaction effect with group [$F(1, 1) = 0.007$; $p = 0.932$; partial eta$^2 = 0.000$]. Analysis of target valence conditions (*negative, positive*) yields no significant effect [$F(1, 1) = 3.582$; $p = 0.063$; partial eta$^2 = 0.058$] and no group interaction [$F(1, 1) = 0.386$; $p = 0.537$; partial eta$^2 = 0.007$] (**Figure 3**).

Correlation analyses

Results of a bivariate correlation analyses within the escitalopram group only do not suggest any apparent relationship between peripheral plasma escitalopram levels, acquired at the final day of intake, with behavioral performance in each of the specific task conditions at the seventh day of intake. Across six correlation analyses for each condition within the congruency, flanker valence, and target valence conditions, peripheral plasma escitalopram did not correlate with performance (all $p > 0.008$) (**Figure 4**).

Bayesian analyses

A series of Bayesian repeated measures ANOVAs show moderate to strong evidence in favor of the null hypothesis for each cognitive and emotional behavioral outcome, compared to the alternative hypothesis (**Table 3**). Model comparisons show increasing likelihood in favor of the null hypothesis for each additional model contribution (i.e., of the group and group by time interaction terms). Consistent with frequentist repeated measures analyses, no evidence for the null hypothesis is observed for the *time* factor.

Discussion

In the present study, we assessed whether 1-week administration of escitalopram modulates the attention-emotion interaction in healthy female participants with regular OC use. Using a mEFT, we tested the hypothesis that escitalopram intake would facilitate the disengagement from negatively valenced task distractors (Harmer et al., 2009a). Against our hypothesis, our results do not suggest an effect of escitalopram on emotional and attentional distraction, either after single dose, or after 7 days continuous intake, or at a 1-month follow-up assessment. While we observed a significant improvement in task performance over time with a decrease in RT to task stimuli, there was no observed significant difference in performance between groups. Moreover, Bayesian analyses yield moderate to strong support in favor of the null, relative to the alternative, hypothesis. Consequently, these results do not suggest an effect of 1-week escitalopram intake on selective attention and inhibition of negatively valenced distractors in this sample of healthy female participants on OCs.

One possible reason for our findings may be the specificity of our task demands. Specifically, unlike previous emotional processing tasks, the mEFT combines emotional and attentional domains using peripheral distractors that simultaneously present emotional and spatial information (Lichtenstein-Vidne et al., 2012). Previous studies, however, typically employed tasks that considered these domains individually, such as a facial recognition task (Harmer et al., 2003) or an attentional network task (Drueke et al., 2009). Furthermore, previous findings were mainly obtained using tasks that present distracting emotional information inside the focus of attention, such as the emotional Stroop task and the dot-probe task. For example, Browning et al. (2007a), employed a visual probe task to show an increase in attention to socially relevant positive words after a single dose of citalopram. In another study, Murphy et al. (2009b)

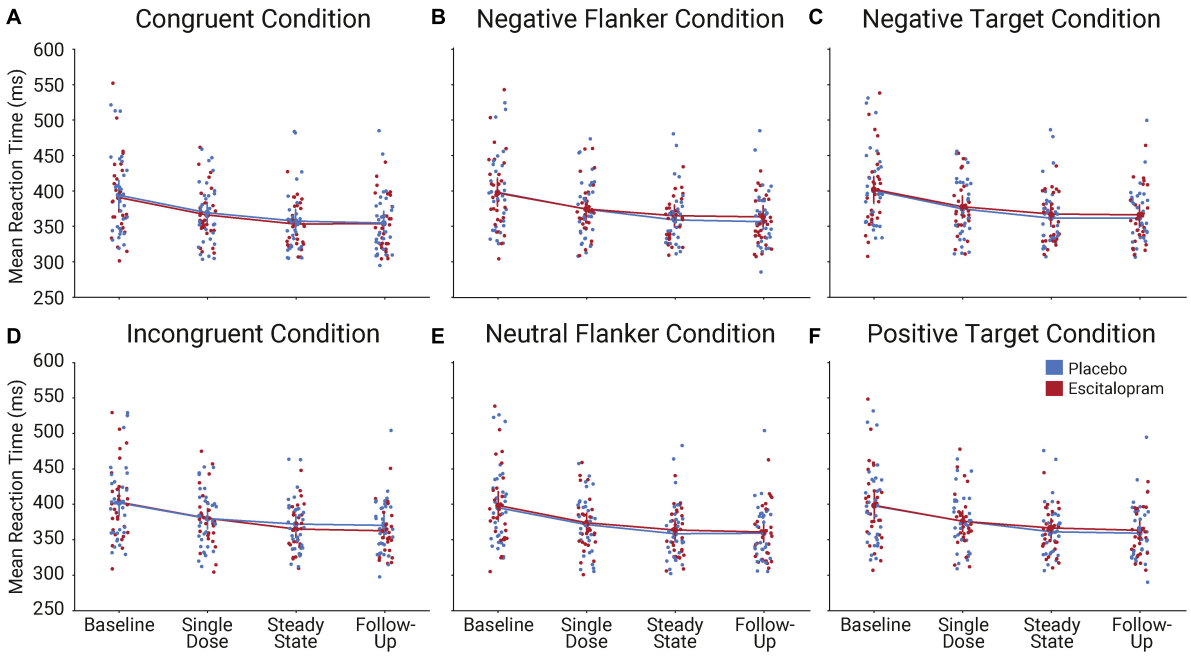

FIGURE 3
Timeline of behavioral performance during 1-week of escitalopram-intake and after a 1-month wash-out period for each task condition: We measured RT [in milliseconds (ms)] performance on the mEFT in both the escitalopram and placebo groups at baseline, single dose, after 1-week of continuous intake, and following a 1-month wash-out period. We assessed performance in each of the **(A)** congruent and **(B)** incongruent conditions, **(C)** the negative and **(D)** neutral flanker conditions, and **(E)** the negative target and **(F)** positive target conditions. While results indicate a significant effect of time, with decreasing RT between the baseline and follow-up assessments, we did not observe any indication of a difference between groups on any measure of performance.

showed reduced attentional orienting to threatening stimuli after 1-week citalopram intake with the use of an attentional probe task. Unlike these paradigms, however, our task presented the distracting emotional information *outside* the focus of attention. Therefore, while previous studies provide evidence for a modulation of an attentional bias toward distracting emotional content that is presented inside the focus of attention, we investigated the effect of escitalopram on an attentional bias to task-irrelevant emotional content, when task settings do not encourage distractors' processing. Investigating such an effect is also of special interest, considering that only a small portion of the visual stimuli in everyday life appear at the visual center (Wandell, 1995). Consequently, our results do not indicate an effect of escitalopram on selective attentional processing of task irrelevant emotional and task relevant spatial information in healthy females on OCs. Our findings are consistent with the concept that emotional processing is not automatic, but highly task and stimuli dependent. Future studies comparing the difference of task-relevant and irrelevant distractors in emotional processing tasks are required, however, to directly assess this possibility.

Another possible reason may be a product of the emotional stimuli presented as part of the mEFT. Evidence suggests that emotional recognition tasks that employ facial stimuli, as used by Ahmed et al. (2021), may be particularly sensitive to changes in emotional processing compared to that of the mEFT, which primarily presents situational stimuli. This interpretation is consistent with other findings that did not suggest an effect of linguistic stimuli (Browning et al., 2019; Ahmed et al., 2021) on emotional processing, stimuli which are thought to have a lower emotional impact relative to pictorial stimuli (Lees et al., 2005). While such illustrations have been described as being less emotionally salient compared to the real-life pictures, presented in our task (Okon-Singer et al., 2013), it is likely that facial stimuli may uniquely stimulate specific brain regions (Britton et al., 2006). In sum, our findings, while not replicating previous findings in healthy volunteers, are consistent with the hypothesis that certain emotional stimuli maybe somewhat specific to certain task demands. Larger studies with a wider array of emotionally salient stimuli are needed to further explore this possibility, however.

Given our longitudinal design and the fact that participants performed the task multiple times, our results may also show a practice or habituation effect. Though we observed no difference in performance between groups, we did observe a significant effect of time with decreased RT, suggesting that all participants responded faster over the course of the experiment. Participants

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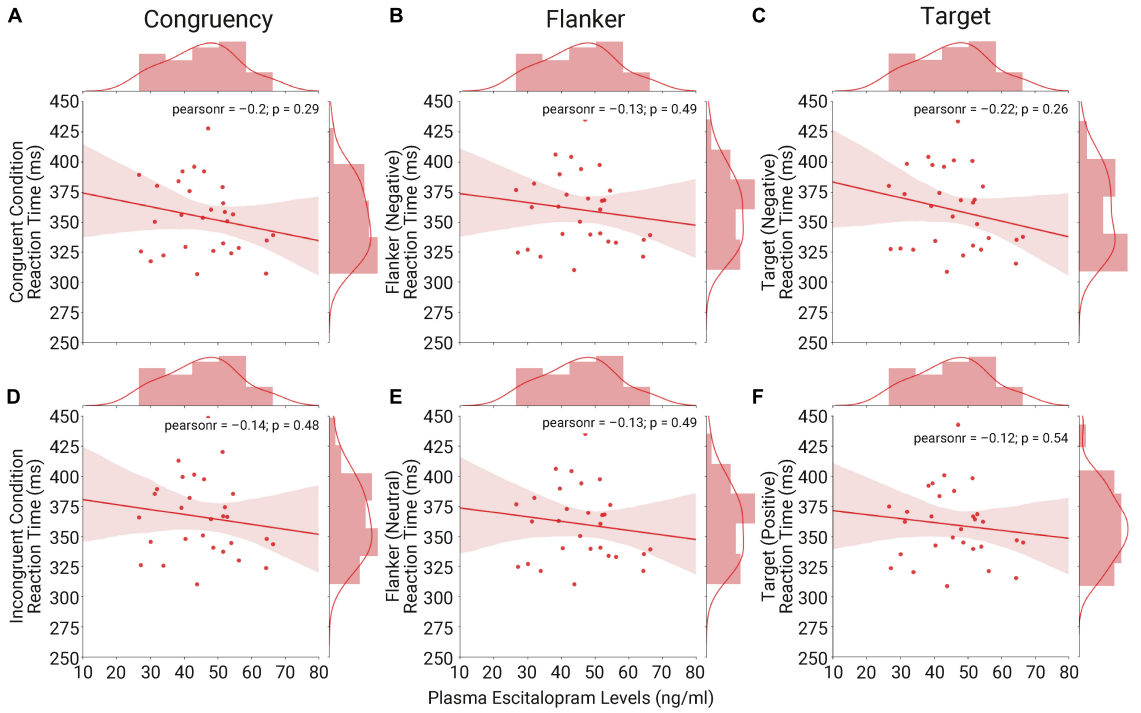

FIGURE 4
Correlation analyses at day seven of escitalopram intake for each task condition: We assessed a potential correlation between peripheral measures of plasma escitalopram and behavioral performance in each task condition category with a bivariate Pearson's correlation. Results for the congruency condition [left column **(A,D)**], flanker condition [middle column **(B,E)**] and target condition [right column **(C,F)**] show no evidence of a correlation. Results considered significant at a Bonferroni-corrected p-value of < 0.008 due to multiple testing.

TABLE 3 Results of Bayesian analysis for each cognitive and valence domain: Repeated measures Bayesian analyses show moderate to strong evidence for the null hypothesis when considering the group and group by time interaction terms for each cognitive and valence-dependent condition.

Congruency	P (M)	BF (M)	BF_{01}	Error (%)	*Incongruency*	P (M)	BF (M)	BF_{01}	Error (%)
Time	0.2	9.42	1	–	Time	0.2	9.57	1	–
Time + group	0.2	1.60	2.4	3.52	Time + group	0.2	1.50	2.58	3.44
Time × group	0.2	0.04	63.5	6.18	Time × group	0.2	0.08	33.09	6.30
Flanker negative					*Flanker neutral*				
Time	0.2	9.45	1	–	Time	0.2	10.20	1	–
Time + group	0.2	1.58	2.47	3.41	Time + group	0.2	1.43	2.72	3.52
Time × group	0.2	0.05	54.52	6.56	Time × group	0.2	0.07	40.54	6.39
Target negative					*Target positive*				
Time	0.2	10.79	1	–	Time	0.2	9.52	1	–
Time + group	0.2	1.38	2.83	3.49	Time + group	0.2	1.54	2.52	3.53
Time × group	0.2	0.05	56.81	6.20	Time × group	0.2	0.06	42.13	6.65

Results indicate the likelihood of the null hypothesis as approximately twice that of the alternative hypothesis for the group factor, and several times that of the alternative hypothesis for the interaction term. P (M), prior model plausibility; BF (M), posterior model odds; BF_{01}, Bayes factor likelihood of the null hypothesis compared to the alternative hypothesis; Error (%), Error computation of Bayes factor.

were asked to respond via a simple button press, making the task relatively easy to perform. With a healthy sample showing no aberrant emotional processing (as measured by our baseline replication analysis), it is possible that participants simply adapted or habituated to the presented stimuli. One possible counterpoint, however, is that the presented stimuli where randomized across each measurement, suggesting that it is likely that different stimuli were presented at each assessment day. However, as this randomization of stimuli was random, we cannot rule out the possibility that similar stimuli were shown

or repeated at different times during the experiment. As a result, the simpler nature of our task in the presence of a healthy sample may have dampened an already small effect of the SSRI, thus leading to our observed outcomes. However, it is also worth mentioning that our task procedure was specifically designed in this less complex manner, given that existing evidence has shown that increased task difficulty may conflict with emotional processing (Pessoa, 2005; Cohen et al., 2011). Regardless, a simple task contributing to habituation and practice effects in a healthy sample present a viable interpretation for our null result, an interpretation which future studies in patients and with more demanding tasks may address.

One final, though admittedly more speculative possibility, is the influence of downregulated endogenous sex hormones via OC use and the associated effects on SSRI responsivity. As we confirmed OC- induced suppression of LH and FSH in comparison to natural cycling women and to control for sex hormone fluctuations during the menstrual cycle (Mihm et al., 2011), it is arguable that this induced downregulation of endogenous sex hormones (Givens et al., 1976) may have dampened, at least in part, the effects of escitalopram on our primary outcome measure, the behavioral response to the mEFT. Evidence in favor of this possibility comes from previous studies showing endogenous estradiol modulates serotonergic transmission, SSRI responsivity and affect (Amin et al., 2005; Michopoulos et al., 2011; Ocampo Rebollar et al., 2017). However, we stress that, as we did not investigate estradiol or an interaction effect of SSRIs, OCs and time on task performance, this interpretation should be taken with great caution. Future interventional studies with multiple groups are required to explore this possibility further.

There are also several limitations to this study that should be considered. First, our results are limited to healthy participants only as we did not include any patients in our sample. This decision was deliberate, however, as we aimed to replicate previous findings in healthy participants and to extend these findings to an underrepresented demographic. While future studies in clinical populations are crucial, our analyses in healthy female participants with long term OC-use provides a much needed extension of previous preclinical studies. Secondly, we cannot exclude that a longer drug administration duration would have resulted in differential effects given that, in patients, antidepressants often take up to 3–4 weeks to exhibit clinically relevant changes in mood (Frazer and Benmansour, 2002). Nevertheless, we again explicitly chose this time frame of administration in order to test our hypothesis of *earlier* alterations in attentional processing in response to SSRIs, which is hypothesized to *precede* the changes in mood often seen at later stages of administration (Harmer and Cowen, 2013). Thirdly, the interpretation of our findings may be limited to escitalopram and thus, we cannot make any comments regarding the potentially differential effects of other SSRIs as escitalopram, unlike other common SSRIs such as paroxetine or fluoxetine, exhibits a unique allosteric binding affinity for the serotonin transporter (Klein et al., 2006). As a result, future studies are needed to assess whether our findings extend to a class-effect or are specific to escitalopram at this specific dose. Fourth, we acknowledge that more experimental groups, in which naturally cycling females are investigated with their endogenous sex hormone fluctuations, would be necessary to clarify whether OC-use, specifically, contributed to our findings. As such, we can only speculate, with great caution, that this is a contributing factor. Future studies with different SSRIs, varying doses, hormonal measures and administration regimes (Frye, 2006), and multiple groups that are specifically designed to assess this possibility, are necessary in order to further discuss this possibility. Finally, our analyses are limited to behavioral outcomes only, which differs to previous studies that assessed neural responses during combined SSRI intake and task performance (Harmer et al., 2006; Murphy et al., 2009a; Norbury et al., 2009). In contrast, we did not assess neural responses to escitalopram during mEFT performance. Future studies employing similar samples (and patient populations) should also consider functional neuroimaging to investigate regional and global effects of escitalopram intake at the neural level during attentional and emotional processing.

In conclusion, our results do not indicate an effect of 7 days escitalopram intake on attentional-emotional interaction in healthy female participants taking OCs. While these outcomes may be a result of the specific task requirements of the modified emotional flanker task, or of our chosen sample of healthy volunteers, another possible explanation may be practice effects resulting from simple task requirements. Nevertheless, our findings provide much needed data on a highly relevant, yet underrepresented sample, thus making a critical contribution toward refining the attention-emotion cognitive model of antidepressant action in healthy volunteers. As a result, these results provide a solid platform for studies in patients, with the ultimate goal of improving personalized treatment for depression.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics committee of the medical faculty of the University of Leipzig. The patients/participants provided their written informed consent to participate in this study.

Author contributions

NB, EM, RZ, and TR contributed to data analysis. NB, EM, RZ, TR, JS, and HO-S contributed on manuscript writing process. NB, EM, RZ, JS, HO-S, FP, US, TR, GZ, RR, and AV contributed on reviewing the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Duration of oral contraceptive use relates to cognitive performance and brain activation in current and past users

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Previous studies indicate effects of oral contraceptive (OC) use on spatial and verbal cognition. However, a better understanding of the OC effects is still needed, including the differential effects of androgenic or anti-androgenic OC use and whether the possible impact persists beyond the OC use. We aim to investigate the associations of OC use duration with spatial and verbal cognition, differentiating between androgenic and anti-androgenic OC. Using functional magnetic resonance imaging (MRI), we scanned a group of 94 past and current OC-users in a single session. We grouped current OC users (N=53) and past OC users with a natural cycle (N=41) into androgenic and anti-androgenic user. Effects of OC use duration were observed for current use and after discontinuation. Duration of OC use was reflected only in verbal fluency performance but not navigation: The longer the current OC use, the less words were produced in the verbal fluency task. During navigation, deactivation in the caudate and postcentral gyrus was duration-dependent in current androgenic OC users. Only during the verbal fluency task, duration of previous OC use affects several brain parameters, including activation of the left putamen and connectivity between right-hemispheric language areas (i.e., right inferior frontal gyrus and right angular gyrus). The results regarding performance and brain activation point towards stronger organizational effects of OCs on verbal rather than spatial processing. Irrespective of the task, a duration-dependent connectivity between the hippocampus and various occipital areas was observed. This could suggest a shift in strategy or processing style with long-term contraceptive use during navigation/verbal fluency. The current findings suggest a key role of the progestogenic component of OCs in both tasks. The influence of OC use on verbal fluency remains even after discontinuation which further points out the importance of future studies on OC effects and their reversibility.

KEYWORDS

oral contraceptives (OC), navigation, verbal fluency, brain activation and connectivity, androgenicity, duration of OC use, sex hormones, progestins

Introduction

It is well documented that endogenous ovarian hormones affect women's brain structure, function and connectivity, resulting in either the maintenance or alteration of various cognitive and emotional functions in fluctuating hormonal milieus (1–3). In particular, the hippocampus and amygdala, as well as the basal ganglia and pre-frontal cortex appear to be particularly sensitive to the effects of endogenous ovarian hormones. For example, it has been repeatedly demonstrated, that the volume (4–6), activation (7) and connectivity (5, 7) of the hippocampus are increased during phases of high estrogen. Vice versa, fronto-striatal networks appear to be sensitive to progesterone, since increased basal ganglia volumes (6; Pletzer et al., 2018), fronto-striatal activation (7) and alterations in connectivity of caudate, putamen and dorsolateral prefrontal cortex (DLPFC) (7–10) have been repeatedly demonstrated. While the effects on the hippocampus and frontal cortex are plausible due to their high density in sex hormone receptors (11–15), the effects by which sex hormones affect the basal ganglia are less understood. However, it has been well documented that sex hormones affect a variety of neurotransmitter systems, including dopaminergic transmission (16).

Combined oral contraceptives (OCs) contain synthetic analogs of endogenous ovarian hormones, including a synthetic estrogen, mostly ethinyl-estradiol, and one of various synthetic progestins. These hormones exert their contraceptive actions *via* activation of the estrogen and progesterone receptors. Accordingly, it is plausible that OCs also influence brain structure, function and connectivity (17–20). Nevertheless, our knowledge about the effects of OCs on the female brain and behavior is still limited. The majority of studies have compared OC users to non-users (20) and a very small number of prospective studies have reported short-term effects of contraceptive use on the brain (21–24). Likewise, potential long-term effects are not well understood and it is still unknown if possible neurocognitive effects are reversible. While the effects of estradiol and progesterone along the female menstrual cycle are short-lived, OC intake usually spans 21-24 days, interrupted by a 4-7 day withdrawal period. Furthermore, synthetic steroids have a higher potency and longer half-life than endogenous hormones (25). It is thus possible that effects accumulate over different treatment cycles. On the other hand, endogenous steroid hormones are suppressed by oral contraceptives (26). Additionally, increased sex hormone-binding globulin levels were found in current OC users compared to never-users (27). In this scenario, it is still unclear which mechanism might be responsible for cognitive changes. We expect our results to be due to a combination of the endogenous suppression and the synthetic hormone intake by OC use.

This is especially of interest since millions of women worldwide take oral contraceptives for extended time periods, spanning years if not decades during their reproductive period. So far, the effects of OCs on brain and behavior are inconsistent and fragmentary. Some studies found an association between OC use and impaired cognitive performance (28), other studies found an improve in performance (29, 30) and some do not find any relationship between OC intake and cognitive performance (31, 32). Furthermore, the reversibility of potential cognitive effects is still unknown. This lack of knowledge raises concerns and questions regarding possible cognitive effects which are unintended side effects of the pill. The lacking evidence of possible OC effects may cause great uncertainty for women starting or using OCs (33). It is, therefore, important to gain a better understanding of the OC effects on the female brain.

One approach to study the long-term effects of OC treatment retrospectively is to focus on the effects of OC treatment duration. While studies using this approach are correlational and cannot make causal inferences, they may generate hypotheses for more costly and time-consuming prospective studies. So far, only a few studies have investigated the effects of OC use duration on brain and cognition (34–36). One study on past users of hormonal contraceptives found an association between longer pill intake and better performance during a speed and flexibility task (34). The authors also observed a tendency of better visuospatial abilities with longer intake duration (34). Another study investigating the impact of pill duration in naturally cycling women found duration of previous OC use positively correlated to gray matter volumes of the hippocampus and basal ganglia (7). When controlling for the time since OC discontinuation, the hippocampal volume effects disappeared but not the effects on the basal ganglia. Both studies question the immediate reversibility of OC effects on brain structure and behavior, but hint at potential neuroprotective effects of OC even after discontinuation of OC use. In current OC users, gray matter volume of bilateral fusiform gyri, hippocampus, parahippocampus, middle frontal gyri and anterior cingulate cortex was found to relate positively to pill duration (35). This effect was dependent on another property of synthetic progestins, their androgenicity, i.e., their actions at the androgen receptor.

Oral contraceptives of older generations exert androgenic actions because they are derived from 19-nortestosterone and, therefore, have a higher binding affinity to androgen receptors compared to newer progestins derived from spironolactone or progesterone (37). These newer progestins (e.g., chlormadinone acetate) bind specifically to progesterone receptors which lead to anti-androgenic actions (37). Unfortunately, only few studies of OC actions on the brain have accounted for the different properties of different progestins. However, given that androgens are responsible for a variety of organizational and activational effects on brain structure and function, agonistic vs. antagonistic actions of progestins at the androgen receptor may result in opposing effects.

Spatial and to a lesser extent also verbal functions have been particularly extensively studied with regards to testosterone actions, since the robust sex differences in these functions make them a likely candidate for organizational sex hormone actions (38, 39). Interestingly, neuroimaging studies focusing on the differential processing patterns underlying these sex differences, found the inferior frontal gyrus (IFG) differentially activated in men and women during both, verbal and spatial processing (40, 41). Results regarding the activational effects of testosterone during adulthood are more controversial (42), but point to an improvement of spatial and decline of verbal functions with higher circulating testosterone levels (though an inverted U-shaped relationship has also been discussed). For example, several studies demonstrated better navigation performance in participants with higher circulating testosterone levels (Burkitt et al., 2007; Müller et al., 2016; but see Nowak et al., 2014). Vice versa, higher levels of testosterone were associated with reduced word production during a verbal fluency task (43) and verbal recall (44) in women (but see 45, 46). Most convincing evidence comes from studies using testosterone administration. Administration of one dose of 0.5 mg sublingual testosterone already improved performance during a mental rotation task compared to a placebo (47) and increased medial temporal lobe activation during successful virtual navigation (47). Conversely, half a year of testosterone treatment in postmenopausal women led to decreased left IFG activation during a verbal fluency task, though no change in task performance (48). Accordingly, androgen administration appears to alter verbal and spatial processing in women. It is thus plausible that progestins with androgenic vs. anti-androgenic properties also have opposing effects on verbal and spatial processing.

Indeed, there is some indication from cross-sectional studies that the use of androgenic OCs is associated with improved spatial abilities (49). Subdividing OC-users in androgenic, less androgenic and anti-androgenic groups revealed improved mental rotation performance in androgenic OC-users compared to anti-androgenic OC users, as well as non-users (49). Another study supported this finding by demonstrating that women taking androgenic OC had an advantage in a mental rotation task compared to non-users (18). In contrast, anti-androgenic OCs seem to be associated with improved verbal fluency performance. Users of anti-androgenic OCs generated significantly more words in a verbal fluency task compared to users of androgenic OCs (50). However, to the best of our knowledge no study has compared the duration effects of androgenic and anti-androgenic OC use on the neural correlates of verbal and spatial processing.

The present study aims to elucidate the effects of OC use duration on spatial and verbal cognition, while controlling for the androgenicity of the progestin component. Estrogenic and progestagenic effects should result in comparable effects across both groups, effects attributable to androgenic vs. anti-androgenic actions should result in opposite effects across groups. However, cross-sectional comparisons present a methodological problem regarding confounding effects. The tolerance for a specific oral contraceptive or the tendencies to side effects leads to the so-called "survivor effect" (51) and hinder cross-sectional comparison interpretations. We, thus, opted for studying those time-dependent associations that may accumulate over OC use duration, while controlling for the androgenicity of OCs. Furthermore, to disentangle immediate effects from effects that persist after discontinuation of OC use, we included both, current OC-users and naturally cycling women with previous OC-use in our study. For the same reasons aforementioned, group comparisons were avoided as well between these two groups since current users differ from previous users with a natural cycle regarding the hormonal status as well as other factors (e.g. relationship status, sexual activity, personality features, socioeconomic status).

To approach the cognitive effects of duration of OC use, we chose a navigation task and a verbal fluency task for which previous neuroimaging data and results regarding sex differences as well as the effects of endogenous sex hormones are available (7, 40) and focused our neuroimaging analyses on brain areas sensitive to estrogenic, progestagenic and/or androgenic actions as described above, i.e. the hippocampus, caudate, DLPFC and IFG. Based on previous results, we hypothesize that longer androgenic pill use is associated with better navigation performance, whereas longer anti-androgenic pill use is linked to better verbal fluency performance. We expect higher activation in hippocampus, caudate, and DLPFC with longer duration of OC use in both tasks irrespective of group and differential effects of androgenic vs. anti-androgenic treatment duration on the IFG.

Methods

Participants

Ninety-four healthy women (53 with current OC use, 41 with past OC use and currently natural cycle) were scanned once during either their menses (past users) or their second or third week of oral contraceptive intake (current users). The participants were subdivided into naturally cycling women with previous androgenic pill use (N=21), previous anti-androgenic pill use (N= 20) and OC users with current androgenic pill intake (N=30) and current anti-androgenic pill intake (N=23). Past OC users had a mean age of 24.61 (SD=3.64) and a mean IQ of 111.17 (SD=7.58) and did not differ significantly in age and IQ between the subgroups of previous androgenic and anti-androgenic users. Current OC users had a mean age of 21.42 (SD=3.28) and a mean IQ of 107.08 (SD=10.62) and differed significantly in age ($t_{(51)}$ = -4.10, $p < 0.0001$), IQ ($t_{(46)}$ = -2.45, $p < 0.05$) and pill duration

($t_{(51)}$ = -2.86, p < 0.01) between androgenic and anti-androgenic pill users (Table 1). In order to control for the age and IQ of the participants, these variables were used as regressors of no interest (or covariables) in subsequent analyses. Past users discontinued OC intake for at least 6 months and provided information about the last three menses start dates to make sure their last natural cycles were regular. We checked if participants complied with the criteria of Fehring etal. (52), i.e., 21 – 35 days of cycle duration with a maximal deviation of 7 days between the cycle lengths. OC users took the pill at least for 6 months.

We categorized past and current OC users into androgenic pill use and anti-androgenic pill intake depending on the OC's progestin. We classified all progestins derived from 19-nortestosterone (except for dienogest) as androgenic because either they or their metabolites (most are rapidly metabolized to Levonorgestrel) demonstrate a binding affinity to the androgen receptor. Progestins derived from spirobolactone or hydroxy-/19-norprogesterone as well as dienogest are classified as anti-androgenic pills. Dienogest is derived from 19-nortestosterone but it exhibits highly selective binding to the progesterone receptor and exerts anti-androgenic activities (53). OC users with levonorgestrel, desogestrel, gestodene, etonogestrel, norelgestromin were counted among androgenic progestins; and dienogest, drospirenone, chlormadinone acetate, cyproterone acetate, nomegestrol acetate as anti-androgenic progestins. Participants had no history of psychological, endocrinological or neurological illness and showed no brain tissue abnormalities. Given that adolescence is a developmental phase, in which the brain seems to be especially sensitive to effects of synthetic steroid hormones (54), we explored the effects of age of first intake in our study. Except for two participants, our sample initiated OC use during adolescence. Since results did not change when those two participants were excluded, we kept the two subjects in our sample.

Ethics statement

The study was approved by the University of Salzburg's ethics committee. Subjects gave their informed written consent to participate in this study. The methods conformed with the Declaration of Helsinki.

Procedure

Before the magnetic resonance imaging (MRI) appointment, a pretest was conducted during which the participants signed the informed consent and completed a training of the cognitive tasks. Additionally, they filled in a screening questionnaire and the Advanced Progressive Matrices by Raven (55). The task-based fMRI was included in a larger session that started with a functional resting-state sequence, followed by task-based scans, high-resolution structural scans and diffusion weighted scans. The MRI session took approximately 1 hour and 30 minutes. Testing sessions were scheduled during the first to seventh day of menses in past users and during the second or third intake week in current OC users. OC duration was based on self-reports and endogenous hormone levels were not analyzed. Oral contraceptive users show reduced level of ovarian hormones which do not reflect the activity of the exogenous hormones at the receptors. Thus, those values would not be informative and were not analyzed in the present work.

Navigation task

The navigation task consisted of 10 levels, for which participants had to navigate through a 3D virtual environment and reach as many goals as possible within 30 seconds. The task was adapted from an earlier version by reducing it from 20 levels to 10 levels (56). The 3D-environment was designed a 10 x 10 field matrix with one of ten different landmarks located on each field. Specific landmarks consisted of a tree, flowers, bushes, bench, stone, house, church, stairs, traffic light and a bridge. In each row and column all ten different landmarks were placed once.

Each level began on a starting field outside of the environment. After a countdown the current cardinal direction they were facing was presented as well as the directions to the first goal. These instructions always contained information

TABLE 1 Demographics between past/current and androgenic/anti-androgenic OC users.

	N	Age (years)		IQ		Education	OC use duration (years)	
		Mean	SD	Mean	SD	N	Mean	SD
Current androgenic pill user	30	20.00*	1.44	104.07*	9.86	28 upper secondary, 2 higher education	3.25	1.82
Current anti-androgenic pill user	23	23.26	4.05	111.00	10.48	16 upper secondary, 8 higher education	5.32**	3.39
Previous androgenic pill user	21	24.24	3.32	111.43	7.22	12 upper secondary, 9 higher education	3.97	3.01
Previous anti-androgenic pill user	20	25.00	4.00	110.90	8.12	11 upper secondary, 9 higher education	3.80	3.50

Number of participants (N), age, intelligence quotient (IQ) and duration of OC use in years separated for the four experimental subgroups. *Age and IQ of the current androgenic users was significantly different to the other subgroups. **Duration of oral contraceptive (OC) use in current anti-androgenic users differed significantly compared to the other three groups.

about the cardinal direction as well as the landmark of the goal field. As soon as the subject reached the first goal, the directions to the second target location were presented. We presented the levels of the navigation and verbal fluency task alternately: the cognitive tasks started with a navigation level and continued with a verbal fluency level.

Verbal fluency task

The verbal fluency task consisted of 10 levels in which participants had 30 seconds per level to think of as many words as possible belonging to a presented semantic category (e.g. farm animals). The consecutive words, which they mentally listed, should relate to the same subcategory. When they could not think of more words within a subcategory, they should continue with words of the next subcategory. To measure the behavioral performance, subjects had to click a remote-control button for each word within a subcategory. As soon as they continued with words of a new subcategory, they had to switch the button. We asked to silently generate the words (covert verbal fluency task) to avoid movement artefacts. It should be noted that neuronal activity during covert and overt verbal fluency might differ (57, 58). Therefore, our results should be interpreted within a framework of covert word production. During the pretest training participants had to vocalize the words that came to their mind to check if they did the task correctly. The presented categories were prescreened for overall difficulty (number of words produced if no specific instruction is given) and clustering difficulty (number of words produced under the clustering instruction) in a sample of 45 men and 45 women (see 41).

fMRI acquisition

The fMRI data were acquired on a Siemens Prisma fit 3.0 Tesla scanner at the Christian Doppler clinic in Salzburg, Austria. A T2-weighted gradient echo planar (EPI) sequence sensitive to BOLD contrast was used for the task-based functional scan (TR = 2250 ms, TE = 30 ms, FOV 192 mm, matrix size 192 × 192, slice thickness = 3.0 mm, flip angle 70°, voxel size 3.0 × 3.0 × 3.0 mm, 36 transversal slices parallel to the AC-PC line). To acquire the high-resolution structural images a T1-weighted sagittal 3D MPRAGE sequence was used (TR = 2300 ms, TE = 2.91 ms, TI delay of 900 ms, FOV 256 mm, slice thickness = 1.00 mm, flip angle 9°, voxel size 1.0 × 1.0 × 1.0 mm, 176 sagittal slices).

fMRI data analysis

The first 6 images of each scanning session were discarded during the preprocessing and the remaining scans were despiked using 3d-despiking as implemented in AFNI (afni.nimh.nih.gov). We used the Statistical Parametric Mapping software (SPM12) to realign and unwrap the images and extract six movement parameters. A biophysically-based model (Functional Image Artefact Correction Heuristic, FIACH) (59) was applied to identify and correct for non-physiological noise. We filtered the images and extracted six regressors of physiological noise *via* principal components analyses from a time-series signal-to-noise ratio (TSNR) map. Furthermore, the standard SPM12 procedures for preprocessing were used including the slice-timing, co-registration of functional to structural images, segmentation of structural images using the computational anatomy toolbox for SPM (CAT12) and normalization of functional images using the parameters obtained by CAT12. Afterwards, the data was resampled to isotropic 3 × 3 × 3 mm voxels and smoothed with a Gaussian kernel of 6 mm.

For the subject-dependent fixed-effects first-level analysis, two regressors of interest were modelled separately to predict blood-oxygen-level-dependent-imaging (BOLD) responses to the different types of events: navigation and verbal fluency with clustering instruction. The following regressors of no interest were entered to the models: episodes during which instructions appeared on the screen, the six realignment parameters and the six physiological noise parameters obtained from the FIACH procedure. All regressors were obtained by convolving the duration of the event with the canonical hemodynamic response function implemented in SPM. A high pass filter cut-off was set at 128s and autocorrelation correction was performed using an autoregressive AR(1) model (60).

For the first level analysis, one statistical contrast was defined for each of the two regressors of interest to compare BOLD-response during the tasks to baseline (blank screen). This resulted in two contrast images (activation maps), one for navigation and one for verbal fluency for each subject. Further, the contrasts were scaled (61) by dividing the contrast image by the amplitude of low-frequency fluctuation (ALFF) map (62) using the Data Processing for Analysis of Brain Imaging (DPABI) toolbox (63) as well as a band-pass filter of 0.01–0.08Hz.

At the second level, we first focus on a region-of-interest-based (ROI) analysis and then explore the whole brain level. For the ROI analysis, we entered the first-level scaled contrast images from each subject and task into two one-sample t-test, separated

for navigation and verbal fluency. We extracted the eigenvalues as measures of BOLD- response from the following bilateral regions of interest (ROIs) for both tasks: hippocampus, caudate, IFG (BA 44/45) and DLPFC (BA46). The ROIs were defined using masks based on Brodmann-areas (BA), which were implemented in the Wake Forest University (WFU) Pickatlas toolbox (64). Subsequent linear models with these variables as dependent variables were run as detailed in section statistical anaylsis.

Additionally, we explored whether duration of OC use affected brain activation at the whole brain level. Therefore, we entered the contrast images into four different full factorial designs; separated for past and current OC users as well as navigation and verbal fluency. For each model, duration of OC use, age and IQ were introduced as regressors and we modelled their interaction with group (androgenic vs. anti-androgenic). For the second level, results were masked with a SPM gray matter template and uncorrected primary threshold of $p < 0.001$ and secondary cluster-level FWE-corrected threshold of $p < 0.05$ were used.

Connectivity analysis

In order to investigate the connectivity of each ROI, bilateral hippocampi, caudate, IFG and DLPFC were used as seeds for the seed-to-voxel connectivity analysis in CONN-toolbox (65). Linear detrending for white matter (WM) and cerebrospinal fluid (CSF) influences, a band-pass filter (0.008-0.09 Hz) and additional motion-correction were executed on the preprocessed functional images. The voxel-wise connectivity maps of each participant for each ROI (hippocampus, caudate, DLPFC, IFG) were entered into a full factorial design separated for past and current users as well as navigation and verbal fluency. For each model, duration of OC use, age and IQ were introduced as regressors of no interest and we modelled their interaction with group (androgenic vs. anti-androgenic). In order to account for multiple testing, the uncorrected p-value threshold was divided by the number of ROIs and therefore set to $p < 0.000125$. Results were masked with a SPM gray matter template, and reported when Family-Wise Error (FWE) corrected $p < 0.05$. For the ROI-to-ROI connectivity analysis, Z-scores were extracted in order to analyze the interhemispheric connectivity of hippocampus, caudate, DLPFC and IFG.

Statistical analysis

We used R Version 1.4.1717 and SPSS Version 26 to compute the statistical analysis. Linear mixed models (LMMs) were performed by using the lm or lmer function from lme4 package (66) in order to assess the *duration of OC use*androgenicity* interactive effect on the following dependent variables: i) performance (words produced for verbal fluency, goals reached for navigation), ii) BOLD response, iii) interhemispheric ROI-to-ROI connectivity. Apart from *duration of OC use* and *androgenicity*, which were the factors of interest, in every model, we included age and IQ in order to control for these variables (e.g., performance ~ *duration of OC use*androgenicity + age + IQ*). For the models including ROI's brain activation, we additionally controlled for hemisphere (e.g., navigation task IFG ~ *duration of OC use*androgenicity + hemisphere + age + IQ*). In case no significant interaction between androgenicity and pill duration was observed, the interaction was removed from the model and the main effect of duration of OC use was assessed (e.g., navigation task IFG ~ *duration of OC use + androgenicity + hemisphere + age + IQ*). The main effect of androgenicity was not reported as cross-sectional comparisons were not the goal of this study. For the models including brain activation, and ROI-to-ROI connectivity, we accounted for multiple testing further FDR-correcting the p-values for the four bilateral ROIs. All continuous variables were scaled prior to analyses to allow for interpretation of effect sizes based on standard deviations.

Results

Behavioral performance

Irrespective of androgenicity, the duration of OC use was not significantly related to navigation performance in current OC users (b = -0.04, SE_b = 0.17, $t_{(47)}$ = -0.27, p = 0.79) or past OC users (b = 0.14, SE_b = 0.22, $t_{(36)}$ = 0.64, p = 0.52). Irrespective of androgenicity, duration of OC use was negatively related to verbal fluency performance in current OC users (b = -0.45, SE_b = 0.17, $t_{(48)}$ = -2.60, p = 0.013) (Figure 1). The longer the current OC use, the less words were produced in the verbal fluency task. In naturally cycling women previous pill duration did not affect the VF performance (b = 0.19, SE_b = 0.22, $t_{(36)}$ = 0.91, p = 0.37).

Brain activation: Overall task-related activation

During the navigation task, and in line with task-related literature (40) we observed significant activation in the bilateral superior frontal gyrus and anterior insula, left superior parietal lobe and supplementary motor cortex (Table 1). Specifically, our results show similar activation compared to the activation of the female sub-group in previous samples (40). During verbal fluency, the activation network was left-lateralized showing a large cluster including the bilateral middle cingulate and superior frontal gyrus with a peak in the supplementary motor cortex and supramarginal gyrus (Supplementary Table 1). The activation network is in line with previous fMRI-studies on covert verbal fluency (67, 68).

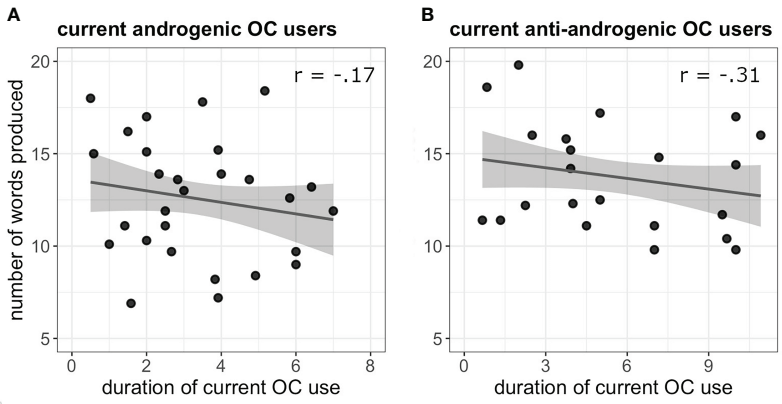

FIGURE 1
(A) Negative association between verbal fluency performance and duration of OC use (in years) in current androgenic users. **(B)** Negative association between verbal fluency performance and duration of OC use (in years) in current anti-androgenic users.

Brain activation: ROI-analysis

During navigation, activation in the hippocampus, DLPFC or IFG was not significantly related to the duration of OC use, irrespective of the androgenicity, in current OC users (all b < 0.58, all t < 2.58, all p > 0.05).

However, there was a significant interaction between the *duration of current OC use* and *androgenicity* on caudate activation (b = 0.83, SE_b = 0.32, $t_{(53)}$ = 2.58, $p_{uncorr.}$ = 0.01, p_{FDR} = 0.04). While androgenic OC-users showed a significant inverse relation of caudate activation and pill duration, showing a stronger deactivation with longer duration of pill use ($r_{(55)}$ = -0.35, p = 0.009); anti-androgenic users showed no pill duration effect ($r_{(41)}$ = -0.15, p = 0.33) (Figure 2).

During verbal fluency, there was no significant association of pill duration, irrespective of the androgenicity, to activation in any of the ROIs in current OC-users (all b < 0.46, all t < 1.36, all p > 0.05).

There was no significant association between duration of previous OC use, irrespective of the androgenicity, and activation in any of the ROIs during navigation or verbal fluency (all b < 0.45, all t < 1.49, all p > 0.05).

Brain activation: Whole brain analysis

During navigation, whole brain analyses revealed a significant negative association between current androgenic OC use and activation in the left postcentral gyrus ([-39, -28, 58], 88 voxels, T = 4.26, p_{FWE} = 0.008) (Figure 3). The longer the duration of androgenic OC use, the stronger was the deactivation in the post-central gyrus. We observed a negative association in androgenic and anti-androgenic OC users between duration of OC use and right superior occipital gyrus activation ([24, -79, 16], 135 voxels, T = 5.08, p_{FWE} = 0.001) as well as right calcarine cortex activation ([18, -76, 7], 73 voxels, T = 4.91, p_{FWE} = 0.02) (Figure 4). The longer the OC use duration, the stronger the deactivation of these areas during navigation.

During verbal fluency, whole brain analyses revealed a significant positive association between the duration of previous androgenic and anti-androgenic OC use and activation in the left putamen ([-24, 8, -8], 48 voxels, T = 4.09, p_{FWE} = 0.047) (Figure 5). The longer women had previously taken OCs, the stronger was their activation in the left putamen during verbal fluency irrespective of androgenicity.

Inter-hemispheric connectivity: ROI-to-ROI analysis

During navigation, the duration of current OC use was significantly associated with stronger connectivity between left and right caudate, irrespective of androgenicity (b = 0.49, SE_b = 0.17, $t_{(48)}$ = 2.84, $p_{uncorr.}$ = 0.007, p_{FDR} = 0.026) (Figure 6).

We observed no effect of pill duration in current and past users on connectivity between left and right hippocampus, left and right DLPFC, left and right IFG as well as in past users on connectivity between left and right caudate during navigation (all b < 0.32, all t < 1.8, all p > 0.05).

Duration of OC use was also not associated with changes in connectivity between left and right hippocampus, left and right caudate, left and right DLPFC and left and right IFG in current nor past users during verbal fluency (all b < 0.36, all t < 1.12, all p > 0.05).

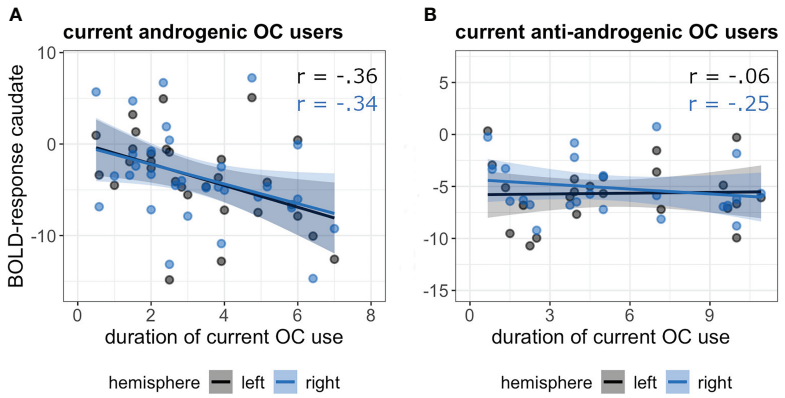

FIGURE 2
Association between duration of current OC use [in years] and BOLD-response in the bilateral caudate in androgenic **(A)** and anti-androgenic **(B)** OC-users during navigation.

Functional connectivity: Seed-to-voxel analysis

Seed to voxel analyses revealed no associations between the duration of pill use and connectivity of the right hippocampus, left and right caudate, left and right DLPFC, as well as left IFG, and irrespective of androgenicity. However, during both tasks, connectivity of the left hippocampus was significantly associated with the duration of OC use, irrespective of androgenicity.

During navigation, longer duration of current OC use irrespective of androgenicity was associated with stronger connectivity between left hippocampus and right cuneus ([12, -85, 22], 44 voxels, T = 5.80, $p_{FWE} < 0.001$), right lingual gyrus ([27, -58, -11], 85 voxels, T = 5.71, $p_{FWE} < 0.001$) and right superior occipital gyrus ([24, -79, 31], 14 voxels, T = 4.68, $p_{FWE} = 0.026$) (Figure 7), as well as weaker connectivity between left hippocampus and right angular gyrus ([42, -55, 34], 49 voxels, T = 5.64, $p_{FWE} < 0.001$) (Figure 8). We observed no associations between the duration of past OC use and left hippocampus connectivity during navigation.

During verbal fluency, longer duration of current OC use, irrespective of androgenicity was associated with stronger connectivity between left hippocampus and bilateral posterior cingulate gyrus (left: [-9, -52, 4], 82 voxels, T = 6.36, $p_{FWE} < 0.001$; right: [18, -46, 1] 19 voxels, T = 4.91, $p_{FWE} = 0.008$), right lingual gyrus ([9, -64, 7], 114 voxels, T = 5.14, $p_{FWE} < 0.001$) and left cuneus ([-9, -73, 19], 22 voxels, T = 4.94, $p_{FWE} = 0.004$) (Figure 9).

Furthermore, duration of previous OC use was related to stronger connectivity between right IFG and right angular gyrus during verbal fluency ([51, -43, 28], 50 voxels, T = 6.06, $p_{FWE} < 0.001$) irrespective of androgenicity (Figure 10).

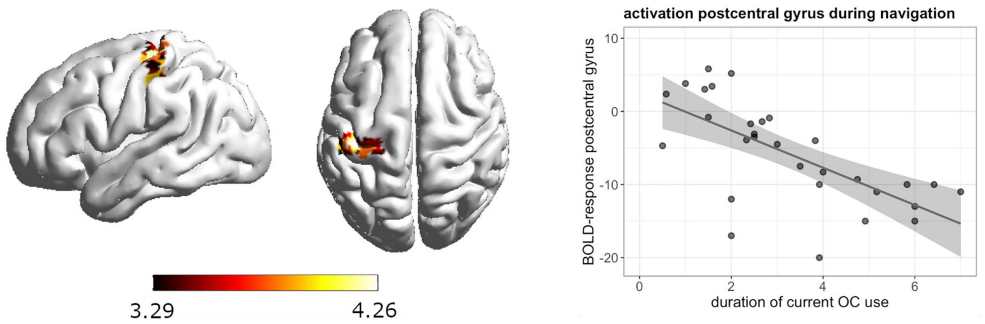

FIGURE 3
Negative association between duration of OC use and left postcentral gyrus activation during navigation in current androgenic OC users.

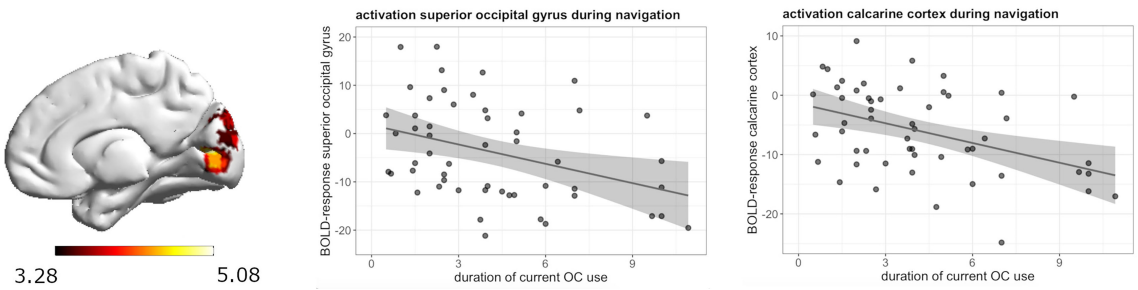

FIGURE 4
Negative association between duration of OC use and right superior occipital gyrus and right calcarine cortex deactivation during navigation in current OC users.

Discussion

The current study explored association between the duration of OC use and cognitive performance, as well as brain activation and connectivity during two different cognitive tasks, that were previously shown to be sensitive to the effects of endogenous sex hormones (7, 40). We further controlled for the androgenicity of the OCs to dissociate effects related to the androgenic activity of progestins from effects related to the estrogenic/progestogenic activity of OCs. Furthermore, we extended the analyses to previous OC users in order to explore the reversibility of OC-dependent effects on the brain. Along these lines, the following commonalities among the results for the navigation and verbal fluency tasks seem noteworthy.

First, several associations to the duration of OC use in the navigation task appear to be modulated by androgenicity with stronger effects in androgenic compared to anti-androgenic OC users, while none of the associations observed for the verbal fluency task were modulated by androgenicity. While the effects of sex hormones to spatial and verbal processing are not entirely understood, several studies hint at an activational role of androgens for spatial processing (69, 70). With regards to verbal memory, results so far point to an important role of estrogens (71–73), while results on the role of androgens during verbal processing remain inconclusive (38, 74–76). In light of these findings, it seems plausible that the androgenicity of progestins affects associations to OC use during spatial processing, but not verbal processing. In particular, deactivation in the caudate and postcentral gyrus appears to be associated to longer androgenic, but not anti-androgenic OC-use. Animal studies hint at different navigation strategies supported by the hippocampus and caudate. While the hippocampus supports "spatial" strategies, the caudate supports stimulus-response learning (77–79). Allocentric landmark-based navigation, as required by the task-instructions in the present study, should per se involve more spatial rather than stimulus-response strategies. However, the current results suggests that the caudate becomes less involved

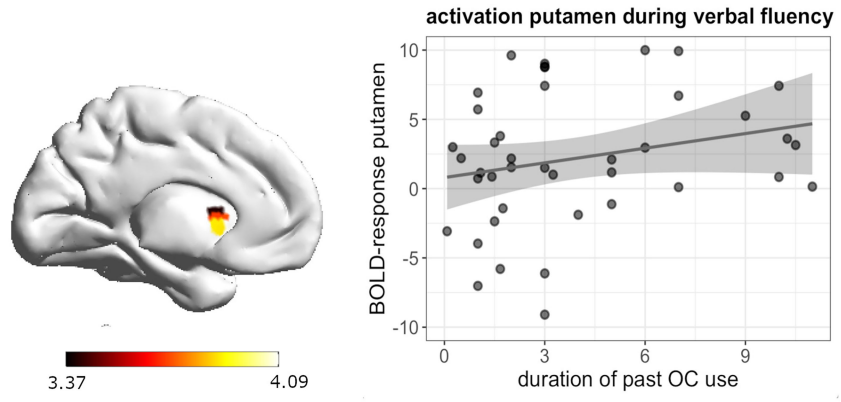

FIGURE 5
Positive association between duration of OC and left putamen activation during verbal fluency in past OC users.

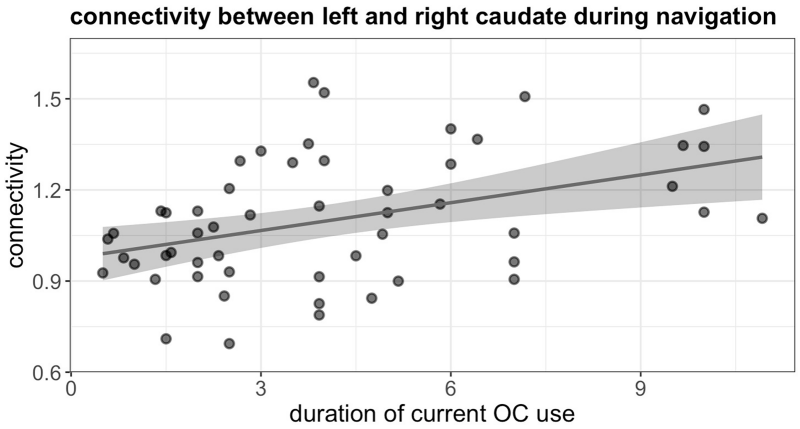

FIGURE 6
Positive association between duration of current OC use and connectivity between left and right caudate during navigation in current users.

with longer duration of androgenic OC use. The results in the postcentral gyrus are particularly interesting since a recent study on rats found a new spatial navigation system in the somatosensory cortex (80). The authors detected functionally distinct spatial cell types like place, grid and head-direction cells in the primary somatosensory cortex which is located in the postcentral gyrus. The cells showed spatially selective firing patterns which are similar to the ones of the hippocampal place cells. This finding indicates the existence of a spatial map system in rodents' sensory cortices (80).

Second, in the navigation task, no associations to previous OC use were observed, while in the verbal fluency task, duration of previous OC use affects a variety of brain parameters, including activation of the left putamen, as well as connectivity between right-hemispheric language areas, i.e. the right IFG and the right AG. This points towards stronger organizational effects of OCs on verbal rather than spatial processing. While it is hard to disentangle organizational from activational effects of sex hormones, several results suggest that hormonal effects on spatial processing are of a more activational, while effects of sex hormones on verbal processing are of a more organizational nature. For instance, the onset of sex differences in spatial abilities appears to be roughly around puberty (though social factors may also contribute to this finding), while sex differences in verbal abilities are observed from a very young age on (38). It is however also possible that results in previous OC

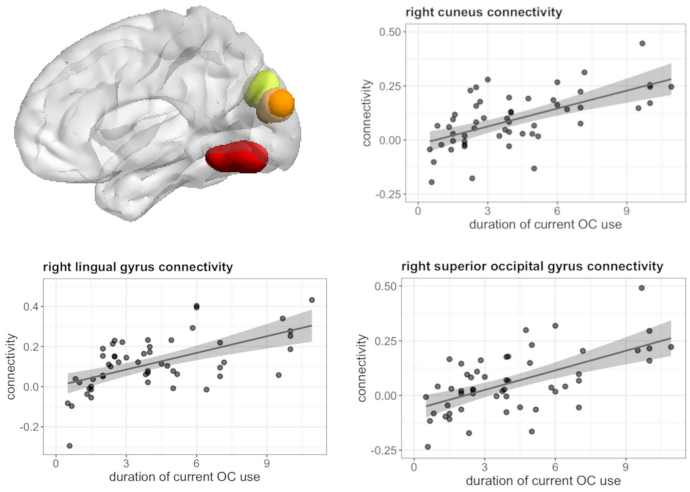

FIGURE 7
Positive association between duration of OC use and connectivity between left hippocampus and right cuneus, lingual gyrus, superior occipital gyrus in current users during navigation.

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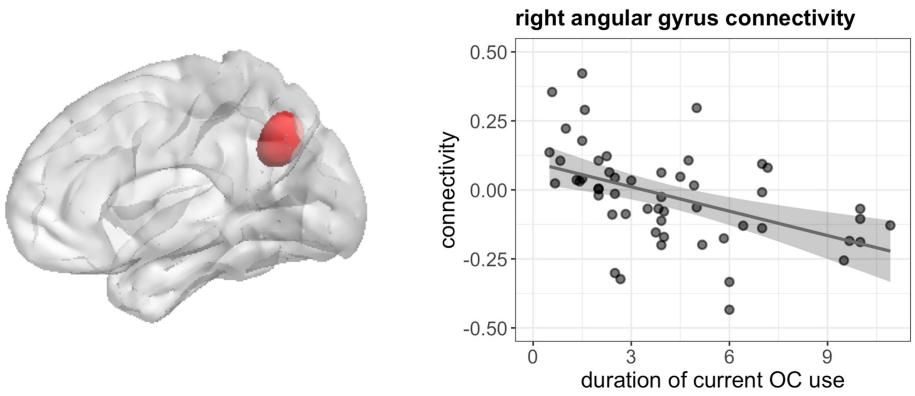

FIGURE 8
Negative association between duration of OC use and connectivity between left hippocampus and right angular gyrus in current users during navigation.

users were missed due to the heterogeneity of progestins used in this group, while current androgenic OC users were all users of levonorgestrel.

A further difference between the navigation and verbal fluency task are the associations of current OC use to task performance. While duration of OC use was not related to task performance in the navigation task, verbal fluency performance was slightly reduced with longer duration of current OC use. It has to be noted that – similar to previous neuroimaging studies using these tasks (e.g. 7) – behavioral results should be interpreted with caution, since our tasks were optimized for the assessment of brain activation and may not be sensitive enough to reflect changes in behavioral performance. For example, the strong inter-individual variability in navigation performance requires a fixed length of navigation trials, which makes an assessment of navigation speed imprecise. Likewise, the verbal fluency task had to be implemented as a covert version to avoid movement artefacts. Nevertheless, the results may hint that OC effects on the brain during navigation are adaptive and may represent altered processing styles to uphold task

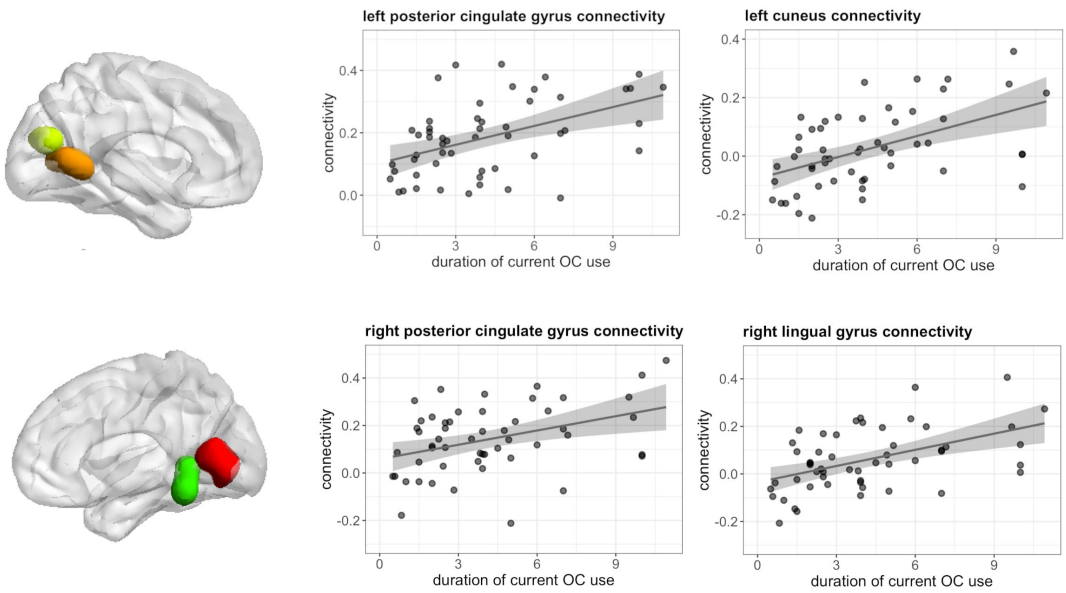

FIGURE 9
Positive association between duration of OC use and connectivity between left hippocampus and bilateral posterior cingulate gyrus, left cuneus and right lingual gyrus in current users during verbal fluency.

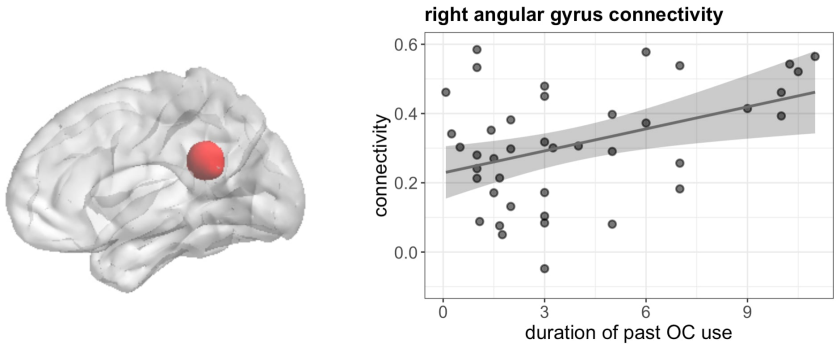

FIGURE 10
Positive association between duration of OC and connectivity between right IFG and right angular gyrus in past users during verbal fluency.

performance. This interpretation seems plausible, given that both caudate activation and connectivity as well as hippocampus connectivity were related to the duration of current OC use. As discussed above, these two areas have been implicated in the balancing of different processing styles during navigation. The fact that the duration of OC use is reflected in task performance during verbal fluency may also be in line with the interpretation of more organizational effects during verbal fluency.

Surprisingly, previous studies indicate protective effects of estrogens on verbal and memory performance (71, 73), while verbal fluency performance was impaired with longer OC use in the current study. However, many studies proposing a protective effect of synthetic estrogens have been conducted in post/menopausal women and the suppression of endogenous estrogen fluctuation by the OC use could be responsible for our findings. It is still difficult to disentangle the effects of the lack of endogenous hormones from effects of synthetic OC hormones. Apart from that, an alternative explanation could be the progestagenic actions of OCs since a study on premenopausal women found an association between decreased verbal memory performance and high dose of progesterone intake (81). This placebo-controlled study on the effects of oral progesterone on verbal memory found that the highest dose of 1200mg was associated with decreased information processing and verbal memory performance (81). However, there are contradicting results on the effect of progesterone on verbal memory (82). The idea of an increased effort with the retrieval of verbal information from long-term memory with longer OC use may also be supported by the increased connectivity between the left hippocampus and PCC, which is a major integrating hub and default mode area associated with cognitive effort and task demands (83, 84). Another factor influencing the cognitive effect of OC use might be the vulnerability to depressive episodes (85) since depressive symptoms are associated with compromised cognition (86). Although recent studies have related previous OC use to the development of mood disorders (87, 88), it is still unclear if OC use causes an increased vulnerability to depression (89).

Finally, the results we observed in the hippocampus deserve some attention, given that this very plastic area has been suggested as most sensitive to the effects of sex hormones by both animal and human studies (90, 91). While previous studies very consistently suggest stronger hippocampal activation during high estrogen phases irrespective of the cognitive task involved (5, 7), no effects of OC use duration on hippocampal activation were observed. This is likely due to OCs representing a combination of estrogenic and progestagenic treatment, as observed, e.g., during the luteal cycle phase, while previous results on hippocampal activation were observed during the pre-ovulatory phase, characterized by high estradiol, but low progesterone. Pre-clinical studies provide numerous examples of opposite effects of estrogens and gestagens on various neurotransmitter systems, including the glutamatergic and GABA-ergic systems. Accordingly, estrogens are ascribed excitatory qualities, while progestogens appear to oppose the excitatory effects of estrogens and display inhibitory effects. It is likely that the progestagenic actions of progestins contained in OCs counteract the excitatory effects of estrogens on the hippocampus.

Even though duration of OC use was not significantly associated with activation in the hippocampus, left hippocampal connectivity was substantially modulated by OC duration in both tasks. Irrespective of the task, a stronger connectivity between the hippocampus and various occipital areas was observed. It can be very carefully speculated that this may be related to the transfer of visual information to long-term memory. The reduced connectivity between the left hippocampus and right angular gyrus during navigation fits to the pattern pointing to a shift in strategy or processing style during navigation. Previous studies using this task suggested a stronger verbal labelling of landmark information in women compared to men, which was also reflected in the stronger recruitment of language areas by the hippocampus (40). It is possible that the reduced connectivity between the hippocampus and angular gyrus reflects a reduced recruitment of language areas by the hippocampus.

The main limitation of this study is its correlational nature. While it goes beyond traditional cross-sectional designs and we controlled by confounds like age, education or IQ, we cannot make inferences about the causality of these effects but rather view our results as hypothesis-generating for future prospective studies. Apart from the restrictions regarding behavioral measures and various progestins used among previous contraceptives, another interesting factor to consider in future studies is the age at first contraceptive intake, since developing brains may be more sensitive to synthetic steroids (e.g., 35).

In summary, the current study reveals adaptive brain associations of current OC treatment duration on spatial processing, which depend on the androgenicity of the progestin and are suggestive of a potential shift in processing style with long-term contraceptive use, particularly in the case of androgenic OCs. Furthermore, we observed brain associations of both current and previous OC treatment duration on verbal processing, which are accompanied by a small, but consistent drop in verbal fluency performance. Compared to previous findings from studies on endogenous sex hormones, the results suggest a key role of the progestogenic component of OCs in both tasks.

Data availability statement

Data and scripts for ROI-analyses are openly available at https://osf.io/q7wth/ and http://webapps.ccns.sbg.ac.at/OpenData/. MR-images for whole-brain analyses are available from thecorresponding author upon reasonable request.

Ethics statement

The studies involving human participants were reviewed and approved by University of Salzburg's ethics committee. The participants provided their written informed consent to participate in this study.

Author contributions

BP designed and made the concept of the study. IN and EH-L were responsible for data acquisition. Analysis of the data was performed by BP, EH-L, and IN. IN drafted the manuscript, which was revised and approved by BP. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.885617/full#supplementary-material

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Weak associations between personality and contraceptive choice

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Prospective randomized controlled trials on hormonal contraceptive (HC) effects on the brain are rare due to a number of methodological challenges. Thus, much of the evidence on HC effects on the brain comes from cross-sectional studies comparing HC-users to non-users. In interpreting these findings, it is of importance to be aware of potential confounds associated with women's contraceptive choices. Previous studies have discussed age, education, social status, sexual orientation, relationship status, and tolerability of HC. Given the current trend toward a reduction in HC use and increased skepticism toward HC it seems relevant to also identify variables associated with women's attitudes toward HC and whether they may represent confounds for neuroscientific studies. In the present study, we investigated whether women's personality characteristics were associated with their choice to use or not use HC in the present, past and future and the type of HC chosen. 1,391 females aged 18–45 years participated in an online survey including the HEXACO-60 personality questionnaire, as well as two different measures of gender role, and provided information about their current and previous contraceptive status, as well as experiences with and attitudes toward contraceptive use. We compared (i) current, previous and never-users of HC, (ii) prospective users of HC to women who opposed future HC use, and (iii) current users of IUDs to current users of oral contraceptives. Results revealed that associations between personality and the decision to use or not use HC were negligible, while differences in personality were observed corresponding to contraceptive type. Current users of IUDs showed higher agreeableness and extraversion compared to current users of oral contraceptives. The results suggest that personality is more strongly associated to the choice of contraceptive type rather than the choice between hormonal and non-hormonal options.

KEYWORDS

hormonal contraceptives, personality, gender role, masculinity, femininity, adverse mood effects

Introduction

Hormonal contraceptives (HC), and in particular combined oral contraceptives (COC), have been linked to changes in brain structure and function (Porcu et al., 2019; Brønnick et al., 2020; Rehbein et al., 2021) and associated with behavioral changes, including women's mental health (Sundström-Poromaa, 2021), cognitive performance (Warren et al., 2014), mate preferences (Alvergne and Lummaa, 2010), and social and emotional functioning (Montoya and Bos, 2017; Lewis et al., 2019). Considering the wide-spread use of HC, societal consequences have been intensively discussed (Alvergne and Lummaa, 2010; Montoya and Bos, 2017; Sundström-Poromaa, 2021). Given the estrogenic actions of ethinylestradiol and other synthetic estrogens (Stanczyk et al., 2013), as well as the progestogenic, androgenic, anti-androgenic, or mineralocorticoid properties of the various synthetic progestins contained in HC (Sitruk-Ware, 2006; Griksiene et al., 2022), the described effects appear plausible. Steroid actions on the brain from neurogenesis over synaptic transmission (Barth et al., 2015) to the modulation of large-scale brain networks are well documented (Hidalgo-Lopez et al., 2021). With the increasing availability and usage of long-lasting methods like hormonal intra-uterine devices (IUD), more studies are also conducted comparing the effects of various methods of HC, e.g., COC vs. IUD on the brain and behavior (Bürger et al., 2021).

However, due to the variety of methodologies employed in studies of HC actions on brain and behavior, it is hard to disentangle the effects of various combinations of synthetic steroids from confounding factors. Most importantly, the majority of HC studies uses a cross-sectional design, comparing COC-users to non-users, different groups of COC-users, or COC-users and IUD-users. These groups may differ in a range of factors correlated with the contraceptive choice, e.g., age, education, socio-economic status, relationship status, or tolerability of HCs (Pletzer and Kerschbaum, 2014). The selection of birth control method might depend on women's socioeconomic, demographic, or partnership characteristics (Eeckhaut et al., 2014) as well as on a woman's personal preferences (Dragoman, 2014). While demographic differences between the groups can usually be well-controlled, tolerability of HC and personality traits are generally not documented. However, those two factors in particular are highly relevant to the dependent variables studied in contemporary HC research, i.e., brain structure and function, as well as mood, cognition, and wellbeing.

Regarding the tolerability of HC, the so-called survivor-effect may introduce a sampling bias in cross-sectional study designs. While long-term users usually tolerate HC well, non-users have usually stopped using HC due to adverse side effects (Oinonen and Mazmanian, 2002). Most commonly, emotional side effects and weight gain are listed as a reason for discontinuation of HC-treatment (Lindh et al., 2009). The neurophysiological factors that determine the tolerability of HC are currently unknown. Accordingly, there may be pre-existing neurophysiological differences between HC-users and non-users, such that the differences found in cross-sectional designs may not actually be a result of HC-use, but rather affect the choice to use HC or not. Accordingly, the contraceptive history of non-users, their reasons for discontinuation, as well as their side effect profiles are relevant factors to consider in cross-sectional studies on HC-use.

Relatedly, HC-use has dropped in the past years, which may on the one hand be related to the availability of non-hormonal options, like copper IUDs. However, concerns about potential long-term effects on (mental) health and fertility have resulted in increasing skepticism among women regarding synthetic steroids (e.g., Fiala and Parzer, 2019; Landersoe et al., 2019; Svahn et al., 2021). Thus, newer studies may well face an additional bias concerning women's attitudes toward HC-use. Accordingly, pre-existing differences between HC users and non-users may not only concern neurophysiological factors determining contraceptive tolerance, but also psychological factors, including personality traits related to women's choice to use HC.

Personality traits have been associated with differences in performance on cognitive tasks (Aschwanden et al., 2020), socioemotional functioning (Canli et al., 2002; Yang et al., 2021), psychopathologies (Kotov et al., 2010) and brain structure (Nostro et al., 2017). For example, open, extraverted, and emotionally stable participants demonstrated better verbal fluency (Sutin et al., 2011); neuroticism (negatively) and openness (positively) affected self-estimates of spatial and logical abilities (Stieger et al., 2010); higher conscientiousness, openness, and extraversion as well as lower neuroticism were associated with better memory performance (Luchetti et al., 2021). With regard to socioemotional functions, high neuroticism scores were related to decreased brain activation in the medial prefrontal cortex during implicit negative emotion processing (Yang et al., 2021), while a higher degree of extraversion correlated positively with amygdala activation to happy facial expressions (Canli et al., 2002). And in terms of "big" personality traits (i.e., BIG-5 neuroticism, extraversion, openness, agreeableness, and conscientiousness), especially neuroticism (high) and conscientiousness (low) were significantly associated with anxiety, depressive, and substance use disorders (Kotov et al., 2010). In addition, women's personality traits may be related to a way of coping with physical and/or psychological discomfort determined by hormonal fluctuations during the menstrual cycle. Therefore, women who are more vulnerable may be choosing HC to avoid menstrual cycle related inconvenience. For example, it was demonstrated that women with high neuroticism score were more likely to use hormone replacement therapy (as a way of coping with menopause symptoms) (Loekkegaard et al., 2002).

To the best of our knowledge, only few studies have examined associations between HC-use and women's personalities, yielding inconsistent results. An early study

by Beard et al. (1974) demonstrated a negative relationship between neuroticism scores and the reliability of contraceptive methods used by study participants. It was demonstrated that women with the lowest neuroticism scores tended to use the most reliable methods of contraception (pills and IUDs), whereas participants scoring highest on neuroticism did not use any form of contraception. Priestnall et al. (1978) reported that OC-users were significantly less positive toward religion, more linked to feminism and less neurotic than non-users. No differences were found between users and non-users with regard to extraversion in that study. Jacobsson et al. (1981) demonstrated that the long-term COC- or IUD-users were more stable psychologically and exhibited a lower neurotic potential. However, a more recent study by Ross et al. (2001) demonstrated the opposite result, i.e., significantly higher neuroticism in COC-users than in non-users. Finally, the most recent studies (Hamstra et al., 2017; Beltz et al., 2019) did not find significant differences in personality scores between OC users and non-users.

There are multiple potential reasons for these inconsistencies. On the one hand, women's attitudes toward HC and the demographic and socio-economic characteristics of HC-users have changed over time and are also subject to cultural differences. Similarly, the composition of the comparison group of naturally cycling women may have contributed to inconsistencies in the results, especially if previous experiences with HC were not controlled for. Accordingly, a clearer differentiation between never users, previous users and prospective users of HC among the naturally cycling group will aid to adequately capture associations between personality and women's attitudes toward HC.

On the other hand, it has been discussed, especially with respect to gender differences, that broad personality factors, like the BIG-5, may not be adequately sensitive to group differences (Del Giudice et al., 2012). They encompass a variety of facets, which may be differentially related to the grouping variable, thereby averaging out the group differences in the overarching factor. It is possible, that a similar situation occurs with respect to HC-use or HC type. Thus, the use of a more fine-grained instrument, allowing the simultaneous assessment of broad personality factors and their underlying facets may provide a clearer picture.

Particularly, the sub-facets of extraversion, like dominance and warmth, show gender differences in opposite directions (Del Giudice et al., 2012), given their association with the gender roles masculinity and femininity, respectively (Eagly and Sczesny, 2019). Indeed, various gender-sensitive facets of extraversion, agreeableness and neuroticism may not only be grouped according to the BIG-5, but also along the overarching dimensions of masculinity and femininity (Gruber et al., 2019). Given that femininity in particular has been associated with gray matter volumes in prefrontal areas (Pletzer, 2019), while masculinity has repeatedly been related to spatial abilities (Reilly and Neumann, 2013; Beltz et al., 2022), it is an interesting question whether HC choice is also associated with femininity or masculinity. So far, two studies have investigated associations between HC-use and women's gender role self-concept with inconsistent results: While one study demonstrates that HC-users rate themselves as more feminine compared to non-users (Pletzer et al., 2015), the other study demonstrates no differences in the gender role self-concept of HC-users and non-users (Nielson and Beltz, 2021). The two studies differ in the gender role measures employed. While Pletzer et al. (2015) used only subjective rating scales, Nielson and Beltz (2021) also asked for instrumental and expressive traits associated with masculinity and femininity. Given that gender roles are a concept with considerable cultural differences (Eagly and Sczesny, 2019), self-concepts may be colored by participants perceptions of what is masculine or feminine. Accordingly, a combination of measures is advisable when assessing gender-role in a cross-cultural context.

To obtain a varied picture of personality traits in current, past and never-users of HC, as well as between COC-users and IUD-users, we chose the HEXACO-60 (Ashton and Lee, 2009), which assesses the BIG-5 personality factors, but allows for differentiation of sub-facets, and further used two different gender role measures. The first question of the present study was whether women's attitudes toward the use of synthetic hormones for contraception in general are associated with personality factors. To address this question, we compared current, past and never-users of HC, as well as prospective HC-users to women for whom future HC-use is not an option. Based on previous work we hypothesized that neuroticism and femininity may differ between those groups, though the directionality is unclear given inconsistent results. The second question was, whether the type of HC chosen is related to women's personality. Given that the regular daily intake of COCs requires a greater amount of organization than long-acting methods like IUDs, we hypothesized that conscientiousness is higher in women who choose COCs compared to women who choose IUDs.

Materials and methods

Participants

1,391 biologically female women aged 18–45 (mean age: 26 years, SD = 8 years) participated in this study and filled out an online questionnaire on their current and previous contraceptive status, as well as experiences with and attitudes toward contraceptives. Demographic information of participants can be found in **Table 1**. To determine sex and gender, participants were independently asked, which sex they were assigned at birth and whether they identified as man or woman. Participants were recruited via social media and

university emails at the Universities of Salzburg, Tübingen, and Vilnius. Due to the anonymous nature of the study, participants were not compensated for their participation. The study was approved by the University of Salzburgs' ethical committee.

Questionnaires

Questionnaires were presented as part of an online survey via LimeSurvey and presented in German or Lithuanian translations. Data were collected between July 20th to November 15th 2021, i.e., during a time when no COVID19-lockdowns were in place in any of the participating countries.

Contraception questionnaire

Participants started the Online Survey by answering several questions about their current and previous contraceptive use. Women who used HC at the time of testing gave information on the duration of their use, side effects, why they chose this form of contraception and whether the start of their use was connected to the beginning of a new relationship.

Naturally cycling women were asked for the reason they decided against using HC. If their response was that there was no need for contraception in general, they were asked whether they could or could not imagine using HC in the future and why. They were also asked to give information on previous HC-use.

HEXACO-60

Personality traits were investigated by the self-report form of the HEXACO-60 (Ashton and Lee, 2009) in its German and Lithuanian translations. The inventory consists of 60 items corresponding to the six dimensions of the HEXACO model of personality structure (Honesty-Humility, Emotionality, Extraversion, Agreeableness, Conscientiousness, and Openness to Experience). Participants rated their agreement to each of the statements about themselves (e.g., "I would be quite bored by a visit to an art gallery.") on a 5-point Likert scale, ranging from 1 (= strongly disagree) to 5 (= strongly agree). The HEXACO also allows a more fine-grained assessment of personality by providing scores of four sub-factors for each of the main personality domains. For the German version of the HEXACO-60, Moshagen et al. (2014) confirmed the 6-factor solution, as well as measurement invariance with respect to gender and report good internal consistencies ranging from 0.74 to 0.83, as well as retest reliability over 7 months of 0.72–0.90. Furthermore, the instrument is well-validated with low correlations between subscales, high correlations to other personality questionnaires (Moshagen et al., 2014), as well as lexical personality factors (Ashton et al., 2007). For the Lithuanian version of the HEXACO-60, the 6-factor solution, as well as construct validity was confirmed by Truskauskaitė-Kunevičienė et al. (2012), who also report good internal consistencies ranging from 0.66 to 0.80. Measurement invariance of the HEXACO-60 with respect to the language/country was recently confirmed in a large-scale confirmatory factor analysis by García et al. (2022).

Gender related attributes scale

The Gender Related Attributes Scale (GERAS) developed by Gruber et al. (2019) was used to assess gender role. This scale measures characteristics that are generally perceived as typically masculine or feminine on the three subscales personality, cognition, and interests. All items are rated on a 7-point Likert scale.

First, participants were asked to compare themselves to the general population in how often they portrayed 10 stereotypically masculine (e.g., brave, dominant) and 10 stereotypically feminine (e.g., compassionate, anxious) personality traits on a scale from 1 (= never) to 7 (= always). On the second subscale, participants rated how easy they would find completing each of 14 cognitive tasks on a scale of 1 (= not at all) to 7 (= very). Seven of these tasks required skills that according to previous research men show stronger performance in (e.g., finding an address), whereas the other seven items are typically easier for women (e.g., remembering names and faces).

Finally, participants stated how much they enjoy each of 16 activities on a scale from 1 (= not at all) to 7 (= very much). Again, eight of these items described activities typically perceived as masculine (e.g., watching sports) and eight items described activities typically perceived as feminine (e.g., Yoga). Averaged scores for masculine and feminine items were computed for each subscale. Overall masculinity and femininity scores were obtained by averaging the masculinity and femininity scores of each subscale.

The factorial structure of global masculinity and femininity scores with subscores in personality, cognition and interests, as well as measurement invariance with respect to gender was confirmed by Gruber et al. (2019) for the German version. Gruber et al. (2019) also reports good reliability with Revelle's Omega, split-half and retest reliability ranging from 0.80 to 0.88. The GERAS was validated against other gender role questionnaires, self- and peer-reports, as well as chosen occupation (Gruber et al., 2019). Translation to Lithuanian was performed by 10 independent German/Lithuanian bilingual native speakers and validated by back-translation. Psychometric properties of the Lithuanian translation have not yet been published.

Six-item-scale

To additionally obtain a subjective measure of masculinity and femininity, gender role was also assessed by a Six Item Scale (Pletzer et al., 2015). Participants directly indicated how masculine or feminine they perceived themselves compared to men, other women, and the general population on a scale of 1 (= not at all) to 9 (= very). By measuring subjective masculinity and femininity, this scale takes into account possible

cultural and personal differences in what the participant views as typically masculine or feminine.

Statistical analysis

Data were analyzed using IBM SPSS Statistics 27. Given the large sample size, normality of distributions was determined graphically, using stem- and-leaf plots, histograms, as well as Q-Q plots. All HEXACO-60 and GERAS scales were normally distributed and thus suitable for parametric analysis. The significance threshold was set to $p_{FDR} < 0.05$ throughout the manuscript.

Taking into consideration the whole sample, significant group differences between current, previous and prospective HC-users as well as never-users were observed in language/country, age, education, employment status, sexual orientation, relationship status, relationship duration, and number of children (compare **Table 1**), resulting in a large number of confounding variables when comparing these groups with respect to personality and gender role. Due to the significant group differences in demographic variables, ANCOVA requirements are violated (Miller and Chapman, 2001; Verona and Miller, 2015). Accordingly, we opted for *a priori* matching of confounding variables between groups using propensity scores. Nevertheless, ANCOVA results are reported in **Supplementary Table 3**. Propensity score matching is particularly useful, when multiple confounding variables need to be considered (see e.g., Benedetto et al., 2018). Given that never-users were the smallest group ($n = 321$), we assessed current and previous HC-users according to their similarity to never-users. To that end, we performed two binary logistic regression analyses with group (never vs. previous; never vs. current) as dependent variable and age, language, sexual orientation, and relationship status as regressors. Education and socio-economic status were collinear to age and additional inclusion of education in the binary logistic regression did not improve matching. Likewise, relationship duration and number of children were collinear to relationship status. Probabilities of belonging to the never-user group based on those variables (propensity scores) were saved and the 321 current and 321 previous users with the highest probabilities were selected for further analysis.

For comparison of potential future HC-users ($n = 73$) and women who said that future HC-use was not an option for them, 73 of 714 women for whom future HC-use was not an option were selected based on propensity scores for belonging to the future HC group based on age, language, sexual orientation,

TABLE 1 Comparison of demographic variables between current and previous HC-users and never users prior to matching.

		Never-users ($n = 321$)	Previous users ($n = 493$)	Current users ($n = 577$)	Comparison
Language[a]	Lithuanian	97 (30%)	94 (19%)	94 (16%)	$\mathbf{p < 0.001}$
	German	224 (70%)	399 (81%)	483 (84%)	
Age[b]		24.85 ± 5.82	27.44 ± 6.64	24.20 ± 5.89	$\mathbf{p < 0.001}$
Handedness[a]	Left-handed	35 (11%)	41 (8%)	48 (8%)	$p = 0.362$
Education[c]	Apprenticeship	3 (1%)	10 (2%)	4 (1%)	$\mathbf{p < 0.001}$
	Middle school	4 (1%)	5 (1%)	14 (2%)	
	High school	143 (46%)	160 (33%)	299 (52%)	
	University	158 (51%)	316 (64%)	247 (43%)	
	Unknown	13 (4%)	2 (0%)	2 (0%)	
Employment status[c]	Employed full time	67 (21%)	142 (29%)	127 (22%)	$\mathbf{p < 0.001}$
	Employed part time	51 (16%)	108 (22%)	104 (18%)	
	In education + part time	31 (10%)	70 (14%)	73 (13%)	
	In education + unemploy.	62 (19%)	53 (11%)	87 (15%)	
	Unemployed	110 (34%)	120 (24%)	186 (32%)	
Sexual orientation[b]	Homosexual	18 (6%)	17 (3%)	18 (3%)	$\mathbf{p = 0.006}$
	Bisexual	72 (22%)	117 (24%)	109 (19%)	
	Heterosexual	231 (72%)	359 (73%)	450 (78%)	
Relationship	In a relationship[a]	179 (56%)	367 (74%)	444 (77%)	$\mathbf{p < 0.001}$
	Duration[b]	5.06 ± 4.72	5.77 ± 5.65	4.13 ± 4.72	$\mathbf{p < 0.001}$
	Satisfaction[b]	8.60 ± 1.65	8.55 ± 1.51	8.72 ± 1.49	$p = 0.320$
Children[a]		42 (13%)	91 (19%)	47 (8%)	$\mathbf{p < 0.001}$

[a]binary variables, compared via X^2-Tests, [b]continuous variable, compared via one-way-ANOVAs, [c]ranked variables, compared via Kruskall-Wallis tests. *p*-values were FDR-corrected for multiple comparisons. Bold *p*-values indicate significant difference.

and relationship status. Finally, for comparison of different HC types, 94 out of 428 COC users were selected as comparison group for 94 IUD users based on propensity scores for belonging to the IUD group based on age, language, sexual orientation, relationship status, education, and employment status. Here education and employment status were included since matching based on age alone did not eliminate differences in education and employment status. Demographics were compared between groups using X^2-tests in case of nominal scales, Mann-Whitney-*U*-Tests or Kruskall-Wallis-tests in case of ordinal scales, as well as *t*-tests or one-way-ANOVAs in case of continuous scales and *p*-values were FDR-corrected for multiple comparisons.

In order to assess whether personality and/or gender role related to participant's choice to use HC, personality measures and gender role measures were compared using one-way ANOVAs between current HC-users, previous HC-users and never-users of HC. Independent samples *t*-tests were used to compare potential future HC-users and women, who said future HC-use was not an option for them, as well as IUD users and their matched group of COC-users. An FDR-correction of *p*-values for multiple comparisons was employed across all scales. Differences between sub-factors were explored, when a significant difference in the main scale was observed. As measures of effect size, η^2 or Cohen's d was calculated for each scale. In case significant differences were observed, we additionally calculated Mahalanobis D, which is a multivariate measure of effect size (Del Giudice, 2017) and has been previously used to compare the difference in personality profiles between groups (Del Giudice et al., 2012). Like in these previous studies, Mahalanobis D was calculated based on the averaged covariance matrix of both groups using the *maha* function of the *GenAlgo* packages in R 4.0.5 (Mardia et al., 1979). Exploratory *t*-tests were performed to compare personality, gender role and demographics between previous HC-users with and without emotional side effect. The dataset is available upon request from the corresponding author.

Results

Demographics

Demographic information of current, previous and never-users of HC prior to and after matching can be found in **Tables 1**, **2**, respectively. Prior to matching, previous HC-users were on average older than current users, current and previous users were more likely in a relationship and more never-users and previous users had children than current users. These data correspond to women's contraceptive history, with contraceptive use during adolescence and young adulthood followed by a period of family planning. Furthermore, HC-use was more common among heterosexual than homosexual women. Average age was around 25 years, education level was generally high and increased with older age and employment status varied. Accordingly, the sample was representative of the university population from which participants were recruited. After matching, no differences between the current, previous and never-users of HC remained in demographic variables. Comparisons of prospective HC-users and their matched group of women, who do not intend to ever use HC, as well as current IUD-users and their matched group of current COC-users can be found in **Supplementary Tables 1**, **2**, respectively. After matching, no differences in demographic variables were observed between the groups, with the exception of women for whom HC-use was a future option being in a relationship significantly more often than women who do not intend to ever use HC.

Health variables, hormonal contraceptive-use and side effects

Comparison of health variables and HC characteristics between current, previous and never-users are summarized in **Table 3**. Health information for prospective HC-users and IUD-users is included in **Supplementary Table 2**. Groups did not differ in health variables, with the exception of alcohol consumption being more common among current and previous HC-users compared to never users.

Current users had used significantly more different HCs than previous users, had used HC for a longer period of time and had started their HC later, resulting in more adolescent starters (before the age of 21) than previous users. Use of COC was significantly more common among previous users compared to current users, while IUD-use was significantly more common among current users. However, when also considering the contraceptives previously used by current users, 91% of those not currently on COC had previously used COC.

Current users reported significantly more commonly that they had started HC-use when they entered a new relationship, though contraception was the most common reason for HC-use in both groups. Current users reported more HC-use for the treatment of gynecological problems like menstrual pain, polycystic ovary syndrome (PCOS) or endometriosis, while previous HC-users reported more HC-use for non-contraceptive benefits, like menstrual cycle control or the treatment of acne. Previous users also listed significantly more other reasons for their HC-use, including non-specified medical reasons, and advice to use HC by parents or gynecologists.

Side effects were significantly more common among previous users than current users. The most pronounced difference emerged for psychological side effects (mood swings, depressed mood, anxiety, and irritability), even though neither current users nor previous users were specifically asked for psychological side effects. While 3% of current users reported psychological side effects of their own accord, 54% of the

TABLE 2 Comparison of demographic variables between current and previous HC-users and never users matched for age, language, sexual orientation, and relationship status.

		Never-users (n = 321)	Previous users (n = 321)	Current users (n = 321)	Comparison
Language[a]	Lithuanian	97 (17%)	71 (22%)	94 (29%)	p = 0.248
	German	224 (83%)	250 (78%)	227 (71%)	
Age[b]		24.85 ± 5.82	24.98 ± 5.56	24.85 ± 6.90	p = 0.954
Handedness[a]	Left-handed	35 (11%)	28 (9%)	24 (7%)	p = 0.441
Education[c]	Apprenticeship	3 (1%)	4 (1%)	2 (1%)	p = 0.248
	Middle school	4 (1%)	5 (1%)	11 (3%)	
	High school	143 (46%)	136 (43%)	155 (48%)	
	University	158 (51%)	175 (55%)	143 (45%)	
	Unknown	13 (4%)	1 (0%)	2 (0%)	
Employment status[c]	Employed full time	67 (21%)	71 (22%)	90 (28%)	p = 0.248
	Employed part time	51 (16%)	46 (14%)	64 (20%)	
	In education + part time	31 (10%)	63 (20%)	22 (7%)	
	In education + unempl.	62 (19%)	40 (12%)	63 (20%)	
	Unemployed	110 (34%)	101 (32%)	104 (32%)	
Sexual orientation[a]	Homosexual	18 (6%)	13 (4%)	18 (6%)	p = 0.297
	Bisexual	72 (22%)	92 (29%)	109 (34%)	
	Heterosexual	231 (72%)	216 (67%)	194 (60%)	
Relationship	In a relationship[a]	179 (56%)	205 (64%)	188 (59%)	p = 0.248
	Duration[b]	5.06 ± 4.72	4.28 ± 4.66	5.28 ± 5.88	p = 0.248
	Satisfaction[b]	8.60 ± 1.65	8.52 ± 1.45	8.46 ± 1.55	p = 0.863
Children[a]		42 (13%)	37 (12%)	40 (12%)	p = 0.954

[a]binary variables, compared via X^2-Tests, [b]continuous variable, compared via one-way-ANOVAs, [c]ranked variables, compared via Kruskall-Wallis tests. p-values were FDR-corrected for multiple comparisons.

previous users listed psychological side effects, most commonly also as reason not to use HCs. Furthermore, weight changes and other medical side effects (hair loss, swollen legs, vaginal dryness, edema, thrombosis, etc.) were significantly more common among previous users compared to current users. Loss of libido and physical side effects (headaches, nausea, and breast pain) were comparable between current and previous HC-users, while skin changes were more commonly reported in current OC-users. However, 2% of current users listed positive rather than negative skin changes.

Regarding the reasons not to use HC among current non-users, never-users more frequently reported concern about potential side effects or a general opposition to hormones, while previous users mostly listed negative experiences with HC as reason not to use HC again. The percentage of prospective future HC-users was comparable among never-users and previous users.

Personality and choice of hormonal contraceptive

For the HEXACO-60, average scale scores were slightly above the scale mean of 3 (honesty/humility: 3.88 ± 0.43; emotionality: 3.59 ± 0.48, extraversion: 3.44 ± 0.48, agreeableness: 3.45 ± 0.47, conscientiousness: 3.86 ± 0.45, openness: 3.62 ± 0.42), which is in accordance with values reported for women in the original English version of the HEXACO-60 (Ashton and Lee, 2009), as well as the German and Lithuanian versions of the HEXACO-60 (Truskauskaitė-Kunevičienė et al., 2012; Moshagen et al., 2014). Accordingly, the sample is representative with respect to personality.

Significant differences between current, previous and never users of HC in personality and gender role emerged only with respect to the Honesty-Humility scale of the HEXACO-60 (see **Table 4**). Subscale-analyses revealed that this difference was driven by the Greed-Avoidance subscale. Sidak *post hoc* comparisons revealed that the difference emerged between current users and never-users (Honesty-Humility: p = 0.002; Greed Avoidance: p < 0.001), with previous users taking intermediate values with no significant differences to the other groups (all p < 0.150). Note, however, that the effect size for this difference was small (Honesty-Humility: η^2 = 0.012, Greed Avoidance: η^2 = 0.015). Mahalanobis D also amounted to very small effects sizes of 0.07 for comparison of previous to never-users and 0.15 for comparison of current to never-users.

Furthermore, no significant differences in personality or gender role were observed between non-users, who saw HC-use as a viable option for the future and non-users, who were strictly opposed to HC-use (compare **Table 5**).

TABLE 3 Comparison of health variables and HC-characteristics between current and previous HC-users and never users matched for age, language, sexual orientation, and relationship status.

		Never-users (n = 321)	Previous users (n = 321)	Current users (n = 321)	Comparison
Health[a]	Smokers	28 (9%)	43 (14%)	29 (9%)	$p = 0.325$
	Alcohol	132 (41%)	179 (55%)	195 (61%)	$\mathbf{p < 0.001}$
	Medication	35 (11%)	46 (14%)	50 (16%)	$p = 0.325$
	Neurological disorder	6 (2%)	7 (2%)	3 (1%)	$p = 0.493$
	Psychological disorder	28 (9%)	42 (13%)	24 (7%)	$p = 0.325$
	Endocrine disorder	23 (7%)	38 (12%)	20 (6%)	$p = 0.092$
	Heart disease	8 (3%)	5 (2%)	10 (3%)	$p = 0.493$
	Stress	142 (44%)	142 (44%)	155 (48%)	$p = 0.493$
Characteristics of HC-use	Number of different HC[b]	N/A	1.12 ± 0.34	1.36 ± 0.58	$\mathbf{p < 0.001}$
	Duration of use[b]		3.36 ± 2.69	4.73 ± 4.45	$\mathbf{p < 0.001}$
	Age at first use[b]		17.81 ± 3.03	19.45 ± 5.30	$\mathbf{p < 0.001}$
	Time since discontin.		4.04 ± 4.31	N/A	
	Start dur. adolescence[a]		263 (82%)	247 (77%)	$\mathbf{p = 0.010}$
	Start with relationship[a]		82 (25%)	135 (42%)	$\mathbf{p < 0.001}$
Type of HC[a]	COC	N/A	306 (95%)	233 (73%)	$\mathbf{p < 0.001}$
	IUD		1 (0%)	57 (18%)	
	Ring		7 (2%)	22 (7%)	
	Other		4 (1%)	9 (3%)	
Main reason for HC-use[a]	Contraception	N/A	223 (69%)	212 (66%)	$\mathbf{p < 0.001}$
	Menstrual pain		25 (7%)	65 (20%)	
	Menstrual cycle control		22 (7%)	6 (2%)	
	Acne		19 (6%)	12 (4%)	
	PCOS		8 (2%)	11 (3%)	
	Endometriosis		2 (1%)	10 (3%)	
	PMS		2 (1%)	0 (0%)	
	Other		12 (4%)	5 (2%)	
Side effects[a]	Psychological	N/A	174 (54%)	9 (3%)	$\mathbf{p < 0.001}$
	Weight changes		72 (22%)	2 (1%)	$\mathbf{p < 0.001}$
	Other		62 (19%)	3 (1%)	$\mathbf{p < 0.001}$
	Loss of libido		70 (22%)	74 (23%)	$P = 0.958$
	Bleeding		30 (9%)	39 (12%)	$P = 0.398$
	Headaches		33 (10%)	43 (13%)	$p = 0.390$
	Nausea		28 (9%)	24 (7%)	$p = 0.477$
	Breast pain		13 (4%)	25 (8%)	$P = 0.084$
	Skin changes		20 (6%)	42 (13%)	$\mathbf{p = 0.010}$
Reason not to use HCs[a]	No need	53 (17%)	31 (9%)	N/A	$\mathbf{p < 0.001}$
	Medical reasons	10 (3%)	19 (6%)		
	Negative experiences	N/A	186 (58%)		
	Worried about side/long-term effects	220 (69%)	69 (22%)		
	Opposed to hormones	36 (11%)	15 (4%)		
	Other	1 (0%)	3 (1%)		
Future HC-use optional[a]		41 (13%)	24 (8%)	N/A	$p = 0.082$

[a]Binary variables compared via X^2-tests, [b]continuous variables compared via t-tests. p-values were FDR-corrected for multiple comparisons, separately for health variables, HC-characteristics and discontinuation characteristics. Bold p-values indicate significant difference.

We did notice, however, that due to the smaller sample size, effect sizes for this comparison were in part larger than effect sizes for the comparisons of current HC-users and non-users, which yielded significant results. Among the personality dimensions, effect sizes were larger than 0.20 for Honesty-Humility and conscientiousness. Honesty-Humility

TABLE 4 Personality differences between current, previous, and never-users of HC.

	Never users (n = 321)		Previous users (n = 321)		Current users (n = 321)		Comparison			
	Mean	SD	Mean	SD	Mean	SD	F	P	p_{FDR}	η^2
Honesty-humility	**3.93**	**0.43**	**3.87**	**0.42**	**3.82**	**0.41**	**5.94**	**0.003**	**0.021**	**0.012**
Greed avoidance	**3.67**	**0.69**	**3.57**	**0.66**	**3.47**	**0.63**	**7.52**	**0.001**	**0.014**	**0.015**
Fairness	4.11	0.62	4.06	0.61	3.98	0.62	3.98	0.019	0.077	0.008
Sincerity	3.69	0.79	3.64	0.81	3.62	0.78	0.75	0.472	0.601	0.002
Modesty	4.28	0.57	4.26	0.62	4.22	0.57	0.73	0.481	0.601	0.002
Emotionality	3.54	0.48	3.57	0.49	3.59	0.45	0.85	0.426	0.601	0.002
Extraversion	3.38	0.48	3.45	0.47	3.42	0.51	1.64	0.194	0.452	0.003
Agreeableness	3.51	0.47	3.46	0.46	3.41	0.47	3.84	0.022	0.077	0.008
Conscientiousness	3.83	0.47	3.86	0.42	3.88	0.46	0.98	0.377	0.601	0.002
Openness	3.63	0.42	3.65	0.41	3.62	0.43	0.58	0.558	0.601	0.001
GERAS_femininity[a]	4.91	0.58	4.93	0.61	4.90	0.59	0.21	0.808	0.808	<0.001
GERAS_masculinity[a]	3.97	0.64	3.98	0.66	4.03	0.70	0.76	0.470	0.601	0.002
SIS_femininity[a]	6.39	1.38	6.52	1.50	6.43	1.50	0.60	0.548	0.601	0.001
SIS_masculinity[a]	2.57	1.56	2.55	1.69	2.81	1.79	2.24	0.107	0.300	0.005

[a]Please not that gender role ratings were missing from 6 current users, 13 previous users and 8 never-users. GERAS, Gender-related attributes questionnaire; SIS, Six-Item-Scale; pFDR, FDR-corrected p-value; η^2, estimate of effect size.

TABLE 5 Personality differences between non-users who did and did not see the use of HC as a potential future option matched for age, language, sexual orientation, and relationship status.

	HC no opt. (n = 73)		HC option (n = 73)		Comparison		
	Mean	SD	Mean	SD	t	p	d
Honesty-humility	**3.99**	**0.39**	**3.87**	**0.43**	**1.75**	**0.082**	**0.29**
Emotionality	3.52	0.50	3.59	0.51	−0.74	0.463	0.12
Extraversion	3.39	0.40	3.36	0.46	0.42	0.673	0.07
Agreeableness	3.51	0.49	3.55	0.50	−0.45	0.650	0.08
Conscientiousness	**3.81**	**0.46**	**3.94**	**0.49**	**−1.61**	**0.109**	**0.27**
Openness	3.61	0.40	3.59	0.39	0.23	0.819	0.04
GERAS_femininity[a]	4.88	0.55	4.82	0.57	0.62	0.538	0.11
GERAS_masculinity[a]	4.05	0.62	3.89	0.63	1.46	0.145	0.26
SIS_femininity[a]	6.70	1.40	6.36	1.62	1.31	0.192	0.22
SIS_masculinity[a]	2.30	1.70	2.51	1.65	−0.77	0.444	0.13

[a]Please note that gender role ratings were missing from 2 women in each group. GERAS, Gender-related attributes questionnaire; SIS, Six-Item-Scale; d, Cohen's d.

was higher in women, for whom future HC-use was not an option, while conscientiousness was higher in women, for whom future HC-use was an option. Mahalanobis D for this comparison was 0.24, which also corresponds to a small effect size.

Finally, the comparison of current IUD-users and a matched sample of COC-users demonstrated significantly higher extraversion (sociability) and significantly higher agreeableness (forgiveness) among IUD-users compared to COC-users. Effect sizes for these comparisons were moderate with Cohen's d ranging from 0.38 to 0.49. Likewise, Mahalanobis D across all HEXACO-60 dimensions was 0.45 for this comparison (compare Table 6).

Side effects and personality

An exploratory comparison of previous HC-users with and without emotional side effects, revealed no significant differences in personality or gender role with no effect sizes larger than 0.20 (all $t < 1.80$, all $p > 0.07$). However, we did observe a number of interesting demographic differences between women with previous emotional symptoms and women without previous emotional symptoms (compare Table 7). Previous adverse emotional side effects were significantly more often reported by women from Germany and Austria than women from Lithuania. Also, women who reported previous adverse emotional side effects were

TABLE 6 Personality differences between IUD-users and COC-users matched for age, language, sexual orientation, relationship status, education, and employment status.

	COC (n = 94)		IUD (n = 94)		Comparison			
	Mean	SD	Mean	SD	t	p	p_{FDR}	d
Honesty-humility	3.83	0.37	3.89	0.44	−0.91	0.344	0.476	−0.15
Emotionality	3.51	0.43	3.57	0.51	−0.84	0.404	0.519	−0.13
Extraversion	**3.34**	**0.45**	**3.57**	**0.60**	**−2.99**	**0.003**	**0.018**	**−0.43**
Self esteem	3.80	0.50	3.90	0.61	−1.22	0.223	0.365	−0.18
Social boldness	3.21	0.72	3.38	0.79	−1.58	0.116	0.236	−0.22
Sociability	**2.61**	**0.95**	**3.11**	**1.10**	**−3.38**	**0.001**	**0.018**	**−0.49**
Liveliness	3.56	0.60	3.80	0.73	−2.46	0.015	0.054	−0.36
Agreeableness	**3.30**	**0.48**	**3.50**	**0.42**	**−2.98**	**0.003**	**0.018**	**−0.44**
Forgiveness	**2.54**	**0.98**	**2.91**	**0.98**	**−2.59**	**0.010**	**0.045**	**−0.38**
Patience	3.65	0.80	3.88	0.67	−2.12	0.035	0.105	−0.31
Gentleness	3.30	0.66	3.45	0.62	−1.59	0.114	0.236	−0.23
Flexibility	3.57	0.50	3.68	0.47	−1.46	0.147	0.265	−0.23
Conscientiousness	3.89	0.46	3.84	0.45	0.77	0.441	0.529	0.11
Openness	3.58	0.40	3.65	0.41	−1.11	0.268	0.402	−0.17
GERAS_femininity[a]	4.85	0.63	4.89	0.60	−0.37	0.713	0.802	−0.07
GERAS_masculinity[a]	3.91	0.68	4.06	0.66	−1.57	0.118	0.236	−0.22
SIS_femininity[a]	6.50	1.33	6.53	1.63	−0.16	0.875	0.926	−0.02
SIS_masculinity[a]	2.84	1.79	2.82	1.80	0.06	0.951	0.951	0.01

[a]Please note that gender role ratings were missing from 1 woman in each group. GERAS, Gender-related attributes questionnaire; SIS, Six-Item-Scale; d, Cohen's d; COC, combined oral contraceptives; IUD, intra-uterine device.

significantly younger and accordingly had lower education, shorter relationship durations and fewer children. Interestingly, the two groups did not differ in any of the health variables, including psychological disorders and stress and no differences were observed in the reasons for HC-use.

Discussion

The aim of the present study, was to identify personality factors associated with women's choice to use or not use HC and the type of HC chosen. The results demonstrate only a very weak association between the willingness to use or not use HC and the *Greed Avoidance* subscale of the *Honesty-Humility* scale, while no association between the classical BIG-5 personality factors (emotionality/neuroticism, extraversion, agreeableness, conscientiousness, openness) or gender role and HC-use was observed. However, participant's personality profile was significantly associated with the type of HC chosen. We observed higher agreeableness and extraversion in users of IUD compared to users of COC. In the following we will first discuss the personality characteristics associated with HC-use and HC-type in more detail and then discuss our exploratory findings regarding adverse emotional side effects.

The fact that participants willingness to use HC was not associated with the BIG-5 or gender role, is in contrast to previous studies suggesting association between HC-use and neuroticism or extraversion (Beard et al., 1974; Priestnall et al., 1978; Jacobsson et al., 1981; Ross et al., 2001), as well as femininity (Pletzer et al., 2015). However, these studies did not differentiate between never users, previous users, and prospective users in the group of naturally cycling women, had smaller sample sizes and did not match HC-users and naturally cycling women for demographic variables or relationship status. Furthermore, the most recent studies reported no associations between HC-use and personality on the one hand (Beltz et al., 2019) or gender role on the other hand (Nielson and Beltz, 2021). These results suggest that if demographic variables and relationship status are controlled for, personality and gender role do not present additional confounds for neurocognitive research on HC. The exception is a small association between the willingness to use HC and lower scores on the *Greed Avoidance* subscale of the *Honesty-Humility* scale. According to the HEXACO authors, *Greed Avoidance* assesses a tendency to be uninterested in signs of high social status (Lee and Ashton, 2009). In the items associated with this scale, current and prospective HC-users reported a higher interest in money and luxury goods compared to non-users. Though only a speculation, one explanation for this finding could be the socio-economic consequences of an unplanned pregnancy (Lersch et al., 2017).

Regarding HC type, IUD-users score higher on the *Forgiveness* subscale of the *Agreeableness* scale and the *Sociability* subscale of the *Extraversion* scale. The findings on both scales

TABLE 7 Comparison of previous HC-users with and without emotional symptoms along demographic variables.

		No previous mood symptoms (n = 217)	Previous mood symptoms (n = 254)	Comparison
Language	Lithuanian	61 (28%)	26 (10%)	$\mathbf{p < 0.001}$
	German	156 (72%)	228 (90%)	
Age		29.31 ± 7.09	25.64 ± 5.41	$\mathbf{p < 0.001}$
Handedness	Left-handed	13 (6%)	27 (11%)	$p = 0.174$
Education	Apprenticeship	4 (2%)	5 (2%)	$p = 0.017$
	Middle school	2 (1%)	2 (1%)	
	High school	55 (26%)	97 (38%)	
	University	154 (71%)	150 (59%)	
	Unknown	1 (0%)	0 (0%)	
Employment status	Employed full time	77 (36%)	58 (23%)	$p = 0.077$
	Employed part time	47 (22%)	57 (22%)	
	In education + Minor employment	22 (10%)	46 (18%)	
	In education + no employment	19 (9%)	34 (13%)	
	Unemployed	52 (24%)	59 (23%)	
Sexual orientation	Homosexual	10 (5%)	4 (2%)	$p = 0.700$
	Bisexual	44 (20%)	70 (28%)	
	Heterosexual	163 (75%)	180 (71%)	
Relationship	In a relationship	162 (75%)	188 (74%)	$p = 1.000$
	Duration	7.13 ± 5.93	4.45 ± 4.88	$\mathbf{p < 0.001}$
	Satisfaction	8.58 ± 1.56	8.52 ± 1.50	$p = 0.880$
Children		58 (27%)	26 (10%)	$p < 0.001$
Health	Smokers	30 (14%)	25 (10%)	$p = 0.303$
	Alcohol	122 (56%)	135 (53%)	$p = 0.700$
	Medication	45 (21%)	36 (14%)	$p = 0.149$
	Neurological disorder	10 (5%)	4 (2%)	$p = 0.149$
	Psychological disorder	26 (12%)	32 (13%)	$p = 1.000$
	Endocrine disorder	36 (17%)	30 (12%)	$p = 0.242$
	Heart disease	4 (2%)	5 (2%)	$p = 1.000$
	Stress	101 (47%)	119 (47%)	$p = 1.000$
Previous HC-use	Duration of use	4.72 ± 3.88	4.27 ± 3.45	$p = 0.185$
	Age at first use	18.47 ± 4.09	17.84 ± 3.21	$p = 0.164$
	Adolescent start (<21)	159 (78%)	211 (85%)	$p = 0.149$
	Time since discontin.	6.05 ± 5.81	4.01 ± 3.65	$\mathbf{p < 0.001}$
Reason for Previous HC-Use	Contraception	145 (67%)	194 (76%)	$p = 0.164$
	Menstrual cycle control	19 (9%)	9 (4%)	
	Menstrual pain	15 (7%)	18 (7%)	
	Acne	11 (5%)	16 (6%)	
	Other	17 (8%)	12 (5%)	
Reasons not to use HCs	No need	39 (18%)	6 (2%)	$\mathbf{p < 0.001}$
	Medical reasons	17 (8%)	12 (5%)	
	Negative experiences	76 (35%)	202 (80%)	
	Worry about side-effects	61 (28%)	27 (11%)	
	Opposed to hormones	22 (10%)	4 (2%)	
	Other	2 (1%)	3 (1%)	

P-values were FDR-corrected for multiple comparisons. Bold *p*-values indicate significant difference.

may be related as they both hint at a more positive attitude toward social interactions in IUD-users compared to COC-users. According to the HEXACO authors, people with high sociability scores enjoy talking, visiting, and celebrating with others. A more permanent HC option may facilitate the participation in a variety of social activities without having

to remember the daily intake regimen all the time. In light of this interpretation, it is an interesting observation, that the other personality factor that we hypothesized to differ, i.e., conscientiousness, does not appear to contribute to the choice of HC type.

It is noteworthy, that the difference in *Greed Avoidance* emerged only between current users of HC and never users of HC, not between previous users and never-users. A trend in the same direction was also observed for prospective users of HC. This hints at a slightly stronger association between personality characteristics and the willingness to use HC now as compared to about 10 years ago, when previous users had on average started HC (compare **Table 3**). This result fits with the observation of a shift in attitudes toward HC (Fiala and Parzer, 2019; Svahn et al., 2021). While 10 years ago, HC was the standard contraceptive choice in Europe and the US, women consider their contraceptive options more carefully today. This interpretation is in line with the results, that current users take their HC primarily for contraceptive and gynecological reasons, while previous users also list a number of non-contraceptive benefits, like menstrual cycle regulation or the treatment of acne. Furthermore, previous users frequently name parents and gynecologists to have recommended HC-use among other reasons to use HC, while current non-users frequently list friends who recommended not to use HC among other reasons not to use HC. The reasons listed not to use HC included negative experiences, worry about side effects, medical reasons or an opposition to synthetic hormones. This compares to a recent systematic review on the reasons for rejecting HC in western countries (Le Guen et al., 2021). Interestingly, while adverse emotional side effects are frequently named by current and previous users of HC, the treatment of premenstrual syndrome (PMS) is rarely mentioned as a reason for HC-use.

We did indeed observe some interesting results regarding emotional side effects of HC, although information on adverse mood effects was not specifically requested from the participants, but entered of their own accord in an open answer field. Adverse emotional side effects were reported significantly more often by previous users than current users. While the frequency of emotional side effects in current users, i.e., 3%, is a little lower than the rate observed in prospective randomized controlled trials (Lundin et al., 2017), more than half of the previous users report mood swings, depressed mood, increased emotionality, irritability, and/or anxiety. This observation is in line with various studies demonstrating reduced positive affect and altered stress responsivity in HC-users (Sanders et al., 2001; Nielsen et al., 2013, 2014; Lewis et al., 2019; Gervasio et al., 2022) and that these side effects have been associated with discontinuing usage (Lindh et al., 2009; Sundström-Poromaa and Segebladh, 2012; Sundström-Poromaa, 2021). Indeed, adverse side effects were often cited as reason not to use HC and not to consider HC-use in the future in the current sample. Accordingly, our observation of higher adverse mood symptoms among previous users compared to current users may be reflective of the well-known "survivor-effect" (Oinonen and Mazmanian, 2002). Due to the retrospective nature of this study, the mechanisms underlying these associations between HC-use and emotional side effects remain to be elucidated. Please note also, that this questionnaire was administered during the Covid-19 pandemic to a sample consisting of mostly university students. Therefore, though data were collected during a time when no COVID-lockdowns were in place in any of the participating countries, the added stress of the pandemic, distance learning and online examinations may have contributed to the reporting of adverse mood effects.

Finally, the socio-demographic differences between women, who did and did not report previous adverse mood effects suggest a shift in how often adverse mood symptoms are attributed to HC. For example, previous HC-users in Lithuania report less adverse mood effects than those in Austria and Germany. Furthermore, younger women, who discontinued their HC more recently (compare **Table 7**) report more adverse mood effects. Apparently, the experience/reporting of adverse mood effects is susceptible to cultural and generational context. It appears that the number of negative mood symptoms attributed to HC has increased. Whether this observation is reflective of increased education about side effects and different contraceptive options resulting in different perceptions of HC across generations or a shift in the prescribed HC formulations with different side effect profiles cannot be determined based on these retrospective reports. Importantly, age at first HC-use did not differ significantly between previous users with and without emotional side effects. This is in contrast to previous observational studies suggesting stronger emotional side effects in adolescent starters (e.g., Skovlund et al., 2016).

There are several methodological aspects that are important to consider when interpreting the results of present study. First, the current study relied on a comparably small sample size remaining for the assessment of future attitudes toward HC and HC type. A more convincing connection between personality and women's attitudes toward HC could have been obtained from reliable results about future contraceptive choices. It is remarkable though, that the vast majority of women, who don't use HC at the moment (∼90%), do not consider future HC-use as an option. It appears that the majority of women, who have neutral or positive attitudes toward HC, are already using them. Negative attitudes toward HC, however, primarily stem from concerns about their adverse effects, either due to personal negative experiences or from reports of others (friends, media, etc.). The large number of current HC-users, previous users and never-users and the close matching for demographic differences among these groups is, however, a major strength of the current study. Second, the sample of our study represents mostly the university population living in Austria, Germany, and Lithuania. Therefore, the absence of full demographic data regarding age, race/ethnicity, and socioeconomic status

may limit generalizability. Third, despite the broad range of associations evaluated in the present study, we are not able to conclude whether the reported adverse mood effects were causally related to HC-use. A longitudinal study is needed for such evaluation. Finally, we were unable to control the environment and time spent to fill in questionnaires due to the setup of the study as an online survey.

In summary, associations between personality and the choice to use or not use HC were negligible, though the type of HC chosen was associated with personality traits. Accordingly, we do not expect confounding effects of personality on neurocognitive experiments regarding COC, provided that other demographic differences between COC-users and non-users are well controlled for. Cross-sectional studies comparing IUD-users and COC-users may, however, consider to take personality into account.

Data availability statement

The raw data supporting the conclusions of this article are publicly available at https://osf.io/rmauq/.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the University of Salzburg. The patients/participants provided their written informed consent to participate in this study.

Author contributions

BP, BD, and RG designed the study. CL created the online questionnaire. BP and RG organized the Lithuanian translation of the questionnaire. BP, BD, RG, and CL acquired the data. BP analyzed the data and wrote the first draft of the manuscript, which was revised and approved by BD and RG. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.898487/full#supplementary-material

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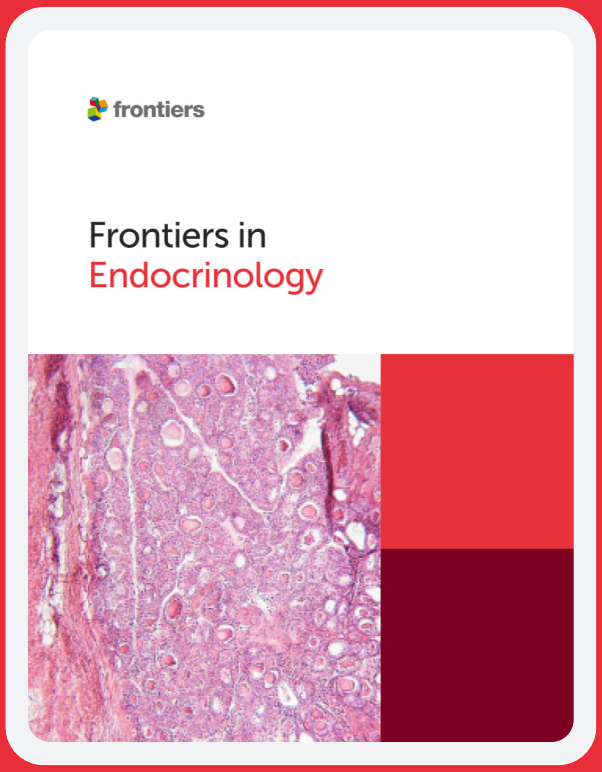

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