ACUTE EFFECTS OF ESTROGEN AND BISPHENOL-A, AN ENVIRONMENTAL CHEMICAL, ON COGNITIVE AND NEURAL FUNCTION

By

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Abstract

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Many natural and synthetic chemicals in environment can mimic or antagonize the effects of endogenous hormones. Bisphenol A (BPA) is one such chemical, with mixed estrogen agonist/antagonist properties. Recent evidence indicates that estrogen and BPA may exert effects on brain functions not only through genomic but also through non-genomic pathways, activating membrane-associcated estrogen receptors. Because at present little is known about how acute BPA may interact with estrogen in the adult brain and affect estrogen mediated behaviors such as memory and learning, this research examined acute effects of estradiol (E2) and BPA, alone, and in combination, on behavior (memory consolidation) and neural function (spine density and monoamine levels) in adult ovariectomized female rats. For behavioral study, acute 17β- and 17α-E2 treatment effects on spatial and non-spatial memory consolidation were tested using object placement (OP) and object recognition (OR) tasks. Both isomers of estradiol facilitated memory consolidation, but enhancement occurred in a time-, dose-, and task specific manner. The dose-response relationship was an inverted-U for both tasks. Co-administration of BPA blocked E2-induced OP memory enhancement far below the current reference safe dose of 50μg/kg/day. A larger BPA dose was needed to

block 17α-E2 induced OR memory enhancement. BPA alone had no effect on OP memory, but high doses enhanced OR memory.

To examine possible neural systems that may contribute to acute E2 and BPA treatment effects, brains were removed for neuromorphological and neurochemical analysis. Golgi impregnation 30 minutes after acute treatments showed that E2 increased apical and basal dendritic spine density in pyramidal neurons of of the prefrontal cortex (PFC), and basal, but not apical, spine density in pyramidal neurons of CA1 region of the hippocampus. Co-administration of BPA with E2 further increased apical and basal spine density in both the PFC and the CA1. Golgi impregnation 4 hours after acute treatments found that co-administration of BPA significantly suppressed E2-induced basal CA1spine density. Neurochemical analyses revealed that acute estrogen increased monoamine and metabolite levels in the PFC, but decreased these chemicals in the hippocampus. Co-administration of BPA further increased monoamine and metabolite concentrations in the PFC.

In summary, the current data provide new information about acute effects of estrogen and BPA on memory and brain function. BPA interacts with E2 at very low doses, and rapidly alters behavioral and neural response to E2. These findings demonstrate BPA behavioral changes at very low doses in the membrane environment and suggest low level exposure of BPA may have a powerful, negative impact, rapidly altering behavioral and neural responses to endogenous estrogen.

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Table of Contents

Title page……..i

Copyright page…….ii

Approval page…….iii

Abstract……...iv

Acknowledgements…….vi

Table of Contents……...vii

List of Tables……..ix

List of Figures……..x

Introduction

Hormones and environmental chemicals…….1

Bisphenol A as an environmental estrogen……..……1

Sensitivity of the developing brain to BPA…….……4

Classic estrogen receptors and distribution…….5

Membrane ER and membrane-initiated action……6

Integration of genomic and nongenomic action……...……7

Estrogen effects on cognitive function……..10

Chronic estrogen effects on memory in laboratory animals……..11

Acute estrogen treatment effects on memory 13

Mechanisms underlying estrogen effect on memory...…….……14

BPA effects on behavior …….……..16

Membrane initiated action of BPA …….……..17

Unique characteristics of BPA action in the brain...…….…….19

BPA and non-monotonic dose-response curves……..……..20

BPA effects in combination with physiological estrogen……..22

Some critical questions about environmental chemicals……..……..25

Specific Research aims…….…….26

Behavioral Study……30
Experiment 1
Effects of acute estrogen treatment on memory consolidation……..……30
Method…….. 32
Results …….. 40
Discussion …… 53
Experiment 2
Acute estrogen and BPA treatment effects, alone and in combination, on memory consolidation and anxiety levels……..……...……...62
Method…….. 63
Results …….. 71
Discussion …… 86

Brain Study …….. 92
Experiment 3
Acute effects of estrogen, BPA+estrogen on dendritic spine density……93
Method…….. 94
Results ……..100
Discussion ……110
Experiment 4
Aute efects of estrogen, BPA and BPA+estrogen treatments on monoamine levels..……...……...……...120
Method…….. 121
Results …….. 124
Discussion …….137

Summary and Conclusion..……..143

References……147

List of Tables

1. Summary of 17β- and 17α-estradiol doses used for the object placement (OP) and recognition (OR) task. ……..……...……....37

2. Summary of groups, treatments and BPA doses used for Experiment 2…….……...67

3. Hormone and BPA treatments used for the anxiety test.….....….68

4. Summary of acute estradiol and BPA treatment effects on memory consolidation...86

5. Hormone and BPA treatment used for spine density and serum estradiol levels.…..94

6. Acute effects of estradiol and BPA+E2 on spine density 30 min and 4 hr after treatment.…...……104

7. Serum estradiol concentrations 30 min and 4 hr after acute treatment…….109

8. Hormone and BPA treatment used for neurochemial analysis ……....122

9. Summary of ANOVA results, *df*, *F* and *p* values for neurochemical analysis…….125

10. Summary of acute E2 and BPA treatment effects on dopamine, metabolite and turnover levels…….……...……..……126

11. Summary of acute E2 and BPA treatment effects on norepinephrine, serotonin, metabolite and turnover levels……...……..……..……....127

List of Figures

1. The chemical structures of estrogen and related compounds ……...…….3

2. Schematic representation of genomic and nongenomic actions and cross talk…...….. 9

3. Non-monotonic dose-response relationships ……...21

4. Synaptic plasticity induced by 17β-estradiol and BPA …...……24

5. The open field chamber and objects for memory tests..…….……..35

6. Timeline for acute post-sampling hormone treatment….……..……...38

7. Acute effects of 17β-E2 on object placement (OP).…...……..43

8. Dose-response for 17β-E2 (object placement)...……..44

9. Immediate vs. delayed post-T1 treatment (object Placement) ……....45

10. Acute effects of 17β-E2 on object recognition (OR).…….…….47

11. Dose-response for 17β-E2 (Object Recognition) ……..48

12. Dose-response for 17α-E2 (Object Placement) …..……50

13. Dose-response for 17α-E2 (Object Recognition)……52

14. Timeline for the two-time point treatment paradigm……...……....66

15. Apparatus used for elevated plus maze …….…..69

16. Effects of BPA alone on memory (exploration ratios) ..……72

17. Effects of BPA alone treatment on OR memory (T2 old vs. T2 New)……..73

18. Effects of BPA+ 17β-E2 on OP memory (exploration ratios) ……..76

19. Effects of BPA+ 17β-E2 on OP memory (T2 old vs. New) …….……....77

20. Effects of BPA+ 17β-E2 on OR memory (exploration ratios). ……...……...78

21. Effects of BPA+ 17α-E2 on OP memory (exploration ratios)…….……..….81

22. Effects of BPA+ 17α-E2 on OP memory (T2 old vs. New)……82

23. Effects of BPA+ 17α-E2 on OR memory. ……..…..83

24. Effect of E2, BPA and their combination on elevated plus maze……...…….85

25. Treatment schedule for neuromorphlogical and neurochemical analyses ……..98

26. Diagram of a pyramidal neuron…….……..99

27. Acute effect of 17β-E2 and BPA on spine density in the PFC and CA1 of the hippocampus: 30 min after treatment……....105

28. Acute effect of 17β-E2 and BPA on spine density in the PFC and CA1 of the hippocampus: 4 hr after treatment……...……..106

29. Acute effect of 17β-E2 and BPA on spine density: 30min vs. 4hr after treatment...107

30. Response patterns in spine density for E2 and BPA+E2 groups: 30 min vs. 4 hr after treatment……....108

31. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in the prefrontal cortex……..……..…131

32 Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in CA1 of the hippocampus…….…..132

33. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in CA3 of the hippocampus…….……..133

34. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in the dentate gyrus of the hippocampus…….……..134

35. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in the vertical diagonal band …….……...135

36. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in the striatum ……..…..…136

Introduction

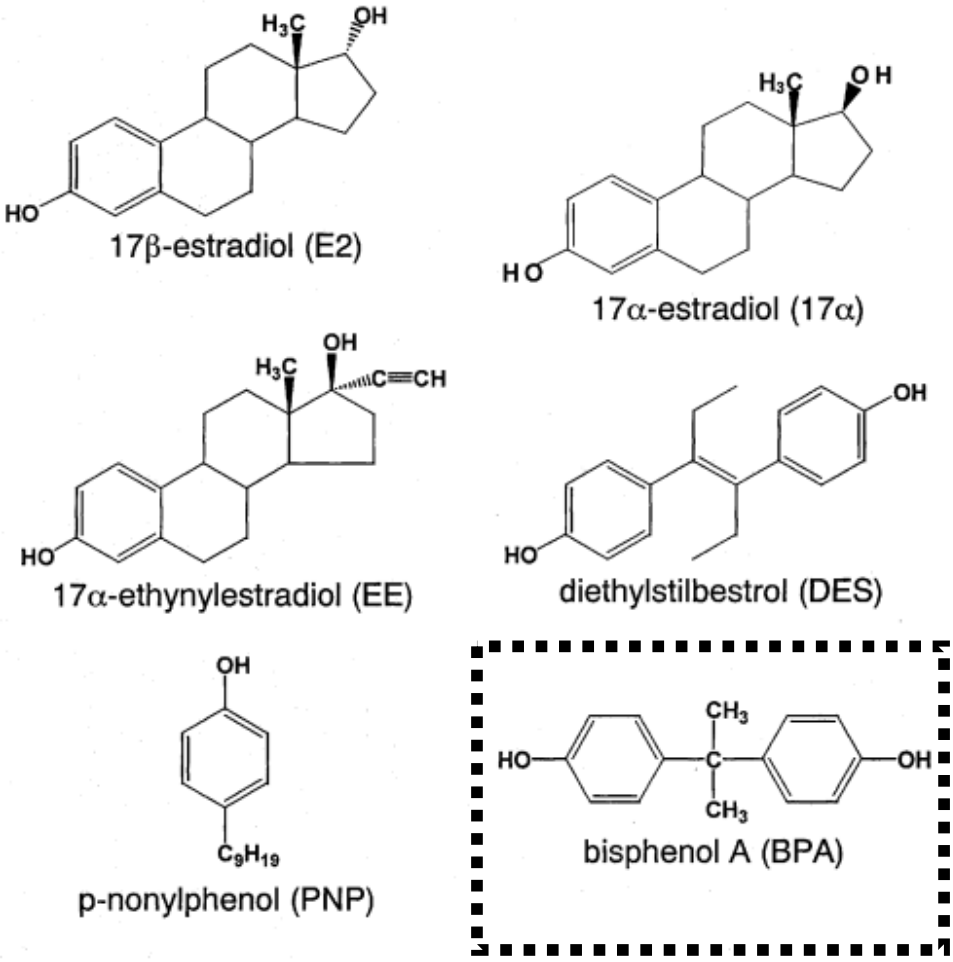
Hormones and Environmental chemicals

Endogenous hormone systems play a crucial role in regulating the behavior and physiology of humans and animals, by maintaining homeostasis, mediating neural signaling, and integrating many functions in the nervous systems. Environmental chemicals, sometimes called endocrine disruptors or environmental estrogens, have chemical properties similar to hormones and therefore may bind to the specific receptors on the cells of target tissues, and hence mimic or, in some cases, antagonize the effects of endogenous hormones. Thus, there has been increasing concern about possible adverse effects of environmental chemicals on human health due to their potential to disrupt the normal action of endogenous hormone systems. Indeed these chemicals have many ways to confuse neural signaling systems. Under normal conditions, for example, hormones activate their receptors at the appropriate level, but these chemicals might inhibit or activate signals at inappropriate levels. The endocrine disrupters also may make signals stronger or weaker than do the endogenous hormones. The action of endocrine disruptors may result in alternation of appropriate cellular responses, which, in turn, could aversively affect many behaviors in humans and animals.

Bisphenol A as an environmental estrogen

Bisphenol-A (BPA), 4,4′-isopropylidene-2-diphenol, is a synthesized non-steroidal environmental chemical with mixed estrogen agonist/antagonist properties. BPA also acts as an antiandrogen (Lee et al., 2003) and as an antagonist to thyroid hormone activity (Moriyama et al., 2002). BPA has a structural homology with the A

ring of estradiol (Fig 1) and was identified as estrogenic in the 1930s (Dodds, 1936). However, BPA never was considered important as an estrogenic compound because its estrogenic activity was considered too weak (vom Saal & Hughes, 2005). Today, according to vom Saal and Hughes, BPA is produced at more than 6.4 billion lb./year and is used widely in manufacture of the polycarbonate plastics, -found in such household products as food and beverage containers (Kang et al., 2003), baby bottles (Brede et al., 2003), and dental sealants (Joskow et al., 2006; Suzuki et al., 2000). Because heat and contact with acidic or basic compounds cause hydrolysis of the ester bonds of these polymer products, BPA leaches out the products and spreads throughout the environment (vom Saal & Hughes, 2005). Studies have found detectable levels of BPA in rivers (Watabe et al., 2004), landfills (Coors et al., 2003), human urine (Calafat et al., 2005), human blood (Ikezuki et al., 2002), and breast milk (Kuruto-Niwa et al., 2007), as well as in the placenta in pregnant women (Schonfelder et al., 2002). In addition, the presence of BPA in saliva, after placement of dental sealants, has been reported (Joskow et al. 2006). Although these data indicate humans are continuously exposed to low levels of this chemical, the low affinity of BPA for the classic nuclear estrogen receptors (ERs), 10000-fold lower than that of 17β-estradiol (Kuiper et al., 1998), and weak bioactivity (Ashby, 2001) have led to the conclusion that low level exposure probably does not elicit significant estrogen responses. As of December 2009, the U.S. Environmental Protection Agency and the U.S. Food and Drug Administration accept 50μg/kg of BPA as the reference safe daily limit, which is calculated based on the lowest dose with observed adverse effects (50mg/kg/day) during the 1980's and this dose was divided by a safety factor of 1000 (vom Saal & Hughes, 2005).



(adapted from Sato, Matsuki, Ohno and Nakazawa, 2002)

Figure 1. The chemical structures of estrogen and related compounds including Bisphenol A.

Sensitivity of the developing brain to BPA

Considerable evidence from the past two decades, however, indicates that BPA could exert powerful hormone mimetic or antagonist activity, specifically when acted on the brain during developmental stages. For example, toxicological studies in laboratory animals have found that low-dose BPA exposures of rodent fetuses produced postnatal estrogenic effects, decreased sperm production and reproductive malfunction in mice (Gupta, 2000), caused disruption of sexual differentiation, alteration in mammary gland development (Vandenberg et al., 2007), vaginal morphology, accelerated growth and puberty. Furthermore, low-dose BPA exposures disrupted meiosis in rats, leading to aneuploidy (see vom Saal 2006 for review). Importantly, when BPA was administrated orally to pregnant female rats, fetal blood BPA was detectable within 10 minutes (Takahashi & Oishi, 2000), which indicates that BPA can cross the placenta and blood-brain barrier easily and reach the brain shortly after exposure.

BPA effects are not limited to the reproductive system and sexual behavior, as estrogen can modulate many functions in the brain including higher mental processes such as memory and learning. There is evidence that BPA could adversely affect not only the brain during development but also the adult brain to alter estrogen mediated central nervous system functions. To provide the necessary background for hormone disrupting activity of BPA, I will briefly review estrogen action on brain, focusing on its effects on memory and learning systems.

Classic estrogen receptors and distribution

Prior to the1980s, estrogen was considered to affect only the female reproductive system and sexual behavior. However, studies over the past three decades have revealed that estrogen has numerous behavioral, neuronal, and physiological effects including the regulation of signaling pathways, body temperature, motor activity, anxiety levels, and physiological homeostasis, such as water and calcium metabolism (McEwen, 2003). The most important finding was that estrogen has functional sites in the adult brain, through which it may influence processes important for higher cognitive function such as acquiring new information and remembering it.

Estrogen exerts a variety of effects through binding specific types of receptors. Currently two types of estrogen receptors (ER), ER α and ER β , have been identified (Kuiper et al., 1997). These receptors are ligand-dependent transcription factors and are located exclusively in the cell nucleus. The classical mechanism of estrogen action, genomic action, involves activation of ERs.

ERα and ERβ show distinctly different binding affinity for different estrogen compounds or ligands. Two isomers of natural estrogen, 17β-estradiol and 17α-estradiol, for example, have different binding affinities. 17β-estradiol binds ERα and ERβ equally, while the binding affinity of 17α-estradiol is much lower - 42% lower for ERα and 89% lower for ERβ compared to 17β-estradiol (Kuiper, et al., 1997). Thus, 17β-estradiol is more potent than 17α-estradiol.

Tissue distributions of ERα and ERβ in the body and the brain are also different. In the brain, localization studies have found that ERα is predominant in the hypothalamus, but relatively more ERβ is outside the hypothalamus, specifically in the

hippocampus, where both ERs are localized including the pyramidal cells (McEwen, 2002). For example, ERα immunoreactivity has been found in the pyramidal cell layer of CA1, CA2, CA3 and in the internuerons, and ERβ has also been localized in CA1, CA2 pyramidal cells and in the glial cells (Azcoitia et al., 1999). One study in the hippocampus reported that a dorsal to ventral gradient and stronger ERα signals than ERβ (Shughrue & Merchenthaler, 2000), which suggests that ERα density in the hippocampus may be greater than previously reported. These two types of receptors have distinct roles on neural systems and behavior, and may exert antagonistic, synergistic and sequential effects (Rissman, 2008).

Membrane ER and membrane-initiated action

In addition to classical estrogen receptors, there are plasma membrane estrogen binding sites (Moriarty et al., 2006). The presence of a significant fraction of nuclear ER on the plasma membrane (Arvanitis et al., 2004) suggests membrane ERs may have similar properties to classic ERs. On the other hand, studies have identified a novel membrane-associated binding site unrelated to the nuclear ERs, called ER-X, which has greater affinity for 17α-E2 (Toran-Allerand et al., 2002) and G-protein coupled receptor, GPR30 (Funakoshi et al., 2006), which rapidly stimulates ERK1/2 phosphorylation (Ding et al., 2009). Moreover, extranuclear ER α (Milner et al., 2001) and ERβ (Milner et al., 2005; Mitra et al., 2003) immunoreactivity were localized in all sub-regions in the hippocampus including unmyelinated axons, dendritic spines, and shafts, which suggests a role of ERs as a membrane-initiated transducer of estrogen effects. Interestingly, a recent study demonstrated that ERα was associated with the clusters of vesicles in

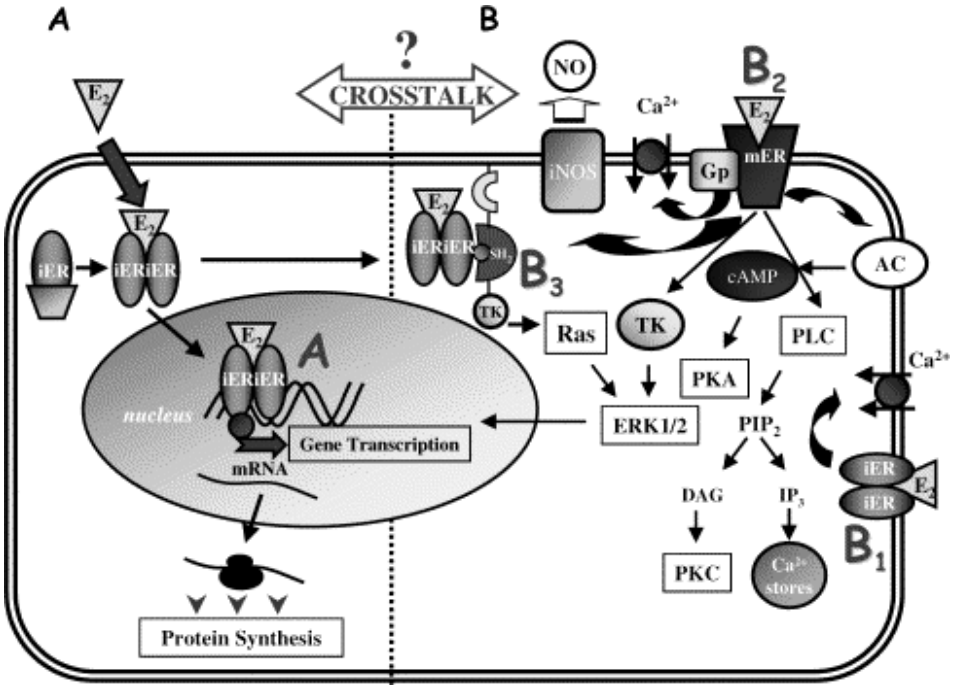
presynaptic inhibitory boutons in the CA1 of the hippocampus, and that estrogen treatment shifts the location of these ERα immunoreactive vesicles toward the synapse (Hart et al., 2007). They suggested a new possible role of estrogen, which is that estrogen may act directly on these ERα-containing vesicles and make them move toward the synapse.

When estrogen binds to membrane receptors, it initiates multiple intracellular signaling cascades within a few seconds to minutes in parallel and in series. For example, studies have shown that estrogen can activate ERK/MARK, phosphoinositide-3-kinase (PI3K)/AKT, and cAMP/PKA signaling pathways, elevate Ca^{2+} levels, induce electrophysiological changes, stimulate growth factor receptors and alter cellular responses (Brinton, 2001; Falkenstein et al., 2000). Importantly, activation of these pathways occurs in a cell-type-specific fashion, which alters downstream effectors and produces specific responses in a target tissue (Moriarty et al., 2006). MAPK pathways, for instance, play a critical role in the mechanisms underlying synaptic plasticity (Komiyama et al., 2002), as a large amount of MAPK is expressed in hippocampal pyramidal dendrites, which can be activated by Ca^{2+} influx via NMDA receptor (Amaranl, 2005).

Integration of genomic and nongenomic action

In the current view, these two types of mechanisms underlying estrogen effects, genomic and nongenomic/membrane initiated actions, are not completely independent, but work together (Levin, 2005) as membrane initiated action may potentiate nuclear transcriptional effects indirectly, which in turn amplify or modify estrogen effects

(Vasudevan & Pfaff, 2007). This integration of genomic and nongenomic actions or cross-talk between the nuclear ERs and membrane ERs, and between the ERs and signaling cascades activated by other neurotransmitters are common features of estrogen effects (Levin, 2005; Luconi et al., 2002; Vasudevan & Pfaff, 2007). A schematic diagram of intracellular estrogen action is shown in Figure 2. Thus, an estrogenic compound with low affinity for the classic ERs, such as BPA, but with high affinity for membrane receptors, could be potent when it activates membrane-associated receptors. In addition, there is evidence that membrane-initiated actions play a dominant role in estrogen- mediated neuronal excitability (Vasudevan & Pfaff, 2007) . For example, Gu et al. (1996) recorded cell activity using the whole-cell voltage-clamp recording, and found that the classical ERs were not necessary for the increase in excitability in CA1 hippocampus neurons in response to kainite. Gu et al suggested that 17β-E2 potentiates kainate-induced currents through a G-protein coupled cAMP-dependent phosphorylation process. These results suggest direct role of membrane-initiated action for the modulation of excitatory synaptic transmission in the brain area important for memory functions (Gu & Moss, 1996).



(adapted from Luconi M, Forti G, Baldi E., 2002)

Figure 2. Schematic representation of genomic and nongenomic actions.
A. Genomic pathway through classical cytosolic/nuclear receptors acting as nuclear transcriptional factors. **B.** Non-genomic pathway mediating rapid effects through membrane receptor (B_2) or classical cytosolic/nuclear receptor spanning through plasma membrane (B_1) or acting through multi-proteic complex associated to the inner part of plasma membrane (B_3).
E_2, estrogens; iER, classical cytosolic/nuclear ER; mER, membrane ER; Gp, G protein; iNOS, inducible nitric oxide synthase; NO, nitric oxide; TK, tyrosine kinases; AC, adenylate cyclase; SH_2, Src homology domain; PLC, phospholipase C; PKA, protein kinase A; ERK1/2, extracellular regulated kinases 1/2; DAG, diacyl glycerol; IP_3, inositol 3-phosphate; PKC, protein kinase C; cAMP, cyclic adenosin monophosphate; PIP_2, phosphatidyl inositol bisphosphate; , mRNA synthesizing ribosome; , ions channel; , heat shock protein associated to ER; , Src.

Estrogen effects on cognitive function

Behavioral effects of estrogen on learning and memory were first reported in animal studies in which gonadectmized male rats with chronic estrogen treatment showed enhanced spatial memory (Luine & Rodriguez, 1994). In human studies, evidence cames from clinical research in aged women with hormone replacement therapy (HT). In one study, researchers found that menopausal women with HT had improved verbal skills and some aspects of memory functions (Maki et al., 2001), suggesting that estrogen may prevent age-related memory loss, decrease risk of Alzheimer's disease, and protect the brain from neurotoxicity (Nilsen et al., 2006).

These growing properties of estrogen have attracted researcher's interests as well as public attention. In 2003, however, the Women's Health Initiative (WHI) reported their cross-sectional and longitudinal studies which found that hormone replacement therapy did not improve cognitive ability; rather it increased the risk for probable dementia (Shumaker et al., 2003). Subsequent research and data analysis revealed that the timing of the initiation of estrogen treatment is critical, which refers to the critical period hypothesis (Morrison et al., 2006). That is, estrogen may exert beneficial effects if treatment is begun closely in time to a natural or surgical menopause but not long after the menopause (Sherwin, 2006, 2007). In the WHI studies, the treatment was initiated in women age 65 or older, which may explain why women with HT showed no beneficial effects of treatment on cognitive function. As this WHI incident implies, exogenous administration of hormones, as well as hormonally active compounds including environmental chemicals such as BPA, could cause adverse effects depending on many factors. Timing is just one of many factors. In fact, extensive studies with laboratory

animals have revealed that estrogen effects on cognition are complex and subtle changes in treatment conditions could result in memory impairment or improvement (Dohanich, 2002).

Chronic estrogen effects on memory in laboratory animals

To date, the majority of existing research on cognitive effects of estrogen has focused on chronic and/or sub-chronic treatments as effects of estrogen on memory were considered to occur via genomic actions, which take hours or even days to occur. These studies have demonstrated that administration of estradiol (E2) to ovariectomized (OVX) animals enhances performance in a variety of, but not all, memory and learning tasks (Dohanich, 2002; Luine, 2006), indicating that estrogen has specific, rather than global, effects on memory systems.

For example, Korol and Kolo (2002) examined effects of the circulating estrogen levels on place learning tasks and response learning tasks. They found that OVX rats treated with estrogen 48 and 24 hour before training learned the place-learning task significantly faster than rats not treated without estradiol. On the other hand, the rats without estradiol did better on the response-learning task than the rats with treatment, which suggested the cognitive effects of estrogen are task-specific, modulating the relative contribution of different learning and memory systems (Korol & Kolo, 2002).

Luine et al. (1998) conducted a series of experiments to test effects of chronic estrogen treatment on radial arm maze performance. The results of their experiment assessing the effects of estrogen on acquisition of the task indicated that estrogen treated rats performed better during acquisition trails. It was also found that estrogen decreases

working memory errors, but had no effects on reference memory (Luine et al., 1998). Thus, these findings suggest estrogen may selectively affect one type of memory but not others when a task tests different types of memory and learning processes. In a typical radial arm maze task, the acquisition phase involves learning processes; "reference memory" refers to the storage of information that is helpful over many trial and lasts longer time such as contingency rules of the task, and "working memory" refers to the storage of information useful within a single trial and lasts shorter time (Dohanich, 2002; Luine, 2006)

The dosage of estrogen treatments is another critical factor that may influence the directions of estrogen effects on memory performance. For instance, Sinopoli et al (2006) studied brain region-specific effects of estradiol on spatial working memory by infusing either saline or high and low concentrations of estrogen into the prelimbic area of the hippocampus or the prefrontal cortex in well-trained rats. It was found that higher doses were required to facilitate memory when infusion was made in PFC. Lower doses, however, facilitated performance when infused into the dorsal hippocampus. Moreover, working memory was significantly impaired 24 h after infusions of estradiol into the hippocampus but not PFC (Sinopoli et al., 2006). Their results suggested that facilitation or impairment of working memory by estradiol is dose, time and brain site specific.

An inverted-U shape dose-response relation has been observed in some studies that used different types of memory tasks. For example, higher levels of estrogen impaired performance in a non-spatial delayed alternation T-maze task, but a lower dose facilitated (Wide et al., 2004). A similar pattern of results was found in working/reference memory version of the radial arm maze task (Holmes et al., 2002), the

hippocampus-dependent spatial working-reference memory task, the prefrontal cortex-hippocampus dependent delayed win-shift task, the striatum-dependent cued win-stay task, and the amygdala-dependent conditioned place preference task (Galea et al., 2001).

These chronic treatment studies have revealed that the beneficial effects of estrogen depend on various factors, including demands of memory tasks (Galea et al., 2001; Korol & Kolo, 2002; McLaughlin et al., 2008), doses (Holmes et al., 2002; Wide et al., 2004), routes of administration (Garza-Meilandt et al., 2006), duration and timing of treatment (Daniel et al., 2006; Gresack & Frick, 2006b), age (Frick, 2009; Wallace et al., 2007), sex (Gibbs & Johnson, 2008; Levy et al., 2005) and stress levels (Bowman et al., 2002; Bowman et al., 2009) of subjects, environmental enrichments (Gresack et al., 2007a, 2007b) and the degrees of daily handling (Bohacek & Daniel, 2007).

Acute estrogen treatment effects on memory

In contrast to the extensively studied chronic treatment effects of estrogen, acute effects of estrogen on memory function are less studied, but recent work has shown that acute treatments can influence memory consolidation processes in rats (Barha et al., 2010; Luine et al., 2003; Packard & Teather, 1997a, 1997b; Rhodes & Frye, 2004, 2006) and mice (Fernandez et al., 2008; Gresack & Frick, 2006a; Harburger et al., 2009; Lewis et al., 2008). Some of these studies demonstrated time-limited effects of acute estrogen on memory consolidation using "post-training treatment paradigms", in which hormone treatment was given after training or the sampling phase of a memory task. This paradigm minimizes possible non-mnemonic or psychological effects of treatments since drug/hormones are not present when animals encounter new information. Packard and

Teather (1997b), for example, trained OVX rats in a Morris water maze and immediately gave intraperitoneal injections of 0.1, 0.2 or 0.4mg/kg of 17β-estradiol. The intermediate dose (0.2mg/kg) improved memory for the hidden platform location when tested 24hr later, but the same dose given 2hr after training was ineffective. The results suggested a "critical time and/or dose window" for acute estrogen enhancements of memory consolidation.

Using pre- and post-sampling injection paradigms, our laboratory has shown that enhancement of memory performance can occur rapidly, within a few hours after acute estrogen treatment (Luine et al., 2003). Interestingly, 17α-estradiol was more potent than 17β-estradiol for hippocampal dependent spatial memory when treatment was given 30 min before a sample trial and tested 4hr later. Affinity of 17α-estradiol for nuclear estrogen receptors, ERα and ERβ, is much lower compared to 17β-estradiol (Kuiper et al., 1997; Kuiper et al., 1998), but recent studies indicate 17α-estradiol shows greater affinity for some membrane-related estrogen receptors (Toran-Allerand et al., 2002; Toran-Allerand et al., 2005). Thus, rapid memory enhancement by acute estrogen treatment and the greater potency of 17α-estradiol suggest that the responses are, at least in part, mediated via non-genomic pathways, activating membrane-associated estrogen receptors and signaling cascades

Mechanisms underlying estrogen effect on memory

The mechanism underlying estrogen effects on memory remains to be elucidated. However, evidence suggests that estrogens affect the biochemical and structural properties of nerve cells in the areas involved in cognitive functions. In one of the early

studies, Luine (1985) demonstrated that estrogen, and sometimes estrogen-progesterone combination could change neurochemical communications, altering the levels of choline acetyltransferase, in the regions that involve higher mental process, such as hippocampus, the basal forebrain, and the cerebral cortex (Luine, 1985). Later, Shughrue et al (2000) found ERα was the predominant estrogen receptor in the cholinergic neuron (Shughrue et al., 2000).

Evidence also indicates that monoamines affect learning and memory and estrogen may influence monoamine activity including monoamine synthesis and release, by regulating activity of various enzymes, receptors, and transporters (Dohanich, 2002). For example, a study showed that D1 receptors in the prefrontal cortex play an important role for working memory (Seamans et al., 1998) while another study demonstrated that hippocampus D2 receptor activity is crucial for spatial working memory (Wilkerson & Levin, 1999).

Dendritic spines are main entrance sites of excitatory inputs, and estrogen has been shown to alter the dendritic spine density and spine synapse density in CA1 hippocampal pyramidal cells. In early studies, McEwen group found that removal of the ovary and hence decreased circulating estradiol level significantly decreased spine density in CA1 pyramidal cells of the hippocampus, and estrodiol replacement prevented this decrease in dendritic spine density (Gould et al., 1990; Woolley & McEwen, 1992). Subsequent studies revealed that estrogen administration to OVX rats could alter dendritic synapse density, and increase CA1 neurons' sensitivity to NMDA receptor mediated synaptic input (Woolley et al., 1997). Importantly, recent study have demonstrated that estrogen mediated synaptic plasticity can occur within 2- and 4hours

after exogenous administration of estrogen (MacLusky et al., 2005b) and even 30 min after hormone exposure (MacLusky et al., 2005a), which suggests responses may be mediated via non-genomic pathways. The changes in spine density were also observed naturally across 4-5 day estrous cycle in the female rat hippocampus; the highest dendritic spine density was in proestrus when estradiol levels are highest, and the lowest density was in estrus when estradiol levels are lowest (Woolley et al., 1990).

Several studies have reported that these structural changes in spine density parallel performance in memory tasks. Vehicle treated OVX animals show memory impairment and low spine density, while estrogen treated OVX animals show memory enhancement and significantly increased spine density in young (McLaughlin et al., 2008; Sandstrom & Williams, 2001) and aged rats (Wallace et al., 2006). These results suggest estrogen-dependent changes in spine density could be a possible mechanism that contributes to estrogen effects on memory. However, there are some studies that found changes in estrogen levels across the estrous cycle were not consistent with memory performance (Berry et al., 1997; Stackman et al., 1997; Warren & Juraska, 1997). Warren et al (1997), for instance, have reported that optimal performance on the spatial memory task occurs during estrus when levels of estradiol were low, whereas the least efficient performance occurs during proestrus when levels of estradiol were high. These results suggest that increased spine density may not underlie improved memory performance.

BPA effects on behavior

The majority of behavioral research on BPA has been focused on the effects of developmental long-term exposure to this chemical on later behavior in adults. Such

studies have found that perinatal exposure to BPA disrupts learning of fear evoked stimuli (Negishi et al., 2004), causes memory impairment associated with dopamine, hippocampus-cholinergic system dysfunction (Miyagawa et al., 2007), affects play (Dessi-Fulgheri et al., 2002), sociosexual (Farabollini et al., 2002), impulsive (Adriani et al., 2003) and maternal behavior (Palanza et al., 2002). BPA exposure also causes hyperactivity (Ishido et al., 2004; Negishi et al., 2005), and alters anxiety level (Ryan & Vandenbergh, 2006). It is critical to investigate possible neural systems that may contribute to behavioral alternations induced by BPA exposure. There are some developmental studies that reported both brain and behavioral effects of long-term exposure to BPA. For example, Miyagawa et al (2007) found poor performance of BPA treated rats in a passive avoidance tests was associated with dramatic reduction of choline acetyltransferase like immunoreactivity in the hippocampus. In another study, Ishido et al (2004) showed correlation between hyper activity and decreased levels of gene expressions of the dopamine D4 receptor and dopamine transporter in BPA treated rats, suggesting BPA affect the central dopamine system.

Membrane initiated action of BPA

Recent evidence indicates BPA may exert effects not only through genomic but also through membrane-initiated action, interacting with estrogen receptors located in the plasma membrane or synaptic terminals, and activating second messenger systems. Such effects may occur rapidly and could be observed within seconds-minutes after BPA exposure.

Since membrane receptors have different ligand specificities and binding affinities from the nuclear estrogen receptors (ER), ERα and ERβ, they provide a new site for ligand actions. Thus, BPA might selectively activate or inhibit responses mediated via estrogen membrane receptors. If so, then low level exposure could adversely affect estrogen-mediated behaviors. Supporting this hypothesis, many in vitro studies have reported powerful non-genomic actions of BPA. Studies have found that BPA rapidly activates cyclic AMP responsive element-binding protein (CREB) in pancreatic β cells(Quesada et al., 2002) and ERK phosphorylation in hippocampus HT-22 cells (Lee et al., 2008) and cerebellar cortex (Zsarnovszky et al., 2005), stimulates nitric oxide synthesis in mouse endothelial cells (Noguchi et al., 2002), increases dopamine release through guanine nucleotide-binding protein and N-type calcium channels (Yoneda et al., 2003), accelerates Ca^{2+} influx via L-type channels and prolactin secretion (Watson et al., 2007a; Watson et al., 2007b), binds and activate the novel seven-transmembrane estrogen receptor, GPR30 (Thomas & Dong, 2006), impairs Ca^{2+} signals in pancreatic α cells (Alonso-Magdalena et al., 2005), and binds with high affinity to a novel plasma membrane binding site unrelated to ERα and ERβ in pancreatic β cells (Nadal et al., 2000). In an *in vivo* study, (Canesi et al., 2005) found BPA rapidly altered the phosphorylation state in tyrosine kinase-mediated cell signaling, in particular MAPK and signal transducers, which are important for development of dendritic spines. Moreover, BPA may rapidly alter receptor responses in opposite directions depending on concentration. For example, low concentrations of BPA rapidly potentiated $GABA_A$ receptor responses, while high concentrations suppressed them (Aoshima et al., 2001).

These findings suggest that BPA is not a "weak estrogen" but could be potent when it acts through nongenomic pathways.

Unique characteristics of BPA action in the brain

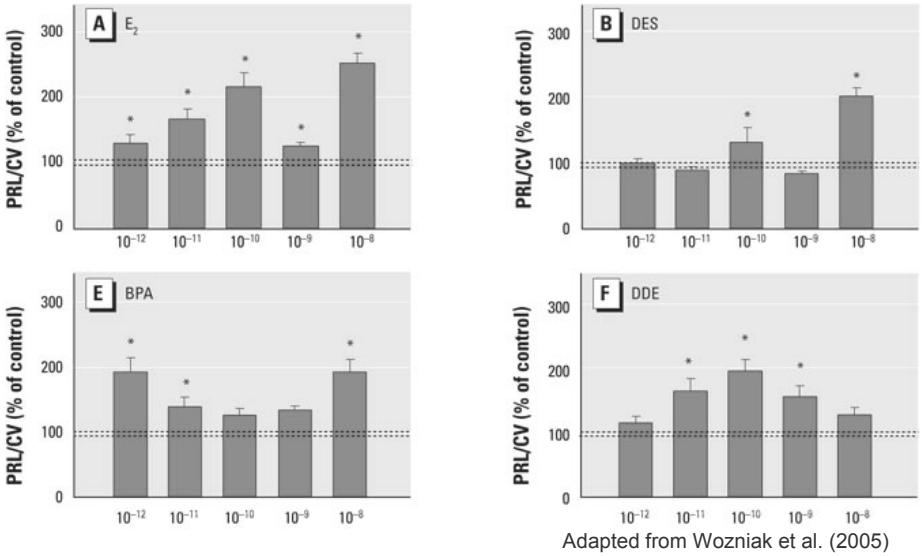
Watson et al. (2007a,b) recently reviewed several estrogenic compounds examined in their laboratory and reported some interesting characteristics of BPA effects. They tested whether several estrogen-related compounds, including BPA, have effects on activation of signaling pathways, induction of Ca^{2+} flux and secretion of peptide hormones in pituitary cells (Wozniak et al., 2005) and found that 17β-E2 and all tested compounds elevated intracellular Ca^{2+} levels at 10^{-12} to 10^{-8} M concentrations within 30s via L-type channels located in the cell membrane. The dose-response relationship for prolactin (PRL) release was bimodal for E2, BPA and DES treatments (shown in Fig. 3A). Thus, both high and low BPA concentrations were effective, but intermediate doses were not. Moreover, data indicated that signal vs. functional response (or response effectiveness) patterns for E2 and tested compounds were markedly different. For example, E2 caused a large PRL release, while it only modestly raised Ca^{2+} levels. On the other hand, nM concentrations of BPA, that did not evoke PRL release, caused large Ca^{2+} elevations. Watson et al. argued that E2 must control some additional responses other than elevation of $Ca2^{+}$ levels that contribute to PRL release. For ERK activation, E2 and other compounds activated ERK at low concentrations, but BPA did not. These results demonstrate that BPA is active in one nongenomic pathway (Ca^{2+} elevation and the subsequence PRL release), but inactive in another (ERK activation) at least in pituitary cells, which suggests that BPA might evoke rapid PRL release through another

signaling pathway. Supporting this idea, Yoneda et al. (2003) found that BPA could activate multiple signaling pathways simultaneously. They examined whether membrane receptors participate in BPA-evoked dopamine release in PC12 cells and found that BPA-evoked dopamine release was suppressed by several inhibitors for specific pathways (Yoneda et al., 2003). Thus, BPA could activate multiple signaling pathways in parallel to release dopamine in PC12 cells.

BPA and non-monotonic dose-response curves

As discussed above, Wozniak et al. (2005) found bimodal dose-response relationships for prolactin release evoked by E2, BPA and DES treatments (Fig. 3A). In the non-monotonic dose responses, the threshold that separates effective or ineffective doses does not exist. Thus, dose response curves may appear like an inverted-U (the highest response at intermediate doses) or may be a U-shape (the lowest response at intermediate doses). These non-monotonic dose responses are a relatively common feature for some drugs, hormones, neurotransmitters and environmental chemicals, and they are observed in many studies (Vandenberg et al., 2009), including BPA and estrogen (Welshons et al., 2003). For example, Wetherill et al. (2002) found an inverted-U dose response for prolification responses induced by BPA in prostate tumor cells as nM doses of BPA induced the maximal responses but higher or lower doses showed less cell proliferation (Fig. 3B). Mechanisms underlying non-monotonic dose responses are not yet known. However, some studies suggest down-regulation of receptors at higher doses, while others suggest involvement of multiple signaling pathways (see Vandenberg et al., 2009 for review).

A.



Adapted from Wozniak et al. (2005)

B.

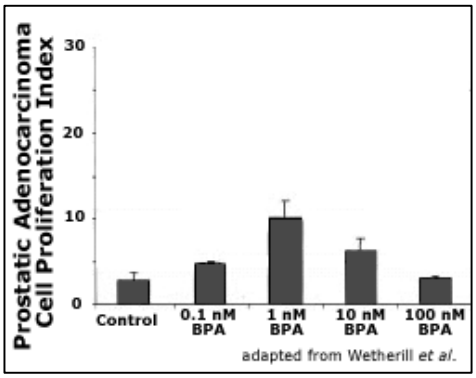
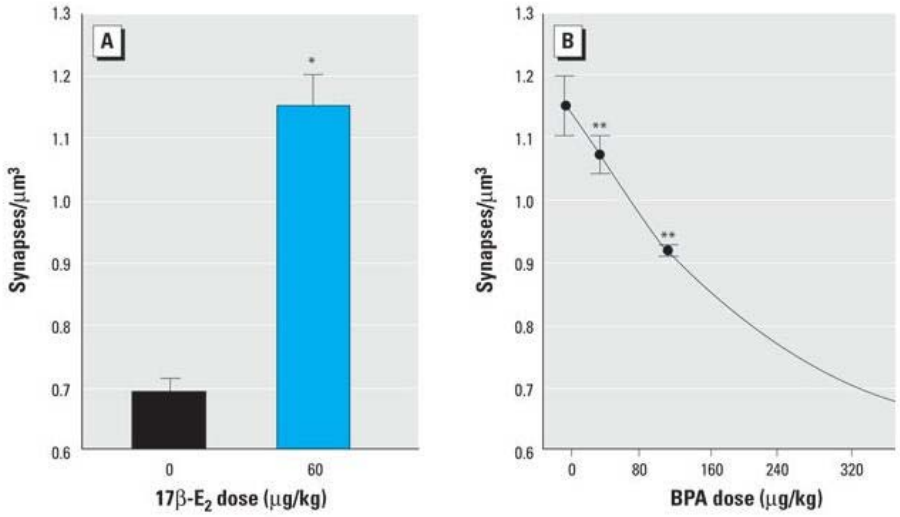


Figure 3. Non-monotonic dose responses for E2, BPA and some environmental chemicals. **A.** Dose-responses for prolactin release appeared monotonic for E2 and E2 related compounds. BPA evoked responses at lower and higher concentrations, but intermediate doses were inactive, showing U shaped curve (Wozniak et al., 2005) **B.** 1nM dose of BPA elicited the strongest proliferation response, but higher and lower doses were ineffective. Dose responses appeared an inverted-U (Wetherill et al., 2002).

BPA effects in combination with physiological estrogen

Recent brain studies reveal another interesting characteristic of rapid BPA actions through non-genomic pathways: in the absence of estrogen, BPA weakly mimics estrogenic actions or has no effect, while in the presence of estrogen, BPA blocks estrogenic activity. Such studies include BPA inhibition of E2 induced long-term potentiation in hippocampal cell culture (Kawato, 2004), suppressive effects of BPA on E2-induced CA1 synaptogenesis in adult rats (MacLusky et al., 2005a) and monkeys (Leranth et al., 2008), and disruption of BPA on E2-mediated ERK1/2 activation in the developing cerebellar cortex (Zsarnovszky et al., 2005). For example, MacLusky et al (2005a) demonstrated that acute administration of 60μg of 17β-E2 to OVX rats increased CA1 pyramidal cell dendritic spine synapse density (PSSD) rapidly, within 30 min, after estrogen treatment and co-administration of BPA (40-400μg/kg) with E2 dose-dependently inhibited E2 induced PSSD (see Fig. 4 for detail). Recently, this antagonistic effect of BPA on E2-induced hippocampal synaptic plasticity was confirmed in chronic treatment studies with monkeys (Lenarth et al., 2008). Interestingly, our laboratory found that 60μg/kg of E2 enhanced spatial memory rapidly when treatment was given 30 min before a sample trial and tested 4 hours later (Luine et al., 2003). Thus, 60μg/kg of E2 alters both behavior (memory enhancement) and neural systems (increased dendritic spine synapse density) within a relatively short time. As discussed earlier, considerable studies show positive correlation between changes in spine (synapse) density and changes in memory performance. Therefore, if BPA is a membrane ER antagonist and if changes in spine density are one of neural systems that contribute to

estrogen mediated memory function, then rapid memory enhancement induced by E2 should be inhibited by co-administration of BPA with E2.



(MacLusky NJ, Hajszan T, Leranth C, 2005)

Figure 4. Synaptic plasticity induced by acute 17β-E2 treatment and BPA inhibition. **A.** 30 min after 60ug/kg of 17β-E2 injection, CA1 pyramidal cell dendritic spine synapse density were increased almost 100%. **B.** Co-administration of BPA inhibited spine synapse density response to 17β-E2 in dose-dependent manner (MacLusky et al. 2005b)

Some critical questions about environmental chemicals

The results in brain studies discussed above raise several important questions about the interaction between environment chemicals and endogenous estrogen. How do environmental chemicals such as BPA act in combination with estrogen via membrane-initiated actions in adult brain? Do they compete against each other? Or do they work in concert? How does BPA affect estrogen mediated specific behaviors? Currently little information is available and few studies address these issues in an experimental setting that examines behavioral significance of acute actions of estrogen and BPA together with measurements of underlying neural systems in adult brains. Thus, the focus of my research is to examine the acute effects of E2 and BPA, alone and in combination, on behavior (hippocampus-dependent memory) and neural function (dendritic spine density and monoamine levels) in adult female rats. Based on findings in past studies, specifically in Maclusky et al (2005a,b) and Luine et al (2003), we predict that acute estrogen treatment will enhance performance in memory tasks and co-administration of BPA with E2 will block behavioral and neural responses to E2 via membrane-initiated actions.

Specific Research Aims

The main objective of this study was to investigate the effects of acute exposure to estrogens and BPA on cognitive and neural functions in adult female rats. Recent brain studies indicate that estrogen and BPA may exert powerful effects on estrogen-mediated central nervous functions in adult brains through non-genomic actions, activating membrane-associated ER systems. Such effects occur rapidly within a few seconds or minutes after acute exposure to hormonally active chemicals, and may alter behavior. To date, the majority of estrogen research has focused on chronic treatment effects, and no published data are available for acute BPA effects on behavior in adults. Thus, this research is one of the first investigations to address these issues and examine both behavioral and neuronal effects of acute estrogen and BPA treatments in adulthood. We designed a series of experiments to specifically address each of the following:

Behavior Study

1. To determine acute effects of estrogen on memory consolidation.

Acute effects of 17β- and 17α-estradiol on spatial and non-spatial memory consolidation were investigated. Of particular interest was to examine dose-response patterns between acute estrogen and memory performance. To achieve this goal, various doses of 17β- and 17α-estradiol were tested using a post-sampling injection paradigm and relatively simple, less-stress memory tasks, the object placement (OP) task, which measures object location memory (hippocampus-dependent spatial memory) and the object recognition (OR) task, which measures object recognition

memory (less hippocampus dependent, but more cortical dependent non-spatial memory). Aim 1 consisted of the following experiments:

EX. 1-1 Acute effects of 17β-E2 on OP memory consolidation

EX. 1-2 Acute effects of 17β-E2 on OR memory consolidation

EX. 1-3 Acute effects of 17α-E2 on OP memory consolidation

EX. 1-4 Acute effects of 17α-E2 on OR memory consolidation

Based on results of previous acute studies, it is hypothesized that post-sampling estrogen treatment would enhance OP and OR memory consolidation in a dose-, time-, and task specific manner. 17α-E2 would be more potent than 17β-E2 in the both memory tasks. Both isomers of estradiol would show an inverted-U dose-response curve.

2: To determine acute effects of E2 and BPA treatments, alone and in combination, on memory consolidation.

The acute effects of BPA treatments on estrogen-induced OP and OR memory enhancement were examined by administering BPA (doses ranged 0.4-400μg/kg) with the most effective E2 doses for memory enhancement found in Aim1. Our goal here was to determine whether BPA alters behavioral responses to E2 below the current reference safety dose of 50ug/kg/day. BPA alone treatment effects on memory performance were also determined. Additionally, E2 and BPA treatment effects on anxiety levels were examined using the elevated plus maze to ensure that acute hormone/BPA treatments do not alter some psychological factors (i.e., anxiety, mood, motor activity), which may mask treatment effects on memory. The experiments in Aim 2 consist of the following:

EX. 2-1 Effects of BPA alone on OP and OR memory consolidation

EX. 2-2 Effects of BPA on 17β-E2 induced OP memory enhancement

EX. 2-3 Effects of BPA on 17β-E2 induced OR memory enhancement

EX. 2-4 Effects of BPA on 17α-E2 induced OP memory enhancement

EX. 2-5 Effects of BPA on 17α-E2 induced OR memory enhancement

EX. 2-6 Effects of E2 and BPA on anxiety levels

Based on previous behavioral and morphological studies, it is hypothesized that BPA alone would show little or no effects on memory performance and co-administration of BPA with E2 woud block E2-induced OP and OR memory enhancement. Anxiety levels would not be affected by acuteE2 and/or BPA treatment.

Brain Study

3. To examine morphological changes underlying acute E2 and BPA treatment effects on memory.

As a possible neural system which may contribute to acute E2 and BPA+E2 treatment effects on OP memory consolidation, dendritic spine density in the PFC and the CA1 of the hippocampus was measured at two time points: 30 minutes (during the critical time window for memory consolidation) and 4 hours (the inter-trial delay when retention was tested) after hormone and/or BPA exposure. A primary goal here was to examine whether BPA effects on neural responses to E2 are antagonistic (inhibitory) or agonistic (additive or synergistic effects). Serum estrogen levels 30 minutes and 4 hours after acute treatments were additionally measured. Aim 3 consisted of the following:

EX. 3-1 Acute E2 and BPA+E2 treatment effects on spine density 30 minutes after treatments.

EX. 3-2 Acute E2 and BPA+E2 treatment effects on spine density 4 hours after treatments.

EX. 3-3 Serum estrogen levels 30min and 4hr after acute treatments)

Based on previous behavioral and morphological studies, we hypothesized that acute E2 treatment would increase spine density at 30 minutes and at 4 hours after treatments. Co-administration of BPA with E2 would suppress E2-induced spine density treatment.

4. To examine neurochemical changes underlying acute E2, BPA and BPA+E2 treatment effects on memory.

Monoamine concentrations (dopamine, serotonin, norepinephrine and their metabolites) in the brain areas known important for memory were examined at 30 minutes after vehicle, E2, BPA, and BPA+E2 treatments, in order to examine whether the same doses of E2 and BPA that alter memory performance also alter neurochemical levels during memory consolidation. Based on previous behavioral and neurochemical studies, we hypothesized that acute E2 and BPA, alone, and in combination, would alter monoamine levels.

Behavioral Study: Memory Consolidation

Experiment 1: Effects of acute estrogen treatment on memory consolidation (Aim 1)

Accumulative evidence suggests that estrogen exerts beneficial effects on some aspects of higher order brain functions. Specifically, administration of chronic estradiol (E2) treatment to ovariectomized (OVX) animals enhances performance in a variety of, but not all, memory and learning tasks (Macbeth et al., 2008a). In addition, long-term deprivation of ovarian hormone in young OVX rats (Wallace, et.al, 2007) and decrease in circulating estrogen levels in aged female rats (Frick, 2008) result in decline in spatial and non-spatial memory, indicating estrogen plays an important role to maintain the ability of remembering new information. However there is inconsistency in data. Some studies found that estrogens have no effects or even impair performance of memory tasks (Chesler & Juraska, 2000; Snihur et al., 2008; Wang et al., 2009). Accumulative evidence have revealed that long-term estrogen treatments has specific, rather than global, effects on memory systems (Korol and Kole, 2002) and the beneficial effects of estrogen depend on many factors, such as demands of memory tasks (McLaughlin et al., 2008), doses (Holmes et al., 2002; Wide et al., 2004), routes of administration (Garza-Meilandt et al., 2006), duration and timing of treatment (Daniel et al., 2006), sex and stress level of subjects (Bowman et al., 2003).

Acute effects of estrogen on memory function are less studied, but recent work has demonstrated that acute treatments can influence memory consolidation processes (Woolley, 2007). Some of these studies have found rapid memory enhancement within few hours after hormone treatments (Luine et al, 2003; Rhodes and Frye, 2006), time-limited effects of acute treatments (Luine et al., 2003; Packard, 1998; Rhodes & Frye,

2004) and inverted-U dose responses (Gresack & Frick, 2006a; Packard & Teather, 1997a; Packard, 1998). Interestingly, 17α-E2 showed greater potency than 17β-E2 in object placement/recognition task (Luine et al., 2003), while it was ineffective in inhibitory avoidance and water maze tasks (Rhodes & Frye, 2006). These results suggest the responses are, at least in part, mediated via non-genomic pathways. Because available data are limited, more studies are necessary to understand properties of acute effects of estrogens on memory systems.

Thus, the main objective of experiment was to examine acute estrogen treatment effects on spatial and non-spatial memory consolidation. Specifically we were interested in examining dose response relationships between acute estrogen treatments and memory performance. To achieve this goal, we tested various doses of 17β- and 17α-estradiol using relatively simple, less stressful memory tasks, object placement and recognition tasks, and a post-T1 injection paradigm, in which hormone treatments were given after a sampling trial. There are several advantages to use this injection paradigm in acute treatment studies, including; 1) it can dissociate estrogen effects on memory and learning (acquisition phase) because hormones are not present during subjects encounter new information; 2) possible non-mnemonic effects of estrogens (e.g., attention, sensory sensitivity and motor activity) can be minimized; 3) it allows to evaluate time course effects of estrogens by manipulating timing of hormone administration (Packard, 1998). Based on previous findigs, we hypothesized that acute estrogen would enhance memory consolidation in a time-, dose-, and task specific manner.

Materials and Methods

Subjects

Thirty-eight female Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN), aged 55-60 days upon arrival, ovariectomized (Ovx) by the vender, served as subjects. All rats were double-housed in cages, maintained on a 12/12 hr light-dark cycle (lights on at 7:00AM). They had ad libitum access to low phyto-estrogen food (Chow 2016, 16% protein rodent diet, Harlan Teklad Global Diets, Madison, WI) and water for the entire period of the experiments. Rats were allowed to acclimate to the housing environment for a week, and received eight days of habituation sessions prior to behavior experiments (see acclimation/habituation schedule). All experiments were conducted in accordance with the NIH Guide for Care and Use of Animals and the Institutional Animal Care and Use Committee of Hunter College of the City University of New York.

Memory Tasks

Two types of memory tasks were used in this experiment, object placement (OP) and object recognition (OR) task, and they were adapted from working memory tasks developed by Ennaceur and Aggleton (1987). These tasks measure object place memory (spatial memory) and object recognition memory (non-spatial memory) based on animals spontaneous exploring behavior and their innate tendency of novelty preference.

The OP and OR tasks provide a relatively simple and stress-free measurement of working memory, and have several advantages to access hormonal effects on memory performance. First, the tasks do not use positive (e.g., food/water rewards) or negative (e.g., electrical shock) reinforcement, which can interact with some factors such as

motivation, appetite, thirst, and anxiety levels, and may interfere with estrogen effects of memory. Second, animals do not require learning the contingency rules of particular tasks. Thus, hormonal effects on memory and the acquisition phase of task learning could be dissociated. Third, the OP and OR tasks can minimize stress related confounding variables on memory performance since animals do not need to encounter stressful circumstances (e.g., forced to swim) during task performance. Finally, because of the nature of OP and OR tasks, animals can be tested repeatedly (Ennaceur et al., 1997).

Object Placement (OP)

Spatial memory was tested using the object placement (OP) task as previously described in young and aged rats (Beck & Luine, 2002; Bowman et al., 2009; Luine et al., 2003). All trials were conducted in a 3 x 3 grid open field made of black plexiglass (70cm wide x 70cm long x 30cm high), which was placed on a 70cm high table, and external spatial cues (e.g., posters, pictures, a video camera) were available on the walls. Intensity of room light was carefully controlled to illuminate the floor of the chamber evenly. A session consisted of a 3-min sample trial (T1) and a 3-min retention trial (T2) with a 4hr inter-trial interval between T1 and T2. During T1, two identical objects were placed at one end of the open field. The total amount of time rats spent exploring the two objects for 3min was recorded. Exploration behavior was defined when the rats were sniffing at, looking at, or whisking at the objects within 2cm distance. Following an inter-trial delay of 4 hr, a T2 retention trial was given, in which one of the objects was moved to a novel location within the open field. The time spent exploring the objects at the old (familiar location) and the new (novel location) was recorded for 3 min. Objects were candleholders, figurines and statues. The novel location was counterbalanced

across treatments. Objects and the floor of the chamber were cleaned with a disinfectant spray after each trial. All trials were videotaped with a SONY camcorder and analyzed later.

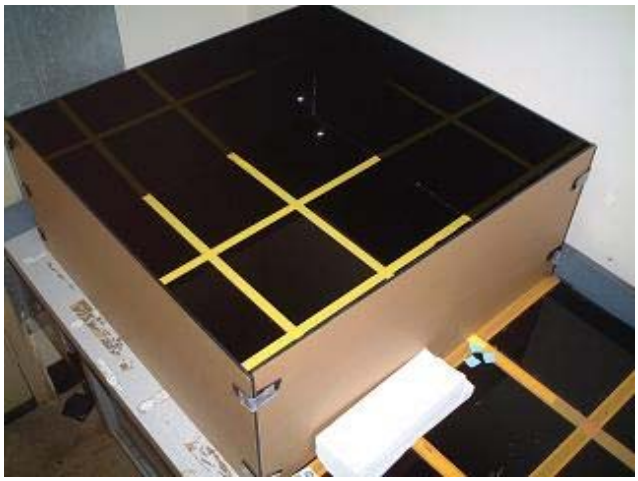
Object Recognition (OR)

Recognition memory was accessed using the object recognition (OR) task. The basic procedure was the identical to the OP task except that one of the objects used during T1 was replaced with a novel/new object in T2. The exploration time around the old (familiar object) and the new (novel object) were recorded. Objects used for the OR tasks were a variety of cans, containers and bottles. The new object and its position in the open field were counterbalanced across groups. The open field chamber and objects used for memory tests are shown in Figure 5.

Acclimation/habituation

Approximately three weeks after ovariectomy and two weeks after arrival, acclimation began and lasted eight days. First, each rat was placed into a 3 x 5 grid open field chamber without objects to explore freely for 6 min. For day 2 through day 5, rats received four habituation sessions to the object recognition task with progressively longer inter-trial intervals (1 min, 15 min, 1 hr, and 2 hr) between T1 and T2. After a 2-day break, three habituation sessions to the object placement were given with a 10 min, 40 min and 1hr inter-trial intervals.

A.



B.

Figure 5. The open field chamber and objects used for memory tests. A. a 3 x 3 grid open field chamber, 70cm width x 70cm length, enclosing walls 30cm high, was used for the object recognition (OR) and object placement (OP) memory test. **B)** Objects used in this study.

Estrogen doses

17α-estradiol and 17β-estradiol were obtained from Sigma-Aldrich Corp (St. Louis, MO). We tested 2-60μg/kg of 17β-estradiol, and 0.5-20ug/kg of 17α-estradiol. See Table 1 for detailed tested doses of estradiol for the OP and OR memory task. These doses were selected based on previous studies in our lab and others. Hormones were dissolved in ethanol for stock solutions (10mg /1ml and 1mg /1ml), and diluted with corn oil for injection. The high concentration stock was used to make 10ug/kg or higher dose injection solution, and the low concentration stock was used for less than 10ug/kg injection in order to control ethanol levels (less than 0.006%) in each solution. Control rats received 1ml/kg of corn oil, which contained the corresponding amount of ethanol.

Estrogen treatment

Hormone or vehicle treatment was given using a post-training/sampling treatment paradigm, which was developed based on memory consolidation hypotheses and has been used in a number of memory studies (Gresack & Frick, 2006a; Luine et al., 2003; McGaugh, 1966; Packard, 1998). OVX rats received either a single subcutaneous (sc) injection of estrogen or corn oil at the nape of the neck immediately after a sample trial (immediate post-T1 injection; see Fig. 6A). To test time-dependent effects of estrogen, a delayed post-sampling treatment paradigm was used for one OP session, and rats received hormone treatment 45min after a sample trial (delayed post-T1 injection; see Fig. 6B).

To determine the lowest effective doses of acute estradiol, rats were tested approximately every 10 days with doses described above. Treatments (vehicle or estradiol) were counterbalanced in every experiment. For the dose-response relationship

study, all doses of estradiol were tested in each experiment using a matched block paradigm (18 or 20 rats per block with equal numbers of rats at each dose). Experiments were repeated every 10 days until each rat received all doses and each dose group contained sample size of at least eight subjects.

Table 1. Summary of 17β- and 17α-estradiol doses for the object placement (OP) and recognition (OR) task.

E2	Memory Task	
	Object placement (OP)	Object recognition (OR)
17β-estradiol	10, 15, 20, 30, 40, 60μg/kg	5, 10, 15, 20μg/kg
17α-estradiol	2, 5, 10, 20μg/kg	0.5, 1, 2, 10μg/kg

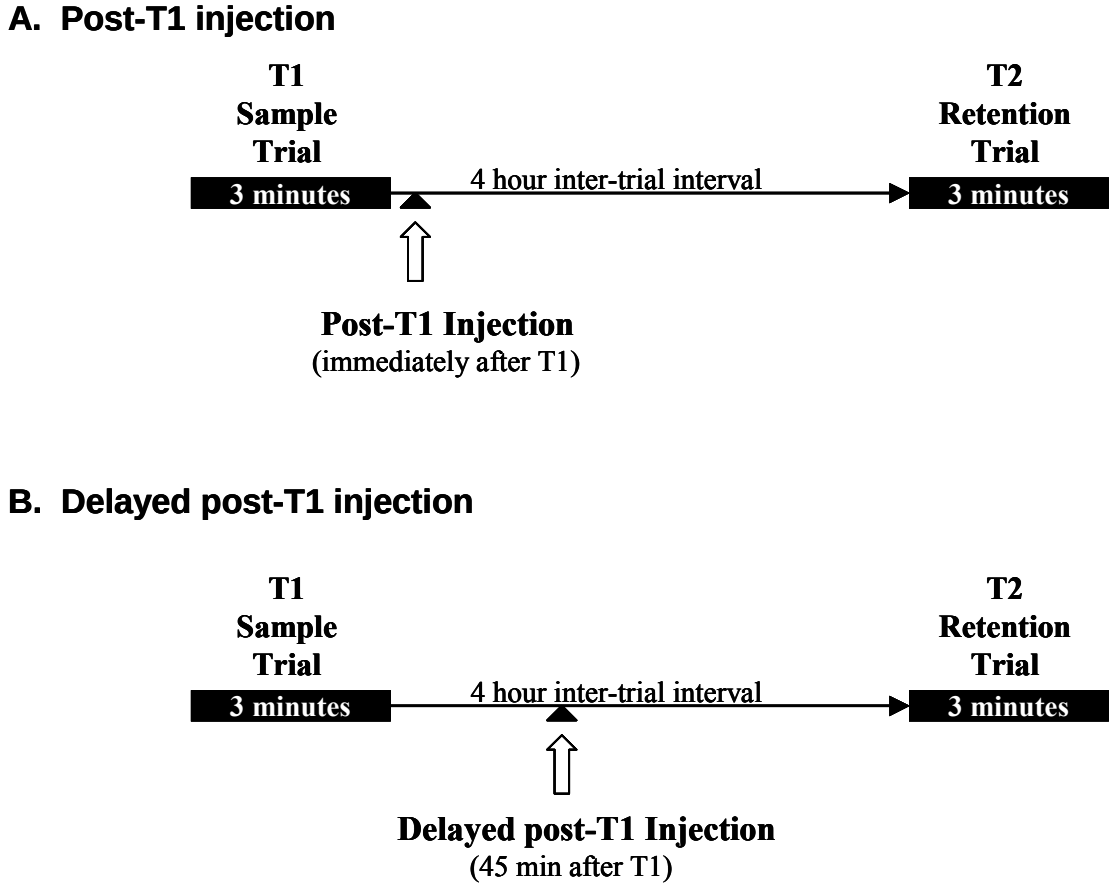


Figure 6. Timeline for acute post-sampling hormone treatment. The memory task consisted of a T1 sample trial, a 4hr inter-trial interval and a T2 retention trial. **A.** Immediate post T1 injection. OVX rats received a single sc injection of either vehicle or estradiol immediately after T1 sampling trial. **B.** Delayed post T1 injection. Subjects received treatment 45 minutes after T1 sampling trials.

Data analyses

All data were analyzed using SPSS software (Systat Inc., Chicago, IL., USA). Group differences in the total exploration times around two objects during T1 were analyzed using independent t-tests (for two groups) or one-way ANOVAs (for more than two groups). For the retention trial (T2), two-way ANOVAs, group (oil, E2) x object exploration (old, new), were used to analyze the time spent exploring the old/familiar objects and the new/novel objects (for OR) or the objects at the old/familiar locations and the new/novel locations (for OP). If significant interaction was found, then paired t-tests tested whether rats significantly discriminated objects between the old and the new locations. Additionally independent t-tests were used to test group differences in exploration ratios (T2 NEW/T2 total time). Exploration ratios show % time spent exploring objects in the new location relative to the total time spent exploring the objects in both locations. Thus an exploration ratio of 0.5 indicates that rats spent an equal amount of time around the old and new object: ratios of above or below 0.5 indicates that the rats spent more time or less time around the new objects than the old objects. When more than two groups were tested (dose-response relationship), one-way ANOVAs were used to analyze group differences in exploration ratios with a post-hoc test (Fisher LSD). Data from rats that did not explore the objects during T1 and/or T2 were excluded from statistical analyses.

Results

EX. 1-1. 17β-estradior effects on object placement (OP)

To determine the most effective dose of 17β-estradiol for OP memory consolidation, we first tested 60ug/kg and 30ug/kg in separate sessions because previous studies indicated these doses injected immediately before or after T1 significantly enhanced OP (Luine et al., 2003), or increased spine synapse density in the CA1 hippocampus (MacLusky et al., 2005a, 2005b). During T1, estradiol- treated and vehicle-treated OVX rats spent similar amounts of time exploring the two identical objects for both sessions (Data not shown). In the T2 retention trials, ANOVA revealed no significant difference between 60ug/kg (n=10) and vehicle treated (n=9) group (Fig. 7A). But a significant interaction was found between the 30μg/kg (n=10) and vehicle treated (n=9) control group ($F_{1,16}$=7.97, p<0.012, Fig. 7C). Post-hoc tests, however, revealed that although the rats in this group spent significantly more time exploring objects at the new location than the old location (Data not shown), group differences in exploration ratios were not significant (t_{16}=1.978, p<0.065: Fig. 7C), suggesting 30μg/kg showed a tread toward significance but was not sufficient to enhance OP memory.

Based on the above results, we reasoned that effective doses for memory consolidation would be between 30ug/kg and 60ug/kg, or below 30ug/kg. Thus, we tested 10, 15, 20 and 40ug/kg of 17β-estradiol (n=9-10). In the T1 sample trials, no significant differences in exploration times were found for all sessions (Data not shown). In the T2 retention trials, ANOVA indicated significant interactions for the 20ug/kg ($F_{1,18}$=10.665, p<0.004), and for 15ug/kg ($F_{1,17}$=8.511, p<0.01) treatments. Post-hoc tests revealed that the rats with these doses spent significantly more time around objects at the new location

(Data not shown), and group differences in exploration ratios were also significantly different, indicating both 20μg/kg (t_{17}=2.988, p<0.008: Fig. 7D) and 15μg/kg (t_{18}=2.943, p<0.009: Fig. 7E) enhanced OP memory consolidation. In contrast, the 40μg/kg (Fig. 7B) and 10μg/kg (Fig. 7F) groups showed chance level performance. Thus, intermediate doses of 17β-estradiol (15and 20ug/kg) enhance OP memory consolidation, but lower (10ug/kg) and higher (40 and 60ug/kg) doses have no effects.

In order to more precisely determine the dose-response relationship, OP memory performance was tested with simultaneous doses of 0, 10, 20, 40, and 60ug/kg of 17β-estradiol (n=10-11 in each group). During T1, no significant group differences in exploring time around the objects were found (data not shown). Fig. 8 shows the mean exploration ratios of the 0μg (ratio=0.47), 10μg/kg (ratio=0.58), 20μg/kg (ratio=0.67), 40μg/kg (ratio=0.57), and 60μg/kg (ratio=0.51) groups during the retention trial. ANOVA indicated a significant group differences ($F_{4, 47}$=3.191, p<0.021), and only the 20ug/kg group was significantly different from the control group (p<0.002). Thus, the dose-response relationship was, based on percent times spent exploring objects at the new locations, approximately inverted U with 20ug/kg of 17β-estradiol exerting the greatest memory enhancing effects.

Next, we determined whether effects of acute estrogen on OP memory were time dependent using the 45min delayed post-sampling injection. Previous studies demonstrated that acute estrogen enhanced place memory when given immediately after T1 but not 2hr later (Luine, 2003). When estrogen treatment was delayed 45 min after T1, memory-enhancing effects of estradiol (Fig. 9A, middle and right panel) were not observed ($F_{1,17}$=0.22, p<0.83), and both control and 20μg/kg group spent similar amount

of time exploring the objects around the new and old locations (Fig. 9B, middle panel), and there was no difference in exploration ratios between two groups (Fig. 9B, right panel). These results indicate that 20μg/kg of estradiol must be present immediately after T1, but not 45min later, to enhance OP memory consolidation.

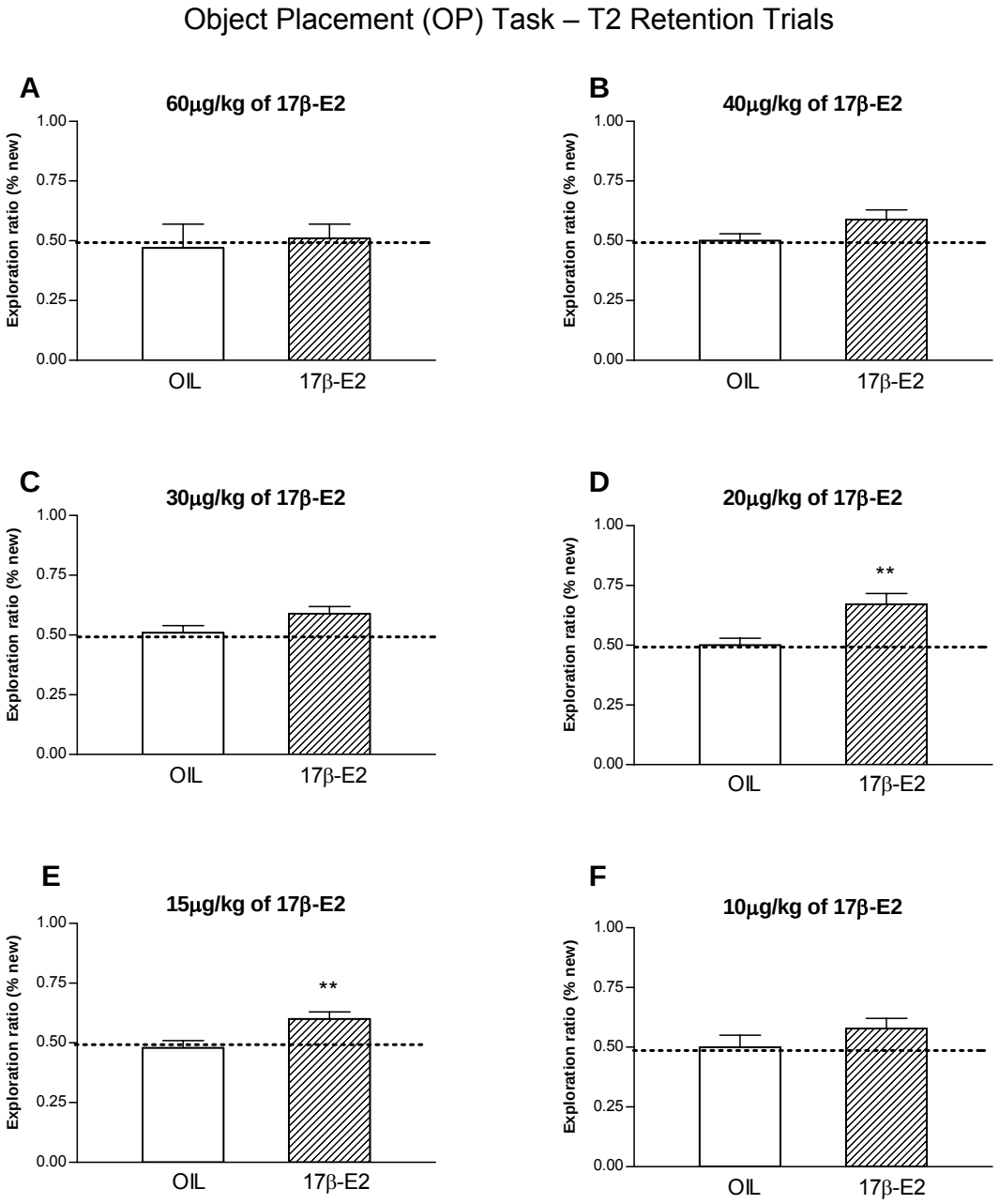


Figure 7. **Acute effects of 17β-E2 on object placement**. Exploration ratios during T2 are shown. Data were analyzed by two-way ANOVA (group x location) with two sample t tests. The dashed line at 0.5 indicates chance level performance (rats spent equal amount of time around objects at old and new locations). n=9-10 in each group. **A.** OIL vs.60ug/kg of E2. Not significant. **B.** OIL vs.40ug/kg of E2. Not significant. **C.** OIL vs.30µg/kg of E2. Significant interaction (p<0.012), but not significant group differences (p<0.065). **D.** OIL vs.20ug/kg of E2. Significant interaction (p<0.004) and group differences (p<0.009) were found. **E.** OIL vs.15ug/kg of E2. Significant interaction (p<0.01) and group differences (p<0.008) were found. **F.** OIL vs.10ug/kg of E2. Not significant. Entries are means ± SEM. *p<0.05; **p<0.01.

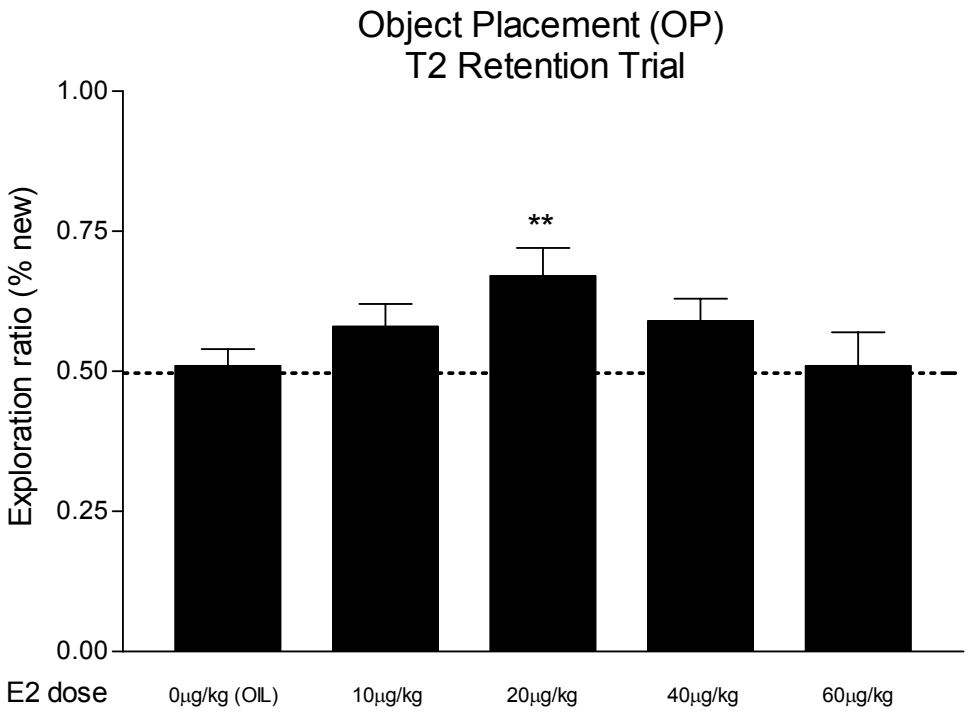
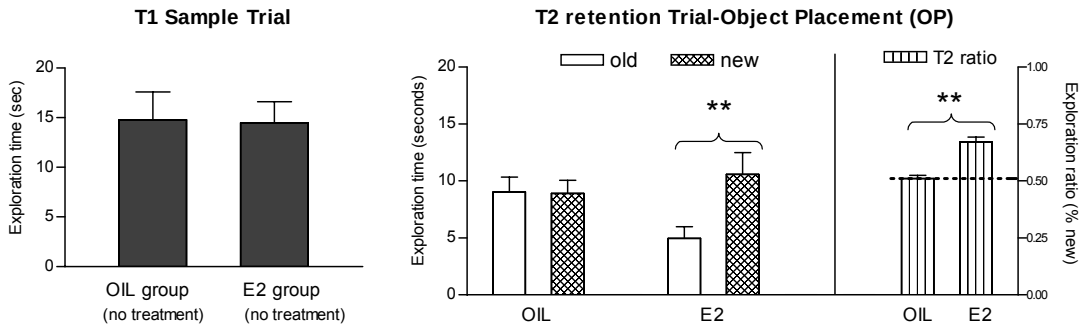


Figure 8. Dose-responses for 17β-E2 (Object Placement). Data were analyzed by one-way ANOVA with post-hoc test (Fisher LSD). The dashed line at the exploration ratio of 0.5 indicates chance level performance (rats spent equal amount of time around objects at old and new locations). A Significant difference was found by ANOVA($F_{4,47}$=3.191, p<0.021). The Fisher LSD indicated only 20μg/kg E2 enhanced OP memory (p<0.002), revealing approximately inverted U dose-response relationship. Entries are means ± SEMs. ** p <0.01.

Immediate vs. Delayed Post-T1 Treatment with 20μg/kg of 17β-E2

A. Immediate Post-T1 Treatment



B. 45 min Delayed Post-T1 Treatment

Figure 9. Immediate vs. delayed post-T1 treatment (object placement). Time-dependent effects of estrogen were examined with 20ug/kg of 17β-E2 using a delayed post T1 injection paradigm. Left panels show exploration times around objects during T1. The time spent exploring objects at old and new locations during T2 were analyzed by two-way ANOVAs (group x location), and shown in the middle panels. Exploration ratios during T2 are shown in the right panels. Dashed lines at 0.5 indicate chance performance (rats spent equal amount of time around objects at old and new locations). **A** 20μg/kg of 17β-E2 given immediately after T1 significantly enhanced OP memory. **B**. When the same dose was given 45min after T1, E2 memory enhancing effects disappeared. Entries are means ± SEM. ** $p < 0.01$

EX. 1-2. Acute 17β-estradiol effects on object recognition (OR)

For OR memory, we first tested 20μg/kg as this dose enhanced OP memory performance. No significant interaction was found by ANOVA (Data not shown). Then we examined 5, 10, and 15ug/kg (n=9-10) based on our previous finding that lower doses of 17β-estradiol were sufficient to enhance OR compared to OP (Luine et al., 2003). During the sampling trials, no significant group differences in exploration times were found (Fig.10, right panels). In T2s, there were significant effects for 5ug/kg ($F_{1,16}$=10.49, p<0.005) and 10ug/kg ($F_{1,17}$=9.038, p<0.008) treatments. Paired t-tests indicated that rats treated with these doses spent significantly more time around the new objects (t_8=3.117, p<0.01, and t_9=4.897, p<0.001, respectively: Fig.10A and Fig.10B, middle panels), while rats treated with 15μg/kg did not (Fig.10C). For exploration ratios, the 5ug/kg group (ratio=0.67) was significantly different from the control group (ratio=0.54) (Fig.10A, right panel, t_{16}=2.188, p<0.044), but 10μg/kg group (ratio=0.64) was not significantly different from the control (ratio=0.56: Fig.10B, right panel), indicating that the lowest tested dose (5μg/kg) enhanced OR memory, but the higher doses (10 and 15ug/kg) had no effects.

The dose-response relationship was examined with 0, 5, 10 and 15ug/kg of 17β-estradiol (n=11-15: Fig.11). There was no difference in explorations in T1 (data not shown). In T2, significant group differences in exploration ratios were found ($F_{3,52}$=3.604, p<0.019). Post hoc test indicated exploration ratio in the 5ug/kg group was different from control (p<0.021). Thus, the dose-response pattern for OR is similar to OP, showing the greatest memory enhancement at 5μg/kg of 17β-estradiol.

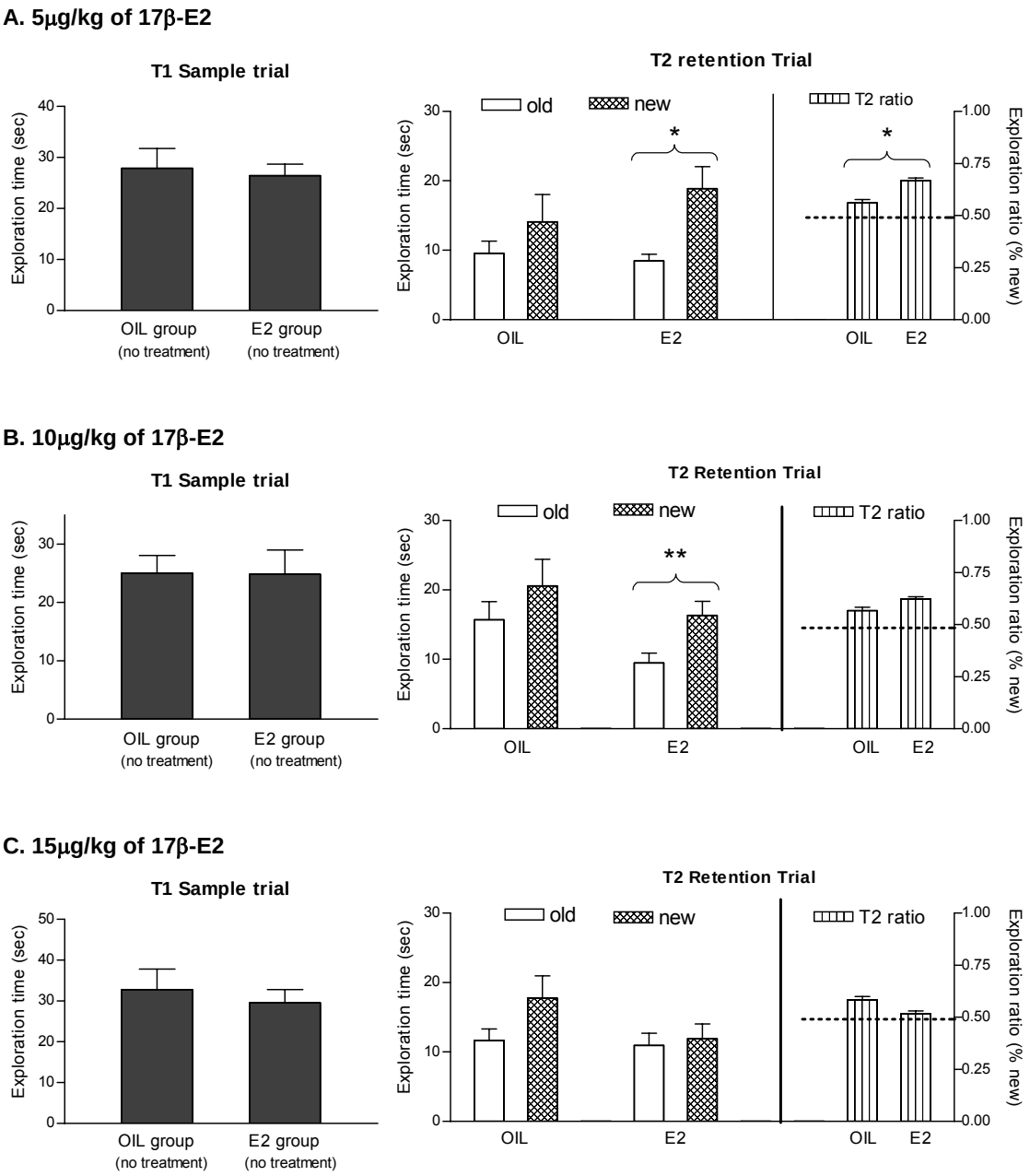


Figure 10. Acute effects of 17β-E2 on object recognition (OR). Time spent around objects during T1 (Left), T2 (Middle) and exploration ratios during T2 (Right). Data were analyzed by two-way ANOVA (group x object) with post-hoc tests. n=9-10. T1 exploration time was not significant for all groups. **A.** OIL vs. 5ug/kg of E2. Significant interaction (p<0.005) was found and both exploration times (p<0.01) and ratios (p<0.04) were different from the control. **B.** OIL vs.10ug/kg of E2. Significant interaction (p<0.008) and exploration times (p<0.001) were found. **C.** OIL vs. 15ug/kg of E2. Not significant by ANOVA. Entries are means ± SEM. * P < 0.05, ** p<0.01

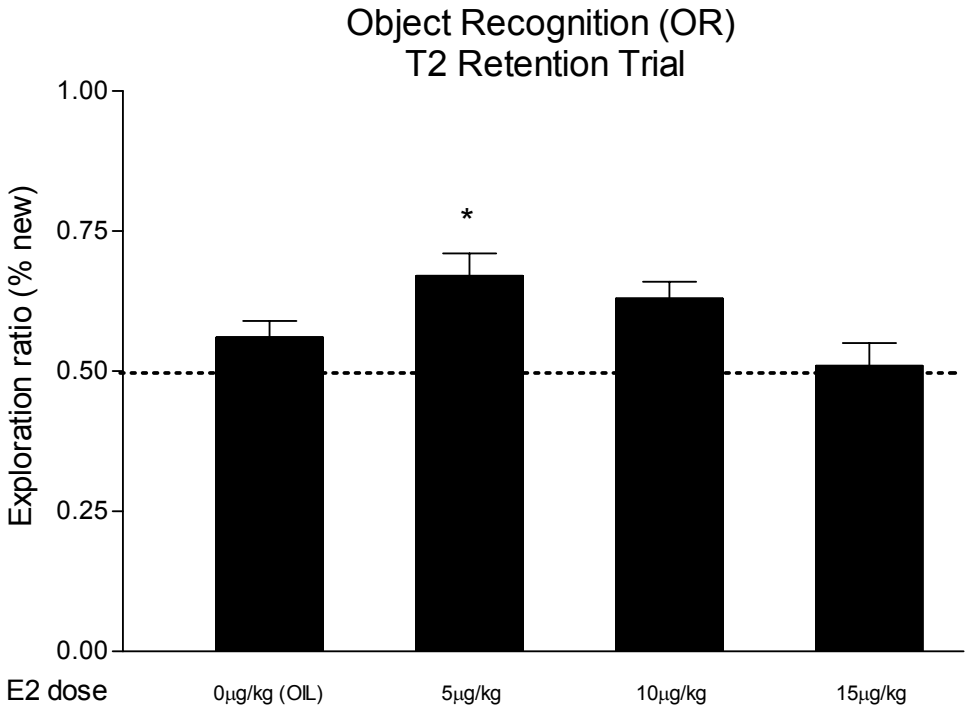


Figure 11. Dose-response for 17β-E2 (Object Recognition) Data were analyzed by one-way ANOVA with post-hoc test (Fisher LSD). The dashed line at the exploration ratio of 0.5 indicates chance level performance (rats spent equal amount of time around objects at old and new locations). A significant difference in exploration ratios was found, $F_{3, 52}$=3.604, p<0.019 and 5μg/kg enhanced memory (p<0.02). Entries are means ± SEMs. *p<0.05.

EX. 1-3. Acute 17α-estradiol effects on object placement (OP)

Because a number of studies showed higher affinity of 17α-estradiol for membrane associated estrogen receptors (MacLusky et al., 2005b; Toran-Allerand et al., 2005; Wade et al., 2001), acute 17α-estradiol effects on memory consolidation were also investigated. As with 17β-testing, we first tested 20μg/kg of 17α-estradiol since this dose of 17β-estradiol was effective for OP memory enhancement. However, no significance was found (data not shown). Since our previous study indicated that OP memory was enhanced at a lower dose of 17α-estradiol than 17β-estradiol when treatment was given 30 min before T1 (Luine et.al, 2003), we tested several lower doses (0, 2, 5, 10μg/kg: n=8-9 in each group) for dose-response relationship between 17α-estradiol and OP memory performance with the post-T1 injection paradigm. We found no significant difference in exploration times during T1 (data not shown). Fig.12 shows the mean exploration ratios of the 0μg (ratio=0.47), 2μg/kg (ratio=0.51), 5μg/kg (ratio=0.68) and 10μg/kg (ratio=0.54) group during the retention trial. One-way ANOVA revealed significant group differences in exploration ratios ($F_{3, 30}=4.06$, $p<0.015$) with only the 5μg/kg dose significantly different from the control group ($p<0.0019$). Thus, the dose-response relationship between 17α-estradiol and OP memory performance is like 17β-estradiol, an inverted U shape, but four-times lower doses of 17α-estradiol showed the greatest effect for OP memory consolidation.

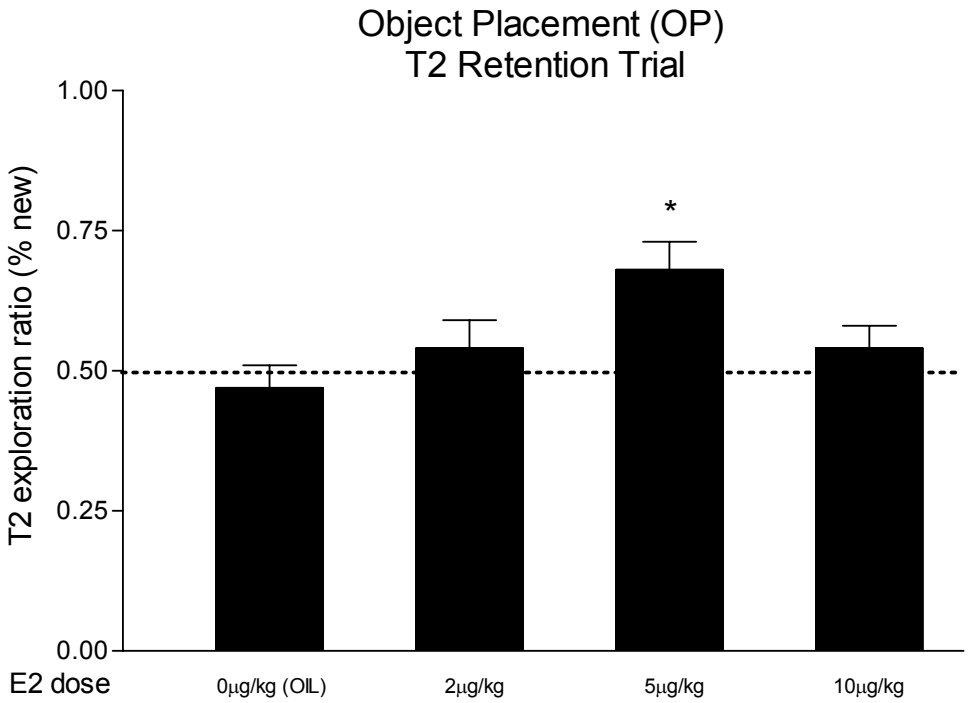


Figure 12. Dose-response for 17α-E2 (Object Placement) Data were analyzed by one-way ANOVA with post-hoc test (Fisher LSD). The dashed line at the exploration ratio of 0.5 indicates chance level performance (rats spent equal amount of time around objects at old and new locations). A significant differences in exploration ratios were found, $F_{3, 30}$=4.06, p<0.015. A post hoc test (LSD) showed the ratio of the 5ug/kg of E2 group was significantly different from the vehicle-treated control group, p=0.019. The dose-response relationship was an inverted U with 5ug/kg of 17α-E2 exerting the greatest OP memory enhancing effects. Entries are means ± SEM. $P < 0.05$.

EX. 1-4. Acute 17α-estradiol effects on object recognition (OR)

Finally, we examined the dose-response relationship between 17α-estradiol and OR memory performance with 0, 0.5, 1, 2, 10ug/kg (n=8-9 in each group), and a similar pattern of results to OP was obtained. During T1, rats in all groups spent similar amount of time around the objects (data not shown). There was a significant group difference in exploration ratios during T2 ($F_{4,38}$=2.763, p<0.041). A post-hoc test revealed that exploration ratio of both 1μg/kg (ratio=0.62) and 2ug/kg (ratio=0.60) groups were significantly different from the control group (p<0.013 and p<0.020, respectively), indicating these doses enhanced OR memory consolidation, but 0.5μg/kg (ratio=0.55) and 10μg/kg (ratio=0.5) were ineffective. Consistent with 17β-E2 experiments, the dose-response patterns between acute 17α-E2 and OR memory performance appeared similar to an inverted-U (Fig.13)

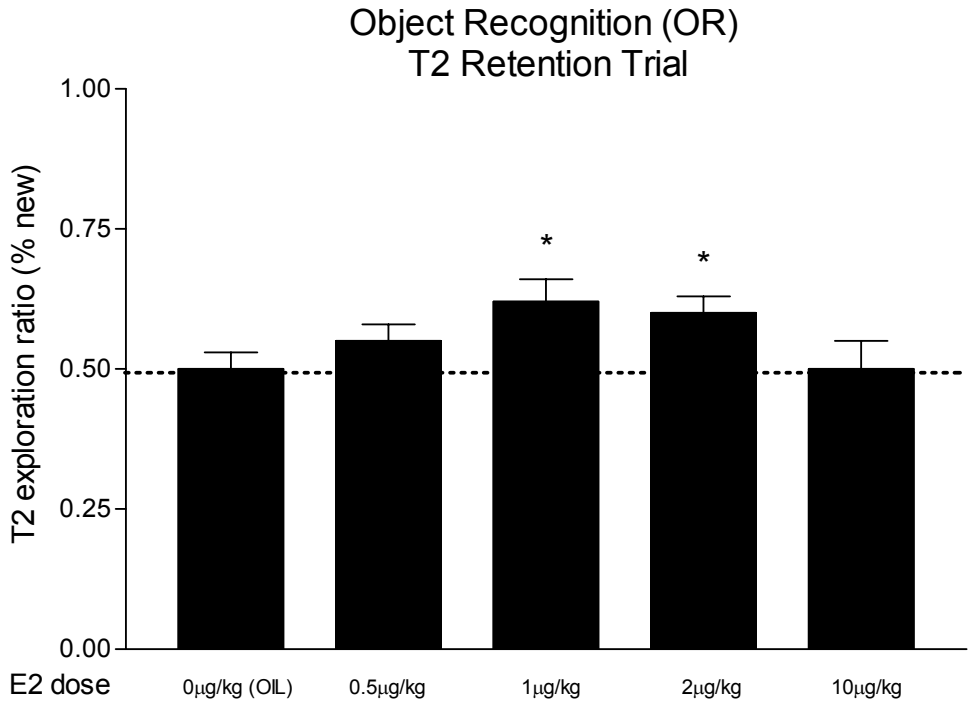


Figure 13. Dose-response for 17α-E2 (Object Recognition) Data were analyzed by one-way ANOVA with post-hoc test (Fisher LSD). The dashed line at the exploration ratio of 0.5 indicates chance level performance (rats spent equal amount of time around objects at old and new locations). A significant differences in exploration ratios were found, $F_{4, 38}$=2.763, p<0.041 and both 1μg/kg (p<0.013) and 2ug/kg (p<0.02) of E2 enhanced memory. The dose-response relationship appeared an inverted U.Entries are means ± SEMs. *p<0.05.

Discussion

The current study examined acute effects of estrogen on memory consolidation and determined dose-response characteristics between hormone treatment and object placement (spatial memory) and object recognition (non-spatial memory) tasks. The present data are consistent with previous results that post-sampling injection of both 17β- and 17α-estradiol facilitates memory consolidation, and they provide novel results that enhancement is dose-, task-, and temporally dependent. Some important relationships between acute estrogen and memory are: (1) Dose-response patterns are an inverted-U for both estrogen isomers in the tasks: (2) The effective window for the post-sampling treatment is narrower (45min) than previously reported (2hr): (3) Non-spatial memory is enhanced at much lower doses of estrogens than spatial memory: (4) 17α-estradiol is more potent than 17β-estradiol in enhancing memory

Dose-response patterns

The present study demonstrates that acute post-sampling exposure to 17-β estradiol and/or 17α-estradiol results in rapid memory enhancement in the hippocampus-dependent memory tasks when retention is tested 4hr later. This result is compatible with previous findings from our lab (Luine et al, 2003) and others (Rhodes & Frye, 2004), and provides further support for the hypothesis that estrogens have the ability to improve memory rapidly, within a few hours after treatment. Moreover, a novel finding is that acute effects of estrogen on OP and OR memories are highly dose dependent, and dose-response patterns are not a classic sigmoid curve, but appear to be an inverted-U. These results are consistent with previous work showing a similar inverted-U dose-response curve using other types of spatial memory tasks, different routes of administration, and

longer inter-trial delays in both male and female rats (Packard and Teather, 1997a: 1997b: 1998) and mice (Gresack and Frick, 2006b). For example, Packard and Teather utilized a Morris water maze to access spatial memory and demonstrated that doses higher and lower than 200μg/kg of 17β-estradiol did not affect retention when tested 24hr after intraperitonial (ip) injection in a post-training treatment paradigm (1997b). This finding was recently replicated by Gresack and Frick (2006b) using the same task, doses and ip injection in OVX mice. Thus, this pattern of hormonal effects may be generalized across memory tasks, routes of administration, lengths of inter-trial delays, and types of species.

Results show that 17α-estradiol, as well 17β-estradiol, is potent and exhibits dose-response patterns similar to an inverted-U in the OP as well as in the non-spatial, object recognition (OR), task (see Fig. 6A and 6B). To our knowledge, this is the first demonstration that post-sampling 17α-estradiol enhances both spatial and non-spatial memory consolidation in a dose-specific manner and that the dose-response patterns are an inverted-U. This response pattern is not surprising (Baldi & Bucherelli, 2005; Calabrese & Baldwin, 2000, 2001a, 2001b, 2003; Calabrese & Blain, 2005; Zoladz & Diamond, 2008) because inverted-U dose-response curves are relatively common characteristics for the post-training injection effects of some hormones, drugs, environmental chemicals and neurotransmitters on performance in memory and learning tasks (Boccia et al., 1998; Clark et al., 1998; Flood et al., 1987; Okuda et al., 2004; Roozendaal, 2000). Packard (1998) argued that an inverted U might represent optimal levels of receptor activation necessary for memory enhancing effects. In this hypothesis, both too little and too much agonist is ineffective because lower doses produce sub-

optimal levels of receptor activation, whereas higher doses produce supra-optimal levels which adversely affect receptor activation.

Although mechanisms underlying estrogen effects on memory are not clear, evidence indicates that estrogens exert direct effects on hippocampal synaptic plasticity (Spencer et al., 2008; Woolley, 2007) and association between estrogen-mediated changes in spine density and performance in memory tasks has been reported (Li et al., 2004; McLaughlin et al., 2008; Wallace et al., 2006; Wallace et al., 2007). A recent brain study demonstrated that both isomers of estradiol altered dendritic spine synapse density rapidly and, interestingly, lower doses of 17α-estradiol (15μg/kg) produce greater increases than higher doses (45μg/kg) of the same hormone (MacLusky et al, 2005b), suggesting dose-response relationships between 17α-estradiol and spine synapse density may also be an inverted U. Thus, their results are consistent with Packard's optimal receptor activation hypothesis, and may account for the inverted U dose-response effects of acute estrogen treatment on memory consolidation observed in previous and the current behavior studies.

In contrast to the similar dose-response patterns, the effective dose of 17β-estradiol for spatial memory consolidation was markedly higher in the previous studies, 200μg/kg, (Packard and Teather, 1997b: Gresack and Frick, 2006b: Fernandez et al, 2008: Lewis et al, 2008: Harburger et al., 2009) than in the current study, 20μg/kg. This discrepancy most likely results from differences in length of inter-trial delays, demands of memory tasks, as well as routes of hormone administration. In the current study, retention was tested 4 hr after hormone treatment, while all previous studies tested memory 24hr and/or 48 hr after treatment. Therefore, it is possible that longer inter-trial

delays require more estrogen to enhance memory. Different demands in memory tasks may also influence the dose-response. In this study, spatial memory was measured on the object placement task, which is a relatively stress-free memory task as it uses animal's novelty preference. On the other hand, the Morris water maze is a relatively stressful task as animals are forced to swim to locate a hidden platform. High levels of corticosterone in the limbic system and the hippocampus during the course of the Morris water maze have been reported (Aguilar-Valles et al., 2005). In such environment, greater levels of receptor activation and hence higher doses of estrogen might be required to enhance memory consolidation. Alternatively, dose-response relationships for acute post-training/sampling estrogen may not be a simple inverted U, but rather a non-monotonic curve with multiple effective doses (ineffective doses between effective doses) or bimodal dose-responses. Some hormones, environmental chemicals and endocrine disruptors have shown these non-monotonic dose-response relationships in cell culture lines, including time and dose-dependent effects of 17β-estradiol on ERK activation levels (Watson et.al, 2007a,b), and membrane-initiated action of Bisphenol A, an estrogenic environmental chemical, on prolactin release (Wozniak et al., 2005). It is important to note that the lowest tested dose of 17β-estradiol in the previous acute post-training studies was 100μg/kg, whereas the present study tested the dose range of 10-60μg/kg of 17β-estradiol. Therefore, there is a possibility that both 20 and 200μg/kg are effective but doses in between are ineffective. Since no study has explored this possibility, further research is necessary to investigate possible bimodal dose-responses between acute estrogen and memory consolidation processes.

Time-limited effects of acute estrogen treatment

Previous studies have shown that acute estrogen treatments enhance memory given up to 2hr after a training or sample trial (Packard et al., 1997b: Luine et al., 2003) We evaluated this effect with 20μg/kg of 17β-estradiol given immediately, or 45 min after the sampling trial. Confirming previous findings, OVX rats given estrogen immediately after the sample trial had better spatial memory than the vehicle-treated control (Fig. 4A, right), but OVX rats receiving the treatment 45 min after the sample trial did not, a narrower critical time window than previously reported 2hr (Fig. 4B, right). The differences between this and previous studies are probably due to different inter-trial delays (e.g., 24hr vs.4hr, respectively) and/or effective estrogen doses (e.g., 200μg/kg vs. 20μg/kg, respectively).

Interestingly, a similar time-dependent effects of acute estrogen treatment was also reported in a neural plasticity study (MacLusky et al., 2005b), which found that increases in dendritic spine synapse density in the hippocampus were greater at 30 min after acute estrogen treatment than that at 4.5 hr regardless of increasing circulating estrogen levels over time. MacLusky et al argued that the decline at 4.5hr and greater potency of the lower dose might reflect down-regulation of the response mechanism. In addition, these biphasic temporal effects of estrogen on hippocampal plasticity also suggest that responses may involve different mechanisms; membrane ER mediated responses for the initial rapid increase in spine synapse density at 30 min after treatment, and nuclear mediated responses or integrated actions of genomic and nongenomic pathways for the subsequent decrease at 4.5hr after treatment. Importantly, these time-dependent effects of estrogen are consistent with memory consolidation hypotheses

(McGaugh, 2000), in which newly acquired information is not stable in short-tem memory and must undergo a series of time- and hippocampus-dependent dynamic processes including protein synthesis (McGaugh, 1966; Rossato et al., 2007), activation of ERK and CaMKII (Cammarota et al., 2008), MAPK, CREB and zif268 (Bozon et al., 2003) , BDNF (Lee et al., 2004) , mRNA synthesis (Igaz et al., 2002) and increase/decrease synaptic efficacy (Bozon et al., 2003) to be consolidated. Typical research in this field uses protein synthesis inhibitors and has shown existance of critical time windows for these alterations during the course of memory consolidation, which may be task specific (Seitz et al., 2005). For example, object recognition memory consolidation was not blocked when a protein synthesis inhibitor was given 360 min after the sampling trial, but was blocked if the same inhibitor was administrated immediately after and/or 180 min after the sampling trial (Rossato et al., 2007). Considerable evidence in recent research suggests there are at least two different time windows for protein systhesis inhibitors: one during or immediately after training/sampling, and the other 3-4hr after training/sampling (Igaz et al., 2002). Most importantly, some drugs and hormones alter these protein synthesis and memory consolidation processes time-dependently. For example, strychnine, a stimulant, has memory enhancing effects only if administrated within 30min after training (McGaugh, 1966). A recent model for NMDA receptor dependent protein synthesis suggested that altered protein synthesis serves as a trigger for memory formation (Klann & Sweatt, 2008). Thus, it is possible that encoding of new information may require activation of rapid, non-genomic pathways, which trigger protein synthesis process, and the presence of adequate levels of estrogen immediately after the sampling trial would facilitate these processes. In other words,

observed time-dependent effects in the current behavioral study suggest that initial membrane mediated responses may be critical for estrogen induced enhancement of memory consolidation and better performance later in retention.

It should be noted that one possible confound of the current study is that injected hormones might not be fully metabolized and present in the circulating system when animals are tested 4 hr later, and therefore, it is possible that estrogen might have enhanced the retrieval process rather than consolidation and that potential non-mnemonic effects of estrogen might have affected memory performance during retention. But this possibility is unlikely because treatments given immediately after the sample trial enhance memory, while treatments administrated 45 min after the sample trial have no effects.

Optimal doses for spatial vs. non-spatial memory consolidation

The current results indicate that the same dose of estrogen which enhances object memory consolidation, 5μg/kg, is not effective for place memory consolidation. Four times the dose of estradiol (20μg/kg) was necessary to enhance OP memory, indicating that non-spatial memory maybe more sensitive to estradiol than spatial memory. These differences may be due to differential effects of estrogen in distinct brain regions critical for these two types of memories. Although both are hippocampal dependent working memory tasks, object placement is primarily dependent on an intact hippocampus and/or fornix (Ennaceur & Aggleton, 1994), and may also rely on prefrontal cortical input (Ennaceur et al., 1997). On the other hand, object recognition is less dependent on the hippocampus and requires prefrontal cortical activity (Ennaceur et al., 1997). This notion is evidenced by the demonstration that lesioning of the hippocampus impairs memory

performance in the object placement task, but caused lesser effects in the object recognition task (Broadbent et al., 2004; Mumby et al., 2002). For example, Broadbent et al showed that damage 30–50% of the dorsal hippocampus caused spatial memory impairment, while lesions of 75–100% were required to impair object recognition memory (Broadbent et al., 2004).

In addition, differences in task demands may also account for different effective doses for object placement and recognition memory, as the cognitive load for spatial memory is greater than non-spatial object recognition memory (Ennaceur et al., 2005). Indeed, objects can be encoded and discriminated through multiple sensory modalities (e.g., vision and tactile) using a variety of cues such as the size, shape, color (wavelength as well as intensity) and texture of objects, while discriminating location of objects involves abstract categorizations and use of "cognitive maps". Thus, it is consistent with the current result that object memory consolidation is facilitated at lower estrogen doses compared to spatial location memory.

17α-estradiol is more potent than 17β-estradiol

Interestingly, the present results indicate that post-sampling 17α-estradiol was more effective than 17β-estradiol for enhancement of memory consolidation. As shown in Fig. 3 and Fig. 6, about 4-5 fold lower doses of 17α-estradiol, as compared to 17β-estradiol, enhance memory. These results are particularly noteworthy because binding affinity of 17α-estradiol for classic nuclear estrogen receptors, ERα and ERβ, is considerably weaker (42% lower for ERα and 89% lower for ERβ) than 17β-estradiol (Kuiper, et al., 1997, 1998), and, therefore, 17α-estradiol is generally considered less active in the brain and does not elicit significant estrogenic responses (Anstead et al.,

1997). Thus, our results challenge the general notion of effectiveness of 17α-estradiol and raise a possibility that observed acute estrogen effects on memory consolidation may be mediated through membrane associated estrogen receptor systems. Supporting this hypothesis, several lines of evidence indicate that 17α-estradiol has an equal or even stronger biding affinities to membrane associated ERs (Green et al., 1997; Toran-Allerand et al., 2005; Wade et al., 2001) and can elicit greater estrogenic responses rapidly (MacLusky et al., 2005b; Toran-Allerand et al., 2005). For example, 17α-estradiol has an equal potency to 17β-estradiol to elicit rapid and sustained activation of the MAPK/ERK and phosphatidylinositol 3-kinase-Akt signaling pathways (Singh et al., 2000) and 17α-estradiol is considerably more potent than 17β-estradiol in rapid elevation of hippocampal CA1 dendritic spine synapse density (MacLusky et al., 2005b), which parallels the current behavior data. Interestingly, recent studies have reported that 17α-estradiol might be synthesized locally in the brain (Toran-Allerand et al., 2005), and affects neural functions rapidly within the timescales of sec to minutes (Hojo et al., 2008; Woolley, 2007). In intact rats, Toran-Allerand et al (2005) measured estrogen concentrations in several tissues including the hippocampus, adrenals, ovaries and uterus and found higher levels of 17α-estradiol than 17β-estradiol in all samples of the brain. In OVX and adrenalectomized mice, 17α-estradiol was also measured in the brain, while 17β-estradiol was not. Although functional effects of elevated 17α-estradiol are not yet known, 17α-estradiol may exert autocrine and/or paracrine effects in the brain (Toran-Allerand et al., 2005), one of which might be on cognitive function.

Experiment 2: Acute estrogen and BPA treatment effects, alone and in combination, on memory consolidation and anxiety levels (aim 2)

Many chemicals in the environment can mimic or antagonize the effects of endogenous hormones. Bisphenol A (BPA) is such a chemical with mixed estrogen agonist/antagonist properties. Recent studies indicate that low dose BPA may exert a powerful impact on neural systems, activating membrane associated ER systems and signaling pathways (see Vandenberg et al, 2009 for review), which suggests that BPA may also alter estrogen-mediated behavior in adults. Currently, almost all existing BPA behavior studies have focused on behavioral consequences of developmental exposure to this chemicals and little is known how BPA interact with E2 in the adult brains and hence affect behavior.

BPA has been reported that it weakly mimics estrogenic activity when E2 is absent, while in the presence of estrogen, BPA blocks estrogen activity (Kawato, 2004; MacLusky et al, 2005a; Zsarnovszky et al, 2005; Leranth et al, 2008). A recent synaptic plasticity study, for example, showed that acute BPA treatments rapidly antagonized estrogen-induced spine synapse density in the CA1 of the hippocampus in adult female rats (MacLuskey et al, 2005a). It is hypothesized that hippocampus plasticity is important for memory and learning (Silva, 2003). Thus, if spine synapse density in the CA 1 region of the hippocampus is one of neural system that contributes to estrogen mediated memory function, then, rapid memory enhancement induced by E2 might be blocked by co-administration of BPA. We addressed this possibility in experiment 2.

The results of experiment 1 showed that acute post sampling treatment of estradiol enhanced both OP and OR memory consolidation. In experiment 2, we

examined acute effects of BPA, alone (Ex.2-1) and combination with E2 (Ex.2-2, 3, 4, and 5) by administrating BPA (doses ranged 0.4-400μg/kg) with the most effective E2 doses for memory enhancement found in aim 1. Of particular interest was whether BPA alters behavioral responses to E2 at or below the current reference safe dose of 50μg/kg/day. Because some studies reported that chronic/subchronic E2 treatments, as well as long-term developmental exposure to BPA, influence anxiety levels (Adriani et al., 2003; Negishi et al., 2004), and therefore affect memory performance, acute E2 and BPA treatment effects on anxiety levels were tested using the elevated plus maze (Ex. 2-6). We hypothesized that BPA treatments alone would have little or no effects on memory performance: Co-administration of BPA with E2 would block E2-induced memory enhancement: Acute E2 and/or BPA treatments would not affect performance in the elevated plus maze.

Methods

Subject

Eighty-three OVX female Sprague Dawely rats, aged 55-60 days, were obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN and served as subjects for the memory tests (59 rats, Experiment 2 and 3), and the elevated plus maze test (24 rats, Experiment 4). Subjects were kept under the same condition as described in Experiment 1. All experiments were conducted in accordance with the NIH Guide for Care and Use of Animals and the Institutional Animal Care and Use Committee of Hunter College of the City University of New York.

Memory task, General procedure, habituation schedule

Apparatus, memory tasks, general procedure, and acclimation/habituation schedule were identical to those described in Experiment 1

BPA and estrogen treatment

A two-time point, acute injection paradigm (Fig. 14) was used to test effects of BPA and estrogen, alone and in combination, on memory and anxiety. Subjects were randomly assigned into one of four groups (vehicle-treated, E2 treated, BPA treated, and BPA+E2 treated) and received two subcutaneous injections --- 30 min before the T1 sample trial (Pre-T1 INJ) and immediately after the T1 sample trial (Post-T1 INJ). We used the most effective doses of 17β- and 17α-estradiol for OP and OR memory enhancement obtained from experiment 1 and tested with a variety of BPA doses (ranged 0.4-400μg/kg). These BPA doses were chosen based on previous findings that showed that acute BPA treatments inhibited E2 induced hippocampal spine synapse density within 30 minutes (MacLusky et al., 2005a). For 17α-estradiol+BPA experiments, only lower doses of BPA (less than the current reference safety dose of 50μg/kg/day). See Table 2 for the specific doses, treatment and groups for the OP and OR memory task.

Injection solutions for 17β- and 17α-estradiol were prepared as described in Experiment 1. For BPA, 10mg, 1mg and 100μg of BPA were dissolved in ethanol for stock solutions (10mg /1ml, 1mg/1ml and 100μg/1ml), and then diluted with saline for injection. The high concentration stock (10mg/1ml) was used to make 120μg/kg or higher dose injection. The medium (1mg/1ml) and low (100μg/1ml) concentration stocks were used for 10-100μg/kg injection solutions and for lower than 10μg/kg injection solution, respectively, in order to control ethanol levels in each solution.

Experimental design

Rats were tested approximately every 10 days with doses shown in Table 2. Treatments were counterbalanced in every experiment. In each OP and OR experiment, all doses of BPA were tested using a matched block paradigm (18-24 rats per block having approximately equal numbers of rats in each dose group). Such experiments were repeated every seven to 10 days until each dose group contained at least sample size of eight subjects. Vehicle-treated and E2 alone treated groups served as negative and positive control.

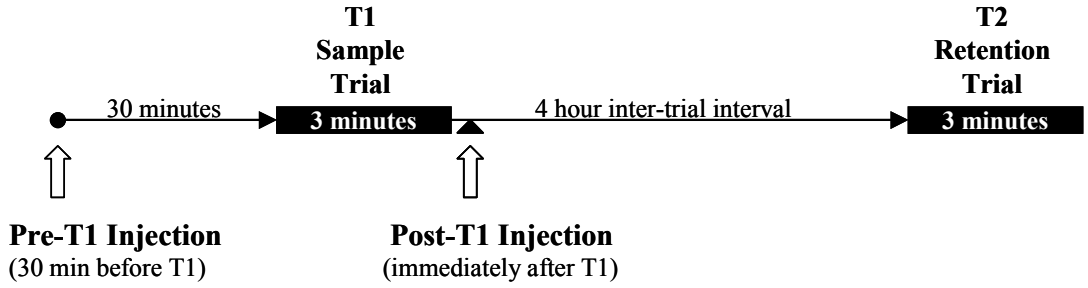


Figure 14. Timeline for the two-time point treatment paradigm. Subjects received a pre-T1 injection 30 minutes before a sample T1 trial. Post-T1 injection was given immediately after T1. Following a 4-hour intertrial interval, subjects performed the T2 retention trial.

Table 2. Summary of groups, treatments and BPA doses tested in Experiment 2

EX. 2-1 BPA alone, OP and OR

Group	Pre T1 INJ	Post T1 INJ
Vehicle-treated control	corn oil	saline
BPA groups	corn oil	1, 4, 40, 120, 240 and 400μg/kg of BPA

EX. 2-2 BPA+17β-E2, Object placement (OP)

Group	Pre T1 INJ	Post T1 INJ
Vehicle-treated control	corn oil	saline
E2-treaed control	saline	20μg/kg of 17β-E2
BPA+E2 groups	0.4, 1, 4, 40, 120, 240, 400μg/kg of BPA	20μg/kg of 17β-E2

EX. 2-3 BPA+17β-E2, Object Recognition (OR)

Group	Pre T1 INJ	Post T1 INJ
Vehicle-treated control	corn oil	saline
E2-treaed control	saline	5μg/kg of 17β-E2
BPA+E2 groups	1, 4, 40, 120, 400μg/kg of BPA	5μg/kg of 17β-E2

EX. 2-4 BPA+17α-E2, Object placement (OP)

Group	Pre T1 INJ	Post T1 INJ
Vehicle-treated control	corn oil	saline
E2-treaed control	saline	5μg/kg of 17α-E2
BPA+E2 groups	0.4, 1, 4, 40μg/kg of BPA	5μg/kg of 17α-E2

EX. 2-5 BPA+17α-E2, Object Recognition (OR)

Group	Pre T1 INJ	Post T1 INJ
Vehicle-treated control	corn oil	saline
E2-treaed control	saline	1μg/kg of 17α-E2
BPA+E2 groups	0.4, 1, 4, 40μg/kg of BPA	1μg/kg of 17α-E2

Elevated plus maze

Anxiety related behavior was tested with 20μg/kg of 17β-estradiol and 40μg/kg of BPA using plus-shaped wooden apparatus painted in gray. The elevated plus maze (EPM) consisted of 10 x 10 cm central open area, two open (50 x 10 cm) and two enclosed arms (50 x 10 x 40 cm), each with an open roof, and elevated 50 cm from the floor (Fig. 15). A new cohort of 24 rats were assigned to one of 4 groups (control, E2, BPA and BPA+E2 group, n=6 in each group) and received Pre- and Post-T1 injections (Table 3). 4hr after the second injection, individual rats were placed in the central open space facing an open arm and allowed to explore for 5 min. The number of entries and time spent in open and closed arms were recorded. A long time spent in closed arms or a high frequency of entries into the closed arms is considered an indicator of high anxiety, while a long duration of time spent in open arms or a low frequency of entries into the open arms is an indicator of low anxiety (Lonstein, 2005; Pellow et al., 1985; Pellow & File, 1986).

Table 3. Hormone and BPA treatments for the anxiety test (elevated plus maze)

Group	Treatment		
	Pre T1 INJ	Post T1 INJ	n
Control	saline	Corn oil	6
E2	saline	20μg/kg of 17β-E2	6
BPA+E2	40μg/kg of BPA	20μg/kg of 17β-E2	6
BPA	Corn oil	40μg/kg of BPA	6

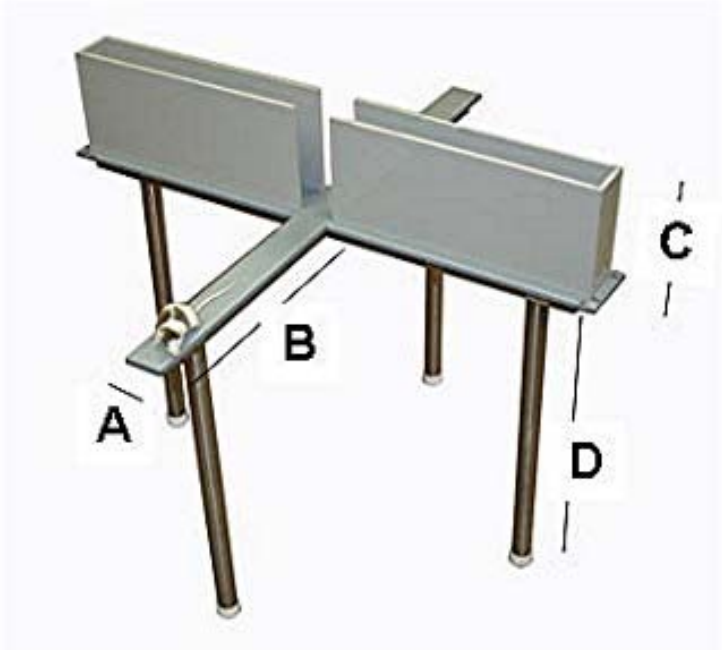


Figure 15. Apparatus for the anxiety test. The drawing shows a typical elevated plus maze, which consists of four arms extended from the center area at 90-degree angles to each other. Two opposing arms are enclosed with walls and the other two are left open. The lengths of the arms and walls used in this study were; A, 10cm: B, 50cm: C, 40cm. The maze was elevated 50cm off the ground (D).

Measurements and Statistical analyses

The basic measurement and statistical analyses were the same as those described in Experiment 1. For experiment 2, one-way ANOVA was used to test group differences in exploration ratios and/or T2 discrimination index during T2 retention trials. To interpret data and identify whether BPA inhibits E2-induced memory enhancement, we used conservative criteria, having both vehicle-treated and E2 alone-treated group as negative and positive control, respectively. Thus, if significant group differences were found by ANOVA, BPA+E2 group data were compared with both vehicle-treated control and E2 alone-treated group in the post-hoc test (Fisher LSD), and the results must meet the following criteria: 1) exploration ratios of the BPA+E2 treated groups and the E2 alone treated control must be significantly different, and 2) exploration ratios between BPA+E2 groups and vehicle-treated control should not be significantly different. If the post-hoc results are not consistent, exploration time data (time spent at the old object/location (T2 OLD) and at the new object/location (T2 NEW) during T2 were additionally tested by paired sample t-tests.

For elevated plus maze data, ratios of open arm entries (numbers of open arm entries/total numbers of entry in open and closed arms) and ratios of time spent in open arms (time spent in open arms/(time spent in open and closed arms) were calculated and group differences were tested by one-way ANOVA. All data were analyzed using SPSS software (Systat Inc., Chicago, IL., USA).

Results

EX. 2-1: Effects of *BPA treatment alone on OP and OR memory*

BPA alone effects on OP and OR memories were examined with 0, 1, 4, 40, 120, 240 and 400μg/kg of BPA (n=6-8). For both OP and OR memory, time spent exploring objects during T1 sample trials were not significant (Data not shown). For T2 retention trials, no significant group differences in exploration ratios for either OP (Fig. 16A) or OR (Fig. 16B) were found, suggesting BPA alone, at these doses, did not affect memory performance. To further test whether rats in vehicle-treated control and BPA groups spent similar amounts of time around old and new objects/locations, paired t tests were carried out to compare T2 exploration data (T2 OLD vs.T2 NEW). For object placement, no significant differences were found in any groups, suggeesting that rats in all groups performed the task at chance level, and therefore BPA did not affect spatial memory. However, for object recognition, analysis revealed significant differences for the 40 μg/kg (p<0.046), 240 μg/kg (p<0.023) and 400 μg/kg (p<0.04) of BPA treated groups and marginal significance for the 120 μg/kg of BPA (p<0.088) group (Fig. 17). These results indicate that rats with higher doses of BPA treatment spent significantly more time around the new object compared to vehicle-treated control and suggest possible memory enhancing effects of BPA on OR memory consolidation.

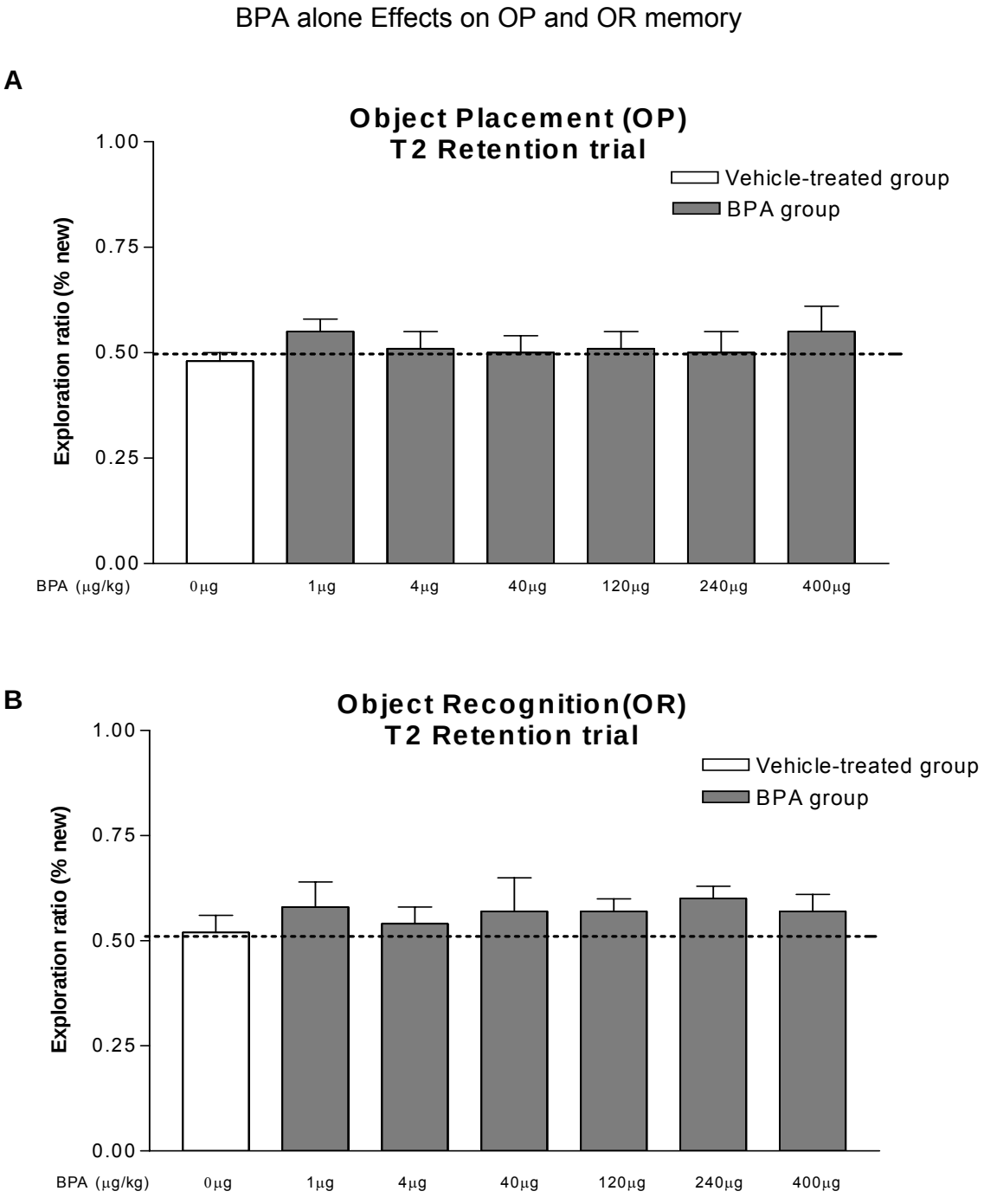


Figure 16. Effects of BPA alone on memory (exploration ratios). Data were analyzed by one-way ANOVA. Not significant for both OR and OP memory. **A**. Exploration ratios for object placement (OP). **B.** Exploration ratios for object recognition. Entries are means ± SEM.

BPA alone Effects on OR memory

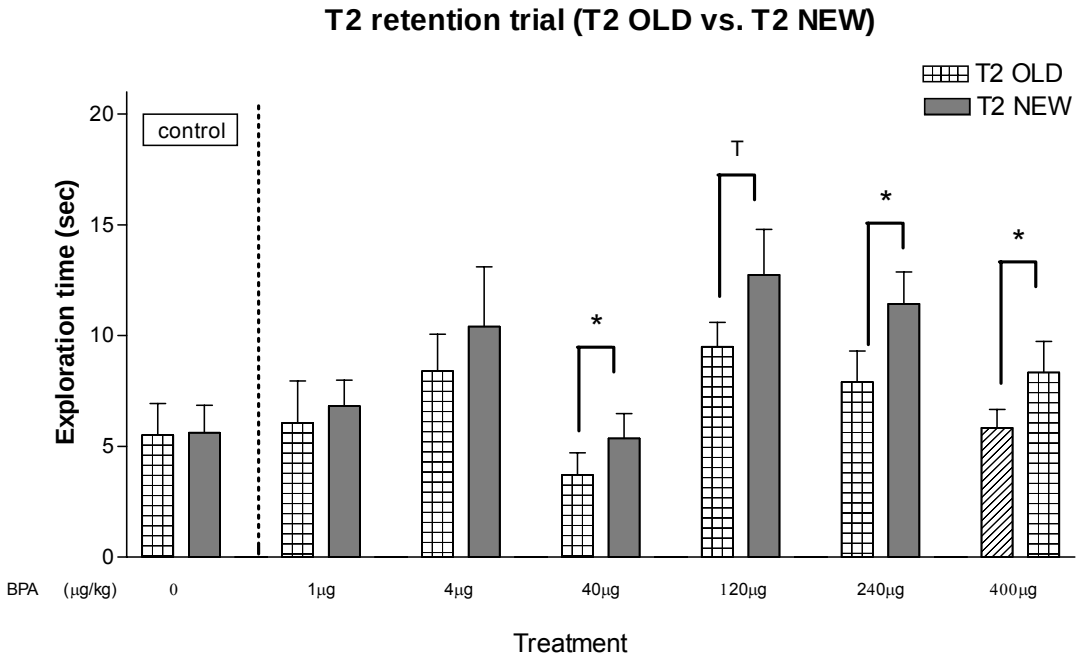


Figure 17. Effects of BPA alone treatment on OR memory (T2 old vs. New). Times spent exploring objects at old and new locations during T2 retention trial were analyzed by paired t-tests. Rats treated more than 40μg/kg of BPA spent significantly more time exploring around objects at the new locations. Data suggest these doses of BPA enhanced OR memory. Entries are means ± SEM. $_{T}$ p <0.088, * p < 0.05.

EX.2-2: BPA+ 17β-E2 effects on object placement (OP)

Time spent exploring objects during T1 sample trials was examined in vehicle-treated, E2 alone (20μg/kg of 17β-E2) treated, and BPA (0.4, 1, 4, 40, 120, 240, 400μg/kg)+E2 treated groups (n= 10-12 in each group). No significant group difference was found, $F_{8,102} = 1.14$, $p<0.35$ (data not shown). Performance on T2 retention trial was analyzed with exploration ratios, and ANOVA found a significant difference among groups, $F_{8, 102}=2.178$, $p<0.035$. Post-hoc testing was first carried out based on comparison with exploration ratios for the vehicle-treated control (ratio=0.48). Consistent with Experiment 1, acute post-sampling 20μg/kg of E2 significantly enhanced OP memory (ratio=0.63, $p<0.002$). The 0.4μg/kg of BPA+E2 group was also significantly different from vehicle-treated control (ratio=0.62, $p<0.015$), but other BPA+E2 groups were not, which suggested that BPA doses above 0.4μg/kg blocked E2-induced memory enhancement (Fig. 18A). Next, the same BPA+E2 data were analyzed based on exploration ratios of the E2 alone treated control. We found that exploration ratios for 0.4μg/kg and 1μg/kg of BPA+E2 groups were not significantly different from the E2 alone group, suggesting that these two doses did not block E2-induced memory enhancement (Fig. 18B). Since the two post-hoc results were not consistent, T2 exploration data (time spent exploring objects at old (T2 OLD) vs. new (T2 NEW) locations) were additionally tested by paired t-tests. The result showed that vehicle-treated, 1, 4, 40, 120, 240, 400μg/kg of BPA+E2 treated rats spent similar amounts of time at old and new locations, while E2 alone treated ($p<0.018$) and 0.4μg of BPA+E2 treated ($p<0.020$) rats spent significantly more time around objects at new locations (Fig. 19). Thus, we concluded that OP memory enhancement by 20μg/kg of 17β-E2 was

blocked by BPA in doses less than 1μg/kg, but above 0.4μg/kg, which is far below the current reference safety dose of 50μg/kg/day.

EX.2-3: BPA+17β-E2 effects on object recognition (OR)

For object recognition, effects of 1, 4, 40, 120, 240, 400μg/kg of BPA+ 5μg/kg of 17β-E2 (n=7-8) on memory performance were tested as Experiment 1 showed 5μg/kg of E2 significantly facilitated OR memory consolidation. Exploration times around objects during T1 were not significant, $F_{7, 54}$= 1.31, p<0.27 (data not shown). In T2, a significant group difference in exploration ratios was found, $F_{7, 54}$=2.844, p<0.013. Post-hoc tests revealed that exploration ratios of vehicle treated controls (ratio=0.52) were significantly different from E2 alone treated (ratio=0.68, p<0.010), 1μg/kg of BPA+E2 (ratio=0.64, p<0.039) and 4μg/kg of BPA+E2 (ratio=0.64, p<0.045) treated groups, while 40, 120, 240, 400μg/kg of BPA+E2 groups had similar exploration ratios to vehicle-treated controls (Fig. 20A). In concert, the exploration ratio of the E2 alone treated group was significantly different from all groups except the 1μg/kg and 4μg/kg of BPA+E2 treated groups (Fig. 20B). Therefore, the two post-hoc analyses based on negative and positive control were consistent. These results indicate that OR memory enhancement induced by 5μg/kg of 17β-E2 was blocked by 40μg/kg of BPA treatment, a lower dose than the current reference safety dose of 50μg/kg/day.

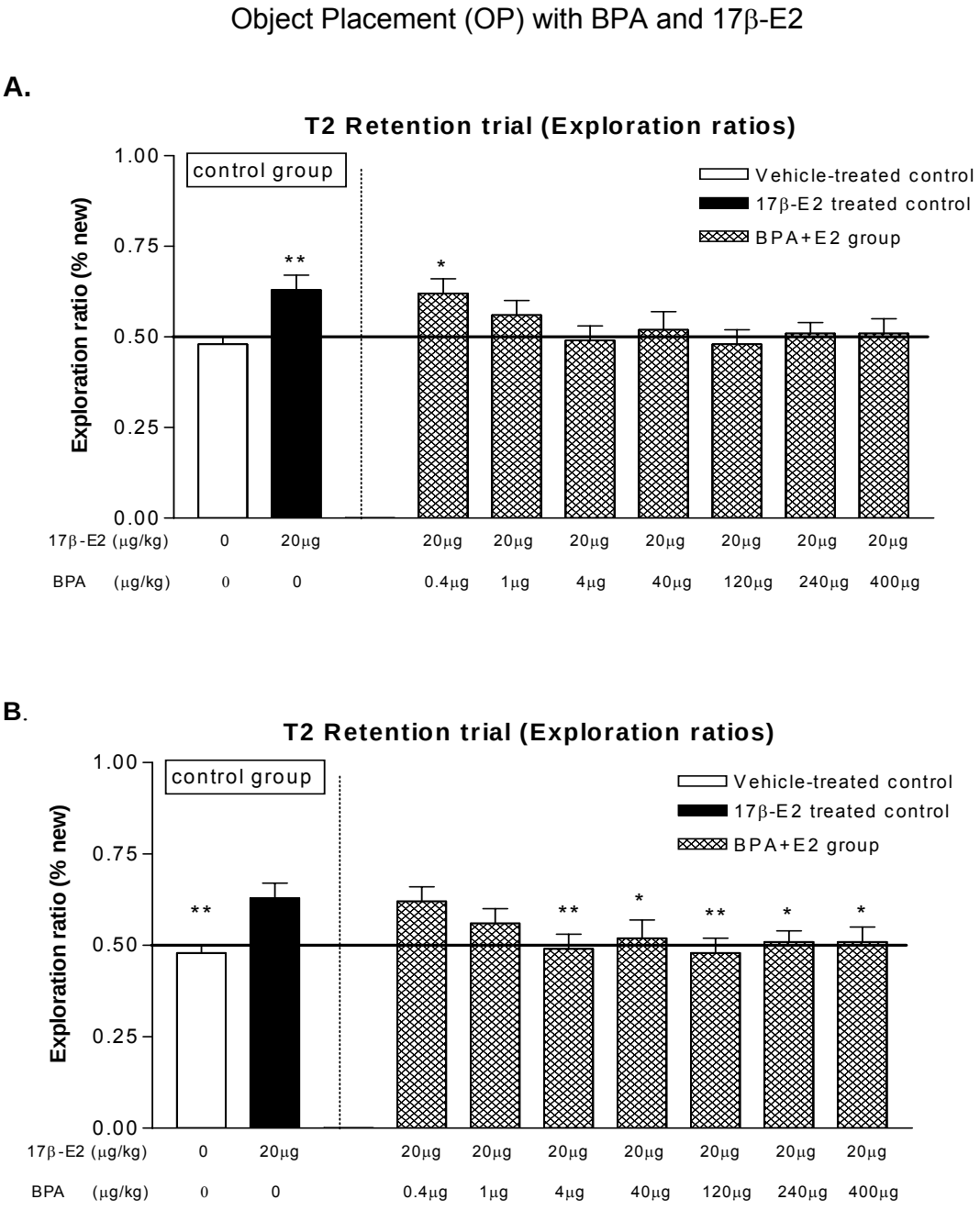


Figure 18. Effects of BPA+ 17β-E2 on OP memory (exploration ratios). Exploration ratios in vehicle-treated, E2-treated, and BPA+E2 treated groups ((n= 10-12 in each group) were tested by one-way ANOVA, which found significant group differences, $F_{8, 102}$=2.18, p=0.035. **A.** Post-hoc comparison based on vehicle-treated control. E2 treated and 0.4μg of BPA+E2 treated groups were different from control. **B.** Post-hoc comparison based on E2-treated control. Vehicle-treated, 4, 40, 120, 240, 400μg/kg of BPA+E2 treated groups were different from control. Entries are means ± SEM. ** p <0.01, * p < 0.05.

Object Placement (OP) with BPA and 17β-E2

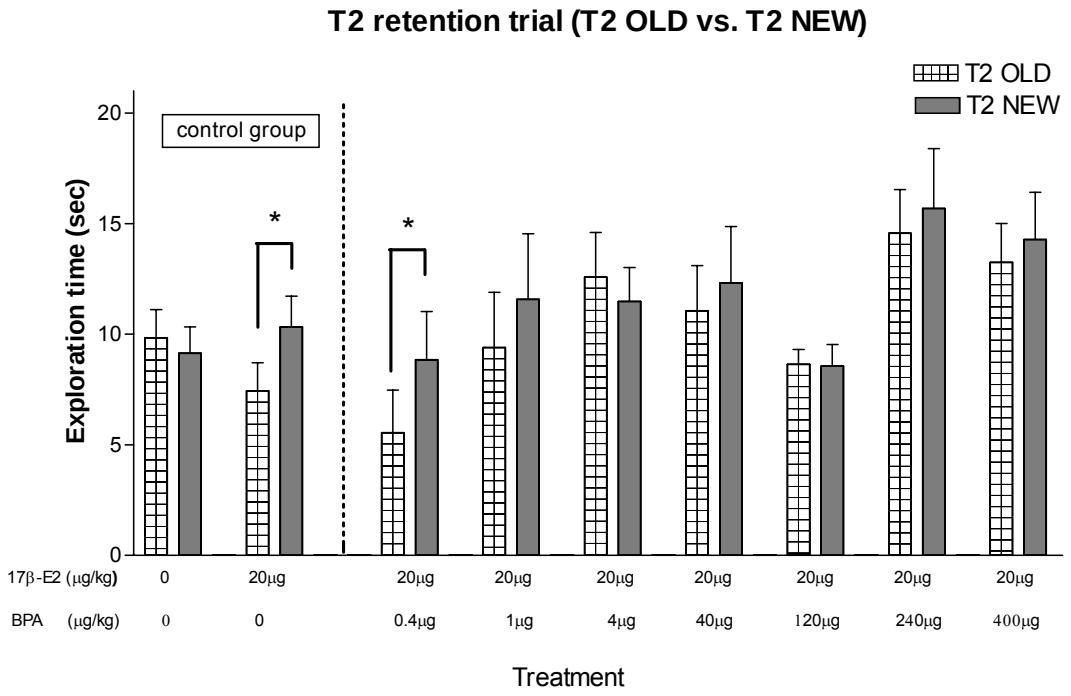


Figure 19. Effects of BPA+ 17β-E2 on OP memory (T2 old vs. New). Times spent exploring objects at old and new locations during T2 retention trial were analyzed by paired t-tests as post-hoc comparison. Rats in E2-treated (p<0.018) and 0.4μg/kg of BPA+E2 treated (p<0.020) groups spent significantly more time around objects at the new location, but rats treated more than 1μg/kg of BPA+E2 were unable to discriminate the objects between new and old location. Entries are means ± SEM. * $p < 0.05$.

Object Recognition (OR) with BPA and 17β-E2

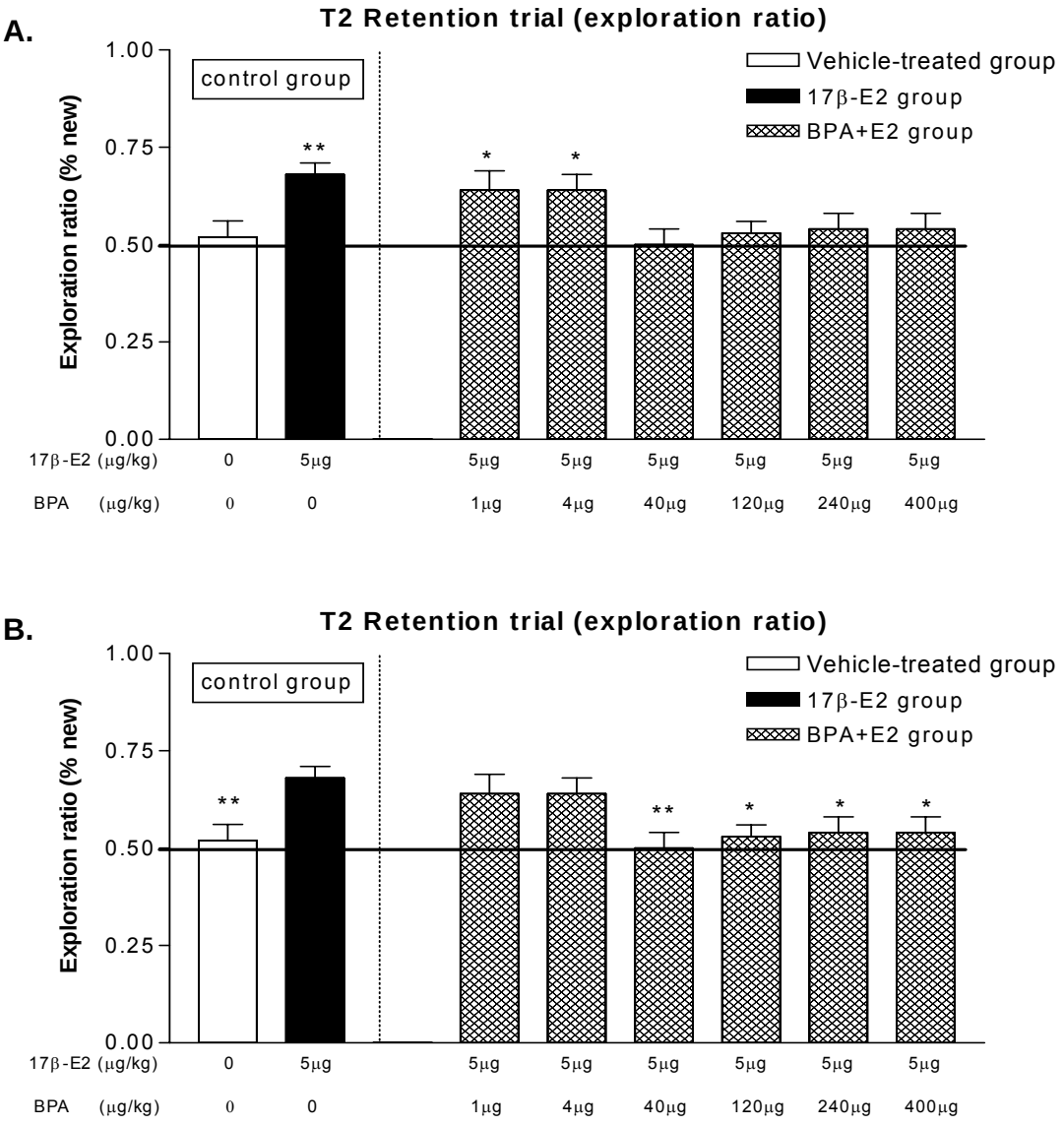


Figure 20. Effects of BPA+ 17β-E2 on OR memory (exploration ratios). Exploration ratios in vehicle-treated, E2-treated, and BPA+E2 treated groups (n=8-9 in each group) were tested by one-way ANOVA. Significant group differences were found, $F_{7, 54}$=2.844, p<0.013. **A.** Post-hoc comparison based on vehicle-treated control. E2 treated, 1 and 4μg/kg of BPA+E2 treated groups were different from control. **B.** Post-hoc comparison based on E2-treated control. Vehicle-treated, 40, 120, 240, 400μg/kg of BPA+E2 treated groups were different from control. Entries are means ± SEM. **P<0.01, * p < 0.05.

EX. 2-4: BPA+17α-E2 effects on object placement (OP)

Since BPA+17β-estradiol experiments (Ex.2-2 and Ex.2-3) showed that 40μg/kg of BPA was sufficient to inhibit E2-induced memory enhancement, only lower doses of BPA (0.4, 1, 4, 40μg/kg, n=8-9) were tested with 5μg/kg of 17α-E2. During T1 sample trials, no significant group differences were found, $F_{5, 48}$ =0.91, p<0.480 (Data not shown). For performance in the T2 retention trials, group differences in exploration ratios were significant, $F_{5, 48}$=2.50, p<0.043. Post-hoc tests revealed that exploration ratios between vehicle-treated (ratio=0.55) and E2 alone treated (ratio=0.71) groups were significantly different (p<0.008), but there were no differences between vehicle-treated and 0.4ug/kg (ratio=0.6), 1μg/kg (ratio=0.54), 4μg/kg (ratio=0.56), 40μg/kg (ratio=0.57) of BPA+E2 groups (Figure 19A), suggesting all tested BPA doses suppressed E2 induced OP memory enhancement (Fig. 21A). But when the same data were compared with E2 alone treated group, exploration ratios of 0.4μg/kg BPA+E2 group were not significantly different (Fig. 21B), suggesting this dose failed to block E2 induced memory enhancement. A paired sample t test revealed that rats in 0.4μg/kg of BPA+E2 group spent similar amount of times around objects at new and old location (p<0.085), suggesting they did not remember the old locations (Fig.22). Thus, we concluded that all tested doses blocked 17α-E2 induced OP memory enhancements.

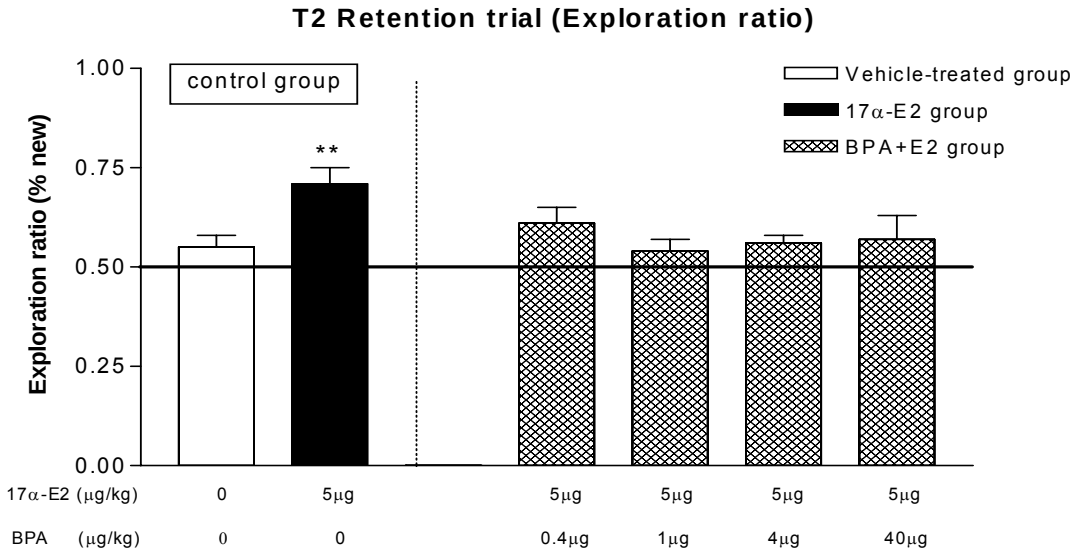
EX. 2-5: BPA+17α-E2 on object recognition (OR)

In Ex. 1-4, we found that both 1and 2μg/kg of 17α-E2 enhanced OR memory (see Fig. 13). Since 1μg/kg treated rats showed better memory performance (ratio= 0.62) than 2μg/kg treated rats (ratio=0.60), we used 1μg/kg of E2 for this experiment. During T1, no significant group differences were found, $F_{5, 57}$ =0.715, p<0.616 (data not shown).

For T2 retention trials, group differences in exploration ratios showed a trend, but were not statistically significant, $F_{5, 57}=2.254$, $p<0.064$. (data not shown). However, the T2 discrimination index was significant, $F_{5, 57}=2.855$, $p<0.023$. Thus, post-hoc analyses were carried out based on the T2 discrimination index, which revealed that performance in all BPA+E2 groups were not significantly different from the vehicle-treated (Fig. 23A) and E2 treated groups except 40μg/kg of BPA+E2 group (Fig. 23B). Paired sample t-tests indicated that all groups except vehicle-treated control spent significantly more time around the new objects (Fig. 23C). These data suggest that all BPA+E2 treated rats remembered the old object, that is, the tested BPA doses did not block 17α-E2 induced OR memory enhancement.

Object Placement (OP) with BPA and 17α-E2

A.



B.

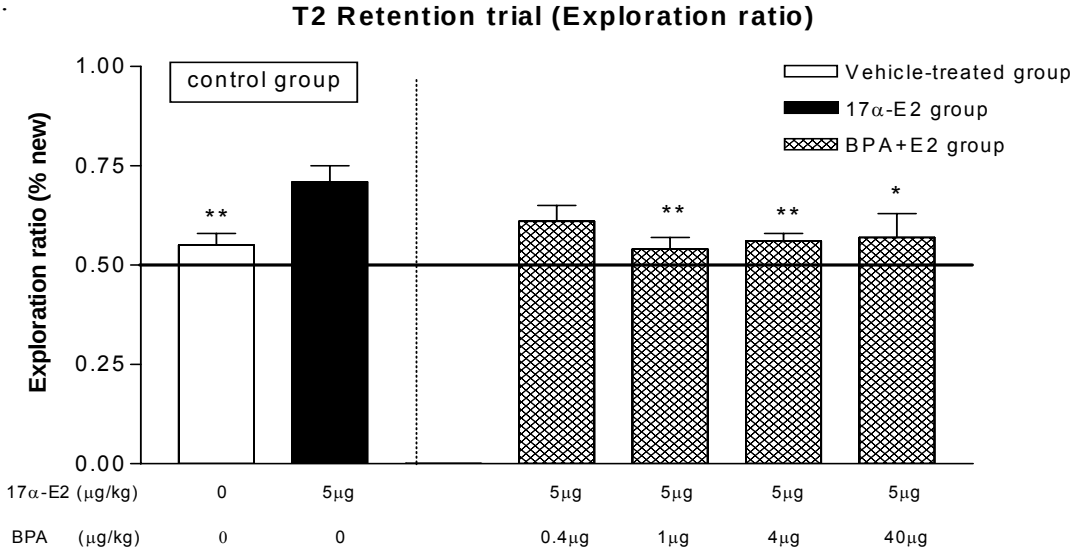


Figure 21. Effects of BPA+ 17α-E2 on OP memory (exploration ratios). Exploration ratios in vehicle-treated, E2-treated, and BPA+E2 treated groups (n=8-9 in each group) were tested by one-way ANOVA. Significant group differences were found, $F_{5, 48}$=2.50, p<0.043. **A.** Post-hoc comparison based on vehicle-treated control. Exploration ratios for all BPA+E2 groups were not different from control. **B.** Post-hoc comparison based on E2-treated control. Exploration ratios for 1, 4, 40μg/kg of BPA+E2 groups were different from control. Entries are means ± SEM. **p<0.01, * p < 0.05.

Object Placement (OP) with BPA and 17α-E2

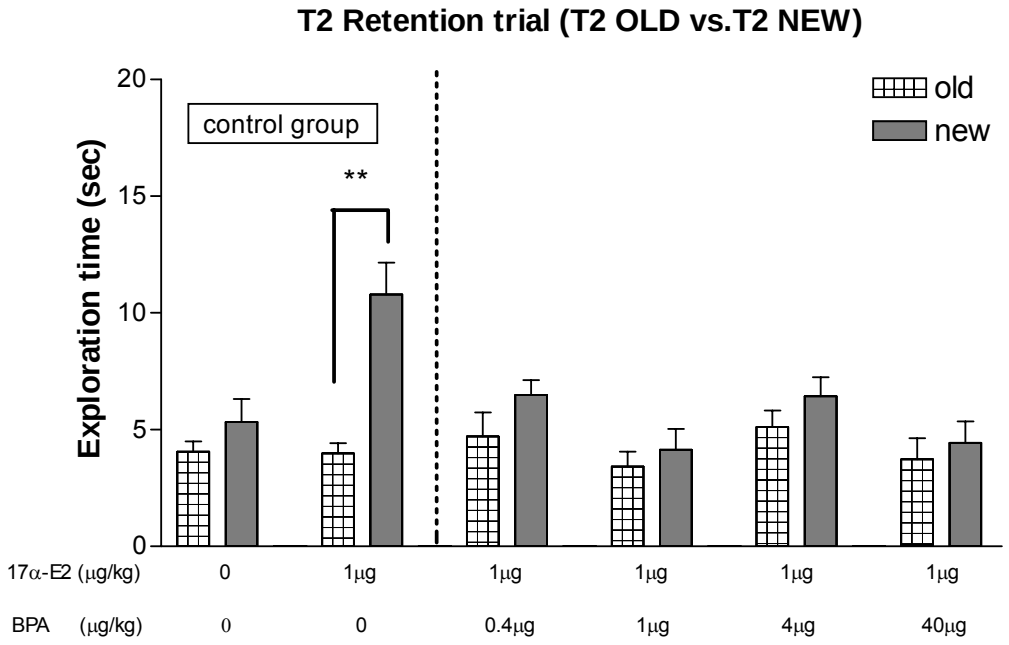


Figure 22. Effects of BPA+ 17α-E2 on OP memory (T2 old vs. New). Times spent exploring objects at old and new locations during T2 retention trial were analyzed by paired t-tests as post-hoc comparison. Rats in all groups except E2-alone treated control spent similar amount of time around objects at the new location. Thus data suggest all tested BPA doses blocked E2 induced memory enhancement. Entries are means ± SEM. ** $p < 0.01$.

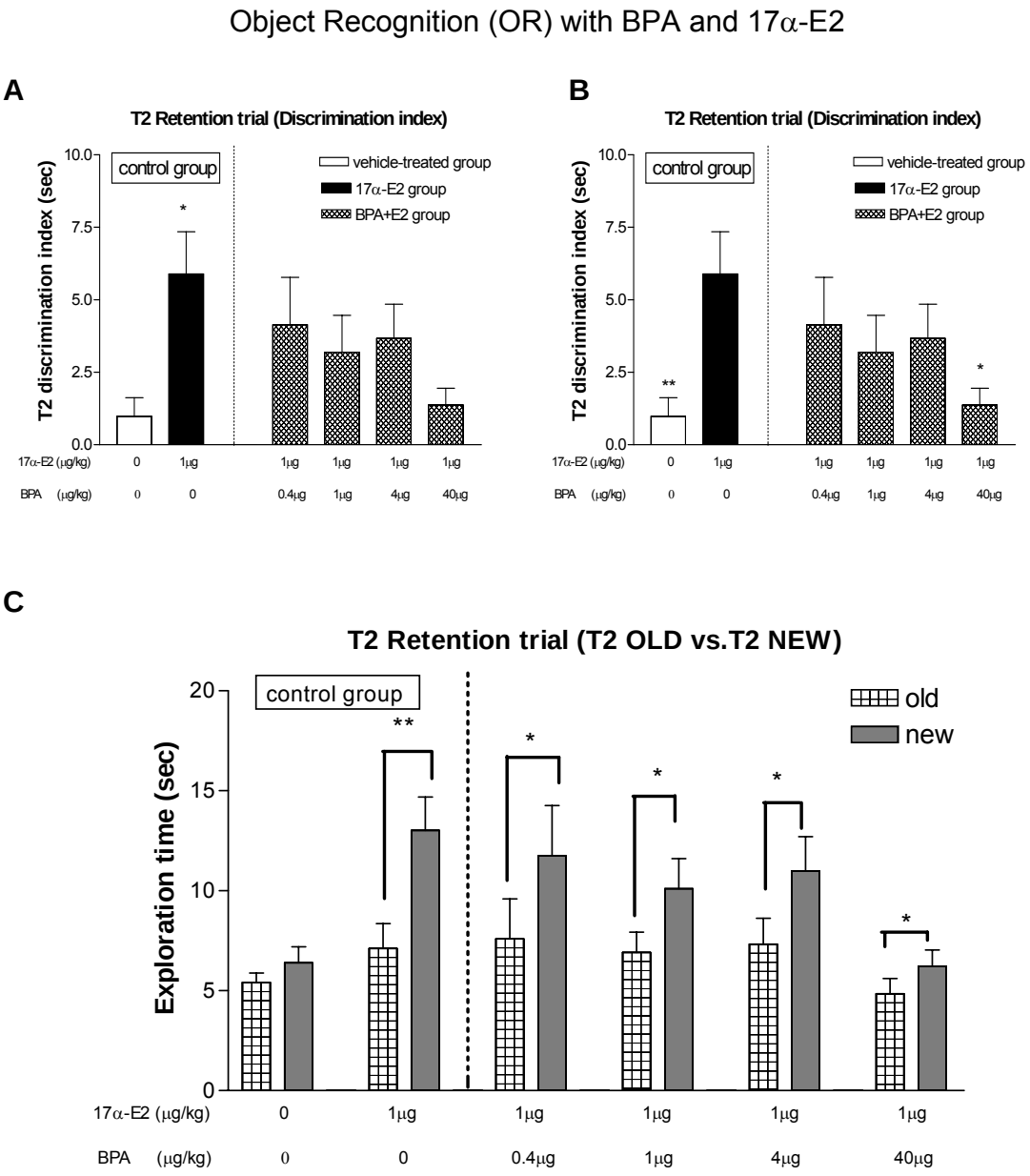
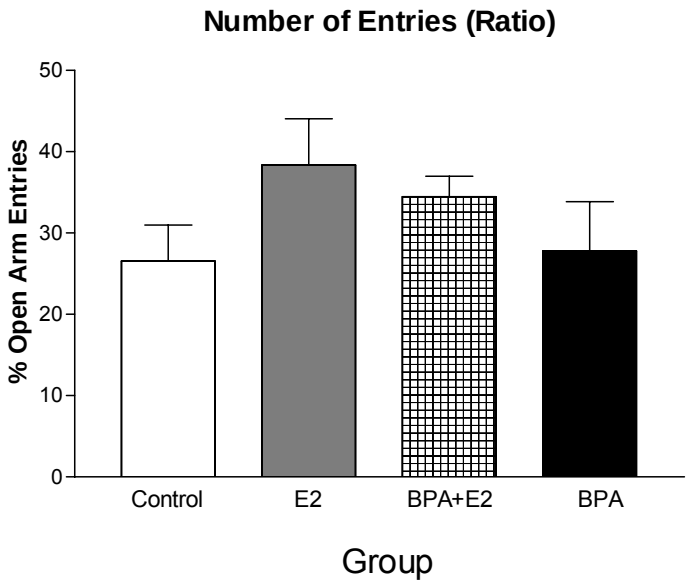


Figure 23. Effects of BPA+ 17α-E2 on OR memory. Discrimination index in vehicle-treated, E2-treated, and BPA+E2 treated groups (n=9-10 in each group) were tested by one-way ANOVA. Significant group differences were found, $F_{5, 57}$=2.855, p<0.023. **A.** Post-hoc comparison based on vehicle-treated control. All BPA+E2 groups were not different from control. **B.** Post-hoc comparison based on E2-treated control. All BPA+E2 groups except 40μg/kg of BPA+E2 were not different from E2 group. C. T2 OLD vs. T2 NEW. Rats in all BPA+E2 groups spent significantly more time at the new objects. Entries are means ± SEM. ** p <0.01, * p < 0.05.

EX.2-6: Effects of E2, BPA and their combinati on elevated plus maze

For the elevated plus maze (EPM), greater percentages of entries in open arms were observed in E2 (38%), BPA (28%), and BPA+E2 (34%) treated rats as compared to vehicle treated subjects (27%), although these small differences were not significant (Fig. 24A). The subjects in all groups had a similar percentage of time spent in open arms (Fig. 24B). Therefore, neither this dose of E2 (20μg/kg) nor BPA (40μg/kg), alone or in combination, influences anxiety levels.

A



B

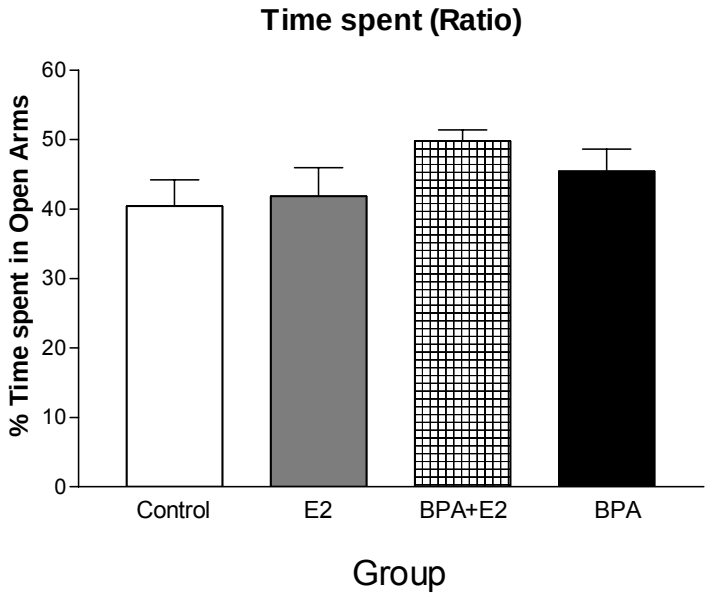


Figure 24. Effect of E2, BPA and their combination on elevated plus maze. A. Percentage of open arm entries ± SEM (number of open arm entries/(number of open + closed arm entries) x 100) is shown. There were no significant differences between groups. **B.** Percentage of time spent in open arms ± SEM (time spent in open arms/(time spent in open + closed arms) x 100) is shown. No significant differences between groups were found.

Discussion

The present experiments examined acute BPA treatment effects on memory and anxiety in the presence and/or absence of estrogen. To determine behavioral effects of BPA on responses to E2, we tested a variety of BPA doses with the most effective E2 doses for OP and OR memory enhancement found in experiments 1.

The results demonstrate, for the first time, behavioral significance of acute BPA effects in adult and provide new information that acute BPA treatment may exert rapid effects on estrogen-mediated hippocampus-dependent memory function in adult female rats. Specifically, 1) in the absence of estrogen, BPA treatment alone differentially influences OP and OR memory as OVX rats treated with higher doses of BPA (more than 40μg/kg) performed better in the OR task, while none of the tested BPA doses had effect on OP memory enhancement; 2) in the presence of estrogen, co-administration of BPA with estrogen blocks estrogen-induced spatial and non-spatial memory enhancement at below the current reference safe dose of 50μg/kg/day. The only exception was BPA+ 17α-E2 effects on OR memory, where none of the tested BPA doses suppressed memory enhancement. These results suggest that there are complex interactions between memory tasks, amounts of estrogen present and BPA doses administrated. Specific BPA doses that antagonized E2-induced memory enhancement are summarized in Table 4.

Table 4. Summary of acute estradiol and BPA treatment effects on memory.

Memory	E2 doses that enhanced memory consolidation	BPA dose that blocked E2-induced enhancement
Object Placement (OP)	17β-E2: 20μg/kg	1μg/kg
	17α-E2: 5μg/kg	Less than 0.4μg/kg
Object Recognition (OR)	17β-E2: 5μg/kg	40μg/kg
	17α-E2: 1μg/kg	-

Acute BPA treatment effects on memory in the absence of estrogen

Previous brain studies have shown that BPA alone treatment has little or no effects when estrogen is absent in the system (Kawato, 2004). The current OP memory experiment is consistent with the brain studies since acute BPA alone treatments (1-400μg/kg) did not enhance OP memory performance. However for OR, higher doses of BPA treatments showed a trend for memory enhancement, as subjects treated with more than 40μg/kg of BPA spent significantly more time around new objects (Fig. 17), suggesting that higher doses enhance OR memory consolidation while lower doses do not. Thus, results show that BPA alone treatments differentially influence non-spatial and spatial memory, and that OR is more sensitive to BPA as compared to OP. This pattern of effects is consistent with findings in experiment 1 that 4-5 times more E2 doses were required to enhance OP memory consolidation as compared to OR. Interestingly, studies have reported that some other environmental chemicals or estrogenic compounds such as DES may exert task-dependent enhancement of memory function. For example, our laboratory has shown that acute DES and/or 16α-iodo-estradiol treatment to OVX rats enhance memory performance within 4 hours, but the doses that enhanced OR memory were ineffective for OP memory facilitation (Luine et al., 2003).

In contrast to similar task-specific effects of hormones and environmental chemicals on memory function, effective E2 doses for OR memory consolidation (5μg/kg of 17β-E2; 1 and/or 2μg/kg of 17α-E2) are markedly lower compared with the effective BPA doses (more than 40μg/kg) observed in the current experiment. These results suggest that BPA is a weaker agonist than both isomers of estradiol, and that it may interact with membrane-associated ER systems. Thus, the current findings provide novel

information that, in the absence of estrogen, BPA weakly mimics behavioral E2 effects at least for OR memory.

Acute BPA treatment effects on memory in the presence of estrogen

Consistent with previous findings that BPA antagonized E2-induced LTP (Kawato, 2004), synaptic plasticity (MacLusky et al, 2005a; Leranth et al, 2008) and ERK activation levels (Zsarnovszky et al, 2005), the present results show that acute BPA treatment blocks 17β-E2 induced OP and OR memory enhancements in adult female rats. In addition, acute BPA treatments also antagonize 17-α induced spatial memory enhancement. Thus, these data provide novel information that low doses of BPA, lower than the current safe daily limit, have ability to rapidly alter behavioral responses to E2.

Several brain studies have demonstrated non-monotonic dose response relationships for estrogen and/or BPA effects on some neural systems, such as Ca^{2+} influx via L-type channels (Watson et al., 2007b) and dopamine efflux through membrane-associated receptors (Alyea & Watson, 2009). In experiment 1, we also found that dose-response patterns between acute estrogen treatments and memory performance were inverted-Us for both isomers of estradiol. The results of experiment 2, however, indicate that antagonistic effects of BPA on E2-induced memory enhancement are not inverted-Us. This result suggests that inhibitory effects of BPA on memory systems may occur dose dependently. Although it is possible that the tested dose range was not broad enough to see non-monotonic responses, two explanations may account for the observed results. First, BPA may be a membrane ER antagonist, and therefore when it occupied all available ER receptors at the dose inhibitory effects were observed, additional BPA+E2 treatment had no effects. Alternatively, BPA may be a membrane-associated ER agonist

and the increases in receptor activation due to E2+BPA treatments might have exceeded appropriate receptor stimulation and subsequent signaling pathways, which resulted in memory impairment. The latter view is consistent with the optimal level of receptor activation hypothesis (Packard, 1998) and can explain inverted-U dose-response relationships for acute E2 treatment on enhancement of memory consolidation observed in experiment 1 and inhibitory effects of BPA+E2 treatments obrained in experiment 2. It should be noted that rapid antagonistic effects of BPA+E2 combinations on E2 induced memory enhancement could occur through ER-independnet manner. Since non-genomic action of E2 and/or BPA may induce rapid, large CA2+ influxes (Sarkar et al., 2008; Wozniak et al., 2005) and increase CA1 excitability (Gu et al, 1996) without activating ERs, these alterations might directly affect neural signaling systems and hence resulted in memory impairment.

Results show that lower doses of BPA are sufficient to block E2 induced OP memory enhancement than OR. For example, 1μg/kg and 0.4μg/kg of BPA suppressed 17β- and 17α-E2 induced OP memory enhancement, respectively, while a dose of 40μg/kg of BPA was required to inhibit 17β-E2 induced OR memory enhancement. In addition, all tested BPA doses failed to suppress 17α-E2 induced OR memory enhancement. These results are noteworthy, as the data from Experiment 1 indicate that lower doses of E2 were sufficient to enhance OR than OP and we also found that BPA alone treatment may enhance OR, but not OP. These results suggest complex interactions between memory tasks, amounts of estrogen in the system and BPA doses administrated may involve in observed BPA+E2 effects. In addition, the results suggest that BPA may interact with each isomer of estradiol differently and that distinct

mechanisms may underlie 17α-and 17β-E2 mediated memory enhancement and BPA inhibition. Interestingly, it has been reported that BPA and E2 interact differently within the ligand-binding domain at ER-α (Gould et al., 1998; Miyakoshi et al., 2009), which suggests that when both BPA and E2 are present in the system, they may induce different molecular effects via membrane associated ER-α. In addition, a study has found that BPA may exert agonistic activity via ER-β, but mixed agonist/antagonist activity via ER-α in a cell specific manner (Kurosawa et al., 2002). Moreover, a recent finding indicates that binding affinity of BPA for GPR30, a G-protein coupled membrane estrogen receptor, is higher than ER-α and ER-β, and that both BPA and 17β-E2 increase cAMP activity through GPR 30 following 30 minutes treatments (Thomas & Dong, 2006). Thus it is possible that BPA may activate cAMP pathways inappropriately through GPR 30, which might affect E2 induced memory enhancement.

Acute E2 and BPA+E2 treatment effects on anxiety levels

Previous chronic treatment studies have reported that estrogens have both anxiogenic and anxiolytic properties (Lund et al., 2005; Tomihara et al., 2009), and that long-term developmental exposure to BPA may cause hyperactivity later in adult (Ishida et al., 2004). Since no studies have examined acute effects of estrogens and BPA on anxiety levels, there is a possibility that acute treatments also affect subject's psychological status, which may influence performance in memory tests. We tested this possibility and found that neither E2-alone nor BPA-alone, or in combinations, influenced anxiety levels. Thus, the observed acute effects of E2, BPA and BPA+E2 treatments on memory performance were not based on changes in anxiety and motor

activity levels as the same treatments did not affect performance in the elevated plus maze task.

In summary, the present results demonstrate that BPA may interact with estrogen at very low doses and alters behavioral response to E2 rapidly, within 30min, in adults. This response pattern suggests that observed acute effects of E2 and BPA, alone and in combination, may be mediated via membrane-initiated actions, and low level exposure of BPA may have a powerful, negative impact in estrogen mediated cognitive function.

Brain Study: Golgi Impregnation and HPLC

One of the current behavior experiments indicate that post-sampling injection of 20μg/kg of 17β-E2 significantly facilitates OP memory consolidation (Ex. 1-1). Co-administration of BPA with this dose of E2 completely blocks the estrogen effect on memory enhancement at a dose far below the current reference safe dose of 50μg/kg (Ex. 2-2). The mechanisms responsible for these 17β-E2 and BPA actions remain to be elucidated, however, evidence indicates that gonadal steroid hormones can activate morphological and cellular changes in the adult brain (Dohanich, 2002). Specifically, many studies have suggested that estrogen mediates synaptic plasticity in the CA1 of the hippocampus (Sandstrom and Williams 2001; Silva, 2003; Li et al., 2004; Maclaughlin et al, 2008; Spencer et al, 2008) and the PFC (Hao et al., 2006; Wallace et al., 2006; Wallace et al., 2007) and changes in monoaminergic activity (Gonzalez-Burgos & Feria-Velasco, 2008; Seamans et al., 1998; Wilkerson & Levin, 1999).

In the current study, we investigated these two parameters (dendritic spine density and levels of monoamines) as possible neural systems that may contribute to the observed acute effects of E2 and BPA+E2 treatments on memory consolidation. Of particular interest was to determine whether neural interactions of BPA with E2 would be antagonistic (inhibitory) or agonistic (additive or synergistic) in mediating the behavioral effects.

Experiment 3: Acute effects of estrogen and BPA+estrogen on dendritic spine density [Aim 3]

Accumulative evidence indicates that fluctuations in estrogen levels across the estrus cycle and administration of estradiol to OVX rats influences synaptic plasticity (Gould et al, 1990; Woolley, 2007; Spencer et al, 2008). Traditionally, estrogen regulation of spine density and spine synapse density have been thought to be mediated through genomic actions, however recent *in vivo* studies have demonstrated that estrogen can alter spine synapse density rapidly, within a few hours and even 30 minutes after hormone exposure, which suggests involvement of non-genomic actions.

To date, little is known whether BPA affects hippocampal plasticity in the absence of estrogen. However, there is one study that found 1nM BPA increases CA3 apical spine density in hippocampal cultured cells, but there are no effects on CA1 spine density (Sato et al., 2002). In the presence of the estrogen, studies have demonstrated that acute and chronic BPA treatments block estrogen-induced increased dendritic spine synapse density in the CA1 of the hippocampus (MacLusky, 2005a; Leranth et al, 2008), findings which support the current behavioral results of acute E2 and BPA treatment effects on memory performance (Experiment 2).

In Experiment 3, we measured spine density at two different time points; 30 minutes (sensitive period for memory consolidation) and 4 hours (the inter-trial interval when retention was tested in the current behavioral study) after acute hormone and BPA treatments. We hypothesized that 1) acute E2 treatments would alter dendritic spine density in the PFC and the CA 1 of the hippocampus, and 2) co-administration of BPA with E2 would suppress E2-induced spine density in both brain areas.

Methods

Subjects and treatments

Two cohorts of OVX rats (39 in total) that were used for behavioral analysis (Sprague-Dawley, four to five-month-old at the time of sacrificed) served as subjects. The subjects were treated and sacrificed approximately one week after the completion of behavior tests. For treatments, 20μg/kg of 17β-E2, which enhanced OP memory, and 40ug/kg of BPA, which suppressed E2 induced memory enhancement were used. We measured spine density and verified serum estrogen levels at two different time points after acute treatments. For the 30 minutes post treatment study, one cohort of 18 rats was assigned to either vehicle-, E2- or BPA+E2-treated group, and received pre-T1 injection, a T1 sample trial and post-T1 injection (Table 5). 30 minutes later, they were lightly anesthetized with carbon dioxide and sacrificed by rapid decapitation. For the 4hr post treatment study, another cohort of 21 rats received the same treatments except that they were sacrificed 4 hours after hormone/BPA injection. See Fig.5 for the treatment timeline of brain analyses.

Table 5. Hormone and BPA treatment used for spine density and serum estradiol levels (Experiment 3)

	Treatment		
Group	**Pre T1 INJ**	**Post T1 INJ**	**n**
Control	saline	Corn oil	6-7
E2	saline	20μg/kg of 17β-E2	6-7
BPA+E2	40μg/kg of BPA	20μg/kg of 17β-E2	6-7

Golgi Impregnation Procedure

Golgi impregnation was used to quantify the number of apical and basal dendritic spines in the hippocampus and the prefrontal cortex as previously described (Luine et al., 2006; Wallace et al., 2006). Briefly, the brains were quickly removed and cut into two blocks: one contained the hippocampus and the other contained the prefrontal cortex. The Golgi impregnation procedure was carried out using FD Rapid GolgiStain ^{TM} kit (FD Neuro-Technologies, inc., MD). Brain blocks were rinsed in a 0.1M phosphate buffer and then immersed in the Golgi-cox solution, a mixture of potassium dichromate and mercuric chloride. The solution was replaced after 24 h of immersion and the brain were kept in the solution for two weeks at room temperature. Then the brain tissues were transferred into a sucrose-based solution and stored at 4 ˚C for 5days. The brain blocks were sliced into 100 μm sections on a cryostat with the chamber temperature set at – 22 C, and placed on gelatin-coated slides. Sections were air-dried at room temperature. Dried sections were rinsed in distilled water, and placed in Golgi solution containing silver nitrate for 10 min. Sections were rinsed again with distilled water, and dehydrated in 50%, 75%, 95%, and 100% ethanol for 5 min each, and cleared in Protocol (Fischer Scientific) three times for 5 min each. Finally sections were cover slipped in Permount (Fischer Scientific).

Spine density analysis

Apical and basal dendritic spine density of pyramidal cells in the medial PFC (layer II/III) and the CA1 hippocampus was analyzed using a Nikon Eclipse E400 microscope and the Spot Advanced Program for Windows (version 3.5, Diagnostic Instruments, inc.) For each rat, six tertiary apical dendrites and six secondary basal

dendrites (Fig.26) were chosen for spine counting according to methods described previously for the hippocampus (Gould et al., 1990), and for the prefrontal cortex (Wellman, 2001). To ensure that the same segments of dendrites and spines in the neurons in the target area were analyzed within and across the subjects, dendrites were chosen based on the following criteria: 1) the cell body of neurons had to be located within the area of interest: 2) the dendritic branch of the neuron had to be isolated well enough from those of nearby neurons: 3) the length of the target dendrite had to be unbroken. Once the neuron was selected, the total length of the target dendrite segment was measured using the Spot Advanced program. Two researchers counted the numbers of spines in a manually using a standard counter, and the mean of these determinations was used to calculate the spine density.

Radioimmunoassay

Serum estradiol concentrations were measured using a commercial radioimmunoassay (RIA) kit (Coat-A-Count®; Diagnostic Products Corp, Los Angeles, CA). Blood samples were collected from trunk blood of subjects, allowed to sit in the refrigerator overnight, centrifuged and serum removed the following day, and frozen in sealed tubes at -80 °C until assay. The Coat-A-Count kit uses estradiol-specific antibody immobilized to the wall of a polypropylene tube. Because this kit is designed for use with human samples, new standards were made by serially diluting the highest estradiol standard from the kit (3600pg/ml) with rat serum collected from OVX rats in order to count for presence of serum binding proteins in rats. The calibrators used for the estradiol assay were 0, 10, 25, 50, 150, and 300pg/ml. Samples were analyzed in duplicates. 100 µl of each calibrator and 100 µl of sample were pipetted into duplicate

tubes. For each sample, 1ml of the isotope ^{125}I-estradiol was added, vortexed briefly, and incubated overnight in a refrigerator at 4°C. The following day, all tubes were decanted thoroughly to separate bound from free estradiol, and bound isotope was counted in a Wizard 1470 Automatic Gamma Counter (Perkin Elmer Life Sciences, Wellesley, MA). The amount of estradiol was calculated by comparison with the standard curve and expressed in pg/ml. All samples were run on the same day.

Measurements and Statistical analyses

Spine density was determined as the number of spines divided by the length of the dendrite being measured and expressed as number of spines/10μm. For each treatment schedule, data were analyzed with three-way ANOVAs (group x areas x branches). If significant effects were found, group differences were tested by one-way ANOVAs with Tukey HSD post-hoc tests. To compare spine density between 30 min and 4hr after treatments, a four-way ANOVA (time x group x area x branch) was conducted to evaluate overall effects. If significance was found, post-hoc tests were carried out.

Serum estradiol levels for each treatment schedule were analyzed by one-way ANOVA with Tukey HSD post-hoc tests.

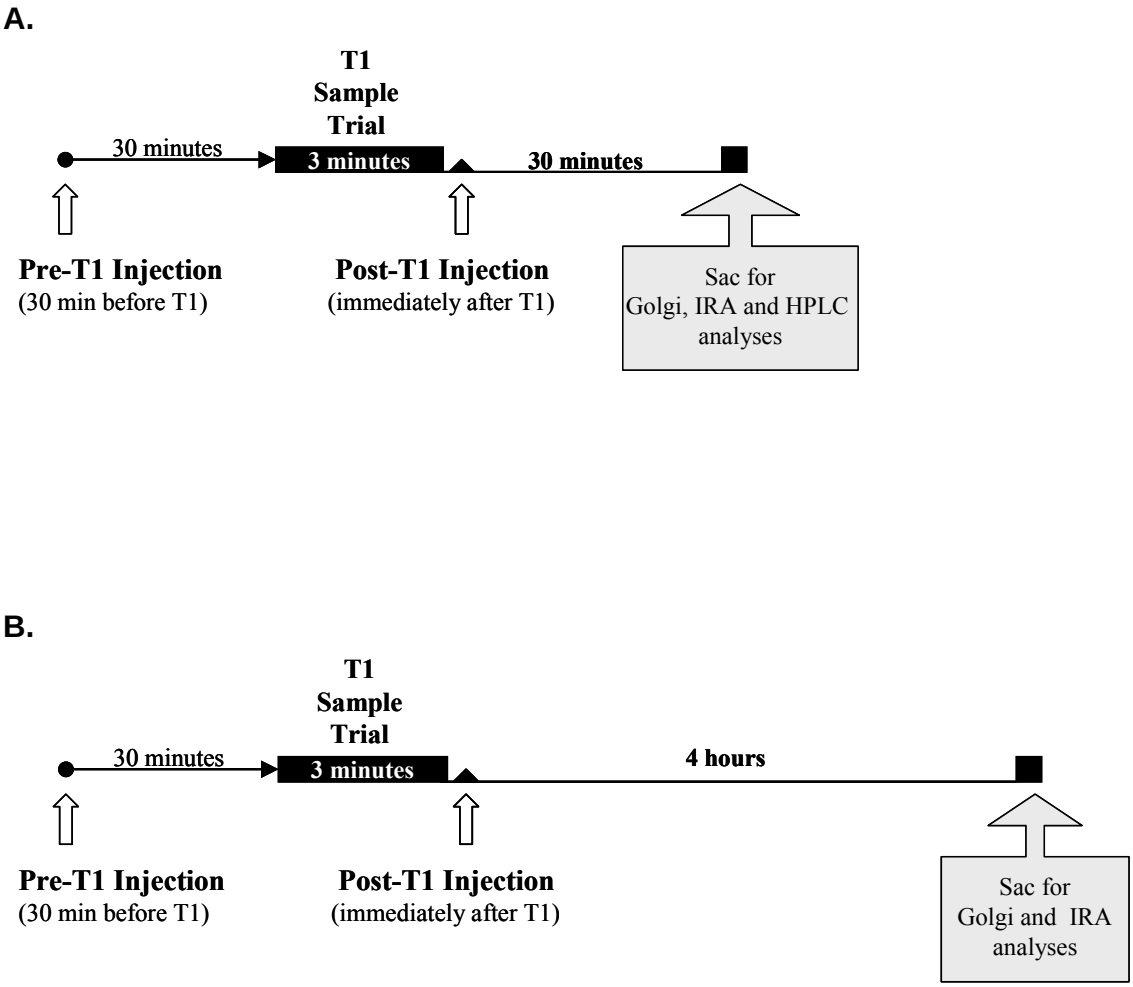


Figure 25. Treatment schedule for neuromorphlogical and neurochemical analyses. All subjects received a pre-T1 injection and T1 sample trials. No retention trials were given. **A.** Treatment schedule for spine density, monoamine concentrations, and serum E2 levels at 30 minutes after post-T1 injection (sensitive period for memory consolidation). **B.** Treatment schedule for spine density and serum E2 levels at 4hr after post-T1 injection (time period for retention trials).

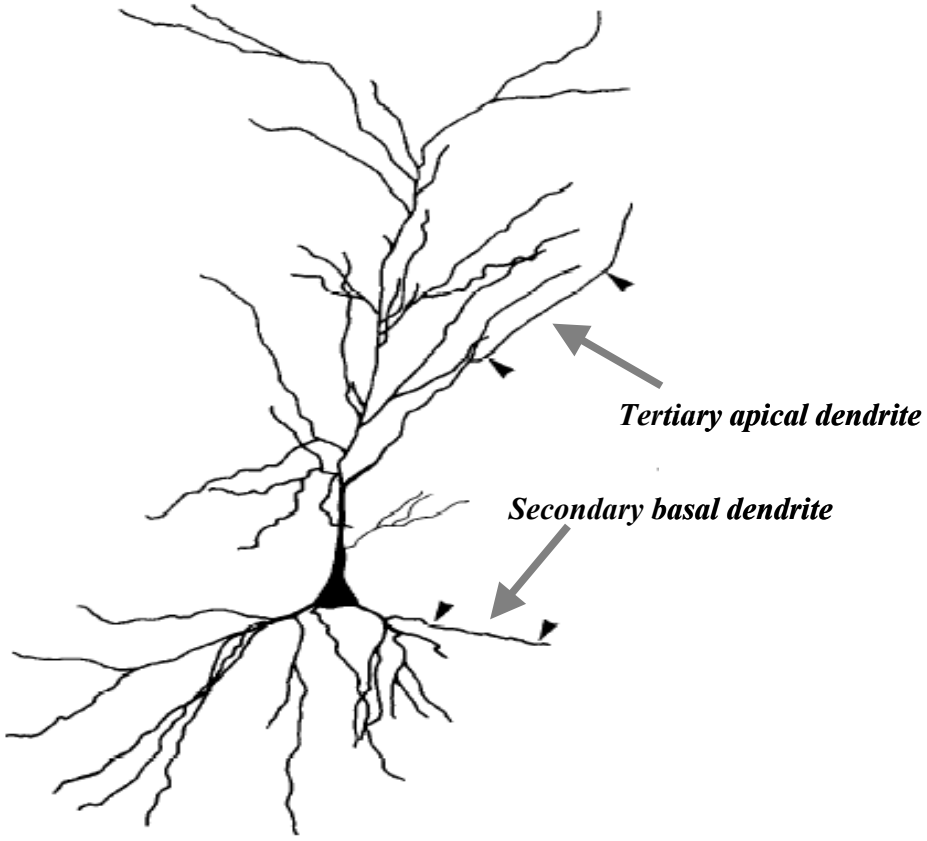


Figure 26. Diagram of a pyramidal neuron. The areas between the arrowheads represent tertiary apical dendrite and secondary basal dendrite segments that were used in spine density analysis.

Results

Spine density 30 minutes post acute treatment (EX.3-1)

Spine density of tertiary apical and secondary basal dendritic branches 30 minutes after acute vehicle, E2 and/or BPA+E2 treatments (n=6 in each group) are summarized in Table 6A. In the prefrontal cortex (Fig 27A), there were significant group differences in spine density for both apical ($F_{2,15}$ =5.817, p<0.013) and basal dendrites ($F_{2,15}$ =13.92, p<0.001). Post-hoc tests revealed that subjects treated with E2 showed significantly higher apical (16% above control, p<0.038) and basal (27% above control, p<0.001) spine densities. BPA+E2 treated subjects also had increased apical (24% above control, p<0.004) and basal (33% above control, p<0.001) spine density as compared with vehicle-treated subjects. Although BPA+E2 treated subjects were higher than E2 treated subject for both apical and basal dendritic branches, these differences were not statistically significant.

In the CA1 of the hippocampus, we found significant group differences for apical ($F_{2,15}$ =5.493, p<0.016) and basal ($F_{2,15}$ =40.523, p<0.0001) dendrites. Post hoc tests revealed that acute E2 treatment significantly increased basal spine density (29% above control, p<0.001), but there were no effects of E2 on apical spines (Fig 27B). In contrast, BPA+E2 treatment increased both apical (25% above control, p<0.006) and basal (48% above control, p<0.001) spines significantly. In addition, the BPA+E2 treated group showed significantly higher basal spine density than E2 alone treated rats (18% above E2 group, p<0.002), indicating co-administration of BPA further increased E2-induced spine density in basal dendritic spines in the CA1. These data demonstrate that acute BPA

effects on spine density in the PFC and the CA1 of the hippocampus are generally additive rather than inhibitory.

Spine density 4 hours post acute treatment (EX. 3-2)

The mean dendritic spine density 4 hours after acute E2 and/or BPA treatments is shown in Table 6B. In the PFC (Fig. 28A), significant group differences were found for basal dendritic spine density ($F_{2, 18}$=5.059, p<0.02), while apical spine density showed a trend ($F_{2, 18}$=3.1, p<0.06). Post-hoc tests revealed that spine density of basal dendrites was significantly increased for both E2 (30% above control, p<0.05) and BPA+E2 (44% above control, p<0.006) treatment. Apical dendritic spine density for BPA+E2 treated subjects was significantly higher than vehicle treated subjects (27% above control, p<0.026), but subjects with E2 treatment (18% above control) failed to reach significance.

In the CA1 of the hippocampus (Fig. 28B), we found significant group differences for both apical ($F_{2,18}$ =4.81, p<0.02) and basal ($F_{2,18}$ =5.14, p<0.02) dendrites. Post hoc tests revealed no significant group difference in apical spine density between vehicle- and E2-treated subjects, but a significant difference was found between vehicle- and BPA+E2 treated rats (12% above control, p<0.006). In contrast, basal dendritic spine density was significantly increased by E2 (12% above control, p<0.01), but not by BPA+E2, suggesting co-administration of BPA suppressed E2-induced spine density in basal dendrites of CA1 neurons 4 hours after treatment.

Spine density 30 min vs. 4hr after acute treatments

Fig 29 shows effects of acute E2 and/or BPA+E2 treatments on spine density at two different time points side by side (30 min vs. 4hr after treatment). As the Figure

clearly shows, spine density measured 4hr after vehicle-, E2- and BPA+E2 treatments appeared to be lower than spine density measured 30min after treatments in both apical and basal branches in the PFC (Fig. 29A) and the CA1 (Fig. 29B) of the hippocampus. To examine whether these differences were statistically significant, we first tested overall effects by 4-way ANOVA (treatment x time x brain area x dendritic branch) and found significant interactions of treatment x time ($p<0.0001$) and treatment x time x brain area ($P<0.008$). In each brain area, significant time x treatment interactions were also found ($p<0.001$, for PFC and $p<0.001$ for CA1). Post-hoc tests revealed that dendritic spine density for vehicle-, E2- or BPA+E2 treated groups measured 4hr after treatments were significantly different from spine density for the corresponding 30 min treatment groups for both apical and basal branches in the PFC (Fig. 29A) and the CA1 (Fig. 29B) of the hippocampus ($P<0.0001$ for all groups), indicating spine density for all groups was decreased over time.

Next, to determine whether E2 and/or BPA treatments specifically affected these changes, response patterns of E2 and/or BPA treatments at 30min and 4hr time points were compared with normalized (within each time point) data and percent changes of spine density relative to control were tested. In the PFC (Fig. 30A), no significant differences in response patterns were found between 30 min and 4hr after treatments, suggesting decrease in spine density 4hr after treatment did not depend on hormone and/or BPA treatment. In contrast, significant treatment x time interaction was found in CA1 of the hippocampus ($F_{2, 73} = 6.40$, $p<0.001$, Fig. 30B). Post-hoc tests revealed that significant response changes occurred in CA1 basal dendritic spine density ($p<0.001$). Two sample t tests indicated significant changes in response patterns between 30min- and

4hr- E2 treated groups ($t_{12} = 3.25$, $p<0.007$) and between 30min- and 4hr BPA+E2-treated groups ($t_{12} = 13.62$, $p<0.0001$), suggesting co-administration of BPA with E2 suppressed E2 induced-spine density 4hr after treatment.

Table 6. Acute effects of estradiol and BPA+E2 on spine density 30 min and 4 hr after treatment

A. Spine density 30 min after treatment (spines/10μm)

Group	Prefrontal cortex		Hippocampus CA1	
	Apical	Basal	Apical	Basal
Control	9.15 ± 0.43	7.88 ± 0.37	11.98 ± 0.34	8.17 ± 0.19
E2	10.65 ± 0.54* [16%]	10.03 ± 0.44** [27%]	12.70 ± 0.54 [6%]	10.57 ± 0.41*** [29%]
BPA+E2	11.35 ± 0.41** [24%]	10.47 ± 0.28*** [33%]	14.98 ± 0.96** [25%]	11.95 ± 0.10*** [48%]

Entries are mean numbers of spines/10μm ± SEM. Percentages of increase/decrease compared with the control groups are also shown in [ ]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

B. Spine density 4 hr after treatment (spines/10μm)

Group	Prefrontal cortex		Hippocampus CA1	
	Apical	Basal	Apical	Basal
Control	5.12 ± 0.49	5.14 ± 0.53	5.57 ± 0.25	5.56 ± 0.21
E2	6.01 ± 0.28 [18%]	6.67 ± 0.18* [33%]	5.41 ± 0.15 [-3%]	6.21 ± 0.11* [12%]
BPA+E2	6.48 ± 0.39* [27%]	7.40 ± 0.70** [44%]	6.27 ± 0.21* [12%]	5.52 ± 0.18 [-1%]

Entries are mean numbers of spines/10μm ± SEM. Percentages of increase/decrease compared with the control groups are also shown in [ ]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

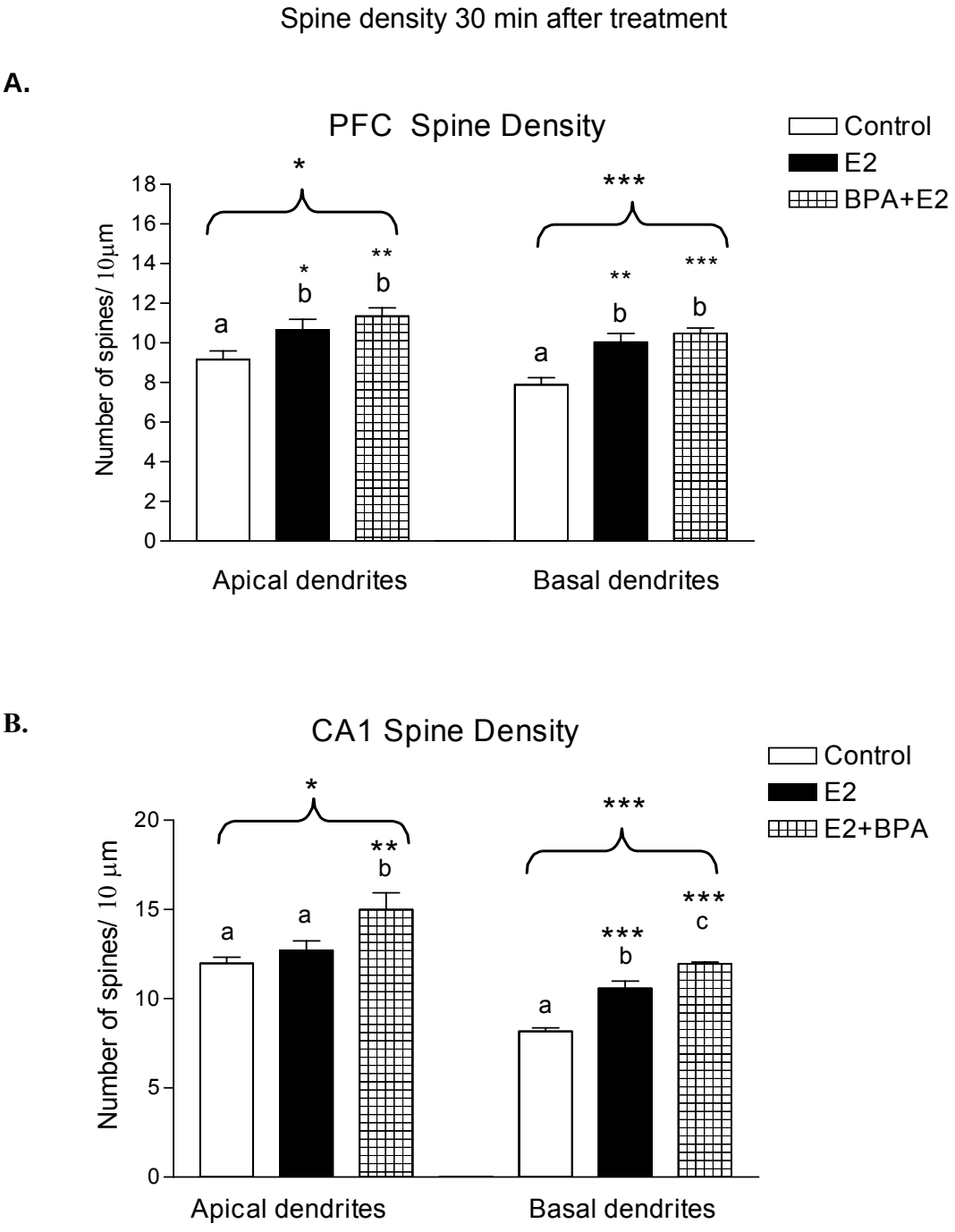


Figure 27. Effect of 17β-E2 and BPA on spine density in the PFC and CA1 of the hippocampus: 30 min after treatment. Data were analyzed by ANOVA with Tukey post-hoc tests. Letter a, b, c indicates significant differences between groups. **A**. Prefrontal cortex. **B.** CA1 of the hippocampus. Entries are mean numbers of spines/10µm ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

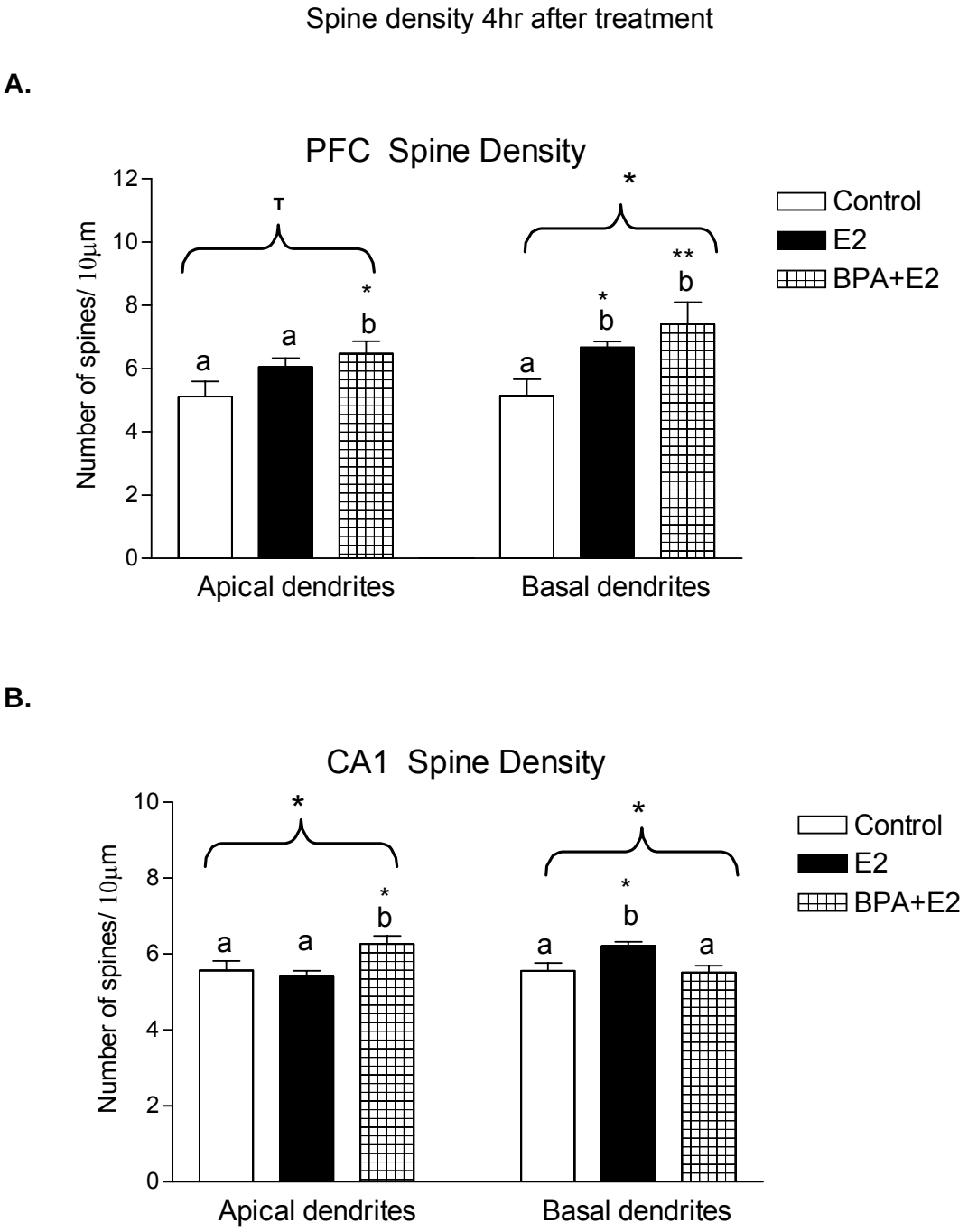


Figure 28. Acute effects of 17β-E2 and BPA on spine density in the PFC and CA1 of the hippocampus: 4 hr after treatment. Data were analyzed by ANOVA with Tukey post-hoc tests. Letter a, b indicates significant differences between groups. **A**. Prefrontal cortex. **B.** CA1 of the hippocampus. Entries are mean numbers of spines/10μm ± SEM. $_{T}$ p <0.069, * p < 0.05, ** p <0.01, *** p<0.001.

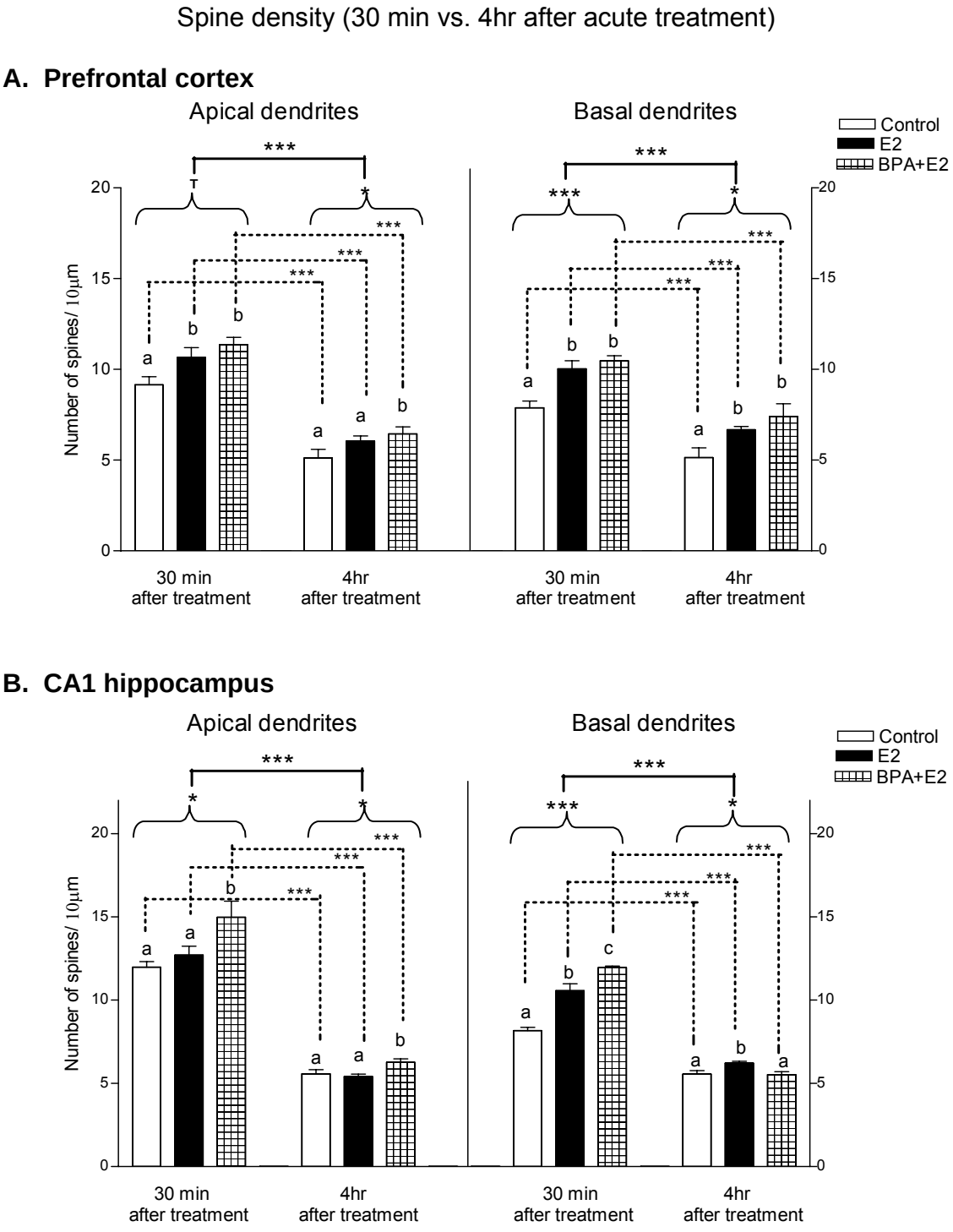
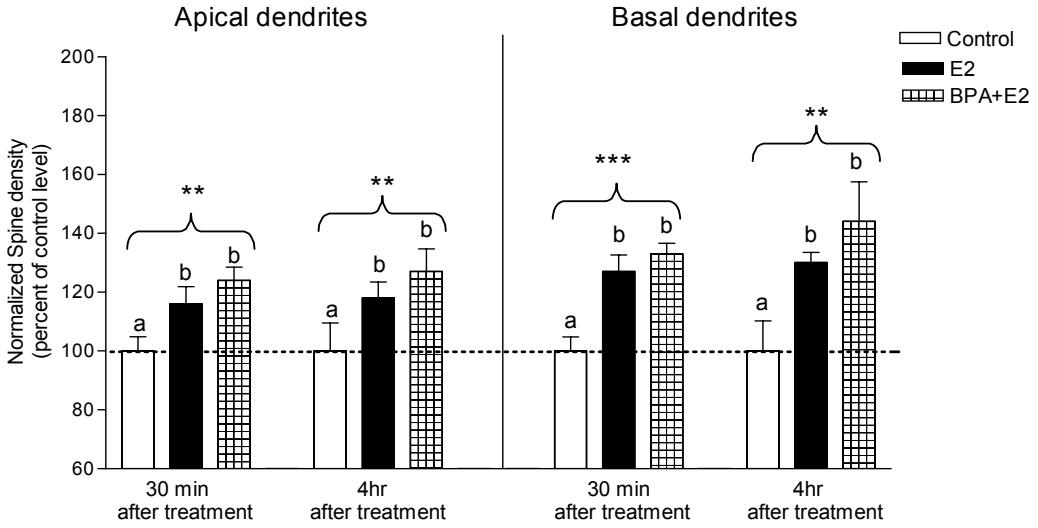


Figure 29. Acute effects of 17β-E2 and BPA on spine density: 30min vs. 4hr after treatment. Apical (left) and basal (right) spines at 2 different time points are shown side by side. Data were analyzed ANOVA with post-hoc tests. Letter a, b, c indicates significant group differences within each treatment condition. Entries are mean numbers of spines/10μm ± SEM $_{T}$ p <0.069, * p < 0.05, ** p <0.01, *** p<0.001.

Normalized Spine density (30 min vs. 4hr after acute treatment)

A. Prefrontal cortex



B. CA1 hippocampus

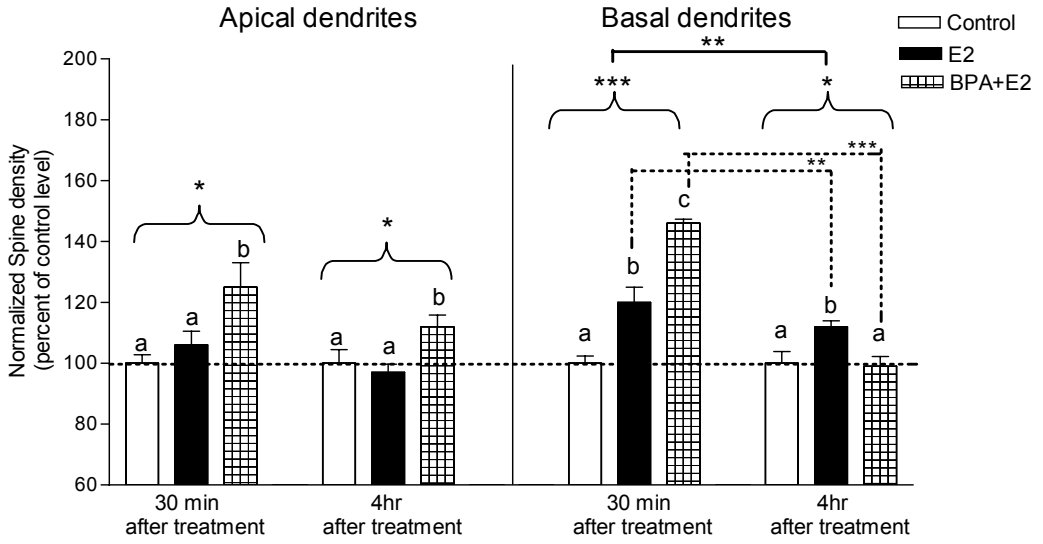


Figure 30 Response patterns in spine density for E2 and BPA+E2 groups: 30min vs. 4hr after treatment. Entries are normalized spine density± SEM. The dashed line and white bars indicate normalized spine density for the control groups. Apical (left) and basal (right) Data were analyzed ANOVA with post-hoc tests. Letter a, b, c indicates significant group differences within each treatment condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Serum estradiol levels (EX. 3-3)

Blood serum estrogen levels for vehicle, E2, and BPA+E2 treated subjects were measured at two different time points: 30 minutes and 4 hours after acute treatments in order to verify levels of circulating hormone. As shown in Table 7, markedly high levels of serum estrogen were observed in the vehicle-treated control groups for both 30 minutes and 4 hours post treatments (see discussion). For serum estradiol levels 30 min after acute treatments, significant group differences were found by a one-way ANOVA ($F_{2,18} = 6.72$, $p < 0.006$). Post hoc analysis with Fisher's LSD revealed that E2 and BPA+E2 treated subjects had significantly higher levels of estradiol compared to vehicle treated subjects. BPA+E2 treated subjects also showed higher serum levels than E2 alone treated subjects, but this difference was not significant.

For serum estradiol levels 4hr after acute treatment, a one-way ANOVA found significant group differences ($F_{2,18} = 24.50$, $p < 0.0001$). Post hoc tests revealed that E2 and BPA+E2 treated subjects had significantly higher levels of estradiol compared to vehicle treated subjects. Serum estradiol levels were similar in both E2 alone and BPA+E2 treated subjects.

Table 7. Serum estradiol concentrations 30 min and 4 hr after acute treatment

(pg/ml)

	Time	
Group	30 min***	4 hr***
Control	48.24 ± 14.8	48.74 ± 4.35
E2	657.55 ± 133.44*	711.09 ± 107.41***
BPA+E2	1081.67 ± 320.11***	900.15 ± 113.66***

Entries are mean ± SEM. Values are expressed as pg/ml. * $p < 0.05$, *** $p<0.001$.

Discussion

The obtained data clearly demonstrate that acute E2 and BPA+E2 act rapidly, within 30 minutes, to alter dendritic spines density in both CA1 and prefrontal pyramidal neurons, which suggests underlying non-genomic mechanisms. Our data indicate BPA effects on morphological response to E2 are additive in the hippocampus, but no effect in the PFC during memory consolidation process, suggesting that BPA alters neural responses to E2 differently depending on the brain areas. These results provide novel information about acute treatment effects on spine density in relation to memory function. Some important relationships include the following; 1) During the memory consolidation process, moderate increase in synaptic transmission in basal CA1 dendrites may be crucial for spatial memory as E2 increases basal, but not apical, spine density and BPA+E2 further increase it; 2) morphological responses of BPA and E2 are additive during memory consolidation, as well as 4 hours after treatment, except in CA1 basal dendritic branches 4hr after treatment as BPA+E2 inhibit E2-induced spine density; 3) apical and basal spines undergo dynamic changes between 30 minutes and 4 hours after hormone and BPA treatments; 4) decreases in spine density 4 hours after treatment is independent of hormone and/or BPA treatments, but changes in response patterns (percents of increase/decrease relative to the vehicle-treated control) depend on the treatments for basal CA1 spine density.

Spine density in the CA1 of the hippocampus

The current results show that acute E2 treatments rapidly alter dendritic spine density in the CA1 of the hippocampus, and that the effects are time and dendritic-branch dependent. These results are consistent with MacLusky et al (2005b) who have reported

that increased spine synapse density in the hippocampus, 30 minutes and 4 hours after acute E2 injection. Thus, our results provide additional support for the hypothesis that estrogen can exert rapid effects on hippocampus plasticity in adult female rats. Rapid onset of responses to E2 shortly after hormone and BPA exposure suggests that effects are probably mediated via non-genomic actions.

Acute E2 effects: apical vs. basal spine density

Interestingly, our results indicate that acute E2 treatment significantly increases basal, but not apical, spine density in the CA1of the hippocampus at both 30 minutes and 4 hours after acute treatment. This result directly contrasts with previous findings that sub-chronic administration of exogenous estrogens to OVX rats increases apical spine density or spine synapse density in the CA1 of the hippocampus (Gould et al, 1990; Woolley et al, 1992; Li et al, 2004; McLauglin, 2008). In a landmark study, Gould et al (1990) showed that ovariectomy and the resulting decreased circulating estrogen levels significantly lowered apical, but not basal, dendritic spine density in CA1 pyramidal neurons, and that this decrease was prevented by subchronic E2 treatment. Gould et al found more dramatic changes in response to E2 in spine density on apical than basal dendrites (Gould et al, 1990).

The differences between previous and the current results are probably due to the different treatment paradigms used in the experiments. The majority of previous studies used chronic/subchronic estrogen replacement, while in our study we used acute E2 treatment. Thus, it is possible that long-term estrogen treatment may act mainly on apical dendrites, which effects are mediated via nuclear ERs and genomic action, while acute treatment may act on basal dendrites through membrane-associated ERs and subsequent

non-genomic actions. Alternatively, testing parameters such as behavior tasks might have influenced estrogen effects on synaptic plasticity differently in these regions, as all subjects in our experiment received a T1 sampling trial prior to hormone and/or BPA treatments, while in the majority of previous studies, treatment effects on spine density were examined without behavior tasks. Indeed, it has been reported that behavioral training, exercise and task-associated stress (Frick et al., 2004) and even simple daily handling (Bohacek and Daniel, 2007) affect hippocampal CA 1 plasticity. In addition, a study found that associative learning-induced increases in spine density were specific to the basal dendrites of the CA1 neurons (Leuner et al., 2003), while another found that olfactory learning-induced increase in spine density was limited to the CA 1 apical branches (Knafo et al., 2004). Although these parameters may mask E2-induced elevation of spine density in the CA1 of the hippocampus, it is unlikely in our experiment, since we used a relatively simple, less-stress memory task which does not require any types of associative learning, and all subjects were carefully handled in a consistent manner. In addition, the elevated plus maze results in Experiment 2 indicate that exploration behavior and anxiety levels were not affected by the acute treatment. Thus, it is reasonable to conclude that basal dendrites are more sensitive to acute E2 treatment in the CA1 of the hippocampus.

Interestingly, this pattern of responses to E2 was also observed in the PFC, although the dendritic branch-specific effects were less obvious compared to those in the CA1 of the hippocampus. In the PFC, while acute E2 treatment significantly increased both apical and basal spine density during memory consolidation process, greater effects were observed in the basal dendrites. However, 4 hours after acute treatment, E2

increased only basal spine density. Thus, it is possible that elevated spine densities in the basal dendrites in PFC and the CA1 of the hippocampus may underlie enhancement of spatial memory consolidation by acute E2 treatment.

Acute BPA+E2 effects on spine density

Surprisingly, the current results show that BPA+E2 injections further increase apical and basal spine density in both the PFC and CA1 of the hippocampus during memory consolidation process. Thus, the present experiment provides the novel information that when estrogen is present, BPA effects on spine density to E2 are additive at 30 minutes after treatment. This result was not consistent with our behavior study in Experiment 2, as well as previous findings of BPA inhibition on E2- induced hippocampus spine synapse density in rats (MacLusky, 2005a) and monkeys (Leranth et al, 2008). However, we also found that the same dose of BPA that increased spine density at 30 minutes after treatment suppressed E2-induced CA1 basal spine density at 4hr after treatment. BPA effects on spine density for other areas (PFC apical and basal; CA1 apical) were additive at 4 hours after injection.

MacLusky et al (2005a) reported that 60μg/kg of 17β-E2 significantly increases hippocampal spine synapse density at 30 minutes after injection and co-administration of 40-400μg/kg of BPA inhibits E2-induced synaptic plasticity. One possible explanation for the different results is that the previous study examined spine synapse density, which measures functional synapses between spines, while the present study tested spine density by counting the number of spines along dendrites. It has been reported that E2 mediated development of spines depend on rapid NMDA receptor activation through c-Src, and that stabilization and maintenance of spines depends on the decrease of GABA inhibition

by interneurons (Brinton, 2001). Although Woolley et al (1992) have demonstrated that changes in dendritic spine density paralleled changes in spine synapse density, it is possible that the newly-developed spines induced by BPA+E2 treatment may be "silent" spines, and did not contribute to active synaptic connection. Indeed, it has been reported that newly generated spines by E2 treatments did not form new synapse of EPSP within 2 hours (Mukai et al., 2007). Alternatively, different E2 doses used in the previous and the current study may be an important factor, as we used 20μg/kg of E2 while MacLusky et al used 60μg/kg of E2. Thus, it is possible that 40μg/kg of BPA differentially interacts with different doses of E2 and may exert temporally distinct effects depending on concentrations of E2 present in the system. Presynaptic boutons afferent to CA1 dendritic spines can be single and/or multiple synapse boutons (Woolley et al., 1996). Since multiple synaptic boutons are connected to more than one dendritic spine (Woolley et al, 1996), it is possible that acute E2 and BPA+E2 treatments may affect differently these existing two types of boutons. Interestingly, several studies have reported that relationships between dendritic spines and their synapse in the hippocampus could be stable, which maintains optimal synaptic activation, or unstable, which changes their frequency based on the activation levels, and the latter may result in reduced synaptic activation, which, in turn, lead to formation of new functional spines and/or filopodia (the precursors of dendritic spines)(Sala et al., 2008; Sorra & Harris, 2000). Thus, it is possible that exogenous administration of the BPA+E2 combination to OVX rats in the current and previous (MacLusky et al, 2005a) studies may have produced unstable spine-synapse relations and resulted in the different outcomes.

In our experiment, the vehicle-treated group showed the lowest spine density, and the BPA+E2 group showed the highest spine density. However, neither treatment improved performance in the memory task. Spine density in the E2 alone treated group, which showed enhanced memory, appeared intermediate. Since the synapses located in dendritic spines are the major sites of excitatory input (Woolley et al, 1992), the excitability of the CA 1 neurons may be increased as spine density increases. Indeed, studies have found that estrogen treatment increases NMDA receptor binding levels in OVX rats (Woolley et al., 1997) and NMDA mediated excitatory neurotransmission (Wong & Moss, 1992) in the CA1 of the hippocampus. Excitatory input is necessary for active synaptic communication; however, excessive levels of excitation could impair many functions. As Packard (1998) discussed in his review of inverted U dose-response relations for acute post-training E2 treatment and memory consolidation, perhaps increased basal spine density (33-48% above control) induced by BPA+E2 treatments during memory consolidation aversively affected synaptic communication, and exceeded 'optimal level of receptor activation' (Packard, 1998) necessary for memory enhancement. Alternatively, changes in spine density may not underlie memory changes or relate linearly to memory functions.

30 minutes vs. 4 hours post acute treatment

Our results showed that significantly decreased spine density at 4 hours post injection for all groups including vehicle-treated control as compared with spine density measured at 30 minutes post injection (see Fig. 29). The statistical test revealed that this decrease in spine density between post 30 minutes and 4hours occured regardless of hormone/BPA treatment. There are some possible explanations that may account for this

result. First, the differences may be due to different cohort and age of rats used for 30 minutes (3-month old) and 4 hours (4-month old) experiment. Although rats in each cohort were treated in the exactly same way during a course of experiments, it is possible that cohort and age differences might affect the results. Alternatively, spine density may have simply decreased over time. Supporting this view, studies have reported that spine density may undergo dynamic changes (i.e., splitting, disappearing and reappearing, changing size and shapes) in relatively short time (Sorra & Harris, 2000; Srivastava et al., 2008). A more likely explanation would be that the sampling trials used prior to treatment might have interacted with E2 and/or BPA+E2 effects on spine density synergistically and increased spine density in 30 minutes post treatment groups. Consistent with this hypothesis, several studies have reported that behavioral tests, stress levels and exercise might influence spine density (Frick et al., 2004). In some cases these parameters could prevent estrogen from increasing spine density and may mask estrogen effects on behavior (Frick et al., 2004). However, this is not the case for the present experiment because we found that response patterns for E2 and BPA+E2 groups relative to control between 30 minutes and 4hours post treatment are strikingly similar (see Fig. 30) except basal CA1 dendritic branches.

Mechanisms underlying acute effects of E2 and BPA+E2 on spine density

The mechanisms underlying rapid changes in spine density by acute E2 and BPA+E2 treatments remain to be elucidated. Several in vitro studies have reported that BPA effects are highly cell-type or target-tissue specific (Watson et al., 2007a; Welshons et al., 2006), and BPA may exert agonistic and/or antagonistic effects depending on ER subtypes in the target cells (Kurosawa et al, 2002). Studies have found that the

distribution of phosphorylated Akt immunoreactivity in CA1 pyramidal neurons, including dendritic spines and shafts, was higher in proestrus rats and estradiol treated OVX rats compared with rats in estrus or diestrus phases (Znamensky et al., 2003), which suggests that estrogen regulation of spine density may involve activation of IP3/Akt pathways. Other studies have found that MAPK pathways play a role since MAPK is highly expressed in hippocampal pyramidal dendrites (Komiyama et al., 2002) and that changes in estrogen levels across the estrus cycle are associated with changes in the activation levels of ERK2 (Bi et al., 2001). Although the mechanisms underlying rapid changes in spine density at 30 minutes after acute treatment may be different from those observed across the 4-5 days of the estrus cycle, the activation of multiple signaling pathways reported in the studies above were likely contributed to the acute E2 and BPA treatment effects on spine density, as previous studies have shown that BPA can rapidly activate several second messenger signaling pathways (Quesada et al., 2002; Yoneda et al., 2003; Thomas et al, 2006; Canesi et al, 2005) and initiate CA2+ influx (Watson et al., 2007b). For effects 4 hours following treatment, it is more likely that the integration of genomic and non-genomic actions is involved. However, gene expression via classic ERs may not be necessary as Gu et al (1997) have demonstrated that estrogen increases the excitability of the CA1 pyramidal neurons through the cAMP-dependent phosphorylation processes and the classic ERs are not involved in this CA1 excitability.

Serum estrogen levels

To examine the physiological impact of acute treatments and verify levels of circulating hormone, serum estrogen levels were measured 30 minutes and 4 hours after vehicle, E2 and BPA+E2 treatments. Our results showed that acute E2 treatment rapidly elevates estrogen levels in the blood at 30 minutes after 20μg/kg of E2 injection (657pg/ml) and that estrogen levels remains constant 4 hours after treatment (711pg/ml). These acute E2 treatment results are consistent with MacLusky et al (2005a) who reported that serum estrogen levels increase rapidly, 10 minutes following acute injection of 45μg/kg of E2 (534pg/ml), remains at similar levels for up to 60 minutes (574pg/ml), and reaches the maximum levels at 270 minutes after treatment (1423pg/ml). The rapid increases in estrogen levels in the blood shortly after hormone administration observed in these two studies may explain the rapid onset of the increase in spine density at the PFC and basal CA1 spine density in the hippocampus, as well as E2-induced facilitation in OP memory consolidation.

Serum estrogen levels in subjects treated with BPA+E2 were higher than those of subjects treated with E2 alone, at 30 minutes (1082pg/ml) and 4 hours (901pg/ml) after the BPA/hormone injections. Although the difference between the E2 alone and E2+BPA groups did not reach a significant level, it is notable that the serum estrogen levels at 30 minutes after injections in BPA+E2 group were almost 1.7-fold higher than those in the E2-alone treated group. Thus, this may account for the greater spine density observed in the BPA+E2 treated group in the PFC and basal dendrites in the CA1 of the hippocampus, which might have caused over stimulation in appropriate synaptic transmission and, hence, suppressed E2-induced enhancement in memory consolidation.

It should be noted that circulating estrogen levels in vehicle-treated OVX subjects (about 48pg/ml for both 30 minutes and 4 hours after treatment) were markedly higher than typically observed estrogen levels in OVX rats (3-5pg/ml) noted in other studies. The difference is probably due to the different ages and weights of subjects, as well as behavioral tests given prior to blood collection. In the present experiment, the subjects were 4-5 months old, ranged in weight from 330-390g at the time of sacrifice, and received a sampling trial 30 minutes or 4hours before blood collection, while many other studies have used younger OVX rats (8-12 weeks old, ranging in weight from 200-250g) and have sacrificed the subjects without any behavior tests. Interestingly, Zhao et al (2005) reported that the blood estrogen levels increase gradually over time after ovariectomy. They measured serum E2 and T levels and extragonadal aromatization expressions for six months and found that 4.06pg/ml of blood E2 levels one month after ovariectomy gradually increased and reached a level of 31pg/ml of E2 along with increases in aromatase proteins and adrenal p450c 17 mRNA expressions at the sixth month after the surgery (Zhao et al., 2005). Zhao et al concluded that adrenal compensation had probably occurred and subsequently contributed to gradually increased blood E2 levels. These results may account for the higher base-line E2 levels observed in the vehicle-treated control subjects in our study.

Experiment 4: Acute effects of estrogen, BPA and BPA+ estrogen treatments on monoamine levels [Aim 4]

It has been shown that monoaminergic neurotransmitters are important for memory formation (Arnsten, 2006, 2007; Murphy et al., 1996a; Murphy et al., 1996b) and estrogen may influences levels of monoamine activity, synthesis and release by regulating enzymes, receptors, and transporters (see Dohanich, 2002 for review). Several behavior studies have reported positive associations between improved memory performance in OVX rats with chronic/subchronic E2 treatment, as well as intact/pregnrant female rats with higher circulating estrogen levels, and increased levels of monoaminergic activity in the hippocampus (Bowman et al., 2002; Luine et al., 1998; Seamans et al., 1998; Wilkerson & Levin, 1999), the PFC (Bowman et al., 2002; Luine et al., 1998) and the olfactory bulb (Macbeth et al., 2008b).

Rapid activation of monoamine systems by acute E2 treatment also has been demonstrated in the hypothalamus, an important region for estrogen-regulated sexual behavior (Cornil et al., 2005; Cornil et al., 2006), but few *in vivo* studies have examined the role of acute E2 treatment in relation to memory function.

In addition, recent *in vitro* studies indicate that low doses of BPA may exert effects on monoamine activity. For instance, in PC12 cells, BPA modulates dopamine release via non-genomic pathways (Yoneda et al, 2003). BPA also inhibits E2-mediated dopamine efflux activating membrane associated ER systems (Alyea & Watson, 2009).

In order to further determine possible relationships between E2 induced enhancements in memory and neural function, experiment 4 examined levels of monoamines (dopamine, serotonin, norepinephrine and their metabolites) in several brain

areas known important for cognitive functions. We hypothesized that acute E2, BPA, and BPA+E2 treatments would alter monoaminergic neurotransmitters and metabolites in the PFC, the CA1, CA3 and dentate gyrus of the hippocampus, the striatum, and the vertical diagonal band (vDB) in some degree, and these changes may contribute to memory enhancements.

Methods

Subjects and treatments.

Two cohorts of OVX rats (38 in total) that were used for behavioral analysis (Sprague-Dawley, four to five-month-old at the time of sacrificed) served as subjects for neurochemical analysis. We measured levels of monoamines and metabolites 30min after 20μg/kg of 17β-E2, which enhanced OP memory, and/or 40ug/kg of BPA, which suppressed E2 induced memory enhancement. Approximately one week after the completion of their behavior tests, one cohort of 18 rats was assigned to either the vehicle-, E2-, or BPA+E2 treated group and received pre-T1 injection, a T1 sample trial and post-T1 injection. 30 min later, the subjects were lightly anesthetized with carbon dioxide and sacrificed by rapid decapitation. This time interval (30min) was chosen to ensure that we examined neurochemical changes induced by acute estrogen and BPA treatment during the memory consolidation process. To add a BPA alone treated group for analysis, an additional cohort of 20 rats were sacrificed later on a different day using the exact same procedure except that the subjects were assigned to either the vehicle-, E2-, BPA+E2- or BPA alone treated groups (Table 8). Data from two cohorts were combined and used for statistical analysis.

Table 8. Hormone and BPA treatment used for neurochemial analysis (Experiment 4)

Group	Treatment		Subjects		
	Pre T1 INJ	Post T1 INJ	cohort 1	cohort 2	total sample size
Control	corn oil	saline	6	4	10
E2	saline	20μg/kg of 17β-E2	6	4	10
BPA+E2	40μg/kg of BPA	20μg/kg of 17β-E2	6	4	10
BPA	corn oil	4μg/kg of BPA	-	8	8

Brain tissue preparation

Following rapid decapitation, the brains were immediately removed, frozen with dry ice and stored at – 70 ^{o}C. We used a similar neurochemical procedure described previously (Beck & Luine, 2002; Bisagno et al., 2003; Luine et al., 1998; Macbeth et al., 2008a; Macbeth et al., 2008b) to measure monoamines and metabolite concentrations. For sampling, the brains were warmed to the point where they were still frozen but could be cut into six serial coronal sections with a razor blade, and mounted on a micro slide. Using a 500μm-diameter cannula, tissue samples in the target areas were obtained from the frozen sections under a dissecting microscope with the stage maintained at approximately –11 ˚C and placed into a 1.5ml Eppendorf tubes. The number of punches obtained from each brain area were as follows; the prefrontal cortex (6-8 punches): CA1 (4 punches), CA3 (4 punches), and dentate gyrus (4 punches) of the hippocampus: vDB (3 punches) and striatum (2 punches).

Monoamine and metabolites were measured by dissolving the punches in 60μl of sodium acetate buffer (pH 5.0) and obtaining the released neurotransmitter through a

process of freezing and thawing, which disrupts cellular structures and releases cellular components including neurotransmitter of interest. α-Methyl-dopamine was added as an internal standard, and samples were centrifuged at 12000r.m.p. for 12 minutes. The supernatant was removed and the pellet was re-suspended in 200 µl (PFC) or 100µl of 2.0N NaOH (other brain areas) for protein analysis using Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA, USA).

High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) with electrochemical analysis quantified neurotransmitter levels. The 40µl supernatant was used in the detection of monoamines and metabolites, including dopamine (DA) and its metabolites, 3-4-dihydroxyphenylalanine (DOPAC) and homovanillic acid (HVA); norepinephrine (NE) and its metabolite 3-Methoxy-4-Hydroxyphenylglycol (MHPG); and serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA). The supernatant was injected into a Waters Associates chromatographic system (Waters 2690), consisting of an alliance module containing an automated refrigerated, injector pump, Symmetry C_{18} 5µm 4.6 X 150mm reverse-phase column (Novapak three micron) and an ESA coulochem III detector with the screening electrode set at +50mV and the detecting electrode set at +480-500mV potential. The mobile phase contained 3% acetonitrile and peak sharpness was increased by the addition of 100% methanol (99.5% mobile: 0.5% methanol).

Millennium software (Waters Associates) was used to run the chromatography system, in which concentrations of neurotransmitters and metabolites were calculated by reference to standards and the internal standard using peak integration. Monoamine and

metabolite concentrations were expressed as pg/μg protein. Turnover ratios (metabolite/monoamine) were calculated as a measure of monoaminergic activity.

Measurements and Statistical analyses

The original pg/μg protein data from two cohorts were normalized within each cohort and then combined for statistical analysis. One-way ANOVAs with Fisher LSD host-hoc tests were used to test group differences in levels of monoamines, metabolites and turnover ratio in each brain regions. Values exceeding more than three standard deviation units were considered as outliers and excluded from analysis.

Results

Monoamine and metabolite concentrations differed among the vehicle-, E2-, BPA+E2-, or BPA treated subjects in some, but not all, brain areas when measured 30 min after acute hormone and/or BPA treatments. The most notable changes were observed in the PFC and the CA1 of the hippocampus. See Table 9 for summary of ANOVA results, df, *F* and *p* values for brain areas important for cognitive function. Table 10 (DA system) and Table 11 (NE and 5-HT system) show normalized neurochemical values for 30 min after acute hormone and/or BPA treatments with the arrows of ↑ ↓, which indicate significant increased/decreased monoamine, metabolites and turnover levels compared to the vehicle-treated control.

Table 9. Summary of ANOVA results, df, F and p values for neurochemical analysis

PFC

	df	F	P value	
DA	3, 34	5.55	0.003	**Sig****
HVA	3, 34	13.34	0.000	**Sig*****
DOPAC	3, 34	8.51	0.000	**Sig*****
HVA/DA	3, 31	7.42	0.001	**Sig****
DOPAC/DA	3, 34	2.66	0.064	**T**
NE	3, 35	3.31	0.031	**Sig***
MHPG	3, 34	15.73	0.000	**Sig*****
MHPG/NE	3, 34	11.42	0.000	**Sig*****
5HT	3, 35	10.96	0.000	**Sig*****
5HIAA	3, 34	12.43	0.000	**Sig*****
5HIAA/5HT	3, 33	9.52	0.000	**Sig*****

CA1

	df	F	P value	
DA	3, 32	2.21	0.106	
HVA	3, 35	0.76	0.524	
DOPAC	3, 32	4.57	0.009	**Sig****
HVA/DA	3, 34	1.45	0.246	
DOPAC/DA	3, 31	5.07	0.006	**Sig****
NE	3, 33	3.19	0.036	**Sig***
MHPG	3, 35	2.94	0.046	**Sig***
MHPG/NE	3, 33	2.37	0.080	**T**
5HT	3, 34	5.05	0.005	**Sig****
5HIAA	3, 35	1.59	0.210	
5HIAA/5HT	3, 35	8.33	0.000	**Sig*****

CA3

	df	F	P value	
DA	3, 22	0.98	0.422	
HVA	3, 34	0.28	0.843	
DOPAC	3, 26	5.65	0.004	**Sig****
HVA/DA	3, 22	2.70	0.070	**T**
DOPAC/DA	3, 27	4.66	0.009	**Sig****
NE	3, 34	0.71	0.554	
MHPG	3, 32	1.76	0.174	
MHPG/NE	3, 30	4.36	0.012	**Sig***
5HT	3, 34	0.10	0.961	
5HIAA	3, 34	1.83	0.161	
5HIAA/5HT	3, 34	1.99	0.134	

Dentate Ggyrus

	df	F	P value	
DA	3, 31	3.26	0.034	**Sig***
HVA	3, 33	0.68	0.569	
DOPAC	3, 34	0.35	0.792	
HVA/DA	3, 33	1.38	0.265	
DOPAC/DA	3, 33	1.92	0.145	
NE	3, 32	1.12	0.355	
MHPG	3, 34	2.82	0.070	**T**
MHPG/NE	3, 33	1.23	0.314	
5HT	3, 35	5.60	0.003	**Sig****
5HIAA	3, 34	2.25	0.100	
5HIAA/5HT	3, 35	3.41	0.028	**Sig***

vDB

	df	F	P value	
DA	3, 28	2.75	0.061	**T**
HVA	3, 31	3.48	0.027	**Sig***
DOPAC	3, 28	1.46	0.247	
HVA/DA	3, 28	1.11	0.361	
DOPAC/DA	3, 24	1.53	0.231	
NE	3, 34	0.74	0.534	
MHPG	3, 25	0.29	0.831	
MHPG/NE	3, 25	0.62	0.606	
5HT	3, 35	0.17	0.917	
5HIAA	3, 34	1.29	0.292	
5HIAA/5HT	3, 34	1.47	0.240	

Striatum

	df	F	P value	
DA	3, 31	18.47	0.000	**Sig*****
HVA	3, 35	0.25	0.862	
DOPAC	3, 34	3.73	0.020	**Sig***
HVA/DA	3,33	3.69	0.021	**Sig***
DOPAC/DA	3, 34	0.70	0.556	
NE	3, 33	0.55	0.649	
MHPG	3, 33	0.61	0.615	
MHPG/NE	3, 32	0.96	0.422	
5HT	3, 33	0.29	0.831	
5HIAA	3, 35	0.58	0.630	
5HIAA/5HT	3, 35	0.45	0.721	

T p <0.08, * p<0.05; **p<0.01; ***p<0.001. See Table 10 and 11 for abbreviations.

Table 10. Summary of acute E2 and BPA treatment effects on dopamine, metabolite and turnover levels

Brain area & Treatment	DA system				
	DA	**HVA**	**DOPAC**	**HVA/DA**	**DOPAC/DA**
PFC					
Control	100 ± 5.3	100 ± 3.5	100 ± 5.1	100 ± 5.1	100 ± 5.8
E2	129 ± 8.1	104 ± 3.3	**142 ± 7.4** ↑	85 ± 4.6	112 ± 1.9
BPA	60 ± 4.2	82 ± 4.9	85 ± 4.2	112 ± 9.0	**143 ± 6.1** ↑
BPA+E2	**143 ± 7.2** ↑	**157 ± 5.1** ↑	**162 ± 5.1** ↑	**146 ± 4.5** ↑	131 ± 7.1
CA1					
Control	100 ± 7.9	100 ± 6.0	100 ± 6.4	100 ± 4.2	100 ± 2.8
E2	101 ± 9.1	98 ± 6.0	91 ± 5.6	118 ± 7.0	94 ± 2.0
BPA	56 ± 4.6	126 ± 7.5	**151 ± 5.9** ↑	180 ± 12.0	**167 ± 5.6** ↑
BPA+E2	60 ± 3.7	109 ± 8.1	79 ± 5.0	110 ± 4.6	89 ± 2.0
CA3					
Control	100 ± 12.2	100 ± 9.2	100 ± 5.4	100 ± 9.1	100 ± 10.6
E2	119 ± 12.6	93 ± 6.0	**172 ± 5.9** ↑	77 ± 13.5	98 ± 11.4
BPA	196 ± 15.2	111 ± 7.3	**161 ± 7.9** ↑	**36 ± 7.2** ↓	**35 ± 3.1** ↓
BPA+E2	116 ± 7.5	103 ± 7.4	107 ± 5.7	65 ± 10.1	**54 ± 4.9** ↓
DG					
Control	100 ± 8.8	100 ± 3.0	100 ± 6.0	100 ± 5.1	100 ± 4.4
E2	87 ± 8.2	98 ± 6.8	99 ± 8.1	104 ± 7.9	126 ± 6.9
BPA	**57 ± 4.5** ↓	116 ± 7.3	110 ± 9.0	100 ± 10.7	140 ± 10.1
BPA+E2	**59 ± 3.7** ↓	97 ± 4.6	91 ± 3.8	133 ± 5.2	**152 ± 7.0** ↑
vDB					
Control	100 ± 6.8	100 ± 6.0	100 ± 7.6	100 ± 3.7	100 ± 2.5
E2	**73 ± 6.6** ↓	134 ± 7.9	98 ± 7.6	144 ± 3.1	129 ± 4.4
BPA	**65 ± 9.9** ↓	**161 ± 7.0** ↑	97 ± 7.7	139 ± 8.6	146 ± 4.8
BPA+E2	74 ± 5.8	105 ± 3.2	**73 ± 5.6** ↓	147 ± 5.1	133 ± 8.8
Striatum					
Control	100 ± 1.9	100 ± 6.1	100 ± 3.7	100 ± 5.6	100 ± 4.7
E2	**112 ± 5.4** ↑	92 ± 2.4	105 ± 3.5	84 ± 2.4	95 ± 4.2
BPA	**70 ± 2.7** ↓	101 ± 6.4	87 ± 3.4	115 ± 5.5	109 ± 6.1
BPA+E2	**117 ± 1.7** ↑	99 ± 6.2	116 ± 4.5	90 ± 3.4	99 ± 3.8

Entries are normalized mean ± SEM. Arrows show significant increase or decrease compared to the control. DA, dopamine; HVA, homovanillic acid; DOPAC, 3-4-dihydroxyphenylalanine. PFC, Prefrontal cortex; DG, dentate gyrus: vDB, vertical diagonal band.

Table 11. Summary of acute E2 and BPA treatment effects on Norepinephrine, Serotonin, metabolite and turnover levels

Brain area & Treatment	NE System			5-HT System		
	NE	**MHPG**	**MHPG/NE**	**5-HT**	**5HAII**	**5HAII/5-HT**
PFC						
Control	100 ± 4.7	100 ± 3.5	100 ± 2.4	100 ± 5.8	100 ± 3.1	100 ± 1.0
E2	102 ± 4.1	**165 ± 3.6** ↑	**162 ± 4.9** ↑	109 ± 3.5	113 ± 2.6	101 ± 1.7
BPA	**78 ± 5.2** ↓	**142 ± 4.5** ↑	**189 ± 4.8** ↑	**57 ± 3.8** ↓	98 ± 4.3	**190 ± 9.0** ↑
BPA+E2	104 ± 4.2	**215 ± 5.4** ↑	**226 ± 6.3** ↑	83 ± 5.7	**153 ± 4.2** ↑	**178 ± 3.3** ↑
CA1						
Control	100 ± 6.7	100 ± 8.5	100 ± 2.9	100 ± 8.2	100 ± 6.2	100 ± 6.1
E2	**65 ± 5.4** ↓	71 ± 3.6	105 ± 4.7	109 ± 7.7	113 ± 7.3	96 ± 2.1
BPA	97 ± 7.6	110 ± 8.4	**136 ± 7.5** ↑	**138 ± 5.0** ↑	**130 ± 1.8** ↑	81 ± 2.3
BPA+E2	91 ± 5.2	74 ± 5.1	92 ± 7.5	73 ± 4.5	109 ± 5.3	**135 ± 5.2** ↑
CA3						
Control	100 ± 8.3	100 ± 6.3	100 ± 8.9	100 ± 6.5	100 ± 4.8	100 ± 5.5
E2	92 ± 5.8	101 ± 6.3	128 ± 6.8	95 ± 5.8	**84 ± 2.4** ↓	95 ± 4.6
BPA	107 ± 8.5	87 ± 9.0	78 ± 8.4	94 ± 5.2	95 ± 4.5	114 ± 5.3
BPA+E2	115 ± 5.6	72 ± 5.9	71 ± 5.8	97 ± 4.3	88 ± 3.0	89 ± 3.2
DG						
Control	100 ± 3.8	100 ± 7.6	100 ± 9.5	100 ± 5.8	100 ± 4.8	100 ± 5.6
E2	79 ± 6.2	**71 ± 6.2** ↓	85 ± 7.3	77 ± 8.1	92 ± 6.5	124 ± 6.4
BPA	90 ± 7.7	**73 ± 6.8** ↓	66 ± 8.6	102 ± 6.7	97 ± 4.4	87 ± 6.9
BPA+E2	86 ± 6.6	79 ± 4.0	86 ± 6.3	**62 ± 1.9** ↓	**77 ± 2.7** ↓	128 ± 4.1
vDB						
Control	100 ± 8.8	100 ± 6.3	100 ± 4.5	100 ± 3.9	100 ± 2.4	100 ± 4.7
E2	123 ± 4.2	114 ± 4.6	86 ± 2.7	102 ± 8.0	115 ± 5.0	120 ± 7.3
BPA	109 ± 9.9	109 ± 10	88 ± 9.8	93 ± 7.2	115 ± 4.4	94 ± 7.3
BPA+E2	102 ± 6.7	117 ± 8.4	103 ± 8.5	101 ± 5.4	107 ± 4.0	100 ± 2.9
Striatum						
Control	100 ± 4.7	100 ± 3.6	100 ± 5.7	100 ± 4.6	100 ± 3.6	100 ± 5.2
E2	114 ± 3.6	104 ± 3.7	80 ± 4.3	99 ± 4.0	104 ± 3.6	110 ± 5.4
BPA	99 ± 5.0	106 ± 4.4	107 ± 10	99 ± 4.1	107 ± 4.9	111 ± 2.7
BPA+E2	114 ± 4.3	97 ± 4.7	86 ± 7.4	94 ± 4.6	97 ± 4.4	103 ± 5.4

Entries are normalized mean ± SEM. Arrows show significant increase or decrease compared to the control. NE, norepinephrine; MHPG, metabolite 3-Methoxy-4-Hydroxyphenylglycol; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole acetic acid.

Prefrontal cortex

As shown in Table 9, significant group differences were found in all monoamine, metabolite and turnover levels in the PFC. The only exception was DOPAC/ DA turnover ratio, which showed a trend ($p<0.064$) but failed to reach significance. In DA system, post-hoc tests (Figure 31A and 32B) revealed E2 alone treatment affected only DOPAC level (43% above control, $p<0.016$), and BPA alone treatment had effect only on DOPAC/DA ($p<0.02$) but not on other monoamines. However, co-administration of BPA with E2 increased DA ($p<0.05$), HVA ($p<0.001$) and HVA/DA turnover ($p<0.01$) levels 40-70% above control, which suggested BPA effects were additive when given with E2.

In NE system (Fig 31C and 31D), similar patterns to DA data were obtained for some neurochemicals. For example, acute E2 alone treatment increased NE metabolite MHPG ($p<0.001$) and MHPG/NE turnover levels ($p<0.01$) about 65% above control and BPA+E2 treatment resulted in more than 2-fold higher levels ($P<0.0001$ for both). Thus, BPA+E2 effects appeared additive and more potent compared to its effect on the DA system. Interestingly, BPA alone treatment showed some distinct effects on NE system, decreasing NE levels (22% below control, $p<0.03$), but increasing MHPG ($p<0.03$) and MHPG/NE ($p<0.001$) levels significantly.

There were no effects of E2 alone treatment on the 5-HT system (Fig 31E and 31F), but BPA+E2 treatment significantly increased 5-HIAA ($p<0.001$, 53% above control) and 5-HIAA/5HT ($p<0.001$, 78% above control) turnover levels. BPA alone treatment showed the same response pattern to NE concentration for 5-HT, that is, BPA alone significantly decreased 5-HT ($p<0.001$, 43% below control), but increased 5-HT to

5-HIAA turnover ratio ($p<0.0001$, 90% above control). In fact, 5-HIAA/5-HT level was the highest in BPA alone treated group.

CA1, CA3 and DG of the hippocampus

In the CA1 of the hippocampus, significant group differences were found in NE, MHPG, 5-HT, 5-HIAA/5-HT and DOPAC/DA levels (Table 9). Post-hoc tests (Fig 32) revealed that acute E2 and BPA treatment effects on CA1 monoamine levels were different from response patterns observed in the PFC. The most notable difference was greater potency of BPA alone treatment. For example, BPA significantly increased levels of DOPAC (51% above control, $p<0.02$, Fig. 32A), DOPAC/DA (67% above control, $p<0.006$, Fig. 32B), MHPG/NE (36% above control, $p<0.05$, Fig. 32D), 5-HT (38% above control, $p<0.03$, Fig. 32E) and 5-HIAA (30% above control, $p<0.04$, Fig. 32E), while E2 alone and BPA+E2 treatments had no effects on these monoamine levels. Co-administration of BPA with E2 was additive only for 5-HIAA/5-HT turnover ratio (35% above control, $p<0.003$). E2 alone treatments did not increased neurochemical levels, but significantly decreased NE concentrations (35% below control, $p<0.008$, Fig. 32E).

In the CA3 of the hippocampus, significant group differences were found in DA and NE systems, but not in 5-HT system (Table 9). Post-hoc tests indicated DOPAC levels (Fig. 33A) were increased by E2 (72% above control, $p<0.004$) and BPA (61% above control, $p<0.009$). In contrast, HVA/DA and DOPAC/DA turnover levels (Fig. 33B) were 65% decreased by BPA ($P<0.01$ and $p<0.007$ respectively). BPA+E2 treatment also significantly decreased DOPAC/DA levels (44% below control, $p<0.02$)

In the dentate gyrus of the hippocampus, significant group differences were found in DA, 5-HT and 5-HIAA/5-HT levels (Table 9). E2 treatments did not alter any

monoamine levels. DA concentrations were decreased by BPA (43% below control, $p<0.01$) and BPA+E2 (41% below control, $p<0.023$) (Fig. 34A), while DA turnover ratio to DOPAC was increased by BPA+E2 (52% above control, $p<0.03$, Fig. 34B). In the 5-HT system, BPA+E2 treatments significantly increased 5-HT (38% below control, $p<0.002$) and 5-HIAA (23% below control, $p<0.02$) levels (Fig. 34E), while the same treatment increased 5-HT turnover ratio to 5-HIAA (28% above control, Fig. 34F). But this increase did not reach significance ($p<0.057$).

vDB and Striatum

Significant group differences were found only in the DA system (Table 9). In vDB (Fig. 35), levels of DA were significantly lowered by both E2 (27% below control, $p<0.04$) and BPA (35% below control, $p<0.02$) treatment. However, HVA concentration was significantly elevated by BPA (61% above control, $p<0.001$). BPA+E2 treatments did not influence monoamine levels.

Finally in the striatum (Fig. 36), DA concentration was significantly increased by E2 (12% above control, $p<0.04$) and BPA+E2 (17% above control, $p<0.005$), but decreased by BPA (30% below control, $p<0.001$). BPA+E2 also elevated DOPAC levels (16% above control), but this difference did not reach significant levels ($p<0.065$).

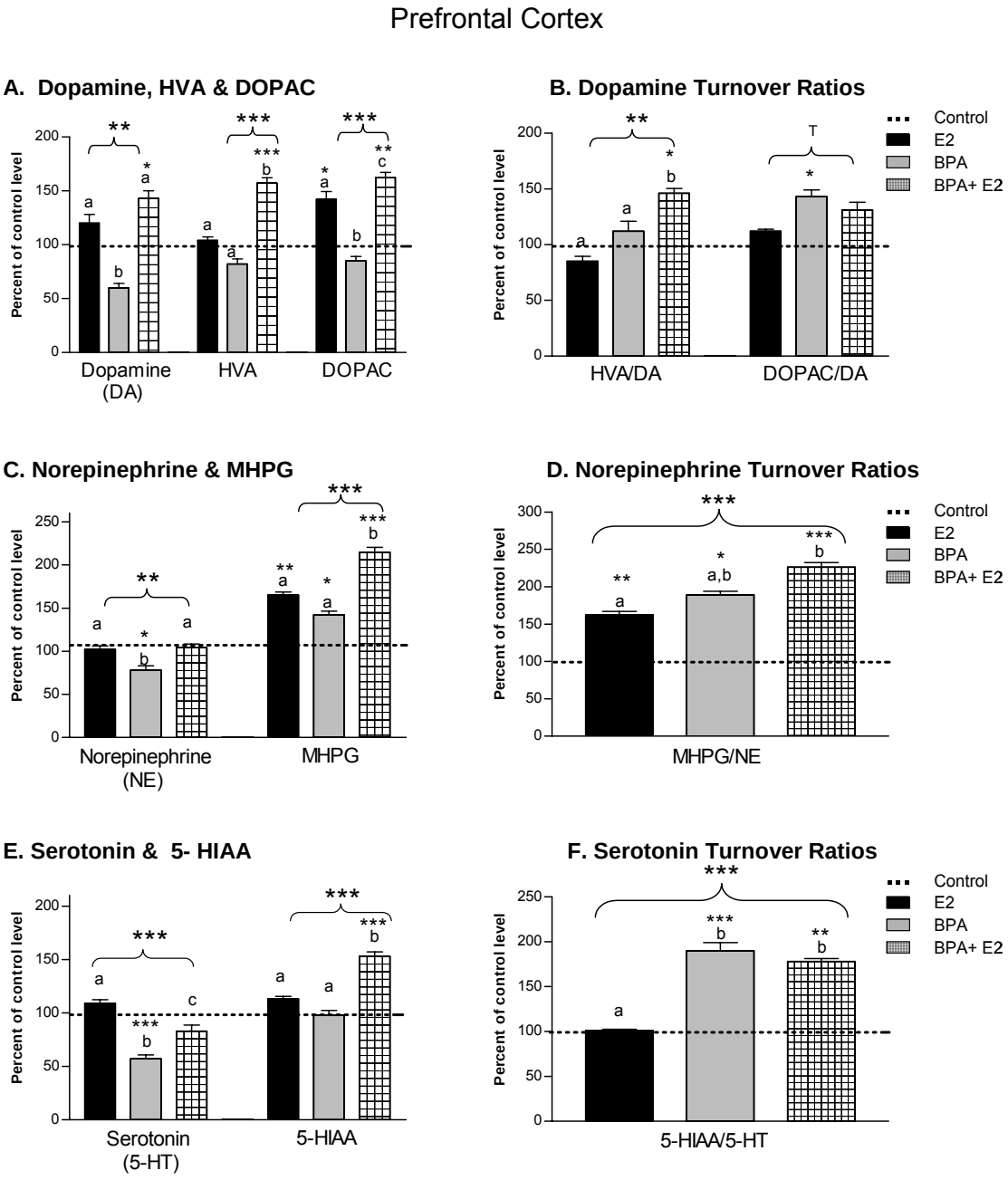


Figure 31. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in the prefrontal cortex Data were analyzed by one-way ANOVAs with Fisher LSD post hoc tests. **A.** Concentrations of DA and metabolites HVA and DOPAC. **B.** Ratios of DA to HVA and DOPAC. **C.** Concentration of NE and metabolite MHPG. **D.** Ratio of NE to MHPG. **E.** Concentration of 5-HT and metabolite 5-HIAA. **F.** Ratio of 5-HT to 5-HIAA. All values are normalized and presented as percentage of control (the dot lines) levels. Letter a, b, c indicates significant differences among 3 treated groups. Entries are mean ± SEM. $_{T}$ p <0.064, * P<0.05; **p<0.01; ***p<0.001.

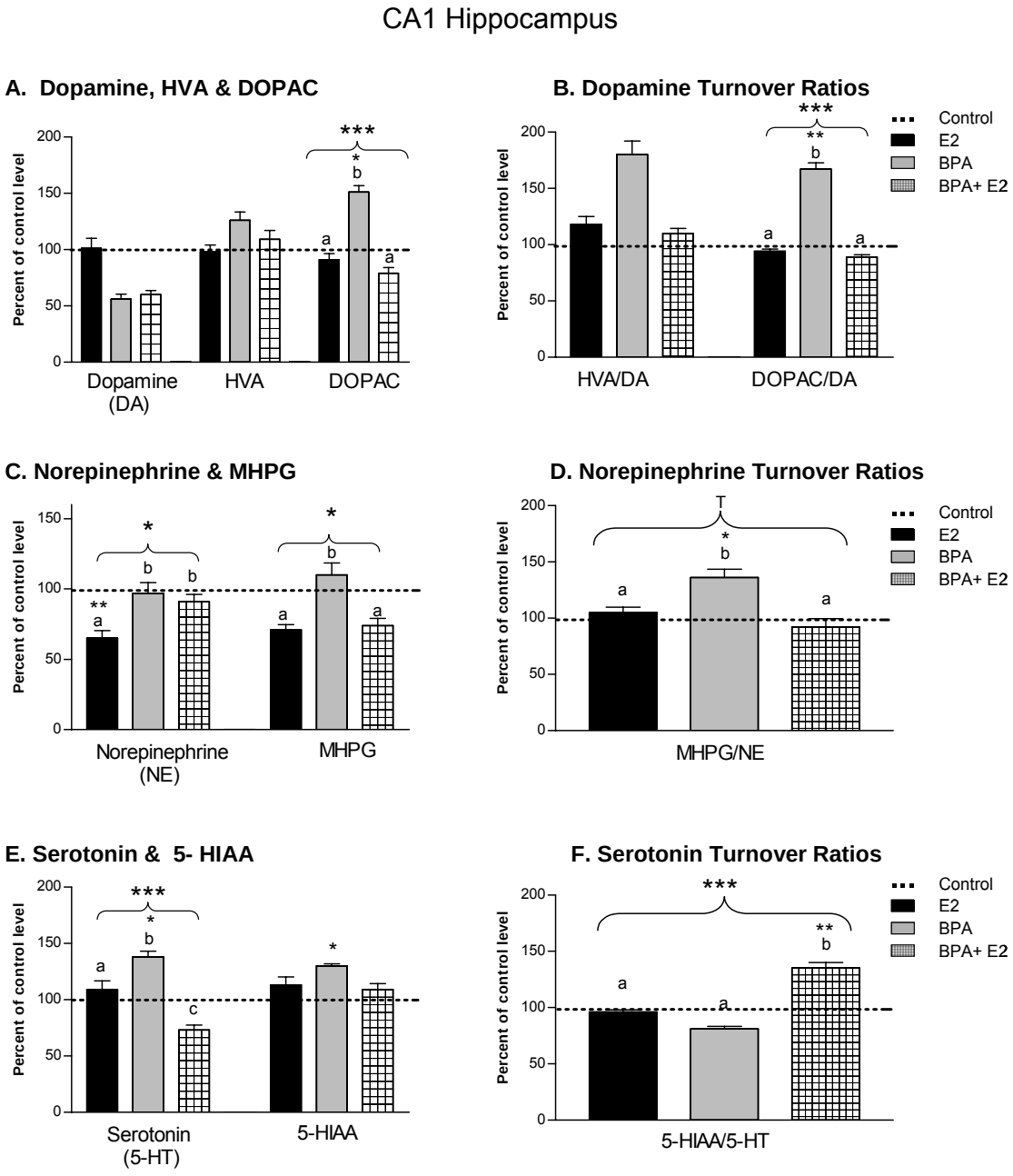


Figure 32. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in the CA1 hippocampus Data were analyzed by one-way ANOVAs with Fisher LSD post hoc tests. **A.** Concentrations of DA and metabolites HVA and DOPAC. **B.** Ratios of DA to HVA and DOPAC. **C.** Concentration of NE and metabolite MHPG. **D.** Ratio of NE to MHPG. **E.** Concentration of 5-HT and metabolite 5-HIAA. **F.** Ratio of 5-HT to 5-HIAA. All values are normalized and presented as percentage of control (the dot lines) levels. Letter a, b, c indicates significant differences among 3 treated groups. Entries are mean ± SEM. $_{T}$ p <0.08, * P<0.05; **p<0.01; ***p<0.001.

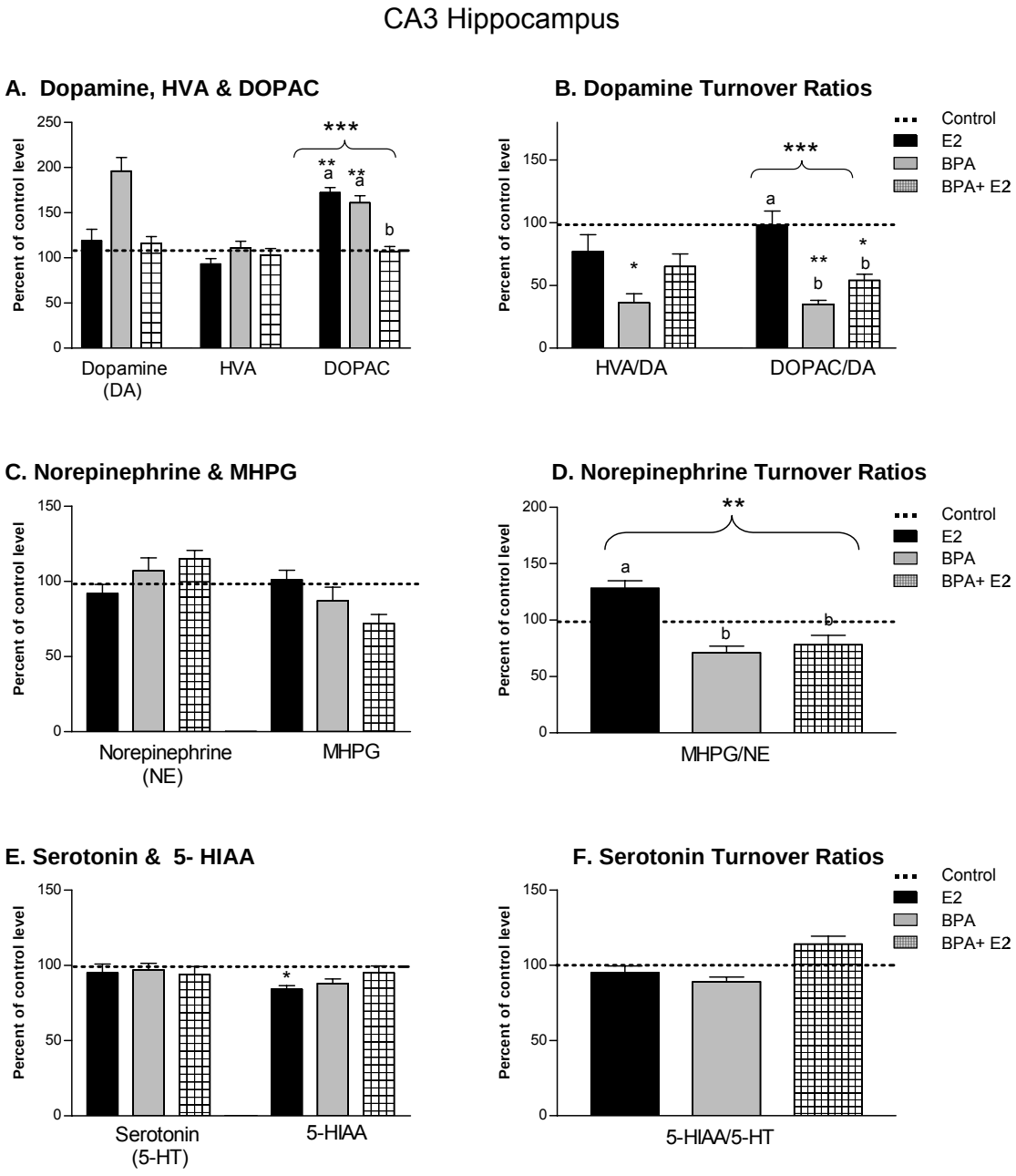


Figure 33. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in CA3 hippocampus Data were analyzed by one-way ANOVAs with Fisher LSD post hoc tests. **A.** Concentrations of DA and metabolites HVA and DOPAC. **B.** Ratios of DA to HVA and DOPAC. **C.** Concentration of NE and metabolite MHPG. **D.** Ratio of NE to MHPG. **E.** Concentration of 5-HT and metabolite 5-HIAA. **F.** Ratio of 5-HT to 5-HIAA. All values are normalized and presented as percentage of control (the dot lines) levels. Letter a, b, c indicates significant differences among 3 treated groups. Entries are mean ± SEM. * $P<0.05$; ** $p<0.01$; *** $p<0.001$.

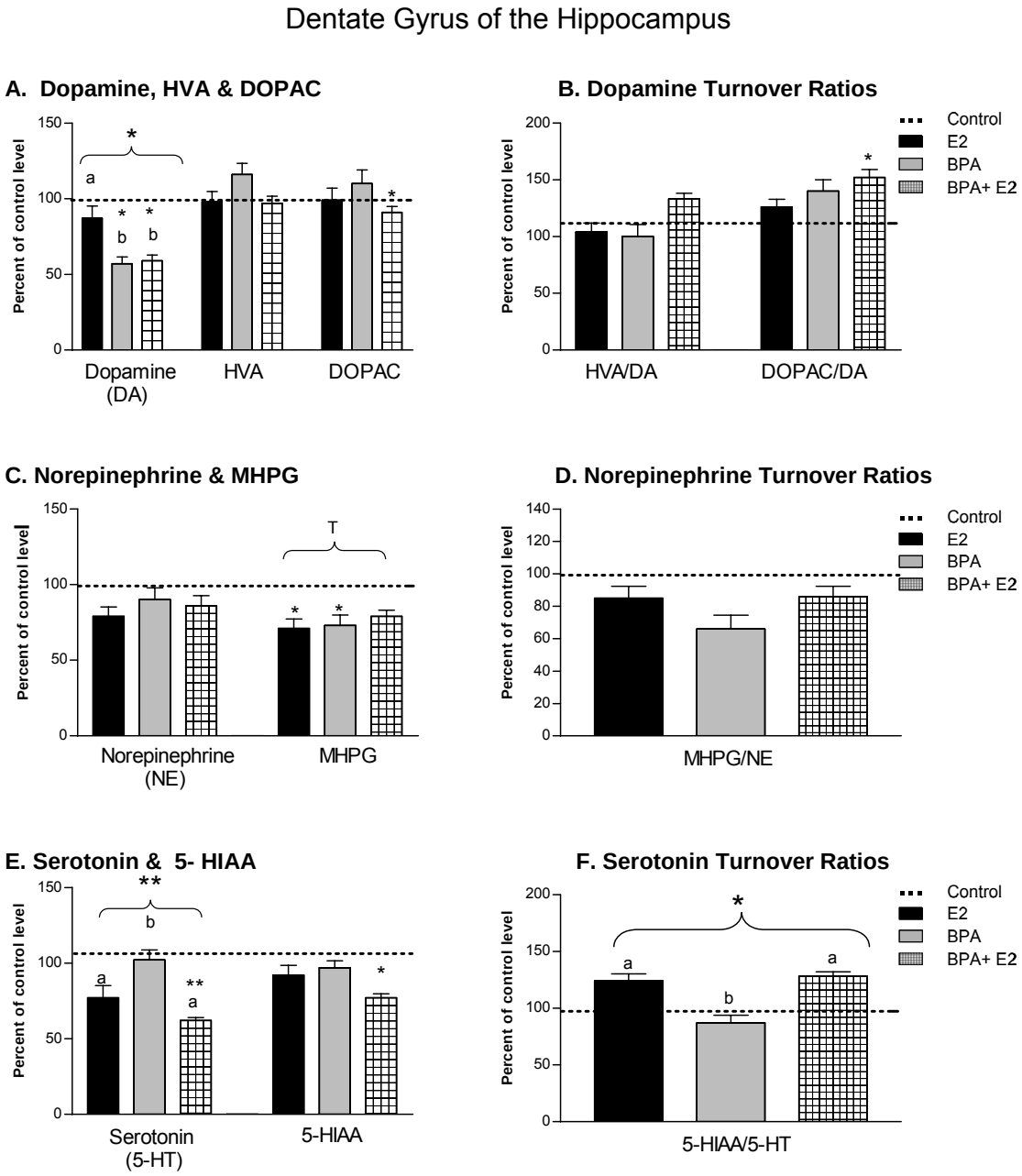


Figure 34. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in the dentate gyrus of the hippocampus Data were analyzed by one-way ANOVAs with Fisher LSD post hoc tests. **A.** Concentrations of DA and metabolites HVA and DOPAC. **B.** Ratios of DA to HVA and DOPAC. **C.** Concentration of NE and metabolite MHPG. **D.** Ratio of NE to MHPG. **E.** Concentration of 5-HT and metabolite 5-HIAA. **F.** Ratio of 5-HT to 5-HIAA. Values are normalized and presented as percent of control (the dot lines) levels. Letter a, b, c indicates significant differences among 3 treated groups. Entries are mean ± SEM. $_{T}$ p <0.08, * P<0.05; **p<0.01; ***p<0.001.

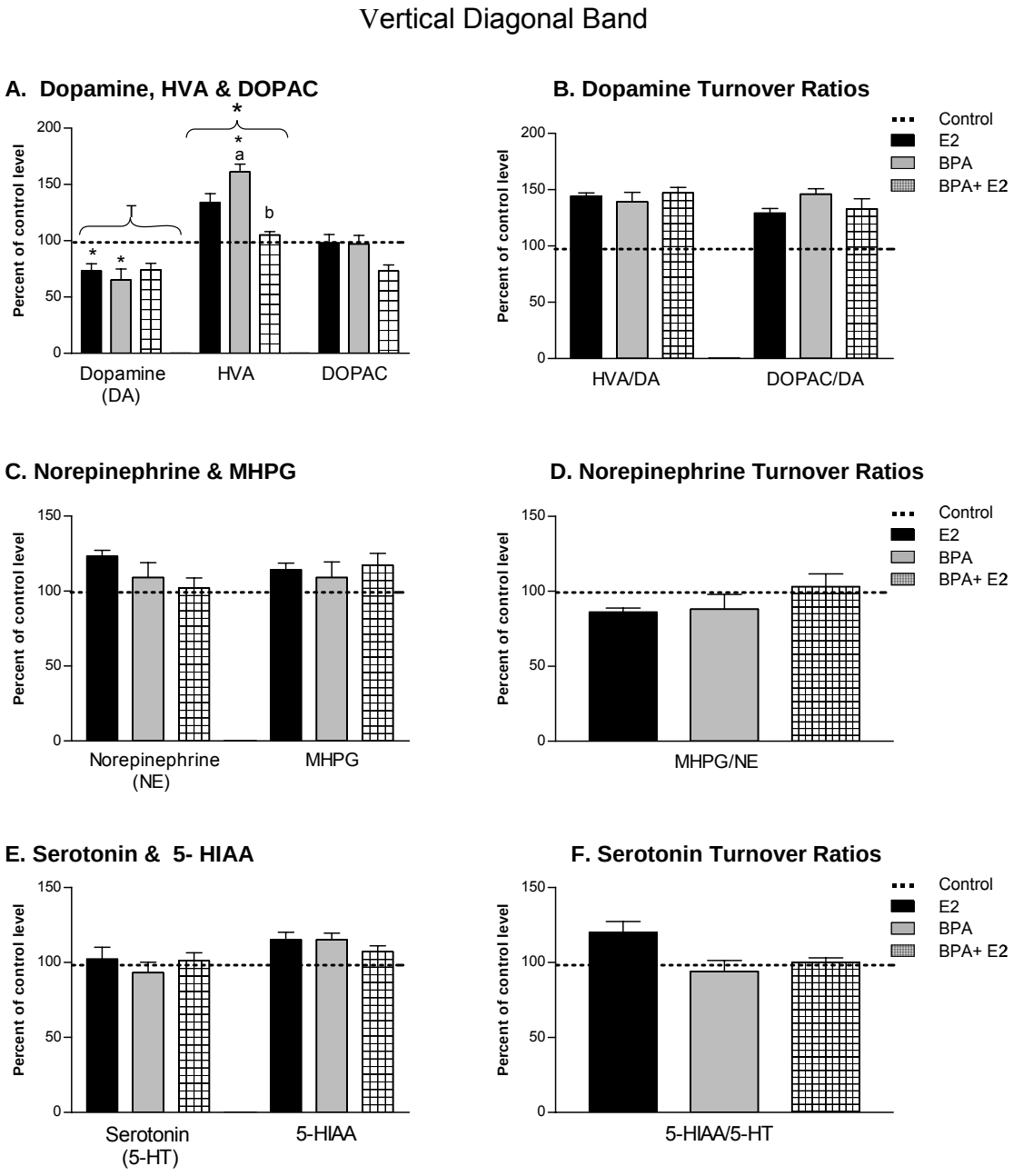


Figure 35. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in the vertical diagonal band. Data were analyzed by one-way ANOVAs with Fisher LSD post hoc tests. **A.** Concentrations of DA and metabolites HVA and DOPAC. **B.** Ratios of DA to HVA and DOPAC. **C.** Concentration of NE and metabolite MHPG. **D.** Ratio of NE to MHPG. **E.** Concentration of 5-HT and metabolite 5-HIAA. **F.** Ratio of 5-HT to 5-HIAA. All values are normalized and presented as percentage of control (the dot lines) levels. Letter a, b, c indicates significant differences among 3 treated groups. Entries are mean ± SEM. $_{T}$ p <0.061, * P<0.05

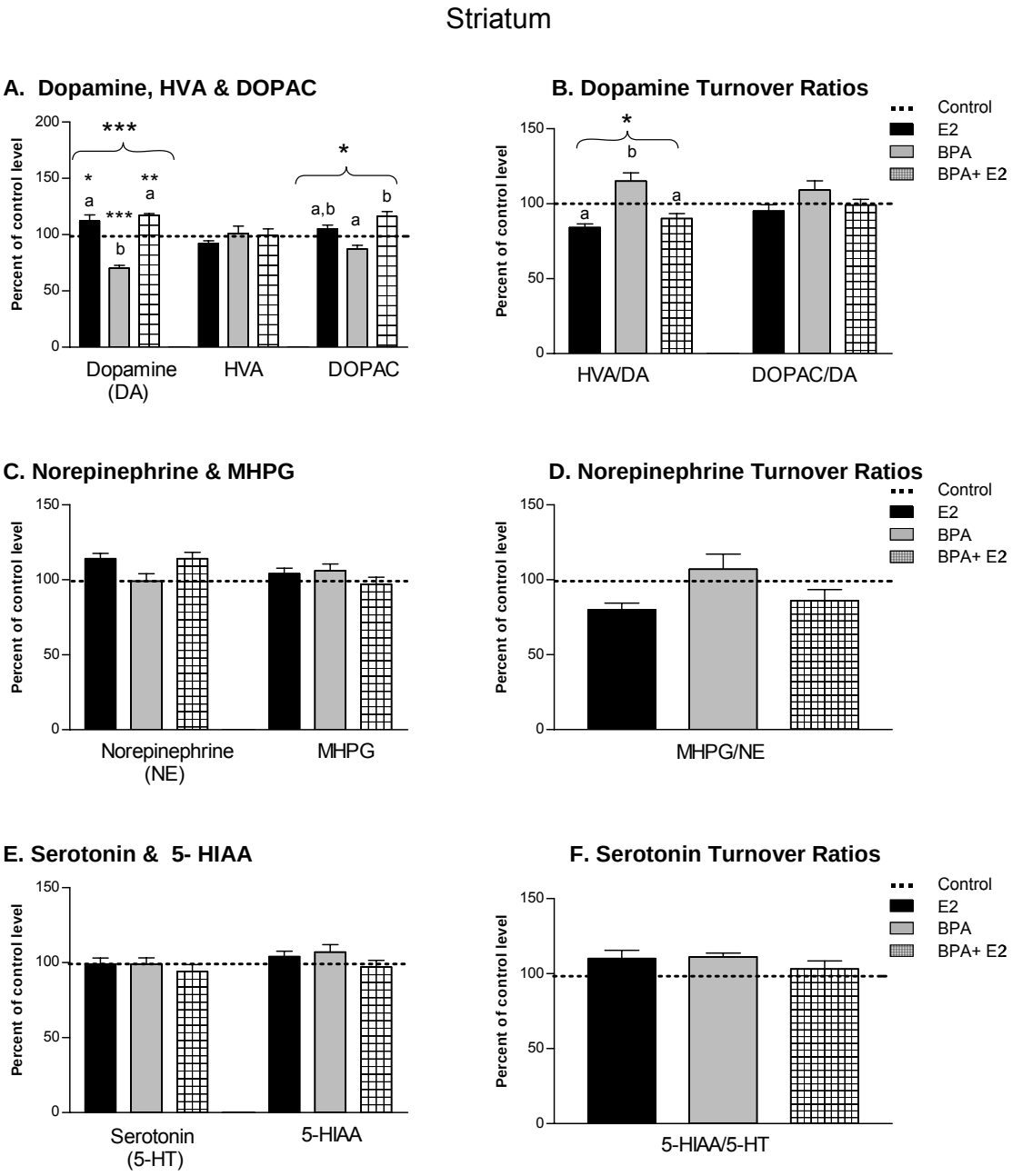


Figure 36. Effect of acute 17β-E2 and BPA on monoamine, metabolite and turnover levels in the striatum. Data were analyzed by one-way ANOVAs with Fisher LSD post hoc tests. **A.** Concentrations of DA and metabolites HVA and DOPAC. **B.** Ratios of DA to HVA and DOPAC. **C.** Concentration of NE and metabolite MHPG. **D.** Ratio of NE to MHPG. **E.** Concentration of 5-HT and metabolite 5-HIAA. **F.** Ratio of 5-HT to 5-HIAA. All values are normalized and presented as percentage of control (the dot lines) levels. Letter a, b, c indicates significant differences among 3 treated groups. Entries are mean ± SEM. * $P<0.05$; ** $p<0.01$; *** $p<0.001$.

Discussion

The results of experiment 4 show that acute treatment with estrogen or the combination of estrogen and BPA differentially alterd monoamine and metabolite levels in the PFC, the CA1, CA3 and dentate gryus of the hippocampus, vDG and the striatum. These results provide novel information that the same dose of 17β-estradiol that enhances spatial memory consolidation and the BPA dose that blocks the E2 effect on memory also influence neurochemical levels, within 30 minutes after a post-sampling treatment, in brain areas known to be important for cognitive function. Notable findings in the present experiment include that; 1) in the PFC, this dose of 17β-E2 generally increases neurochemical levels and co-administration of BPA with E2 further increases almost all neurochemical activity. These findings indicates that neurochemical responses of BPA to E2 in the PFC are additive; 2) in the CA1 of the hippocampus, acute E2 treatment does not alter monoamine levels aside from a significant decrease in NE and, interestingly, BPA-alone treatment exerts the strongest effects; and, 3) in the striatum, acute treatments rapidly alter DA levels, supporting the view that responses are mediated via membrane-associated ER as this region contains few nuclear ERs.

Acute E2 and BPA effects in the PFC and the hippocampus

The results of Experiment 4 show that activity of monoaminergic terminals in the PFC and the CA1 of the hippocampus are differentially affected by acute E2, BPA and BPA+E2 treatment at the doses and during the time when memory consolidation occurs. In the CA 1 of the hippocampus, E2 treatment resulted in a 35% reduction of NE levels, while BPA-alone and BPA+E2 treatments did not change NE levels. In the PFC, estrogen treatment resulted in 142% higher levels of DA metabolite DOPAC, 165%

higher MHPG levels, 162% higher MHPG/NE turnover ratios, and 113% higher levels of 5HT as compared with the vehicle-treated control. BPA+E2 treatment further elevated these neurochemical levels up to 162%, 215%, 226%, 153% respectively. These rapid changes in monoaminergic activities may contribute to acute E2 and BPA+E2 treatment effects on spatial memory consolidation.

Effects of DA and NE systems in the PFC

Several lines of evidence indicate that the PFC is extremely sensitive to changes in the neuromodulatory inputs from the NE and DA systems (Arnsten, 1997; Arnsten et al., 1998; Arnsten, 2006; Zahrt et al., 1997). Both DA and NE have beneficial effects on working memory functions in the PFC, however, studies have shown that both excessive and insufficient levels of DA and NE in the PFC induce cognitive impairment, including deficits in spatial working memory in humans, rats, and monkeys (Murphy et al., 1996: Arnsten 1997: Zahrt et al 1997). DA has been shown to enhance PFC function via the D1 receptors, which stimulation increases production of cAMP, while stimulation of the D2 receptors decreases it (Trantham-Davidson et al., 2004). Similarly, Arnsten (1998) has shown that NE enhances working memory via the α-2 adrenergic receptors and that it has opposing effects in the α-1 receptors (Arnsten, 1998). In addition, evidence suggests that DA has an inverted-U dose-response relationship at the D1 receptors; that is, both too little and too much stimulation of the D1 receptors impair working memory in rats (Arnsten, 2007; Hotte et al., 2005). Arnsten (1998) argues that memory impairment occurs with the endogenous release of both DA and NE during stress, as well as the exogenous administration of high and low doses of D1 and α-1 agonists. These observations suggest that critical levels of DA and NE are crucial for optimal PFC

functions. In our study, we observed the highest levels of DA and NE metabolites in the PFC following BPA+E2 treatment, which suggests that intensive activity during memory consolidation, while E2-alone treatment increased these metabolites to intermediate levels. Thus, it is possible that neurochemical activity in the PFC is important for memory consolidation, but BPA+E2 treatment causes excessive levels of activity, which result in the suppression of E2-induced memory enhancement.

These results are particularly noteworthy as they show that altered neurochemical levels in the PFC may play an important role in spatial memory consolidation. Although spatial memory is considered to be primarily hippocampal rather than PFC dependent, several studies have reported that PFC-hippocampal projections may be critical for both spatial and non-spatial memory (Floresco et al., 1997; Seamans et al., 1998; Thierry et al., 2000; Wang & Cai, 2006), and suggest possible bi-directional regulation of synaptic strength, based on the specific demands of memory tasks (Laroche et al., 2000). Our neurochemical and behavior results are consistent with this hypothesis, as this dose of 17β-E2 did not enhance non-spatial OR memory.

Acute E2 and BPA effects on NE system in the hippocampus

Perhaps most importantly, the present study shows that enhancement of spatial memory consolidation is not directly associated with increased levels of monoaminergic activity in the hippocampus. This result is surprising, because the hippocampus is a key structure for formation of spatial memory (Morris et al., 1982; Olton et al., 1978). Nonetheless, in our experiment, most of the monoamines, metabolites and turnover ratios of the E2-alone group did not differ significantly from the BPA+E2 treated and vehicle-treated groups. The only exception was NE concentration, which was significantly

decreased by E2 treatment in the CA1 of the hippocampus. One possible interpretation of these data would be that acute estrogen treatment may temporarily suppress NE synthesis and activity in the hippocampus, and that these alterations may be necessary to facilitate OP memory consolidation.

Although the mechanisms underlying the function of NE in memory are not clear, it has been reported that NE modulates the efficacy of glutamate transmission, activating G-protein coupled adrenergic receptors (Scheiderer et al., 2004). Some electrophysiological studies have shown that NE promotes long-term potentiation or LTP in the adult rat hippocampus (Izumi & Zorumski, 1999) and also induces long-term depression or LTD (Scheiderer et al., 2004). In addition, rapid acute administration of E2 has been shown to induce both LTP (Kawato, 2004) and LTD (Mukai et al., 2007; Ogiue-Ikeda et al., 2008) in hippocampal cell cultures. Kawato (2004) has also shown that co-administration of BPA suppresses E2-mediated LTP induction, while Ogiue-Ikeda et al (2008) found that 10-100nM BPA enhances LTD. Evidence indicates that both LTP and LTD are important for certain types of memory formation and are considered as the cellular model of learning and memory (Kemp & Manahan-Vaughan, 2004; Scheiderer et al., 2004). Specifically, novelty acquisition is associated with induction of hippocampal LTD (Kemp & Manahan-Vaughan, 2004; Manahan-Vaughan & Braunewell, 1999), and performance in a spatial memory task is significantly correlated with the magnitude of LTD in hippocampus (Nakao et al., 2002). Moreover, using a behavior test similar to the OP/OR task, Kemp et al (2004) demonstrated that induction of LTD is correlated with the encoding of the object location within a spatial context rather than the recognition of the object themselves, and not triggered by exploration behavior in space. Kemp et al also

showed the LTD/LTP induction was regulated by 5-HT_4 receptor activation. These findings are of particular interest as in our behavior study, the vehicle-treated OVX rats and BPA+E2 treated rats did not remember the locations of the objects, and showed significantly higher levels of NE metabolite MHPG and MHPG/NE turnover ratios in the PFC and higher levels of NE in the CA1 of the hippocampus as compared with E2 treated rats, whereas rats received acute estrogen treatment immediately after novelty acquisition did remember the locations of objects and had moderate levels of MHPG/NE turnover ratios and lower levels of NE. It therefore appears that these neurochemical changes observed in the E2 group may exert appropriate "buffering" effects on LTD expression, which, in turn, resulted in better memory performance in the OP task.

Acute vs. chronic treatment effects

It is important to note that acute and chronic estrogen treatment may influence neurochemical levels differently. Luine et al (1998), for example, have demonstrated that a 27-day estrogen treatment to OVX rats resulted in 56-80% of monoamine activity levels in the NE and DA systems in the PFC as compared with vehicle-treated OVX rats and E2-treated rats performed better in the radial maze task, a spatial working memory task. Whereas in the current acute study, we found that OVX rats with a single post-sampling treatment had higher levels of monoamines and metabolites than the vehicle-treated control in the PFC and E2-treated rats showed enhanced memory performance in OP task, another spatial memory task. Thus, it seems that chronic and acute estrogen treatment may differentially alter monoamine and metabolite levels in the PFC (reduction for chronic treatment and elevation for acute treatment), and that the optimal neurochemical levels for memory enhancement may be different depending on the length

of estrogen treatment and/or the demands of memory tasks. Yanagihara et al (2006, 2008) have found that acute estradiol rapidly stimulates catecholamine synthesis via activation of p44/42MAPK through membrane-associated estrogen receptors (Yanagihara et al., 2006; Yanagihara et al., 2008). Activation of MARK pathways has been reported important for memory consolidation (Harburger et al., 2009; Kelly et al., 2003), as well as for synaptic plasticity (Komiyama et al., 2002). It appears that observed acute treatment effects on neurochemical levels are probably mediated, at least in part, through these pathways.

Summary and Conclusion

The current study examined effects of acute administration of estrogen and BPA, alone and in combination, on behavior (object recognition and object placement memory consolidation) and neural functions (spine density and levels of monoamines).

Our results demonstrate that BPA interacts with E2 at very low doses and rapidly alters E2 effects on performance in memory tasks, spine density, and levels of monoamines. These results cannot be explained with the classic genomic action of ER because biding affinity of BPA for nuclear ER receptors is extremely low (Kuiper et al., 1997). Rather, the pattern of the combined results suggest that rapid effects of BPA and E2 are probably mediated via membrane-initiated mechanisms, or the integrated action of genomic and nongenomic mechanisms (Vasudevan & Pfaff, 2007, 2008).

The data obtained provide some crucial information about how acute estrogen treatment alters memory consolidation. In addition the interaction between estrogen and environmental chemicals in the adult have the following effects; 1) acute estrogen treatment rapidly enhances both spatial and non-spatial memory consolidation in dose-, time-, task- and estradiol isomer-specific manner: 2) dose-response relationships between acute estrogen treatments and memory performance are inverted-U for both spatial and non-spatial memory: 3) 17α-estradiol is more potent than 17β-estradiol for memory enhancing effects: 4) Co-administration of BPA with E2 blocks E2 induced-memory enhancement of memory consolidation below the current reference safe dose of 50μg/kg. Thus, behavioral BPA responses to E2 are inhibitory: 5) acute estrogen and BPA+E2 treatments alters apical and basal dendritic spine density in the PFC and the CA1 of the hippocampus: 6) morphological responses of BPA to E2 are brain region-, dendritic

branch- and temporally dependent. During memory consolidation process, BPA+E2 treatment further increases apical and basal dendritic spine density in both brain areas, but blocks E2-induced spine density in the basal dendrites of CA1 of the hippocampus at 4hr after treatment. Thus, morphological responses of BPA to E2 are against during memory consolidation, but inhibitory in CA1 basal dendritic branches at 4hr after treatment; 7) acute estrogen and BPA treatments alters monoamine levels in some, but not all, brain areas known to be important for cognitive function

These results suggest that the dosage of acute E2 treatment is a critical factor for direction of the beneficial effects of estrogen on spatial and non-spatial memory consolidation, and both higher and lower doses may result in memory impairment depending on types of memory tasks, isomers of estradiol and timing of hormone administration. Our data are consistent with Packard's "optimal levels of receptor activation" hypothesis (1998) for acute estrogen effects on memory consolidation, which also may accounts for our experiment 2 findings that BPA may be a weak membrane-associated ER agonist and co-administration of BPA with E2 inhibits E2-induced memory enhancement when the doses of hormone+BPA combinations are exceeded the task specific optimal levels. The current neuromorphological (experiment 3) and neurochemical (experiment 4) analyses basically support the behavioral results and show that spine density and neurochemical levels in both PFC and the hippocampus are critically involved in acute estrogen and BPA+E2 treatment effects on memory.

Although it is impossible to determine precisely which receptor systems are responsible for the observed acute estrogen and BPA effects based on the current data, the rapid onset of responses, within 30 minutes, suggests that membrane-associated ERs

and non-genomic actions probably mediate the observed effects. Several studies with ER-βKO and ER-αKO mice have shown that the beneficial effects of estradiol on cognition and hippocampal synaptic plasticity may be mediated by ER-β (Liu et al., 2008), while inhibitory effects of large doses of estradiol may be mediated by ER-α (Rissman, 2008). The relationships between two types of ERs are complex, but Rissman (2008) suggests that, based on evidence from ER-βKO and ER-αKO mice studies, when low-intermediate levels of E2 or ER-β agonist are administrated, these chemicals bind to ER-β, which produces better spatial memory performance; but when large doses of E2 or ER-α agonists are administrated, excess E2 and ER-α agonist bind to ER-α, which results in poor spatial memory performance. Thus, if BPA is a membrane ER-α agonist, Rissman's model explains an inverted-U dose-response relationship between acute E2 treatment and memory performance observed in the present study.

For future research, it would be interesting to examine whether and how acute BPA interacts with fluctuations in estrogen levels across the estrus cycle in intact rats, as effects of BPA in combination with exogenous hormones and endogenous hormones might be different. Another possible future research would be to investigate how BPA interacts with locally synthesized estrogen in the brain. Recent studies have revealed that estrogens are actually produced in the hippocampus at high level, and these locally synthesized estrogens may produce acute effects on synaptic physiology (see Woolley, 2007 for review). Although functional effects of neurosteroids are poorly understood, locally synthesized high level of estrogen in the hippocampus, independent of the ovary, opens up new approach to investigate effects of estrogen and estrogenic chemicals such as BPA on behavior and neural function.

Taken together, the present experiments demonstrate that BPA interacts with estrogen at very low doses and alters behavioral and neural response to E2 rapidly, within 30min. This pattern suggests that observed acute effects of E2 and BPA may be mediated via membrane-initiated actions, and BPA may be a membrane associated ER agonist. Thus our results provide novel information as to the mechanisms of action and functional effects of environmental estrogens, and raise additional concerns about the use of BPA in manufacture of plastics and household products. Low level exposure to BPA may have a powerful, negative impact in brain and estrogen mediated cognitive function, rapidly altering behavioral and neural responses to endogenous estrogen.

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