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THE EFFECTS OF ESTROGEN ON ANXIETY-LIKE BEHAVIOR IN RODENTS: A SYSTEMATIC REVIEW AND META-ANALYSIS

by
GEORGE SALIM MERHEJ

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BY

GEORGE SALIM MERHEJ

Approved by:

Dr. Nada Lawand, Assistant Professor
Department of Neurology
Advisor

Dr. Abdo Jurjus, Professor
Department of Anatomy, Cell Biology,
and Physiological Sciences
Member of Committee

Dr. Georges Daoud, Associate Professor
Department of Anatomy, Cell Biology,
and Physiological Sciences
Member of Committee

Dr. Firas Kobeissy, Associate Professor
Department of Biochemistry and Molecular Genetics
Member of Committee

Date of thesis defense: January 15, 2021

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ABSTRACT
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The mechanisms by which estrogen hormone affects anxiety are unclear, but clinical observations related to increased anxiety related symptoms around menopausal age suggest that work in rodents may clarify important mechanistic details about this association. A key challenge in studying the effects of estrogen on defensive behavior in rodents is the plethora of inconsistent results. To address this issue, we conducted a systematic review and meta-analysis, examining the effects of estrogen injection on exploratory-defensive behavior in mice and rats using the elevated plus maze (EPM). This search yielded a total of 5 studies that satisfied our search criteria. Estrogen injection was not associated with changes in defensive behavior in rats (SE of the overall effect= 0.86, Variance V= 0.75, and effect size ES= 1.13). There was a considerable amount of publication bias between the studies. Importantly, in one study, where older rodents were used, the sensitivity of anxiety to estrogen injection was the highest. This suggested an age- dependent effect for estrogen on anxiety. Together, these findings suggest that the effects of estrogen on anxiety are more complicated that once thought. Although some studies show significant correlation between anxiety and estrogen, bias and inconsistent results suggest the conduction of more studies. Moreover, the age of the animal should be studied as an independent variable in future studies.

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CHAPTER I

INTRODUCTION

A. Estrogen Forms

Estrogens are a group of steroid hormones, largely known to be responsible for the development and regulation of the female reproductive system. Estrogens exist in both genders, in men as well as women, however, they are found in higher amounts in women, especially during the reproductive age. In females, estrogen can be synthesized in the ovaries as well as the corpus luteum and in non-gonadal sites. The granulosa cells of the ovaries produce pregnenolone, which is converted to androstenedione, which is converted to estrone by aromatase, which is then converted to estradiol by 17β-HSD. Aromatase is expressed in non-gonadal sites such as fat cells and bones and facilitates the peripheral aromatization of androgens to estrone (Cedard, 1975) (S. C. Hewitt, Hewitt, & Korach, 2016).

Three major forms of estrogen exist in females. These are estrone (E1), estradiol (E2), and estriol (E3). The most potent form of estrogen before menopause is E2. E1 is, on the contrary, potent after menopause. E3 is the least potent of the 3 forms, and is synthesized directly from E1. The same 3 forms of estrogen are detected in male bodies too (Cui, Shen, & Li, 2013). However we will focus our discussion to female estrogen, since male estrogen is beyond the scope of this thesis work.

B. Estrogen Synthesis

In women, estrogen is produced from cholesterol, mainly in the ovaries, placenta and corpus luteum. The production of estrogen is not restricted to gonadal organs. Some non- gonadal organs produce estrogen as well. These include: the heart, liver, skin, and the brain (Kotov, Falany, Wang, & Falany, 1999) (Cui et al., 2013).

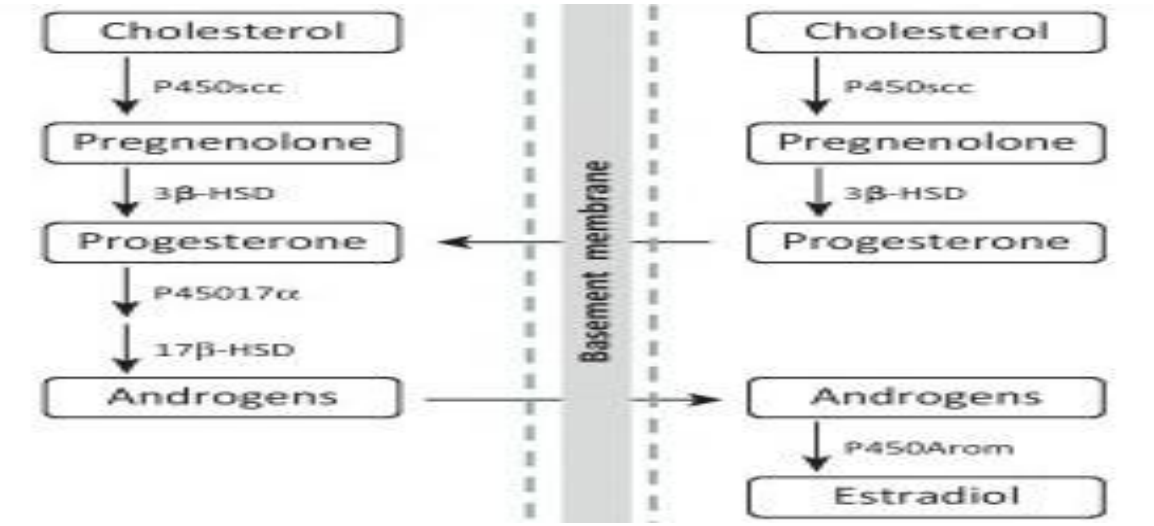

Figure 1: Synthesis of androgens and estrogens from cholesterol. Adapted from (Cui et al., 2013).

It is very important to note that the synthesis of estrogen is different between reproductive and non- reproductive females. What is meant with "non-reproductive" is pre and post-menopausal years. During these years, extra-gonadal sites are the main sites of estrogen synthesis. This extra-gonadal synthesis is usually followed by local release rather than release into the blood stream, like it is the case with gonadal synthesis/ secretion (Inoue et al., 2012).

All enzymes required for E2 synthesis are present in the brain (Naftolin, Ryan, & Petro, 1971). Moreover estrogen receptors are abundant on some brain regions including the caudate nucleus (Osterlund, 2000) (Creutz & Kritzer, 2004).

P450scc, or the enzyme required for the conversion of cholesterol to pregnenolone, which as is an intermediate in the synthesis of E2 from cholesterol, is highly abundant in the hypothalamus, hippocampus, amygdala, and caudate nucleus (Naftolin et al., 1971).

C. Estrogen Functions

Estrogen has a list of functions, which are related to many body organs and homeostatic functions. These include:

- ***Reproductive cycle***

Estrogen is known for its vital roles throughout the female's reproductive life.

Estrogen is essential for a normal uterine growth throughout the growth phase of the menstrual cycle. Moreover, it is needed for normal glandular growth in the uterus, and for maintenance of pregnancy (An et al., 2004). Estrogen also maintains normal levels of follicular stimulating hormone (FSH) and luteinizing hormone (LH), both of which are essential for a regular follicular development in the ovaries (Glidewell-Kenney et al., 2007). Mice with abnormal estrogen receptor activity αER were incapable of having since their follicles were unable to differentiate. Moreover, these mice have shown increased numbers of unruptured follicles and decreased number of oocytes (Drummond & Fuller, 2010).

- ***Functions related to the mammary gland***

In the mammary glands, estrogen has roles in the development of the mammary gland tissue, and in milk production. Mice with abnormal estrogen and/or abnormal estrogen receptor functions show insufficient maturation of the mammary gland and reduced release of prolactin (Silberstein, Van Horn, Hrabeta-Robinson, & Compton, 2006) (Hewitt, 2003).

- ***Male reproduction***

Although what is considered as "the male sex hormone" is androgen, estrogen also has basic roles related to many aspects of the **male** reproductive life. Moreover balance between androgen and estrogen is essential for normal development of male reproductive organs (McPherson, Ellem, & Risbridger, 2008). In mice, abnormalities in estrogen receptor alfa (ERα) were associated with smaller testes, lower sperm counts, and lower fertility rates. Estrogen receptor beta (ERβ), however, was not shown to be as important as its alfa counterpart in regards to the aforementioned functions (K. H. Lee, Park, Bunick, Lubahn, & Bahr, 2009). This sheds the light on the importance of different types of receptor signaling in controlling the different functions of estrogen.

- ***Bone development and maintenance***

Bone is an actively viable tissue, where different types of cells interact with one another and with some blood chemicals (Imai, Kondoh, Kouzmenko, & Kato, 2009). The high prevalence

of bone diseases following menopause has always shed the light on the possible roles of female hormones in regulating bone related functions (Cauley, 2015). Moreover estrogen was shown to have receptors in the bone tissue, through which it can stimulate the release of insulin like growth factor (IGF-1) which influences bone resorption (Vico & Vanacker, 2010). Mice lacking (ERα) had decreased bone size and length as compared to normal controls (Chilibeck & Cornish, 2008).

- ***Cardiovascular functions***

Although understanding the involvement of estrogen in cardiovascular regulation might be complicated, the fact that the rate of cardiovascular disease increases among women after menopause is significant and clear (Knowlton & Lee, 2012). The metabolic functions of estrogen that are related to cholesterol and glucose might be involved in this aspect of estrogen's function. Moreover, the hormone has receptors on smooth muscle cells and on blood vessels; a fact which can explain many of estrogen's involvement in cardiovascular control (Kim, Moriarty, & Bender, 2008)

- ***Inflammation***

The deficit in endogenous estrogen has been suggested to facilitate the onset of inflammation. Therefore, the anti-inflammatory effects of estrogen are discussed in the literature. In one study, inflammation scores related to colitis were reduced upon treatment of male rats' colitis with estrogen. The levels of some critical inflammatory molecules, such as NF-alpha, IL-6 and IL-1beta were decreased (Hajj Hussein et al., 2014). Some other studies have shown that

estrogen receptor- initiated pathways can affect innate immune cells and their signaling pathways. While estrogen can activate the release of interferon 1 by innate immune cells, it can also affect the division/ differentiation of innate immune cells through epigenetic mechanisms. Estrogen receptor alfa (ERα) was shown to affect intrinsic pathways related to the cell cycle of hematopoietic stem cells (HSCs). Treated with estrogen, HSCs differentiation to granulocytes was shown to be negatively affected (Kovats, 2015). Indeed the mechanisms through estrogen regulates different aspects of inflammation need to be further discussed.

- ***Brain and behavior***

Although we will discuss functions related to the nervous system in a separate section, it would be worth summing up here the general nervous and cognitive functions that estrogen has, without dwelling on the biological details of these functions (which we will discuss in a coming section).

These roles include:

- Roles in dendrite- axon development
- Roles in regulation of the sexual behavior
- Roles in regulation of the limbic system
- Roles in regulation of stress/anxiety levels in both humans and lab animals (H.-R. Lee, Kim, & Choi, 2012) (Witt, 2005) (M. M. et al., 2013).

D. Estrogen and the Nervous System

As mentioned before, estrogen has been shown to have roles related to the nervous system (H.-R. Lee et al., 2012) (Witt, 2005) (M. M. et al., 2013). For example, estrogen has been shown to influence verbal fluency, verbal memory skills, movement, and performance on spatial tasks in lab animals (Sherwin & Tulandi, 1996) (Hampson, 1990). Moreover, estrogen has been shown to affect Parkinson's disease and tardive dyskinesia's symptoms in human subjects (Dziedziejko, Białecka, Machoy-Mokrzyńska, Kłodowska-Duda, & Chlubek, 2009) . These facts, in addition to gender differences in many nervous functions as well as the abundance of estrogen receptors on many brain structures, have always suggested the involvement of estrogen in controlling many functions of the human brain (Cersosimo & Benarroch, 2015).

In addition to the hypothalamus, where gonadal hormones exert their reproduction-related effects, the hippocampus, amygdala, olfactory lobe, caudate, dorsal raphe and cerebellum are bran areas with abundance in estrogen receptors (McMillan & Dorsa, 1999).

The effects of estrogen on the human brain are similar to those on other tissues and cells in regards to being either genomic or non- genomic. With "genomic", we mean that it involves intracellular receptors. With non-genomic, we mean mechanisms involving neuronal physiological functions, such as excitability and action potential (McEwen, Krey, & Luine, 1978).

In the cerebellum, for example, estradiol was shown to have a non-genomic role through rapidly exciting cerebellar neurons (Sheryl S. Smith, Waterhouse, & Woodward,

1987) (S. S. Smith, 1997). The same applies to CA1 neurons of the hippocampus. However, the presence of mRNA for estrogen receptor beta in the cells of the aforementioned brain structures has suggested an estrogen-receptor mode of control governing brain functions related to these areas (McMillan & Dorsa, 1999).

Examples on genomic mechanisms of action of estrogen in the brain are numerous. These mechanisms usually take longer durations of time to happen as compared to the non-genomic mechanisms. Genomic control involves manipulation of genes and gene expression through the activation/ blockade of intracellular receptors. Examples on genes that are regulated by estradiol include: oxytocin receptor, serotonin 2A receptor, and the estrogen receptor alfa. The regulation of Serotonin and Oxytocin receptors might explain, at least in part, the roles of estrogen in mood, anxiety, and psychotic disorders (Cornil, Ball, & Balthazart, 2006) (McMillan & Dorsa, 1999).

Another interesting effect for estrogen on the nervous system is related to neuronal excitability (Kelly, Moss, & Dudley, 1977) (Dufy et al., 1979) (Woolley, 2007) (Verrotti, Sebastiani, Scaparrotta, & Verrotti, 2014). Estrogen was shown to affect neuronal excitability, by both: genomic and non-genomic mechanisms. The direct application of 17b-estradiol decreases firing of neurons of medial pre-optic area. Moreover, in the similar experiment, the application of 17b-estradiol increased the firing rate in pituitary cells. Another example of the effects of estrogen on neuronal excitability was observed in the hippocampus. The application of 17b-estradiol increased the amplitudes of excitatory post synaptic potentials. This was achieved through increasing currents coming from Kainate receptors of hippocampal neurons(Kelly et al., 1977) (Dufy et al., 1979). Indeed, such

effects on the hippocampus might explain many of the cognitive gender differences that have been already identified and discussed.

Not all effects on neuronal excitability are rapid. Some effects occur within minutes. Adrenal steroids, for example, act on CA1 pyramidal neurons in order to inhibit Serotonin inhibition on these neurons (Joëls & Karst, 2009). A similar mechanism occurs in long term potentiation (LTP) (Martin & Shapiro, 2000). This needs further investigation.

Another aspect for the effects of estrogen on the nervous system is: "neuro-protection". In vitro cultural studies have shown that estrogen can protect neurons from cell death by inhibiting pathways of programmed cell death that operate in neurons. In one study, estrogen protected culture neurons from death in the absence of serum (serum- free medium) (Faivre-Bauman, Rosenbaum, Puymirat, Grouselle, & Tixier-Vidal, 1981) (Green, Bishop, & Simpkins, 1997). Picomolar levels of 17 beta estradiol were used, and the effects of neuro-protection were reversed by Tamoxifen (Chowen, Torres-Aleman, & Garcia-Segura, 1992). Some studies have shown that estrogen can protect neurons against death from oxidative (radical) damage. This needs further investigation (Mooradian, 1993) (Roy & Liehr, 1999). Among the genes whose expression levels have been increased by estradiol are Bcl-2 family genes. It is thought that through up-regulating the expression of those genes, estrogen can inhibit programmed cell death of neurons (Garcia-Segura, Cardona-Gomez, Naftolin, & Chowen, 1998).

Whether these neuro-protective effects can explain the cognitive/ behavioral effects of estrogen still needs further investigation.

E. Estrogen and Anxiety

Among the different effects that estrogen has on the nervous system, cognitive/ mood effects have been thoroughly suggested and studied. For different reasons/ observations, estrogen has been suggested to have effects on cognition, mainly on mood and anxiety disorders (Borrow & Handa, 2017) (Schoenrock et al., 2016) (Dohanich, 2003):

First gender differences do exist in regards to mood disorders and anxiety disorders, with anxiety and depressive disorders being more common in females than in males (Usall I Rodié, 2001).

Second, changes in the mood and anxiety levels accompanying the menstrual cycle in females are evident, with days preceding the menstrual phase being associated with higher anxiety levels as compared to other days of the month (Golub & Harrington, 1981) .

Third, menopausal women were shown to suffer from higher anxiety levels as compared to premonopausal women (Maki, 2008).

Forth, the presence of estrogen receptors in brain regions known for their roles in anxiety, such as limbic structures and recently, the caudate nucleus (Borrow & Handa, 2017).

Fifth, and although this needs extensive investigation, suggesting the effectiveness of estrogen replacement therapy for treatment of anxiety disorders (Misra et al., 2013).

- ***Definition of anxiety***

According to the diagnostic and statistical manual of psychiatric disorders- 5th edition (DSM-5), anxiety is defined as an "anticipation of future threat, that is associated with muscle tension and vigilance in preparation for future danger and cautious or avoidant behaviors". DSM-5 distinguishes between anxiety and fear by considering fear as an emotional response to "real" or perceived threat, that is different that anxiety, which, as mentioned before, an "anticipation of future fear" (American Psychiatric Association, 2013).

Indeed DSM-5 differentiates between different forms of anxiety, which although can overlap with one another, can be significantly different in terms of features and even treatment options (American Psychiatric Association, 2013). The different forms of anxiety are beyond the scope of this thesis work. In fact, the form of anxiety studied in lab animals of our studies of interest do not belong to a specific anxiety type. It would, however, mostly resemble "generalized anxiety disorder".

Similar to the diagnosis of most psychiatric disorders, anxiety needs to persist for 6 months or more, for a diagnosis of "anxiety disorder" to be made, according to DSM-5(American Psychiatric Association, 2013).

- ***Estrogen and anxiety***

As mentioned earlier, the effects of estrogen on anxiety have been thoroughly suggested by neuroscientists as well as by scientists interested in the functions of hormones different than those related to their direct targets (Balthazart & Ball, 2013). Studies investigating the effects of estrogen on anxiety can be found in the literature, and the results of these studies are sometimes contradicting with one another in regards to the effects of estrogen on anxiety and anxiety- like behavior (Witt, 2005) (Yen et al., 2018).

Clinical studies haven't yielded clear results in regards to the effects of estrogen on anxiety. Some studies, however, have shown an important effect for estrogen in relieving the symptoms of anxiety in post- menopausal women. However, some other studies have shown no effect for estrogen treatment on anxiety levels (Borrow & Handa, 2017).

Although most animal studies have shown that estrogen can have anti-anxiolytic effects in the study subjects, some discrepancies in these studies have also been noticed and discussed (P. M., 2014).

Many studies have shown that the risk of developing an anxiety disorder increases around menarche, where the level of estradiol increases relative to pre-pubertal age (Patton et al., 1996). Surprisingly, an increase in anxiety severity and symptoms is also observed around menopause, when estrogen levels drop (Maki, 2008). Moreover, the period close to the end of the luteal phase (pre-menstrual), which is characterized by a dramatic decline in estrogen level is also characterized by anxiety symptoms in women with premenstrual disorders (Gingnell, Morell, Bannbers, Wikström, & Sundström Poromaa, 2012).

More confusing were the results of pre-clinical and clinical trials of estrogen prescription for the treatment of anxiety disorders. Prescribing estrogen to postmenopausal women for treatment of anxiety has either decreased their anxiety levels or kept it unaffected (Misra et al., 2013). Indeed differences in the used doses as well as the type of anxiety symptoms might have contributed to the aforementioned result discrepancies in clinical trials. As for animal studies, those have also been heterogeneous in regards to the anxiety tests that were used as well as the administered dose of estrogen (Borrow & Handa, 2017). One study published by Kastenberg et al has shown that high dose of estrogen, but not low dose could alleviate anxiety symptoms and improve the performance of animals in the Elevated Plus Maze (Kastenberger, Lutsch, & Schwarzer, 2012). Some studies have shown that the low dose isn't just ineffective but even anxiety inducing (Tomihara et al., 2009). These conflicting results can be attributed to the diversity in estrogen receptor subtypes, distribution, and function (Borrow & Handa, 2017).

In addition to the classically identified and discussed receptor subtypes: ERα and ERβ (Burris et al., 2013), the G protein coupled estrogen receptor (GPR30 or GPER1) is also discussed in the literature for its involvement in rapid estrogen induced signaling as well as in transcriptional activation (Prossnitz et al., 2008). Other receptor subtypes are also discussed in the literature. These include STX-sensitive receptor and ER-X . The data collected about these receptors is, however, scarce (Borrow & Handa, 2017). Therefore we will not discuss them any further.

Serotonin and the serotonergic system has long been known for its roles in anxiety and anxiety like symptoms in both humans and animal models (Handley, McBlane,

Critchley, & Njung'e, 1993). Estrogen can affect this system in different ways that involve ERβ and GPR30. The roles of these receptors in the release of serotonin have been confirmed by studies where agonists/ antagonists of these receptors were prescribed and release of serotonin was examined. In 1 study, treatment of male mice with WAY-200070, which is an ERβ agonist, has resulted in increase in the amount of released serotonin in the striatum (Hughes et al., 2008). Knockout of ERβ gene has resulted in decreased level of 5-hydroxytryptophan, the precursor of serotonin in the frontal cortex (Hughes et al., 2008). Moreover, activation of ERβ was shown to activate the expression of tryptophan hydroxylase, the enzyme that starts the process of serotonin synthesis from tryptophan (Donner & Handa, 2009).

Oxytocinergic system is another system that is modulated by estrogen and estrogen receptors. Oxytocin is a poly-peptide that is produced by magnocellular neurons of the supraoptic and PVN nuclei of the hypothalamus. Oxytocin is released in 3 ways: release into the circulation, where it acts as a hormone, release by dendrites into local brain regions such as the amygdala and hippocampus, and release by axons within the PVN (Murgatroyd et al., 2004).

Although it has been classically known for its roles in mating behavior and lactation, oxytocin has recently been thoroughly discussed for its roles in anxiety and anxiety disorders (Gottschalk & Domschke, 2018) (Burbach, Young, & Russell, 2006). Nasal administration of Oxytocin was shown to alleviate social anxiety symptoms in patients diagnosed with social anxiety disorder (Hoge, Pollack, Kaufman, Zak, & Simon, 2008) (Labuschagne et al., 2010). Furthermore, central administration of oxytocin or its agonist

decreased anxiety and suppressed the HPA axis's response to stress (Sabihi, Durosko, Dong, & Leuner, 2014) .

Similar to the serotonergic system, the oxytocinergic system is modulated by estrogen and estrogen receptors by more than 1 manner. These involve estrogen receptor ERβ and ERα. Interestingly, the transcription of oxytocin gene is also controlled by ERβ (Borrow & Handa, 2017).

The direct involvement of estrogen in anxiety has recently been fairly investigated (Li, Cui, & Shen, 2014) (Gasbarri & Tomaz, 2012). One of the most significant findings related to estrogen is that estrogen levels affect anxiety in both: human subjects taking estrogen replacement therapy, and in lab animals injected with estrogen (P. M., 2014) (Misra et al., 2013). Studies have shown that Estrogen can significantly decrease anxiety levels, whether these levels were assessed by elevated plus maze or other types of anxiety tests (Holm, Liang, Thorsell, & Hilke, 2014) (Walf, Koonce, & Frye, 2008).

The exact mechanisms through which estrogen affects anxiety and anxiety related behavior, however, need further investigation. These mechanisms might involve genetic as well as epigenetic factors (McEwen & Alves, 1999). Moreover gender specific factors were shown to control the effects of estrogen on the human brain (Zalta & Chambless, 2012). It is worth noting here that linking estrogen exposure to structural and/ or functional brain changes affecting anxiety pathways isn't easy to replicate in humans. Therefore animal models can provide vital details about the mechanisms through which estrogen can regulate defensive/anxiety behavior in humans, especially that defensive circuits are conserved between rodents and humans.

The link between estrogen and anxiety is suggested by previous studies that have shown that estrogen has receptors in different brain parts, and that estrogen affects anxiety in humans (Keshavan & Yeragani, 2018) (Witt, 2005) (McEwen & Alves, 1999). Translational research work, conducted on animals, does indeed help in clarifying how estrogen affects anxiety, and in suggesting/ clarifying which brain pathways are involved or might be involved in this link. Moreover, such research work will clarify whether estrogen replacement therapy is a good candidate for treatment of anxiety and anxiety-like disorders. Finally, work on rodents allows scientists to accurately control for different variables like genes and life stressors.

F. Hypothesis and Aim of the Study:

Experiments aiming at identifying the effect of estrogen on anxiety levels have used either rats or mice as animal models. In these experiments, female rodents (mice or rats) are chosen. All animals are subjected to ovariectomy, or ablation of ovaries prior to any experimental work. After ovariectomy, experimental animals are injected with Estradiol, while control animals are injected with vehicle. In most studies the injection is done only once, and the route is in most experiments the subcutaneous route. Afterwards elevated plus maze is used to assess for anxiety and anxiety-like behavior in animals injected with estradiol vs control animals (Holm et al., 2014) (Rauhut & Curran-Rauhut, 2018). These studies have yielded contradicting results in regards to the effects of estrogen on anxiety and anxiety like behavior in rodents; with some studies showing highly significant effect

for estrogen in alleviating anxiety, and other studies showing low to null effect. After identifying the aforementioned studies, we raised two main questions:

1- Can estrogen be used to treat anxiety in humans?
2- Is the relationship between estrogen and anxiety replicable among different studies conducted in different settings?

To answer these questions, we have conducted a meta-analysis that aims at examining the effects of estrogen injection on defensive-exploratory behavior in the EPM in mice and rats. Although meta-analysis is commonly used in clinical research for collection of evidence, it is rarely used in animal studies. Our meta-analysis will use animal studies in order to collect data related to a topic that is of interest to both: scientific and clinical communities.

CHAPTER II

MATERIALS AND METHODS

A. Search Strategy

Two reviewers have searched the electronic data base of Pubmed and Web of Science from February 1st 2019 till July 10 2019 for relevant studies using the following search: (estrogen) AND (mice OR mouse OR rodent OR rats OR rat) AND (elevated plus maze). Studies were limited to English language studies. References of included studies and relevant reviewers were searched for additional citations. The titles and abstracts of the different studies were isolated and examined for inclusion by the two reviewers.

B. Study Selection

For preliminary inclusion, studies were investigated in order to confirm that the inclusion criteria were met. Studies that didn't meet the inclusion criteria were excluded, and a final list of papers satisfying the inclusion criteria was obtained.

To be eligible for meta- analysis, the papers satisfied the following 5 criteria:

1- The rodents were injected with estradiol either peritoneally or subcutaneously
2- The rodents were older than 10 days
3- EPM was used to assess anxiety
4- Number of entries to the open arm of EPM was measured

5- Studies were published in English

C. Data Extraction

Data collected from each article included year of publication, authors, rodent species, sample size, types of outcomes tested (EPM, Open arm entries), estradiol form, estradiol concentration, route of injection. Test outcomes were collected as mean and variance measure (SEM or SD). When these data were available only in graphical form, the program WebPlotDigitizer (Ankit Rohatgi, 2019) was utilized to convert graphically represented data into numerical values.

D. Data Analyses

All statistical analyses were completed in Pro-meta 3- Version 3. Our outcomes of interest were anxiety indications tested by Elevated Plus Maze (EPM). These were the number of entries to the open arm of the maze. The random effects model was used as the main method of meta- analysis.

Publication bias was assessed by Egger's test, and presented by the funnel plot. Heterogeneity was measured by the I square statistics for heterogeneity.

CHAPTER III

RESULTS

A. Studies Included in the Meta-Analysis

The list of studies included in the meta-analysis and the procedures performed in the different studies are summarized in **Table 1**

Table 1: List of studies included in the meta-analysis

Reference	Species tested	Form of estradiol	Route of injection	Age of testing	Sample size
Lund et al	Spague Dawley rats	17β-estradiol	Subcutaneous	(60-90) d	9
Holm et al	Sprague Dawley rats	17β-estradiol	Subcutaneous	(60-90) d	10
Huaihai et al	C57 BL/6 Mice	17β-estradiol	Intraperitoneal	(300-390) d	8
Fedotova	Albino- Wistar rats	17β-estradiol	Subcutaneous	400 d	8
Marcondes et al.	Wistar rats	Estradiol	Intraperitoneal	90 d	20

Figure 2 shows a Prisma flow diagram that depicts the selection of studies for our meta- analysis. After 92 studies were identified, the number of records decreased to 40, after duplicates were removed. Among the 40 studies, 20 were excluded by title or abstract. Among the 20 studies that remained at the end, 15 studies were excluded due to insufficient data, young age of animals, or inconsistent EPM outcome (number of entries to the open arm vs duration of entry).

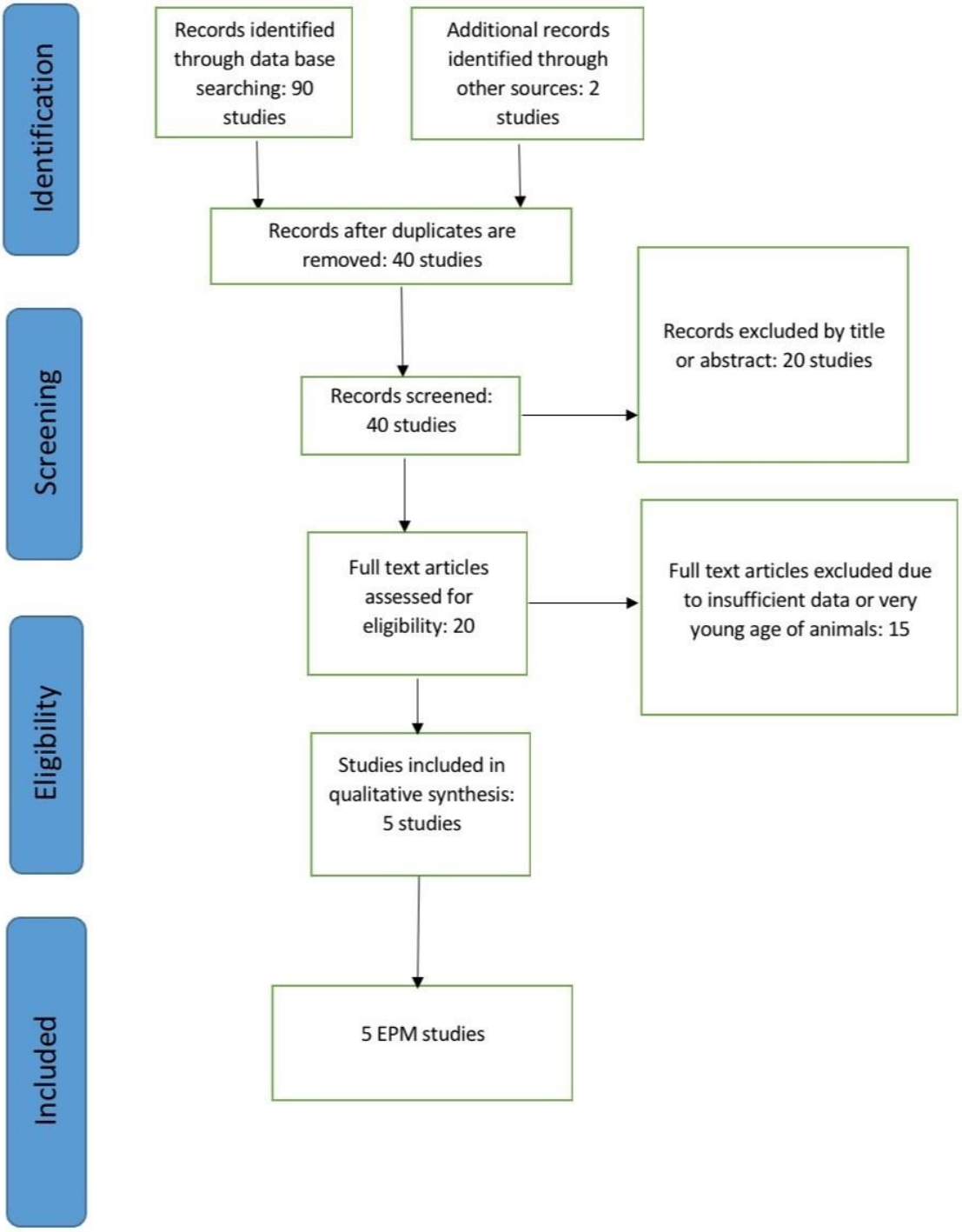

Figure 2: A Prisma flow diagram depicting selection of studies

B. Overall Effect Size

Figure 2 is the Forest Plot that shows the relationship between anxiety levels, tested via the Elevated Plus Maze, and estrogen treatment. Although some studies show significant association between estrogen and anxiety levels, The overall effect was not significant. The Standard error SE of the overall effect= 0.86, Variance V= 0.75, and effect size ES= 1.13.

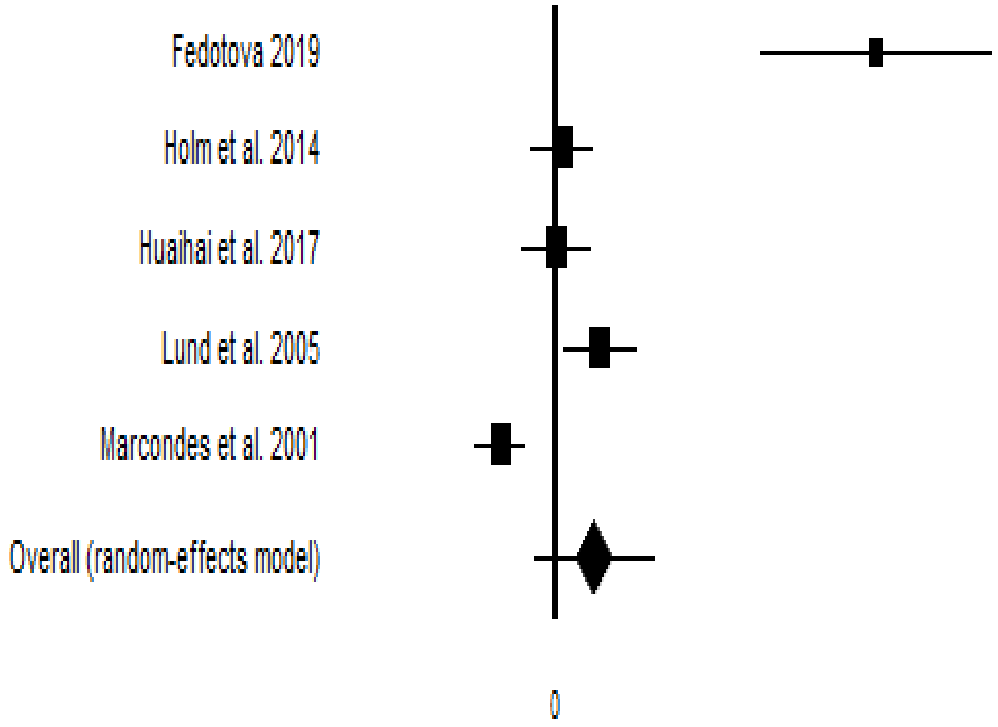

Figure 3: Forest Plot of the relationship between anxiety levels, tested with EPM and estrogen treatment

C. Publication Bias

Possible publication biases affecting the selected articles were evaluated using the funnel plot.

by definition publication bias is “an editorial predilection for publishing positive results, which leads to the failure of authors to submit negative findings for publication. It is assumed that small studies are more likely to be susceptible to publication bias than large ones, and it is this difference which is detectable.

Many factors contribute to publication bias and these include: the different aspects of the design or execution of a study, including sample size and the method of reporting the data. The investigator's own expectations also influence the outcome and lead to bias.

In the funnel plot, the weighted mean of effect sizes (pooled estimate of effects) corresponds to the funnel axis. The larger the n of a primary study, the larger its corresponding weight in a pooled estimate of effects determination.

In ideal funnel plot, where no bias is present, the included studies should scatter either side of the overall effect line in a symmetrical manner.

Asymmetry to either side, however, is an indication that publication bias may be present, which is the case in our meta-analysis study. This was confirmed by the Egger’s test which produced a P value less than 0.05.

There was significant evidence of publication bias among the studies. This was indicated by the egger’s test (rat: p=0.021) and by funnel plot asymmetry **(Figure 3).**

Z value for kendall’s tau was 1.96 with significance= 0.05

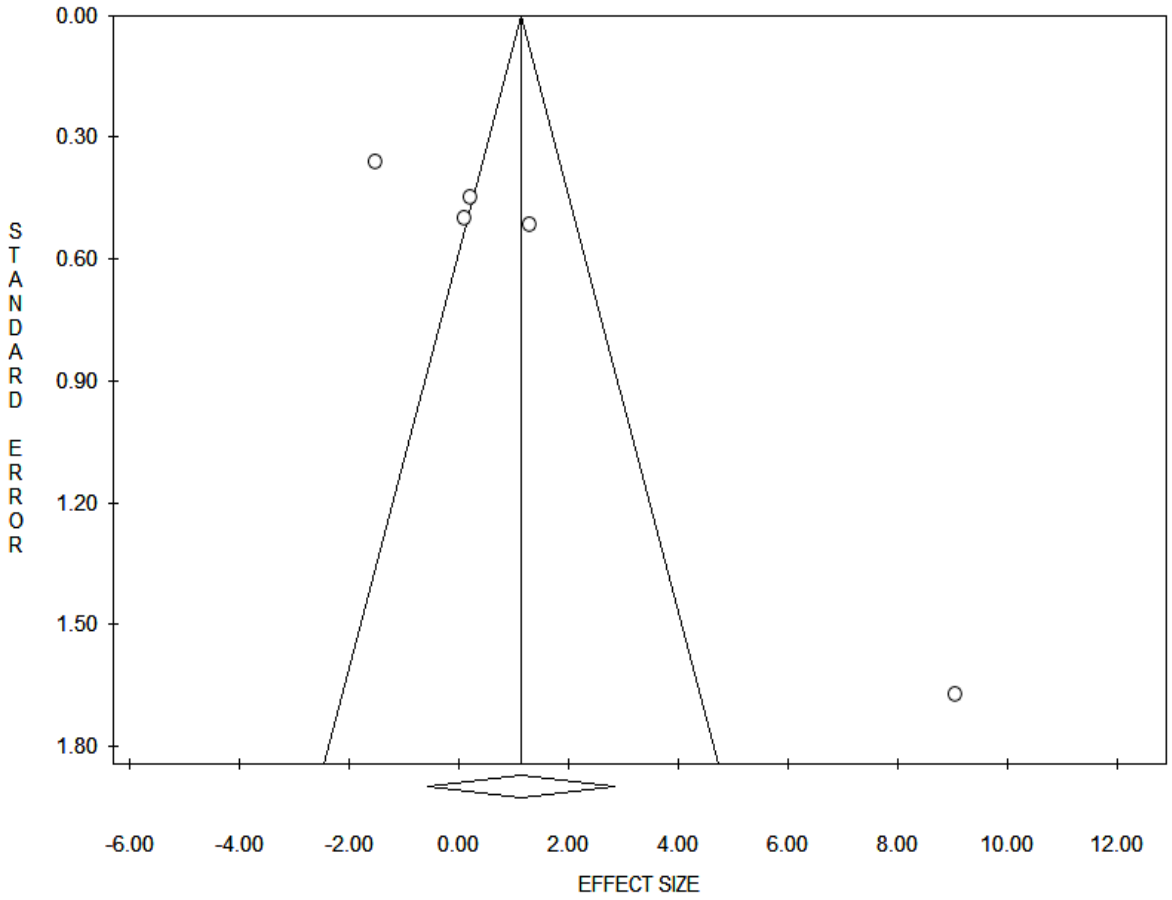

Figure 4: Funnel Plot, which depicts publication bias

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After investigating the Forest Plot again **(Figure 2)**, a question can be asked:

What is it about "Fedotova" that might be responsible for this significant effect as compared to other variables?

As we can see in the Table **(Table1)**, in “Fedotova”, older rats have been used. (400 days old). Whether the age of rats was responsible for the unique results observed in this study, is discussed further in our “discussion” section.

Table 1: List of studies included in the meta-analysis

Reference	Species tested	Form of estradiol	Route of injection	Age of testing	Sample size
Lund et al	Spague Dawley rats	17β-estradiol	Subcutaneous	(60-90) d	9
Holm et al	Sprague Dawley rats	17β-estradiol	Subcutaneous	(60-90) d	10
Huaihai et al	C57 BL/6 Mice	17β-estradiol	Intraperitoneal	(300-390) d	8
Fedotova	Albino- Wistar rats	17β-estradiol	Subcutaneous	400 d	8
Marcondes et al.	Wistar rats	Estradiol	Intraperitoneal	90 d	20

CHAPTER IV

DISCUSSION

This meta-analysis investigates the effects of estrogen on anxiety in animals. Given the contradictive effects of estrogen on anxiety in women that is discussed in the literature (Borrow & Handa, 2017), we focused on the effects of estrogen on anxiety-related behavior in rodents.

The involvement of estrogen in cognitive regulation has recently gained the huge attention of the medical as well as the scientific communities (Li et al., 2014) (Gasbarri & Tomaz, 2012). One of the most significant findings related to estrogen is that estrogen levels affect anxiety in both: human subjects taking estrogen replacement therapy, and in lab animals injected with estrogen (P. M., 2014) (Misra et al., 2013). Estrogen has been shown to significantly decrease anxiety levels, whether these levels were assessed by elevated plus maze or other types of anxiety tests (Holm et al., 2014) (Walf et al., 2008).

The exact mechanisms through which estrogen affects anxiety and anxiety related behavior need further investigation. These mechanisms might involve genetic as well as epigenetic factors (McEwen & Alves, 1999). Moreover gender specific factors were shown to control the effects of estrogen on the human brain (Zalta & Chambless, 2012). It is worth noting here that linking estrogen exposure to structural and/ or functional brain changes affecting anxiety pathways isn't easy to replicate in humans. Therefore animal models can provide vital details about the mechanisms through which estrogen can regulate

defensive/anxiety behavior in humans, especially that defensive circuits are conserved between rodents and humans.

The link between estrogen and anxiety is suggested by previous studies that have shown that estrogen has receptors in different brain parts, and that estrogen affects anxiety in humans (Keshavan & Yeragani, 2018) (Witt, 2005) (McEwen & Alves, 1999). Translational research work, conducted on animals, does indeed help in clarifying how estrogen affects anxiety, and in suggesting/ clarifying which brain pathways are involved or might be involved in this link. Moreover, such research work will clarify whether estrogen replacement therapy is a good candidate for treatment of anxiety and anxiety-like disorders. Finally, work on rodents allows scientists to accurately control for different variables like genes and life stressors.

Experiments aiming at identifying the effect of estrogen on anxiety levels have used either rats or mice as animal models. In these experiments, female rodents (mice or rats) are chosen. All animals are subjected to ovariectomy, or ablation of ovaries prior to any experimental work. After ovariectomy, experimental animals are injected with Estradiol, while control animals are injected with vehicle. In most studies the injection is done only once, and the route is in most experiments the subcutaneous route. Afterwards elevated plus maze is used to assess for anxiety and anxiety-like behavior in animals injected with estradiol vs control animals (Holm et al., 2014) (Rauhut & Curran-Rauhut, 2018).

While the studies gathered in our meta-analysis were all rodent studies, one of them was conducted in mice, 2 were conducted in Sprague- Dawley rats, 1 in Wistar rats, and another one in Albino- Wistar rats. We think that these discrepancies don't affect the

eligibility of the different studies for our meta-analysis, mainly due to previous results showing similar results among different rodent species and strains (Wada et al., 2018) (Pandaranandaka, Poonyachoti, & Kalandakanond-Thongsong, 2009).

More important are the size/weight of the animals used and its effect on the observed results regarding estrogen and anxiety. Although some animal group were smaller than other groups used in other studies, the amount of estrogen that was injected was relatively the same, when discussed in terms of the body weights of the different animal groups.

In addition to the species, strain, and weight, the route of estrogen injection can be a very important factor affecting the observed results. In the studies included in our meta-analysis, the route of injection was either the intra-peritoneal or the sub-cutaneous. Taking into account the chemical structure of estrogen (Steroid hormone) (McMillan & Dorsa, 1999), which we have elaborated on in our literature review section, we think that the route of injection doesn't affect the effects of estrogen on anxiety. Moreover, and since neither of the routes affects the transport of estrogen to the brain (Yao & Diaz, 2014), we think that estrogen's effects on the brain are the same with either of the 2 routes of injection.

The Elevated Plus Maze (EPM) is a behavioral test that has been used by many previous studies for assessment of anxiety in rodents (Carobrez, Kincheski, & Bertoglio, 2015) (Manuscript, 2013). Although other tests, including Open Field Test, have been used in some anxiety-estrogen studies (Sinha & Ghosh, 2019), EPM has been the only common test used by all of the studies we have picked for our meta-analysis. Moreover, the number of entries to the open arm has been a common parameter used by all the studies. Therefore

the number of entries to the open arm has been used as an end result for our analysis of anxiety in the studies of interest.

Our study showed no significant effect for estrogen on anxiety tested via the elevated plus maze. Although many previous studies have shown that estrogen alters anxiety in rodents, our meta-analysis showed the opposite. This sheds the light on the importance of conducting future studies using larger sample sizes and precisely identified variables. The candidacy of estrogen as an anxiety medication that could be used to treat in humans has been discussed in previous studies. We think, however, that more rodent studies are needed before any recommendation can be given in regards to the candidacy of estrogen as a medication used to treat anxiety disorders in humans or in menopausal women.

Although this was the main finding of our study, we would like to focus on another major finding of our study. This is related to one study published by "Fedotova", and which shows uniquely significant effects for estrogen administration on anxiety. Since in this study older animals were used , relative to other studies incorporated in our meta-analysis, we hypothesize that older animals might have unique anxiety pathophysiology, which might be responsible for the observed effects of estrogen in alleviating anxiety-related behavior tested by the elevated plus maze (EPM). Indeed this suggests future studies, aiming at dissecting the exact role of age in the etiology of anxiety, as well as in the roles of estrogen/ estrogen deprivation, and perhaps estrogen receptors in the pathophysiology of anxiety. Interestingly, some previous studies have shown that estrogen receptors, mainly ERα and ERβ undergo age related changes in availability and function (Maffucci & Gore, 2006). In a study published in 2014, Mott et al have suggested that the age dependent

effects of 17β-estradiol on the human brain are due to changes in estrogen receptor availability and physiology that might occur with age. E2 treatment, known for its role in controlling the association of ERβ to its target proteins, was shown to do this in an age dependent manner. The older the animal was, the less the number of proteins that precipitate with ERβ would be. The authors also showed that ERβ undergo changes in interaction with their target proteins. These effects were studied in vivo, in the ventral hippocampus (Mott et al., 2014). Although the exact molecular roles of the proteins investigated in this study requires further investigation, the findings related to changes in their interaction with ERβ might also shed the light on possible behavioral changes in estrogen response that might be age-dependent. In addition to the distribution of estrogen receptors, the observation of higher anxiety levels in menopausal women also sheds the light on possible effects for estrogen on anxiety and anxiety like behavior that might be age- dependent (Anna, Baikova, & Dashkova, 2014). In other words, the same estrogen therapy that might treat anxiety in older women, might fail to do so, or might even worsen anxiety in younger women. Indeed this requires further investigation.

Discussing anxiety changes with age, it would be very important to note that anxiety symptoms also show up around the menarche (Patton et al., 1996), or the maturation period of females. Whether this is due to the elevation in estrogen levels characteristic of the maturation of ovaries, or due to the fluctuation in estrogen levels characteristic of the menstrual cycle, also requires further investigation.

Indeed the differences in the effects of estrogen on anxiety and anxiety like behavior can also be attributed to changes that are not directly related to estrogen and its receptors.

Changes in the physiology of the serotonergic system for example can be responsible for this variation in the response of the brain to estrogen (Moses-Kolko et al., 2011). Similarly, changes in the dopaminergic system might be responsible for changes in the pathophysiology of anxiety, and the response of anxiety to hormones/ medications (Dreher, Meyer-Lindenberg, Kohn, & Berman, 2008). In this case, the differences might not be "estrogen specific". In addition, changes in cellular proliferation/ differentiation might also contribute to the estrogen-anxiety relationship. Recently, many studies have confirmed changes in neurogenesis as well as in cell death levels that are age dependent (G et al., 2005). Interestingly, neurogenesis was shown to alter the neurons of the hippocampus (Christie & Cameron, 2006), a region well known for its roles in anxiety and in psychiatric disorders in general (Cha et al., 2016) (Prior, Schwegler, Marashi, & Sachser, 2004).

Significant funnel plot asymmetry with egger's test p=0.021 characterized our investigated studies **(Figure 3)**. Although these findings raise the possibility that the significant effect of estrogen on anxiety-like behavior in the EPM might be driven by a publication bias (i.e., the tendency not to publish negative results), Funnel Plot asymmetry can occur in the absence of a publication bias. This is especially true in analysis with large variability, using under-powered samples, and relatively few studies, such as in our case. Moreover, the specificity of our research question limits the option of choosing a huge number of articles, and hence of lowering the significance of publication bias.

In conclusion, our analysis highlights two important findings. First, estrogen doesn't alter exploratory behavior in the EPM in rodents, suggesting that experiments that have shown the opposite need to be replicated with bigger sample sizes and higher level of

variable control. Second, the effects of estrogen on anxiety might be more significant in older animals. This result suggests more mechanistic studies that compare the effects of estrogen on circuits that regulate defensive- exploratory behaviors in rodents from different age groups.

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