Universidade do Minho

Escola de Medicina

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Implementation of a chronic social stress rat model and the effects of allopregnanolone to reverse depressive and anxiety-like signs in nulliparous and primiparous females

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Implementation of a chronic social stress rat model and the effects of allopregnanolone to reverse depressive and anxiety-like signs in nulliparous and primiparous females

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

A depressão é uma doença psiquiátrica multidimensional, que afeta vários domínios do comportamento, e a mais prevalente a nível mundial. Além disso, é também a principal causa de incapacidade, sendo a exposição ao stress, nomeadamente stress social, um dos principais fatores desencadeadores do seu desenvolvimento. A maioria dos estudos pré-clínicos de avaliação da génese e tratamento na depressão são realizados em modelos animais de roedores do sexo masculino; no entanto, a depressão é duas vezes mais prevalente em mulheres do que em homens. A desregulação dos neuroesteróides, incluindo a alopregnanolona, está associada à depressão, nomeadamente em mulheres. A brexanolona, uma formulação aquosa e proprietária da alopregnanolona (Sage Therapeutics, Inc) foi recentemente aprovada pela FDA (Food and Drug Administration) como primeiro tratamento específico para a depressão pós-parto, abrindo a hipótese de que esta também possa ser utilizada para o tratamento de distúrbios depressivos especificamente em mulheres.

Neste trabalho, implementou-se uma versão modificada de um protocolo de stress crónico de instabilidade social (CSIS) em ratos fêmea, com o objetivo de estudar a suscetibilidade de fêmeas nulíparas e primíparas para o desenvolvimento de sintomatologia depressiva. Implementaram-se 3 experiências distintas em fêmeas com idades e níveis de stress diferentes. Além disso, explorou-se o potencial da alopregnanolona enquanto tratamento agudo para depressão induzida pelo stress, especificamente em fêmeas.

O nosso estudo demonstrou que o protocolo de CSIS utilizado não induz comportamento do tipo depressivo ou anedonia em fêmeas em nenhuma das condições testadas, tendo, no entanto, produzido alterações fisiológicas descritas na depressão e comportamento do tipo ansioso, uma co-morbilidade importante na depressão. Verificou-se ainda que a idade, intensidade do stress e experiência reprodutiva afeta diferencialmente a resposta das fêmeas nulíparas e primíparas a este protocolo de stress.

Tendo em consideração as diferenças fenotípicas encontradas, este estudo demonstra a necessidade de aprofundar o estudo dos mecanismos fisiopatológicos específicos da depressão em fêmeas.

Palavras chave: Modelos animais; Stress social; depressão; fêmeas; alopregnanolona.

Summary

Depression is a multidimensional psychiatric disorder, affecting several behavioral domains, and the most prevalent psychiatric disorder worldwide. Moreover, it is the leading cause of disability, with exposure to stress, namely social stress, being one of the main triggers for its development. Whereas depression is reportedly twice more prevalent in women than in men, most preclinical studies are performed in male rodents. Of notice, females' physiology and reproductive experience are associated with changes to endocrine profiles, brain and behavior that may influence both stress and treatment response, supporting the need for animal models of depression considering these factors.

Dysregulation of neuroactive steroids, including allopregnanolone, is associated with depression. Interestingly, a proprietary formulation of allopregnanolone – brexanolone (Sage Therapeutics, Inc.) - has been recently approved specifically for the treatment of postpartum depression, opening the hypothesis of whether it could also be used for treating depression, specifically in females.

In this study, we have implemented a modified version of a chronic social instability stress (CSIS) protocol in female rats, to assess the susceptibility of nulliparous and primiparous female rats for the development of depressive-like signs. Moreover, we have explored the potential use of allopregnanolone for the acute treatment of stress-induced depressive-like behavior specifically in females.

The CSIS protocol used did not induce depressive-like behavior or anhedonia in females under any of the conditions tested, but produced anxiety-like behavior, an important co-morbidity in depression, and physiological changes described in depression.

Molecular and cellular parameters were also assessed revealing that exposure to social stress affects neuronal morphology, gene expression and neuronal connectivity, specifically in primiparous females. Given the phenotypic differences we found linked with age, stress intensity and reproductive experience and the little information concerning female animal models, this work emphasizes the need for further studying the specific pathophysiological mechanisms of depression in females.

Key words: Social stress; animal models; MDD; females; allopregnanolone

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Abbreviations

5-HT- serotonin

5HTR2A- Serotonin receptor 2

A

ACC- Anterior cingulated cortex

AD- Alzheimer´s disease

B

B2M- Beta-2-Microglobulin

BDNF- Brain derived neurotrophic factor

C

CSIS- Chronic social instability stress

D

DCX- Doublecortin

DG- Dentate gyrus

DGAV- Direção-Geral de Alimentação e Veterinária

DHEA- Dehydroepiandrosterone

DLPFC- Dorsolateral prefrontal cortex

DSM-5- Diagnostic and Statistical Manual of Mental Disorders

E

EPM- Elevated plus maze

F

FDA- Food and Drug administration

FST- Forced swimming test

G

GLT1- Glutamate Transporter 1

GR- Glucocorticoid receptor

H

HPA- Hypothalamic-pituitary-adrenal

L

LOPFC- Lateral orbital prefrontal cortex

M

MAOi- Monoamine oxidase inhibitor

MDD- Major depressive disorder

MRNA- Messenger RNA

N

NAS- Neuroactive Steroids

NCS- Neural Stem Cells

NOR- Novel Object Recognition

NSF- Novelty suppressed feeding

O

OF- Open Field test

P

PPD- Postpartum depression

PROG- Progesterone

PSA-NCAM- Polysialylated-Neural cell adhesion molecule

R

RNA- Ribonucleic acid

RT-PCR- Reverse transcription polymerase chain reaction

S

SCT- Sucrose Consumption test

SDT- Sweet drive test

SI- Social Isolation

SNRI- Selective serotonin-norepinephrine inhibitor

SSRI- Selective serotonin reuptake inhibitor

T

TCA- Tricyclic agent

TCT- Three Chamber test

THPROG- Tetrahydrogesterone

U

USV- Ultrasonic vocalizations

V

VMPFC- Ventromedial prefrontal cortex

1. Introduction

1.1. Major depressive disorder

Major depressive disorder (MDD), also referred to as clinical depression, is the most common psychiatric disorder affecting 1 in 5 individuals at some point in their lifetime and prevails as the leading cause of disability worldwide (DSM-5, 2013). The symptoms of depression as stated in the Diagnostic and Statistical Manual of Mental Disorders (DSM–5) include: depressed mood, markedly diminished interest or pleasure in activities (anhedonia), significant weight loss or weight gain, loss of energy, excessive guilt and diminished ability to think or concentrate when present for at least 2 weeks (DMS-5, 2013). Moreover, depression can significantly increase the risk of other causes of mortality, such as heart failure and chronic diseases (Gathright et al., 2017). First episode of depression can occur at any time. Most diagnosed depressions first appear in adolescence and early adulthood (Andrade et al., 2003), especially among those born in more recent decades (Kessler et al., 2003). Some individuals may experience a single depressive episode in response to an acute stressor and recover without further complications. However, many others will have recurrent episodes and significant residual symptoms (Kessler et al., 2003) (**Fig. 1**).

In fact, MDD is highly recurrent, with at least 50% of those who recover from a first episode of depression being prone to have one or more additional episodes in their lifetime (Burcusa and Iacono, 2007). MDD also has significant repercussions for families of affected individuals, which poses additional burden for the society in general (England MJ., 2009).

Though the etiology of depression remains elusive, the interplay between genetics and environmental factors, such as stress, drugs intake and health problems appear to underly its development (England MJ., 2009).

Figure 1. Symptomatology and incidence of major depressive disorder.

1.2 Neurobiology of depression

A complex range of biological processes has been linked to the etiology and course of MDD. These include genetic predisposition, alterations in brain structure and function, neurotransmitter and neuroendocrine processes, and immune system processes (England MJ., 2009; Sullivan, Neale, and Kendler, 2000; Pacák and Palkovits, 2001; Danese, 2008; Miller and Blackwell, 2006).

From the early understanding that MDD was triggered by 'chemical imbalance' in the brain, research has now established a more complex etiology involving neuronal networks and plasticity (Castrén et al., 2005). Though much data still needs to be achieved, imaging has begun to clarify the neurobiological anomalies associated with MDD. Several prefrontal and limbic structures have been implicated in mood regulation. These neuroanatomical areas include the lateral orbital prefrontal cortex (LOPFC), ventromedial prefrontal cortex (VMPFC), dorsolateral prefrontal cortex (DLPFC), ventral striatum, anterior cingulated cortex (ACC), hippocampus and amygdala (Drevets, 1998). The prefrontal cortex, amygdala, cingulate, and hippocampus serve not only for mood regulation, but also learning and contextual memory processes (Swanson, 1987).

In patients with MDD, studies have suggested hyperactivity in the LOPFC and VMPFC and hypoactivity in the DLFPC compared with controls. Given the functions of these regions this atypical activity pattern may be accountable for the manifestations many MDD symptoms (Drevets, 1998).

The hippocampus may be particularly susceptible in MDD. Imaging studies of hippocampal volume have been of particular interest. In a meta-analysis by Videbech and Ravnkilde (2004), hippocampal volume was found to be suggestively reduced in patients with MDD compared with controls (Videbech and Ravnkilde, 2004).

Within each adult human hippocampal dentate gyrus (DG), around 700 newborn granule cells are generated daily (Spalding et al., 2013). The neurogenic theory of depression postulates that impaired adult hippocampal neurogenesis (AHN) triggers MDD and treatment of AHN leads to recovery (Jacobs et al., 2000). This theory originally came from several correlations between MDD and AHN such as: elevation of glucocorticoids can trigger MDD and impair AHN (Cameron and Gould, 1994), depressed patients on average have smaller hippocampi (Kempton, 2011), and serotonergic agents were used to treat MDD along with the ability to boost AHN (Malberg et al., 2000).

Importantly, and considering the heterogeneity of depression, research evidence to date has failed to agree on a single answer for the biological processes responsible for the onset and course of depression. However, evidence supports the role of several mechanisms in the central nervous

system and in the periphery. Consistent throughout the various lines of research is the importance of the interactions between biology and exposure to stress which will be further described (England MJ., 2009; McLaughlin et al., 2010; Stegenga et al. 2012).

1.2.1. Genetics

It is well established that depression runs in families, a phenomenon that implicates both environmental and genetic processes. The risk of developing MDD increases about 2.5–3 times for those with a first-degree relative with depression, while having a stress incident increases the risk from 5 to 16 times in the months after the event (Sullivan, Neale, and Kendler, 2000).

Numerous genetic polymorphisms have been associated with a higher risk of developing MDD in response to stress. Primarily among these are genes of the serotoninergic system. The neurotransmitter serotonin exerts functions on a large range of physiological functions, such as sleep, emotions, thermoregulation, circadian rhythm, appetite, aggression, sexual behavior, pain sensitivity and sensorimotor reactivity (Neumeister, Young, and Strastny, 2004). Deficits in the central 5-HT system, such as reduced 5-HT concentrations, impaired uptake and altered receptor binding, have been linked to several psychiatric disorders and psychological problems, namely MDD (Neumeister, Young, and Strastny, 2004).

In addition to polymorphisms in the serotoninergic system, also genes involved in the function of the hypothalamic-pituitary-adrenal (HPA) axis (Keller et al., 2016) have been described in the context of MDD.

It is unlikely that genetic testing will be effective as an MDD biomarker but continuing analysis of the genetic links to depression will provide a better understanding of the neurobiology of MDD, and it may play a role in the conception of pharmacotherapeutic agents.

1.2.2. Neuroendocrine system

Many studies have reported the subsequent dysregulation of the body in response to stress, which involves, but it is not limited to, the neuroendocrine system and brain responses (Pacák and Palkovits, 2001). One of the key components of the stress response regulation is the HPA axis (Pacák and Palkovits, 2001). Cortisol, the primary glucocorticoid hormone in humans, is responsible for initiating a cascade of functions upon stress exposure, which are adaptive in the acute phases of the stress response, rapidly establishing homeostasis through inhibitory feedback processes within the HPA axis (Herman et al., 2016). Failure to normalize the levels of cortisol

after termination of the stress exposure, results in sustained high cortisol with negative effects (Herman et al., 2016). Numerous studies have shown that around 50% of depressed individuals present higher levels of cortisol compared with nondepressed individuals (Dienes, Hazel and Hammen, 2013). Moreover, individuals who display evidence of abnormal cortisol regulation even after antidepressant treatment are more likely to relapse and usually have a poorer clinical prognosis than patients whose cortisol levels returned to normal after treatment (Dienes, Hazel and Hammen, 2013). Sustained hypercortisolism has been shown to induce neuroplastic changes in several brain regions (Kim, Pellman and Kim, 2015) with generalized effects on the circuits underlying emotion regulation. Both genetic and environmental factors have been shown to account for individual differences on how individuals respond to HPA axis activation (Claessens et al. 2010).

1.2.3. Immune system processes

The role of the immune system, in particular proinflammatory cytokines, the signaling molecules that coordinate inflammation in response to pathogens, in the context of stress and depression has been addressed in the recent years (Danese, 2008; Miller and Blackwell, 2006). It has been proposed that chronic stress activates the immune system leading to inflammation, and that in turn chronic inflammation leads to symptoms of depression (Miller and Raison, 2015). Levels of IL-6 have been reported to be elevated in individuals exposed to chronic stress (Segerstrom and Miller, 2004), as well as in animal models of depression (Remus and Dantzer, 2016). In this context, a bidirectional correlation has been hypothesized, either chronic stressors primes the immune system thus promoting a heightened response to stress or, otherwise, chronic stress interferes with the ability of the immune system to return to baseline after stress exposure, triggering the dysregulation of the HPA axis response and the increased production of glucocorticoids (Miller and Blackwell, 2006). The inflammatory response may also exacerbate symptoms of MDD by evoking sickness-like behaviors, including disruptions in sleep, appetite and social activity (Miller and Blackwell, 2006).

Further research using potential longitudinal designs is needed to clarify the relationship between stress, depression and inflammation. Nevertheless, research on inflammatory response suggests an additional biological process explaining the link between stress and depression.

1.2.4. Stress

MDD is commonly construed as a response to negative environmental circumstances, that include early life events, traumatic experiences and stress exposure. Several studies have shown that the onset of mood disorders such as depression is impacted by stressful life events (McLaughlin et al., 2010). Factors such as relationship problems, lack of social support, lower levels of education, lifetime alcohol problems, anxiety syndrome and financial strain, are important risk factors on recurrent, as well as on first onset of MDD (Stegenga et al. 2012).

Stegenga et al (2012) observed that the impact of risk factors was generally stronger on recurrent MDD than on a first onset of MDD. This finding suggests that the impact of risk factors may be dependent on the progression of the pathology, which may be in accordance with the kindling hypothesis. This hypothesis suggests that first onset MDD may largely depend on the level of stress, whereas recurrent MDD may occur independent of stress.

Most risk factors studied also have a greater impact in women than in men on the risk of onset of MDD at 6 or 12 months of follow-up, independent of confounding factors, which will be discussed further ahead.

Stress as an etiological factor for mood disorders has stimulated the development of several animal models that have been used as surrogates of depression (Kessler, 1997). These models use very different types of stress and are based on the environmental challenges that rodents meet, such as social defeat and unpredictable stressors over a long time period (Koolhaas et al., 2017).

Social defeat is a frequently used animal model of social stress in male rodents (Miczek, 1979). This model is grounded on the resident-intruder paradigm, where animals interact aggressively to establish dominance over the territory (Miczek, 1979). Nevertheless, this paradigm is not adequate to induce social defeat in females as they do not exhibit the same territorial aggression as males do (Haller et al., 1999). Female hierarchies are less linear and despotic compared to male hierarchies, though they still establish consistent social structures (Williamson et al., 2019). There are two pre-clinical models of depression that use social stress in females, the social isolation (SI) and chronic social instability paradigms (Goñi-Balentziaga et al., 2018). Chronic social instability stress (CSIS) includes both periods of isolation and overcrowding. This chronic stress paradigm has been shown to induce HPA axis activation and anxiety-like behavior in females (Herzog et al., 2009). On the other hand, social isolation consists in the total isolation of an animal in regular home cages with access to water and food *ad libitum*. SI results in neurochemical and

neuroendocrine changes, as well as anatomical, physiological and behavioral alterations (Goñi-Balentziaga et al., 2018).

1.3. Animal models of depression

Given the limitations of studying the pathophysiology of MDD in humans, most of the constructs for therapeutic development come from animal models, namely rodent models. Considering the numerous environmental, biological and social risk factors for depression, preclinical research implies the use of multivariable models that integrate these factors. The validity of an animal model may be attained by the evaluation of four major criteria: predictive validity, face validity, construct validity and etiological validity (Belzung and Lemoine, 2011; Abelaira, Reus and Quevedo, 2013). Briefly, animal models of depression should mimic the human condition in the following aspects, including, 1) improvement or reduction of behavioral signs by clinical effective antidepressant therapies (predictive validity); 2) resemblance between the clinical-symptom profile and the behavioral phenotype (face validity), 3) similarity between neurobiological substrates (construct validity), and 4) triggering of the disease in the same manner as the human disorder (etiological validity) (Abelaira, Reus and Quevedo, 2013). The more criteria an animal model fulfills, the more precise and consistent is the data it produces (Belzung and Lemoine, 2011; Abelaira, Reus and Quevedo, 2013). Though fully recapitulating the complexity of the human disease is not possible, specific symptoms or a subset of symptoms can be successfully modeled in animals. Moreover, animal models of depression can be developed by exposure to known etiological factors of depression, such as chronic stress, selective breeding, genetic manipulations and pharmacological administration (Wang et al., 2017).

The most widely used animal models of MDD generally rely on exposure to stressful stimuli and aversive psychosocial experiences, for example neglecting, interpersonal violence, or separation, that induce behavioral or physiological changes, similar to those of the human disease (Berton et al., 2012).

Sex differences have been a commonly disregarded factor in most of these models, with most basic and preclinical studies being conducted in male animal models, putatively leading to a biased characterization of the disease and treatment response (Wang et al., 2017; Halbreich, 2000). Neurodevelopmental determinants, as well as reproductive maturation and experience, are

important mediators of changes in neural plasticity, circuitry and behavior that may influence both stress and treatment response (Halbreich, 2000; Pooley et al., 2018). For this reason, studies in female animal models of depression, should be prioritized for a better understanding of the disease pathophysiology.

Previous studies have also suggested the need to better define clinically relevant symptoms and risk factors based on sex disparities to improve the translation and design of clinical trials (Harro, 2018; Rygula et al., 2018; Wang et al., 2017; Yin et al., 2016; Planchez et al., 2019). Because animal models have, on numerous occasions, been unsuccessful in modeling depression and ineffective in predicting response to therapy, reviewing animal-based research literature may lead to a more comprehensive understanding of the field (Greek and Menache, 2013). We sought to provide an overview of female animal models of depression in a review paper to highlight the major findings in depression models but also the major gaps in the field, attending to factors such as protocol variability and sex differences. The manuscript to be submitted can be found in **Appendix III**.

1.3.1. Behavioral outcomes

Establishing an animal model of such complex disorder may be a challenging task, given that animal models may not be able to display the complex cognitive and emotional traits that characterize the human disease, even because some of these are presumably limited to humans. Notwithstanding, preclinical models can display some of the core behavioral and physiological traits of depression, referred to as endophenotypes (Gould and Gottesman, 2006), including anhedonia, appetite and sleep disturbances, behavioral despair and anxiety-like behaviors (Krishnan et al., 2011). Moreover, a large variety of behavioral tests can be used to evaluate such traits (Krishnan et al., 2011). One of the core symptoms of depression, anhedonia, or the diminished capacity to experience pleasure, is usually assessed in preclinical models using the state-of-the-art sucrose preference test (Eagle et al., 2016). This test is frequently used along with measures of depressive-like behavior. Testing depressive-like behavior in rodents can be attained through the forced swim and tail suspension tests; Though this concept has been highly disputed, some authors assume immobility in these tests as a measure of "behavioral despair" (Belovicova et al., 2017). Anxiety is highly comorbid with depression. Most behavioral tests used to evaluate anxiety-like behavior in rodents, rely on the conflict between the natural willingness to explore novel environments and the

potential threat it may pose (Belovicova et al., 2017); these include the novelty suppressed feeding test (NSF), the elevated plus maze test (EPM) or the open field test (OF).

Memory and learning deficits are also found in depressed individuals and these have been shown to play a significant role in the risk of relapse and therapeutic response, suggesting a correlation between these changes and the pathophysiology of the disease. Importantly, these deficits can also be measured in animal models of depression using cognitive tasks to assess different types of memory and behavioral flexibility such as the novel object recognition (NOR) or the Morris Water Maze (MWM) test.

MDD can significantly change the hierarchy in human social groups and this may be reflected in social behavior of animal models of depression (Ellenbroek and Youn, 2016). A simple test of sociability, the three chambers test (TCT), comprehends measuring the time a rodent, mouse or rat, spends exploring either a novel conspecific versus an empty container.

1.4. Sex differences in depression

Before adolescence, depression is rare and occurs at about the same rate in girls and boys (Albert, 2015; Breslau et al., 2017). However, with the onset of puberty, the risk of developing depression increases dramatically in women and in adulthood, with MDD being reportedly twice more prevalent in women than in men (Albert, 2015; Breslau et al., 2017). Reproductive hormones, genetics, environmental variables and social pressures that are unique to women's life experiences may be contributing factors for this disparity (Stegenga et al. 2012; Martin el al. 2013) (**Fig.2)**.

PredictD, a prospective cohort study developed to predict the risk of onset of MDD in primary care attendees in six European countries and Chile showed that risk differences for the onset of MDD after 6 or 12 months of follow-up were estimated for 35 risk factors from 7101 participants, of both sexes, without MDD at baseline. Twenty-eight risk factors (80.0%) had a greater impact in women than in men on the risk of onset of MDD at 6 or 12 months of follow-up (Stegenga et al. 2012). Lower levels of education, lifetime alcohol problem, non-European ethnicity, two or more recent life events, anxiety syndrome, financial strain, a neighborhood perceived as not being safe, and problems with someone close were the risk factors with greater impact in women. In men, living alone and a nonprofessional occupation had a significantly greater impact on the risk of onset of MDD (Stegenga et al. 2012).

Interestingly, at least one study showed distinct molecular signatures of MDD between men and women, with gene expression alterations in 52 genes occurring in opposite directions (Seney et al. 2018). Gene Ontology reveals that men with MDD present decreased synapse-related genes, whereas women with MDD exhibit transcriptional increases in this pathway. Cell type–specific analysis indicates that men with MDD exhibit increased oligodendrocyte- and microglia-related genes, while women with MDD have decreased expression of markers of these cell types (Seney et al. 2018) (**Fig.2**).

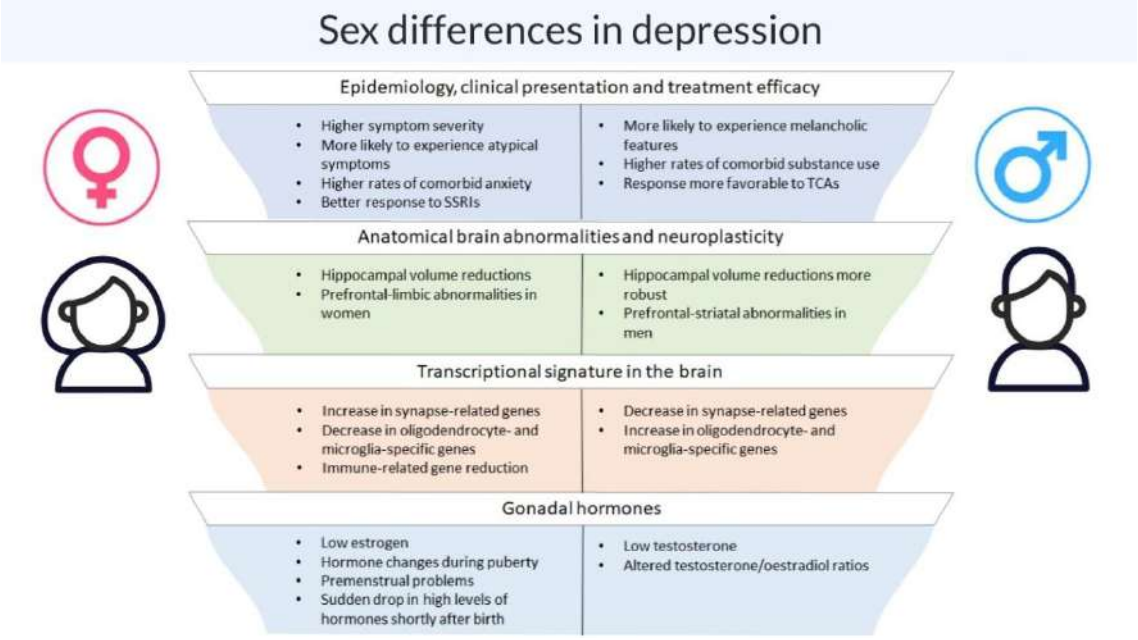


Figure 2. Summary of clinical studies providing evidence for sex differences spanning multiple aspects of depression (Adapted from Eid et al., 2019).

It has been repeatedly shown that women are at a greater risk of developing MDD compared to men, particularly during puberty, prior to menstruation, following pregnancy and at perimenopause (Halbreich U. 2000) (**Fig.2**). This disease prevalence correlation suggests that female hormonal fluctuations may be an important trigger for depression development (Halbreich U. 2000). However, most basic and preclinical studies are conducted in male animal models leading to a biased characterization of the disease and treatment response (Wang et al., 2017; Halbreich, 2000). Preclinical animal models have provided insights into the possible neuro-psychopathological causes of clinical depression over the years. Nevertheless, very few studies attempt to develop animal models specific for females, or therapies sensitive to sex differences.

1.5. Current pharmacological therapies

The use of pharmacotherapy has been the first line of treatment for MDD since the first classical antidepressants were introduced in the clinics in the 1950s (Santarsieri and Schwartz, 2015).

Antidepressants can be subdivided in several classes, including tricyclic antidepressants (TCA), monoamine oxidase inhibitors (MAOi), serotonin reuptake inhibitors (SSRI), selective serotonin-norepinephrine reuptake inhibitors (SNRI) and atypical antidepressants, such as trazodone, vilazodone, ketamine and brexanolone (Santarsieri and Schwartz, 2015; Stahl, 2013; Millan et al., 2015).

TCAs and MAOIs comprise the earliest specific antidepressants. Their clinical use in the 1950s marked the use of psychopharmacotherapy as a vital tool in the treatment of MDD (Santarsieri and Schwartz, 2015; Millan et al., 2015). TCAs are very effective antidepressants acting primarily by elevating serotonin and norepinephrine levels via reuptake inhibition (Santarsieri and Schwartz, 2015). They also function as muscarinic acetylcholine receptors antagonists and are thus prone to a large range of side effects (Santarsieri and Schwartz, 2015). MAOIs act by inhibiting the activity of the monoamine oxidase enzyme, thereby preventing the breakdown of monoamine neurotransmitters (Stahl, 2013). Second-generation antidepressants, namely SSRIs and SNRIs, are currently the most commonly prescribed treatment for MDD, with most evidence-based guidelines recommending these as a first-line therapy. These agents work through the same serotonin reuptake inhibition property found in the majority of the TCAs, but are more selective, avoiding many of the side effects of the TCAs (Stahl, 2013).

SNRIs inhibit both serotonin and norepinephrine reuptake pumps, being also very widely used in the clinical practice (Block and Nemeroff, 2014; Santarsieri and Schwartz, 2015).

Atypical antidepressants include those that do not fit either structurally or mechanistically into any of the previous classes (Santarsieri and Schwartz, 2015). These include, for instance, trazodone, an atypical antidepressant similar to SSRIs (Santarsieri and Schwartz, 2015; Thase, 2008), vilazodone and vortioxetine (Santarsieri and Schwartz, 2015; Thase, 2008).

MDD symptoms begin to resolve around 4 to 6 weeks after the beginning of the treatment with most classical antidepressants, suggesting that an acute rise in the level of neurotransmitter is necessary but insufficient to reverse the depressive symptoms (Santarsieri and Schwartz, 2015; Thase, 2008).

In the more recent years, researchers reported the use of ketamine as a fast-acting agent for the treatment of treatment-resistant depression (Hashimoto, 2019).

1.6. Allopregnanolone: therapeutics and mechanisms

Brexanolone is a proprietary aqueous formulation (Sage therapeutics, Inc.) of the human hormone allopregnanolone (Apα; 3α-hydroxy-5α-pregnan-20-one), a neurosteroid and the major metabolite of progesterone (Azhar and Din, 2019). It became the first drug to have ever been approved specifically for the treatment of postpartum depression (PPD) in adult females (U.S. Food and Drug Administration, 2020; Azhar and Din, 2019). PPD is a severe condition which affects 10 to 20% of women worldwide (Schmied et al., 2013) and one of the leading causes of maternal mortality and morbidity, which ultimately affects the cognitive, behavioral, emotional, and physical wellbeing of the mother, infant, and their siblings (Schmied et al., 2013). Brexanolone is administered as a continuous IV infusion over a period of 60 hours (Azhar and Din, 2019). In clinical studies, bexanolone administration relieved symptoms of PPD (Azhar and Din, 2019), with depression scores improved by 3.7 to 5.5 more points than those of women taking a placebo (Azhar and Din, 2019). Its mechanism of action as an antidepressant is not fully understood. Neurosteroids are primarily synthesized in astrocytes, controlled by the incorporation of cholesterol in the mitochondria (Benarroch et al., 2007). Cholesterol is then converted to pregnenolone by action of the P450 cholesterol side-chain cleavage enzyme (P450scc). In the cytosol, pregnenolone is converted to progesterone (PROG) and PROG is converted to 3,5- tetrahydroprogesterone (THPROG), or allopregnanolone. Pregnenolone is also converted to 17—hydroxypregnenolone, a precursor of dehydroepiandrosterone (DHEA). DHEA serves as a precursor of testosterone, which is converted to estradiol by aromatase (Benarroch et al., 2007) (**Fig. 3**).

In healthy women, allopregnanolone fluctuates similarly to PROG throughout the menstrual cycle with higher levels in the luteal phase than in the follicular phase. During the luteal phase, women with premenstrual dysphoric disorder have significantly increased levels of allopregnanolone, and lower levels of cortisol (Schüle, Nothdurfter and Rupprecht, 2014).

Because pregnancy is associated with a significant increase in the levels of progesterone-derived neurosteroids, which decline rapidly after delivery, it has been hypothesized that neuroactive steroids (NAS) may play a key role in the pathophysiology of depression (Schüle, Nothdurfter and Rupprecht, 2014). Moreover, neurosteroids, such as allopregnanolone, regulate both regeneration and repair systems in the brain. For instance, allopregnanolone treatment was shown to reduce amyloid-beta and microglial activation, and to increase markers of myelin and white matter generation in a mouse model of Alzheimer's disease (Schüle, Nothdurfter and Rupprecht, 2014).

Allopregnanolone also promotes proliferation of rat hippocampal neural progenitor cells and of human cerebral cortical neural stem cells (NSCs), while inhibiting the proliferation of the neural cell adhesion molecule (PSA-NCAM) (Gago et al., 2004). In mature neurons, allopregnanolone increases chloride influx via allosteric modulation of the GABA-A receptor, thereby hyperpolarizing the neuronal membrane potential and decreasing neuron excitability (Calogero et al., 1998). In contrast, in immature neurons, it leads to an efflux of chloride which causes depolarization of the membrane (Wang et al., 2005; Perrot-Sinal, Auger and McCarthy, 2003). Together, this can trigger calcium-dependent mechanisms of mitosis in early precursor cells and human NSCs to promote neurogenesis (Ashworth and Bolsover, 2002).

Allopregnanolone also presents anxiolytic and sedative-hypnotic properties with no indicated toxicological adverse events in healthy human volunteers and in children with refractory infantile spasms (Brinton, 1994; Monaghan et al., 1997; Kerrigan et al., 2000). Further investigations will be needed to identify the full neurobiological and pharmacological potential of allopregnanolone.

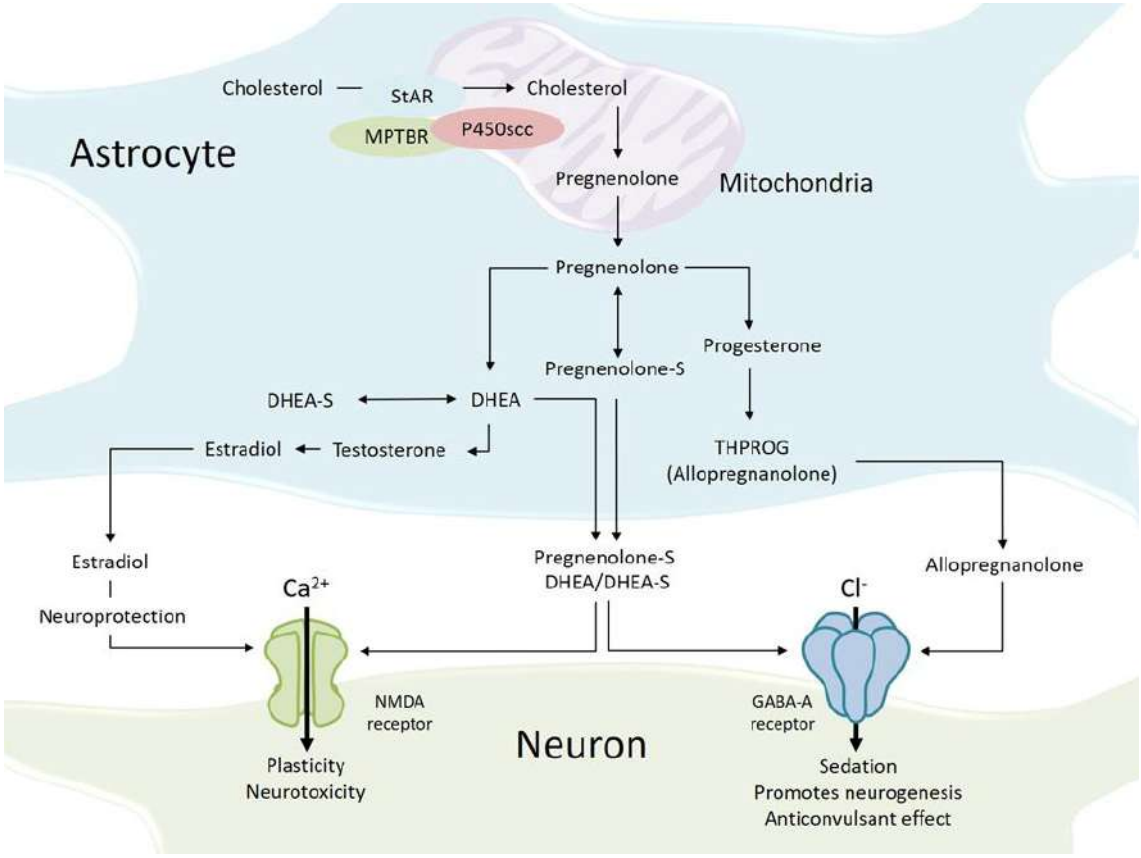


Figure 3. Synthesis of allopregnanolone (Adapted from Benarroch et al., 2007). Neurosteroids are primarily synthesized in astrocytes, controlled by the incorporation of cholesterol in the mitochondria (Benarroch et al., 2007). Cholesterol is then converted to pregnenolone by action of the P450 cholesterol side-chain cleavage enzyme (P450scc). In the cytosol, pregnenolone is converted to progesterone (PROG) and PROG is converted to 3,5-tetrahydroprogesterone (THPROG), or allopregnanolone (Benarroch et al., 2007).

2. Rationale and objectives

The assumption that animal models are predictive of human disease and treatment outcomes is the foundation for much of their use in biomedical research and the basis for the clinical development of many lead compounds for the treatment of neuropsychiatric disorders, including depression. Preclinical animal models have been invaluable in providing insights into the possible neuro-psychopathological causes of clinical depression over the years. Nevertheless, very few studies have attempted to develop animal models specific for females, or therapies sensitive to the sex differences, specifically in the context of MDD.

Chronic stress, namely social stress, is an important precipitating factor for the development of depression and to which females have been shown to be particularly sensitive. Moreover, given that females reproductive experience is associated with a unique set of changes to brain, behavior and endocrine profiles allowing for gestation, parturition and lactation, we hypothesize that it may impact on the susceptibility to develop depressive-like behavior upon exposure to chronic social stress. Additionally, we addressed whether allopregnanolone treatment could reverse the chronic social stress-induced deficits, specifically in females.

To tackle these topics and further advance the knowledge on the neurobiology of depression specifically in females, we proposed the following aims (**Fig. 4**):

1. Assess potential differences in the susceptibility of nulliparous and primiparous females for the development of depressive-like behavior and neurophysiological changes upon exposure to a chronic social instability stress (CSIS);

2. Evaluate the potential of allopregnanolone to reverse depressive-like behavior and neuroplasticity deficits in the hippocampus, in nulliparous and primiparous females, upon acute treatment with allopregnanolone.

The specific objectives were to:

1) Implement a CSIS protocol in females;
2) Evaluate the susceptibility of nulliparous and primiparous females to develop depressive- and anxiety-like phenotype upon exposure to CSIS;
3) Evaluate the impact of CSIS on neural plasticity, particularly in the hippocampus;

4) Evaluate the short-term effects of acute allopregnanolone antidepressant action after exposure to CSIS in terms of behavior and hippocampal neural plasticity.

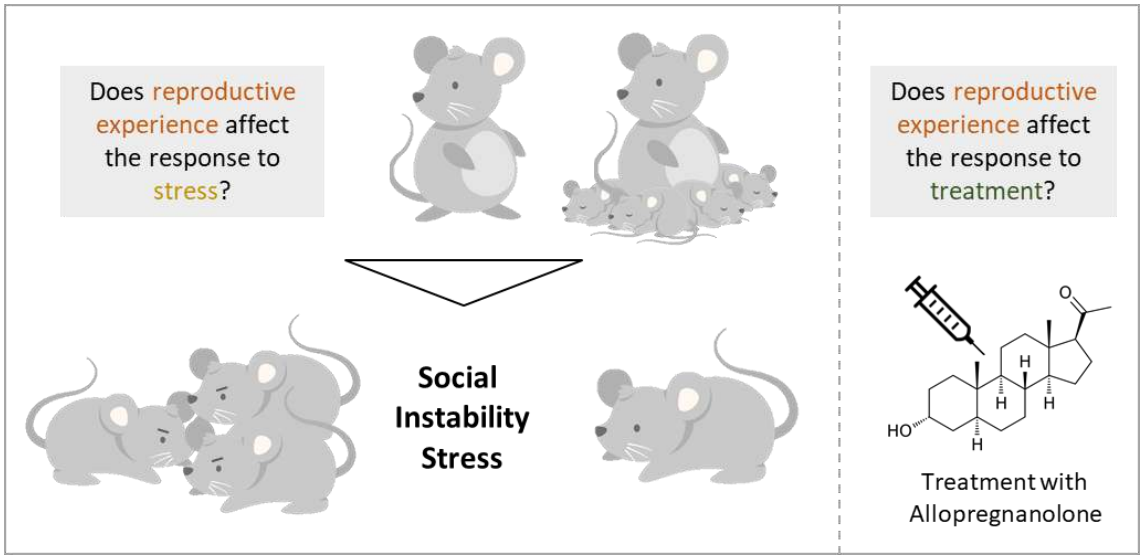


Figure 4. Graphical summary of the proposed objectives.

3. Materials and Methods

3.1 Animals

Four-month-old and nine-month old female nulliparous and primiparous Wistar Han rats (bred at the ICVS's animal house) were used in this study. Nulliparous (animal who has never carried a pregnancy) and primiparous (animal who has given birth once) female rats were bred within the ICVS's animal facility at 10 weeks old. Details on experimental groups and treatments are described below.

All procedures were carried out in accordance with the EU Directive 2010/63/EU and the Portuguese guidelines for animal experimentation. This project has been approved by the EM/ICVS and I3Bs Committee for Animal Welfare (Orgão Responsável pelo Bem-estar Animal – ORBEA; ref. ORBEA EM/ICVS-I3Bs 008/2019) and submitted to the National Competent Authority (Direção Geral de Alimentação e Veterinária, DGAV) for further ethical approval.

3.2 Chronic social instability stress protocol (CSIS)

This model has been proven useful to study depression as it induces alterations in behavior, physiology and neuronal plasticity related to the human disease, and may be also useful to evaluate therapeutic efficacy (Herzog C. et al., 2009). To evaluate the effects of social instability we performed 3 different experiments:

- CSIS experiment 1: 9-month old female rats exposed to protocol no. 1;
- CSIS experiment 2: 4-month old female rats exposed to protocol no. 1;
- CSIS experiment 3: 4-month old female rats exposed to protocol no. 2.

The protocols performed were modified from Herzog C. et al., 2009 and were used to evaluate the effects of CSIS in different age groups and different reproductive experiences. We performed two different CSIS protocols with small changes to evaluate the impact of the stress duration and unpredictability in the behavioral response.

The protocols consist in continuous exposure to social isolation or overcrowding for periods between 12 and 60 hours for 4 weeks (**Fig. 5**). Because unpredictability is a major feature of this and other chronic stress protocols, the sequence of the exposure to stressors and the cages pairings in overcrowding were changed weekly.

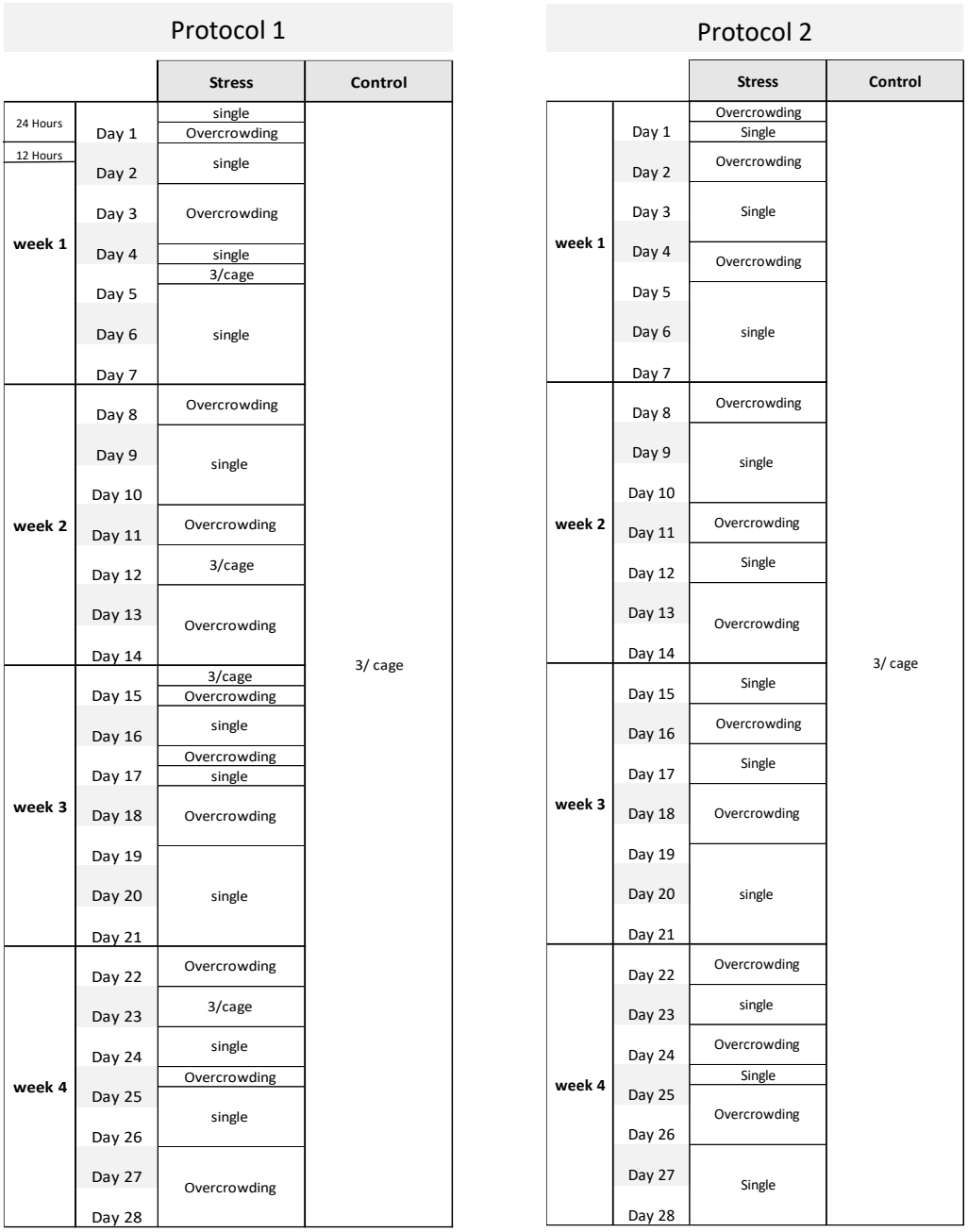


Figure 5. Chronic social instability stress: modified versions of Herzog C. et al., 2009. Animals were exposed to periods between 12 and 60 hours of social isolation or overcrowding for 4 weeks. The sequence of the exposure to stressors and the cages pairings in overcrowding were changed weekly. Animals in experiment 1 and 2 were exposed to protocol no 1. Animals in experiment 3 were exposed to protocol no 2.

3.3 Experimental groups and treatments

For the CSIS experiment 1, four experimental groups were defined to assess stress-related deficits in nulliparous and primiparous females at nine-months old. For the CSIS experiment 2 and 3, six experimental groups were defined to assess stress-related deficits and the short-term (immediately after the end of treatment) effects of allopregnanolone treatment in nulliparous and primiparous

females at four-months old (**Fig. 6**). Allopregnanolone dose and vehicle were defined based on a previous study (Ahmad I. et al., 2005).

3.3.1 CSIS experiment 1

In order to evaluate the feasibility of CSIS, we conducted a preliminary study with a small number of animals per group (**Fig. 6**). Nine months old female rats (n=17, n=2-6 per group) were exposed to protocol no 1. Animals were randomly divided into the following experimental groups:

- Nulliparous females:
    - Control (CTR) - weekly handled animals (not exposed to CSIS), treated with vehicle (sesame oil, i.p.)
    - CSIS + Vehicle (sesame Oil, i.p.)
- Primiparous females:
    - Control (CTR) - weekly handled animals (not exposed to CSIS), treated with vehicle (sesame oil, i.p.)
    - CSIS + Vehicle (sesame Oil, i.p.)

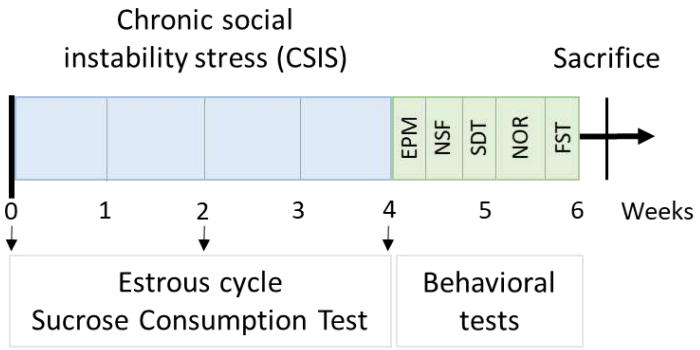


Figure 6. Timeline of *CSIS experiment 1*. Data was collected from physiological, behavioral and molecular assessments. Weight gain was assessed once a week. Anhedonia (sucrose consumption test) and estrous cycle regulation was assessed at baseline (prior to the beginning of the CSIS protocol), and at week 2 and 4 of CSIS. Food and water intake were assessed at week 4 of CSIS. Behavioral assessment was performed after exposure to CSIS by the elevated plus maze (EPM), novelty suppressed feeding test (NSF), sweet drive test (SDT), novel object recognition (NOR), and forced swimming test (FST). At sacrifice, hippocampal dorsal and ventral DG, amygdala and prefrontal cortex were collected for monoamine and mRNA expression analysis. Whole brains were also collected for Golgi-Cox staining and immunohistochemistry (immunostainings not performed in the context of this thesis' work).

3.3.2. CSIS experiments 2 and 3

Four months old female rats of experiment 2 were exposed to protocol no 1 (n=52, n=7-10 per group) and animals of experiment 3 were exposed to protocol no 2 (n=55, n=8-10 per group) (**Fig. 7**). Animals were randomly divided into the following experimental groups:

- Nulliparous females:
    - Control (CTR) - weekly handled animals (not exposed to CSIS), treated with vehicle (sesame oil, i.p.)
    - CSIS + Vehicle (sesame Oil, i.p.)
    - CSIS + Allopregnanolone - 3 days administration at the end of CSIS week 4 (dose=12.4 mg/kg, intraperitoneal, i.p.)
- Primiparous females:
    - Control (CTR) - weekly handled animals (not exposed to CSIS), treated with vehicle (sesame oil, i.p.)
    - CSIS + Vehicle (sesame Oil, i.p.)
    - CSIS + Allopregnanolone - 3 days administration at the end of CSIS week 4 (dose=12.4 mg/kg, intraperitoneal, i.p.)

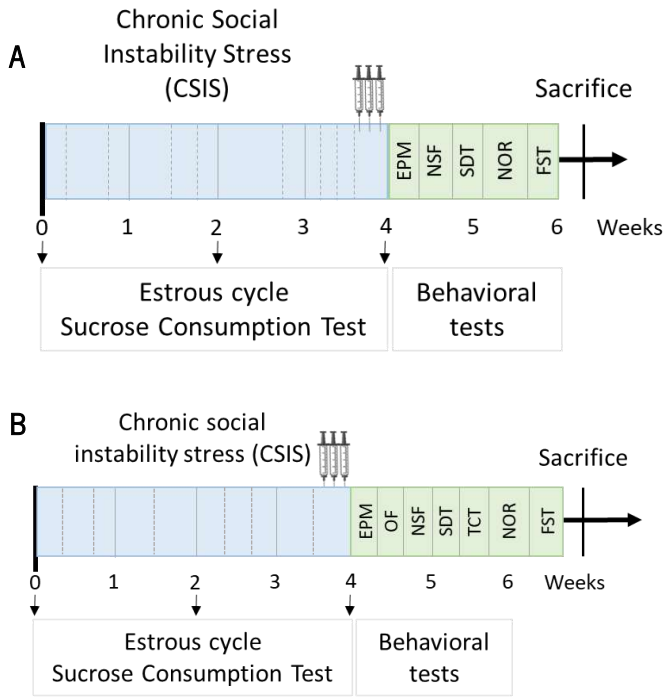


Figure 7. Timeline of CSIS experiments 2 and 3. **A and B)** Data from physiological, behavioral and molecular assessments was collected. Weight gain was assessed once a week. Anhedonia (sucrose consumption test) and estrous cycle regulation was assessed at baseline (prior to the beginning of the CSIS protocol), and at week 2 and 4 of CSIS. Food and water intake were assessed at week 4 of CSIS. Behavioral assessment was performed after exposure to CSIS by the elevated plus maze (EPM), novelty suppressed feeding test (NSF), sweet drive test (SDT), novel object recognition (NOR), and forced swimming test (FST). **B)** In experiment 3, behavioral assessment was additionally performed by the open field (OF) and three chambers test (TCT). At sacrifice, adrenal glands, hippocampal dorsal and ventral DG, amygdala and prefrontal cortex were collected for monoamine and mRNA expression analysis. Whole brains were also collected for Golgi-Cox staining and immunohistochemistry (immunostainings not performed in the context of this thesis' work).

3.4 Social isolation experiment

SI results in neurochemical and neuroendocrine changes, as well as anatomical, physiological and behavioral alterations in both animal and humans (Goñi-Balentziaga et al., 2018). To evaluate the effects of only social isolation, we housed nulliparous and primiparous female rats (4-months old) individually for 4 weeks and performed behavioral tests afterwards (**Fig. 8**).

Animals were randomly divided into the following experimental groups:

- Nulliparous females:
    - Control (CTR) - weekly handled animals (not exposed to SI)
    - Social isolation (SI)
- Nulliparous females:
    - Control (CTR) - weekly handled animals (not exposed to SI)
    - Social isolation (SI)

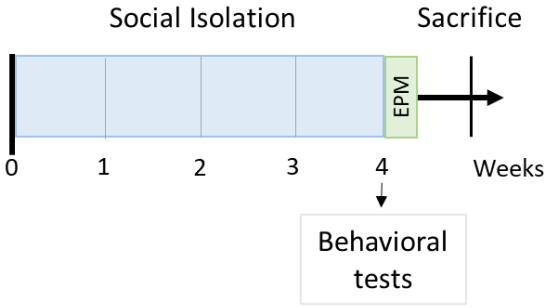


Figure 8. Social isolation experiment timeline. Data was collected from physiological and behavioral assessments. Weight gain was assessed once a week. Food and water intake were assessed at week 4 of CSIS. Behavioral assessment was performed after exposure to CSIS by the elevated plus maze (EPM).

3.5 Behavioral analyses

3.5.1. Sucrose consumption test (SCT)

Anhedonia was assessed at baseline (prior to the beginning of the CSIS protocol) to establish a baseline sucrose preference level, and at week 2 and 4 of CSIS by the SCT. Early studies have demonstrated that the gustatory value of sucrose, or glucose, is concentration dependent and food deprivation dependent. Therefore, animals were food and water deprived for 12h and then presented with two pre-weighed drinking bottles, containing water or 2% (m/v) sucrose (Mateus-Pinheiro et al., 2013). Anhedonic behavior was measured as a decrease in sucrose consumption compared to the baseline.

3.5.2. Sweet drive test (SDT)

Anhedonia was also assessed by the SDT. Ten hours food-deprived animals were allowed to choose between regular and sweet food pellets for 10 min. During the test trials, 50 kHz ultrasonic vocalizations (USVs), associated with positive experiences, were recorded as previously described (Mateus-Pinheiro A. et al 2014). Anhedonic behavior is expressed as a decreased preference for sweet food pellets and decreased number of 50 kHz USVs.

3.5.3. Elevated-plus maze (EPM)

Briefly, rodents were placed in the intersection of the four arms of the elevated plus maze, two open and two closed, and their behavior was recorded for 5 min (Mateus-Pinheiro et al., 2013). An apparatus with a floor elevation of 72.4 cm and a central square area of 100 cm^2 which connects perpendicularly two opposing open-arms (50.8 x 10.2 cm) to two opposing closed-arms (50.8 x 10.2 x 40.6 cm) was used. Anxiety-like behavior (increased closed arm time) was determined from the time spent in the closed arms using a tracking software Ethovision (Noldus L. et al. 2001).

3.5.4. Novelty suppressed feeding test (NSF)

Anxiety-like traits were assessed through the novelty suppressed feeding test (NSF) paradigm. After an 18-hour period of food-deprivation, animals are placed in an open-field arena with a single food pellet at the center of the arena. After biting the pellet animals are returned to their home cage, where they are allowed to feed for 10 min (Stedenfeld et al. 2011). The latency to feed in the open-field arena is used as an anxiety-like behavior measurement, whereas the food consumption in the home cages measures appetite drive.

3.5.5. Open Field test (OF)

Anxiety-like traits and locomotor behavior was investigated using the OF test. The animal was placed in the center of an arena (43.2 X 43.2 cm closed by a wall of 30.5 cm high) and their locomotion was monitored over a period of 5 min by a software (Activity Monitor software, Med Associates, Inc.). Diverse activity parameters were obtained from the OF test including total distance travelled, time spent and distance travelled in the center and periphery of the arena, as well the vertical counts (rearings). Additionally, it can also be determined the average velocity. Furthermore, due to the exploratory activity of rodents, the ratio between the distance travelled and

time spent in the center and in the periphery can also be used as an indication of anxiety-like behavior (Prut and Belzung, 2003).

3.5.6 Three Chamber sociability test (TCT)

The social behavior apparatus is a rectangular, three-chamber acrylic cage with dividing walls that allow free access to each chamber. Recording equipment was mounted above the cage at a distance that provides a complete coverage of the arena but does not interfere with the test environment.

The test animal was first placed in the middle chamber and allowed to explore the whole cage for five minutes. Each of the two sides contained an empty wire cage. After the habituation period, an unfamiliar female rat, with similar weight and age that had no prior contact with the tested subject, was enclosed in the wire cage and placed on the right chamber. For the sociability task, each rat was scored on measures of exploration for 10 minutes in the middle chamber, a side chamber containing an unfamiliar female animal in a wire cage, or an empty side chamber. Parameters scored included the amount of time spent in direct interaction with the stranger rats, first zone entered and the number of transitions between chambers. An entry was defined as all four paws in one chamber. Decreased interaction with the unfamiliar animal and decreased number of entries was considered as an indication of decreased sociability (Kaidanovich-Beilin et al., 2011).

3.5.7. Forced swimming test (FST)

Depressive-like behavior was assessed through the FST. Assays were conducted 24 h after a 5 min pretest session, by placing the rats in transparent cylinders with water during 5 min (Yankelevitch-Yahav et al., 2015). Glass cylinders (50 cm depth) were filled with water at 23 ± 1 °C. Trials were video-recorded, and the immobility time was analyzed using Ethovision. Depressive-like behavior is considered as an increase in the immobility time.

3.5.8. Novel object recognition (NOR)

Long-term memory and object location memory was assessed using the NOR test. On day one, in the habituation phase, each animal is allowed freely exploring the open-field arena in the absence of objects for 8 minutes. The animal is then removed from the arena and placed in its home cage.

On day 2, during the familiarization phase, a single animal is placed in the open-field arena containing two identical sample objects (A + A), for 10 minutes. After a retention interval of 1 hour the animal is returned to the open-field arena for 3 minutes with two objects (test phase), one is identical to the sample and the other is misplaced (A + A-displaced).

On day 3, and to assess long -term memory, 24 hours after the previous test, the animal is returned to the open-field arena with two objects (test phase), one is identical to the sample and the other is novel (A + B), for 3 minutes.

On day 4, to assess short-term memory, a single animal is placed in the open-field arena containing two identical sample objects (C + C), for 10 minutes (familiarization phase). After a retention interval of 1 hour the animal is returned to the open-field arena containing two objects (test phase), one identical to the sample and a novel object (C + D), for 3 minutes.

Recognition memory was expressed by the discrimination index (D), which was defined as D = (time of exploration novel object – time of exploration familiar object)/total time of exploration) (Mathiasen and DiCamillo, 2010).

3.5.9. Corticosterone levels assessment

Blood was collected for corticosterone analysis at baseline and after allopregnanolone treatment by tail venipuncture. Corticosterone levels were measured in the blood serum using a commercially available ELISA Kit (Abcam elisa kit, ab108821, lot nr GR3254889-1).

3.5.10. Estrous cycle assessment

To evaluate a possible influence of the estrous cycle on behavior or disrupted cycle after stress, vaginal smears were collected one week before CSIS, week 2 and week 4 of CSIS and after each behavioral test. To collect cells from the vaginal epithelium, animals were restrained and a 10 µl inoculation loop was used. The sample was then spread evenly on a glass slide and allowed to airdry for 30 min before being fixed with ethanol 70%. The vaginal smears were then stained using Papanicolau staining (Cora et al 2015). The stages of the estrous cycle are identified by the absence, presence, or proportion of leukocytes, nucleated epithelial cells and cornified epithelial cells as well as by the proportion of the cells. The cycle is divided into four stages: proestrus, estrus, metestrus, and diestrus. Proestrus is a short stage, lasting an average of 14 hr with predominance

of nucleated epithelial cells of relatively uniform appearance and size. Estrus duration ranges between 24 and 48 hr in rats and it is characterized by the presence of cornified epithelial cells. Metestrus is a short stage of 6–8 hr in rats presenting a combination of cornified epithelial cells and leukocytes. Diestrus is the longest stage of the estrous cycle with an average duration of 48–72 hr in rats characterized by a combination of leukocyte and nucleated epithelial cells (Cora et al 2015).

3.6 Neuronal morphology analysis

For neuronal morphology analysis, rats (n=7-10 per group) were deeply anaesthetized with pentobarbital (100 mg/kg) and transcardially perfused with saline (0.9% NaCl). Brains were immersed in Golgi-Cox solution for 15 days; transferred to a 30% sucrose solution and cut on a vibratome. Coronal sections of 200 μm were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak), fixed in Kodak Rapid Fix, dehydrated and xylene cleared before coverslipping.

Dendritic morphology of neurons from dorsal/ventral DG were analyzed using Neurolucida™ software (MBF Bioscience Inc., Williston, VA, USA). The analysis accounted for dendritic length and neuronal branching. For each brain, 10 neurons were selected according to the following criteria: (1) Total impregnation of the stain across the dendrites, (2) presence of complete dendrites without truncated ramifications and (3) neurons isolated from blood vessels or other neurons. For each selected neuron, the cell body and dendritic ramifications were drawn using the Olympus BX53 microscope (Olympus, Tokyo, Japan) with a magnification of 1000x.

Dendritic length and sholl analysis were performed using the software NeuroExplorer 3.3 (MicroBright, Williston, United States of America). Sholl-analysis calculates spatial complexity according to the number of dendritic intersections located in concentric circles 20 μm apart, starting from the cell body (Alves ND et al 2017).

3.7 Computer-generated neuron models

The NEURON simulation environment is a powerful engine for performing simulations of neurons and biophysical neural networks. It permits the construction of biologically realistic membranes with active and passive ion channels, combined with virtual connectivity and electrophysiology tools to drive and measure neuron and network behaviors. The neuron models obtained here were created in the NEURON v7.4 simulation software as a module in Python. Several Python modules

were used along with neuron, namely pyplot, numpy, pylab, neurom and eFEL. The basic computational model of neurons was used to simulate their activity.

The neuron models were created based on the morphology from 3D reconstructed granule neurons obtained from the Golgi analysis. Only granule neurons from the dorsal dentate gyrus were analyzed. Sodium and potassium ion channels were added to the soma and dendrites using Hodgkin and Huxley type models. The reversal potential leak current was set to -65 mV. The protocol consists of a set of stimuli and a set of responses. Two square current pulses with different amplitudes were applied to the soma. For every response we defined a set of features and calculated the scores from the protocol response. When combining the two, we could evaluate the cell for a set of parameters: number of spikes, spike with (ms), spike amplitude (mV), soma capacitance, sodium and potassium conductance, time to first spike, time to last spike, voltage values after hyperpolarization, membrane voltage, voltage attenuation along dendrites and synapse transmission. Optimizations were also performed for 5 generations. The module hall of fame evaluates the best individual that ever lived in the population during the evolution and sorts the individual that has the best fitness value ever seen, according to the weights provided to the fitness at creation time. The best individual was evaluated by the program and plotted. The code for this analysis can be consulted in **Appendix II**.

3.8 Gene expression analysis

Ventral DG (other brain regions were collected but, due to time constraints, not analyzed in the context of this work) was collected during sacrifice (n=52, n=7-10 per group). Animals were first anesthetized with pentobarbital and transcardially perfused with 0.9% saline. Immediately after dissection tissues were frozen and stored at -80°C until further analysis. To avoid experimenter-dependent bias, brains were macrodissected by a single investigator.

RNA purification was performed using the Direct-zol RNA purification Kits (Zymo research), and then reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences), according to the manufacturer's instructions. For real time RT-PCR, oligonucleotide primers for selected genes of interest were designed (NCBI PrimerBLAST software), namely brain-derived neurotrophic factor (*Bdnf*), glutamate transporter-1 (*Glt-1*), serotonin receptor (*5htr2*), glucocorticoid receptor (*Gr*) and doublecortin (*Dcx*) (**Table 1**).

Real-time RT-PCR was performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, LLC, CA, USA) using 5X HOT FIREPol EvaGreen qPCR Mix Plus. Target gene

expression levels were normalized against the housekeeping gene Beta- 2-Microglobulin (*B2m*) for mRNA expression analysis. The relative expression was calculated using the DDCt method. Results are presented as mRNA expression levels between the respective experimental groups after normalization to B2M levels.

3.9 Statistical analysis

Statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA). After confirmation of homogeneity, data was subjected to appropriate statistical tests. Two-way ANOVA followed by Bonferroni's post-hoc multiple comparison test was used to determine differences between groups. Two-way repeated measures ANOVA was also used to evaluate differences between the two groups where appropriate, namely for weight gain and sholl analysis. Statistical significance was accepted for $P<0.05$ (For details on statistical analysis please go to **Appendix I**)

Table 1. Oligonucleotide primers for selected genes, product size and annealing temperature of interest used in the real time polymerase chain reaction (RT-PCR).

Gene name	Gene symbol	Fw and Rw Sequence (5´-3´)	Product size (bp)	Annealing Temp. (°C)
Brain-derived neurotrophic factor	*Bdnf*	Fw: GGACCCTGAGTTCCACCA Rw: CGTGCTCAAAAGTGTCAGCC	233	60
5-hydroxytryptamine (serotonin) receptor 2A	*5htr2a*	Fw: ACCGACATGCCTCTCCATTCTTC Rw: CAAAGGCCACCGGTACCCATACA	244	60
Doublecortin	*Dcx*	Fw: CTCAGGTAACGACCAAGACGCAAAT Rw: ACTTCCAGGGCTTGTGGGTGTA	100	60
Nuclear receptor subfamily 3, group C, member 1: glucocorticoid receptor	*Gr*	FW: AGGCCGGTCAGTGTTTTCT RW: CAATCGTTTCTTCCAGCACA	234	60
Beta-2-microglobulin (Housekeeping gene)	*B2m*	FW: GTGCTTGCCATTCAGAAAACTCC RW: AGGTGGGTGGAACTGAGACA	136	60
Solute carrier family 1, member 2: glial high affinity glutamate transporter (GLT1a)	Slc1a2	FW: CCATGCTCCTCATTCTCACAG RW: CAAAAGAATCGCCCACCACA	120	60

4. Results

To evaluate the behavioral, cellular and molecular effects of exposure to CSIS or SI during adulthood, primiparous and nulliparous female rats were exposed to a modified version of these two previously described protocols of social stress (Herzog C. et al., 2009; Goñi-Balentziaga et al., 2018), for 4 weeks. Immediately after protocol exposure, animals were evaluated at the behavioral level to assess the effects of social stress. A subset of the females exposed to CSIS was also treated with allopregnanolone to assess its potential to reverse the effects of stress exposure. Three CSIS experiments in different conditions, and one SI experiment were performed (**Fig. 9**).

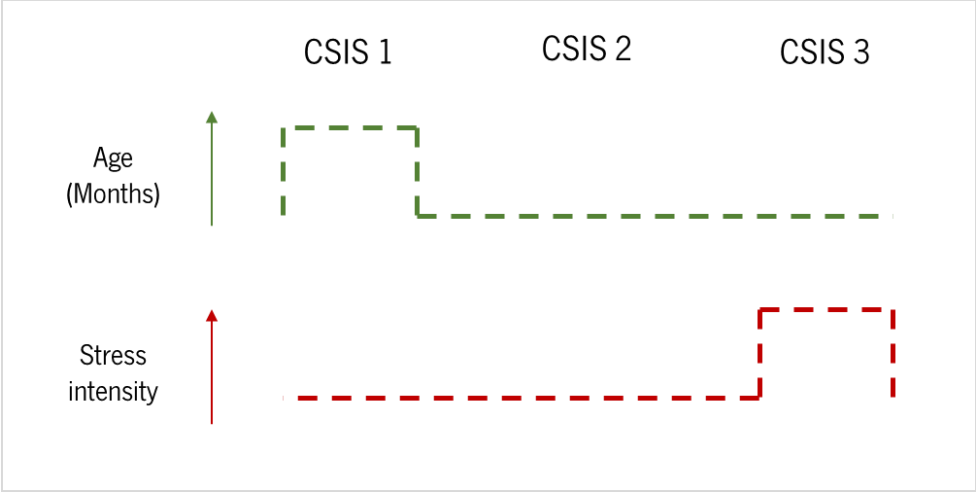


Fig. 9. Schematic summary of the different conditions of CSIS experiments 1, 2 and 3. In CSIS 1, nine months old female rats (n=17, n=2-6 per group) were exposed to CSIS protocol 1. In CSIS 2, four months old female rats (n=52, n=7-10 per group) were exposed to CSIS protocol 1 (CSIS 2). In CSIS 3, four months old female rats (n=55, n=8-10 per group) were exposed to protocol 2. Stress intensity was maintained between CSIS 1 and 2, and increased in CSIS 3.

4.1. Behavioral Effects of Chronic Social Instability stress (CSIS)

4.1.1. CSIS Experiment 1

To investigate anxiety-related behaviors caused by CSIS exposure, CSIS-exposed and control animals were tested immediately after the end of the protocol in behavioral assays designed to measure anxiety-like behavior, namely in the EPM and NSF (**Fig. 10A, B,** and **C**).

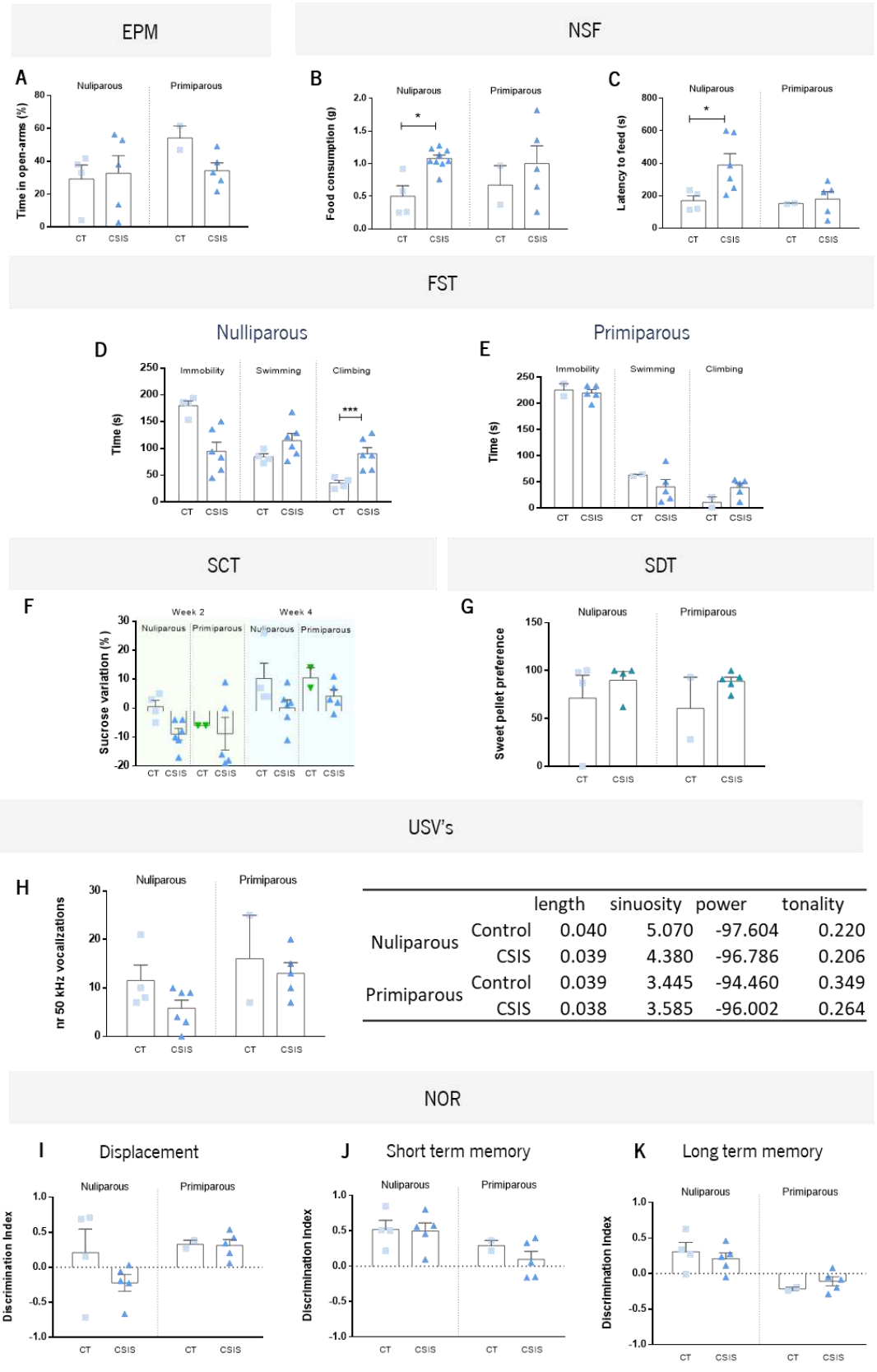
In the EPM, CSIS-exposed female rats, both nulliparous and primiparous, showed no significant differences compared to controls ($F_{(1, 12)} = 1.546$; $p = 0.2374$) (**Fig. 10A**). In contrast, in the NSF, nulliparous females, but not primiparous, showed a higher latency to grab the food pellet in the

center of the apparatus, suggesting a higher anxiety-like behavior in comparison with control ($F_{(1, 13)}$ = 2.169; p = 0.0319) (**Fig. 10C**). Though the food consumption of nulliparous CSIS-exposed females was also higher compared to CT females, this does not invalidate the test, as it indicates an increased appetite in females that, despite this, took longer to reach the pellet in the center of the arena (**Fig. 10B** and **C**).

To study depression-related behaviors caused by CSIS exposure, animals were tested in the FST. Typically, the FST is evaluated for mobility and immobility, under the assumption that an increase in immobility times is correlated with depressive-like behavior (**Fig. 10D** and **E**). Here, we also evaluated other parameters, namely climbing. CSIS-exposed nulliparous females did not show increased immobility, but instead revealed an increase in climbing activity ($F_{(2, 24)}$ = 16.20; p < 0.0001). This behavior can be read as an anxiety-like behavior but not as a depressive-like behavior. CSIS-exposed primiparous females showed no significant differences compared to controls ($F_{(2, 15)}$ = 2.177; p = 0.1479).

To explore anhedonic behavior, the SCT and SDT were performed (**Fig. 10F** and **G**). In the SCT, females showed a tendency for decreased sucrose consumption two weeks after the beginning of the protocol (**Fig. 10F**). However, at the end of the protocol, this difference was not observed (**Fig. 10F**, $F_{(3, 26)}$ = 0.3049; p = 0.8216). In the SDT, no significant differences were found in either nulliparous or primiparous CSIS-exposed females compared with control female rats (**Fig. 10G**, $F_{(1, 11)}$ = 0.08476; p = 0.7764). During the SDT, ultrasonic vocalizations (USVs) were also recorded and analyzed in the 50 kHz; however, no differences were found among experimental groups (**Fig. 10H**, $F_{(1, 13)}$ = 0.1770; p = 0.6809).

Lastly, to evaluate cognitive deficits, animals were tested in the NOR (**Fig. 10I, J** and **K**). CSIS-exposed nulliparous females showed a tendency for decreased spatial memory during the displacement task; however, no differences were found among experimental groups (Displacement: $F_{(1, 12)}$ = 1.033; p = 0.3294; Short-term: $F_{(1, 12)}$ = 0.4141; p = 0.5320; Long-term: $F_{(1, 12)}$ = 1.027; p = 0.3309.



		length	sinuosity	power	tonality
Nuliparous	Control	0.040	5.070	-97.604	0.220
	CSIS	0.039	4.380	-96.786	0.206
Primiparous	Control	0.039	3.445	-94.460	0.349
	CSIS	0.038	3.585	-96.002	0.264

Figure 10. Behavioral results of CSIS experiment 1. **A)** Elevated plus maze (EPM): Time spent in open arms in CSIS-exposed females. Novelty suppressed feeding (NSF): Food consumption **(B)** and latency to feed **(C)** in CSIS-exposed females. Forced swim test (FST): Time spent immobile, swimming or climbing in nulliparous **(D)** and primiparous **(E)** females. **F)** Sucrose consumption test (SCT): sucrose consumption of females in week 2 and week 4 of the CSIS

protocol. **G)** Sweet drive test (SDT): Sweet pellet preference of nulliparous and primiparous females. **H)** Ultrasonic vocalizations (USVs): number, length, sinuosity, power and tonality of 50 kHz vocalizations during the SDT. Novel object recognition (NOR): Discrimination index of novel object in the displacement **(I)**, short-term **(J)** and long-term **(K)** memory tasks. Abbreviations: CT – control; Veh – vehicle; CSIS – Chronic social instability stress. Results are presented as mean±SEM. *p<0.05; ***p<0.001. n=2-6 animals per group.

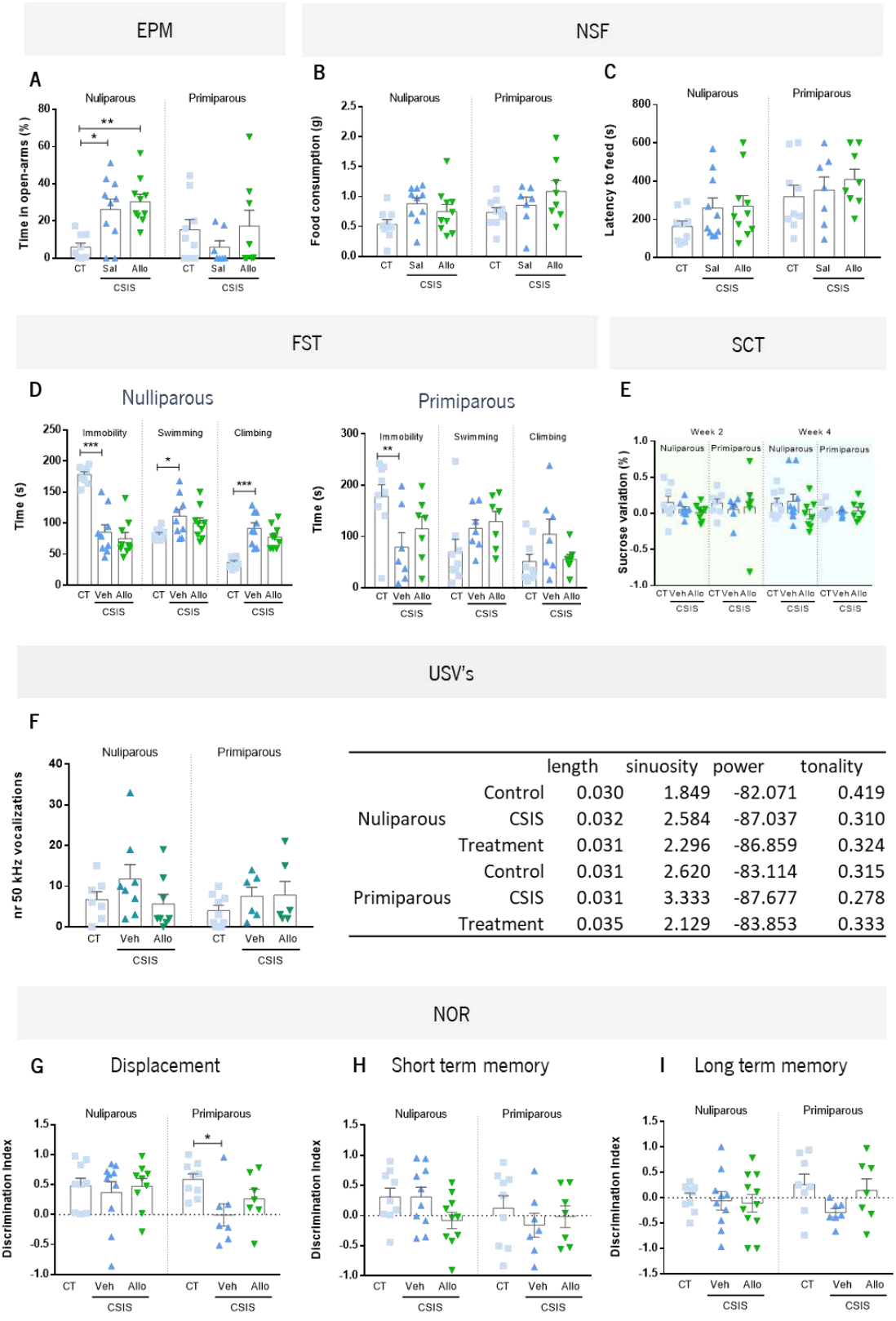
4.1.2 CSIS Experiment 2

To investigate stress-related behaviors caused by CSIS exposure in younger females, four-month-old rodents were exposed to the same protocol as in CSIS experiment 1. Additionally, the effects of acute allopregnanolone treatment were evaluated in terms of behavior and neural plasticity.

In the EPM, CSIS-exposed primiparous females showed no statistically significant differences compared with controls ($F_{(2,47)}$=4.371; p=0.4665); CSIS-exposed nulliparous females spent more time in the open arms than the controls ($F_{(2,47)}$=4.371; p=0.0167) (**Fig. 11A**). Allopregnanolone treatment did not produce any effect on CSIS exposed females ($F_{(2,47)}$=4.371; Nulliparous: p=0.8235; Primiparous: p=0.3442). In the NSF test, CSIS-exposed females showed no significant differences compared with controls when evaluated for food consumption ($F_{(2, 47)}$= 1.205 p= 0.3087) and latency to feed ($F_{(2,47)}$=0.1761; p=0.3985) (**Fig. 11B** and **C**) ; Allopregnanolone treated females were also not different from CSIS-exposed female rats ($F_{(2,47)}$=0.1761; p=0.8391) (**Fig. 11C**).

In the FST, nulliparous and primiparous CSIS-exposed females presented decreased immobility (Nulliparous: $F_{(4,72)}$=30.71; p<0.0001; Primiparous: $F_{(4,60)}$=4.736; p=0.0057), and increased climbing activity, which was also observed in CSIS Experiment 1. Statistical results were only observed in nulliparous females (Nulliparous: $F_{(4,72)}$=30.71; p<0.0001; Primiparous: $F_{(4,60)}$=4.736; p=0.0022) (**Fig. 11D**). Treatment with allopregnanolone slightly reduced climbing but did not reach statistical significance (Nulliparous: $F_{(4,72)}$=30.71; p=0.5362; Primiparous: $F_{(4,60)}$=4.736; p=0.3398) (**Fig. 11D)**.

In the SCT, CSIS exposure produced no significant changes in sucrose variation, related to the baseline, when compared with CT ($F_{(5,87)}$=0.7194; p=0.6106) (**Fig. 11E**). In this experiment, we could not evaluate the SDT sweet pellet preference results, due to a problem with the habituation of the animals to the pellets. No differences were found in the 50 kHz USVs recorded during the SDT ($F_{(2,38)}$=0.8421; p=0.4387) (**Fig. 11F** and **G**).



		length	sinuosity	power	tonality
	Control	0.030	1.849	-82.071	0.419
Nuliparous	CSIS	0.032	2.584	-87.037	0.310
	Treatment	0.031	2.296	-86.859	0.324
	Control	0.031	2.620	-83.114	0.315
Primiparous	CSIS	0.031	3.333	-87.677	0.278
	Treatment	0.035	2.129	-83.853	0.333

Figure 11. Behavioral results of CSIS experiment 2. **A)** Elevated plus maze (EPM): Time spent in open arms in CSIS-exposed females. Novelty suppressed feeding (NSF): Food consumption **(B)** and latency to feed **(C)** in CSIS-exposed females. Forced swim test (FST) **(D)**: Time spent immobile, swimming or climbing in nulliparous and primiparous females. **E)** Sucrose consumption test (SCT): sucrose consumption of females in week 2 and week 4 of the CSIS protocol. **F)** Ultrasonic vocalizations (USVs) in the sweet drive test (SDT): number, length, sinuosity, power and tonality of 50 kHz vocalizations during the SDT. Novel object recognition (NOR): Discrimination index of novel object in the

displacement **(G)**, short-term **(H)** and long-term **(I)** memory tasks. Abbreviations: CT – control; Veh – vehicle; CSIS – Chronic social instability stress. Results are presented as mean±SEM. *p<0.05, **p<0.01; ***p<0.001. n=7-10 animals per group.

Lastly, in the NOR test, we observed that primiparous females, specifically, presented impaired spatial memory during the displacement task and a tendency for impaired long-term memory task (Displacement task: $F_{(2,45)}$ =1.383; p=0.025; Long-term task: $F_{(2,46)}$ =1.324; p=0.0944) (**Fig. 11G, H** and **I**). Memory impairments were slightly recovered by allopregnanolone treatment, but results were not statistically significant (Displacement task: $F_{(2,45)}$ =1.383; p=0.4887; Long-term task: $F_{(2,46)}$ =1.324; p=0.2450).

4.1.3 CSIS Experiment 3

To evaluate the efficacy of a slightly more intense CSIS protocol, with longer sustained periods of isolation and overcrowding compared to protocol nr 1, animals with the same age as in CSIS experiment 2 (4-month-old) were exposed to protocol nr 2 (**Fig. 5**).

CSIS-exposed females showed no significant differences compared with CT in the EPM or NSF (**Fig. 12A**, **B** and **C**, respectively; NSF: $F_{(2,42)}$ =2.254; p=0.1175; EPM: $F_{(2,49)}$ =4.247; p=0.0199).

In the OF, nulliparous and primiparous CSIS-exposed females showed no significant differences compared with control (**Fig.12. D - H**) ($F_{(4, 81)}$ = 2.041; (D) p = 0.0964; (E) p= 0.6064; (F) p=0.3089; (G) p=0.8690; (H) p=0.7141)

In the FST, nulliparous and primiparous CSIS-exposed did not show increased immobility when compared to controls (Nulliparous: $F_{(4, 78)}$ = 83.99; p< 0.0001; Primiparous: $F_{(4, 69)}$ = 36.04; p< 0.0001), but instead revealed an increase in climbing activity which was also seen in CSIS experiments 1 and 2 (Nulliparous: $F_{(4, 78)}$ = 83.99; p< 0.0001; Primiparous: $F_{(4, 69)}$ = 36.04; p< 0.0001) (**Fig. 12I** and **J**).

As in the two previous experiments, neither CSIS-exposed or treated females revealed anhedonic behavior both in the SCT or in the SDT when compared to control females (SDT: $F_{(2,49)}$ =0.4136; p=0.6636; SCT: $F_{(5,90)}$ = 0.03720; p=0.9992) (**Fig. 13A** and **B**). Regarding the 50 kHz USVs recorded during the SDT, we could observe an increase in the number of 50kHz vocalizations in both nulliparous and primiparous CSIS females compared to CT, which was reversed by allopregnanolone treatment (Nulliparous: $F_{(2,49)}$ =0.6560; CT vs CSIS: p=0.0040; CSIS vs Allo: p=0.0106; Primiparous: $F_{(2,49)}$ =0.6560; CTvsCSIS: p=0.1221; CSIS vs Allo: 0.0350). Subtle

differences in vocalizations length, power and tonality were observed though not reaching statistical significance (**Fig. 13C**).

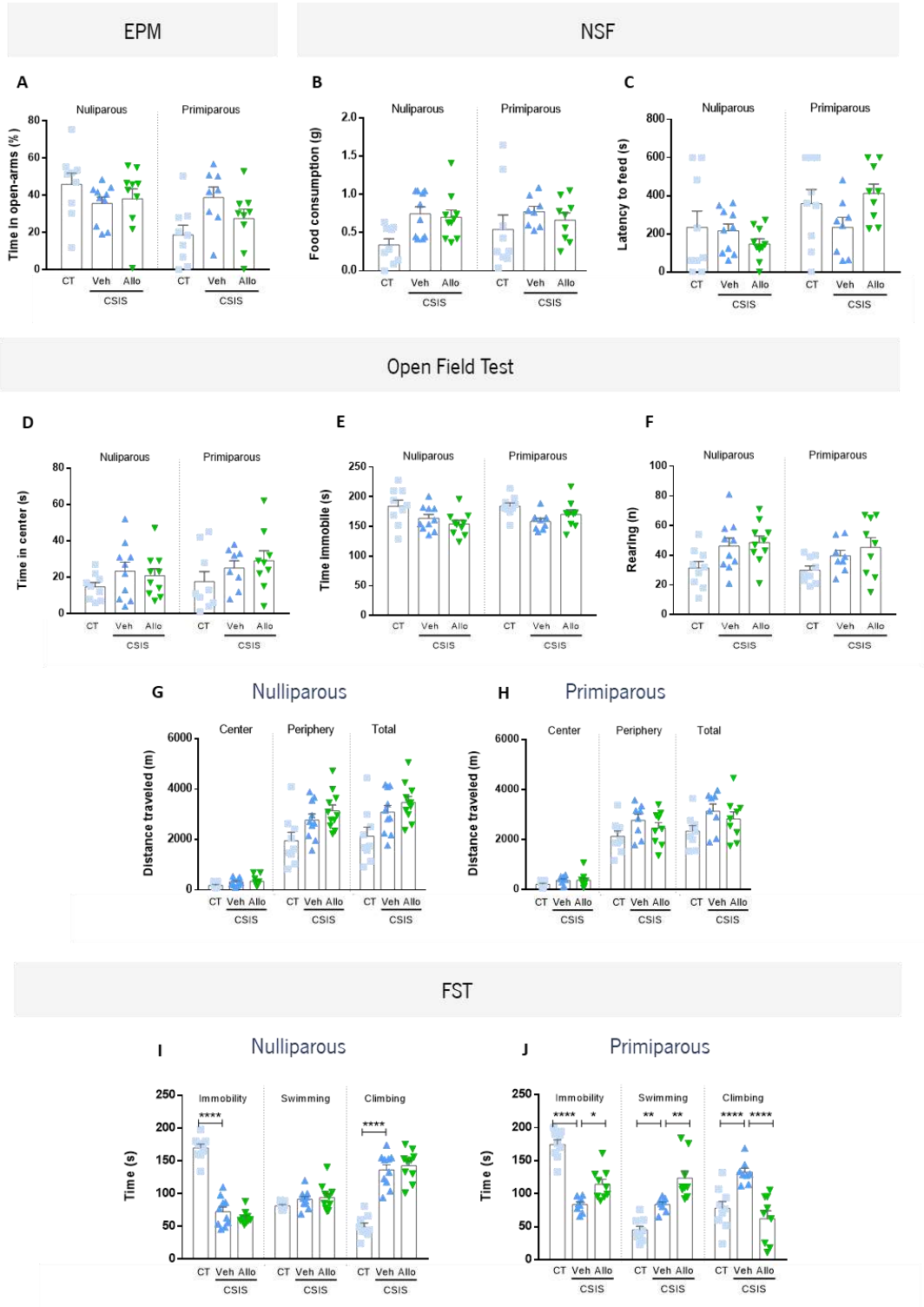
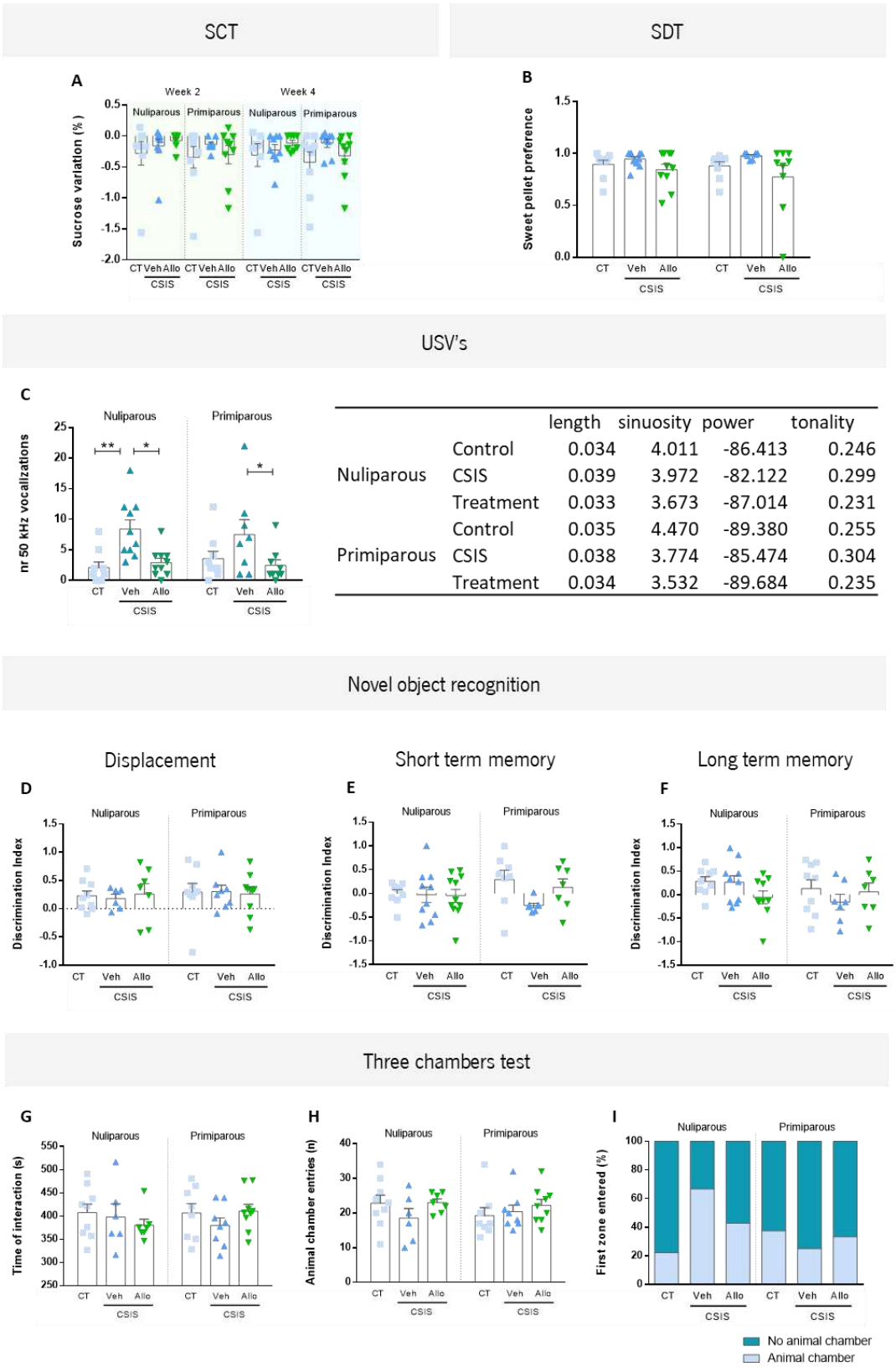


Figure 12. Behavioral results of CSIS experiment 3 (Part 1). **A)** Elevated plus maze (EPM): Time spent in open arms in CSIS-exposed females. Novelty suppressed feeding (NSF): Food consumption **(B)** and latency to feed **(C)** in CSIS-exposed females. Open field test (OF): Time spent in the center of the arena **(D)**, immobile **(E)**, rearing **(F)** and distance travelled **(G** and **H)**. **I)** and **J)** Forced swim test (FST): Time spent immobile, swimming or climbing in nulliparous **(I)** and primiparous **(J)** females Abbreviations: CT – control; Veh – vehicle; CSIS – Chronic social instability stress; Allo –

allopregnanolone. Results are presented as mean±SEM. *p<0.05; **p<0.01, ****p<0.0001. n=8-10 animals per group.



		length	sinuosity	power	tonality
Nuliparous	Control	0.034	4.011	-86.413	0.246
	CSIS	0.039	3.972	-82.122	0.299
	Treatment	0.033	3.673	-87.014	0.231
Primiparous	Control	0.035	4.470	-89.380	0.255
	CSIS	0.038	3.774	-85.474	0.304
	Treatment	0.034	3.532	-89.684	0.235

Figure 13. Behavioral results of CSIS experiment 3 (Part 2). **A)** Sucrose consumption test (SCT): sucrose consumption of females in week 2 and week 4 of the CSIS protocol. **B)** Sweet drive test (SDT): Sweet pellet preference of nulliparous

and primiparous females. **C)** Ultrasonic vocalizations (USVs): average number, length, sinuosity, power and tonality of 50 kHz vocalizations during the SDT. Novel object recognition (NOR): Discrimination index of novel object in the displacement (**D**), short-term (**E**) and long-term (**F**) memory tasks. Three chambers test: Time of interaction (**G**), animal chamber entries (**H**) and first zone entered (I). Abbreviations: CT – control; Veh – vehicle; CSIS – Chronic social instability stress; Allo – allopregnanolone. Results are presented as mean±SEM. *p<0.05; **p<0.01. n=8-10 animals per group.

In the NOR test, no significant differences were found in either displacement, short- or long-term memory between experimental groups (Displacement: $F_{(2,42)}$=0.0995; p=0.9055; Short-term: $F_{(2,46)}$=1.504; p=0.2330; Long-term: $F_{(2,46)}$=1.649; p=0.2034) (**Fig.13D, E** and **F**).

In order to observe the impact of CSIS on social behavior, we additionally performed the three chambers test; however, no significant effects were found between experimental groups, in any of the parameters assessed, including time of interaction, nr of entries and zone of first entry (**Fig. 13G - I**; Time interacting: $F_{(2,41)}$=0.8443; p=0.4372; Number of entries: $F_{(2,41)}$=0.8634; p=0.4292).

4.2 Physiological Effects of CSIS exposure and allopregnanolone treatment

4.2.1 CSIS Experiment 1

In this first experiment, animals were monitored from the baseline until sacrifice to explore the effects of CSIS exposure in physiological parameters, such as weight gain, food and water intake, estrous cycle and corticosterone levels.

Body weight was weekly monitored. We observed a decrease in weight gain in both primiparous and nulliparous females after CSIS exposure from week 2, compared to control females (Nulliparous: $F_{(3,32)}$ =4.244; p=0.0124; Primiparous: $F_{(3,20)}$ =1.558; p=0.2306) (**Fig. 14A** and **D**).

Food and water intake were measured at baseline and at the end of the CSIS protocol. CSIS-exposed primiparous females showed a decrease in water intake ($F_{(2,15)}$ =1.064; p=0.0305) (**Fig. 14B** and **D**) and nulliparous females exposed to stress presented a tendency for increased food intake when compared to control females ($F_{(2,24)}$ =4.970; p=0.0156) (**Fig. 14C** and **E**). Regarding the estrous cycle, we observed that nulliparous females were not affected by CSIS, whereas primiparous females seemed to be affected by CSIS exposure, when compared with the control, with half of the females from the CSIS group presenting an irregular cycling pattern (**Fig. 14G**).

Lastly, serum corticosterone was collected at baseline and at the end of the protocol. We observed that nulliparous and primiparous females did not differ in corticosterone levels when compared to controls at the baseline or after exposure to stress (Baseline nulliparous vs primiparous: $F_{(1,14)}$ =0.4689); p=0.5047; Nulliparous CT vs CSIS: $F_{(1,21)}$ =1.106; p=0.3049; Primiparous CTvsCSIS: $F_{(1,10)}$ =0.6449; p=0.4406) (**Fig. 14H-J**).

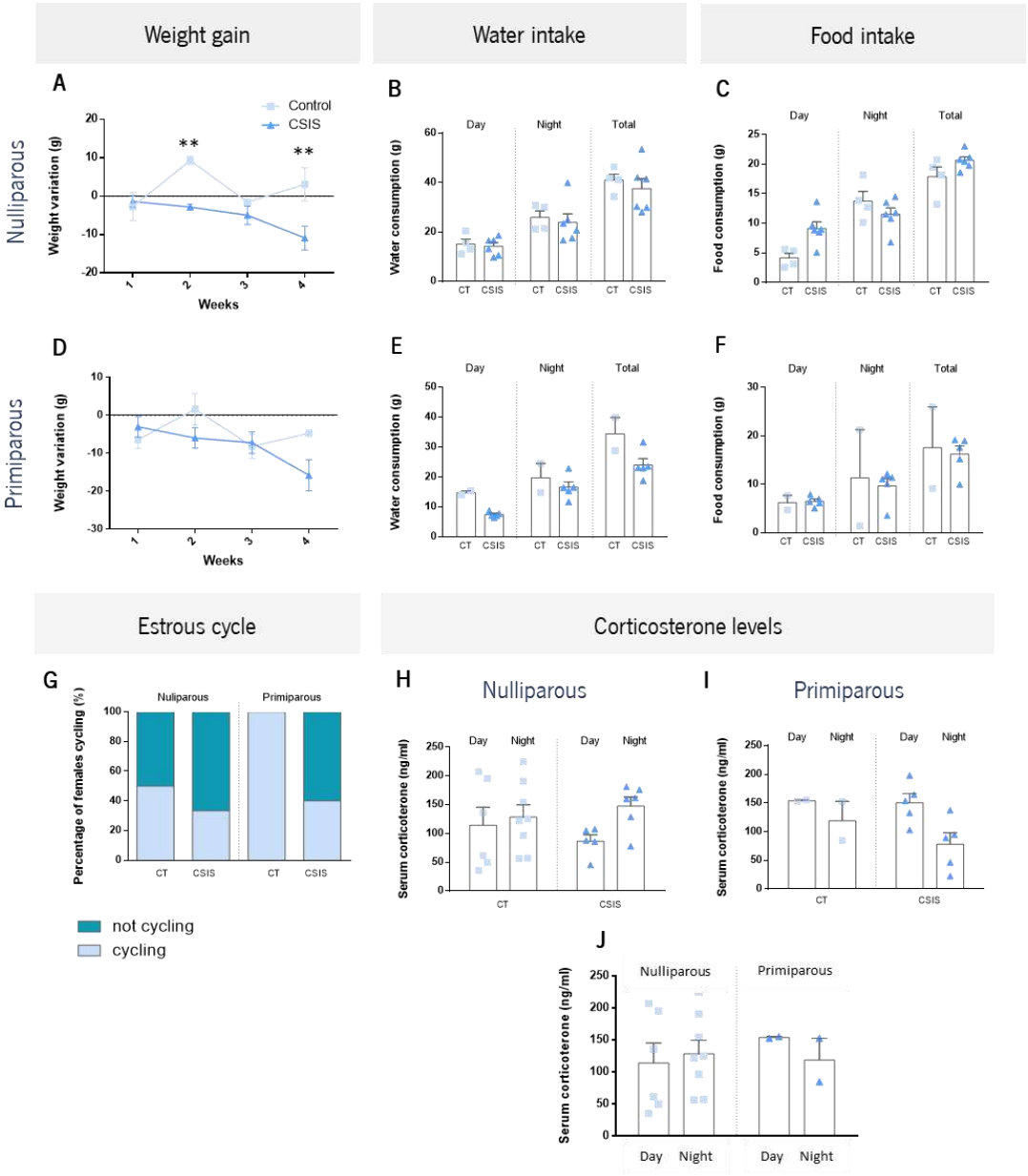


Figure 14. Physiological measurements of animals from CSIS experiment 1. **A)** and **D)** Weight gain of nulliparous (A) and primiparous (D) females. **B)** and **E)** Water intake of nulliparous (B) and primiparous (E) females. **C)** and **F)** Food intake of nulliparous (C) and primiparous (F) females. **G)** Estrous cycle. **H)**, **I)** and **J)** Corticosterone assessment of nulliparous and primiparous females. Comparison of nulliparous and primiparous baseline corticosterone (J). Abbreviations: CT – control; Veh – vehicle; CSIS – Chronic social instability stress; Allo – allopregnanolone. Results are presented as mean±SEM. **p<0.01. n=2-6 animals per group.

4.2.2 CSIS Experiment 2

As in CSIS Experiment 1, body weight was measured every week. A decrease in body weight gain was observed in both primiparous, from week 6, and nulliparous, from week 4, CSIS females in comparison to controls (Nulliparous: $F_{(5,142)}$ =41,49; p<0.0001; Primiparous: $F_{(5,116)}$ =20.44; p<0.0001). Treatment with allopregnanolone did not reverse the weight gain changes observed in CSIS-exposed females (**Fig. 15A** and **D**).

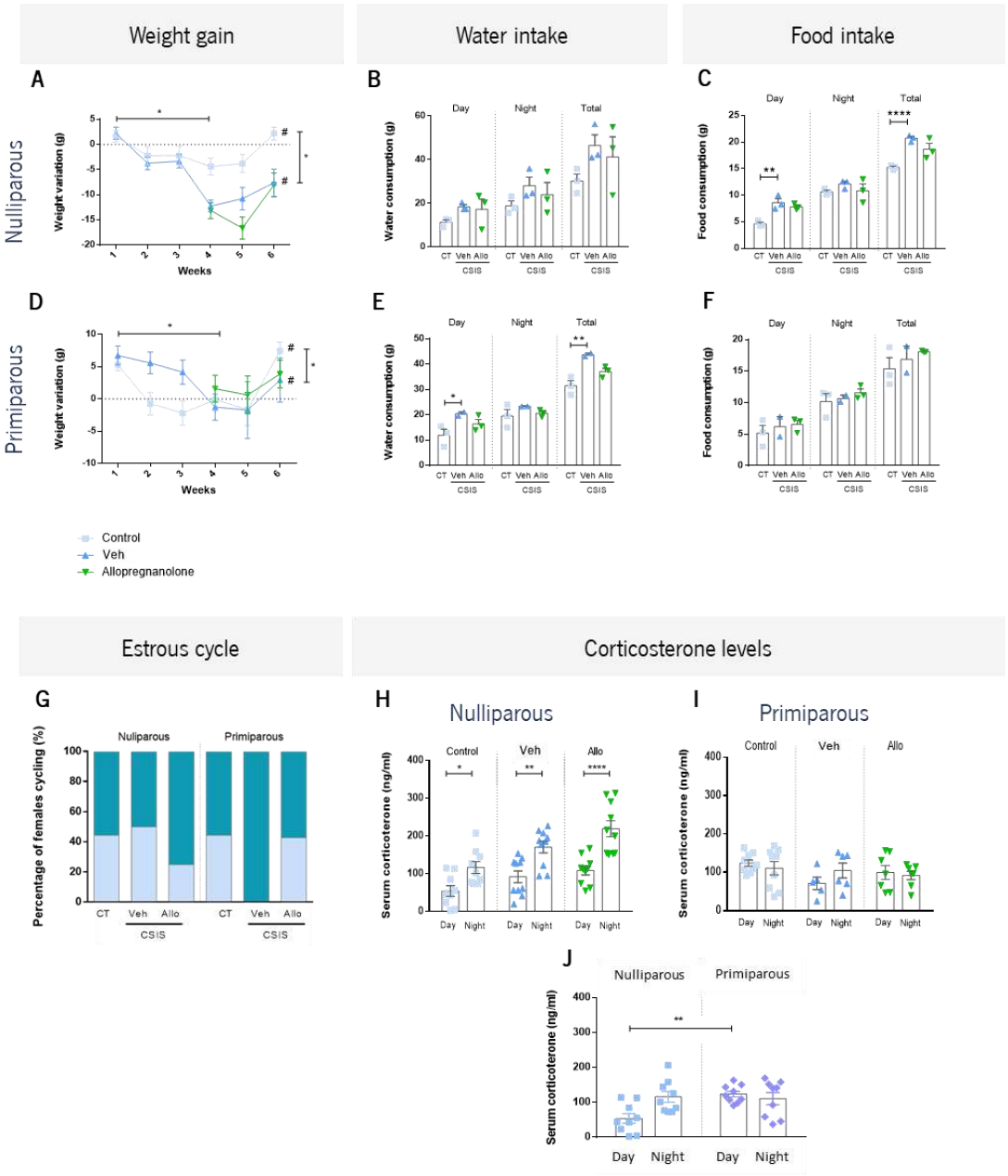


Figure 15. Physiological measurements of animals from CSIS experiment 2. **A)** and **D)** Weight gain of nulliparous (A) and primiparous (D) females. **B)** and **E)** Water intake of nulliparous (B) and primiparous (E) females. **C)** and **F)** Food intake of nulliparous (C) and primiparous (F) females. **G)** Estrous cycle. **H)**, **I)** and **J)** Corticosterone assessment of nulliparous and primiparous females. Comparison of nulliparous and primiparous baseline corticosterone (J). Abbreviations: CT – control; Veh – vehicle; CSIS – Chronic social instability stress; Allo – allopregnanolone. Results are presented as mean±SEM. *p<0.05; **p<0.01; ****p<0.0001, p<0.05 between 4th and 6th week. n=7-10 animals per group.

Food and water intake were also measured at baseline and at the 4th week of the CSIS protocol. CSIS-exposed primiparous females showed an increase in total water intake ($F_{(4,15)}$ =1.254; p=0.0012). In contrast, CSIS nulliparous females presented increased total food intake ($F_{(4,18)}$ =2.835; p<0.0001). Treatment with allopregnanolone slightly reversed the food and water intake values to normal, but not reaching statistical significance (Nulliparous water intake: $F_{(4,15)}$ =1.245; p=0.0688; Primiparous food intake: $F_{(4,18)}$ =2.835; p=0.1081) (**Fig. 15B** and **E**).

Regarding the estrous cycle, we observed that nulliparous CSIS females were not affected, whereas primiparous CSIS-exposed females seemed to be affected when compared with the control, with all females from this group presenting an irregular cycling pattern. Treatment with allopregnanolone recovered the cycling pattern to normal (**Fig. 15G**).

Lastly, serum corticosterone was collected at baseline and at the end of the CSIS protocol. Nulliparous and primiparous control females differed in corticosterone levels at the baseline, with primiparous females presenting almost twice as much corticosterone levels at the day timepoint (N, Nadir) ($F_{(1,32)}$ =6.969; p=0.0030). CSIS primiparous females presented a disruption of the corticosterone circadian secretion pattern, but not nulliparous females, presenting sustained high levels of corticosterone between day and night ($F_{(2, 52)}$= 1,173; Control: p=0.031; CSIS: p=0.0025; Allo: p<0.0001). Treatment with allopregnanolone did not reverse the changes in serum corticosterone levels (**Fig. 15H - J**).

4.2.3 CSIS Experiment 3

Weekly body weight measurement revealed a decrease in body weight gain in both primiparous, from week 2, and nulliparous, from week 4, CSIS females when compared to control females (Nulliparous: $F_{(5,142)}$ =41.49; p<0.0001; Primiparous: $F_{(5,116)}$ =20.44; p<0.0001). Treatment with allopregnanolone slightly recovered weight gain to normal but results were not statistically significant (**Fig. 16A** and **D**).

Food and water intake were also measured at baseline and at the 4th week of the CSIS protocol. CSIS nulliparous and primiparous females showed an increase in water intake (Nulliparous: $F_{(4,18)}$ =0.4707; p=0.0211; Primiparous: $F_{(4,15)}$ =3.379; p=0.0368) (**Fig. 16B** and **E**). Moreover, CSIS-exposed nulliparous females presented increased food intake compared to controls ($F_{(4,18)}$ =0.8054; p=0.0056) (**Fig. 16C** and **F**). Treatment with allopregnanolone fully reversed water intake levels in CSIS primiparous females ($F_{(4,15)}$ =3.379; p=0.0002) while not recovering the remaining changes,

namely water intake, food intake and weight gain (Water intake: $F_{(4,18)}$ =0.0211; p=0.7478; Food intake: $F_{(4,18)}$ =0.8054; p=0.3490) (**Fig. 16A-F**).

Lastly, blood was collected for serum corticosterone levels evaluation and vaginal smears were performed for estrous cycle analysis at baseline and at the end of the protocol. Due to time constraints these samples were not analyzed yet and are not presented here.

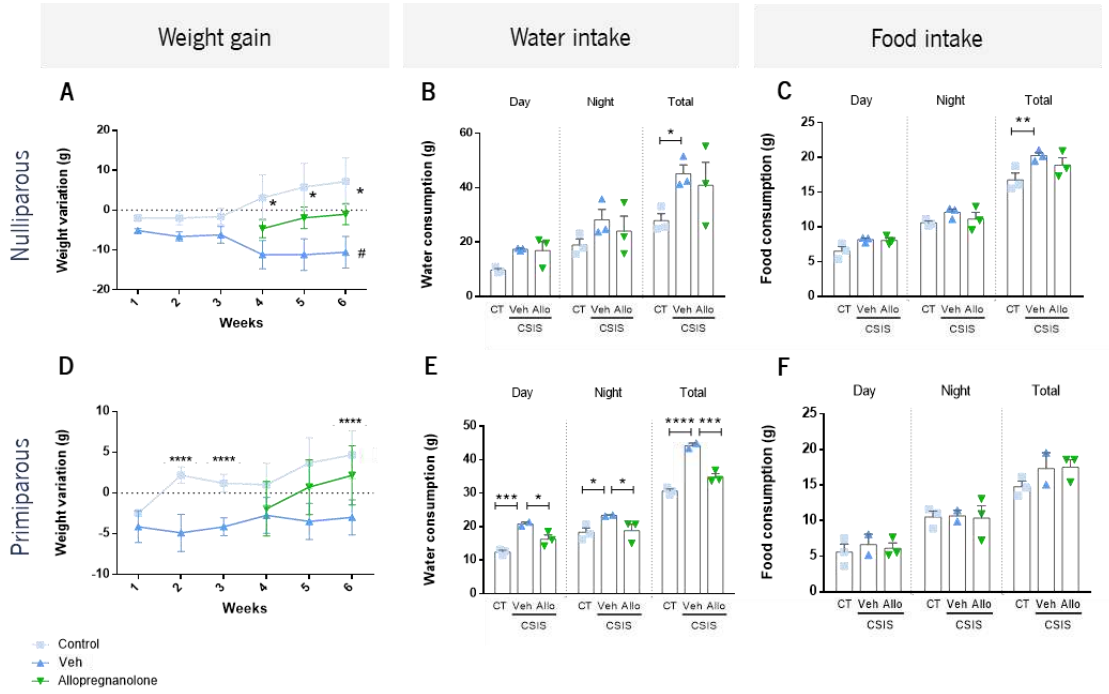


Figure 16. Physiological measurements of animals from CSIS experiment 3. **A)** and **D)** Weight gain of nulliparous (A) and primiparous (D) females. **B)** and **E)** Water intake of nulliparous (B) and primiparous (E) females. **C)** and **F)** Food intake of nulliparous (C) and primiparous (F) females. CT – control; Veh – vehicle; CSIS – Chronic social instability stress; Allo – allopregnanolone. Results are presented as mean±SEM. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, # p<0.05 between 4th and 6th week. n=8-10 animals per group.

4.3 Morphological analysis of hippocampal DG neurons and predicted electrophysiological effects of CSIS

To explore morphologic and predicted electrophysiological alterations caused by CSIS exposure, Golgi stained neurons from the dorsal and ventral dentate gyrus (DG) were reconstructed and used as a foundation of electrophysiological simulations. An *in silico* approach was used to predict the electrical activity from the morphologic 3D reconstructions, as previously described (Hines, Davison and Muller, 2009). Only neurons from animals of the CSIS experiment 2 were evaluated for these parameters.

4.3.1 Dorsal DG

The length and branching of the dendritic tree of granule neurons were assessed (**Fig. 17A-F**). We observed that primiparous CSIS females had an increased number of branches (**Fig.17B**) and complexity (**Fig.17F**) compared with control females ($F_{(2,24)}$ =12.86; p<0.0001) (**Fig.17B)**, whereas neurons from nulliparous females were only affected in terms of complexity in the sholl analysis (**Fig.17E**; $F_{(2,24)}$ =1.576; p=0.0054).

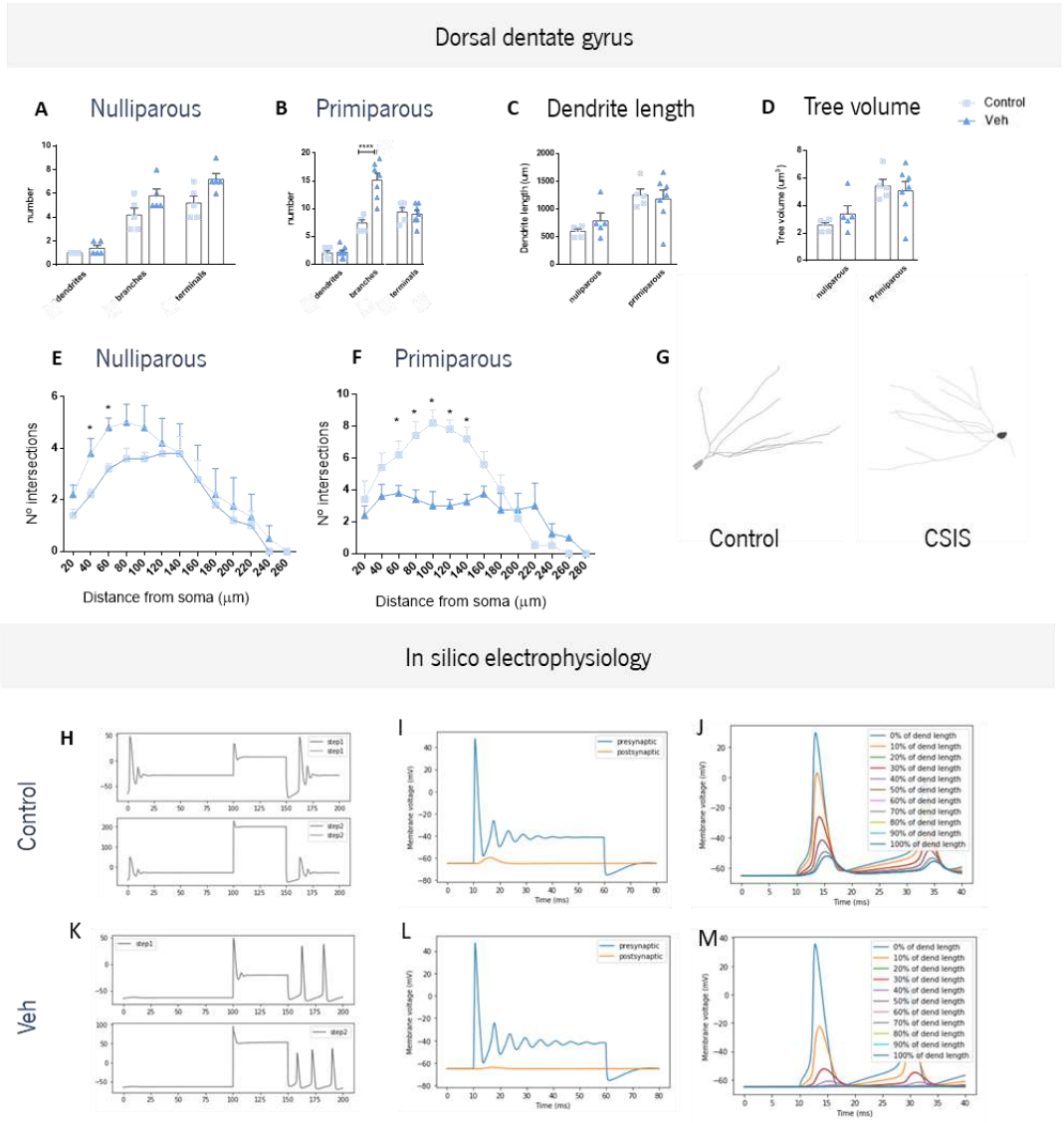


Figure 17. Morphological analysis and *in silico* electrophysiological simulations of dorsal DG neurons of animals from CSIS experiment 2. **A-D)** Morphological analysis of the dorsal dentate gyrus according to number of branches, dendrites, terminals, as well as dendritic length and tree volume. **E)** and **F)** Sholl analysis of dorsal DG reconstructed neurons in nulliparous and primiparous females. **G.** Representation of a reconstructed Golgi-Cox stained neuron from each experimental group analyzed. **H- M)** *In silico* electrophysiological simulation of current clamp recordings (50 ms stimulation), synaptic transmission and morphological reconstruction from primiparous females. Voltage attenuation along the dendrite in the dDG. Voltage attenuation is greater in CSIS due to the increased dendrite length. CT – control; Veh – vehicle; CSIS – Chronic social instability stress; Allo – allopregnanolone. Results are presented as mean±SEM. *p<0.01; ****p<0.0001. n=7-10 animals per group.

No differences were observed in the dendritic length of neurons between the control and CSIS-exposed females (**Fig. 17C)**

Membrane voltage and synaptic transmission were evaluated in computer-generated simulations. The NEURON simulator allows us to stimulate and record neurons in response to different protocols. For this experiment, two protocols were applied: 1) A square current injection was applied to the soma of the neuron in order to evaluate the intrinsic firing of the neuron and firing in response to stimulation ; 2) synaptic transmission between neurons was observed from the membrane voltage of both pre- and post-synaptic neurons.

Intrinsic firing without stimulation was observed in control primiparous females, but not in CSIS-exposed untreated females. The firing pattern during stimulation was not affected by CSIS exposure. However, membrane potential of CSIS primiparous females was lower in comparison to CT. Synaptic transmission was also not affected by CSIS (**Fig. 17H-M)**.

4.3.2 Ventral DG

In the ventral DG, primiparous CSIS-exposed females neurons presented increased number of branches (**Fig. 18B)** and complexity (**Fig. 18 F)** compared to those of CT females ($F_{(4,33)}$ =4.510; p=0.0051) (**Fig. 18B)**. Neurons from nulliparous CSIS-exposed females were not affected (**Fig. 18**; $F_{(2,15)}$ =0.2595; p=0.7748). The dendritic length and the tree volume of vDG neurons from primiparous CSIS-exposed females were also increased, although it did not reach statistical significance ($F_{(4, 42)}$ = 12.86; p=0.6340) (**Fig. 18A-G)**.

Intrinsic firing without stimulation was observed in CT primiparous females, but not in CSIS females (**Fig. 18H-M)**. Regarding synaptic transmission, CSIS primiparous females' neurons lacked the ability to send action potentials to post-synaptic neurons (**Fig. 18I** and **L**).

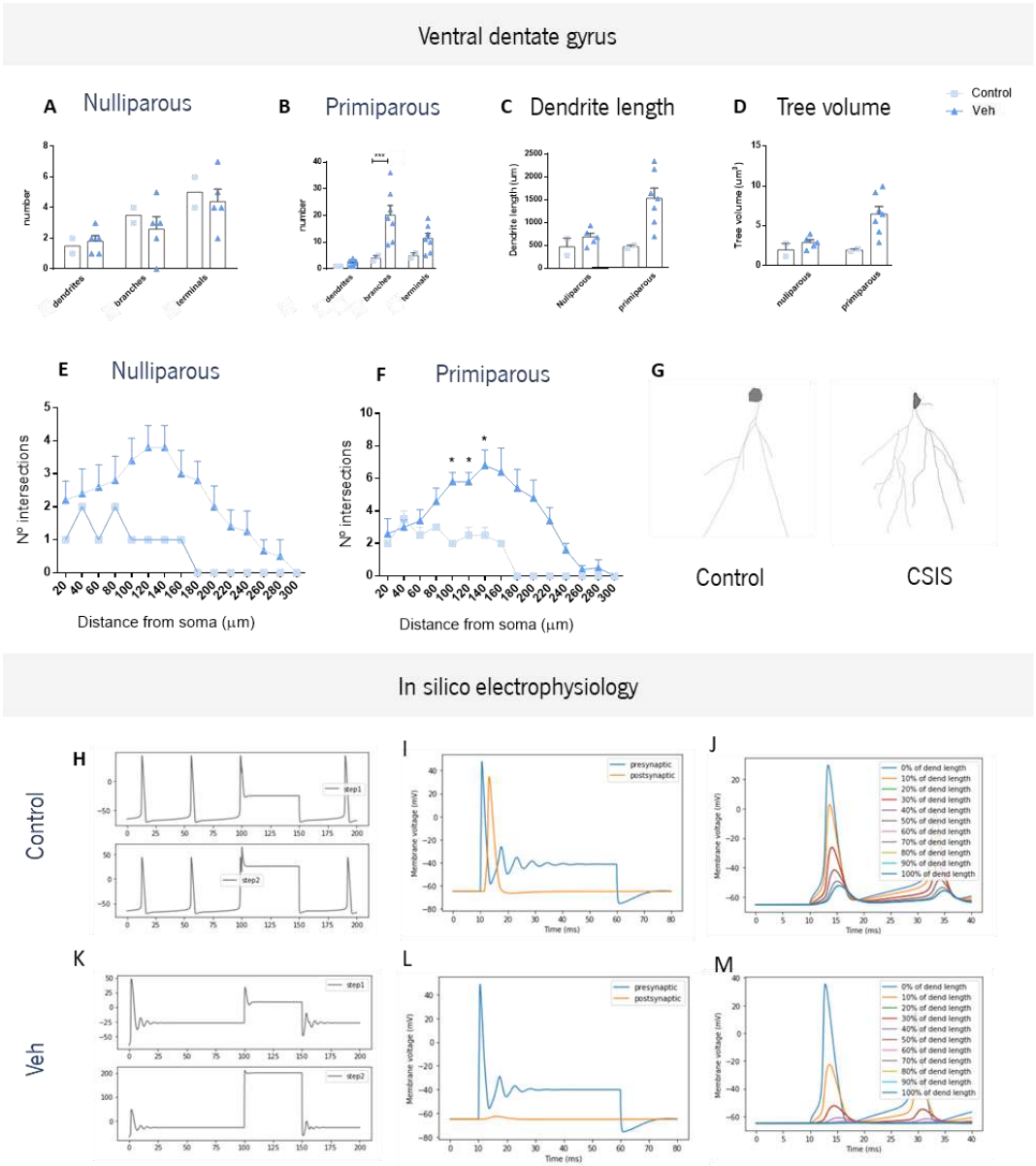


Figure 18. Morphological analysis and *in silico* electrophysiological simulations of dorsal DG neurons of animals from CSIS experiment 2. **A-D)** Morphological analysis of the dorsal dentate gyrus according to number of branches, number of dendrites, number of terminals, dendritic length and tree volume. **E)** and **F)** Sholl analysis of nulliparous and primiparous females. **G.** representation of a reconstructed Golgi-Cox stained neuron from each experimental group analyzed. **H- M)** *In silico* electrophysiological simulation of current clamp recordings (50 ms stimulation), synaptic transmission and morphological reconstruction from primiparous females. Voltage attenuation along the dendrite in the vDG. Voltage attenuation is greater in CSIS due to the increased dendrite length. CT – control; Veh – vehicle; CSIS – Chronic social instability stress; Allo – allopregnanolone. Results are presented as mean±SEM. ****p<0.0001. n=7-10 animals per group.

4.4 Molecular Effects of CSIS and allopregnanolone treatment

To explore the molecular alterations caused by CSIS exposure, that could be underlying some of the observed neuromorphological changes, gene expression levels were evaluated in the ventral DG from animals from CSIS experiment 2 (**Fig. 19**). Brain-derived neurotrophic factor (BDNF) expression levels showed a tendency to increase in CSIS-exposed primiparous females ($F_{(2,15)}$

=1.932; p=0.2372) and recovered to the levels of CT animals after treatment with allopregnanolone (F(2,15)=1,932; p=0.3554) (**Fig. 19A)**. Doublecortin (DCX) expression showed a tendency to reduce in primiparous females upon CSIS exposure ($F_{(2,15)}$ =3.296; p=0.0651) and recovered by allopregnanolone treatment ($F_{(2,15)}$ =3.296; p=0.2116) (**Fig. 19B)**. The serotonin receptor 5HTR2 was also altered upon CSIS exposure, showing a tendency to increase mRNA expression levels in CSIS-exposed primiparous females ($F_{(2,14)}$ =3.357; p=0.064) and a reduction after treatment ($F_{(2,14)}$ =3.357; p=0.1600) (**Fig. 19C)**. Glutamate transporter-1 (Glt-1) expression levels in CSIS primiparous females had a tendency to reduce ($F_{(2,15)}$ =1.515; p=0.3970) and also showed a tendency to recover by treatment with allopregnanolone ($F_{(2,15)}$ =1.515; p=0.2060) (**Fig. 19D)**.

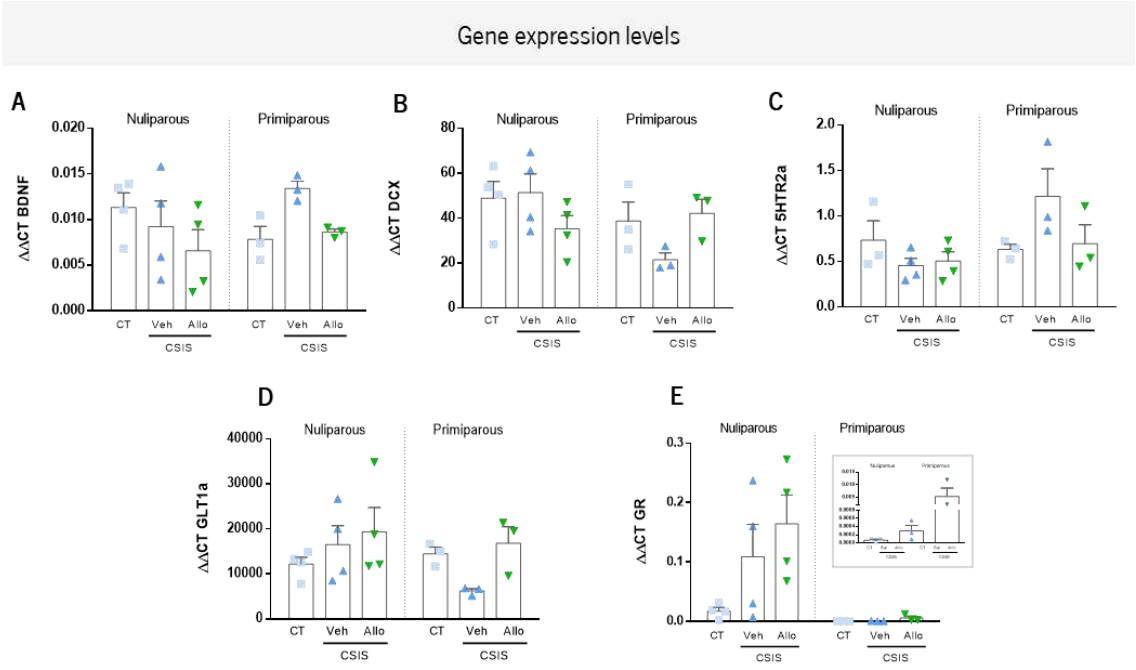


Figure 19. Gene expression analysis evaluated by RT-PCR in the vDG of animals from CSIS experiment 2. **A)** Gene expression levels of BDNF. **B)** Gene expression levels of DCX. **C)** Gene expression levels of 5HTR2a. **D)** Gene expression levels of GLT1a. **E)** Gene expression levels of GR. CT – control; Veh – vehicle; CSIS – Chronic social instability stress; Allo – allopregnanolone. Results are presented as mean±SEM. N=3-4 animals per group.

Lastly, the glucocorticoid receptor (GR) on the other hand, was affected both by stress exposure and reproductive experience; primiparous females had a tendency to present lower levels of control GR expression than nulliparous females, but CSIS females presented very heterogeneous levels among animals, leading to inconclusive results (**Fig. 19E)**. CSIS exposure and allopregnanolone treatment did not affect the expression of any of the genes analyzed in nulliparous females (**Fig. 19A-E**).

4.5 Behavioral Effects of a SI protocol

To evaluate the effects of a SI stress protocol, we performed a pilot experiment in which nulliparous and primiparous females were isolated during 4-weeks and assessed for anxiety-like behavior in the EPM test. Additional behavioral and molecular tests were planned but due to time constraints they were not performed.

In the EPM, SI-exposed nulliparous and primiparous females showed no statistically significant differences when compared with controls ($F_{(1,42)}$ = 0.8196; p = 0.3705) (**Fig. 20**).

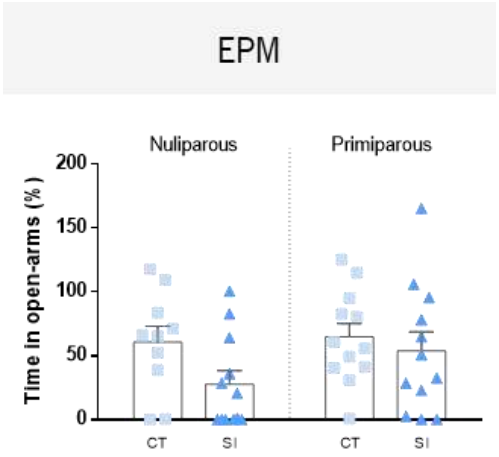


Figure 20. Behavioral evaluation of SI effects in the EPM: percentage of time spent in open arms. Abbreviations: CT – control; SI – Social isolation stress. Results are presented as mean±SEM. n=10-12 animals per group.

5. Discussion

In this study we aimed to implement and validate an animal model of depression for female rats based on the exposure to social stress, the chronic social instability stress (CSIS) paradigm, assessing the impact o reproductive experience in stress susceptibility. Additionally, we wanted to assess the effects of acute treatment with allopregnanolone, which has been recently used as an antidepressant in post-partum depression.

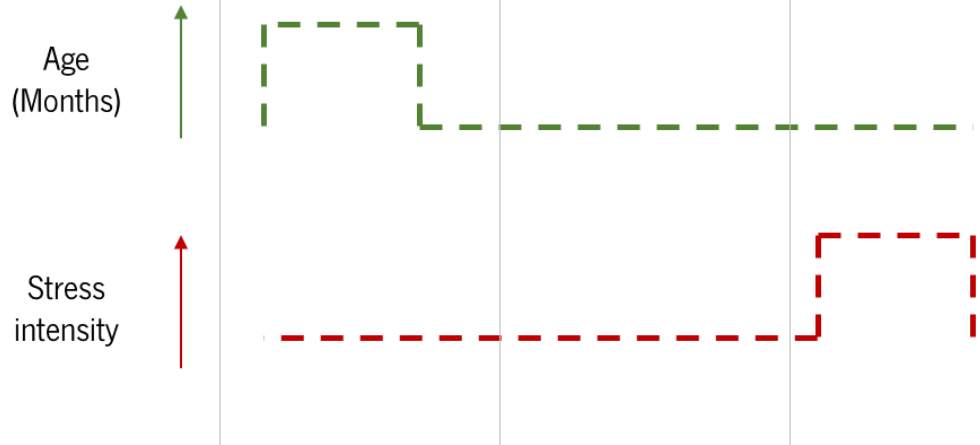
5.1 Chronic social instability stress as an animal model of depression for rodent females

Women may experience depression in response to difficulties in their close social network and personal life events, with depression in women showing a major association with social stress (Hammen, 2003). Social stress is also considered an important factor for the development of depressive-like signs in animal models, but most of the currently available stress protocols are only validated in male animals, revealing the need for the development of more representative models to study the neurobiological basis of the disease in women. The validity of an animal model may be attained by the evaluation of four major criteria: predictive validity, face validity, construct validity and etiological validity (Belzung and Lemoine, 2011; Abelaira, Reus and Quevedo, 2013). Though fully recapitulating the complexity of human depression is not possible, animal models can be developed by exposure to known etiological factors of depression, such as chronic stress (Wang et al., 2017), and display some of the core behavioral and physiological traits of the human disease, referred to as endophenotypes (Gould and Gottesman, 2006). These behavioral signs include anhedonia, appetite and sleep disturbances, behavioral despair and anxiety-like behaviors (Krishnan et al., 2011). Additionally, a large variety of behavioral tests can be used to evaluate such traits (Krishnan et al., 2011).

To assess face validity of the CSIS model, we characterized the female rat's physiology, behavior and molecular signature, after 4 weeks of exposure in 3 different conditions. First, in a pilot experiment, nine months-old female rats were exposed to a modified version of a previously validated CSIS protocol to assess deficits associated with social stress (Herzog C. et al., 2009). To investigate stress-related behaviors in younger females, four months-old female were exposed to the same CSIS protocol and subjected to allopregnanolone treatment. Lastly, and because the results of the first implemented protocol were not so prominent, namely regarding the development of depressive-like and anhedonic behavior, 4 months-old females were exposed to a more intense version of the CSIS protocol.

Different behavioral responses and impairments were observed according to the age and to the CSIS protocol used, revealing a complex interaction between these factors (**Table 2**).

Table 2. Summary of CSIS experiment 1, 2 and 3. Assessment of anxiety- and depressive-like behavior, cognition, food and water intake, weight gain, estrous cycle, corticosterone, neuronal morphology, *in silico* electrophysiology and gene expression. N - Nulliparous; P - Primiparous; FI - Food intake; WI - Water intake.



	CSIS 1	CSIS 2	CSIS 3
Anxiety-like behavior	↑ N	↑ N and P	↑ N and P
Depressive-like behavior	Not present	Not present	Not present
Cognition	Tendential ↓ spatial memory in N	Spatial memory ↓ in P	Not affected
Food (FI) and water (WI) intake	Not affected	↑ FI in N ↑ WI in P	↑ FI in N ↑ WI in N and P
Weight gain	↓ in N and P	↓ in N and P	↓ in N and P
Estrous Cycle	Dysregulated in P	Dysregulated in P	-
Corticosterone	Not affected	Dysregulation in P	-
Neuronal Morphology	-	↑ nº branches, dendrite length and tree volume in vDG of P	-
***In silico* electrophysiology**	-	↓ intrinsic activity and synaptic transmission of P	-
Gene expression	-	↑ BDNF and 5HTR2a as well as ↓ DCX and GLT1a in P	-

Though none of the experiments revealed an effect of CSIS in the development of anhedonic or depressive-like phenotype, irrespective of age and protocol intensity, exposure to this stress was able to produce important behavioral and physiological deficits, akin to human depression, in both nulliparous and primiparous females.

Nine-month old CSIS-exposed nulliparous female rats revealed deficits linked with anxiety, memory and physiology. Increased climbing activity during the FST can be read as an anxiety-like behavior but not as a depressive-like behavior, as seen in Labaka et al. (2017). When exposed to a cognitive behavioral test, we observed an impairment in spatial recognition memory in the novel object recognition test. Food intake and weight gain were also affected (**Table 2**).

The significantly higher food intake by nulliparous females in a stressful environment is consistent with other studies showing that exposure to chronic stress increases food intake (Michopoulos et al., 2012). Interestingly, this was a companied by a decreased body weight gain, another important feature of depressive-like behavior in stress-induced animal models (Mateus-Pinheiro *et al.* 2013; Patrício *et al.* 2015)

Nine-month old CSIS-exposed primiparous females revealed increased anxiety-like behavior and physiological changes (**Table 2**), namely weight loss, decreased water intake and estrous cycle regulation impairments. Changes in food and water intake patterns have been linked to depression and depressive-like behaviors. Additionally, hypo-hydration (water loss <1.0% of body weight) produces decreased activity in the ventral cingulate gyrus, orbito-frontal cortex, hypothalamus, dorsal cingulate cortex, amygdala, post-central gyrus, right striatum and superior parietal cortex, which in turn, and consistent with our results, were associated with anxiety (Young et al., 2019).

Regarding the effects of stress on the estrous cycle, in this study we have observed that all analyzed control primiparous females were cycling normally, in contrast with the CSIS exposed primiparous females that presented a disruption in normal cycling. Nulliparous females were not affected whatsoever. Previous studies have shown that HPA axis activation by stress exerts an inhibitory effect on the female reproductive system (Kalantaridou et al., 2004). Corticotropin-releasing hormone (CRH) inhibits hypothalamic gonadotropin-releasing hormone secretion and glucocorticoids inhibit pituitary LH, ovarian estrogen secretion and progesterone secretion (Kalantaridou et al., 2004). These effects may be responsible for the “hypothalamic” amenorrhea of stress, which is observed in women suffering from anxiety and depressive disorders (Kalantaridou et al., 2004), and similarly produce a dysregulation of the estrous cycle in female rats (Lüscher and Möhler, 2019).

When evaluating stress-related behaviors in younger females, nulliparous and primiparous females exposed to protocol number 1 showed increased anxiety and weight loss (**Table 2**). Primiparous females also showed a tendency for impaired spatial recognition and long-term memory, increased water intake, disruption of the corticosterone circadian variation and dysregulation of the estrous cycle. Hypersecretion of glucocorticoids is associated with human depression. In fact, around 50% of depressed patients have increased levels of cortisol in the blood (Dallman et al., 2008). Under non-stressful conditions, corticosterone, the major glucocorticoid in rodents, is held at low levels during the day and have a peak at night-time (Dallman et al., 2008). During our study, CSIS-exposed primiparous females showed sustained high levels of corticosterone during day and night as observed in clinical conditions (Dallman et al., 2008).

The ability to learn and recall spatial locations, and to link them with other stimuli, is a vital adaptive behavior that is essential for survival. Spatial memory and spatial navigation are largely associated with the hippocampus, both in humans and rodents (Bannerman et al., 2014), a region also majorly associated with the regulation of anxiety and depression (Bannerman et al., 2014). Our results on spatial memory may suggest an impairment of the hippocampal function possibly correlated with the altered dendritic length and tree volume observed in the hippocampal DG, as well as molecular alterations.

On the third experiment, with the aim of intensifying the phenotype observed in the first 2 protocols, animals were exposed to longer overcrowding and isolation periods. Nulliparous and primiparous females exposed to this more intense version of the CSIS protocol, presented increased number of 50 kHz vocalizations, increased water intake and decreased body weight gain. Rats and related rodents emit a large number of ultrasonic vocalizations in the 50-kHz range (Burke et al., 2017). There have been two major hypotheses advanced to account for these calls: the movement byproduct hypothesis and the affective communication hypothesis. The movement byproduct hypothesis postulates that the 50-kHz vocalizations emitted by rodents are mostly produced by the biomechanical forces on the thoracic area generated during movement (Blumberg et al., 1992). More recently, in the affective communication hypothesis, it has been suggested that these vocalizations convey the animal's affective state to surrounding animals (Burke et al., 2017). Here, we could not detect changes in the hedonic profile of the females exposed to CSIS neither decreased 50KHz USVs emission, as expected. This, together with the increased activity of CSIS exposed females observed during the FST, leads us to hypothesize that the increased number of

USVs may be due to an increased activity and it might not be a good measure of the female's affective state.

Because our conclusions are mostly based on the assessments from the behavioral tests, other, more sensitive behavioral tests for females were also made on the last experiment, such as the OF and TCT; nevertheless, no deficits were found in any of these two behavioral tests in CSIS-exposed rats.

Our prediction for the current study was that both forms of chronic social stress would induce behavioral, physiological, and neuronal changes when compared to controls, and that prolonged periods of overcrowding and isolation would be a more potent chronic social. Nevertheless, we could observe that higher level of unpredictability enhances the response to the stressor.

Differences between experiments were though linked to age, reproductive experience and stress type. Age was a strong factor guiding the way stress impacted nulliparous and primiparous females differently. We observed that in older females, nulliparous females seemed to be more affected regarding cognition and anxiety, whereas, in younger rats, primiparous females were much more affected (**Table 2**). In fact, the natural hormonal variations occurring across female's lifespan are accompanied by differences in the stress response. Regarding reproductive experience, adult nulliparous females have been shown to adapt differently to stress when compared with dams and multiparous females (Rima et al., 2009). The hormonal environment experienced through pregnancy and post-partum are known to change hormonal receptors in the brain, hippocampal neurogenesis and spatial memory (Barha et al., 2015). Age, nevertheless, affected the response to stress. In older animals, nulliparous females were more prone to develop anxiety-like behavior, in contrast, primiparous females were more prone to develop anxiety-like behavior in younger ages. However, studies in multiparous females show that pregnancy confers stress resilience, suggesting the number of pregnancies also affects the stress response.

Symptom profiling in major depression may differ significantly in men and women indicating that there are variances in neurobiological mechanism of the disorder between genders. Nevertheless, very few studies have attempted to develop animal models specific for females, or sex differences' sensitive therapies. Moreover, we may hypothesize that due to the lack of information regarding symptom profiling in women and behavioral assessment in female rodents, the behavioral tests used may not be the most appropriate, leading to inconclusive results.

5.2 Allopregnanolone as an antidepressant in a female animal model of depression

We also aimed to evaluate the short-term effects of acute allopregnanolone treatment after exposure to CSIS. Brexanolone, an analog of the endogenous human hormone allopregnanolone, became the first drug to have ever been approved specifically for the treatment of postpartum depression (PPD) in adult females but its action as a putative antidepressant in major depression in females is not yet known.

In our study, the action of allopregnanolone on the recovery of behavioral deficits remained inconclusive. This can be explained by a myriad of factors, such as the mild phenotype induced by CSIS exposure, but also the dosage and duration of treatment. Nevertheless, we could observe the reversion of some physiological alterations induced by CSIS exposure. For instance, allopregnanolone treatment could slightly restore the anxiety-like and memory impairments observed in primiparous females (**Table 3**). Treatment with allopregnanolone also seemingly lacked the ability to reverse the changes in serum corticosterone levels, though it recovered the estrous cycle pattern in CSIS exposed primiparous females (**Table 3)**. Previous studies have shown that allopregnanolone affects the process of follicle maturation and gonadal steroidogenesis, thus disrupting follicular development and decreasing LH levels (Pelegrina et al., 2017; Canipari et al. 2012). Because social stress activates the HPA axis, which in turn induces the release of LH under relatively high plasma levels of estradiol (Tarín et al., 2010), we may hypothesize that allopregnanolone works by counteracting this effect, thus restoring the estrous cycle.

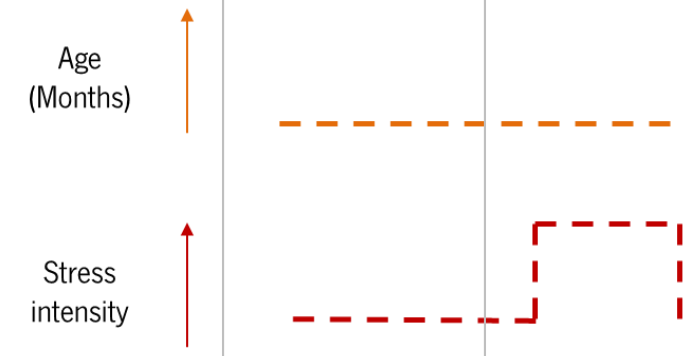
5.3 Neurobiological correlates of CSIS and allopregnanolone effects

Chronic stress exposure has been shown to affect neuronal morphology of hippocampal DG neurons (Dioli et al., 2019). To explore the morphological and predicted electrophysiological alterations caused by CSIS exposure and allopregnanolone treatment, Golgi stained neurons from the dorsal and ventral DG, were reconstructed and used as a foundation of electrophysiological simulations.

Electrophysiological alterations are important biomarkers to assess neural connectivity. Mainen and Sejnowski have shown a causal relationship between dendritic structures and intrinsic firing patterns observed from *in vitro* electrical recordings for a wide variety of cell types (Mainen and Sejnowski, 1996). Moreover, the branching pattern has been shown to be strongly affect the propagation of action potentials, which links information processing at different regions of the dendritic tree of neurons (Vetter, Roth and Hausser, 2001). More recently, Yi et al. have

demonstrated a crucial role of neuronal morphology in determining field-induced neural response (Yi et al., 2017). These results, among others, have contributed to a now widespread acceptance that neuronal morphology plays a critical role in neurons' activity and function.

Table 3 Summary of allopregnanolone treatment in experiment 2 and 3. Assessment of anxiety- and depressive-like behavior, cognition, food and water intake, weight gain, estrous cycle, corticosterone, neuronal morphology, *in silico* electrophysiology and gene expression. N - Nulliparous; P - Primiparous; FI - Food intake; WI - Water intake

	CSIS 2	CSIS 3
		
Anxiety-like behavior	Tendential recovery in P	Recovery in P
Depressive-like behavior	Not affected	Not affected
Cognition	Tendential recovery in P	Not affected
Food and water intake	Tendential recovery in P	Recovery in P
Weight gain	Not recovered	Tendential recovery in N and P
Estrous Cycle	Recovery in P	NE
Corticosterone	Not recovered	NE
Neuronal Morphology	NE	NE
electrophysiology	NE	NE
Gene expression	Tendential recovery of BDNF, 5HTR2a, DCX and GLT1a in P	NE

In our study, CSIS exposed primiparous females showed increased dendritic length and tree volume in the ventral DG specifically (**Table 2**). This might be correlated with the observed behavioral changes in anxiety-like behavior in primiparous females, since the hyperactivity in this brain region might lead to the morphological changes and is linked to anxiety behavior. Interestingly enough, although nulliparous females also presented anxiety-like behavior, they did not show neuromorphological changes in the vDG.

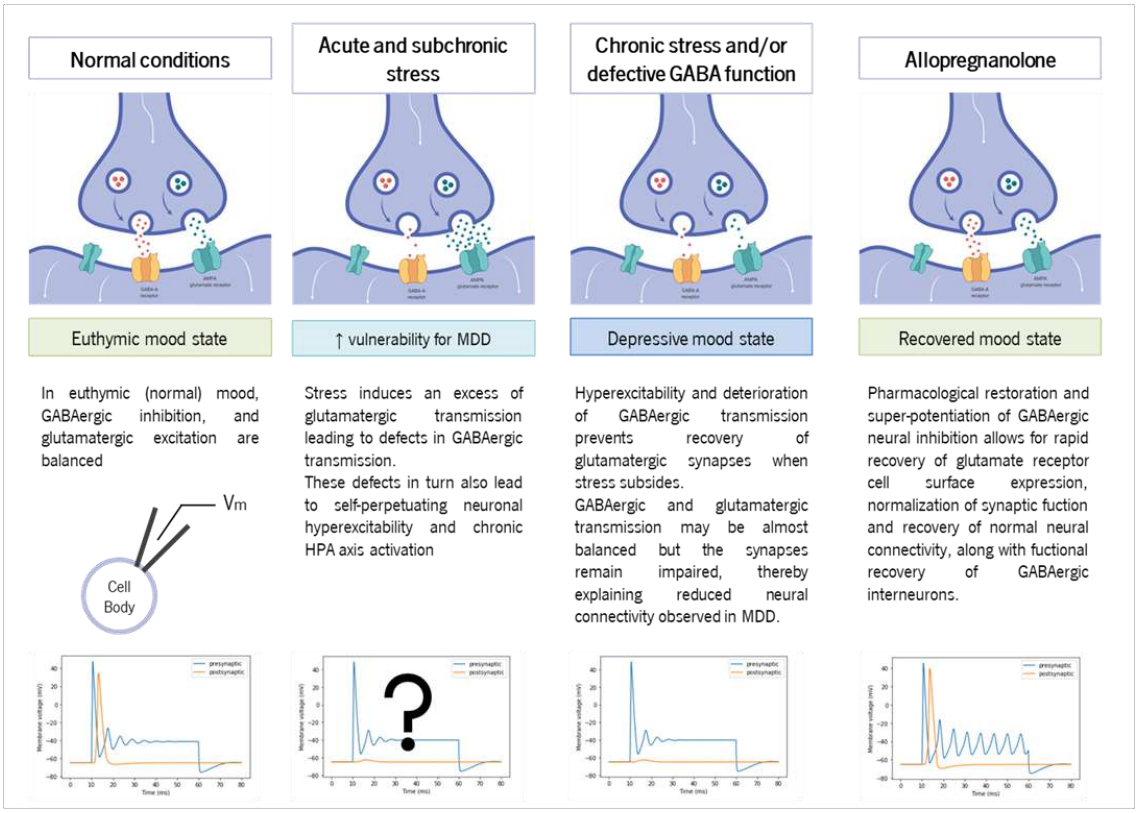


Figure 21. Predicted electrophysiological alterations caused by CSIS exposure. Link between stress, exposure, treatment and GABAergic transmission (Adapted from Lüscher and Möhler, 2019).

Although we could not observe the manifestation of depressive-like behavior upon CSIS exposure in these females, we observed a decrease of intrinsic energy and synaptic transmission in CSIS-exposed primiparous females when we analyzed the electrical properties of the reconstructed neurons (**Table 2**). Interestingly, others have shown that the reduced neural connectivity observed in stress-induced depression can be explained by disruption of the GABAergic transmission. In euthymic (normal) mood, GABAergic inhibition, and glutamatergic excitation are balanced (Lüscher and Möhler, 2019). Upon stress-exposure, there is an excess of glutamatergic transmission leading to defects in GABAergic transmission. These defects in turn lead to self-perpetuating neuronal hyperexcitability and chronic HPA axis activation (Lüscher and Möhler, 2019). Hyperexcitability and

deterioration of GABAergic transmission over long periods of time, such as chronic stress, prevents recovery of glutamatergic synapses when stress decreases. Subsequently, GABAergic and glutamatergic transmission may be almost balanced, but the synapses remain impaired, thereby explaining reduced neural connectivity observed in MDD (Lüscher and Möhler, 2019). Pharmacological restoration of GABAergic neural inhibition allows for rapid recovery of glutamate receptor cell surface expression, normalization of synaptic function and recovery of normal neural connectivity. Here, we showed a loss of synaptic activity when female animals were exposed to CSIS. Even though it was not evaluated due to time constraints, a recovery by treatment with allopregnanolone, a potent positive allosteric modulator of GABA, might be expected.

Because stress exposure is known to affect gene expression in several brain regions, which is often causally related to observed neuromorphological changes, we further evaluated gene expression levels in the ventral DG. Although no statistically significant changes were observed, possibly due to the small number of samples analyzed per group, we could observe slight tendencies for change in many molecules that have been previously reported to be altered by chronic stress exposure, namely BDNF, DCX, 5HTR2a and GLT1a (**Table 2**). BDNF, for instance, has been implicated in development, neural regeneration, synaptic transmission, synaptic plasticity and neurogenesis (Yu and Chen, 2010). The increased expression of BDNF in the vDG of CSIS-exposed primiparous females, a region involved in the modulation of anxiety-like behaviors, suggests that there was an increased activity in this region, coherent with the observed increase in anxiety-like behavior and increased neuronal length of neurons (Weeden et al., 2015). We also observed a tendency for increased expression of 5HTR2A and decreased expression of DCX in CSIS-exposed primiparous females. Several genetic studies have shown an association between 5HTR2A and depression (Khait et al., 2005), and also with suicidal behavior (Correa et al., 2002). DCX, on the other hand, is a microtubule-associated protein expressed by neuronal precursor cells and immature neurons in embryonic and adult cortical structures (Brown et al., 2003). Due to the nearly exclusive expression of DCX in developing neurons, this protein has been used increasingly as a marker for neurogenesis (Brown et al., 2003) suggesting CSIS-exposed primiparous females may present decreased neurogenesis. In the future, it is also of relevance to study neurogenesis in these females. GLT1a, the major glutamate transporter of the brain, was also tendentially decreased in CSIS-exposed primiparous females (Chen et al., 2004). GLT1a is expressed in excitatory axon terminals of mature hippocampal neurons, contributing significantly to glutamate uptake into excitatory terminals. Our results might suggest that glutamate uptake is significantly diminished in CSIS-

exposed primiparous females, perhaps due to the hyperexcitability and deterioration of GABAergic transmission during exposure to stress. According to the neuronal morphology and gene expression analysis, we could suggest that CSIS-exposed females reveled decreased neuronal activity and decreased neurogenesis.

6. Conclusions and Future perspectives

Here, we have shown that age, reproductive experience and stress intensity are important factors to consider when implementing a novel model of depression in female rodents. Though not the focus of this work, the type of stressor might also be an important factor to have into account. Moreover, we have observed that the behavioral and physiological changes were accompanied by cellular and molecular alterations in the hippocampal DG, which are worth further exploration in the future. Treatment with allopregnanolone was successful in recovering some of the physiological, molecular and cellular deficits. Still, its limited therapeutic effects in reversing behavioral deficits suggest that a longer treatment period or dose should be revised in future studies.

In order to further assess the impact of age, it would also be of interest to further explore the deficits induced by CSIS in adolescence and in older adult females. Electrophysiological recordings *in vivo* should be performed to confirm our results and further asses the role of CSIS and allopregnanolone on brain connectivity. Other parameters such as the effect of age, reproductive experience, hormonal status, among others, should also be considered in future studies.

SI was also assessed in a pilot study but due to time constraints many behavioral tests were not evaluated. Nevertheless, it would be of interest to evaluate the efficacy of different protocol of SI in females. Because behavioral assessments are the primary outcomes measured in animal models of depression, and may differ between sexes, we propose that it would be of interest to develop novel or refine the existing behavioral paradigms specifically for females.

In regard to the validity of this animal model as a model of human depression, we might state this despite being a model with a strong etiological validity, we have only achieved partial construct and face validities, because only a limited number of endophenotypes and physiological alterations were observed upon exposure to the chronic stressful stimulus. On the other hand, because allopregnanolone is an experimental drug in this context, and because no gold standard treatment was used, we cannot definitely conclude about the predictive validity of this model.

Though this study allowed some new insights into the neurobiology of depression in females, it also raised many questions on how a new animal model for such a complex disorder as depression, should be developed, validated and implemented. We expect that the work presented herein, adds to the literature and encourages further experiments in this field, leading to a more comprehensive understanding of depression's pathophysiology and treatment in women.

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Appendix I

Statistical analyses were done using SPSS software (SPSS, Chicago, IL, USA). After Confirmation of homogeneity, data was subjected to appropriate statistical tests. Two-way ANOVA followed by Bonferroni's post-hoc multiple comparison test was used to determine differences between groups. Two-way repeated measures ANOVA was also used to determine differences between the two groups where appropriate, namely for weight gain and sholl analysis. Statistical significance was accepted for $p<0.05$.

Behavioral outcomes	
EPM	
NSF	
OF	
SCT	
SDT	Two-way ANOVA
TCT	
NOR	
USV's	
FST	
Physiological outcomes	
Weight gain	Two-way repeated measures ANOVA
Water/ Food intake	Two- way ANOVA
Corticosterone	
Molecular outcomes	
RT-PCR	Two-way ANOVA
Morphological outcomes	
Sholl analysis	Two-way repeated measures ANOVA
Morphology	Two-way ANOVA

Appendix II

NEURON simulation environment. Neuron models were created based on the morphology from 3D reconstructed granule neurons obtained from the Golgi analysis. Only granule neurons from the dorsal dentate gyrus were analyzed. Sodium and potassium ion channels were added to the soma and dendrites using Hodgkin and Huxley type models. The reversal potential leak current was set to -65 mV. The protocol consists of a set of stimuli and a set of responses. Two square current pulses with different amplitudes were applied to the soma. For every response we defined a set of features and calculated the scores from the protocol response.

In [1]:
```
%matplotlib inline
import matplotlib.pyplot as plt
%load_ext autoreload
%autoreload
```

In [2]:
```
import bluepyopt as bpop
import bluepyopt.ephys as ephys
```

Setting up the cell model First a template that will describe the cell has to be defined. A template consists of:

the shape of the cell (morphology) the description (equations) of the ion channels on the membrane (mechanisms) the parameters of the model Adding a morphology The shape of a neuron is loaded from a file (SWC or ASC format). The description of the SWC format can be found here.

Below you can see an SWC description of a morphology with a soma with a radius of 10 micron, and a single dendritic section with a length of 100 micron and a radius of 2 micron.

Let's write this to a file so that we can load it in BluePyOpt.

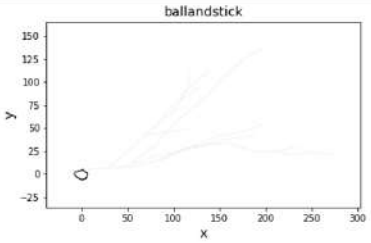
In [4]:
```
morph_swc_string = """
1 1 -7.448 2.109 0.000 0.037 -1
 2 1 1.549 4.806 0.000 0.037 1
 3 1 6.563 0.904 0.000 0.037 2
 4 1 5.310 -4.444 0.000 0.037 3
 5 1 0.590 -6.025 0.000 0.037 4
 6 1 -4.867 -3.465 0.000 0.037 5
 7 1 -8.038 0.678 0.000 0.037 6
 8 4 4.425 2.787 0.000 0.037 7
 9 4 0.661 5.408 0.000 0.037 8
 10 4 29.056 6.854 -0.800 0.037 9
 11 4 41.638 7.640 -1.700 0.037 10
 12 4 52.703 7.682 0.300 0.037 11
 13 4 65.595 10.770 2.150 0.037 12
 14 4 80.197 14.235 2.850 0.037 13
 15 4 84.548 15.516 2.700 0.037 14
 16 4 97.192 18.836 6.100 0.037 15
 58 4 180.937 128.128 1.000 0.037 57
 59 4 196.866 135.434 1.000 0.037 58
 60 4 39.012 15.130 -3.200 0.037 10
 61 4 47.566 21.993 -6.100 0.037 60
 62 4 62.545 36.534 -8.500 0.037 61
 63 4 70.215 42.861 -9.850 0.037 62
 64 4 78.595 43.862 -11.650 0.037 63
 65 4 87.592 44.530 -12.150 0.037 64
 66 4 102.931 46.874 -14.300 0.037 65
 67 4 115.246 49.661 -14.800 0.037 66
 68 4 80.392 52.728 -11.950 0.037 63
 69 4 85.112 56.418 -12.750 0.037 68
 70 4 89.600 61.223 -14.450 0.037 69
 71 4 93.751 60.473 -18.400 0.037 70
 72 4 103.707 78.963 -24.500 0.037 71
 73 4 107.247 82.127 -25.650 0.037 72
 74 4 115.949 84.838 -26.250 0.037 73
 75 4 123.250 90.638 -33.900 0.037 74
 76 4 128.485 95.985 -35.750 0.037 75
 77 4 100.975 87.248 -25.650 0.037 73
 78 4 118.087 95.232 -35.150 0.037 77
 79 4 127.748 102.900 -40.400 0.037 78
 80 4 135.934 110.597 -49.050 0.037 79
 81 4 138.146 112.103 -50.750 0.037 80
 82 4 101.325 69.131 -14.700 0.037 70
 83 4 100.650 82.830 -18.750 0.037 82
 84 4 114.600 99.597 -22.350 0.037 83
 85 4 117.571 103.894 -28.700 0.037 84
 86 4 117.202 113.685 -35.900 0.037 85
"""
with open('ballandstick.swc', 'w') as swc_file:
    swc_file.write(morph_swc_string)
```

In [5]:
```
import neuron
```

In [6]:
```
import neurom as nm
```

In [7]:
```
import pylab as plt
```

In [8]:
```
import neurom
import neurom.viewer
fig, ax = neurom.viewer.draw(neurom.load_neuron('ballandstick.swc'))
```



In [9]:
```
import neurom.viewer
```

In [10]:
```
morph = ephys.morphologies.NrnFileMorphology('ballandstick.swc')
```

By default a Neuron morphology has the following section lists: somatic, axonal, apical and basal. Let's create Location objects that point to the somatic and basal sectionlist. This object will be used later to specify where mechanisms have to be added etc.

In [11]:
```
somatic_loc = ephys.locations.NrnSeclistLocation('somatic', seclist_name='somatic')
dend_loc = ephys.locations.NrnSeclistLocation('basal', seclist_name='basal')
```

Creating a mechanism¶ Now we need to specify which ion channels are present on the membrane of this morphology. The NEURON simulator has a builtin implementation of the Hodgkin-Huxley Sodium (Na), Potassium (K) and leak channels. Let's add this mechanism (called 'hh') to the soma.

The 'name' field can be chosen by the user, this name should be unique. The 'suffix' points to the same field in the NMODL file of the channel. 'locations' specifies which sections the mechanism will be added to.

In [12]:
```
hh_mech = ephys.mechanisms.NrnMODMechanism(
        name='hh',
        suffix='hh',
        locations=[somatic_loc, dend_loc])
```

Creating parameters Next we need to specify the parameters of the model. A parameter can be in two states: frozen and not-frozen. When a parameter is frozen it has an exact value, otherwise it only has some bounds but the exact value is not known yet. Let's define first a parameter that sets the specific capacitance of soma and dendrites to a frozen value. In this tutorial we'll focus on optimizing the parameters the soma. So we also fix the leakage conductance of the dendrite, and we make it passive by disabling the Na and K conductances.

In [13]:
```
cm = ephys.parameters.NrnSectionParameter(
        name='cm',
        param_name='cm',
        value=1.0, # in microfarad/cm2
        locations=[somatic_loc, dend_loc],
        frozen=True)

# Fix leak conductance dendrite
gl_dend = ephys.parameters.NrnSectionParameter(
        name='gl_dend',
        param_name='gl_hh',
        value=1e-5,
        locations=[dend_loc],
        frozen=True)

# Disable Na and K
gnabar_dend = ephys.parameters.NrnSectionParameter(
        name='gnabar_hh_dend',
        param_name='gnabar_hh',
        locations=[dend_loc],
        value=0,
        frozen=True)
gkbar_dend = ephys.parameters.NrnSectionParameter(
        name='gkbar_hh_dend',
        param_name='gkbar_hh',
        value=0,
        locations=[dend_loc],
        frozen=True)
```

Now we set the bounds for the maximal conductances of the sodium and potassium channels in the soma. These two parameters will be optimised later.

In [14]:
```
gnabar_soma = ephys.parameters.NrnSectionParameter(
        name='gnabar_soma',
        param_name='gnabar_hh',
        locations=[somatic_loc],
        bounds=[0.0, 1.0],
        frozen=False)
gkbar_soma = ephys.parameters.NrnSectionParameter(
        name='gkbar_soma',
        param_name='gkbar_hh',
        bounds=[0.0, 1.0],
        locations=[somatic_loc],
        frozen=False)
```

Creating the cell model We put all the components (morphology, mechanisms, parameters) together into a CellModel.

In [15]:
```
ballandstick_cell = ephys.models.CellModel(
        name='simple_cell',
        morph=morph,
        mechs=[hh_mech],
        params=[cm, gnabar_soma, gkbar_soma])
```

In [16]:
```
print(ballandstick_cell)
```

```
simple_cell:
  morphology:
    ballandstick.swc
  mechanisms:
    hh: hh at ['somatic', 'basal']
  params:
    cm: ['somatic', 'basal'] cm = 1.0
    gnabar_soma: ['somatic'] gnabar_hh = [0.0, 1.0]
    gkbar_soma: ['somatic'] gkbar_hh = [0.0, 1.0]
```

Setting up a cell evaluator To optimize the parameters of the cell we need to create an object that will translate parameter values into fitness scores.

This object will need to know which protocols to inject, which parameters to optimize, etc.
Creating the protocols A protocol consists of a set of stimuli, and a set of responses (i.e. recordings). These responses will later be used to calculate the score of the parameter values. Let's create two protocols, two square current pulses at somatic0 with different amplitudes. We first need to create a location object

In [17]:
```
soma_loc = ephys.locations.NrnSeclistCompLocation(
        name='soma',
        seclist_name='somatic',
        sec_index=0,
        comp_x=0.5)
```

and then the stimuli, recordings and protocols. For each protocol we add a recording and a stimulus in the soma. The NrnSquarePulse stimulus represents a current clamp with a square current injection.

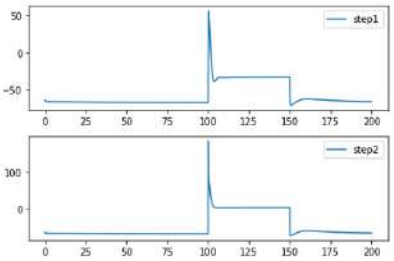
In [18]:
```
sweep_protocols = []
for protocol_name, amplitude in [('step1', 0.1), ('step2', 0.5)]:
    stim = ephys.stimuli.NrnSquarePulse(
                step_amplitude=amplitude,
                step_delay=100,
                step_duration=50,
                location=soma_loc,
                total_duration=200)
    rec = ephys.recordings.CompRecording(
            name='%s.soma.v' % protocol_name,
            location=soma_loc,
            variable='v')
    protocol = ephys.protocols.SweepProtocol(protocol_name, [stim], [rec])
    sweep_protocols.append(protocol)
twostep_protocol = ephys.protocols.SequenceProtocol('twostep', protocols=sweep_protocols)
```

Running a protocol on a cell Now we're at a stage where we can actually run a protocol on the cell. We first need to create a Simulator object (an abstraction of the NEURON simulator).

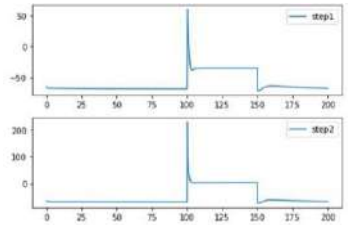
In [19]:
```
nrn = ephys.simulators.NrnSimulator()
```

In [20]:
```
default_params = {'gnabar_soma': 0.25, 'gkbar_soma': 0.1}

responses = twostep_protocol.run(cell_model=ballandstick_cell, param_values=default_params,
sim=nrn)
```

In [21]:
```
# Complete the code below to plot voltage along time
def plot_responses(responses):
    plt.subplot(2,1,1)
    plt.plot(responses['step1.soma.v']['time'], responses['step1.soma.v']['voltage'], label=
'step1')
    plt.legend()
    plt.subplot(2,1,2)
    plt.plot(responses['step2.soma.v']['time'], responses['step2.soma.v']['voltage'], label=
'step2')
    plt.legend()
    plt.tight_layout()

plot_responses(responses)
```



In [22]:
```
other_params = {'gnabar_soma': 0.1, 'gkbar_soma': 0.1}
plot_responses(twostep_protocol.run(cell_model=ballandstick_cell, param_values=other_params,
sim=nrn))
```

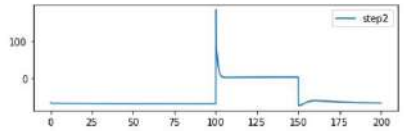


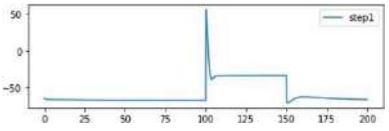
Defining eFeatures and objectives Instead of visually inspecting the traces, we can make use of eFEL (eFeature Extraction Library) to analyse the traces for us. This library can e.g. calculate the number of spikes in a trace, the height of these spikes, etc. https://efel.readthedocs.io/en/latest/eFeatures.html#implemented-efeatures-to-be-continued

In [23]:
```
responses = twostep_protocol.run(cell_model=ballandstick_cell, param_values=default_params,
sim=nrn)
# show where these names come from
step2_time = responses['step2.soma.v']['time']
step2_voltage = responses['step2.soma.v']['voltage']

# Define this dictionary
trace = {'T': step2_time, 'V': step2_voltage, 'stim_start': [100], 'stim_end': [150]}

import efel
# Explain AP_width (from where to where is AP_amplitude...
feature_values = efel.getFeatureValues([trace], ['AHP_depth', 'Spikecount', 'AP_width', 'AP_
amplitude', 'time_to_first_spike', 'time_to_last_spike'])[0]

plot_responses(responses)
print ('Number of spikes in 2nd trace: %s' % feature_values['Spikecount'])
print ('Spike widths (ms) in 2nd trace: %s' % feature_values['AP_width'])
print ('Spike amplitude (mV) in 2nd trace: %s' % feature_values['AP_amplitude'])
print ('time_to_first_spike (ms) in 2nd trace: %s' % feature_values['time_to_first_spike'])
print ('time_to_last_spike (ms) in 2nd trace: %s' % feature_values['time_to_last_spike'])
print ('AHP_depth (mV) in 2nd trace: %s' % feature_values['AHP_depth'])
```

```
C:\Users\susan\anaconda3\lib\site-packages\efel\api.py:480: RuntimeWarning: Error while calcu
lating feature AP_amplitude: AP_amplitude: Error calculating AP_begin_indicies
  RuntimeWarning)
```

```
Number of spikes in 2nd trace: [1]
Spike widths (ms) in 2nd trace: [5.8]
Spike amplitude (mV) in 2nd trace: None
time_to_first_spike (ms) in 2nd trace: [0.1]
time_to_last_spike (ms) in 2nd trace: [0.1]
AHP_depth (mV) in 2nd trace: [71.85729706]
```





Now we will, for every response, define a set of eFeatures we will use for the fitness calculation later. Let's use the SpikeCount feature: the first trace should have 1 spike, the second trace 5.

Different features can be combined together into objectives that will be used by the optimisation algorithm. In this case we will create one objective per feature.

Let's first set our goal. Let's ask for 4 spike in the first trace, and 6 spikes in the second trace:

In [24]:
```
efel_feature_means = {'step1': {'Spikecount': 4}, 'step2': {'Spikecount': 6}}
```

And then we define the eFELFeature and Objective objects:

In [25]:
```
objectives = []
features = []

for protocol in sweep_protocols:
    stim_start = protocol.stimuli[0].step_delay
    stim_end = stim_start + protocol.stimuli[0].step_duration
    for efel_feature_name, mean in efel_feature_means[protocol.name].items():
        feature_name = '%s.%s' % (protocol.name, efel_feature_name)
        feature = ephys.efeatures.eFELFeature(
                    feature_name,
                    efel_feature_name=efel_feature_name,
                    recording_names={'': '%s.soma.v' % protocol.name},
                    stim_start=stim_start,
                    stim_end=stim_end,
                    exp_mean=mean,
                    exp_std=0.05 * abs(mean))
        features.append(feature)
        objective = ephys.objectives.SingletonObjective(
            feature_name,
            feature)
        objectives.append(objective)
```

Creating the cell evaluator We will need an object that can use these objective definitions to calculate the scores from a protocol response. This is called a ScoreCalculator.

In [26]:
```
score_calc = ephys.objectivescalculators.ObjectivesCalculator(objectives)
```

Combining everything together we have a CellEvaluator. The CellEvaluator constructor has a field 'parameter_names' which contains the (ordered) list of names of the parameters that are used as input (and will be fitted later on).

In [27]:
```
cell_evaluator = ephys.evaluators.CellEvaluator(
        cell_model=ballandstick_cell,
        param_names=['gnabar_soma', 'gkbar_soma'],
        fitness_protocols={twostep_protocol.name: twostep_protocol},
        fitness_calculator=score_calc,
        sim=nrn)
```

Evaluating the cell Now we can ask evaluator to calculate the scores for a set of parameters. (The lower the score the better the model). Let's calculate the score of the default_params set we used before, we know that this parameter set generates 1 spike in the first trace, and 5 spikes in the second, so we know that this should generate a perfect score of 0 for step1. For step2 we are searching for a solution with 6 spikes, so the score of our default_params won't be perfect for that trace:

In [28]:
```
print ('Scores:', cell_evaluator.evaluate_with_dicts(default_params))
```

```
Scores: {'step1.Spikecount': 15.0, 'step2.Spikecount': 16.666666666666664}
```

Setting up and running an optimisation As you might have seen in Exercise 1, it's not always trivial to find a parameter value set that matches the objectives. Let's now use the BluePyOpt parameter optimisation algorithm to find a set of parameter values that generates a perfect score of 0 on both traces.

The evolutionary algorithm used by BluePyOpt will sequentially generate of population of individual parameter sets (generations). In every generation a set of parents is selected from the previous population. These parents are used to generate an offspring, and a new population is created based on parents and offspring.

Let us specify that we want to have an offspring size of 10 individuals. This means every population will have 20 individuals (10 parents, 10 offspring).

In [29]:
```
optimisation_algorithm = bpop.deapext.optimisations.IBEADEAPOptimisation(
        evaluator=cell_evaluator,
        offspring_size = 10)
```

Now the optimisation algorithm can be run for a certain number of generations.

Executing the next cell will take some time (a couple of minutes). There will be a [*] shown next to line while it is running.

In [30]:
```
final_pop, hall_of_fame, logs, hist = optimisation_algorithm.run(max_ngen=5)
```

The optimisation has return us 4 objects: final population, hall of fame, statistical logs and history

The final population contains a list of tuples, with each tuple representing the two parameters of the model

In [31]:
```
print('Hall of fame: ')
for ind in hall_of_fame:
    print ('gnabar_soma=%f, gkbar_soma=%f' % tuple(ind))
```

```
Hall of fame:
gnabar_soma=0.748723, gkbar_soma=0.022405
gnabar_soma=0.207757, gkbar_soma=0.028347
gnabar_soma=0.498938, gkbar_soma=0.000384
gnabar_soma=0.703079, gkbar_soma=0.104288
gnabar_soma=0.823264, gkbar_soma=0.025338
gnabar_soma=0.760910, gkbar_soma=0.124975
gnabar_soma=0.694447, gkbar_soma=0.104288
gnabar_soma=0.575417, gkbar_soma=0.359763
gnabar_soma=0.658400, gkbar_soma=0.786689
gnabar_soma=0.228762, gkbar_soma=0.945271
```

The best individual found during the optimisation is the first individual of the hall of fame

In [32]:
```
best_ind = hall_of_fame[0]
print('Best individual:  ', best_ind)
```

```
Best individual:    [0.7487227668107452, 0.022404960210796798]
```

We can evaluate this individual and make use of a convenience function of the cell evaluator to return us a dictionary of the scores.

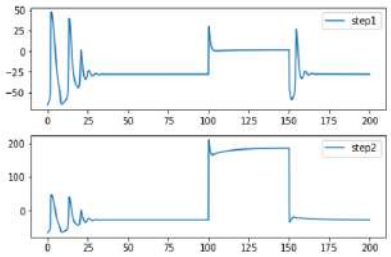
In [33]:
```
best_ind_dict = cell_evaluator.param_dict(best_ind)
print (best_ind_dict)
```

```
{'gnabar_soma': 0.7487227668107452, 'gkbar_soma': 0.022404960210796798}
```

As you can see the evaluation returns the same values as the fitness values provided by the optimisation output. We can have a look at the responses now.

In [34]:
```
responses = twostep_protocol.run(cell_model=ballandstick_cell, param_values=best_ind_dict, s
im=nrn)
print ("Score: ", score_calc.calculate_scores(responses))
plot_responses(responses)
```

```
Score:  {'step1.Spikecount': 5.0, 'step2.Spikecount': 3.333333333333333}
```



And indeed, as requested, the algorithm found a solution with 4 spikes in step1 and 6 spikes in step2

Let's have a look at the optimisation algorithm statistics. We can plot the minimal score (sum of all objective scores) found in every generation.

In [35]:
```
import numpy
gen_numbers = logs.select('gen')
min_fitness = logs.select('min')
max_fitness = logs.select('max')
plt.plot(gen_numbers, min_fitness, label='min fitness')
plt.xlabel('generation #')
plt.ylabel('score (# std)')
plt.legend()
plt.xlim(min(gen_numbers) - 1, max(gen_numbers) + 1)
plt.ylim(0.9*min(min_fitness), 1.1 * max(min_fitness))
```

Loading the NEURON simulator

In [1]:
```
import neuron
```

In [2]:
```
print (neuron.h)
# Load external files
neuron.h.load_file("stdrun.hoc");
```

```
<TopLevelHocInterpreter>
```

In [3]:
```
neuron.h.stdinit();
```

Constructing a neuron consisting of one compartment

In [4]:
```
soma = neuron.h.Section(name='soma')

print ("Soma object:", soma)
print ("Soma object name: ", soma.name())

print ("Number of segments in the soma:", soma.nseg)
```

```
Soma object: soma
Soma object name:  soma
Number of segments in the soma: 1
```

In [5]:
```
soma.L = 14.6
soma.diam = 10.1
print ("Soma length: %f micron" % soma.L)
print ("Soma diameter: %f micron" % soma.diam)
```

```
Soma length: 14.600000 micron
Soma diameter: 10.100000 micron
```

In [6]:
```
soma_area_eq = 2 * neuron.h.PI * soma.L * soma.diam / 2
print ("Soma area according to cylinder surface area equation: %f micron^2" % soma_area_eq)

# The 0.5 refers to the segment in the middle of the soma
# Because there is only one segment, in this case it refers to the entire soma
soma_area = neuron.h.area(0.5, sec=soma)
print ("Soma area according to NEURON: %f micron^2" % soma_area)

print ("Both values match: %s" % (soma_area_eq == soma_area))
```

```
Soma area according to cylinder surface area equation: 463.259253 micron^2
Soma area according to NEURON: 463.259253 micron^2
Both values match: True
```

In [7]:
```
soma_sphere_area_eq = 4 * neuron.h.PI * pow(soma.diam / 2, 2)
print ("Soma area according to sphere surface area equation: %f micron^2" % soma_sphere_area
_eq)
```

```
Soma area according to sphere surface area equation: 320.473867 micron^2
```

In [8]:
```
print ("Specific capacitance: %f uf/cm2" % soma.cm)
```

```
Specific capacitance: 1.000000 uf/cm2
```

In [9]:
```
soma_tcap = (soma.cm * (soma_area / pow(1e4, 2)))
print ("Total soma capacitance: %f uf" % (soma.cm * (soma_area / pow(1e4, 2))))
```

```
Total soma capacitance: 0.000005 uf
```

Running a simulation

In [10]:
```
print ("Membrane voltage soma: %f mV" % soma(.5).v) # mV
```

```
Membrane voltage soma: -65.000000 mV
```

In [11]:
```
print ("Current time: %f ms" % neuron.h.t) # ms
```

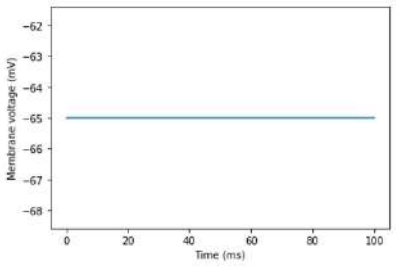
```
Current time: 0.000000 ms
```

In [12]:
```
neuron.h.tstop = 100
print ("Simulation stop time: %f ms" % neuron.h.tstop)
print ("Integration time step: %f ms" % neuron.h.dt)
```

```
Simulation stop time: 100.000000 ms
Integration time step: 0.025000 ms
```

In [13]:
```
time = neuron.h.Vector()
voltage = neuron.h.Vector()

time.record(neuron.h._ref_t)
voltage.record(soma(.5)._ref_v);
```

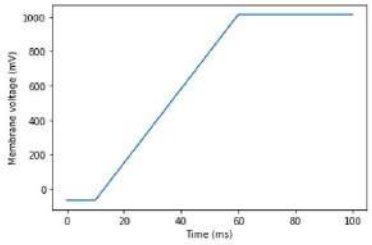
In [14]:
```
neuron.h.run()

def plot_tv(time_array, voltage_array, show=True, label=None, constants=[]):
    import matplotlib.pyplot as plt
    import numpy
    plt.plot(time_array, voltage_array, label=label)
    for constant in constants:
        plt.plot(time_array, constant*numpy.ones(len(time_array)))
    plt.xlabel('Time (ms)')
    plt.ylabel('Membrane voltage (mV)')
    if show:
        plt.show()

plot_tv(time, voltage)
```



Injecting a current

In [15]:
```
iclamp = neuron.h.IClamp(.5, sec=soma)
iclamp.amp = 0.1 # nA
iclamp.delay = 10 # ms
iclamp.dur = 50 # ms
```

In [16]:
```
neuron.h.run()

plot_tv(time, voltage)
```



In [17]:
```
V1 = -65 # Voltage before stimulus, mV
V2 = soma.v # Voltage after stimulus, mV
deltaV = V2 - V1 # Voltage difference, mV
Im = iclamp.amp # nA
deltaT = iclamp.dur # ms
soma_tcap # total soma membrane capacitance, uF

deltaV_eq = Im * deltaT / soma_tcap # in nA * ms / uF == microvolt
deltaV_eq /= 1e3 # Correction factor to get mV

print ('Observed dV: %f mV' % deltaV)
print ('Calculated dV: %f mV' % deltaV_eq)
print ('Simulated dV matches equation dV: %s' % (deltaV - deltaV_eq < 1e-6))
```

```
Observed dV: 1079.309257 mV
Calculated dV: 1079.309257 mV
Simulated dV matches equation dV: True
```

Adding a leak conductance

In [18]:
```
soma.insert('hh');
```

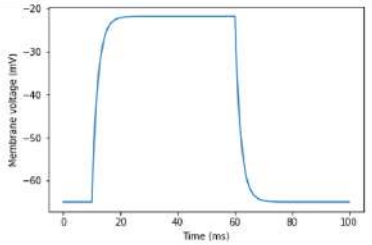
In [19]:
```
soma.gkbar_hh = 0.0
soma.gnabar_hh = 0.0
```

In [20]:
```
soma.gl_hh = 5e-4 # Leak conductance, S/cm^2
```

In [21]:
```
el = soma.el_hh = -65 # Reversal potential leak current, mV
print ("Reveral of leak current: %f mV" % el)
```

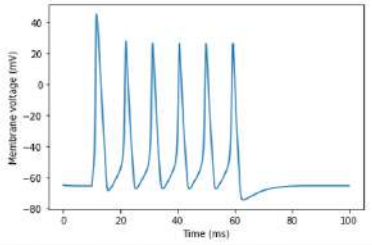
```
Reveral of leak current: -65.000000 mV
```

In [22]:
```
soma.gkbar_hh = 0.0
soma.gnabar_hh = 0.0

neuron.h.tstop = 100

neuron.h.run()

plot_tv(time, voltage)
```



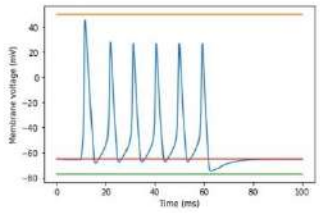
Adding active ion channels

In [23]:
```
soma.gkbar_hh = 0.01 # in S/cm^2
soma.gnabar_hh = 0.1

neuron.h.run()

plot_tv(time, voltage)
```



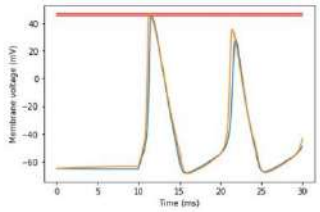
In [24]:
```
ena = soma.ena # explain e_na
ek = soma.ek
el = soma.el_hh

print ("Na reversal: %f" % ena)
print ("K reversal: %f" % ek)
print ("Leak reversal: %f" % el)

plot_tv(time, voltage, constants=[ena, ek, el])
```

```
Na reversal: 50.000000
K reversal: -77.000000
Leak reversal: -65.000000
```

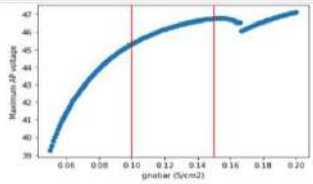


Parameter sensitivity

In [25]:
```
import matplotlib.pyplot as plt
import numpy

for gnabar in [0.1, 0.15]:
    soma.gkbar_hh = 0.01
    soma.gnabar_hh = gnabar

    neuron.h.tstop=30

    neuron.h.run()

    plt.plot(time, max(voltage)*numpy.ones(len(time)), 'r')
    plot_tv(time, voltage, show=False)

plt.show()
```



In [26]:
```
soma.gkbar_hh = 0.01

# definitely mention critical value where state changes
# show something with percentages
# show value we were using before on plot

max_voltages = []
import numpy
gnabar_range = numpy.arange(.05, 0.2, 0.001)
for gnabar in gnabar_range:
    soma.gnabar_hh = gnabar

    neuron.h.run()

    max_voltages.append(max(voltage))

plt.plot(gnabar_range, max_voltages, 'oC0')
plt.xlabel('gnabar (S/cm2)')
plt.ylabel('Maximum AP voltage')
for xs in [0.1, 0.15]:
    plt.axvline(x=xs, color='r')
plt.show()
```



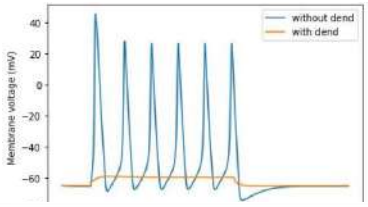
In [27]:
```
# linear_coef = numpy.polyfit(gkbar_range, max_voltages, 1)

# print 'Linear equation max_voltage = %f*gkbar + %f' % tuple([x for x in linear_coef])
```

Extending the model with a dendrite

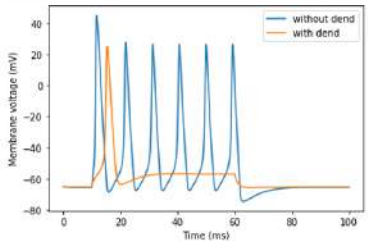
In [28]:
```
dend = neuron.h.Section(name='dend')

dend.connect(soma)

dend.L = 1079 # micron
dend.diam = 2.0 # micron
dend.nseg = 8 # number of segments in the dendritic section
```

In [29]:
```
dend.insert('hh')
dend.el_hh = -65 # Reversal potential leak current, mV
dend.gl_hh = 5e-4 # Leak conductance, S/cm^2

dend.gkbar_hh = 0.0
dend.gnabar_hh = 0.0
```

In [30]:
```
for with_dend in [False, True]:
    neuron.h.tstop = 100

    soma.gkbar_hh = 0.01
    soma.gnabar_hh = 0.1

    if with_dend:
        dend.connect(soma)
    else:
        neuron.h.disconnect(sec=dend) # disconnect dend for now

    neuron.h.run()

    plot_tv(time, voltage, show=False, label='with dend' if with_dend else 'without dend')

plt.legend()
plt.show()
```

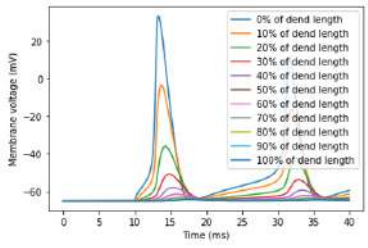


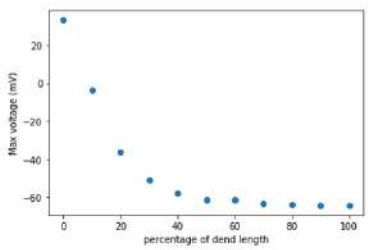
In [31]:
```
dend_ra = 100
dend_gl = 5e-4

for with_dend in [False, True]:
    # For every addition of mechanism create figure show newer model
    # Lines for reversal potentials Na, K and leak
    neuron.h.tstop = 100

    soma.gkbar_hh = 0.01
    soma.gnabar_hh = 0.1

    dend.el_hh = -65 # Reversal potential leak current, mV
    dend.gl_hh = dend_gl # Leak conductance, S/cm^2
    dend.Ra = dend_ra

    if with_dend:
        dend.connect(soma)
    else:
        neuron.h.disconnect(sec=dend) # disconnect dend for now

    neuron.h.run()

    # Convert the NEURON vectors to numpy arrays
    time_py = time.to_python()
    voltage_py = voltage.to_python()

    plot_tv(time_py, voltage_py, show=False, label='with dend' if with_dend else 'without de
nd')

plt.legend()
plt.show()
```



Voltage attenuation along the dendrite

In [32]:
```
dend.Ra = 200
dend.gl_hh = 5e-4

voltage_dend = {}

# distances are in percentage of dendritic length
# 1.0 is at end of dendrite, 0.0 at connection with soma
distance_range = numpy.arange(0, 1.1, 0.1)
for distance in distance_range:
    voltage_dend[distance] = neuron.h.Vector()
    voltage_dend[distance].record(dend(distance)._ref_v);

neuron.h.tstop = 40
neuron.h.run()

for distance in distance_range:
    plot_tv(time, voltage_dend[distance], show=False, label='%.0f%% of dend length' % (dista
nce*100))

plt.legend()
plt.show()

max_voltage_dend = []
for distance in distance_range:
    max_voltage_dend.append(max(voltage_dend[distance]))

plt.plot(distance_range*100, max_voltage_dend, 'o')
plt.xlabel('percentage of dend length')
plt.ylabel('Max voltage (mV)')
plt.show()
```





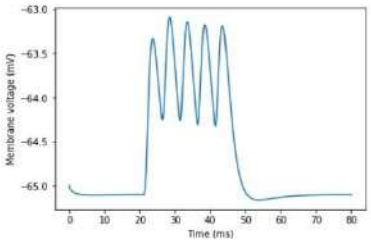
Adding a synapse

In [33]:
```
expsyn = neuron.h.ExpSyn(.5, sec=dend) # We add a synapse to the middle (.5) of the dendrite
```

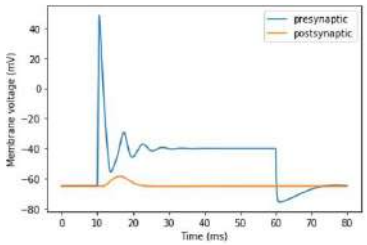
In [34]:
```
netstim = neuron.h.NetStim()
netstim.interval = 5
netstim.number = 5
netstim.start = 20
netstim.noise = 0
```

In [35]:
```
netcon = neuron.h.NetCon(netstim, expsyn)
netcon.weight[0] = 1.0
```

In [36]:
```
iclamp.amp = 0
neuron.h.tstop = 80
neuron.h.run()

plot_tv(time, voltage)
```



In [37]:
```
soma_pre = neuron.h.Section(name='soma')
soma_pre.L = 14.6
soma_pre.diam = 10.1
soma_pre.insert('hh')

iclamp_pre = neuron.h.IClamp(.5, sec=soma_pre)
iclamp_pre.amp = 1.0 # nA
iclamp_pre.delay = 10 # ms
iclamp_pre.dur = 50 # ms

time_pre = neuron.h.Vector()
voltage_pre = neuron.h.Vector()

time_pre.record(neuron.h._ref_t)
voltage_pre.record(soma_pre(.5)._ref_v);

expsyn.tau = .9
netcon_pre = neuron.h.NetCon(soma_pre(.5)._ref_v, expsyn, sec=soma_pre)
netcon_pre.weight[0] = 1

if 'netstim' in locals():
    del netstim
if 'netcon' in locals():
    del netcon

neuron.h.run()

time_py = time.to_python()
voltage_py = voltage.to_python()

plot_tv(time_pre, voltage_pre, show=False, label='presynaptic')
plot_tv(time, voltage, show=False, label='postsynaptic')
plt.legend()
plt.show()

del netcon_pre
```



Appendix III

The neglected gender: a review on female animal models of depression

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Abstract

Major depression (MD) is the most common psychiatric disorder predicted to affect around 264 million people worldwide. Though the etiology of depression remains elusive, the interplay between genetics and environmental factors, such as early life events, stress, exposure to drugs and health problems appears to underly its development. Whereas depression is twice more prevalent in women than in men, most preclinical studies are performed in male rodents. In fact, females' physiology and reproductive experience are associated with changes to brain, behavior and endocrine profiles that may influence both stress, an important etiological factor for depression, and treatment response.

In this review, we provide an overview of female animal models of depression highlighting the major findings in depression-related models but also the major gaps in research, attending to factors such as the role of protocol variability and sex differences. Given the phenotypic differences found between models and the little information concerning female animal models, we aim to emphasize the need for focusing on specific pathophysiological mechanisms of depression in females.

Keywords (3-12): Major depression, Females, Animal models, Chronic stress, Social stress, Genetic models, sex differences.

Introduction

Major depression (MD) is the most common psychiatric disorder predicted to affect more than 260 million people worldwide (GBD 2017). The symptoms of depression, as stated in the Diagnostic and Statistical Manual of Mental Disorders (DSM–V), include, depressed mood, markedly diminished pleasure or interest in activities (anhedonia), significant weight gain or weight loss, excessive guilt, loss of energy and diminished capacity to think or concentrate (DMS-5, 2013). To be diagnosed with major depression, the individual must be experiencing five or more symptoms during the same 2-week period and at least one of the symptoms should be either depressed mood or anhedonia (DMS-5, 2013). Moreover, depression can significantly increase the risk of other causes of mortality (Gilman et al., 2017).

Though the etiology of depression remains elusive, the interplay between genetics and environmental factors, such as early life events, exposure to stress and/or drugs and other health problems appear to underly its development (Bembnowska et al., 2015). People whose first-degree relatives suffer from MD are estimated to be 1.5 to 3 times more probable to develop depression. However, heritability alone cannot justify the development of major depression, as 60% of the factors involved in its etiology are explained by other factors (Flint and Kendler., 2014). Additionally, there is evidence of a bidirectional correlation between depression and other disorders, including diabetes, drug or alcohol addiction and cancer, as each condition seems to impact negatively on the other (Katon, 2003; Ramsubbu et al., 2012).

Before puberty, depression is rare and develops at about the same frequency in girls and boys. However, with the onset of adolescence, the risk of developing MD increases in women (**Fig.1**.; Albert, 2015; Breslau et al., 2017). In adulthood, MD is twice more prevalent in women than in men (Albert, 2015). Reproductive hormones, genetics, environmental variables and also the social context, which may be unique to women's life experience, seem to be contributing factors for this disparity (**Table 1;** adapted from Stegenga et al., 2012 and Martin el al., 2013).

A previous study has identified risk factors for MD in patients of a cohort study - PredictD (Stegenga et al., 2012). Risk differences for the onset of MD in male and female were assessed for 35 risk factors from 7101 participants, of both genders, without MD at baseline (Stegenga et al., 2012). Twenty-eight risk factors (80.0%) had a stronger impact in women than in men (Stegenga et al., 2012). Risk factors such as non-European ethnicity, lower levels of education, religious or spiritual beliefs, lifetime alcohol problem, two or more recent life events, a neighborhood perceived as not being safe, financial strain and problems with someone close, had a significantly stronger impact in women. In men, living alone and a nonprofessional occupation had a significantly larger impact (Stegenga et al., 2012).

Gender differences in symptom profiles of major depression in Korea were explored in a study by kim et al. (2015) (Kim et al., 2015). Women with MD were associated with increased incidence of hypersomnia, fatigue, psychomotor retardation and suicidal attempts, suggesting a higher occurrence of atypical

symptoms. In the same analysis, men with MD seemed more susceptible to reduced libido.

Other studies have also proposed that the increased incidence of MD in women is linked with hormonal changes, mainly throughout puberty, previous to menstruation, subsequent to pregnancy and at perimenopause (Halbreich, 2000). This disease-incidence correlation implies that female hormonal fluctuations could also be a trigger for MD (Albert, 2015). Given that hormones have been hypothesized to interfere in modulation of endocrine, immune and neurotransmitter systems, it can be hypothesized that female prevalence of atypical depression is associated with the action of those hormones (Antonijevic, 2006; Angst et al., 2002; Silverstein, 2002).

The serotonergic system is also controlled by female gonadal steroid hormones. These hormones have de ability to modify density and function of pre- and post-synaptic serotonin receptors and transporters (Klink, Robichaud and Debonnel, 2002). Remarkably, women have increased somatodendritic 5-HT1A receptor binding than men (Parsey et al., 2002). Serotonin 5-HT1A heteroreceptors also influence neuroplasticity in the dorsal raphe 5-HT nerve cells and in the hippocampus (Borroto-Escuela et al., 2018). Oestrogen stimulates GABAergic transmission, suggesting that females, particularly during the pre-menopausal years, are at a bigger risk for decreased serotonergic neurotransmission (Herbison and Fenelon, 1995). This sequentially might benefit HPA hypoactivity and a bigger risk to develop atypical depression (Antonijevic, 2006).

The molecular signature of MD is also different between men and women (Seney et al., 2018). Gene expression alterations were found to be regulated differently

in men and women in 52 genes; men with MD had decreased synapse-related genes, while women displayed expression increases in this pathway (Seney et al., 2018). Cell type analysis also showed that men with MD exhibited increased expression of microglia- and oligodendrocyte-related genes, wheras women had decreased expression of these cell types (Seney et al., 2018).

Given the limitations of studying the pathophysiology of MD in humans, most of the constructs for therapeutic development come from animal models, namely rodent models. Considering the numerous environmental, biological and social risk factors for depression, preclinical research implies the use of multivariable models that integrate these factors. The validity of an animal model may be attained by the evaluation of four major criteria: predictive validity, face validity, construct validity and etiological validity (Belzung and Lemoine, 2011; Abelaira, Reus and Quevedo, 2013). Briefly, animal models of depression should mimic the human condition in the following aspects, including, 1) improvement or reduction of behavioral signs by clinical effective antidepressant therapies (predictive validity); 2) resemblance between the clinical-symptom profile and the behavioral phenotype (face validity), 3) similarity between neurobiological substrates (construct validity), and 4) triggering of the disease in the same manner as the human disorder (etiological validity)(Abelaira, Reus and Quevedo, 2013). The more criteria an animal model fulfills, the more precise and consistent is the data it produces (Belzung and Lemoine, 2011; Abelaira, Reus and Quevedo, 2013). Though fully recapitulating the complexity of the human disease is not possible, specific symptoms or a subset of symptoms can be successfully modeled in animals. Moreover, animal models of depression can be developed by exposure to known etiological factors of depression, such as chronic stress,

selective breeding, genetic manipulations and pharmacological administration (Wang et al., 2017).

The most widely used animal models of MD generally rely on exposure to stressful stimuli and aversive psychosocial experiences, for example neglect, interpersonal violence, or separation, that induce behavioral or physiological changes, similar to those of the human disease (Berton et al., 2012).

Sex differences have been a commonly disregarded factor in most of these models, with most basic and preclinical studies being conducted in male animal models, putatively leading to a biased characterization of the disease and treatment response (Wang et al., 2017; Halbreich, 2000). Neurodevelopmental determinants, as well as reproductive maturation and experience, are important mediators of changes in neural plasticity, circuitry and behavior that may influence both stress and treatment response (Halbreich, 2000; Pooley et al., 2018). For this reason, studies in female animal models of depression, should be prioritized for a better understanding of the disease pathophysiology.

Previous review articles have discussed the existing models and suggested the need to better define clinically relevant symptoms based on sex disparities, clinically relevant risk factors and improved the translation and design of clinical trials (Harro, 2018; Rygula et al., 2018; Wang et al., 2017; Yin et al., 2016; Planchez et al., 2019). Because animal models have, on numerous occasions, been unsuccessful in modeling depression and ineffective in predicting response to therapy, reviewing animal-based research literature may lead to a more comprehensive understanding of the field (Greek and Menache, 2013). In this review, we sought to provide an overview of female animal models of depression

to highlight the major findings in depression models but also the major gaps in the field, attending to factors such as protocol variability and sex differences.

Material and Methods

PubMed and Cochrane Library database were used to identify relevant studies describing animal models of depression in females, published before November 2019. Potentially pertinent papers were primarily identified through title and abstract searches, the full text and reference list of the selected articles was then assessed. Two search strategies were used: 1) Combination of MeSH Major Topic "animal disease models" and Mesh Major Topic “depressive disorder", filtering the results to only females and other-animals; 2) Combination of Mesh term "Animals" and search terms "depressive-like AND females" (**Fig. 2**). The inclusion criteria were: 1) Studies regarding only females or in which females and males were analyzed separately; 2) Studies in which depression was validated by behavioral outcomes; 3) Studies regarding only major depression. Different species were found across studies and included, namely *Phodopus sungorus, Microtus ochrogaster, Macaca mulatta, Rattus norvegicus and Mus musculus.*

The study exclusion criteria were: 1) Animal models of depression following reproductive experience, namely pregnancy or post-partum; 2) Ovariectomized rodents; 3) Articles which made no distinction between males and females on the behavioral outcomes; 4) Studies with endophenotypes representing more than one psychiatric disorder.

Data was extracted using a multistep process of study selection centered on search strategy, abstract screening, full-text article screening and eligibility of the studies based on the defined inclusion and exclusion criteria. The following data was extracted from each study: animal model, method of induction, animal-related variables (species, strain, age and diet), sample size, protocol duration and outcome measures.

Results

Forty-three studies were included in this review after a careful selection of the studies (**Fig. 2**). Since behavioral and physiological traits of major depression can be induced with different paradigms, female animal models of depression were divided in six main classes based on the nature of the etiological factor used (**Table 2**):

1. Stress-based animal models
    i) Maternal separation animal models;
    ii) Social stress animal models;
    iii) Acute and chronic stress animal models;
2. Pharmacologically-induced animal models;
3. Pathology-induced animal models;
4. Genetic animal models;

Thirty-one studies reporting stress-based animal models in females were found, of which twenty-one are stress animal models, five are maternal separation animal models and another five are from acute and chronic stress animal models. Pharmacologically-induced animal models were reported in five studies,

pathology-induced animal models in two studies and genetic animal models in five studies.

Behavioral outcomes in animal models of depression

Establishing an animal model of such complex disorder may be a challenging task, given that animal models may not be able to display the complex cognitive and emotional traits that characterize the disease, even because some of these are presumably limited to humans. Notwithstanding, preclinical models can display some of the core behavioral and physiological traits of depression, referred to as endophenotypes (Gould and Gottesman, 2006), including anhedonia, appetite and sleep disturbances, behavioral despair and anxiety-like behaviors (Krishnan et al., 2011). Moreover, a large variety of behavioral tests can be used to evaluate such traits (Krishnan et al., 2011). One of the core symptoms of depression, anhedonia, or the diminished capacity to experience pleasure, is usually assessed in preclinical models using the state-of-the-art sucrose preference test (Mateus-Pinheiro et al., 2013). This test is frequently used along with measures of depressive-like behavior. Testing depressive-like behavior in rodents can be attained through the forced swim and tail suspension tests; Though this concept has been highly disputed, some authors assume immobility in these tests as a measure of "behavioral despair", (Belovicova et al., 2017). Anxiety is highly comorbid with depression. Most behavioral tests used to evaluate anxiety-like behavior in rodents, rely on the conflict between the natural willingness to explore novel environments and potential threat it may pose

(Belovicova et al., 2017); these include the novelty suppressed feeding test, the elevated plus maze test or the open field test.

Memory and learning deficits are also found in depressed individuals and these have been shown to play a significant role in the risk of relapse and therapeutic response, suggesting a correlation between these changes and the pathophysiology of the disease (Castaneda et al., 2015). Importantly, these deficits can also be measured in animal models of depression using cognitive tasks to assess different types of memory and behavioral flexibility (Bushnell, 1999).

MD can significantly change the hierarchy in human social groups and this may be reflected in social behavior of animal models of depression (Ellenbroek and Youn, 2016). A simple test of sociability comprehends measuring the time a mouse spends exploring either a novel mouse or an empty container.

All animal models were evaluated according to the phenotype and methodology used in an attempt to clarify the impact of different etiological factors in the development of MD.

1. Stress-based animal models

Stress is one of the most widely used etiological factors in the development of rodent models of depression (Kessler, 1997). Stress-based models use very different types of stress and use environmental challenges that rodents meet, including social defeat and unpredictable stressors over a long time period

(Koolhaas et al., 2017). The severity, duration and unpredictability of the applied stressors are essential parameters to consider in these models (Koolhaas et al., 2017). Only a few protocols of stress are currently validated for females, namely social isolation, chronic social instability stress, unpredictable chronic mild stress, photoperiod manipulation, single prolonged stress, chronic juvenile stress and maternal separation. In the next section, we will approach some of these models of stress in females.

i) Maternal Separation

In animal models of depression, the timing of stress exposure throughout the rodent's lifetime seems to be one of the most relevant factors for the development of the maladaptive responses leading to dysfunction and behavioral alterations akin to depression (Novais et al., 2009). Prolonged periods of maternal separation (MS) induce high levels of stress in the offspring (Lehmann and Ferdon, 2000). The consequences of maternal separation are expressed in adulthood and persist for life, and include, among others, an hyperreactivity of the hypothalamic-pituitary-adrenal (HPA) axis.

Female rats exposed to MS for 12 to 22 days, immediately after birth (0-2 days) were not affected differently according to the time of the behavioral tests. Behavioral tests performed in stressed females at 36 days old reported increased depressive-like behavior following MS (Leussis et al., 2012). However, behavioral tests performed between 62 and 67 days old observed decreased anxiety and depressive-like behavior with very similar protocols (Dimatelis et al., 2015; Donmez et al., 2015).

In a different protocol, maternal separation for 24h in 9 days-old male and female mice led to changes in expression of about 347 genes (Malki et al., 2014).

The results suggest that MS causes molecular and behavioral alterations up until 36 days after stress, but at 62 days the animals may be capable of recovering.

ii) Social stress animal models

Chronic social stress is possibly the most common etiological factor of depressive disorders in humans (Kessler, 1997). In preclinical studies, social defeat is a frequently used social stress in male rodents (Miczek, 1979). This model, based on a resident-intruder paradigm, where male rodents interact aggressively to establish dominance (Miczek, 1979), is not suitable to induce stress in females rodents since they do not exhibit territorial hostility (Haller et al., 1999). Though female rodents establish consistent social structures, their hierarchies seem less steep and despotic when compared to male hierarchies (Williamson et al., 2019).

Pre-clinical models of depression based on social stress in females mostly rely on social isolation and chronic social instability (Goñi-Balentziaga et al., 2018). Social isolation (SI) consists in the total isolation of an animal in regular home cages with access to water and food *ad libitum*. SI results in neurochemical and neuroendocrine changes, as well as anatomical, physiological and behavioral alterations in both animal and humans (Goñi-Balentziaga et al., 2018).

Praire voles (*Microtus ochrogaster*) isolated for 28 days developed anhedonia, increased aggression, as well as depressive- and anxiety-like behavior (Grippo

et al., 2008). Contrarily, female rats (*Rattus norvegicus*) isolated for only 5 days already develop anxiety-like behavior that was accompanied by decreased levels of spinophilin in the prefrontal cortex, a protein important for the regulation of the synaptic cytoskeleton and depressive-like behavior (Allen, Ouimet, & Greengard, 1997; Leussis and Andersen, 2008). When isolated for 30 days they develop high corticosterone levels and deficiency of glucocorticoid receptor (GR) and Corticotropin Releasing Hormone Receptor 1 (CRHR1) (Pisu et al., 2016). Female mice (*Mus musculus*) also had increased depressive-like behavior when isolated for 35 days (Martin and Brown, 2010). Russian dwarf hamsters (*Phodopus sungorus*) isolated for 21 days revealed decreased sociability along with increased norepinephrine levels in the diencephalon (Jacqueline, 1984). While other physiological parameters, such as food intake and body weight, were not affected by these social isolation paradigms, none of these studies evaluated cognitive performance. Overall, chronic but not acute, social isolation appears to be an effective stressor across rodent species, causing both behavioral and molecular deficits akin to human depression.

Yet, another commonly used social stress protocol is the chronic social instability stress (CSIS), which not only includes periods of isolation but also overcrowding. This chronic stress paradigm has been shown to induce HPA axis activation and anxiety-like behavior in females (Herzog et al., 2009). At least two different protocols duration have been described in rats, either alternation between isolation and overcrowding during 15 days or during 28 days, with impacts in terms of behavioral and other physiological outcomes. Adult female rats exposed to CSIS for 15 days revealed decreased weight gain and social interaction, decreased neurogenesis, neuronal atrophy and increased number of

vocalizations (Baranyi et al., 2015; McCormick et al., 2013). Another study using the same protocol but in adolescent female rats, showed similar impairments with decreased neurogenesis and decreased short-term memory (McCormick et al., 2009). None of these 15 days-protocols found anxiety-like deficits.

On the other hand, female rats exposed to the 28 days CSIS protocol presented decreased food intake, increased anxiety and decreased rearing times, accompanied by increased corticosterone levels and adrenal gland weight, increased body temperature and dysregulated estrous cycle (Herzog et al., 2009; Nowacka et al., 2014; Nowacka-Chmielewska et al., 2017a; Nowacka-Chmielewska et al., 2017b; Nowacka-Chmielewska et al., 2017c; Pittet et al., 2017). Contrary to what was observed on the 15-days protocol, weight gain changes were not detected. Moreover, while BDNF and NGF expression in the amygdala and hippocampus was not affected, VEGF expression in the amygdala, hippocampus and hypothalamus was increased (Herzog et al., 2009; Nowacka et al., 2014; Nowacka-Chmielewska et al., 2017a; Nowacka-Chmielewska et al., 2017b). VEGF has been shown to impact synaptic transmission and serve as a neurotrophic factor in hippocampal neurogenesis (Jin et al., 2002; McCloskey, 2005), while also influencing memory and learning (Cao et al., 2004).

In mice, CSIS protocols duration ranges from 28 days to 49 days, with consistent results, including decreased weight gain and food intake, increased anxiety and decreased sociability, that is accompanied by increased levels of plasma corticosterone and decreased hippocampal neurogenesis (Saavedra-Rodríguez and Feig, 2013; Jarcho et al., 2015; Labaka et al., 2017; Yohn et al., 2019). At the molecular level, expression of GR in the hypothalamus and IL-10 in the

hippocampus were reduced (Labaka et al., 2017). The increased plasma corticosterone and the lower hypothalamic GR expression are suggestive of an HPA hyperactivity, which is also a common feature of human depression. Interestingly, and similar to rats, the estrous cycle was also dysregulated (Labaka et al., 2017). Together, these results suggest that the duration of the CSIS protocol is determinant for the development and installation of anxiety-like behavior, as well as other physiological alterations that were only observed when longer protocol periods were applied. This is in line with other studies showing that, not only the type but also the duration of stress exposure, is a major factor; it's only when stress exceeds a certain duration or intensity that it starts eliciting maladaptive responses thus triggering the development of neuropathological scenarios (Sousa, 2016; Sousa and Almeida, 2012).

Moreover, SI and CSIS seem to produce slightly different phenotypes in female rodents. While SI did not affect physiological measures but induced anhedonia, depressive- and anxiety-like behavior and decreased sociability, CSIS induced anxiety-like behavior, weight gain, estrous cycle regulation, temperature and neurogenesis, but not depressive-like behavior. Though being both social-based stressors, they present fundamental differences and seem to impact on distinct neural substrates.

iii) Acute or chronic stress animal models

Apart from socially-induced stress, other stressful experiences have been also shown to be critical to the pathogenesis and development of numerous

psychiatric disorders, namely major depression, schizophrenia, and anxiety (Heim and Nemeroff, 1999).

Animal models that intend to assess behavioral changes induced by stress exposure vary in the nature of stressors and their duration (Campos et al., 2004). Here, we gathered data from both acute and chronic stress models, namely unpredictable chronic mild stress (uCMS), photoperiod manipulation, single prolonged stress and chronic juvenile stress.

The unpredictable chronic mild stress (uCMS) is one of the most widely used models to study the neurobiological underpinnings of depression, however, mostly described in male rodents (Frisbee et al., 2015).

Amongst the collected studies, uCMS protocols performed in female mice were applied during 14 or 28 days. UCMS induced the dysregulation of 350 genes after 14 days and anhedonia, anxiety- and depressive-like behavior after 28 days (Malki et al., 2014; Zhu et al., 2014). Female rats exposed to uCMS during 42 days also developed depressive-like behavior, anhedonia, increased GFAP and CB1R protein expression, as well as a decrease of CB2R (Marco et al., 2017). Even though only three studies were evaluated, the deficits were consistent across studies and species indicating that this protocol may also be suitable for females.

Photoperiod manipulation is another protocol capable of inducing depression-related symptoms (Qin et al., 2015), as the length of the lighting period per day is considered as one of the main etiological factors of a subtype of depression – the seasonal affective disorder (SAD) (Shelton, Hartmann and Allen, 2002). In

fact, changes in circadian rhythm have been shown to strongly impact on the individual's psychological and physical homeostasis (Shelton, Hartmann and Allen, 2002). Though it is a good protocol for the induction of short-term stress responses, chronic exposure can lead to adaptation. One study where manipulation of the photoperiod was performed in *Macaca mulatta* females revealed the development of anhedonia, decreased active behavior and body weight, as well as increased cortisol levels, which matches clinical reports of SAD (Qin et al., 2015).

The timing of stress exposure is also an important factor to be considered in animal models of depression, as adverse experiences during neural developmental phases can negatively impact adult health (Chang et al., 2019). Chronic juvenile stress, mainly through exposure to chronic restraint stress, can mimic this condition in female rat unraveling immediate and lasting effects in adulthood that include, increased corticosterone levels, anhedonia and neuronal atrophy (Eiland et al., 2012). Interestingly enough, this protocol did not induce depressive- or anxiety-like behavior, so it might not be accurate to consider it a model of depression (Eiland et al., 2012).

The three different types of stress protocols evaluated in this section were shown to induce deficits related to human depression, though important differences were observed in outcomes and between species. Nevertheless, still a very small number of studies are available for each protocol in females, reinforcing the need to replicate these findings.

2. Pharmacologically-induced animal models

Pharmacologically-induced models of depression are based on the direct application of clinical observations, namely on hormonal changes in HPA axis, monoamine depletion and dysregulation of the immune system (Krishnan et al., 2011).

Four different types of drug-induced models are presented in this review, including administration of lipopolysaccharides (LPS), Pegylated interferon-alpha, TNF-alpha and corticosterone.

Multiple intermittent LPS administration in female mice was shown to produce anhedonia, increase in corticosterone levels and a decrease of spleen cells proliferation and spleen weight (Kubera et al., 2013). The etiological basis of this animal model of depression comes from observations that changes in immune-inflammatory pathways may play a significant role in the pathophysiology of MD (Leonard and Maes, 2012), and might be a suitable animal model of depression also in females.

Other studies have also shown the association between inflammation and depression to impact on the levels of tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) (Dowlati et al., 2010). The potential of TNF-α to induce depressive-like symptoms was also evaluated in females, with a reported depressive-like phenotype in the Tail Suspension Test (Manosso et al., 2013). On the other hand, chronic administration of pegylated IFN-α, which can lead to the development of depressive disorders in hepatitis C patients (Loftis and

Houser, 2004), was not able to induce depressive-like symptoms in female rodents (Loftis et al., 2006).

Stress and depressive-like behaviors are widely recognized to be linked to changes in the HPA axis leading to corticosterone hypersecretion in response to stress (Bao and Swaab,2019). Interestingly, and for the sake of etiological validity, the hypersecretion of cortisol also holds true for around half of depressive patients. At least two studies have reported the development of anxio-depressive-like behavior in female rats and mice upon chronic administration of corticosterone (Mekiri et al., 2017; Koot et al., 2016), attesting at least a partial validation of this model. Nonetheless, corticosterone exposure in female mice revealed no impact on neurogenesis.

3. Pathology-induced models

MD that is secondary to other medical conditions, such as cancer and stroke, can be clinically indistinguishable from primary depression (Kang et al., 2015). Depression is, for instance, a common comorbid condition in many cancer cases, affecting more than 10% of patients (Young et al., 2018). Moreover, there is substantial clinical evidence that psychological alterations related to MD precede the diagnosis of cancer (Passik et al 1999), with more than 25% of women displaying symptoms of depression prior to being informed of their cancer diagnosis (Van Esch et al., 2012). Though yet to be defined, mechanisms such as inflammation, HPA axis dysregulation and glutamate excitotoxicity may play a role in the onset of MD in the context of cancer (Young et al., 2018).

The first successfully validated cancer induced depression (CID) model, developed by injecting 4T1 mammary carcinoma cells in female rodents, has shown to induce the development of anxiety and depressive-like behavior, as well as a decreased dendritic morphology in the prefrontal cortex (Nashed et al., 2015).

MD is also a secondary medical condition to diabetes, with evidence that the occurrence of MD is increased in diabetic patients (Eker, 2018). This link may be explained by the activation of brain renin angiotensin system (RAS), brain inflammatory events and HPA axis dysregulation (Aswar et al., 2016). Persistent hyperglycemia in female rats was shown to induce depressive- and anxiety-like behavior as observed by increased immobility and reduced exploration of the open arm (Aswar et al., 2016).

These pathology induced models highlight a strong correlation between the development of MD and inflammation and HPA axis regulation in the context of other medical conditions. This suggests that many other disorders involving these mechanisms are a potential a target in the context of MD development and should be further studied as they have crucial implications for women's health and disease management.

4. Genetic models

There is a significant influence of genetic factors in the pathogenesis of depression (Shi et al., 2011). Even though it is largely acknowledged that genetic and environmental factors are key to depression pathophysiology, there is still limited understanding, namely on how robust is the genetic predisposition, and

on how genetics shapes adult depression and regulates the susceptibility to stressors (Will, Aird and Redei, 2003).

Many transgenic mouse lines have been developed to target genes of pathways traditionally associated to the pathophysiology of MD (Planchez et al., 2019). Genetic models also include selectively bred mouse and rat lines, selected according to stress sensitivity based on the manifestation of depressive-like behavior in specific tests (Will et al., 2013).

For instance, helpless female mice in the TST display high levels of corticosterone (Popa et al., 2005), anhedonia, anxiety- and depressive-like behavior (Yacoubi et al., 2013). Moreover, they present changes in sleep patterns, including decreased levels of wakefulness, basal locomotor activity, and Rapid Eye Movement (REM) sleep, as well as decreased power density of delta frequencies (Popa et al., 2005). Sleep alterations are also common in depressed patients and include, sleep fragmentation, sleep onset insomnia and disturbances of the REM sleep (Adrien, 2002).

In wistar-kyoto rats, hypoactivity in the open field test and decreased genetic variability were observed. However, it is dubious that this hypoactivity shows increased fear/anxiety since there were no significant differences in the defensive burying test (Will et al., 2003).

Concerning transgenic lines, at least two have been assessed as animal models of depression in females, namely Crtc1-/- and Pitx3-/- mice (Meylan et al., 2016; Kim et al., 2014). CREB-regulated transcription coactivator 1 (CRTC1) has an association with behavioral and molecular depressive-like endophenotypes

(Meylan et al., 2016). Female mice lacking CRTC1 present depressive-like phenotype along with an upregulation of agmatine-degrading enzyme (AGMAT) (Meylan et al., 2016). Agmatine, has been previously showed to have antidepressant potential in animal models of depression and has been implicated in mood regulation (Meylan et al., 2016). Pituitary homeobox 3 (*Pitx3*) is a transcription factor with a crucial role on survival and development of midbrain dopaminergic neurons in mammals (Smith et al., 2008; Chung et al., 2005). Behavioral, biochemical and pharmacological data validate parkinsonism phenotypes in Pitx3-deficient mice (Hwang et al., 2003; Nunes et al., 2003). Because nearly 40–50% of Parkinson's disease (PD) patients display depressive symptoms (Lemke, 2008), Pit3x-deficient mice, have been used to evaluate acute stress responses and depressive-like behaviors (Kim et al., 2014). Pit3x deficient female mice showed increased anhedonia, increased c-fos expression in the prefrontal cortex, striatum, Nac and hippothalamus, and high corticosterone levels in accordance with a depressive-like phenotype (Kim et al., 2014).

Major conclusions

In this review, we aimed to highlight the major findings in depression-related female models but also the major gaps in research, attending to the different available protocols and sex specificities.

Literature has consistently reported that women are at a higher risk of developing MD compared to men. Nevertheless, whether female MD prevalence data is accurate, or if women report more frequently the disorder is also a question of

debate. Nevertheless, this leads to the question whether the clinical presentation of the disorder varies between men and women.

Likewise, emerging studies have shown how males and females respond differently to stress at least at the cellular, molecular and circuitry levels (Bangasser and Valentino, 2012; McEwen, 2015), which does not necessarily leads to different behavioral outcomes. Nevertheless, our limited analysis may be missing different substrates and pathological targets that would lead to a more accurate understanding and treatment of depression in women. In fact, sex is a commonly disregarded factor in most of depression preclinical models (Wang et al., 2017; Halbreich, 2000) and it is thus urgent to re-orient the focus in the field and invert this trend.

Considering the previously gender differences described, we focused on clarifying the impact of diverse etiological factors in the development of MD, specifically in females, as well as the subsequent impact on clinical diagnosis in patients.

In this review we focused on 4 different major types of models, mostly based on the etiological factors pertaining depression, including Stress-based, pharmacologically- and pathology-induced, and genetic models. Whereas most of these models were able to fulfill at least one of the validity criteria previously identified for considering an animal model of depression, we identified fundamental differences between them. These include differences in the degree of manifestation of the depressive-like signs, namely the core behavioral deficits of anhedonia and depressive-like behavior, but also on additional physiological parameters, that are well-known physiopathological deficits of human depression.

Some of these deficits are exclusive to female models and mught be worth further exploring in the context of their relevance for the understanding of depression in women patients, namely hormonal changes and their relationship with estrous cycles dysregulation.

Another important message from this review is the need for protocols' standardization in order to diminish inconsistencies between laboratories and consequently attain more reliable results. This problem is not exclusive to female's models but may be even more relevant in the context of research in female neurobiology due to the additional number of variables to consider, namely hormonal fluctuations and reproductive experience.

Moreover, for all of the analyzed models and protocols, it was patent that only a small number of studies was available, reinforcing the need to replicate research in this field.

Preclinical animal models have provided insights into the possible neuro-psychopathological causes of clinical depression over the years. Nevertheless, very few studies attempt to develop animal models specific for females, or therapies sensitive sex differences. Given the phenotype differences between models and the lack of information concerning female animal models, we hope to raise awareness for the need to focus on the specific pathophysiological mechanisms underlying depression on female rodents.

Limitations of this review

Translation of animal models to humans may be compromised by a number of factors, including, preclinical studies design, methodological differences, interpretation of the results and the design of the clinical trials. In fact, despite their invaluable importance for medical research in neuropsychiatry, animal models have, on several instances been ineffective in foreseeing human response to disease and drugs (Greek and Menache, 2013). This has been particular challenging in the field of psychiatry, where only a small percentage of preclinically developed new drugs actually come to market.

One of the major concerns might be our ability to infer outcomes from behavioral tests; For that, researchers need to use adequate measures to reinforce the validity of their claims. In this context, it has been discussed how methodological limitations in existing animal-based research may determine the withdrawn conclusions, including, disparities in drug treatment schedules, discrepancy in the way animals are chosen for each study, the randomization and reporting processes, small experimental groups with insufficient power, differences among laboratories and experimenter's techniques, inconsistent selection of outcomes, among others (Greek and Menache, 2013).

Differences and inconsistencies between animal models were found during our analysis which precluded us from taking further conclusions. For the specific case of female animal models, very little studies are available thus making it harder to get validates and replicated models. For instance, stressor duration, outcome, measurement and raw data availability were some of the problems we had to cope with during this analysis. Data replication and improved reporting are

needed to validate these animal models and study methodologies that will certainly improve the understanding of the disease pathophysiology and hel develop better therapeutics.

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Figures

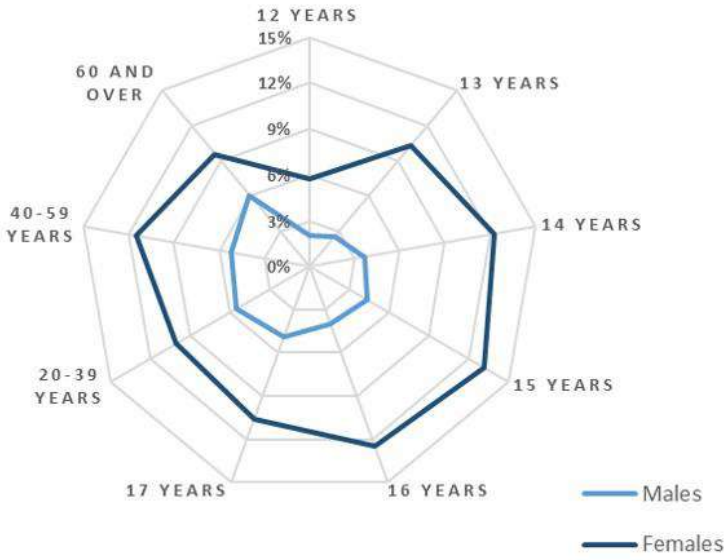

Figure1. Schematic representation of first-onset depression, by age and sex (Data source - NCHS, National Health and Nutrition Examination Survey, 2013–2016; Breslau et al., 2017)

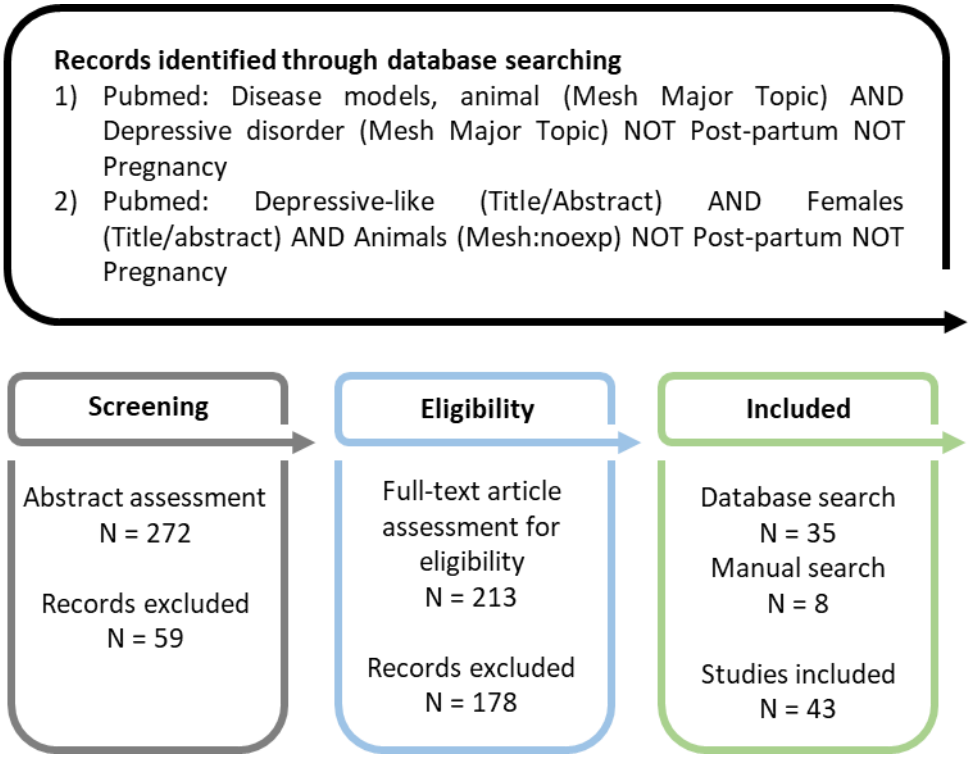

Figure 2. Diagram depicting the flow of information throughout the different phases of the bibliography selection and inclusion process. Data was extracted using a multistep process of study selection centered on online articles search,

abstract screening, full-text article screening and eligibility of the studies based on previously defined inclusion and exclusion criteria.

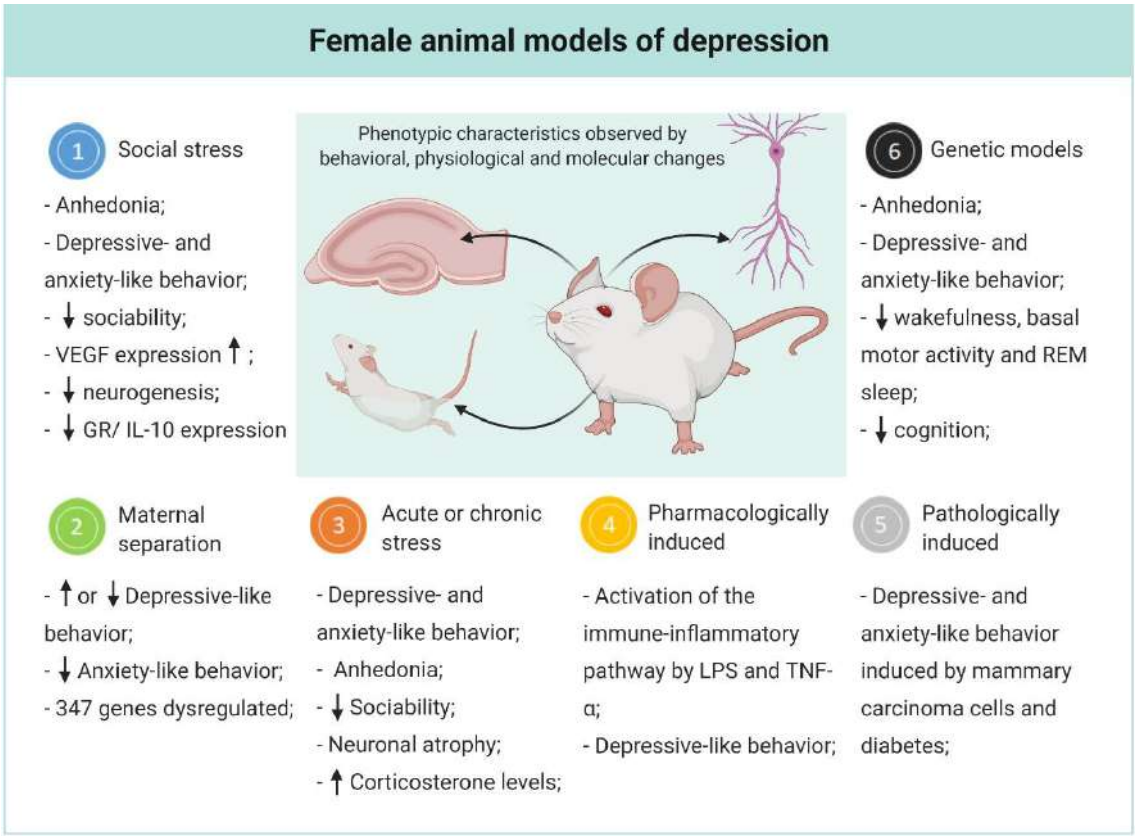

Figure 3. Overview of the identified female animal models of depression and corresponding phenotypic characteristics of each animal model.

Table 1. Compilation of the main risk factors for depression in women versus men (Stegenga et al. 2012; Martin el al. 2013; Seney et al. 2018; Halbreich U. 2000).

	Women	Men
Sociodemographic	Lower Education	None
Psychiatric comorbidity/function	Alcohol problem Other anxiety problem	None None
Adverse experiences/life events	Two or more negative life event	None
Work, living and environment	Neighborhood perceived not safe Financial strain	Nonprofessional occupation Living alone
Family and friends	Problems with someone close	None
Genetic factors	Increase in synapse-related genes Decrease in oligodendrocyte- and microglia-specific genes Immune-related gene reduction	Decrease in synapse-related genes Increase in oligodendrocyte- and microglia-specific genes
Gonadal hormones	Low estrogen Hormone changes during puberty Premenstrual problems Sudden drop in high levels of hormones shortly after birth	Low testosterone Altered testosterone/oestradiol ratios

Table 2. Relevant studies published before November 2019 describing animal models of depression in females. Data was extracted using a multistep process of study selection centered on search strategy, abstract screening, full-text article screening and eligibility of the studies based on the defined inclusion and exclusion criteria. The following information was extracted from each study independently: animal model, method of induction, animal-related variables (species, strain, age and diet), study sample size, protocol duration and outcome measurements.

Title	Animal Variables		Protocol details			Outcome measurements	Stressor impact
	strain		age	Stressor	duration		
			Social stress animal models				
Jacqueline NC. 1984	*Phodopus sungorus*		42 days	Social isolation	21 days	social behavior	↓ encounters with unfamiliar hamster
						body weight	No impact
						Monoamine levels	↑ Norepinephrine levels
Kim JW et al. 1996	*Microtus ochrogaster*		35 days	social isolation	24h	body weight	No impact
						corticosterone levels	No impact
Grippo AJ et al. 2008	*Microtus ochrogaster*		60-90 days	social isolation	28 days	Sucrose cunsumption test	↑ anhedonia
						Elevated plus maze	↑ anxiety
						Forced swim test	↑ depressive like symptoms
						pup exposure	↑ agression
						body weight	No impact
Leussis M. and Andersen S. (2008)	*Rattus Norvegicus*	sprague dawley	30 days	Social isolation	5 days	Forced swim test	No effect
						Elevated plus maze	↑ anxiety like symptoms
						learned helpessness	↑ Helplessness following inescapable shock
						protein content	↓ spiNophilin in pre-frontal cortex
Martin A. And Brown R. (2010)	*Mus musculus*	C57BL/6J	23 weeks (161 days)	Social isolation	35 days	Light/dark box test	No effect
						Forced swim test	↑ depressive like symptoms

						Tail suspension test	↑ bouts of immobility
						cued and context fear conditioning	No effect
						Weight gain	↑ weight gain
						corticosterone levels	↑ corticosterone levels
Pisu M. et al 2016	*Rattus Norvegicus*	sprague dawley	adolescence	social isolation and foot shock	30 days	SPT	No impact
						protein content	↓ CRHR1 and GR levels
						corticosterone levels	↑ corticosterone levels
						allopregnaNolone levels	No impact
Baranyi et al., 2005	*Rattus Norvegicus*	Wistar-han	Adult	Chronic social instability stress	15 days	Weight gain	↓ weight gain
						Social interaction test	↓ social interaction
							↑ agonistic interaction
						Elevated plus maze	No impact
McCormick et al., 2009	*Rattus Norvegicus*	Long-evans	Adolescence (30 days)	Chronic social instability stress	15 days	BrdU cell count	↓ Neurogenesis
						Ki-67 cell count	No impact
						Spatial location test	↓ short-term memory
Herzog et al., 2009	*Rattus Norvegicus*	Wistar-han	Adult	Chronic social instability stress	28 days	Estrous cycle	Animals Not cycling
						Weight gain	No impact
						Food intake	↓ intake
						Adrenal glands	↑ weight
						corticosterone levels	↑ levels

						Prolactine levels	↑ levels
						Luteinizing hormone levels	↑ levels
						Core body temperature	↑ temperature
						Sucrose cunsumption test	No impact
						Forced swim test	No impact
						NGF and BDNF levels in hippocampus	No impact
McCormick et al., 2013	*Rattus Norvegicus*	Long-evans	22 or 62 days	Chronic social instability stress	15 days	Vocalization	↑ number
						Contextual fear condition	Higher sensitivity to stress
Saavedra-Rodríguez and Feig, 2013	*Mus musculus*	-	Adult	Chronic social instability stress	49 days	Elevated plus maze	↑ anxiety
						Social interaction	↓ interaction
						Social Novelty	↓ interaction
						corticosterone levels	↑ levels
Nowacka et., 2014	*Rattus Norvegicus*	sprague dawley	56 days	Chronic social instability stress	28 days	body weight	No impact
						Adrenal glands	↑ weight
						corticosterone levels	No impact
						BDNF expression	No impact
Jarcho et al., 2015	*Mus musculus*	CD1	Adult	Chronic social instability stress	35 days	Weight gain	No effect
						corticosterone levels	↑ levels
						Rearing frequency	No effect
Labaka A. Et al. 2017	*Mus musculus*	CD1 mice	56 days	Chronic social instability stress	28 days	Forced swim test	↑ climbing activity
						Sucrose cunsumption test	↑ anhedonia
						Food intake	No impact
						Weight gain	No impact

						mRNA expression in the hippocampus	↓ GR and IL-10 expression
						corticosterone levels	↑ corticosterone levels
						Estradiol levels	No impact
						Estrous cycle	↑ time estrous cycle phases
						whiskers length	↓ length whiskers
						Monoamines levels in Hipocampus	↓ 5HT, DA and DOPAC ↑d 5HIAA
Nowacka-Chmielewska et., 2017a	*Rattus Norvegicus*	sprague dawley	63 days	Chronic social instability stress	28 days	BDNF expression	No effect
						protein content	No effect
Nowacka-Chmielewska et., 2017b	*Rattus Norvegicus*	sprague dawley	63 days	Chronic social instability stress	28 days	Sucrose cunsumption test	↑ sucrose preference
						Open field	↓ rearing time
						Adrenal weight	↑ weight
						corticosterone levels	No effect
						Plasma ACTH levels	No effect
						VEGF expression	↑ levels in hippocampus, amygdala and hypothalamus
						Serum VEGF levels	↓ levels
Nowacka-Chmielewska et., 2017c	*Rattus Norvegicus*	sprague dawley	60–63 days	Chronic social instability stress	28 days	Open field	Decresed rearing and grooming
						Elevated plus maze	↑ anxiety
						Weight gain	No impact

						Estrous cycle	No impact
						ACTH/ Corticosterone ratio	↑ ratio
						Gene expression	Alterations in the hippocampus, amygdala, prefrontal cortex and hypothalamus
Pittet et al., 2017	*Rattus Norvegicus*	sprague dawley	60-70 days	Chronic social instability stress	28 days	Social interaction	↓ agression
						Grooming behavior	No effect
						Maternal care	No effect
Dadomo et al., 2018	*Mus musculus*	CD1	70 days old	Chronic social instability stress	28 days	Weight gain	↓ weight
						Food intake	↓ intake
						Sucrose cunsumption test	No impact
						Adrenal weight	No impact
						corticosterone levels	No impact
						Plasma ACTH levels	No impact
Breach et al., 2019	*Rattus Norvegicus*	sprague dawley	Adolescence (30 days)	Chronic social instability stress	15 days	Weight gain	No impact
						Adrenal glands	No impact
						Dendrites in prelimbic cortex	↓ branch number
							↓ apical branch length
						Spine density	No impact
Yohn et al., 2019	*Mus musculus*	C57BL/6J	56 days	Chronic social instability stress	49 days	Open field	↑ anxiety
						Elevated plus maze	No impact

						Forced swim test	No impact
						Novelty supressed feeding	↑ anxiety
						corticosterone levels	↑ levels
						Dcx cell count	↓ Neurogenesis
Maternal separation animal models							
Mourlon V. et al. 2010	*Rattus norvegicus*	long evans	o days	Maternal separation	22 days	Forced swim test	no effect
						OSST	↓ swimming activity
						Sucrose cunsumption test	no effect
						Novel object recognition	no effect
Leussis M. et al. 2012	*Rattus norvegicus*	sprague-dawley	2 days	Maternal separation	18 days	Learned helplessness	↑ latency to escape
						protein content	no effect
						cell survival	no effect
Malki K. et al 2014	*Mus musculus*	129S1/SvImJ, C57LB/6 J, DBA/2 J and FVB/NJ	9 days	Maternal separation	24h	gene expression	347 dysregulated genes
Dimatelis J. et al 2015	*Rattus norvegicus*	sprague dawley	2 days	Maternal separation	12 days	Ultrasonic vocalizations	↓ 22kHrz vocalizations
						Open field test	↑ time in inner zone
						Forced swim test	↓ immobilibity time
						protein content	↓ phospho-ERK levels in ventral hippocampus

Donmez R. et al. 2015	*Rattus norvegicus*	Wistar Han	8 days	Maternal separation	13 days	home cage activity	↓ time grooming
						Elevated plus maze	Decrease anxiety
						Novel object recognition	no impact
						Forced swim test	↓ immobility
						USVs	no impact
						BDNF protein levels	no impact
Acute or chronic stress animal models							
Malki K. et al 2014	*Mus musculus*	129S1/SvImJ, C57LB/6 J, DBA/2 J and FVB/NJ	70 days	Unpredictable Chronic Mild Stress	14 days	gene expression (whole-genome oligonucleotide arrays)	350 dysregulated genes
Zhu S. et al 2014	*Mus musculus*	C57BL/6 mice	adult	Unpredictable chronic mild stress and chronic restraint stress	28 days	Open field test	↑ anxiety-like behavior
						Elevated plus maze	↑ anxiety
						Novelty supressed feeding	↑ anxiety
						FST	↑ depressive-like behavior
						Sucrose cunsumption test	↑ anhedonia
Marco EM et al 2017	*Rattus norvegicus*	wistar han	56 days	Unpredictable chronic mild stress	42 days	holeboard test	no effect
						Sucrose cunsumption test	no effect
						Forced swim test	↑ depressive-like behavior
						two bottle choice test	↑ preference alchool
						Food intake	no effect
						Weight gain	no effect

						protein content	↑ GFAP and CB1R levels ↓ CB2R and NCAM-140KDa
						corticosterone levels	no effect
Eiland L. et al 2012	*Rattus norvegicus*	sprague-dawley	20 days	Chronic juvenivel stress	21 days	Sucrose cunsumption test	↑ anhedonia
						Elevated plus maze	No effect
						Forced swim test	↓ immobility
						corticosterone levels	↑ corticosterone levels
						neuronal morphology	↓ length
Qin D. et al 2015	*Macaca mulatta*	-	11-14 years	Photoperiod manipulation	-	Sucrose cunsumption test	↑ anhedonia
						Active Behavior	↓ behavior
						Locomotion	↓ locomotion
						body weight	↓ body weight
						Cortisol levels	↑ cortisol levels
Pharmacologically-induced animal models							
Loftis J. et al. 2006	*Rattus norvegicus*	Lewis rats	adult	Pegylated interferon-alpha (IFN-a) administration	21 days	locomotor activity	no impact
						Forced swim test	no impact
						Body weight	no impact
						protein content and phosphorilation	no impact
Kubera M. et al. 2013	*Mus musculus*	C57BL/6 mice	3 months	Lipopolysaccharide administration	4 months	Sucrose consumption test	↑ anhedonia
						food and water intake	↓
						Cytokine levels	no impact
						corticosterone levels	↑ level

						Spleen cells proliferation	↓
						Spleen and thymus weight	↓ thymus wheight
Manosso L. et al. 2013	*Mus musculus*	swiss mice	45-55 days old	TNF-alpha administration	3 days	Tail suspension test	↑ immobility
						Open field	no impact
						protein content and phosphorilation	no impact
Kott J. et al. 2016	*Rattus norvegicus*	sprague-dawley	77-84 days	Corticosterone administration	30 days	Open field test	no impact
						Forced swim test	↑ immobility
						estrous cycle	no impact
						Body weight	↓ weght
						Cell density	no impact
Mekiri M. et al. 2017	*Mus musculus*	C57BL6/Ntac mice	56-70 days	Corticosterone administration	28 days	Elevated plus maze	no impact
						Open field	↓ time in center
						Novelty supressed feeding	↑ latency
						Splash test	↓ grooming
						weight gain	↓ weight
						fur coat state	Altered
						cell proliferation, survival and maturation	no impact on neurogenesis
			Pathologically induced				
Nashed et al. 2015	*Mus musculus*	BALB/c mice	28-42 days	cancer induced depression	28 days	sucrose consumption test	↑ anhedonia
						forced swim test	↑ immobility
						tail suspension test	↓ immobility
						dendritic morphology	↓ complexity
Aswar et al. 2016	*Rattus norvegicus*	wistar rat	adult		21 days	forced swim test	↑ immobility

				diabetes induced depression		open field test	↓ ambulation, rearing and grooming activities
						↑ plus maze test	↓ time in open arms
						cortisol levels	↑ levels
				Genetic			
Popa et al 2006	*Mus musculus*	CD1	2-3 months	Selectively bred: Helpless and nonhelpless mice selected across 12-14 generations	60 days	locomotor activity	↓ basal locomotor activity
						Sleep disturbances	↑ Slow Wave Sleep 2 ↓ REM sleep Disregulation of sleep and wakefulness across the estrous cycle
						corticosterone	↑ Corticosterone
						Body temperature	no effect
						EEG spectra	↓ power density in the delta frequency range (0.5–4.99 Hz)
						wakefulness monitoring	↓ levels of wakefulness
Will C. et al 2003	*Rattus norvegicus*	Wistar-kyoto	70 days	Selectively bred: Males and females with the highest immobility and lowest climbing scores in the FST	28 days	Open field	hypoactivity
						Defensive burying test	no effect
						genetic heterogeneity	Reduced variability

				were mated for 7 generations			
Yacoubi M. et al 2013	*Mus musculus*	CD1	3-4 months	Selectively bred: High, intermediate and low spontaneous helplessness in the tail suspension test (TST)	28 days	Forced swim test	↑ immobility
						Elevated plus maze	↑ anxiety
						Open field	↑ anxiety
						sucrose preference test	↑ anhedonia
						Light/dark box	↑ anxiety
Kim KS et al 2014	*Mus musculus*	C57BL6/J	10–16 weeks (70-112 days)	Pitx3 -/- mice: Selective nigrostriatal dopaminergic neurodegeneration and motor dysfunction	20 days	sucrose preference test	↑ anhedonia
						C-fos expression	↑ c-fos expression in PFC, Striatum, Nac, hippothalamus
						corticosterone	↑ corticosterone
Meylan E et al 2016	*Mus musculus*	-	35 days	CRTC1 mice: Mice lacking CREB-regulated transcription coactivator 1	21 days	FST	↑ immobility
						protein content	Agmat upregulation in the prefrontal cortex and hippocampus
						Gene expression	Agmat upregulation in the cortex
						Agmat-expressing cells	↑ number of Agmat-expressing cells