Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Animal

Effects of Stress on CA3 Pyramidal Neurons in the Pregnant Female Rat

Andreia Barbosa Valença

Mestrado em Biologia Humana e Ambiente

2010

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Acknowledgements

I would like to thank everyone that in one way or another took part in this big journey:

Dr. Jodi Pawluski, for her guidance, knowledge, encouragement, patience, time and wise advices throughout the elaboration of this thesis. Besides being my supervisor, her friendship along the way was very important.

Dr. Tatyana Strekalova, for believing in me, making this project possible and all her support and time.

Professor Harry Steinbusch, for the great opportunity that was working in the Department of Neuroscience and for his support throughout the time in Maastricht.

Marisela, for her endless patience, support, friendly smile and the awesome mexican dinner.

My mom, with all my heart, for her unconditional love, never ending support and patience and for always believing in me and in my future endeavours and giving me strength to go ahead and follow my dreams. She is the best mom anybody could wish for! And my sister Cristina for supporting me in her own way. Thank you both for being by my side, always.

Margarida, for being the best rommie I could ever wish for! In health and sickness, in joy and tears, we made it through and survived to this big journey and kind of adventure, with distinction. Together we laughed, learned, fell and raised again, and shared really good and not so good moments but together we kept always the best side (which really brought on my psychologist skills and made me see how big my zen side is). Together we grew and we were stronger and made the best of it! And Margarida's parents, for their endless support and good mood. And Bruno "pops", for taking part in a bit of everything, listening, laughing, cooking, biking, flying.

Bianca, for always being there for me along the way for so many years. I know I can count on her for everything. Thank you.

Rodrigo “Zukinha”, for being one in a million. It was just impossible not to laugh around him! Countless moments and healthy discussions to remember by a lucky chance that made us cross paths, through Maastricht flying all the way to Amsterdam. Friends have the gift to turn everything richer, fuller, better. Tot volgende keer!

Julie and João “Xangue”, for sharing worries and making everything easier with their help. Julie, our favorite Belgian girl, even with all the work, there was always time for fun and Pecherese! Xangue, in the middle of Dutch, we rocked it in Portuguese! Together we made it, thank you guys!

Marta, Edgar, Joana and Rocha, for endless afternoons in Starbucks and dinners, and really special funny bike rides, just having fun and not driving crazy with the thesis work. Their support and friendship mean the world to me.

Paulo for being a great friend and such an informatics genius!

All my friends, family, lab mates and peers. Thank you.

“DSU room”, for being our “office” throughout our time in Maastricht and kind of the Portuguese speakers’ place. There we were happy and sometimes less happy. There we laughed a lot, made friends and spent great moments, and even celebrated 7 goals!

All the awesome people I met along the way that sure made the best of Maastricht, with great discovery weekends, protocol nights, crazy Amsterdam feelings, lunches and dinners to laugh and remember.

The organizations and people that made my work possible: International Stichting Alzheimer Onderzoek (ISAO), The Netherlands, grant N 09501 to Dr. Tatyana Strekalova; Buddha Biopharm, Finland; Fundação para a Ciência e a Tecnologia (FCT) and Centro de Biologia Ambiental (CBA), Portugal; Professor Ana Santos, Dr. Martti Vallila, Professor Deodália Dias, Professor Margarida Reis, Dr. Cláudia Oliveira, Luís Marques and Raquel Vaz.

Abstract

Stress is one of the primary factors leading to many disorders, including depression, one of the most prevalent psychiatric disorders. Additionally, it has been well documented that hippocampal plasticity is vulnerable to the effects of stress and these effects are often sexually differentiated. Women are twice as likely as men to experience stress-related disorders during the lifespan. In fact, a growing number of women experience psychological stress, such as depression and anxiety, during pregnancy and the postpartum period. This maternal stress may have detrimental effects on maternal mood and maternal care of offspring. In turn, recent research has documented a significant impact of pregnancy and motherhood on hippocampus plasticity in the mother. However, very little research has focused the impact of stress during gestation on the neurobiology of mother. Therefore, the present study investigated how stress affects dendritic morphology of CA3 pyramidal neurons in the hippocampus of pregnant females, and whether these effects differ from those in virgin females. Age-matched pregnant and virgin female Wistar rats were divided into two conditions: 1) Stress and 2) Control. Females in the stress condition were restrained for 1 hour/day for 2 weeks, beginning on gestation day 8 and at matched time-points in virgin females. Females were sacrificed the day after the last restraint session, prior to giving birth, and the brains were processed using Golgi impregnation technique. The results obtained show that repeated restraint stress results in dendritic atrophy in the apical region of CA3 pyramidal neurons in both pregnant and virgin females. Moreover, pregnant females resulted in less complex CA3 pyramidal neurons compared to virgin females. Stress had no effect on weight gain in virgin and pregnant, or litter characteristics and sex of fetuses in pregnant females. These factors were also not associated with CA3 dendritic morphology. Further work is needed to determine how restraint stress affects dendritic morphology in other regions of the hippocampus.

Key words: Stress, hippocampus, CA3 pyramidal neurons, dendritic morphology, pregnancy.

Resumo

O quotidiano é preenchido por diversos episódios *stressantes* que podem representar uma grande ameaça ao bem-estar físico e emocional. De facto, o *stress* é um dos principais factores que leva a diversos transtornos, incluindo depressão, um dos transtornos psiquiátricos mais prevalentes. Assim, para lidar adequadamente com situações de *stress*, ajustes fisiológicos ou estratégias comportamentais são de extrema importância e são normalmente acompanhadas pela activação da resposta ao *stress*, com a intenção de manter ou alcançar a homeostase interna. Uma activação e desactivação da resposta ao *stress* bem sucedidas são, então, vitais para a sobrevivência. A resposta ao *stress* é coordenada pelo cérebro, que interpreta as experiências como ameaçadoras ou não e, de acordo com a situação, determina as respostas comportamentais e psicológicas. Portanto, quando uma ameaça real ou percebida ocorre, a resposta ao *stress* é activada no cérebro e envolve a libertação de hormonas pelo sistema nervoso simpático e pelo eixo hipotálamo-pituitária-adrenal (HPA). Os glucocorticóides (GC), cortisol nos humanos e corticosterona em roedores, desempenham um papel central na mediação de aspectos essenciais à resposta ao *stress* e retorno à homeostase. A duração do *stress* também está implicada nesta resposta neuronal, sendo que uma duração prolongada por mais de uma semana acarreta efeitos mais profundos ao nível dos neurónios. O hipocampo, constituído principalmente pelas regiões do *cornu ammonis* (CA) e pelo giro denteado (DG), para além de desempenhar um papel essencial na aprendizagem e memória, tem também a função de regulação de "feedback" negativo da resposta ao *stress* através do eixo HPA. A grande concentração de receptores de GC na formação hipocampal sugere que os efeitos desta hormona no hipocampo sejam directos, tornando esta área do cérebro particularmente sensível ao *stress* e aos GC. De facto, tem sido bem documentado que a plasticidade do hipocampo é vulnerável aos efeitos do *stress*, através de níveis elevados de GC, causando alterações estruturais e funcionais no hipocampo. Os neurónios piramidais da região CA3 do hipocampo são particularmente sensíveis ao efeito do *stress* crónico, apresentando remodelação dendrítica. Sendo que esta região está envolvida na formação de memórias e processamento espacial, é interessante que eventos *stressantes* repetitivos resultem em atrofia dos neurónios piramidais CA3, caracterizada pela redução da complexidade dendrítica e do comprimento dendrítico total em machos, o que igualmente afecta a função do hipocampo, incluindo perda de memória espacial. Esta remodelação

dendrítica pode ter duas interpretações: uma resposta mal adaptada, com a retracção dendrítica a contribuir para uma maior vulnerabilidade do hipocampo a outros eventos, como doenças, e factores *stressantes* crónicos, ou uma resposta compensatória para protecção contra efeitos neurotóxicos. É também importante ter em consideração que estes efeitos do *stress* são muitas vezes sexualmente diferenciados. A propensão para desenvolver transtornos relacionados com *stress* é estimada em duas vezes mais para mulheres em relação aos homens, durante a vida. Esta tendência é marcada pelo envolvimento das hormonas gonadais femininas, progesterona e estradiol, e a sua acção no eixo HPA. Tendo em consideração que, para além de desempenharem um papel chave no desenvolvimento diferencial do cérebro, estas hormonas estão também envolvidas na formação da plasticidade cerebral nos principais centros emocionais e podem exercer um papel importante na modulação da resposta ao *stress*, é cada vez mais reconhecida uma ligação entre género e transtornos relacionados com *stress*, com as discrepâncias entre géneros atribuídas ao efeito das hormonas gonadais. O ciclo reprodutivo da mulher está intimamente relacionado com os níveis de GC, com elevada libertação desta hormona e elevada sensibilidade ao *stress* durante a fase folicular do ciclo menstrual bem como da fase proestro do ciclo estral em roedores, quando os níveis de estrogénio estão elevados. Assim, uma potencial combinação de GC e hormonas gonadais pode levar a uma maior incidência de transtornos relacionados com *stress* em fêmeas. De facto, um número crescente de mulheres sofre *stress* psicológico, como depressão e ansiedade, durante a gravidez e o período pós-parto. Por outro lado, pesquisas recentes têm documentado um impacto significativo da gravidez e maternidade na plasticidade do hipocampo da mãe. Este impacto pode estar relacionado com o envolvimento do hipocampo nas importantes adaptações hormonais, neurológicas e comportamentais necessárias na mãe para assegurar a sobrevivência da prole, na transição para a maternidade. A placenta, os ovários e o feto contribuem para as flutuações dramáticas de hormonas esteróides e peptídicas que ocorrem durante a gravidez e o período pós-parto e são importantes para a indução do circuito maternal e o inicio dos comportamentos maternos. Além disso, visto os efeitos que as hormonas esteróides têm nas propriedades estruturais do hipocampo, estas flutuações hormonais no período reprodutivo podem ter também um impacto na plasticidade desta área do cérebro. O *stress* e os níveis de GC têm também um impacto na mãe. Apesar das alterações normais nos níveis de GC serem importantes para diversos aspectos da maternidade, o *stress* durante a gestação leva ao aumento da concentração basal de GC

e pode ter efeitos prejudiciais sobre o humor materno e os cuidados maternos da prole. No entanto, pouca pesquisa tem focado o impacto do *stress* durante a gestação sobre a neurobiologia da mãe. Assim, o presente estudo investigou o efeito do *stress* sobre a morfologia dendrítica dos neurónios piramidais da região CA3 do hipocampo de fêmeas grávidas e se, estes efeitos, diferem em fêmeas virgens.

Ratos Wistar fêmeas, grávidas e virgens de idades correspondentes, foram divididos em duas condições: *Stress* e Controlo. As fêmeas na condição de *stress* foram contidas em caixas de contenção uma hora/dia durante duas semanas, começando no oitavo dia de gestação e em tempos correspondentes em fêmeas virgens. As fêmeas foram sacrificadas no dia a seguir à última sessão de contenção, antes do parto. O útero das fêmeas grávidas foi dissecado para permitir a contagem dos fetos, tendo também em conta o seu sexo. Os cérebros foram processados usando a técnica de impregnação de Golgi, que consiste numa impregnação metálica e permite detectar as árvores dendríticas e as espinhas dendríticas. Para a análise da morfologia dendrítica, seis células piramidais CA3 por cada cérebro foram escolhidas e o número de pontos de ramificação, bem como o comprimento total da árvore dendrítica, foram avaliados separadamente para a região apical e basal. A distribuição e complexidade das dendrites foram analisadas recorrendo à contagem das intersecções das dendrites com círculos concêntricos equidistantes (análise de Sholl).

Os resultados obtidos mostraram que as fêmeas grávidas e virgens, na condição de *stress*, tiveram atrofia dendrítica significativa na região apical dos neurónios piramidais CA3, em comparação com as fêmeas controlo. Para além disso, as fêmeas grávidas apresentaram neurónios piramidais CA3 significativamente menos complexos, em comparação com as fêmeas virgens. O *stress* não teve efeito sobre o peso em virgens e grávidas, nem afectou as características das ninhadas. Este estudo forneceu novas evidências de que o *stress* e a gravidez têm um impacto na morfologia dendrítica dos neurónios piramidais CA3. Pesquisa futura irá avaliar a morfologia dendrítica e a densidade das espinhas dentríticas na região CA1 e DG bem como o possível papel do *stress* e da maternidade no desempenho de tarefas dependentes do hipocampo na fêmea adulta.

***Palavras-chave**: Stress*, hipocampo, neurónios piramidais CA3, morfologia dendrítica, gravidez.

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Abbreviation Index

ACTH	Adrenocorticotropin hormone
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
CA	Cornu ammonis
CBG	Corticosterone binding globulin
CRH	Corticotropin-releasing hormone
CUMS	Chronic ultramild stress
DG	Dentate gyrus
DSU	Disk spinning unit
EC	Entorhinal cortex
e.g.	*exempli gratia*
FCM	Faculdade de Ciências Médicas
GABA	Gamma-aminobutyric acid
GC	Glucocorticoids
Glu	Glutamate
GR	Glucocorticoid receptors
HPA axis	Hypothalamic-pituitary-adrenal axis
LSD	Least significant difference
LTP	Long-term potentiation
MR	Mineralocorticoid receptors
NMDA	N-methyl-D-aspartate
SEM	Standard error of the mean
SNS	Sympathetic nervous system
Sub	Subiculum
vs.	versus

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Chapter I Introduction

A growing number of women experience stress-related diseases, such as depression and anxiety, during pregnancy and the postpartum period. In fact, more than 20% of women suffer from mood disorders during this time (Bennett *et al*., 2004a, b; Oberlander *et al*., 2006). These stress-related mood disorders can have detrimental effects on the mother and offspring, resulting in difficulties in mother-offspring bonding and increased susceptibility to mood disorders and cognitive problems in adult offspring (Lindgren, 2001; Smith *et al*., 2004; Maccari & Morley-Fletcher, 2007; Darnaudéry & Maccari, 2008).

Unfortunately our understanding of how stress affects the neurobiology of the mother is limited as very little research in humans and rodent model has aimed to determine the effect of stress during gestation on neurobiology of the maternal brain.

Stress in the Brain

The body's ability to physiologically regulate its inner environment, ensuring its stability in response to changes in the outside environment, can be defined as homeostasis. One of the major threats to homeostasis is stress (Bartolomucci & Leopardi, 2009). Life is manifested by single or recurring stressful episodes that can be a major threat to one's physical or emotional health. Events, such as the loss of a spouse or the onset of disease, may set in motion fear, helplessness and emotional distress that can develop into stress-related disorders, such as depression and anxiety (Kessler, 1997; Kim *et al*., 2007). To adequately cope with major stressful events, adjustments in physiology or behavioral strategies are of major importance and are usually accompanied by activation of the stress response (Joëls & Baram, 2009), intending to maintain the initial homeostasis or achieve a new homeostasis (Bartolomucci & Leopardi, 2009). In fact, as stress has an impact in emotional states and cognitive abilities, leading to variety of mental disorders and diseases (McLaughlin *et al*., 2009; Strekalova & Steinbusch, 2010), a successful activation and termination of a stress response is vital for survival (de Kloet *et al*., 2005; Conrad, 2008).

The brain is the organ that interprets experiences as threatening or nonthreatening and, according to the situation, determines the behavioral and psychological responses (McEwen, 2007). Thus, when a real or perceived threat (stressor) occurs, the stress

response is activated in the brain and includes the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis (Fuchs & Flügge, 2002; McEwen, 2002; Conrad, 2008). A rapid stress response occurs through the SNS, involving the release of catecholamines within seconds of the onset of the stressor, providing the regulation of blood pressure, heart rate and cardiovascular tone. On the other hand, the slower onset stress response by the HPA axis involves the release of glucocorticoids (GC: the primary glucocorticoid is cortisol in humans and corticosterone in rodents) within minutes of stressor onset (Conrad, 2008). The release of GC occurs via the release of the corticotropin-releasing hormone (CRH) in the hypothalamus that stimulates synthesis and release of the adrenocorticotropin hormone (ACTH) from the pituitary into the blood circulation (Cannon, 1914; Selye, 1936a, b). ACTH activates the adrenal glands where subsequently a variety of hormones, such as GC, are released into the blood to mediate essential aspects of the stress response and help the body return to homeostasis (de Kloet *et al*., 1998; Miller & O'Callaghan, 2002). For example, the GC hormones, derived from the cortex of the adrenal glands, convert proteins and lipids into carbohydrates, which can be directly used as energy resources (Sapolsky *et al*., 2000).

Despite the fact that a stress response is crucial for a successful adaptation to a threat, detrimental consequences can result from a persistent stress response or from inefficient management of *allostasis,* the active process by which the body responds to daily events and maintains homeostasis (McEwen 2001, 2007; McEwen & Gianaros, 2010). In the brain, the limbic system is particularly vulnerable to the effects of stress (Kim *et al*., 2007; Conrad 2008). For example, the nature of the neuronal responses is closely linked to the duration of the stressor. When acute stressors are present, a rapid surge of neurotransmission, neuronal activation and hormone release is established, followed by rapid return to baseline levels (McEwen, 2007). On the other hand, when stress is prolonged for a week or more (chronic stress), more profound changes will be induced, such as expression of particular genes, structural alterations in neurons and changes in neuronal firing patterns throughout the brain (Joëls *et al*., 2007; Krugers *et al*., 2010). In addition, the diversity of the stressors and their impact on an individual's brain also relies on many factors, such as interaction of sex and genetic factors with life events (Magariños & McEwen, 1995; Joëls & Baram, 2009).

The Structure of Hippocampus

Multiple brain regions are likely involved in the organization of responses to stressful stimuli. In the limbic system, the hippocampus (Figure 1) has long been studied for its potential involvement in mental illness in general. The hippocampus is a neural structure comprised of three main areas: the cornu ammonis (CA) regions, CA1 and CA3, and the dentate gyrus (DG). The hippocampal formation also includes the entorhinal cortex, subiculum, parasubiculum, and the presubiculum (Amaral & Lavenex, 2007; Scharfman, 2007). The CA regions are primarily constituted of pyramidal neurons, while the DG, where neurogenesis occurs throughout adulthood, consists mainly of granule neurons (Amaral & Witter, 1989; Leuner & Gould, 2010). The hippocampus is comprised of a trisynaptic circuitry (Figure 2) where fibres from the entorhinal cortex project to the distal granule cell dendrites of the DG, via the perforant path (synapse 1), and projections from the DG granule cells project, via the mossy fibers, to the proximal dendrites of CA3 pyramidal cells (synapse 2). The pyramidal cells of the CA3 region send their major input via the Schaffer collaterals to the CA1 (synapse 3) (Amaral & Witter, 1989; Amaral & Lavenex, 2007; McEwen, 2007; Scharfman, 2007).

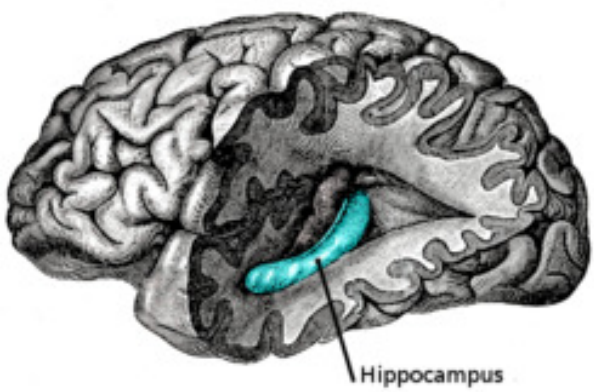


Figure 1. The human hippocampus.
(From http://medlibrary.org/medwiki/Cornu_Ammonis_region_3)

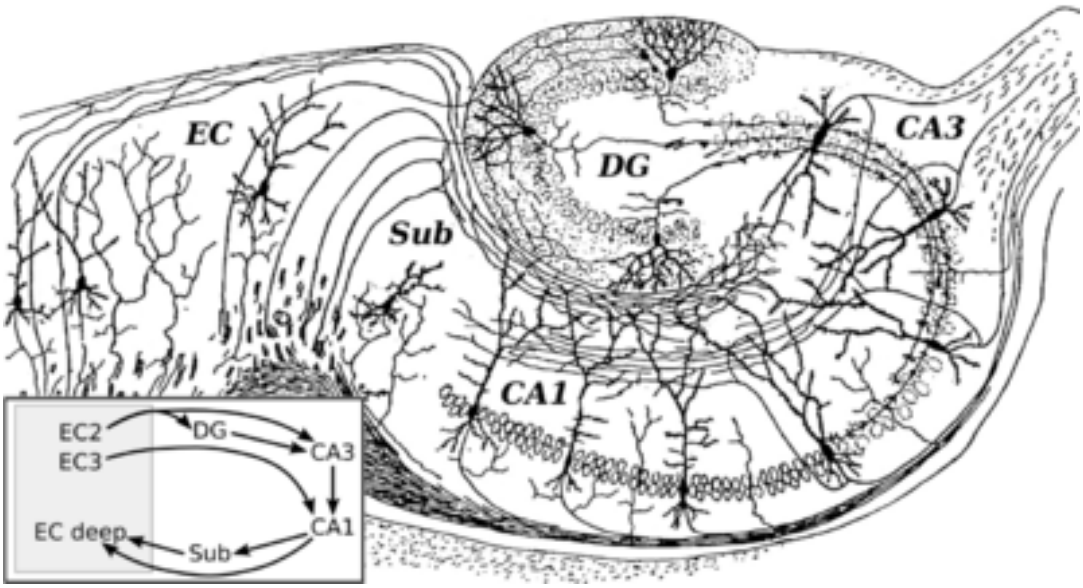


Figure 2. Basic circuitry of the hippocampus, shown using a modified drawing by Ramon y Cajal. CA3: cornu ammonis region 3; CA1: cornu ammonis region 1; DG: dentate gyrus; Sub: subiculum; EC: entorhinal cortex.
(From http://medlibrary.org/medwiki/Cornu_Ammonis_region_3)

The Role of Hippocampus in the Stress Response

This hippocampus has been primarily established to play a critical role in learning and memory, but it also has an important role in general cognition, mood regulation, and even in encoding predictions for future events (Dranovsky & Hen, 2006; Conrad, 2008; DeCarolis *et al*., 2010). However, a less well known function of the hippocampus is its role as a negative feedback regulator of the stress response via the HPA axis (Vyas *et al*., 2002; Dranovsky & Hen, 2006; Leuner & Gould, 2010). The high concentration of GC receptors in the hippocampal formation suggests that the effect of GC on the hippocampus may be direct, therefore making this brain area particularly sensitive to stress and GC (Lucassen *et al*., 2001; McEwen, 2001; Kim *et al*., 2007; Conrad, 2008; Leuner & Gould, 2010). In the rat, binding sites for the high-affinity mineralocorticoid receptors (MR) and low-affinity glucocorticoid receptors (GR) are located in the nuclei of granule cells in the DG and pyramidal neurons in the CA1 and CA3 subfields of the hippocampus, where corticosterone is highly taken up and retained (de Kloet *et al*., 1998; Jöels & Baram, 2009). These two types of receptors are responsible for translation of GC into specific cellular actions (Joëls, 1997; de Kloet *et al*., 1998). In fact, as a target for adrenal "stress" hormones, the hippocampus provides a crucial

model for studying neurobiological consequences of stress (Magariños *et al*., 1997). An increasing body of research suggests that stress, via elevated levels of GC, can cause changes in the hippocampus, affecting both hippocampal structure and function (McEwen, 2006; Jöels *et al*., 2007). These changes include decreasing neurogenesis, alterating spine density, hindering synaptic output, impeding long-term potentiation (LTP), changing inhibitory and excitatory tone, altering pyramidal cell morphology, and reducing dendritic complexity (McEwen, 2007; Conrad, 2008).

Stress and the CA3 Region of the Hippocampus

Stress greatly influences CA3 pyramidal neurons in the hippocampus. In particular, the neurons of the CA3 region are very sensitive to chronic stress effects and show dendritic remodeling after chronic stress has been experienced (Magariños & McEwen, 1995; Sousa *et al*., 2000). The CA3 region is crucial to memory formation and spatial processing (Cerasti & Treves, 2010) and presents remarkable anatomical features: CA3 neurons are the largest pyramidal neurons within the hippocampus (Fitch *et al*., 1989) and are homogenously placed in cell bands that are easily identifiable, without interneurons and glial cells (Newrzella *et al*., 2007). Despite the fact that the main projections are from the DG, via a few, but very strong, tens of inputs from the granule neurons, the CA3 pyramidal neurons also receive many thousands of weak inputs from other sources, such as perforant path connections from the entorhinal cortex. This scarcity in the connectivity is unclear, however it is purposed to help the DG drive CA3 activity during the storage of new memories (McLaughlin *et al*., 2009; Cerasti & Treves, 2010).

The pyramidal neurons are comprised of two regions, apical and basal (see Figure 3). The apical and basal regions differ in functionality (Spratling, 2002), but the extent of this difference in the hippocampal pyramidal neurons is not well documented. The CA3 apical dendrites receive input from all parts of the DG while the basal dendrites receive input mainly from the infrapyramidal blade of the DG (Witter, 1989).

Considering these significant roles and inputs of CA3 cells, it is interesting that repeated stressful experiences result in atrophy of hippocampal CA3 pyramidal neurons, characterized by reduced dendritic complexity and reduced total dendritic length in males, shown in several studies (Watanabe *et al*., 1992; Magariños & McEwen, 1995; Conrad, 2008). Therefore, stress-induced changes in hippocampal CA3 neurons are

consistent with deficits in hippocampal function, including spatial memory impairment in male rats (McLaughlin *et al*., 2007).

CA3 atrophy found in rats is a relatively slow process, taking normally at least three weeks to develop under daily stress, but the atrophy produced by stress is reversible, within a week or so after the termination of stress, and factors, such as physical exercise, can speed up this process (McEwen & Magariños, 1997). Chronic stress-induced CA3 dendritic remodeling may be a maladaptive response and this dendritic retraction may contribute to hippocampal vulnerability to other life events and chronic stressors (Conrad 2006, 2008). For example, cognitive dysfunction can result when chronic stress precedes or coincides with other conditions, such as AIDS (Oberfield *et al*., 1994), depression (Sheline *et al*., 1996), and Alzheimer's disease (de Leon *et al*., 1993). However, another interpretation is that CA3 dendritic remodeling may be a compensatory response to protect against extended excitatory amino acid stimulation, which can compromise and kill neurons (Conrad 2006, 2008).

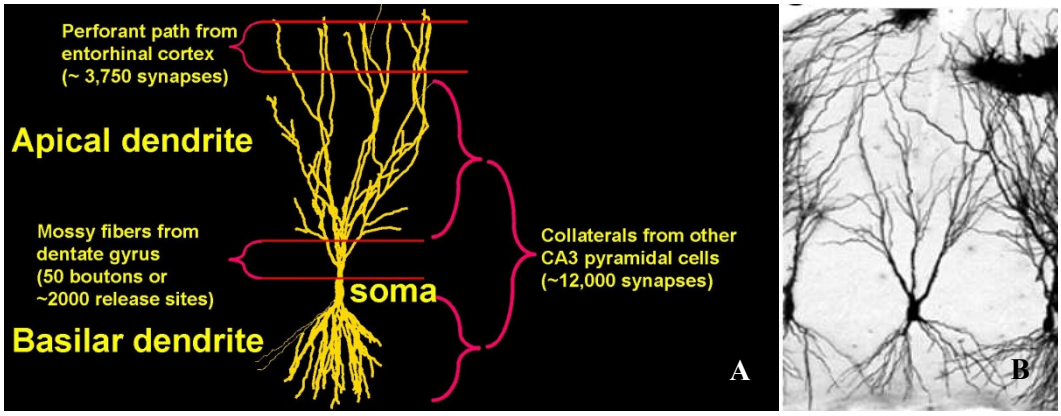


Figure 3. A) A schematic representation of a CA3 pyramdidal cell and its inputs and B) a photomicrograph of a representative Golgi stained CA3 pyramidal cell.
(From http://www.pitt.edu/~german/ (A) and Magariños *et al*., 2006 (B))

An illustration of the mechanism underlying stress-induced CA3 dendritic retraction is in Figure 3. Moreover, as shown in previous studies, dendritic retraction typically occurs on the CA3 apical region while the basal region seems not to be affected (Watanabe *et al*., 1992; Magariños & McEwen, 1995; Magariños *et al*., 1996). The mossy fiber input to the CA3 region at the stratum lucidum appears to drive the dendritic remodeling, as it is the apical dendrites above this input that retract (McEwen, 1999). In addition, the middle part of the apical CA3 dendritic tree, corresponding to the

region expressing chronic stress-induced changes in the N-methyl-D-aspartate (NMDA) glutaminergic receptor sensitivity, suffers drastic remodeling by chronic stress (Kole *et al*., 2002, 2004). Electrophysiological investigations in the CA3 region have shown that after chronic stress, NMDA-receptor mediated responses are enhanced (Kole *et al*., 2002), whereas LTP, long-lasting enhancement in signal transmission between two neurons that results from stimulating them synchronously, is largely impaired (Pavlides *et al*., 2002). Consequently, the commissural-associational collaterals are implicated as contributing to CA3 dendritic retraction (Conrad, 2006). Aside from the vulnerability of hippocampal morphology in the CA3 region to the effects of stress, it is also important to take into consideration that often these effects are sexually differentiated (McLaughlin *et al*., 2009).

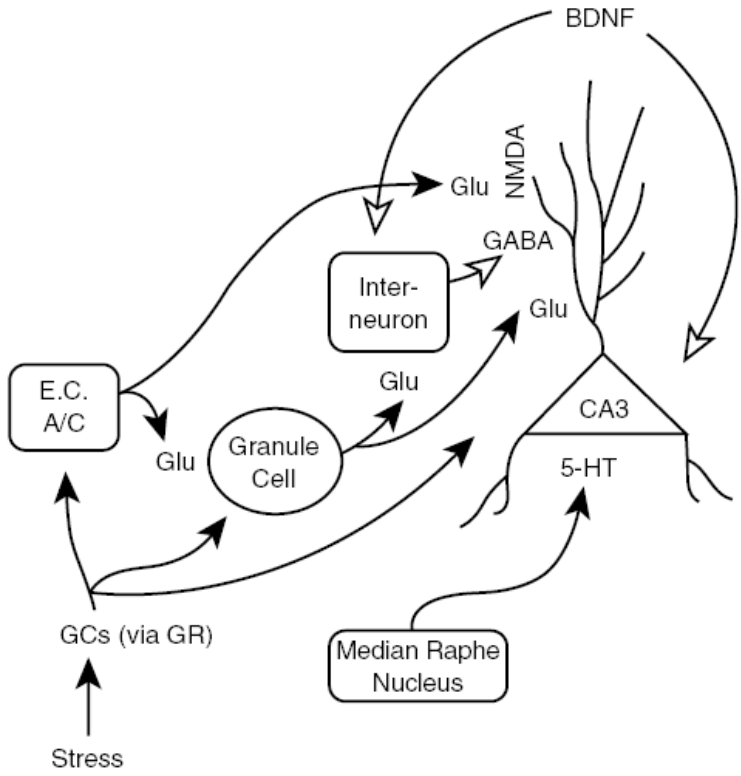


Figure 4. Mechanism of CA3 dendritic retraction following chronic stress. Repeated GC elevations from chronic stress directly influence the CA3 pyramidal cells and CA3 afferents (dentate gyrus granule cells, commissural/associational fibers [C/A], entorhinal cortex [E.C.]) because all of these cells express receptors for GC. The GR most likely mediates dendritic retraction in rodents, but the MR probably plays a role in primates. Excess glutamate (Glu via N-methyl-D-aspartate [NMDA] receptor) and serotonin (5-HT) as well as altered inhibitory tone from interneurons and gamma-aminobutyric acid (GABA) modulate CA3 dendritic retraction. Reduced levels of brain-derived neurotrophic factor (BDNF), which is retrogradely transported to CA3 neurons, may permit CA3 dendritic remodeling. Solid arrows = enhanced tone permits CA3 dendritic retraction; open arrows = reduced tone permits CA3 dendritic retraction (From Conrad, 2006).

Sex Differences and Stress Effects in the CA3 Region of the Hippocampus

There is a clear pattern for the sex-specific prevalence rates of mental and physical disorders (Wang *et al*., 2007). In general, men are more prone to infectious diseases, cardiovascular disease, aggressive behavior, abuse of drugs or alcohol and schizophrenia, which has been associated with prenatal and early life exposures to stress (Wang *et al*., 2007). Women are more susceptible to autoimmune diseases and chronic pain, and tend to show heightened stress sensitivity and an increased predisposition to affective disorders, such as depression and anxiety (Wang *et al*., 2007; Goel & Bale, 2009; Lin *et al*., 2009). In rodents, the initial response of the HPA axis to a stressor is similar between males and females, however adult females generally have elevated levels of GC compared to males (Romeo, 2003). Prior to puberty, when the activation of gonadal hormones has not occurred, males and females also have a similar predisposition to stress-related disorders (Arnold & Gorski, 1984; Romeo & McEwen, 2006). However, the presence of an increase of testosterone beginning in puberty can affect active coping behaviors and stress physiology by exerting additional modulatory actions on serotonergic and GABAergic systems (Goel & Bale, 2009). In fact, during adolescence, a blunted male responsiveness, as a result of maturation of stress neurocircuitry, is likely associated with an increase in testosterone (Gomez *et al*., 2004).

Following adolescence, there is an increased predisposition to affective disorders in females compared to males (Romeo & McEwen, 2006). This may be due to the effects of female gonadal hormones, estradiol and progesterone, and their action on the HPA system. These gonadal hormones can act in the HPA responsiveness with sluggish cortisol feedback on the brain and less or delayed containment of the stress response (Young & Altemus, 2004). For example, it has been proposed that a compromised cortisol feedback effect on HPA arousal in women plays a role in the neurobiological pathway mediating the greater tendency of women to develop depression (Young & Altemus, 2004). These findings suggest that gonadal hormones besides having a key role in differential brain development (Gomez *et al*., 2004), are also involved in shaping brain plasticity in key emotional centers, and may play an important role in modulating stress responsivity (Romeo & McEwen, 2006; Goel & Bale, 2009; Lupien *et al*., 2009). Thus, gender discrepancies may be partly attributed to the effect of gonadal hormones and a link between gender and stress-related disorders is gaining recognition.

In animal models, chronic stress or stressful life events often lead to depressive-like symptoms, with females and males coping differently with stressful situations (Luine, 2002; Bowman *et al*., 2003; Westenbroek *et al*., 2003). For example, female rodents exhibit a greater physiological stress response than males, as seen by higher release of GC (Handa *et al*., 1994) and decreased corticosterone binding globulin (CBG) (Galea *et al*., 1997), following a variety of stressors through-out the estrous cycle, with greater peaks in proestrous rats (Viau & Meaney, 1991; Conrad *et al*., 2004). Fluctuations in estradiol and prolactin can also stimulate corticosterone secretion (Lo & Wang, 2003; McLaughlin *et al*., 2005). Furthermore, women's reproductive cycle is intimately linked to GC levels, as increased GC release and stress sensitivity is commonly observed during the follicular phase of the menstrual cycle as well as in the proestrous phase of the estrous cycle in rodents, when estrogen levels are high (Viau & Meaney, 1991; Kajantie & Phillips, 2006).

Importantly, sex differences may also be present in innervations of the CA3 region (Galea *et al*., 1997). As previously mentioned, the main input to the CA3 region is from the DG, and interestingly male rats have a larger DG than female rats (Madeira *et al*., 1991). Furthermore, sex differences exist in central NMDA receptor function, with a stronger NMDA receptor activation in the DG after high frequency stimulation of the perforant path in adult male rats compared to adult female rats (Maren *et al*., 1994). Sex differences are also found in the apical tree of short-shaft pyramidal neurons of the CA3 area, with dendritic trees being more complex in the proximal portion in females, while the distal dendritic tree is more complex in males (Juraska *et al*., 1989). The pattern of sex differences in the proximal region of the apical dendritic tree may be influenced by the principal afferents to this strata, the mossy fibers from the granule cells, and appears to be more active in females (Juraska *et al*., 1989). Galea *et al*. (1997) documented that chronic stress resulted in dendritic atrophy in the apical CA3 pyramidal cells in adult male rats while in females atrophy occurred in the basal region. Thus, stress appears to differentially affect hippocampal morphology in the CA3 pyramidal cells of males and females.

Interestingly, estrogens buffer the SNS and HPA arousal (Kajantie & Phillips, 2006) and the effects of these gonadal hormones on the structure and function of the hippocampus of the female have been well documented (for review see Woolley & McEwen, 1993; McEwen, 2002). Therefore, it is likely that these hormone induced changes contribute significantly to the activation of neural circuits necessary for certain

behaviors (Gould *et al*., 1990; Kinsley *et al*., 2006). Taken together, a potential combination of GC and gonadal hormones leads to a higher incidence of stress-related disorders in females, contributing to gender discrepancies in developing stress-related disorders (McLaughlin *et al*., 2009). However, care and treatment of women has been derived predominantly from studies performed on males. Therefore, more research on females is necessary to better understand the effects of stress on the brain and thus, improve women's health.

Visualization of Dendritic Morphology via Golgi Impregnation

The Golgi technique has been widely used in many studies to examine dendritic structure and dendritic spines in brain sections (for review see Leuner & Gould, 2010). The technique, discovered by Camillo Golgi in the late 1800s, was used to provide the first reports on morphology of neurons throughout the brain (Cajal, 1909). Over the past several decades Golgi impregnation has been used widely to investigate behavioral-morphological relationships (Galea *et al*., 1997; Gibb & Kolbe, 1998; Pawluski & Galea, 2006) There are several variations of Golgi's method of impregnating nerve cells (Golgi, 1873) but all with the same metallic impregnation principle. This staining technique is achieved by impregnating fixed nervous tissue with potassium chromate and silver nitrate, resulting in microcrystallization of silver chromate, according to the reaction illustrated in Figure 5. The microcrystalline precipitate either grows directly from the surface of the tissue block into transected neuronal processes or spreads from nucleation centers inside the block into nerve cell processes like in preformed channels - until the neuron has been completely filled. Finally, dendrites, as well as the cell soma and spines, are clearly stained in brown and black (Figure 6) and can be followed in their entire length (Harry *et al*., 1980; Spacek, 1989, 1992). The popularity of this technique is due to the fact that standard histopathological methods are not able to stain dendrites and/or spines while Golgi impregnation detects the soma along with entire dendritic arbors and dendritic spines of the neurons. Moreover, it is less expensive and less time consuming compared to other techniques, such as cell filling, that also detect dendritic arbors and dendritic spines (Gibb & Kolb, 1998). Furthermore, the ability to detect early and progressive neuronal atrophy and show neuroplasticity and recovery from injury (e.g., re-growth of branching and re-gain of spine density) is also of great importance. This technique is really effective, however it is also capricious

and unpredictable, as it only stains a limited number of cells, approximately 5% at random, and the mechanism by which this happens is still unknown (Smit & Colon, 1969; Shimono & Tsuji, 1987).

$$2\,AgNO_3(aq) + K_2CrO_4(aq) \longrightarrow Ag_2CrO_4(s) + 2\,KNO_3(aq)$$

Figure 5. Golgi impregnation reaction. When aqueous solutions of silver nitrate ($AgNO_3$) and potassium chromate (K_2CrO_4) are mixed, insoluble silver chromate (Ag_2CrO_4) forms, leaving potassium nitrate ($AgNO_3$) in solution.

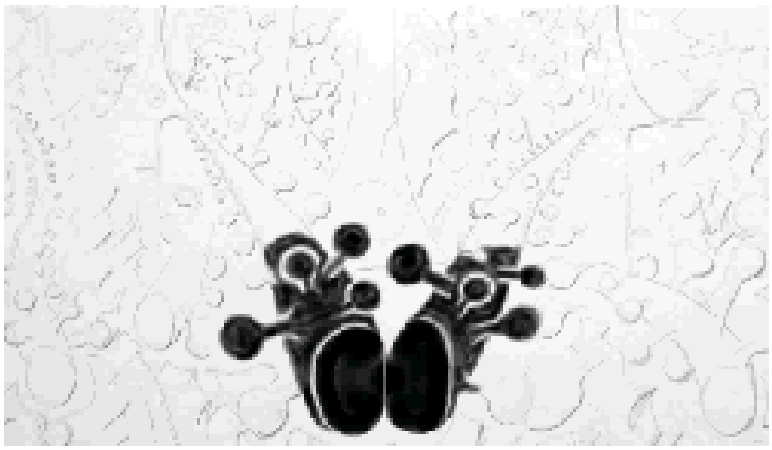


Figure 6. Representation of dendrites and spines during impregnation with Golgi technique. (From http://synapses.clm.utexas.edu/learn/visualize/visualize.stm#GolgiEvol)

Impact of Pregnancy and Motherhood in the Hippocampus

Pregnancy and mothering are major biological events that can have dramatic effects on the physiology and psychology of the mother. Recent research has documented a significant impact of pregnancy and motherhood on the hippocampus, an area not traditionally associated with the "maternal circuit" and maternal behavior, in the mother (Kinsley *et al*., 1999; Pawluski & Galea, 2006). For example, there is a decrease in the hippocampus volumes during pregnancy in both the human and rodent (Galea *et al*., 2000; Oatridge *et al*., 2002) and previous motherhood enhances both hippocampus-dependent learning and memory (Kinsley *et al*., 1999, Pawluski & Galea, 2006; Pawluski *et al*., 2006a, b) and LTP (Tomizawa *et al*., 2003). This may be due to an involvement of the hippocampus in the remarkable number of hormonal, neurological and behavioral adaptations required in the mother to ensure offspring survival in the transition to motherhood (Kinsley *et al*., 2006; Kinsley & Lambert, 2006; Pawluski & Galea, 2006, 2007; Numan, 2007; Pawluski *et al*., 2009a, 2010).

Pregnancy and the postpartum period are accompanied by dramatic fluctuations in the levels of steroid (estrogen, progesterone and corticosteroids) and peptide (oxytocin and prolactin) hormones (Numan, 1988). During pregnancy, the ovaries, placenta, and fetus contribute to these fluctuations (Kinsley & Lambert, 2006), which are continued following parturition and throughout lactation (Russell *et al*., 2001). In rodents, estradiol levels increase from day 11 until the end of pregnancy (Rosenblatt *et al*., 1979; Nelson, 2000), while progesterone remains elevated throughout pregnancy (Rosenblatt *et al*., 1979, 1988). Prior to parturition, progesterone levels fall drastically followed by a decreased in estradiol levels during the postpartum period (Rosenblatt *et al*., 1979; Garland *et al*., 1987). Basal corticosterone levels increase during late pregnancy and remain elevated during the postpartum period, during the first two weeks of lactation (Atkinson & Waddell, 1995; Fisher *et al*., 1995) (See Figure 5). Prolactin levels increase at the onset of pregnancy, followed by a decrease until parturition and a new increase in response to the suckling stimulation during lactation (Rosenblatt *et al*., 1979). Similarly, increased levels of oxytocin are present primarily during parturition and lactation (Russel *et al*., 2001). These fluctuations in circulating hormones during late pregnancy, parturition, and the early postpartum (Numan *et al*., 2006), as well as the response of receptors in several brain areas to these hormones (Numan, 1988; Numan *et al*., 2006), are important for the induction of the maternal circuit and onset of maternal behaviors (Numan, 1988; Rosenblatt *et al*., 1988).

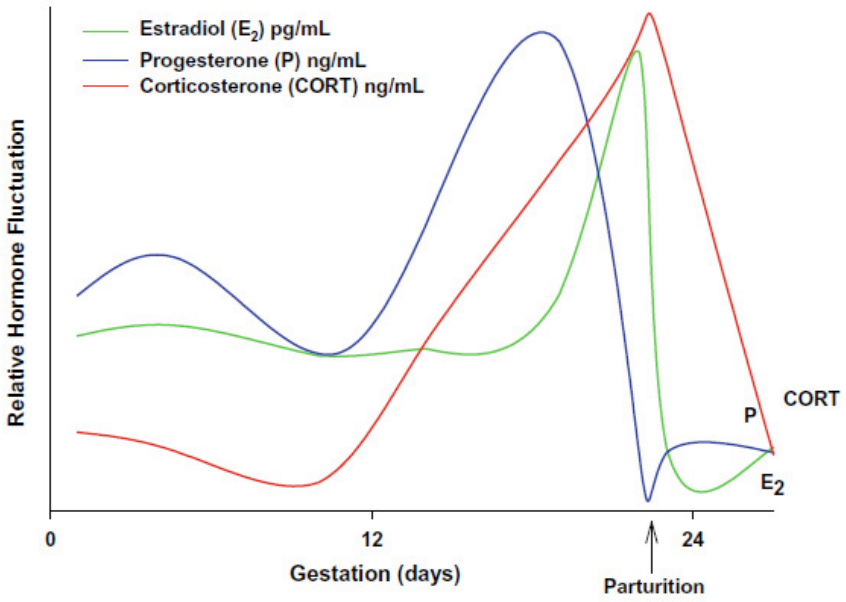


Figure 7. A profile of relative levels of estradiol (pg/mL), progesterone (ng/mL) and corticosterone (ng/mL) across pregnancy and parturition in the female rat (From Pawluski *et al*., 2009a).

Given that, steroid hormones markedly affect structural properties of the hippocampus (Gould *et al*., 1990; Galea *et al*., 1997), it is not surprising that the great hormonal fluctuations that occur during in pregnancy and postpartum period may have an impact in hippocampus plasticity. Recent work has shown that neurogenesis in the DG of the hippocampus is affected by motherhood and reproductive experience, with regards to a decrease in cell proliferation and survival during the early postpartum period. (Pawluski & Galea, 2007; Darnaudéry *et al*., 2007; Leuner *et al*., 2007; Pawluski *et al*., 2009b, 2010). In addition, motherhood significantly impacts dendritic morphology in the hippocampus: primiparous rats (first time pregnancy) showed significant dendritic atrophy in CA3 and CA1 pyramidal neurons compared to multiparous (having been pregnant and mothered at least twice) and nulliparous rats (Pawluski & Galea, 2006). This dendritic remodeling seen in primiparous rats is similar to the one seen following chronic stress, leading to a significant role of corticosterone, as high levels of this hormone are present in both pregnancy and prolonged stress (Woolley *et al*., 1990a; Magariños & McEwen, 1995; Galea *et al*., 1997).

Stress Effects in the Mother

Stress and elevated levels of GC have also been shown to impact the mother. However, normal changes in GC are very important for many aspects of motherhood. For example, in human mothers, cortisol is important for a mother's attraction to her infant, particularly in a first pregnancy (Fleming *et al*., 1997). Studies from rodents have also shown an important role for the elevation in GC during pregnancy and postpartum in maternal pup-directed behaviors (Graham *et al*., 2006; Pawluski *et al*., 2009b). In addition, increased GC levels late in pregnancy are important for mobilization of maternal energy stores to be able to stand fetal demands (Knopp *et al*., 1973; Metcalfe *et al*., 1988) and for milk production (Tucker, 1988; Casey & Plaut, 2007). The elevation in GC during late pregnancy is also very important for many aspects of fetal growth and development, such as development and maturation of fetal organs before birth (Smith & Shearman, 1974).

Exposure to stress can significantly impact GC and maternal and fetal health. Unfortunately, a growing number of women experience severe and chronic stressors during pregnancy (Bennett *et al*., 2004a, b). Nowadays, life events, such as problems at work, domestic issues, financial instability, young age, and unplanned pregnancy

(Pajulo *et al*., 2001; Ryan *et al*., 2005), together with problems with the pregnancy and the responsibilities and challenges that come with a care of a newborn, can be overwhelming for the mother. This can lead to an increased incidence of psychological stress, such as depression and anxiety, during pregnancy and the postpartum period (Bennett *et al*., 2004a, b). Stress can have detrimental effects on maternal mood and maternal care of offspring (Smith *et al*., 2004). Moreover, maternal stress during gestation can also have a negative impact on the offspring (Maccari & Morley-Fletcher, 2007; Darnaudéry & Maccari, 2008). For example, gestational stress during critical periods of fetal brain development can result in increased anxiety-like and depressive-like behavior, increased HPA axis reactivity, and memory deficits in adulthood (Welberg & Seckl, 2001; Kofman, 2002; Weinstock, 2008). Taken together, it is of great importance to fully determine and understand how stress affects the maternal brain, and thus improve the health and well being of the mother and child.

Chronic stress models using immobilization, administration of high levels of corticosterone, or chronic ultramild stress (CUMS), have recently been used to investigate the effects of gonadal hormones and stress on the affective-like behavior and physiology of the mother during pregnancy and postpartum (Darnaudéry *et al*., 2004; Smith *et al*., 2004; Brummelte *et al*., 2006). For example, repeated restraint stress of pregnant rodents during gestation can induce a postpartum depressive-like state in female rats (Smith *et al*., 2004) and dams stressed during gestation show an increase in basal corticosterone concentrations and a decrease in corticosteroid binding globulin during the late pregnancy (Takahashi *et al*., 1998; Maccari *et al*., 2003). Gestational and postpartum stress also affects maternal care of offspring (Pardon *et al*., 2000; Smith *et al*., 2004; Brummelte *et al*., 2006; Brummelte & Galea, *in press*) and persistently affects the affective-like behavior of the mother long after the stress has stopped (Darnaudéry *et al*., 2004; O'Mahony *et al*., 2006). For example, dams stressed during pregnancy are more anxious (Darnaudéry *et al*., 2004) and can exhibit increased depressive-like behavior (O'Mahony *et al*., 2006; Brummelte & Galea, *in press*) one month after the last restraint stress session has occurred (Maccari *et al*., 2003; Darnaudéry *et al*., 2004).

Unfortunately, very little research has investigated the effect of gestational stress on hippocampal plasticity in the mother. A recent study has shown that administration of elevated levels of corticosterone during late pregnancy and postpartum results in decreased neurogenesis in the hippocampus of the mother (Brummelte & Galea, *in*

press). Clearly, further work is needed to understand how stress during gestation affects other measures of neural plasticity in the maternal brain.

Thesis Objectives

The present thesis aims to determine the affects of stress on dendritic morphology of CA3 pyramidal neurons in the hippocampus of pregnant female rats, and whether these effects during pregnant females differ from those in virgin female rats. In order to do this, a repeated restraint stress paradigm will be applied and, through Golgi impregnation, dendritic morphology of the CA3 region of the hippocampus will be assessed to evaluate the effects of stress.

This study will increase our understanding of how stress affects the maternal brain, and thus contributes to improve the health and well being of the mother and child.

Chapter II Material and Methods

Animals and Housing

Twenty-one adult female Wistar rats (four months old) obtained from Faculdade de Ciências Médicas (FCM) da Universidade Nova de Lisboa, were used in this study. The breeding colony at FCM originated from Charles River Laboratories in Barcelona. Rats in the present study were individually housed in clear polyurethane cages with absorbent bedding throughout the study (from impregnation to decapitation and at matched time points in virgin females). The animals were kept isolated in order to strictly control for enriched social environmental influences on brain morphology. All rats were given pellet food (maintenance chow) and tap water *ad libitum*. All rats were maintained in a 12h:12h light/dark with lights on at 5 a.m. in a standard laboratory environment (18-24°C, 55% humidity, ventilation: 8-10 changes/hour). Cages and water bottles were changed weekly. All protocols were in accordance with the European Union's Directive 86/609/EEC and Council Directive 93/119/EC, Portuguese law Law-Decrees DL129/92 (July 6th), DL197/96 (October 16th) and Ordinance Port.131/97 (November 7th) and approved by FCM's ethical committee board.

Breeding

For breeding, one female and one male were housed together in a wire mesh cage until a vaginal plug was released. Upon release of a vaginal plug, indicating copulation had occurred, females were individually housed in clear polyurethane cages for the duration of the experiment. Virgin females were singly housed throughout the experiment.

Group Formation

This study required twenty-one adult females, divided over two groups (Virgin versus Pregnant females), and two conditions/group (Control versus Stress) (Table 1). For all groups a n=5-6 per group was used as this is a minimum required for histological measures utilizing Golgi impregnation, based on previous investigations (Galea *et al*., 1997; Pawluski & Galea, 2006).

Table 1. Group information. Virgin and Pregnant female were divided into control or stressed conditions.

Group	Condition	Number of animals
Virgin	Control	6
	Stress	5
Pregnant	Control	5
	Stress	5

Restraint Stress Procedure

For this experiment, pregnant and virgin females assigned to the stress condition were subjected to restraint stress by being placed in transparent plastic cylinders (diameter 6 cm: See Figure 8). Restraint took place between gestation days 8-21 and at matched points in virgin females. This was done to determine how reproductive status may account for changes in neuroplasticity in response to stress. Briefly females were subjected to daily, 1 hour restraint stress that occurred once between 11 am and 2 pm. Control groups were left undisturbed except for regular weight measurements.

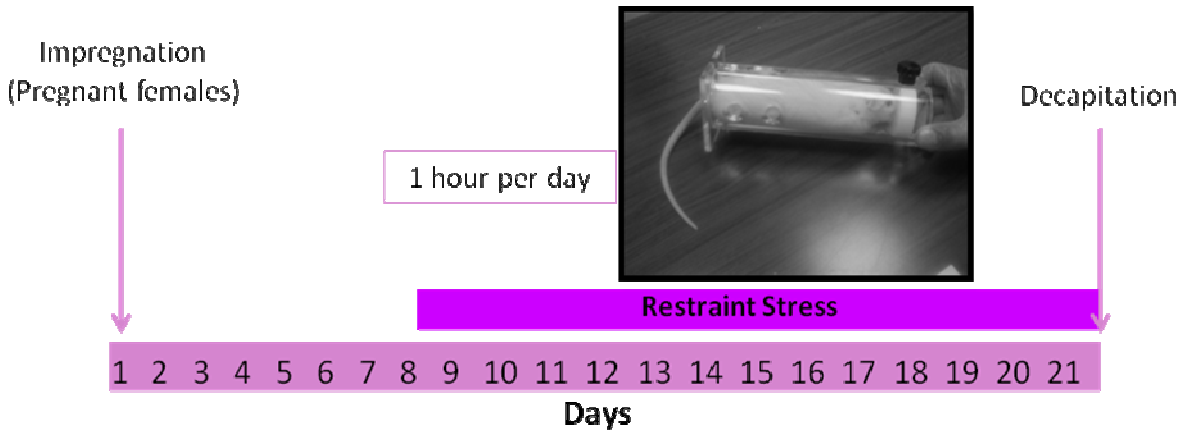


Figure 8. Timeline of the experiment for pregnant and virgin females assigned in stress condition.

Sacrificing and Dissection

On gestation day 21, and at matched time points in virgins, females were deeply anesthetized with pentobarbital (100 mg/kg, intra-peritoneal) and decapitated.

Brain Removal

For the removal of the brain, the dorsal portion of the skull was skinned and the skin-flaps were peeled to the right and left side, from the back of the head forward to between the eyes. After localization of the foramen magnum, a large opening at the back of the head where the spinal column enters the skull, the rongeurs were placed into it and used to cracked and pull away the bone and tissue on either side of the opening. Then, the tips of the rongeurs were placed in the eye sockets, and were used to crack the piece of skull that lies between them. Using the rongeurs, the skull was carefully removed, starting at the top of the foramen magnum and chipping away the bone, up over the cerebellum and then forward toward the eyes. The skull bone was removed by breaking up the bone piece by piece always holding the rongeurs horizontally until the dorsal brain was exposed on three sides. Holding the head upside down, the brain was carefully pried away from the base of the skull with a flat metal spatula. The optic and trigeminal nerves attaching the brain to the skull were severed with the edge of the spatula and the brain was removed and longitudinally sectioned with a sharp scalpel (Schneider, 2007).

Uterine Horns Dissection in Pregnant Females

In order to quantify the possible effect of stress on litter size and number of male and female fetus, the uterine horns were dissected after decapitation. To do this, a vertical 2 cm abdominal skin incision was made with a scalpel. The skin was pulled apart toward the head and tail to expose the abdomen. The peritoneum was grasped with forceps and cut to expose the abdominal cavity. The reproductive organs in the dorsal region of the body cavity were located: two uterine horns, the oviduct and the ovaries (Figure 9). The uterine horns were removed by grasping the uterus below the oviduct and cutting it free along the mesoterium. A vertical incision was made in the uterus at the union of the two horns and the pup–placental units were delivered. Each embryo was separated by cutting between implantation sites along uterine horn. The muscular uterine lining was grasped by sliding watchmaker's forceps between the surrounding

muscle layer and enveloped decidua tissue. The muscle layer was pulled back, exposing the decidua. A portion of the exposed decidua at the apex was clipped off (approximately 1/5 of the decidua tissue) exposing the midventral or distal tip of the enclosed embryo. The embryos were shelled out using the tips of forceps. The decidua was pierced with forceps surrounding the embryo and open forceps to tear decidua apart. The number of fetuses in a litter was measured, taking in account as well the number of male and female fetuses (Shea & Geijsen, 2007).

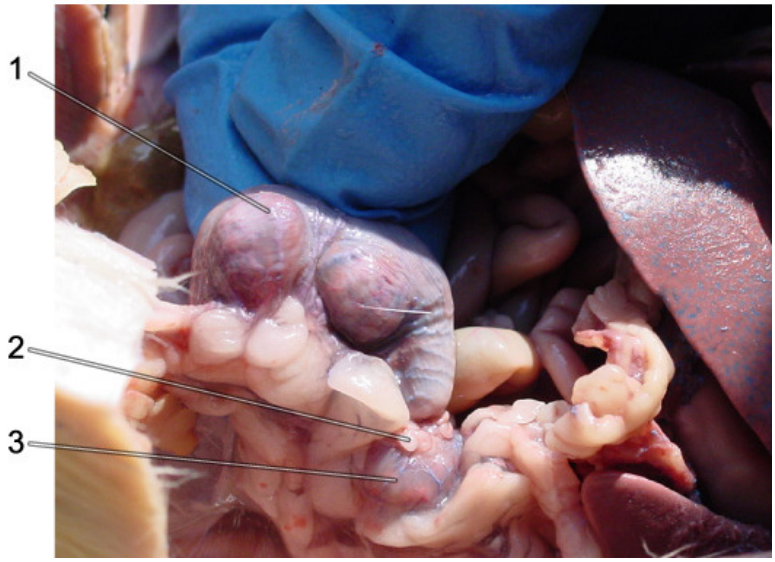


Figure 9. Close-up of the left side of a pregant female rat, preserved and dissected.
1. Embryo in left uterine horn; 2. Oviducts (fallopian tubes, uterine tubes); 3. Ovary (greatly enlarged from normal, non-pregnant, state).
(From http://faculty.orangecoastcollege.edu/mperkins/zoo-review/rat-repro/rat-repro3.html)

Histological Procedures

Golgi Impregnation Technique

After brain removal, the left hemispheres of the each brain were processed for Golgi impregnation using the FD Rapid GolgiStain Kit™ (FD Neurotechnologies Consulting & Services, Elliot City, MD, U.S.A.) adapted for Vibratome (as previously described in Dalla *et al*., 2009; Gibb & Kolb, 1998). The right hemisphere was used in a separate analysis not discussed here. For the Golgi impregnation, 1 cm blocks of brain tissue including the hippocampus were rinsed with distilled water and immersed in an impregnation solution containing potassium dichromate, mercuric chloride and

potassium chromate (provided in the kit). Brains were left undisturbed in the dark for 2,5 weeks. After the 2,5 weeks, brains were immersed in 30% of sucrose at 4°C to protect them from drying. Two to four days later coronal sections (200µm) of the entire hippocampus were cut using a vibratome (Leica VT6000, Leica Microsystems, Germany) in a bath of 15% sucrose and the slices stored in the dark at 4°C in 15% sucrose solution until mounting. Sections were mounted on gelatin coated Superfrost slides (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) and firmly pressed using moist filter paper to prevent the slices from falling off the slide during development (Gibb & Kolb, 1998). Slides were placed in a humidity chamber in the dark and were stored overnight at 4°C. For development, slides were rinsed with distilled water twice for 2 minutes and were then placed in developing solutions (provided in the FD GolgiStain Kit). The slides stayed for 10 minutes in the developing solution, then were rinsed in distilled water twice for 2 minutes, taken through a graded alcohol series (50%-96%, 4 minutes each rinse), cleared with xylene for 8 minutes, and coverslipped with Permount (Fisher) (Figure 10).

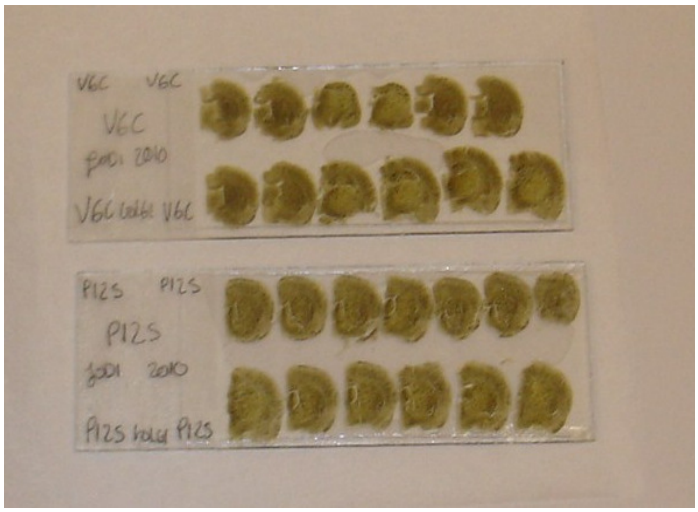


Figure 10. Final slides of the Golgi impregnation technique.

Dendritic Morphology

Dendritic morphology in the CA3 region of the hippocampus was analyzed blind to experimental conditions as previously described (Galea *et al*., 1997; Pawluski & Galea, 2006). For analysis of dendritic morphology, a pyramidal cell was chosen using the following criteria: 1. the cell body and its dendrites were fully impregnated; 2. the cell was relatively isolated from surrounding impregnated cells to obtain a clear image of

the entire cell; 3. the cell was located in the CA3 region of the dorsal hippocampus (Figure 11).

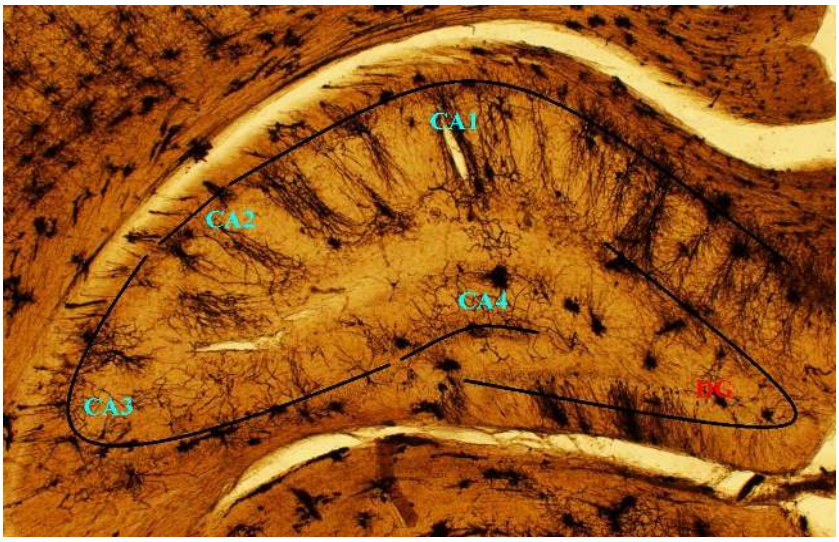


Figure 11. Photomicrograph of Golgi impregnated dorsal hippocampus showing the CA regions and the dentate gyrus (DG). The main focus of this thesis is the CA3 region. The photomicrograph is taken under 40x magnification. CA = cornu ammonis.

Six CA3 pyramidal cells from each brain were analyzed. For each cell the following variables were measured separately in the apical and basal regions of each cell: the number of branch points - total number of branch points in the dendritic arbor; and the total dendritic length - total length of dendrites connecting to a given cell body. Sholl analysis (Sholl, 1953) was also used to estimate the distribution and complexity of the dendrites by counting numbers of intersections of dendrites with an overlay of concentric rings centered at the cell body (Figure 6). This consecutive-circles (cumulative intersections) analysis is a method for quantifying a specific scaling property of the dendritic tree and specifies dendritic geometry, ramification richness, and dendritic branching patterns. The Sholl analysis consists of: (i) construction of concentric and equidistantly organized spherical shells (in 3 dimensions (3D) case), which are centered in the cell body, (ii) counting the numbers of intersections of dendrites with the circles of increasing radii (10 μm).

To quantify dendritic length, branch points and Sholl analysis, the Neurolucida program (MicroBrightField, Inc., Williston, VT, U.S.A.) was used. When a cell of interest was identified, a 3D morphological image of the cell was manually obtained using the Neurolucida neuronal tracing system (made under 400x) attached to a DSU

microscope (Olympus BX51WI, Olympus America Inc., Center Valley, PA, U.S.A.) (Figure 12). For example, Figure 12 depicts a Golgi impregnantion CA3 pyramidal neuron (A) and the corresponding neuronal tracing (B).

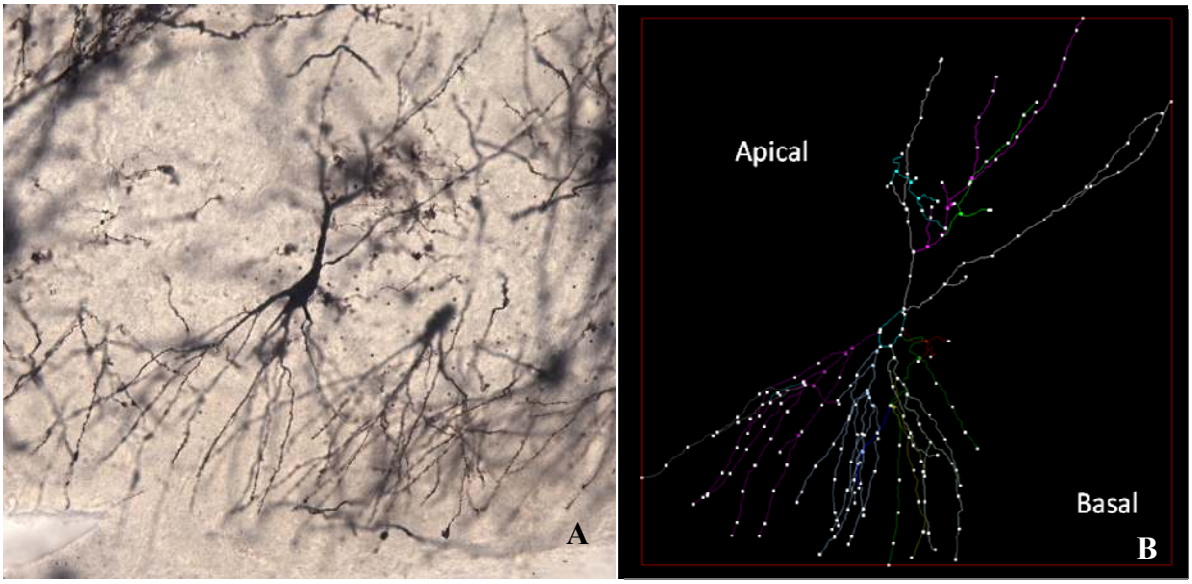


Figure 12. A. CA3 pyramidal neuron (made under 400x); B. CA3 pyramidal neuron drawing obtained using the Neurolucida neuronal tracing system (made under 400x).

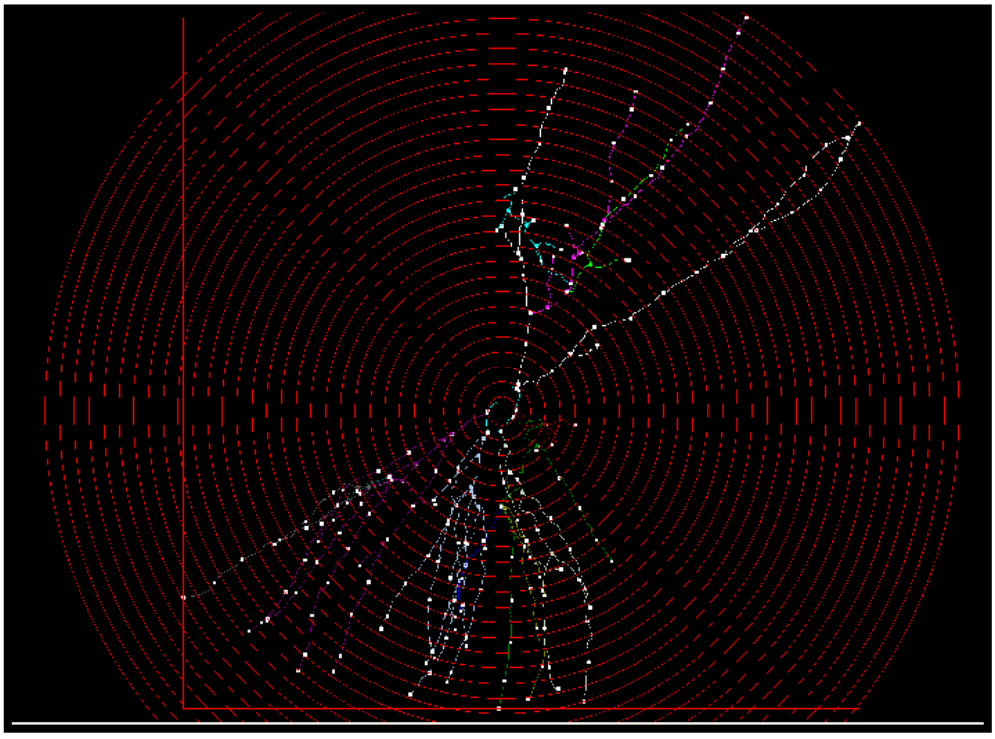


Figure 13. Scholl analysis showing the overlay of concentric rings centered at the cell body.

Statistical Analysis

The number of CA3 branch points and dendritic length were each analyzed using repeated-measures analysis of variance (ANOVA) with two factors (pregnant vs. virgin, stress vs. control) as the between-subjects factors and region (apical vs. basal) as the within-subjects factor. For the Sholl analysis, the number of dendritic intersections was analyzed using repeated-measures analysis of variance (ANOVA) with two factors (pregnant vs. virgin, stress vs. control) as the between-subjects factors. *Post hoc* comparisons utilized the Fisher's LSD procedure. Independent t-tests were conducted on litter size, number of male and female pups in pregnant females. Pearson product moment correlations were performed between apical and basal CA3 morphology and litter size, number of male pups, number of female fetuses. All statistical procedures were set at $\alpha = 0.05$. All statistical analysis was performed using the software Statistica 9 (StatSoft, Inc., Tulsa, OK, U.S.A.).

Chapter III Results

Pregnant Females Gained Significantly More Weight than Virgin Females

A factorial ANOVA on the weight change between groups revealed a significant main effect of reproductive state ($F_{1,17} = 42.25$, $P \leq 0.0001$; Figure 14), with pregnant females gaining significantly more weight than virgin females, as expected. There was no significant main effect of stress or a significant interaction between the effect of stress and reproductive state on weight ($0.1 \leq P \leq 0.5$).

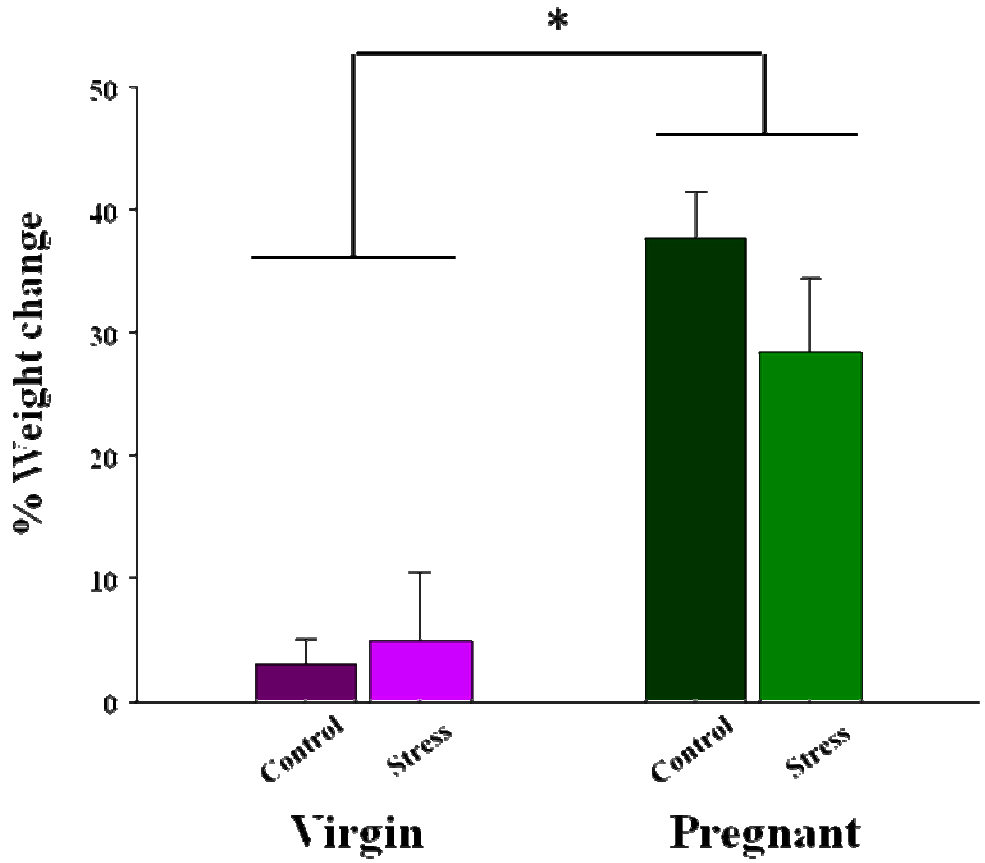


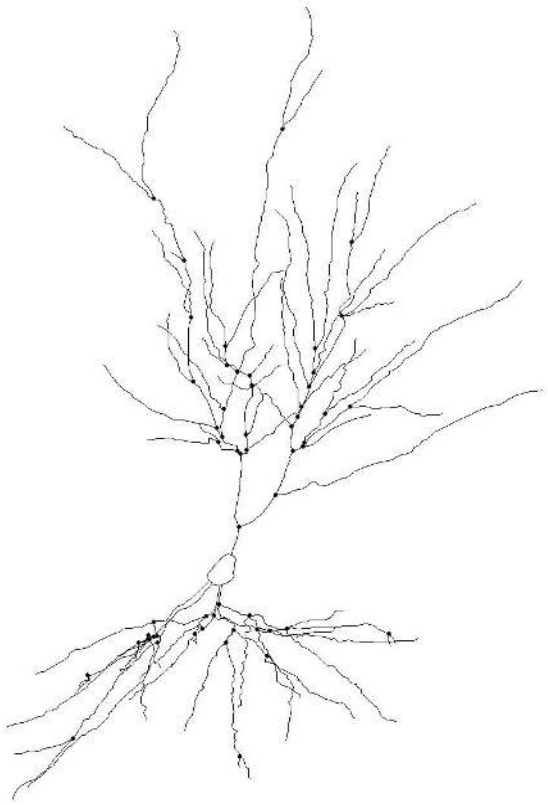
Figure 14. Mean (± SEM) percentage of weight change across the duration of pregnancy and at matched time points in virgin females. Pregnant females gained significantly more weight than virgin females ($P \leq 0.0001$), regardless of stress. *denotes pregnant females significantly different from virgin females (n=5-6/group).

Regardless of Reproductive State, Stressed Females Showed Dendritic Atrophy in the Apical Tree of CA3 Pyramidal Neurons

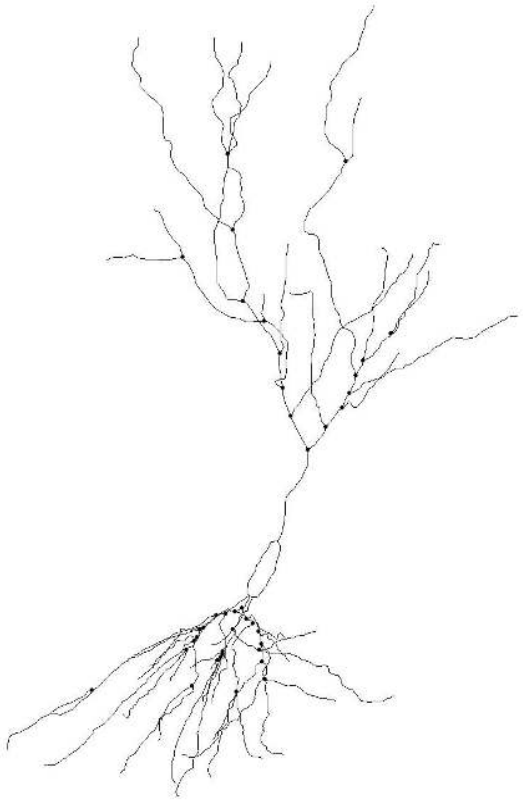
There were region differences in the effects of repeated restraint stress on dendritic length, with stressed females showing a decrease in the number and length of apical dendrites. Figure 15 represents neurolucida drawings of a representative cell for each of the conditions of female rats. The mean dendritic length of pyramidal cells in the CA3 region of the hippocampus of stressed and control virgin and pregnant female rats is shown in Figure 16. For dendritic length, there was a significant interaction between the effect of stress and region (apical vs. basal) ($F_{1,17}$ = 6.03, $P \leq 0.025$), with stressed pregnant and virgin females having shorter apical dendritic lengths than control pregnant and virgin females. *Post hoc* tests revealed that pregnant and virgin females had shorter apical dendritic lengths compared to control pregnant and virgin females ($P \leq 0.05$) and there was no difference between groups in basal dendritic lengths ($P \leq 0.37$). There was also a significant main effect of region ($F_{1,17}$ = 6.23, $P \leq 0.023$), resulting in significantly longer dendrites in the apical region compared to the basal region, but no significant main effect of stress ($P \leq 0.5$). There was also no significant main effect of reproductive state ($P \leq 0.096$) and no significant interactions between reproductive state and stress ($P \leq 0.14$), reproductive state and region ($P \leq 0.46$), or reproductive state, region and stress ($P \leq 0.85$).

Figure 17 shows the mean number of branch points of pyramidal cells in the CA3 region of the hippocampus of pregnant and virgin female rats. There was a significant interaction between the effect of stress and region ($F_{1,17}$ = 7.29, $P \leq 0.008$), with stressed pregnant and virgin females having fewer apical branch points than control pregnant and virgin females. *Post hoc* tests revealed that there were fewer apical branch points in stressed females, compared to control females ($P \leq 0.04$), regardless of reproductive state. There was also a significant main effect of region on the number of branch points ($F_{1,17}$ = 10.65, $P \leq 0.005$), with a greater number of branch points in the apical region than in the basal region, but no significant main effect of stress ($P \leq 0.32$). There was no significant difference between stressed and control females in the total number of basal branch points ($P \leq 0.81$). There was a tendency towards a significant interaction between the effect of reproductive state and region ($F_{1,17}$ = 3.81, $P \leq 0.068$). There was also no significant main effect of reproductive state ($P \leq 0.28$) and no

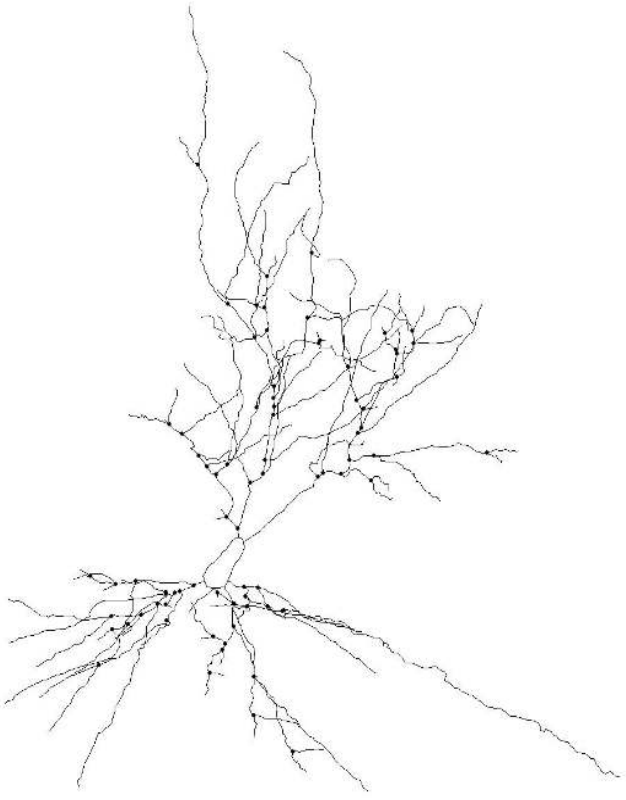
significant interactions between reproductive state and stress ($P \leq 0.68$) or reproductive state, region and stress ($P \leq 0.95$).



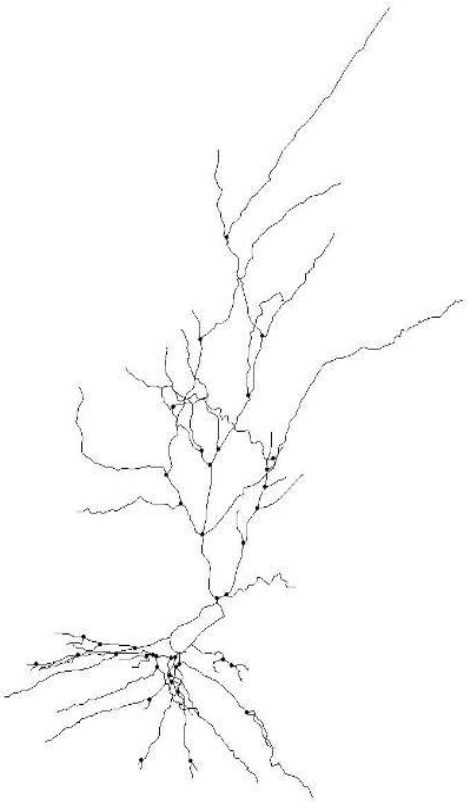
Control virgin



Stress virgin



Control pregnant



Stress pregnant

Figure 15. Neurolucida drawings of representative CA3 pyramidal neurons from each of the four groups of animals. Female rats, regardless of reproductive state, showed a significant atrophy in the apical dendrites as well as a decrease in the number of apical branch points after repeated restraint stress.

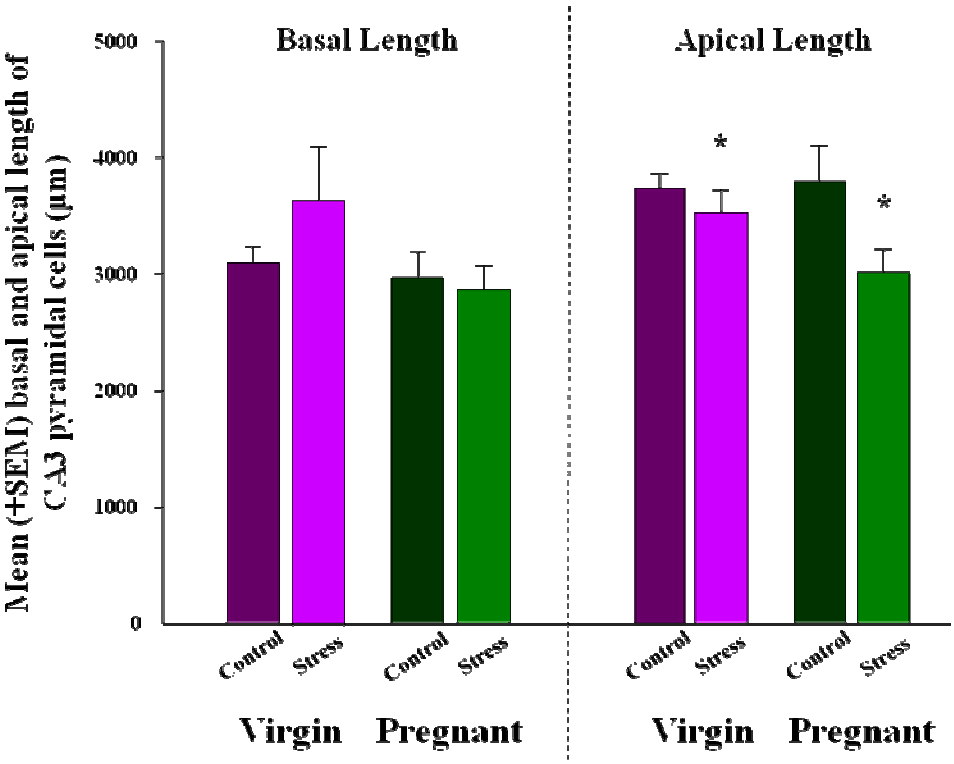


Figure 16. Mean (± SEM) total dendritic length in basal and apical regions of CA3 pyramidal neurons. Stressed females had significantly shorter apical dendritic lengths ($P \leq 0.025$) of CA3 pyramidal neurons than control females and dendrites were longer in the apical region compared to the basal ($P \leq 0.023$), regardless of reproductive state. *denotes stressed females significantly different from control females (n=5-6/group).

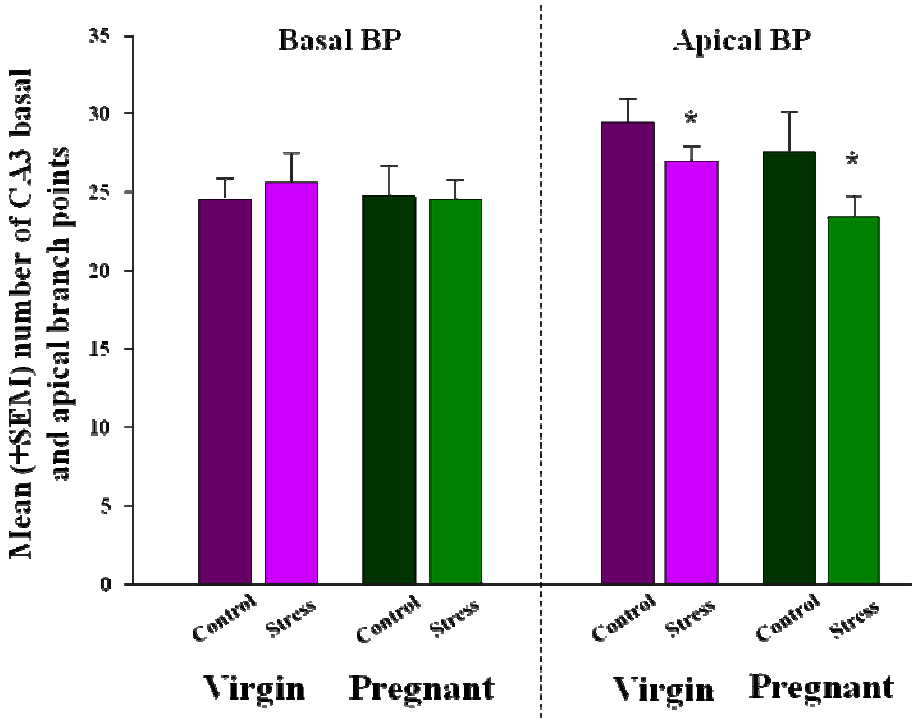


Figure 17. Mean (± SEM) total number of branch points in basal and apical regions of CA3 pyramidal neurons. Stressed females had significantly fewer CA3 apical branch points ($P \leq 0.008$) than control females, and the number of branch point was greater in the apical region than in the basal region ($P \leq 0.005$), regardless of reproductive state. *denotes stressed females significantly different from control females (n=5-6/group).

CA3 Pyramidal Neurons Are Less Complex in Pregnant Female Rats

Using Sholl analysis for CA3 pyramidal neurons, Figure 18 shows the mean total number of dendritic intersections at increased distance from the soma for the four groups/conditions of female rats, using Sholl analysis for CA3 pyramidal neurons. This quantitative analysis demonstrates a significant main effect of reproductive state ($F_{1,17} = 6.37$, $P \leq 0.02$), with pregnant females having significantly fewer dendritic intersections than virgin females. There was no significant main effect of stress ($P \leq 0.28$) or a significant interaction between the effect of reproductive state and stress ($P \leq 0.46$).

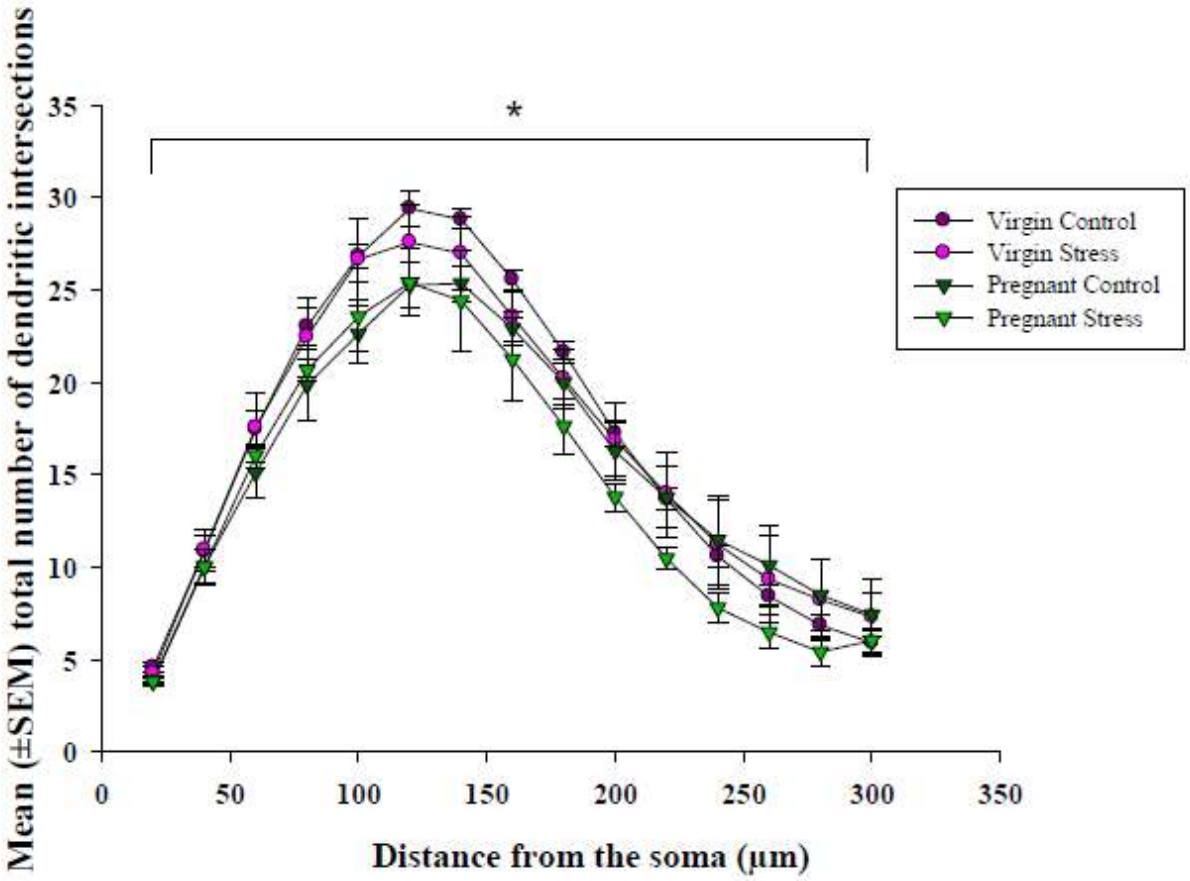


Figure 18. Mean (± SEM) total number of dendritic intersections from the soma using Sholl analysis for CA3 pyramidal neurons. Overall pregnant females had significantly fewer intersections than virgin females, regardless of stress ($P < 0.02$). *denotes pregnant significantly different from virgin (n=5-6/group).

There Was No Significant Effect of Stress on Litter Characteristics

Table 1 shows the size and sex ratio of litters in stressed and virgin pregnant female rats. A one-way ANOVA revealed no significant differences between stressed and control pregnant females in the litter size ($P \leq 0.78$), number of male fetuses ($P \leq 0.35$) or number of female fetuses ($P \leq 0.66$) at the time of perfusion during late pregnancy.

Table 2. Mean (± SEM) total litter size and number of male and female fetuses in pregnant female rats assigned in each condition. There were no significant differences between groups ($0.35 \leq P \leq 0.78$).

Condition	N	Total litter size	Male fetuses	Female fetuses
Stress	5	10.2 ± 0.8	5.6 ± 0.5	4.6 ± 0.5
Control	5	9.6 ± 1.9	4.4 ± 1.1	5.2 ± 1.2

Size of Litter and Sex of Fetuses Was Not Associated with CA3 Dendritic Morphology in Pregnant Female Rats

The size of the litter and the number of male and female fetuses in the litter did not significantly correlate with the number of branch points or dendritic length of pyramidal cells in the CA3 region of the hippocampus of either control and stressed pregnant female rats ($0.1 \leq P \leq 0.9$, Table 2, 3 and 4). However, there was a trend toward a significant negative correlation between the number of male fetuses and the number of basal branch points ($r = - 0.57$, $P \leq 0.086$), indicating that an elevated number of male fetuses in a litter was associated with fewer number of basal branch points.

Table 3. Correlations between CA3 branch points and dendritic length with litter size and number of male and female fetuses of stressed and control pregnant female rats. There were no significant correlations between CA3 branch points and dendritic length with litter size and number of male and female fetuses of stressed and control pregnant female rats ($0.08 \leq P \leq 0.9$).

	Total litter size	**Male fetuses**	**Female fetuses**
Apical branch points	r = - 0.22, $P = 0.54$	r = - 0.51, $P = 0.13$	r = 0.15, $P = 0.68$
Basal branch points	r = - 0.30, $P = 0.40$	r = - 0.57, $P = 0.09$	r = 0.08, $P = 0.82$
Apical dendritic length	r = - 0.06, $P = 0.88$	r = - 0.36, $P = 0.30$	r = 0.26, $P = 0.47$
Basal dendritic length	r = - 0.09, $P = 0.81$	r = - 0.39, $P = 0.26$	r = 0.24, $P = 0.51$

Table 4. Correlations between CA3 branch points and dendritic length with litter size and number of male and female fetuses of stressed pregnant female rats. There were no significant correlations between CA3 branch points and dendritic length with litter size and number of male and female fetuses of stressed pregnant female rats ($0.1 \leq P \leq 0.9$).

	Total litter size	**Male fetuses**	**Female fetuses**
Apical branch points	r = - 0.41, $P = 0.49$	r = 0.06, $P = 0.92$	r = - 0.70, $P = 0.18$
Basal branch points	r = - 0.30, $P = 0.63$	r = 0.12, $P = 0.85$	r = - 0.59, $P = 0.30$
Apical dendritic length	r = 0.07, $P = 0.92$	r = 0.04, $P = 0.94$	r = 0.06, $P = 0.93$
Basal dendritic length	r = - 0.19, $P = 0.76$	r = 0.15, $P = 0.81$	r = - 0.44, $P = 0.46$

Table 5. Correlations between CA3 branch points and dendritic length with litter size and number of male and female fetuses of control pregnant female rats. There were no significant correlations between CA3 branch points and dendritic length with litter size and number of male and female fetuses of control pregnant female rats ($0.1 \leq P \leq 0.9$).

	Total litter size	**Male fetuses**	**Female fetuses**
Apical branch points	r = - 0.15, $P = 0.82$	r = - 0.55, $P = 0.33$	r = 0.27, $P = 0.66$
Basal branch points	r = - 0.30, $P = 0.62$	r = - 0.81, $P = 0.10$	r = 0.26, $P = 0.68$
Apical dendritic length	r = - 0.01, $P = 0.99$	r = - 0.30, $P = 0.63$	r = 0.25, $P = 0.68$
Basal dendritic length	r = - 0.04, $P = 0.95$	r = - 0.64, $P = 0.25$	r = 0.51, $P = 0.38$

Chapter IV Discussion

The present study found that repeated restraint stress resulted in apical dentritic atrophy (a decrease in the number of apical branch points and dendritic length) in CA3 pyramidal neurons of pregnant and virgin females, compared to non-stressed virgin and pregnant rats. Pregnant rats also showed a significant decrease in overall complexity of CA3 pyramidal neurons, as evidenced by fewer dendritic intersections, compared to virgin rats. There was also a tendency toward a significant correlation between male fetuses and the number of basal branch points, indicating that a great number of male fetuses in a litter was associated with fewer basal branch points on CA3 pyramidal neurons. There were no significant effects of stress on dendritic morphology in the basal region of CA3 pyramidal cells, in litter characteristics in pregnant females, or in weight gain.

Stress Decreased Dendritic Morphology in the Apical Region of CA3 Pyramidal Neurons in Pregnant and Virgin Female Rats

The present work is the first demonstration that repeated restraint stress during pregnancy results in marked morphological changes in the CA3 region of the hippocampus of the pregnant mother. The initial transition to motherhood (primiparity) also results in significant dendritic atrophy in CA3 and CA1 pyramidal neurons compared to multiparous and nulliparous (Pawluski & Galea, 2006). Pawluski & Galea (2006) showed that parity and mothering have an impact in hippocampal morphology during the postpartum period, with primiparous rats showing a decrease in the number of branch points and dendritic length in CA3 and CA1 pyramidal neurons compared to multiparous and nulliparous rats. In the present study, basal dendritic atrophy was not found in pregnant females, regardless of stress condition, perhaps due to different times of testing (pregnancy vs. postpartum period), different rat strains and age. Interestingly, the dendritic atrophy in primiparous rats does not last into aging: multiparous, primiparous, and nulliparous females at older ages do not differ in dendritic morphology in pyramidal cells in CA1 region (Love *et al*., 2005).

The present work also found that repeated restraint stress results in apical dendritic atrophy in cycling virgin female rats. Previous studies have consistently found that chronic repeated stress results in apical dendritic atrophy in CA3 pyramidal cells in

male rats (Watanabe *et al*., 1992; Magariños & McEwen, 1995; Galea *et al*., 1997). For example, Galea *et al*. (1997) report a significant decrease in the number of apical branch points and dendritic length in CA3 pyramidal neurons of male rats that had undergone 21 days of restraint stress. However, there have been inconsistent findings with regards to the effects of repeated stress on dendritic atrophy of CA3 pyramidal neurons in virgin female rats. For example, McLaughlin *et al*. (2005) found that stressed virgin female rats had apical dendritic atrophy in CA3 neurons whereas Galea *et al*. (1997) found that repeated stress resulted in dendritic atropy in the basal region of CA3 neurons of virgin female rats.

Several factors, such as rat strain, age, stress paradigm, and estradiol levels, may explain the different findings with regards to the effects of repeated stress on dendritic morphology of CA3 pyramidal neurons in virgin female rats. In the present study, Wistar female rats were used, whereas Galea *et al*. (1997) used Sprague-Dawley rats, and behavioral and neuroendocrinal differences may underlie these two rat strains (Kühn *et al*., 1983; Gatewood *et al*., 2005; Love *et al*., 2005). For example, Kühn *et al*. (1983) have documented that Wistar and Sprague-Dawley rats differ in endocrine responses and this may lead to differences in the stress responsivity and CA3 pyramidal neuron morphology between rat strains. Furthermore, there were also age differences in the animals of both studies, with younger females (50-56 days old) in the study performed by Galea *et al*. (1997) than in the present study (4 months old), which can also influence the differences observed. Age is also involved in changes in the estrous cycle (Fentie *et al*., 2004). Importantly, estradiol levels can stimulate GC secretion (Lo & Wang, 2003; McLaughlin *et al*., 2005), and regulate NMDA receptor antagonist binding in the DG, the main afferent to the CA3 pyramidal cells (Weiland, 1992). Thus, estradiol levels may be involved in CA3 morphological changes after exposure to stress in females. Estradiol levels were not assessed in this study, however Galea *et al*. (1997) found a decrease in plasma estradiol levels in stressed females, pointing towards a "shut down" of gonadal function.

It is well documented that stress affects the estrous cycle (Pollard & Cairncross, 1977; Ma *et al*., 1998). Therefore, the duration and intensity of the stress may also account for the differences between studies. In the present study, 1h of restraint stress was applied per day, for 14 days, accordingly to the same stress paradigm applied by Smith *et al*. (2004), while Galea *et al*. (1997) restrained 6h/day for 21 consecutive days. The stress paradigm applied by Galea *et al*. (1997) lasted for more days and each

restraint session was longer. However, Smith *et al*. (2004) reported that the stress paradigm used in their study was sufficient to induce a state of increased basal corticosterone levels, thus indicating stress effects of restraint (Smith *et al*., 2004). Taken together, the apical dendritic atrophy in CA3 pyramidal cells seen in this study (as well as previous work) and not in Galea *et al*. (1997) study may be due to many procedural differences.

Furthermore, the present study found that apical dendritic atrophy in CA3 pyramidal cells was similar in virgin stressed and pregnant stressed females. This may indicate that, at least on a neural level, pregnant and virgin females are similar in their stress response. However, the present study also found that CA3 neurons were less complex in pregnant female rats compared to virgin female rats, regardless of stress condition. This is perhaps not surprising given the different hormone profiles in pregnant females compared to virgin. It has been well documented that physiological adaptations of neuroendocrine and behavioral stress responses exist in the female brain, during pregnancy and with the onset of motherhood (for review see Slattery & Neumann, 2008). These neuroendocrine changes are needed to ensure the healthy development of the offspring by preventing excess prenatal GC exposure, and appropriate maternal care (Stern *et al*., 1973; Neumann *et al*., 1998; Russell *et al*., 1999; Lightman *et al*., 2001; Kammerer *et al*., 2002; de Weerth & Buitelaar, 2005; Slattery & Neumann, 2008).

The dendritic remodeling seen in this study is similar to dendritic remodeling seen in primiparous rats during the postpartum period and after exposure to chronic restraint stress. Higher corticosterone levels are associated to both pregnancy (Atkinson & Waddell, 1995; Fisher *et al*., 1995) and stress (Conrad, 2008). Thus, it seems likely that corticosterone plays an important role in the remodeling of dendritic trees (branch points and dendritic length) in stressed pregnant and virgin rats, as prolonged increased levels of corticosterone, stress, or pregnancy, induce dendritic remodeling of CA3 pyramidal neurons (Woolley *et al*., 1990a; Magariños & McEwen, 1995; Galea *et al*., 1997; Pawluski & Galea, 2006). Despite the fact that corticosterone levels were not assessed in the present study, Galea *et al*. (1997) reported higher levels of corticosterone in stressed females. Moreover, Smith *et al*. (2004) also reported higher levels of corticosterone in pregnant rats, following the same restraint stress paradigm used in this study.

Corticosterone and its GR are considered one of the main factors that mediate dendritic atrophy (Magariños & McEwen, 1995; Takahashi *et al*., 1998). The high levels of corticosterone can enhance the amplitude of the high-voltage-activated Ca^{2+} current and the synthesis and release of Glutamate (Glu) (Zhou *et al*., 1993; Akaishi *et al*., 2004), which can result in intracellular Ca^{2+} overloading of CA3 neurons (Jia *et al*., 2010). This Ca^{2+} overloading may result in a disaggregation or hydrolysis of skeleton proteins, which may lead to the apical dendritic atrophy (McEwen & Sapolsky, 1995). Glutamic acid, as an excitatory neurotransmitter, may also be involved in this selective damage. Restraint stress is reported to increase Glu high-affinity uptake and release in hippocampus (Gilad *et al*., 1990), as well as increase hippocampal lactic acid release by an NMDA receptor mechanism (Schasfoort *et al*., 1988), supporting that excitatory amino acid release is activated in response to stress and can be one possible explanation of the enhanced susceptibility of the apical dendrites of CA3 neurons to stress. Moreover, CA3 pyramidal neurons receive excitatory inputs from DG via mossy fibers and the apical region, importantly, receive input from all parts of the DG (Amaral & Witter, 1989; Witter, 1989). Thus, damage in the apical dendrites of CA3 neurons can be a result of excitation of the granule cells in the DG by repeated restraint stress. Apical dendritic atrophy of CA3 pyramidal neurons probably relieves the excitotoxic damage from the DG (Jia *et al*., 2010), leading to the possibility that it is not only a consequence but also an adaptation to Glu excitotoxicity and the intracellular Ca^{2+} overloading. Other factors, such as the density and affinity of GC receptors in the hippocampus, as well as levels of CBG and CRH, may also play a role in dendritic remodeling. In fact, Pawluski *et al*. (2009b) found a decrease in CBG levels in primiparous and multiparous rats throughout the postpartum period. Moreover, Takahashi *et al*. (1998) reported that stress during pregnancy resulted in a decrease of maternal levels of CBG, which was similar to decreased plasma CBG levels in virgin females after exposure to repeated restraint stress reported by Galea *et al*. (1997). Thus, these findings may suggest increased circulating levels of free corticosterone.

Litter Size or Sex of the Fetuses Was Not Affect by Stress and Not Associated with CA3 Dendritic Morphology in Pregnant Female Rats

Consistent with past literature, litter characteristics in the present study were not affected by stress (Smith *et al*., 2004). On the other hand, there was a trend towards a

significant correlation between male fetuses and the number of basal branch points, indicating that a great number of male fetuses in a litter was associated with fewer basal branch points on CA3 pyramidal neurons, suggesting that testosterone *in utero* may contribute to dendritic remodeling. Previous studies have also looked into the role of litter characteristics on hippocampal dendritic morphology. Pawluski & Galea (2006) reported a positive correlation between number of male pups and spine density of the basal region of CA1 pyramidal neurons. Interestingly, in that study, multiparous rats had greater spine density in the basal region of CA1 pyramidal neurons and gave birth to more male pups compared to primiparous. Thus, the great spine density in multiparous rats may have been the result of more male pups (Pawluski & Galea, 2006). In the present study, spine density was not assessed. However, spines have been proposed to be plastic and have a role in memory acquisition, as they transform into large, or mushrooms spines after memory acquisition (Kasai *et al*., 2003). Further, spine density is also associated with hormonal fluctuations throughout the female estrous cycle, with a highest density during proestrus, when estradiol levels are increased (Woolley *et al*., 1990b; Woolley & McEwen, 1992, 1993; Shors *et al*., 2001). Progesterone and testosterone are also responsible for fluctuations in spine density in CA1 region of the female rat (Gould *et al*., 1990; Woolley *et al*., 1990b; Leranth *et al*., 2004). For example, testosterone *in utero* may increase CA1 spine density in the mother (Pawluski & Galea, 2006), while progesterone, which is also increased during pregnancy, seems to initially further amplify and subsequently suppress the effect of estradiol on spine density (Gould *et al*., 1990; Woolley & McEwen, 1993).

There is also an effect of stress on spine density, with an increase of CA1 basal spines, following chronic stress in females, which is associated with enhanced spatial learning and memory (McLaughlin *et al*., 2005, 2010). Importantly, high spine density is associated with high density of excitatory synapses (Anderson *et al*., 1996), which contribute to LTP (Matsuzaki, 2007). Moreover, given that LTP has a role on the induction of learning and memory and also increases spine density itself, dendritic spines may have a central role in learning and memory related synaptic plasticity (Matsuzaki, 2007). Gould *et al*. (1990) found no changes in dendritic spine density in CA3 pyramidal cells, which may suggest a specific effect in CA1 neurons. Thus, further work should be performed in CA1 region, with regards to the effects of stress and reproductive state on spine density.

Stress Had No Significant Effect on Body Weight in Pregnant and Virgin Female Rats

In this present study, stress did not significantly affect body weight in pregnant and virgin females. Previous studies have shown that stressed males exhibited a slow rate of weight gain (D'Aquila *et al*., 1997; Bielajew *et al*., 2003; Konkle *et al*., 2003; Dalla *et al*., 2005; Strekalova & Steinbusch, 2010), while stressed virgin females exhibited the same weight gain as controls (Dalla *et al*., 2005; Bowman *et al*., 2009). Thus, a sex-specific fashion may underlie the weight gain in stress conditions, with stress affecting weight in males but not in virgin females. Galea *et al*. (1997) reported higher body weight levels in the control group in comparison with the stress group during the stress paradigm, regardless of sex, and lower weight levels in females compared to males, regardless of stress. Despite an attenuated weight gain in stressed animals, both males and females gained weight over the duration of the stress paradigm, regardless of stress (Galea *et al*., 1997). Thus, rat strain, age and duration or intensity of the stress paradigm may also contribute for differences in weight gain.

As expected, pregnant females used in this study gained significantly more weight across the time than virgin females. Previous research has consistently documented an effect of stress during pregnancy on maternal weight gain, with stressed pregnant females gaining less weight than controls (Darnaudéry *et al*., 2004; Baker *et al*., 2008). In the present study there was no significant difference in weight gain between stressed and control pregnant females, however stressed pregnant females did have attenuated weight gain compared to control pregnant females. Differences between these findings and those of others may be due to differences in stress protocol or rat strain used. As discussed previously, Wistar rats have difference physiological reactions than Sprague-Dawley rats.

Possible Consequences of Hippocampal CA3 Dendritic Remodeling in Response to Repeated Stress for Maternal Behavior

The hippocampus has a well-documented role in learning and on components of spatial memory, such as location memory (Biegler *et al*., 2001), location details (Rosenbaum *et al*., 2000), topographical maps (Teng & Squire, 1999), and navigation (Jacobs *et al*., 1990). The role of estradiol and corticosterone in hippocampus-dependent spatial memory is also documented (Conrad *et al*., 1996; McEwen, 2002). Low levels of

estradiol facilitate while high levels impair spatial working memory in rodents (Holmes *et al*., 2002). Additionally, cognitive deficits in rodents, such as deficits in spatial memory, are a result of high levels of corticosterone (Luine *et al*., 1993; Conrad *et al*., 1996).

Previous studies have found that high levels of corticosterone, resulting in CA3 dendritic atrophy, and also CA1 in a lesser extent, were correlated with significant impairment on spatial learning and memory in the laboratory rat (Sousa *et al*., 2000). Moreover, others have shown worse performance on several spatial tasks in male rats that undergone repeated stress, including radial arm maze, object placement, and Morris Water maze (Luine *et al*., 1994; Conrad *et al*., 1996, 2003; Bowman *et al*., 2001). However, it seems that chronic stress may have a contrary effect in female rats, shown by a performance either enhanced or not affected on the same tasks compared to male rats (Bowman *et al*., 2001, 2002; Conrad *et al*., 2003; Kitrai *et al*., 2004). Thus, male impairment and apparent female resistance in response to stress have been documented to occur in connection with morphological (Watanabe *et al*., 1992; Galea *et al*., 1997) and neurochemical changes (Beck *et al*., 2002; Bowman *et al*., 2002, 2003; Luine, 2002; Luine *et al*., 2007). Conrad (2006) hypothesized that spatial ability in male rats is influenced by a compromised hippocampal ability to regulate the HPA axis, as a result of CA3 dendritic atrophy. Female resistance may be attributed to ovarian hormones, mainly estrogen neuroprotective action (Bowman *et al*., 2001; Lee & McEwen, 2001; McLaughlin *et al*., 2005), which may have a role in hippocampal morphology and function via separate mechanisms than stress (Conrad, 2006).

Furthermore, there has been a growing interest in the impact of motherhood in hippocampus-dependent learning and memory and LTP of the mother (Kinsley *et al*., 1999; Pawluski *et al*., 2006a, b). Previous studies reported that multiparous rats had enhanced working memory performance and primiparous rats had enhanced reference memory performance, following maternal experience, compared to nulliparous rats (Kinsley *et al*., 1999). Pawluski *et al*. (2006a, b) also compared performances between primiparous and multiparous rats after full mothering, reporting that primiparous rats had also enhanced reference memory compared to multiparous and, consistent with Kinsley *et al*. (1999), nulliparous rats. Moreover, enhanced working memory performance in primiparous rats compared to nulliparous rats was also reported in that study (Pawluski *et al*., 2006a, b). These findings are consistent with the facilitating action of low levels of estradiol in spatial working memory in rodents (Holmes *et al*.,

2002), as after delivery, there is a decrease in estradiol levels (Atkinson & Waddell, 1995). Interestingly, the third trimester in primiparous rats, when levels of estrogen are increased, is associated with a decline in spatial working memory performance compared to nulliparous (Galea *et al*., 2000). Thus, performance on spatial memory working tasks, steroid hormone levels and dendritic arborization in the pregnant female rat may have a positive correlation (Woolley *et al*., 1990; Magariños & McEwen, 1995; Galea *et al*., 1997; Sousa *et al*., 2000; Isgor & Sengelaub, 2003). In fact, dendritic atrophy as a result of chronic stress (Magariños & McEwen, 1995; Galea *et al*., 1997; Sousa *et al*., 2000) is associated with enhanced learning and memory performance in the female rat (Bowman *et al*, 2001; Kitraki *et al*., 2004), which is in agreement with the apparent female resistance in response to stress.

Importantly, some studies have found that stress during pregnancy impairs maternal behavior, which may have profound impact in offspring HPA axis function and behavior (Moore & Power, 1986; Melniczek *et al*., 1994; Maccari *et al*., 1995; Smith *et al*., 2004). Smith *et al*. (2004) reported that, after exposure to the same stress paradigm used in the present study, arched-back nursing times were reduced in the stressed mother and stressed mothers spent less time gathering and grouping their litters under them. However, the importance of each behavior, or set of behaviors, in offspring development remains unclear. Furthermore, a chronic stress paradigm during pregnancy is sufficient to induce a state of post-natal depression, as gestationally stressed mothers exhibited greater immobility, which is a suggestive of enhanced depression-like symptoms (Alonso *et al*., 1997; Smith *et al*., 2004). Thus, post-natal depression, which is often accompanied by poorer maternal behaviors and offspring care (Smith *et al*., 2004), may be directly linked with stress during pregnancy, and this deficient maternal care may have a detrimental effect on the offspring.

Chapter V Conclusion and Future Directions

The present study provides new evidence that stress and pregnancy have an impact in dendritic morphology of pyramidal neurons in the CA3 region of the hippocampus. Stressed female rats, regardless reproductive state, had significant apical dendritic atrophy of pyramidal neurons in the CA3 region of the hippocampus when compared to non-stressed female rats. Overall, pregnant female rats, regardless of stress, had significantly less complex pyramidal neurons compared to virgin female rats. Moreover, stress did not affect body weight in pregnant and virgin female rats, however stressed pregnant females did not gain as much weight as control females. Stress also did not affect litter characteristics, but there was a trend toward a significant negative correlation between the number of male fetuses and the number of basal branch points.

Care and treatment of women has been derived predominantly from research on males, however recent research has begun to focus on sex differences, reporting that understanding the stress response and cognitive ability in males may not extrapolate to females. Given statistics on mental health in women, it is alarming that still few studies use females to investigate the influence of stress on brain morphology and cognitive ability; including also the impact of stress during periods of reproduction and its implications for mother and offspring well-being. Therefore, this present study is a first demonstration of the effect of stress and reproductive state on dendritic morphology in CA3 region of the adult female rat. Further research will assess dendritic morphology and spine density in the CA1 and DG regions of the hippocampus, as well as elicit the role of steroid hormones mediating these effects and the possible role of parity and stress exposure on performance of hippocampal dependent tasks in the adult female.

Chapter VI Bibliographic References

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