“The impact of prenatal stress and neonatal handling on the neuronal development of the rodent limbic system”

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Dedicated to my wonderful father, Dominic Murmu and mother, Marium Soren

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ABSTRACT

The way in which experience sculpts developing neural circuitry is one of the most intriguing questions in developmental neurobiology. Evidence has been accumulated that epigenetic factors affect the development of brain and behavior in a much more pronounced way than previously appreciated. However, the cellular mechanisms of such experience-driven developmental changes are far from being understood. The present study provides the first evidence that stress *in utero* represents an epigenetic factor that can considerably interfere with the development of neuronal morphology in the rodent limbic system.

In recent years, the role of psychobiology in understanding abnormal behaviors has become increasingly important. There has been increased awareness that various forms of pathological behaviors in humans might be the outcome of adverse or traumatic experiences, such as stress, occuring early in life. For instance, stress experienced by a mother during pregnancy can act as a predisposing risk factor in the development of schizophrenia, depression, autism and attention-deficit-disorder in the offspring. Similarly, there is considerable evidence indicating that the separation of infant from the mother during the first weeks of their life may lead to behavioral problems at adulthood. Our working hypothesis postulates that exposure to stress during critical developmental periods interferes with the development of neuronal morphology and the establishment and refinement of synaptic circuits. Based on our hypothesis, the aim of this study was to identify the impact of prenatal stress and neonatal handling on the development of neurons and their synaptic networks in the rodent limbic system. The limbic system is a target for hormones involved in stress response and has been implicated in several behavioral and psychiatric disorders that are exacerbated or precipitated by stress exposure. Thus the assessment of the effects of stress on the limbic system may have important implications for the causes and prevention of disorders due to dysfunctional limbic system.

This study shows pronounced changes in the morphology of pyramidal and granular neurons in response to prenatal stress. Prenatal stress resulted in significantly lower spine density in the orbitofrontal and anterior cingulate cortices relative to the untreated control animals. In addition, there was a significant reduction in the total dendritic length and arborization in the orbitofrontal and anterior cingulate cortices of males, and the CA3 and CA1 hippocampal areas of both sexes as well as in the basolateral nucleus of males. The present study also provides evidence that the effect of prenatal stress is sexually dimorphic. The neuronal morphology of males and females is

altered differentially by prenatal stress. This study further indicated that the neuronal alterations induced by prenatal stress are prevented and/or reversed by neonatal handling. Neonatal handling prevented and/or reversed prenatal stress-induced neuronal alterations in a sex, region and dendrite-specific manner. The present study also demonstrated that the separation of infants from the mothers during the early weeks of their life caused significant alterations in the morphology of pyramidal and granular neurons, which markedly differed between the sexes. Finally, this study revealed that there are considerable sex differences in the neuronal morphology of untreated control animals.

The findings of this study provide a neuroanatomical substrate for the behavioral deficits described in prenatally stressed animals. Stress-induced morphological alterations might underlie or contribute to the behavioral impairments caused by stress.

1.0 INTRODUCTION

The ability of an organism to adapt to its environment is integral to its survival. Daily life involves confrontation with changing situations that can be physiologically and psychologically challenging. In biological systems, the term “stress” is used to define any condition that seriously perturbs the physiological/psychological homeostasis of an organism. Stressful stimuli have a significant impact on brain function and integrity. Brief periods of moderate, predictable stress are not problematic; indeed the body's survival actually depends upon the ability to mount a response to stress. On the other hand, exposure to prolonged, severe, or unpredictable stress has been shown to be potentially injurious to both humans and experimental animals. In the recent decade, an important line of neuroscience research has shown that stressful experiences that occur during the critical periods of pre - and postnatal development can have a negative impact on brain function. These studies have shown that the brain's development can be altered by these experiences, resulting in impaired or delayed physical, cognitive, emotional and social maturation. Consequences of stress, such as its negative influences on cognition and emotion are an issue of major relevance to human health. However, the mechanisms by which stress in early life provokes these long-term effects remain unsolved. Gaining insight into the biological underpinnings of the consequences of early life stress might help us to understand specific processes activated by stressful stimuli at the cellular level.

1.1 Stress during pregnancy: risk factor for developing psychopathology in the offspring at adulthood

In recent years, it has become increasingly evident that the antecedents of many illnesses begin in fetal life and that prenatal events can modulate our development towards either health or disease later at adulthood. In particular, there is considerable evidence indicating that exposure of mothers to psychosocial stress or adverse life events during pregnancy may act as a predisposing risk factor in the development of several psychiatric and neurological disorders in the offspring. Such emotional traumas include

natural disasters, such as floods (Selten et al 1999) or earthquakes (Watson et al 1999), and man-made disasters like war (Van OS J & Selten 1998) as well as unpredictable aircraft noise (Jones & Tauscher 1978) and the death of loved ones (Huttunen & Niskanen 1978). A study by Van OS J and Selten (1998) indicated that the 1941 invasion of the Netherlands by the German army led to a higher incidence of schizophrenia in those offspring whose mothers were pregnant during that time. Similarly, women that were bereaved of their husbands during pregnancy due to the Second World War had children with a higher risk of suffering from schizophrenia, depression and neurotic disorders later in life (Huttunen & Niskanen 1978, Van OS J & Selten 1998). Maternal psychosocial stress during pregnancy has also been linked with Attention-deficit-disorder (Linnet et al 2003) and autism in the offspring (Beversdorf et al 2005). Furthermore, adverse life events during pregnancy are related to delays in the motor and mental development (Huizink et al 2003) and a higher incidence of behavioral problems in the offspring (Neugebauer et al 1999, Papousek & Hofacker 1998, Ward 1991). Stressful events during pregnancy are also associated with adverse obstetric outcomes, such as increased risk for preterm delivery, lower birth weight and small head circumference of infants (Austin & Leader 2000, Lederman et al 2004, Wadhwa et al 1993).

Since it is rarely possible to study brain development in humans, most studies showing the effects of stress *in utero* have been undertaken using rats, with fewer studies in primates and guinea pigs. Pregnant animals have been exposed to variety of mild, moderate and severe stressors at different times during gestation including restraint (McCormick et al 1995), crowding (Dahlof et al 1977), electric tail shocks (Takahashi et al 1998), footshocks (Shalev & Weiner 2001), noise (Clarke & Schneider 1993), saline injections (McClure et al 2004), and immobilization (Dahlof et al 1977). The effects of prenatal stress (PS) are modulated by the nature, duration and timing of the occurrence of stress during gestation. Stress during the last week of pregnancy has been shown to significantly alter brain development and the behavior of the offspring (Weinstock 2001a).

Animal research has indicated that exposure to prenatal stress induced persistent

behavioral aberrations. In particular, adult rats and primates exposed to prenatal stress continued to exhibit anxiety (Fride & Weinstock 1988, Patin et al 2005, Vallee et al 1997) depressive-like behavior (Alonso et al 2000, Secoli & Teixeira 1998), fearfulness (Dickerson et al 2005, Ward et al 2000), reduced social interaction (Clarke & Schneider 1993), exaggerated behavioral response to stress (Clarke et al 1994), impaired learning (Lemaire et al 2000, Szuran et al 2000) and cognition (Bowman et al 2004) as well as a delay in the motor development (Patin et al 2004).

Neuroendocrine functions are also altered in response to prenatal stress. Exposure to *in utero* stress impaired the regulation of the hypothalamus-pituitary-adrenal (HPA) axis in the rodents (Maccari et al 2003, Weinstock 1997). Elevated concentrations of ACTH, corticosterone and β-endorphin have been described in prenatally stressed animals in comparison to controls (Koenig et al 2005, Weinstock 2001a). Furthermore, prenatal stress has been linked with alterations in glucocorticoid receptors in the offspring (Henry et al 1994, Koehl et al 1999).

Experimental studies in animal models have indicated that prenatal stress affects the biogenic amine system. For example, prenatally stressed animals have exhibited alterations in the plasma concentration of norepinephrine, dopamine and their metabolites (Alonso et al 1994, Alonso et al 1997, Muneoka et al 1997, Schneider et al 1998). Changes in the asymmetric patterns of dopamine neural circuitry in the CNS of prenatally stressed rats have also been reported (Fride & Weinstock 1987, Fride & Weinstock 1989). Furthermore, prenatal stress has been associated with long-term alterations in the serotonergic activity (Peters 1988, Peters 1990).

Although prenatal stress has been shown to affect behavioral development in laboratory rodents and non-human primates, relatively few studies have investigated the impact of prenatal stress on brain development. Nevertheless, these few studies have indicated that stress experienced by a mother during pregnancy can also produce significant morphological alterations in the offspring. A study by Hayashi et al (1998) indicated that exposure to prenatal restraint stress decreased hippocampal synaptic density (approx. -

30%) in the rat offspring. Prenatal stress has also been found to suppress the process of neurogenesis and reduce the density of nitric oxide producing neurons in the hippocampus of rodents (Lemaire et al 2000, Vaid et al 1997). In addition, exposure to prenatal stress caused an expansion of the lateral amygdaloid nucleus (Salm et al 2004) and reduced the volume of nucleus accumbens (McClure et al 2004). Prenatal stress has also been shown to alter pre- and postsynaptic gene expression in the rat frontal pole (Kinnunen et al 2003).

In spite of the potentially strong effects of prenatal stress on brain morphology and functions, it is not known how exposure to prenatal stress influences the development of neurons and their synaptic network in the offspring. This is one of the first comprehensive studies investigating the development of synaptic network in the limbic brain areas of the male and female rats exposed to stress *in utero.*

1.2 Gender and the Brain: Effect of prenatal stress is sexually dimorphic

The idea that male and female brains are organised differently has been around for a long time. After all, since males and females are dissimilar in size, behave differently and respond differently to environmental events, it is not too surprising that the brain organisation also differs between the genders. For instance, the cytoarchitecture of human cerebral cortex is sexually dimorphic. Language-associated cortical regions such as Wernicke and Broca as well as the volume of orbitofrontal cortex, anterior commissure etc. is larger in women compared to men (Allen & Gorski 1991, Gur et al 2002, Harasty et al 1997). On the other hand, men have larger corpora callosa than women (Sullivan et al 2001). Morphometric analysis of human brains have shown differences in neuronal architecture in the cortex with men having more cortical neurons and women having more neuronal processes (De Courten-Meyers 1999, Rabinowicz et al 1999).

Like the studies in humans, animal research has detected substantial sex differences in

the neuroanatomy of different cortical regions. Seymore and Juraska (1992) have shown sex differences in the rat visual cortex with females having greater total dendritic length and longer terminal branches than males. Similarly, Bartesaghi et al (2003) have shown that the males had more dendritic branches than females in the innermost dendritic tree whereas the females had more branches over the middle/outer dendritic tree and a longer dendritic length. It has also been shown that the anterior commissure and the arcute nucleus of the hypothalamus are larger in male rats compared to females (Leal et al 1998, Noonan et al 1998). On the other hand, female rats have larger volume and greater number of neurons than males in the locus coeruleus (Pinos et al 2001). Recently, Markham and Juraska (2002) have shown that males have greater dendritic spine density as well as arborization than females in the anterior cingulate cortex.

With respect to prenatal stress, sexually dimorphic alterations in brain and behavior have been reported. A study by McCormick et al (1995) indicated that prenatal stress increased the plasma concentration of adrenocorticotropin (ACTH) and corticosterone in the female rats but not in the males. In addition, exposure to prenatal restraint stress reduced the number of hippocampal granular neurons in the female rats but not in the male rats (Schmitz et al 2002). Szuran et al (2000) have shown that the prenatally stressed males performed worse in the water-maze test compared to the females. Also, Gue et al (2004) have described sex differences in the passive avoidance test, with significant impairments in the prenatally stressed female rats relative to the males. Additionally, prenatal stress alters dopaminergic activity in a sex-specific manner with the PS males showing decreased HVA and DOPAC levels whereas the stressed females had elevated HVA and DOPAC levels in the prefrontal cortex (Bowman et al 2004). Furthermore, prenatal stress has been shown to impair latent inhibition in a sex-specific manner. For example, *in utero* exposure to inescapable footshock and constraint stress disrupts latent inhibition in the prenatally stressed males but not in the female rats (Bethus et al 2005, Shalev & Weiner 2001).

It is clear from the above observations that prenatal stress can affect brain development and behavior in a sex-specific manner. This study was designed to investigate the

possibility that sex difference exist in the influence of prenatal stress on the neuronal and synaptic development of the offspring.

1.3 Behavioral effects of prenatal stress are prevented and/or reversed by neonatal handling

It has been shown that it is possible to prevent and/or reverse some of the behavioral abnormalities induced by prenatal stress by appropriate manipulations in the neonates. Neonatal handling is one of the most common experimental paradigms used to study the reversal effects of prenatal stress. In this type of handling, the rat pups are removed from the nest for a short period during the first days of their life. A study by Wakshlak and Weinstock (1990) demonstrated that exposure of pregnant rats to random light and noise stress led to enhanced emotionality and timidity whereas daily handling for 21 days lowers behavioral reactivity in the rat offspring. Similarly, Vallee et al (1997) have shown that prenatal stress resulted in higher anxiety whereas neonatal handling reduced anxiety levels in the adult rats. Handling of prenatally stressed rats has also been shown to prevent the dysregulation of hypothalamic-pituitary-adrenal (HPA) axis at adulthood (Weinstock 2002). Furthermore, there is substantial literature documenting that neonatal handling can influence brain development and behavior in the opposite directions to that induced by prenatal stress. It has been shown that prenatal stress elevates the plasma concentration of corticosterone and ACTH in response to stressful stimuli (Clarke et al 1994, Koenig et al 2005, Weinstock 2001a) whereas corticosterone response to a wide variety of stressors are reduced in rats that were handled during the first 3 weeks of their life (Meaney et al 1989, Viau et al 1993). Also, exposure to prenatal stress induced fear of novelty (Dickerson et al 2005, Ward et al 2000) whereas neonatal handling is reported to decrease fear in rats exposed to novel intimidating situations (Vallee et al 1997). Finally, it has been shown that prenatal stress impaired learning and memory (Lemaire et al 2000, Szuran et al 2003) but handling during the early weeks of life improved various aspects of learning and memory in the adult rats (Escorihuela et al 1994, Escorihuela et al 1995). In addition, disruption of normal mother-infant interaction

in the form of neonatal handling is associated with long-term alterations in emotional and behavioral regulations, cognitive functions, and neuroendocrine response to stress, brain morphology and neurochemistry (Anisman et al 1998, Papaioannou et al 2002, Smythe et al 1994, Wilson et al 1986) in the rodents.

Since neonatal handling was reported to prevent and/or reverse behavioral and endocrine impairments in the rodents, I wanted to examine if the prenatal stress-induced structural alterations are also prevented and/or reversed by neonatal handling. In addition, I have tried to find out how handling alone influence the neuronal morphology of the rodent limbic system.

1.4 Limbic system: Structure, Function and Psychopathology

The term “Limbic” is derived from the Latin word “Limbus” meaning border and was coined by Paul Broca in 1878. He used the term limbic lobe to describe parts of cerebral cortex that formed a rim around the corpus callosum on the medial surface of the hemisphere. In the year 1937, James Papez postulated that emotions were the product of activity within the limbic cortex; arguing that the emotional brain consisted of a circuit in which information flowed from the mammillary bodies in the hypothalamus to the anterior thalamic nucleus to the cingulate cortex, then to the hippocampus and back to the mammillary bodies. In 1949, Paul D. MacLean expanded the Papez circuit and proposed that there are three main "sub circuits" of the limbic system: the **Survival Circuit,** consisting of the amygdala and hippocampus; the **Pleasure Circuit,** consisting of the cingulate gyrus, septum and part of the hypothalamus; and the **Social Circuit,** consisting of the thalamus and hypothalamus. Although there is no universal agreement on the structures which comprise the limbic system, it can be considered to be consisting of the hippocampal formation, amygdala, prefrontal cortex, cingulate gyrus, fornix, thalamus and the hypothalamus.

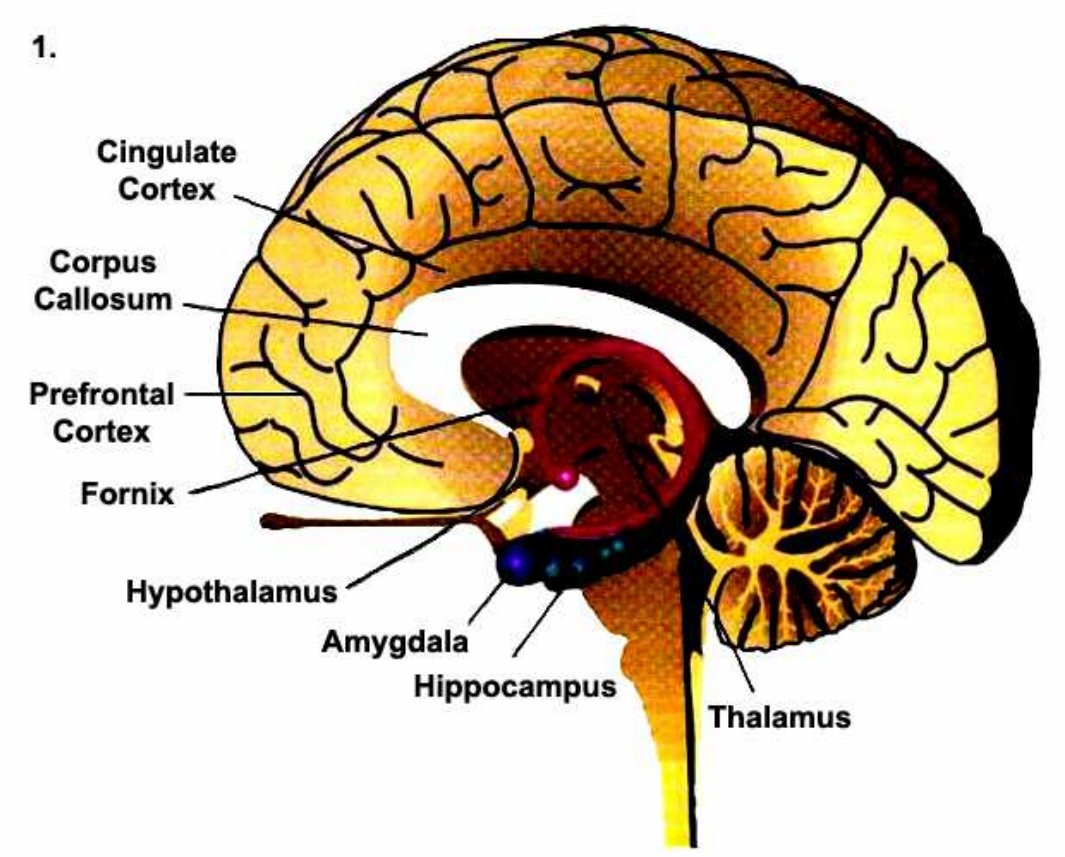


Figure 1. Diagram illustrating the major components of the limbic system of human brain. This figure is adapted from the website: general.rau.ac.za.

More detailed accounts of the morphological and functional aspects of those limbic brain areas which were analysed in this study are provided below:

Hippocampal Formation: The hippocampal formation is composed of two distinct structures, known as Ammon's Horn (often referred to as the hippocampus) and the dentate gyrus. **Hippocampus:** The hippocampus is located within the inferior medial wall of the temporal lobes between the thalamus and cortex, towards the posterior of the forebrain. It forms a part of the limbic system and plays a part in learning and memory. The hippocampus can be divided into three substructures on the basis of their cytoarchitecture and connectivity, known as CA1, CA2 and CA3. The main input to the hippocampus arises from the entorhinal cortex and passes through the dentate gyrus. The dentate gyrus projects via mossy fibers to the hippocampal CA3 area which in turn projects via schaffer collaterals to field CA1. These connections form the major circuit of

the hippocampal formation. Signals transmitted from the entorhinal cortex are processed within this circuit and are then transmitted by CA3 and CA1 outside the hippocampal formation. CA1 neurons also receive input directly from the perforant path and send axons to the subiculum. These neurons in turn send the main hippocampal output back to entorhinal cortex forming a loop. **Dentate gyrus:** The dentate gyrus is part of the hippocampal formation consisting of granule cells as the principal excitatory neurons. Granule cells project to the pyramidal cells and interneurons of layer CA3 of the hippocampus and receive inputs from areas in the neocortex. It plays an important role in formation of new memories (Morgane & Mokler 2005).

Psychopathology: Brain imaging studies show smaller hippocampal volume in several psychiatric disorders, including post-traumatic stress disorder (Lindauer et al 2004), major depressive disorder (Lange & Irle 2004), borderline personality disorder (Driessen et al 2000, Tebartz et al 2003) and schizophrenia (Sachdev et al 2000). Hippocampal atrophy is also evident in Alzheimer disease (Callen et al 2001) and in Down syndrome (Aylward et al 1999) as well as in epilepsy (Marchetti et al 2003). Recently, Schumann et al (2004) have shown that the hippocampal volume is enlarged in autism. Besides alterations in the volume, the hippocampal formation also displays synaptic and molecular abnormalities in several psychiatric disorders. A study by Kolomeets et al (2005) indicated that the size and dendritic arborization of CA3 pyramidal neurons are altered in people with schizophrenia. Similarly, there is a selective loss of interneurons and parvalbumin-containing cells in the hippocampus of schizophrenics (Heckers & Konradi 2002, Knable et al 2004). An abnormal synaptic terminal with lower values/ratio of the presynaptic proteins has been described in the hippocampus of schizophrenics (Sawada et al 2005). A decreased amount of reelin protein is reported in the dentate gyrus of patients suffering from schizophrenia, bipolar disorder and depression (Knable et al 2004). In the animal model of epilepsy, there was a significant impairment of long-term potentiation in area CA1 of the hippocampus (Schubert et al 2005).

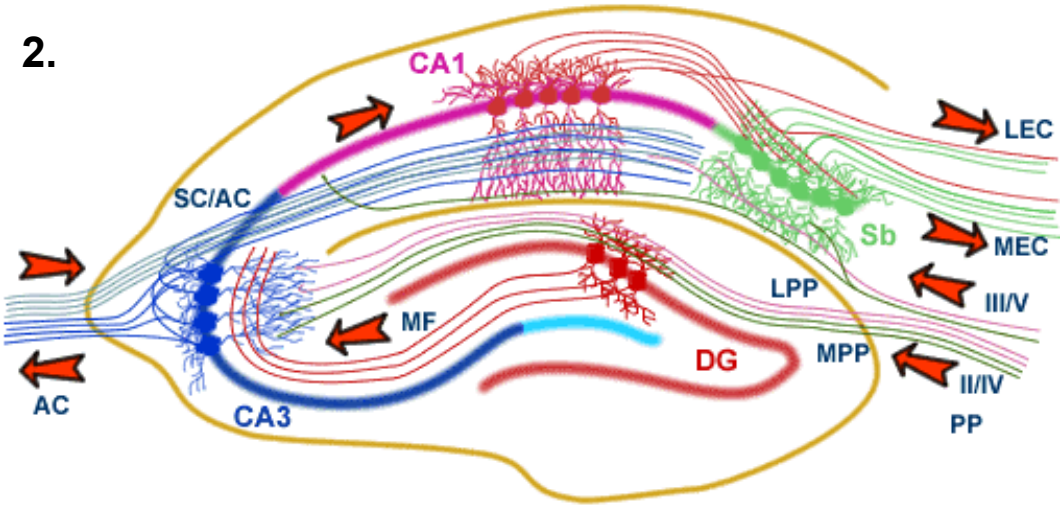


Figure 2. The circuitry and connections of the hippocampal formation. Abbreviations: EC - Entorhinal cortex; DG - Dentate gyrus; SC - Schaffer collateral pathway; AC - Associational commissural pathway; PP - perforant pathway; Sb - Subiculum; MF - Mossy fibers; LPP - Lateral and MPP - Medial perforant pathway; LEC - Lateral and MEC - Medial entorhinal cortex; CA1 & CA3 areas of the hippocampus. This figure is adapted from the website: www.bris.ac.uk.

Prefrontal cortex: The prefrontal cortex (PFC) is the anterior part of the frontal lobes of the brain, located anterior of motor and premotor areas. The rat prefrontal cortex in general is divided into three parts – i) medial prefrontal cortex, ii) orbital prefrontal cortex and iii) lateral prefrontal cortex. Medial prefrontal cortex constitutes the major portion of the medial wall of the hemisphere anterior and dorsal to the genu of corpus callosum. In rodents, medial PFC is divided into four distinct areas: medial precental area, anterior cingulate area, prelimbic area and the infralimbic area. The medial prefrontal cortex can be further subdivided into a dorsal component that includes the dorsal anterior cingulate cortex and dorsal part of prelimbic area, and a ventral component including the ventral prelimbic, infralimbic and medial orbital areas. The orbital prefrontal cortex lies dorsal to the caudal end of the olfactory bulb. Lateral prefrontal cortex or agranular insular cortex is located in the anterior part of the rhinal sulcus. Efferent and afferent projection patterns of dorsally located prefrontal areas are characterized primarily by somatosensory cortical associations, and more ventrally located areas are characterized by cortical relationships with limbic and associational

areas. Limbic regions like the hippocampus and amygdala are predominantly connected with the ventrally located areas. Although as a whole the amygdaloid complex is connected with the entire medial prefrontal cortex, there appears to be clear predominance for interconnections with the more ventrally located areas. Medial PFC is involved in attentional processes and in working memory as well as in social behavior. The orbital cortex on the other hand is thought to play an important role in social and emotional behaviors. The lateral prefrontal cortex is involved in cognition (Fuster 2001, Heidbreder & Groenewegen 2003, Uylings et al 2003).

Psychopathology: Neuroimaging studies have shown prefrontal atrophy in schizophrenia (Molina et al 2005, Ragland et al 2004), obsessive-compulsive disorder (Szeszko et al 1999), post-traumatic stress disorder (Rickert et al 2006) attention-deficit-disorder (Seidman et al 2005), depression (Lai et al 2000) and personality disorders (Tebartz et al 2003). Altered synaptic connectivity in the PFC has also been reported in schizophrenics. For e.g. spine density is reduced in the PFC pyramidal neurons of schizophrenics (Glantz & Lewis 2000). Elevated neuronal densities have been described in the PFC of schizophrenics as well (Selemon et al 1995, 1998). GABA-nergic neurotransmission and concentration of presynaptic protein is also altered in the PFC of schizophrenics (Glantz & Lewis 1997, Halim et al 2003, Karson et al 1999, Lewis et al 1999). Similar effects are reported in the animal model of schizophrenia (Halim et al 2003).

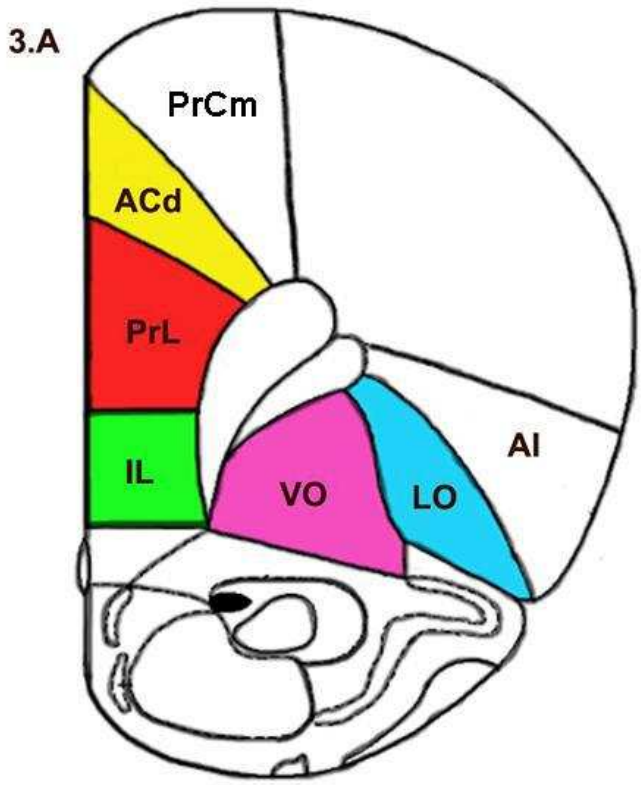


Figure 3.A Major divisions of the prefrontal cortex. Abbreviations: IL - Infralimbic cortex; PrL - Prelimbic cortex; ACd - anterior cingulate cortex; VO - Ventral orbital cortex; LO - Lateral orbital cortex; AI - Agranular cortex. PrCm - Precentral area. This figure is adapted from the Rat Brain by Paxinos & Watson (1998).

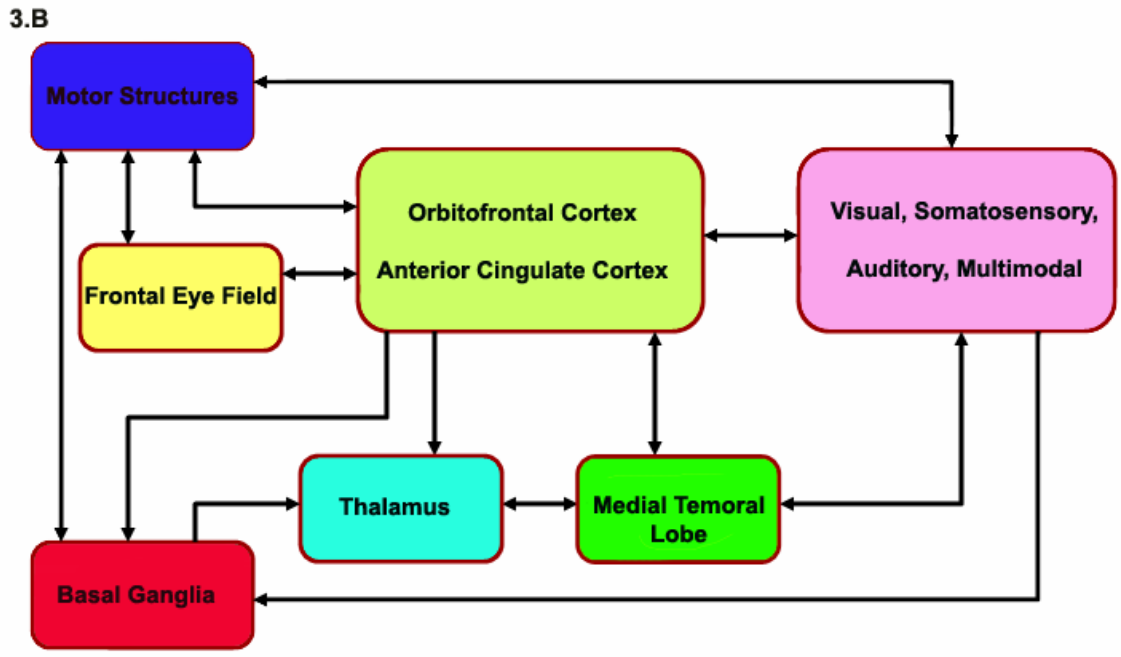


Figure 3.B Diagram illustrating the major connections of the orbitofrontal and anterior cingulate cortices. Here the double-headed arrows represent reciprocal connections whereas the single-headed arrows represent non-reciprocal connections. This figure is adapted from Miller and Cohen (2001), Annu. Rev. Neurosci., 24:167-202.

Amygdala: The amygdala was first identified by Burdach in the early 19th century. It is an almond-shaped structure lying deep within the temporal lobe. In rats, the amygdaloid complex is structurally diverse and comprises 13 nuclei, each with different inputs and outputs and with different functions. These nuclei are further divided into subdivisions that have extensive internuclear and intranuclear connections. These nuclei and subnuclei are distinguised on the basis of cytoarchitectonics, histochemistry and the connections they make. Amygdala nuclei are divided into three groups: i) Deep or basolateral group, which includes the lateral nucleus, basal nucleus and the accessory basal nucleus; ii) Cortical-like nuclei, which includes the cortical nuclei and nucleus of lateral olfactory tract; and iii) Centromedial group composed of medial and central nuclei. Inputs to the amygdala can be separated into those arising from cortical and thalamic structures and those arising in the hypothalamus and brain stem. Brain stem inputs arise from regions involved in behavior and the autonomic system. Cortical and thalamic inputs supply information from sensory areas and structures related to the memory system. The prefrontal cortex is a major source of cortical projection to the amygdaloid complex. The amygdala receives inputs from all modalities: olfactory, somatosensory, gustatory, viseral, auditory and visual. Based on Golgi studies, two main types of neurons are described in amygdala, pyramidal and stellate. About 70% of the cell population in the amygdala are pyramidal. The amygdala is primarily involved in producing and responding to signs of anxiety, fear and aggression. It has also been shown to play an important role in emotional processing and memory (Sah et al 2003).

Psychopathology: Clinical research has indicated that abnormalities in amygdala may lead to autism (Wand 2005), panic disorders (Massana et al 2003) as well as epilepsy (Trimble & Van Elst 2003). The amygdala is hyperactive in anxiety and mood disorders and may undergo a biphasic change in the structure - such as increasing in size in acute depression and shrinking in long-term depression (Frodl et al 2003, Lange & Irle 2004). Furthermore, magnetic resonance studies have shown amygdala atrophy in cases of bipolar disorder (Blumberg et al 2003) as well as in the borderline personality disorders (Tebartz et al 2003). In the animal model of epilepsy, significant impairments of long-term potentiation in the lateral amygdala have been reported (Schubert et al 2005).

Serotonergic transmission is altered in the rat model of depression (Hasegawa et al 2006, Zangen et al 1997). In addition, synaptic hyperexcitability has been described in the lateral nucleus of amygdala of the epileptic rats (Benini & Avoli 2006).

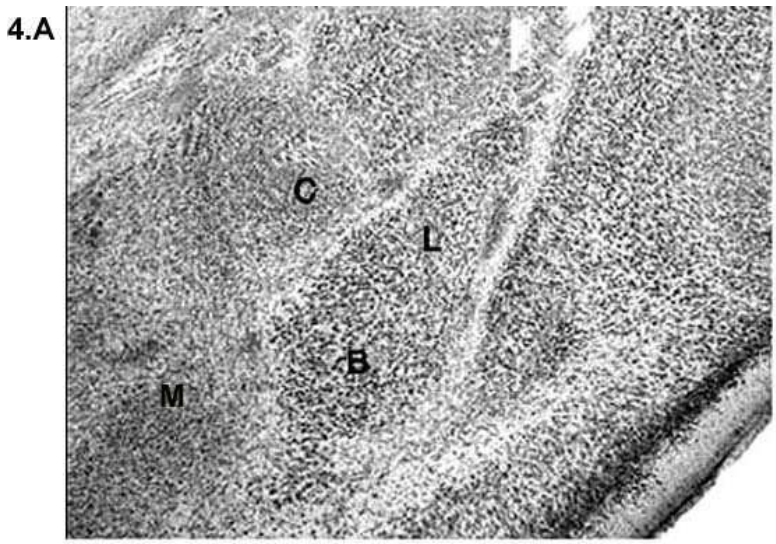


Figure 4.A Nissl stained picture of amygdaloid complex showing the central (C), basolateral (B), lateral (L) and medial (M) nucleus of rodents. This figure is adapted from Salm et al (2004), Deve. Brain Res., 148:159-167.

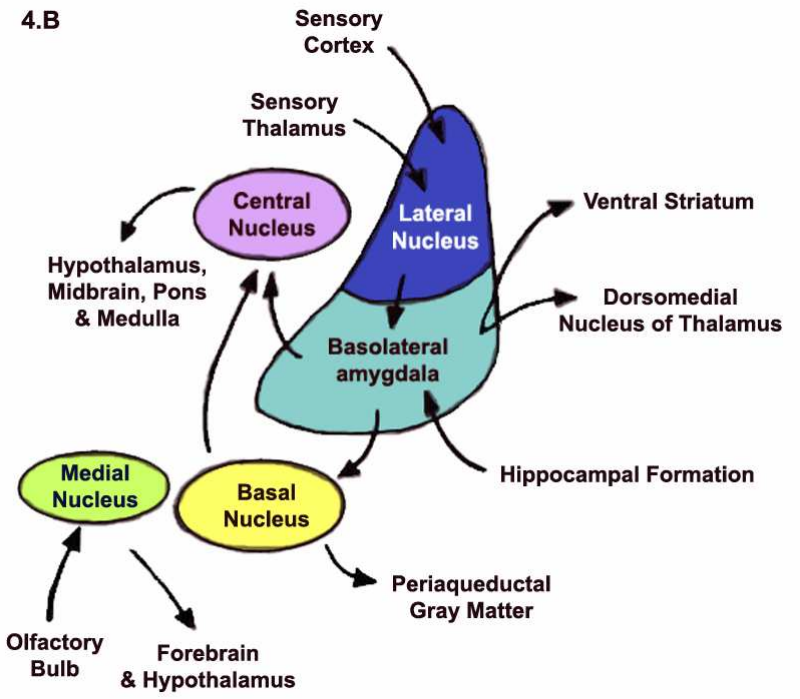


Figure 4.B Diagram illustrating the major connections of the amygdala. This figure is adapted from the website: homepage.psy.utexas.edu.

1.5 Stress and neuronal plasticity in the limbic system

Neuronal and synaptic plasticity is an intrinsic property of the mammalian brain and represents evolution's invention to enable the nervous system to escape the restrictions of its own genome and thus adapt to environmental pressures, physiologic changes and experiences. The adult nervous system is not static, but instead can change and be reshaped by experiences. In general, there are two ways by which experience can alter the brain: either by modifying existing circuitry or by creating novel circuitry. Both positive and negative experiences characterized for examples as “enriched” and “improvised” environments have been shown to variously affect brain size, cortical thickness, neuron size, spine density, dendritic branching as well as glial structure and density. For instance, rodents that were placed in complex environmental conditions had increased dendritic arborization in the visual cortex relative to cage-reared animals (Green et al 1983, Kolb & Whishaw 1998). Similar changes were found in the animals trained in complex motor tasks (Greenough et al 1985). There was also an increase in the number of synapses per neuron (Turner & Greenough 1985), astrocytic processes (Jones & Greenough 1996, Sirevaag & Greenough 1987, 1991) as well as cortical thickness and size in the brains of enriched versus cage-reared animals (Beaulier & Colonnier 1987, Rosenzweig & Bennett 1996). The olfactory system in rodents also seems to be sensitive to experience-dependent changes. It is generally found that olfactory deprivation leads to restricted morphological development of the olfactory system, whereas olfactory training or olfactory enrichment leads to enhanced development (Doving & Pinching 1973, Meisami & Safari 1981, Rosselli-Austin & Williams 1990). Analogous to experience-dependent changes in the olfactory and visual systems, the development of the limbic system appears to be sensitive to emotional experiences as well (Braun et al 2000, Poeggel et al 1999, 2000). Experience-dependent changes are thought to be modulated by various factors such as stress, aging, gonadal hormones and brain injury (Kolb & Whishaw 1998).

Experimental studies have demonstrated that stress can induce plasticity in the nervous system, particularly in the limbic system. The hippocampal formation expresses high levels of adrenal steroid receptors and is vulnerable to the effects of stress and trauma

(McEwen 1999). Studies have shown that stress and stress hormones impair hippocampus-dependent forms of memory in both humans and animals (Conrad et al 1996, Conrad et al 2003, Kirschbaum et al 1996). Electrophysiological studies indicate that stress and stress hormones can impair LTP and enhance the induction of LTD in area CA1 of adult rat (Pavlides et al 2002, Xu et al 1997). In addition to affecting synaptic plasticity and memory, stress and corticosterone have been shown to alter hippocampal dendritic morphology (Lambert et al 1998, Sousa et al 2000, Watanabe et al 1992 a.b) and to inhibit neurogenesis in the adult brain (Gould et al 1997). Similarly, the amygdala displays high concentration of glucocorticoid receptors (Johnson et al 2005) and is one of the most essential components of the neural circuitry mediating stress responses. Exposure to stress facilitates aversive learning (Shors et al 1992) and the basolateral amygdala has been shown to be essential for stress-induced facilitation of aversive learning (Liang et al 1994, Shors & Mathew 1998). In addition, stress has been shown to alter dendritic morphology in the amygdala of rodents (Mitra et al 2005, Vyas et al 2002). Furthermore, lesions or pharmacological suppression of the amygdala prevents stress-induced analgesia and anxiety-like behavior (Helmstetter 1993). Finally, the prefrontal cortex is thought to be a target for glucocorticoid involved in the stress response (Diorio et al 1993, Meaney & Aitken 1985) and shows neurochemical changes in response to stressors (Moghaddam 1993). Stress and stress hormones alter spine density and dendritic morphology of PFC pyramidal neurons in the rodents (Brown et al 2005, Cook & Wellman 2004, Radley et al 2004, 2006, Seib & Wellman 2003, Wellman 2001). Exposure to stress also alters dopaminergic and serotonergic neurotransmission in the prefrontal cortex of rodents (Bekris et al 2005).

1.6 Dendritic spines: modulated by activity/experiences

The principal neurons of most brain regions are covered with small protrusions known as dendritic spines. Dendritic spines were first described by Ramon y Cajal in 1888, who proposed that spines were the primary sites of synaptic contact between dendrites and axons and that they were involved in learning and memory. Dendritic spines are the site of excitatory input in the neocortex, but in addition can also

carry inhibitory input (Jakab et al 1997). Dendritic spines are thought to play an important role in neuronal information processing. Spines are also known to compartmentalize Ca^{2+} and other signalling components (Korkotian & Segal 2000). The morphology of the spine is highly variable and has been roughly categorised into three main types: thin, mushroom and stubby spines. Thin spines are most common and have a thin, long neck. Spines with a large head are called mushroom spines, and spines that are devoid of a neck are called stubby spines. In mature neurons of the hippocampus, dendritic spines are found at a linear density of 1-10 spines per μm of dendritic length (Sorra & Harris 2000). The density of spines on the dendrite is related to the degree of connectivity between these neurons and the axons that pass through their dendritic arbors. (Hering & Sheng 2001, Tashiro & Yuste 2003). It has been shown that dendritic spines are modifiable by activity and/or experiences. Changes in dendritic spines are generally categorised into two groups - i) changes in spine number and distribution, evidenced by either dramatic decrease or increase in spine density; and ii) changes in spine size and morphology.

Dendritic spine number and distribution is shaped by activity. *In vivo* studies have shown that increases of neural activity produce more spines. For instance, spine density is increased after visual stimulation (Parnavelas et al 1973). On the other hand, light deprivation in mice caused a reversible reduction in the number of spines (Globus & Scheibel 1967, Valverde 1967). Activation of NMDA receptor antagonist led to the formation and prunning of spines in the rodents (Goldin et al 2001). Exposure to stimuli which increases neuronal activity has been shown to increase spine density on secondary dendrites of cultured hippocampal neurons (Papa & Segal 1996). Studies by Popov and his colleagues have shown that squirrels lose 40% of their spines during hibernation and recover them in few hours after arousal from hibernation (Popov & Bocharova 1992, Popov et al 1992). A study by Kirov & Harris (1999) indicated that synaptic blockade increases spine number in the hippocampus. In birds, spine morphological plasticity is observed during postnatal development (Rausch & Scheich 1982) and during learning tasks (Patel et al 1988). It has also been shown that auditory imprinting caused significant spine loss in the chick forebrain (Wallhausser & Scheich 1987). Similarly, flial

imprinting reduced spine frequencies in the chick forebrain (Bock & Braun 1999a). Furthermore, spatial training of adult rats increased the number of excitatory spine synapses on basal dendrites of CA1 pyramidal neurons (Moser et al 1997).

There are few studies which indicate changes in the spine morphology after activity and/or experience. A study by Papa & Segal (1996) has shown that exposure to stimuli which decreased neuronal activity caused a marked elongation of dendritic spines. Environmental manipulation, such as rearing animals in a complex environment, has also been shown to alter spine morphology (Greenough & Volkmar 1973). A reduction in the size of spines has been observed after the first orientation flight in honey bees (Brandon & Coss 1982). It has also been shown that blockade of synaptic transmission affects spine size (Kirov & Harris 1999).

It is well documented that stress and chronic elevation of corticosterone can alter spine distribution in different regions of the cortex. In the anterior cingulate cortex of rats, repeated maternal separation alters spine density (Bock et al 2005). Also, daily handling for few seconds resulted in higher spine densities in the apical dendrites of layer III ACd pyramidal neurons (Helmeke et al 2001a.b). Similarly, exposure to repeated maternal separation resulted in higher spine densities in the same region (Helmeke et al 2001a.b). Higher spine densities are reported in the parentally deprived animals as well (Helmeke et al 2001b). Furthermore, exposure to repeated restraint stress decreased apical dendritic spine density in the medial prefrontal pyramidal neurons of rodents (Radley et al 2006). Chronic restraint stress increased apical and basal spine density of CA3 pyramidal neurons (Sunanda et al 1995). In amygdala, stressful experiences led to the formation of new spines in the rodents (Mitra et al 2005). Likewise, the administration of corticosterone has been shown to alter spine density in the medial prefrontal cortex of rats (Seib & Wellman 2003). In this study, I have investigated how *in utero* stress and neonatal handling affects the development of dendritic spines in the rodent limbic system.

1.7 Stress and dendritic plasticity

Synapse formation involves two partners: axons and dendrites. Dendrites are the prime site of synaptic connectivity between neurons. Dendrites form a large fraction of the neuropil and hence they are considered as an important indicator of the functional capacity of neuronal network. In particular, dendrites represent 95% of the receptor surface with which neurons form connections (Schade & Baxter 1960). It is assumed that the extent and pattern of dendritic branching determines the range and scope of synaptic input a neuron can process. For example, the number of inputs to a sympathetic and parasympathetic ganglion in an adult animal is related to the number of dendritic branches (Purves & Hume 1981, Purves et al 1981). It has been shown that the size and complexity of the dendrite varies according to the task of the neuron. Pyramidal neurons in the prefrontal cortex that are responsible for associating sensory, motor and affective variables in learning and memory have much more elaborate dendrites relative to cortical neurons involved in simple sensory processing tasks (Elston & Rosa 1997, Elston 2000, Poirazi & Mel 2001).

Dendrites are highly plastic in nature and can undergo rapid changes in response to physiological stimuli. The dendrite grows and retracts in response to various events including neuronal activity, various chemicals and exposure to stressors. For example, placing rodents in complex environmental conditions increased dendritic arborization in the visual cortex relative to cage-reared animals (Green et al 1983, Kolb & Whishaw 1998). Similarly, it has been shown that the dendritic morphology of cortical and hippocampal neurons are significantly altered by stressful experiences. In rodents, stress caused apical dendritic atrophy of CA3 pyramidal neurons (Magarinos et al 1996, Watanabe et al 1992a). Stress-induced dendritic atrophy has also been reported in the CA1 region (Lambert et al 1998). In the prefrontal cortex, stress-induced dendritic alterations are well documented. Exposure to stress reduced the number and length of apical dendrites of layer II/III pyramidal neurons of the medial prefrontal cortex (Brown et al 2005, Cook & Wellman 2004, Radley et al 2004). It has also been suggested that exposure to excessive glucocorticoid can alter the morphology of layer II/III prefrontal pyramidal neurons (Wellman 2001). Similarly, it has been shown that excessive

glucocorticoid decreased apical dendritic length and numbers of branch points of CA3 pyramidal neurons of the rodents (Watanabe et al 1992b, Woolley et al 1990a). In rodents, treatment with drugs has been shown to alter dendritic morphology of the spiny neurons in the nucleus accumbens and the pyramidal neurons of the prefrontal cortex (Robinson & kolb 1997, Robinson & Kolb 1999a, Robinson & Kolb 1999b). In this study, I have examined how *in utero* stress and neonatal handling affects the dendritic morphology of pyramidal and granular neurons in the rodent limbic system.

1.8 Questions and aims

The following questions were addressed in this study:

1. Does stress experienced by a mother during pregnancy influence the development of neurons in the offspring?

Developing fetal brain is sensitive to maternal experiences and influences. Stress experienced by a mother during pregnancy can adversely affect behavior, brain structure and functions in the offspring (See section 1.1). Recently, much progress has been made in understanding the neurobiological basis for altered adult behavior resulting from adverse experiences in the prenatal period. However, these studies mainly have focussed on the hormonal and neurochemical alterations and the impact of prenatal stress on neuronal development is largely unexplored. Therefore, this study was designed to investigate the possibility that stress experienced by a mother during pregnancy can alter dendritic and synaptic organization in the offspring.

2. Does prenatal stress affect the structure of neurons in the males and females in a different way?

It is well accepted that the males and females behave differently. Morphometric studies indicate that male and female brains differ in size, cortical thickness, neuronal architecture and synaptic density (See section 1.2). Similarly, studies investigating the effects of prenatal stress on brain and behavior have shown that prenatal stress caused

behavioral, structural and neurochemical alterations in a sex-specific manner (See section 1.2). In view of the qualitatively different effects of prenatal stress in the males and females, one of the aims of this study was to investigate if stress experienced by a mother during pregnancy can differently alter the morphology of neurons in the male and female offspring. In addition, I also wanted to examine whether there were sex differences in the neuronal morphology of untreated control animals.

3. Are the structural alterations induced by *in utero* stress prevented by handling?

The components of the nervous system continue to develop after birth and therefore it is possible to prevent and/or reverse prenatal stress-induced alterations by appropriate manipulations during the neonatal period. Neonatal handling, which involves removal of pups from the mother for a brief period is reported to prevent and/or reverse some of the behavioral and endocrine abnormalities caused by prenatal stress (See section 1.3). Therefore, in this study I was interested to find out if the dendritic & synaptic changes caused by prenatal stress are prevented and/or reversed by neonatal handling.

4. How does handling affect neuronal development in the rodent limbic system?

It is generally accepted that early experiences have profound influences on brain development and thereby can affect adult function and behavior. Neonatal handling, which modifies normal mother-pup interactions, has been shown to alter neuroendocrine responsiveness to stress, brain morphology and neurochemistry (See section 1.3). In this study, I wanted to examine if handling during the early weeks of life can alter dendritic morphology and synaptic density in the male and female brains and also to search for differences in these effects.

2.0 MATERIALS AND METHODS

2.1 Experimental animals

Male and female Sprague-Dawley rats were used in this study. All experiments were

carried out in accordance with the guidelines of the university committee for Institutional Animal Care, based on those of the National Institutes of Health, USA. Female pathogen-free Sprague-Dawley rats weighing 280-300g (Harlan, Biotech, Jerusalem) on day 1 of pregnancy (detected by the presence of vaginal plug) were randomly assigned to stress and control groups and housed in individual cages in the animal house at an ambient temperature of 22 $\pm$ 1°C and a 12 hr diurnal light cycle (lights on 1700 hr, off 1900 hr). Food and water was provided ad libitum and the cages were changed twice weekly.

2.2 Experimental groups

The male and female offspring of the stressed and control dams were assigned into 4 experimental conditions:

- **Untreated control (C) -** This group consisted of rats that were born from the unstressed mothers and were left undisturbed after birth (n = 8; Male = 4; Female = 4).
- **Prenatal stress (PS) -** This group consisted of rats that were stressed *in utero* (n = 8; Male = 4; Female = 4).
- **Prenatal stress + Handling (PSH) -** This group consisted of rats that were stressed *in utero*, in addition were separated from their mothers during the first weeks of their life (n = 8; Male = 4; Female = 4).
- **Neonatal handling (H) -** This group consisted of rats that were briefly separated from their mother during the first weeks of their life (n = 8; Male = 4; Female = 4).

5.

Figure 5. Photograph of the experimental animal. Male and female Sprague-Dawley rats were used in this study.

2.3 Gestational stress and Handling procedure

Gestational stress

Pregnant rats were stressed during their last week of pregnancy. From day 15-20 of gestation, the pregnant rats experienced three different stressors. The dams were exposed to each stressor twice, on every alternate day in order to prevent them from adapting to the stressors. **i) Restraint** - On days 15 and 18, each rat was placed in transparent cylindrical restrainers (8 cm wide & 20 cm long) in normal light and at room temperature 21° – 22° C. This process lasted for 45 minutes. **ii) Crowding** - On days 16 and 19, the pregnant rats were placed in one joint cage for 8 hours. **iii) Forced-swimming** - On days 17 and 20, each pregnant rat was placed individually for 15 minutes in a glass cylinder (21 cm wide & 60 cm high) filled with lukewarm water (Temp. 25°C). The containers were filled with water up to 30 cm, so that the rats were unable to stand and were forced to swim for most of the time. The gestation period lasted for 21 days. Control pregnant females were left undisturbed throughout their gestation.

Handling

Immediately, after the birth, the pups were weighed and culled to 4 males and 4 females in each litter. The process of weighing and culling on the first day was also considered as handling. For the next 9 days the pups were separated from their mother, once a day for 3 minutes. During the separation, the pups were placed individually in single cages. After the separation, the pups were returned to their home cages.

2.4 Histological Procedure

At the age of 23, the animals were killed. Brains were removed quickly and were stained using a modified Golgi-Cox procedure.

2.4.1 Golgi-Cox method

For almost a century, Golgi method has been recognised as one of the best and most elegant procedure in investigating the fundamental structures of the nerve cells.

Using Golgi impregnation subtle morphological alterations in neuronal dendrites and dendritic spines have been discovered in the brains of animals treated with drugs as well as the post mortem brains of patients with neurological disorders (Graveland et al 1985, Robinson & Kolb 1997). Originally, the method was developed by Camillo Golgi in the year 1873. In the course of testing the effects of silver nitrate on nervous tissue he had unexpectedly discovered this method. When he tried to impregnate the innermost membrane of pia matter with silver, he observed that in the adjacent brain substances there were occasional nerve tissue stained in dark brown. Due to the capricious nature of the method, many modifications have been employed to improve the reliability and quality of the technique. The most significant improvement to this method was made by Cox in the year 1880 and 1891. Cox modifications were appreciated by several scientist including Cajal and Lorente de No who had used his method in their studies of the hippocampus and many other regions of the brain (Ramon-Moliner 1958, Valverde 1968).

2.4.2 Principles of Golgi-Cox method

The Golgi-Cox method is used to study the nervous system of any animal species and is applicable equally to central nervous structures, peripheral sense organs and free nerve endings. This technique elaborates the shape and spatial arrangement of isolated nerve units in unsurpassed clarity as no other method can. Both the original Golgi method as well as the modified Golgi-Cox method includes four essential features:

i) This technique stains only a few cells, approx. 1- 4% of cells are stained and thus it allows complete studies of individual cells and their processes.

ii) Those cells that take the stain, clearly displays soma or perikaryon as well as the ramifying processes that originates from it. Nerve cells and the dendrites are clearly outlined and the dendritic spines are clearly and consistently stained.

iii) Golgi method stains nerve cells and dendrites in dark brown/black colour. The remaining unstained structures compose a transparent background and therefore the stained cells stand out very clearly.

iv) There are no staining gradations, i.e. either the structures are completely dark or perfectly transparent.

2.4.3 Protocol for Golgi-Cox Staining

Step I: Prepare the Golgi-Cox solution

3 different aqueous solutions are taken:

- Solution A: 5% solution of Potassium dichromate ($K_2Cr_2O_7$) in distilled water.
- Solution B: 5% solution of Mercuric chloride ($HgCl_2$) in distilled water.
- Solution C: 5% solution of Potassium chromate (K_2CrO) in distilled water.

From the above solutions, 2 new solutions are prepared.

i) Mix solution A + solution B (1:1) into a glass beaker to prepare solution D

ii) Mix solution C + Distilled water (1:2,5) to prepare solution E

From these solutions, the actual Golgi-Cox solution is produced. Mix 1000 ml of solution D into 1400 ml of solution E while stirring continuously. Store in a glass stopper bottle for 5 days in dark until precipitates form. The unfixed brains are kept in this Golgi solution for 14 days at dark room temperature. Determining the optimal impregnation time the sample cuts can be made.

Step II. Embedding

After completion of step I, the brains are dehydrated and embedded in celloidin in the following steps:

- Washed in distilled water, 3 x 2 minutes.
- Slowly dehydrated in the refrigerator (4-6°C) as follows:

50 % alcohol - 4 hours; 70 % alcohol - overnight; 80 % alcohol - overnight; 96 % alcohol - overnight; 96 % alcohol - overnight.

- Then the brains are embedded at the dark room temperature as follows:

100 % alcohol - Diethyl ether (1:1) - 2 hours

2 % Celloidin-3 days

4 % Celloidin-3 days

8 % Celloidin- 3 days

The brains are then inserted in 9% celloidinmass and are placed in the evaporating glass, which is then kept overnight in the excitator with open faucet. Subsequently, a second glass with closed faucet containing phosphorous pentaoxide is placed until the celloidin is reduced to half. This usually lasts for 24 hours. During this process, it is important that the celloidin remains free of bubbles. Phosphorous pentaoxide is removed and chloroform is placed in the bowl so that the celloidin out polymerizes in 1-2 days. Then the celloidin block is cut into a desired form and is placed in 70% alcohol. This block can be kept in the refrigerator for a long time. For cutting, the celloidin block containing the brain is fixed into a wooden block with the help of adhesives. While cutting in the microtome, the knife should be continuously moistened with 70% alcohol in order to prevent the slices getting dried.

III. Staining

- 150µm coronal sections were cut at the level of prefrontal cortex, hippocampus and amygdala using a sliding microtome.
- Tissue sections were collected serially and immersed in 70 % alcohol until all the necessary sections were obtained.
- Washed in distilled water, 3 x 1 minutes.
- Alkalinized with ammonia (NH_3:H_20, 1:1), 1 x 60 minutes.
- Treated with 0, 5 % phenyldiamine solution (Merck, Germany) – prepared by dissolving 0.5 gm phenyldiamine in 100 ml distilled water, 1 x 1 minutes.
- Treated with 0, 5 % phenyldiamine solution (Merck, Germany), 1 x 4 minutes. This treatment deepens the colour of the staining.
- Washed in distilled water, 2 x 2 minutes.
- Treated with 1 % dektol (Kodak, Germany) – prepared by dissolving 0.5 gm dektol in 50 ml distilled water, 1 x 2 minutes. This treatment stablizes the tissue impregnation.
- Washed in distilled water, 2 x 1 minutes.
- Treated with 5 % tetenal (Kodak, Germany) - prepared by dissolving 2.5 gm tetenal in 50 ml distilled water, 1 x 10-15 minutes. This treatment is important for fixing the tissue impregnation.
- Washed in distilled water, 3 x 5 minutes.

- Dehydrated in the ascending grades of alcohol as follows: 70 % alcohol - 3 minutes or overnight; 80 % alcohol - 10 min; 96 % alcohol - 10 min; 99 % alcohol - 10 min.
- Cleared in Isopropanol (Optal, Roth, Germany), 1 x 10 minutes.
- Cleared in Xylene, 2 x 5 minutes.
- Mounted with merckoglas (Merck, Darmstadt, Germany) and was cover slipped for further microscopic observation. The slides were coded with numerical which was only broken after the analysis was completed.

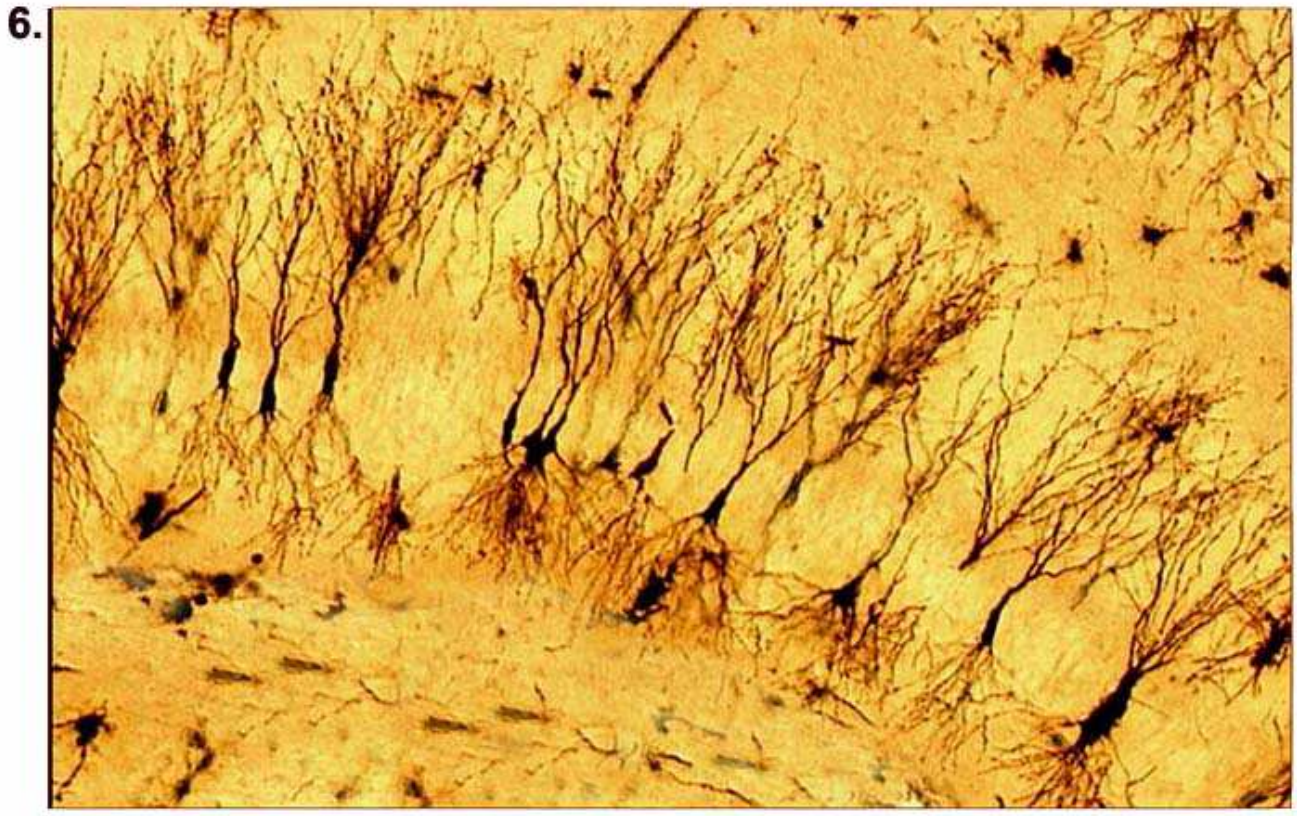


Figure 6. Photomicrograph of pyramidal neurons from area CA3 of the hippocampus. The cells are stained with modified Golgi-Cox method.

2.5 Data acquisition and statistical analysis

2.5.1 Neuronal types

In this study, 2 types of neurons were analyzed – i) Pyramidal; and ii) Granular. Only those neurons that satisfied the following criteria were selected. i) neurons that exhibited complete dendritic tree, evidenced by well-defined endings; ii)

dark and consistent impregnation along the entire length of the dendrite; and iii) relatively isolated from neighbouring neurons in order to prevent interference with the analysis.

Pyramidal neurons: Pyramidal neurons were analyzed from the orbitofrontal cortex, anterior cingulate cortex, hippocampal CA3 and CA1 as well as the basolateral nucleus of the amygdala. The pyramidal neurons were defined by the presence of their characteristic triangular shaped soma, a distinct, single apical dendrite arising from the apex, dendritic spines and some basal dendrites (fig.7.A) In the orbitofrontal & anterior cingulate cortices, only those pyramidal neurons whose soma was located near the layer II/III border was selected. The apical dendrites of these neurons typically started to branch in layer II. Neurons with longer primary apical dendrites possessing oblique dendrites, which typically are found deeper in layer III, were not included in this study.

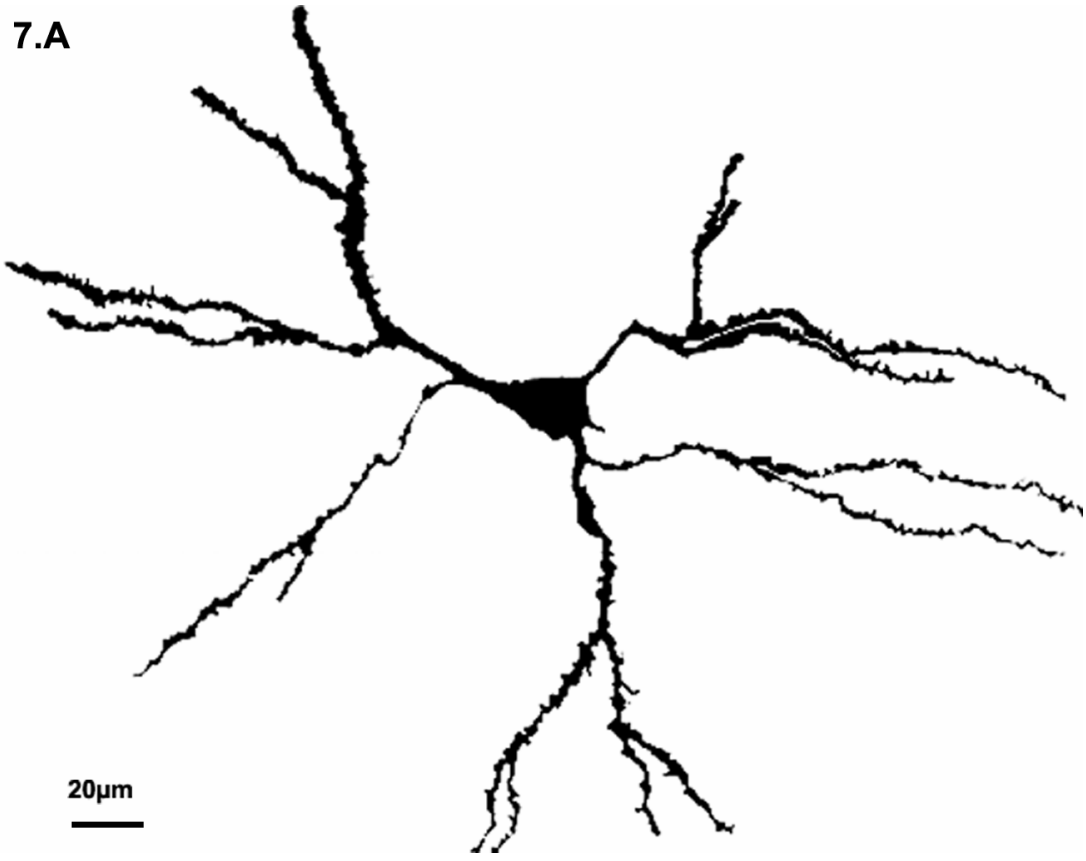


Figure 7.A Photomicrograph of Golgi-impregnated pyramidal neuron from layer II/III anterior cingulate cortex. Scale bar 20µm.

Granular neurons: Granular neurons were analyzed in the upper and lower blade of dentate gyrus. Granular neurons were characterized by single or pair of dendrites originating from its cells body as the main dendrite and displaying complete secondary and tertiary branches and have numerous dendritic spines. (Fig.7.B).

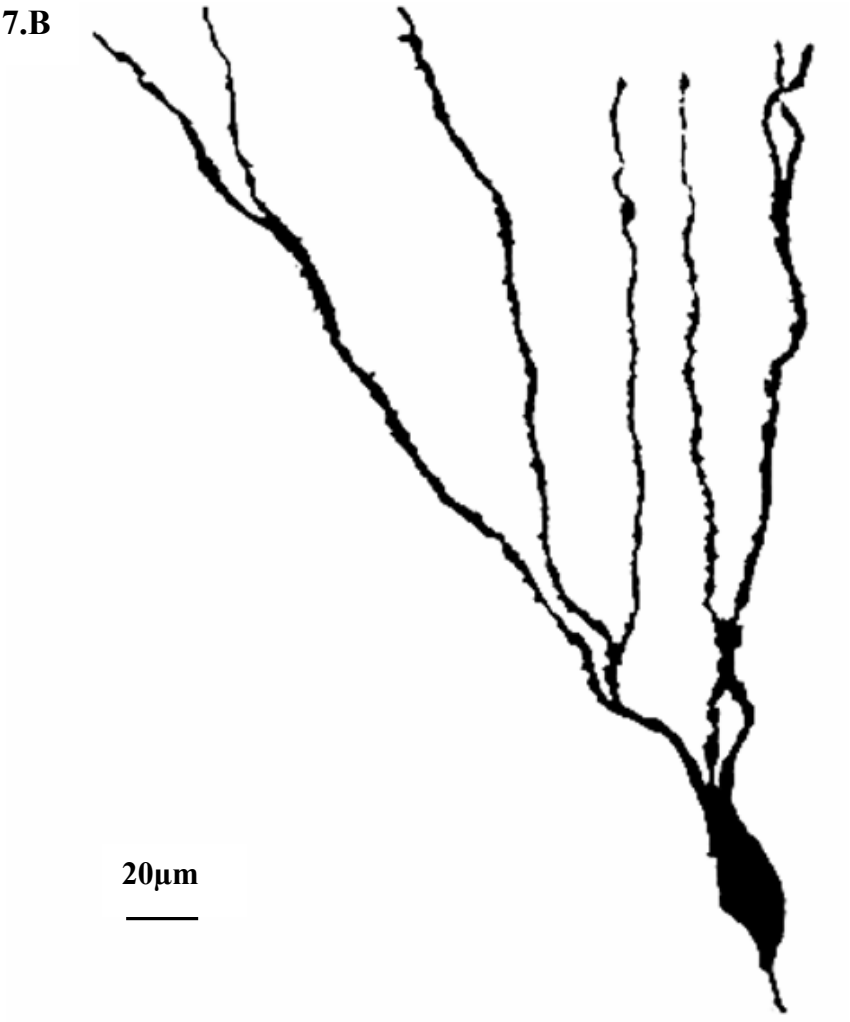


Figure 7.B Photomicrograph of Golgi-impregnated granular neuron from dentate gyrus. Scale bar 20 µm.

2.5.2 Investigated regions:

Prefrontal cortex: Following subregions of the prefrontal cortex (PFC) were investigated in this study.

a) Orbitofrontal Cortex (OFC): Orbitofrontal cortex was defined according to Paxinos & Watson (1998). The orbitofrontal cortex lies dorsal to the caudal end of the olfactory bulb. Neurons were analyzed in two subregions of the orbitofrontal cortex – i) Ventral orbital cortex (VO); and ii) Lateral orbital cortex (LO). No distinction was made between these two subregions. On the basal surface of the frontal pole VO is found. LO is located laterally. Only those neurons that were located in layer II/III were analyzed.

b) Anterior Cingulate Cortex (ACd): Anterior cingulate cortex was defined according to Uylings et al (2003) and the commonly used nomenclature of Krettek & Price (1977). This area corresponds to Cg1 of Paxinos and Watson (1998) or Zilles and Wree (1995). Only those neurons that were located in layer II/III were analyzed.

In the orbitofrontal as well as anterior cingulate cortices, layer II and III was identified based on their following characteristic features - i) it's position is immediately ventral to the cell poor layer I; ii) higher cell density; and iii) smaller somata of pyramidal neurons in layer II / III relative to deeper layers.

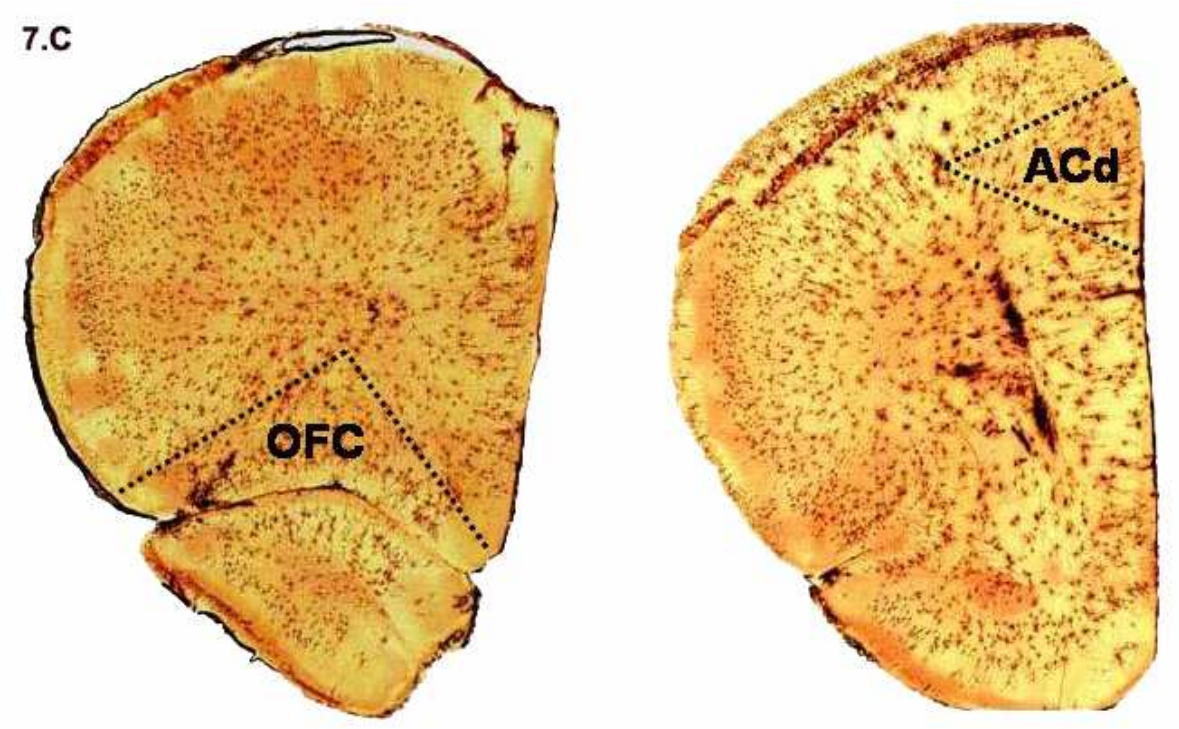


Figure 7.C Photomicrograph of coronal brain slices showing the regions of prefrontal cortex analyzed in the study. Abbreviations: OFC - Orbitofrontal cortex; ACd - Anterior cingulate cortex.

<u>Hippocampal formation</u>: The hippocampus was defined according to Paxinos & Watson (1998). The hippocampus was identified by interfolded layers of dentate gyrus and cornu ammonis located in the inferior medial wall of the temporal lobes between the thalamus and cortex, towards the posterior of the forebrain (fig.7.D). Neurons were analyzed in 3 subdivisions of the hippocampal formation.

a) **CA3 region**
b) **CA1 region**
c) **Dentate gyrus:** in two layers; i) Outer blade; and ii) Inner blade.

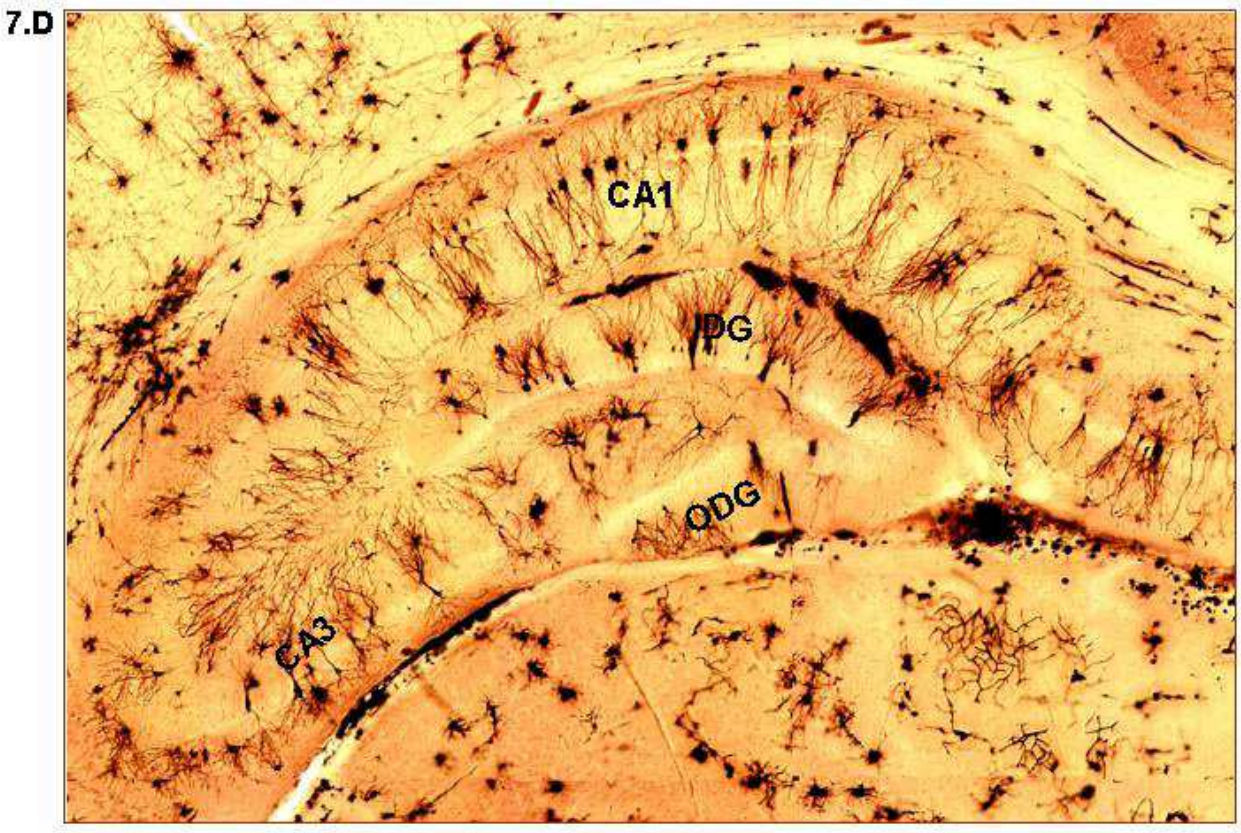


Figure 7.D Photomicrograph of the hippocampal formation showing the areas that were analyzed in this study. Abbreviations: CA1 & CA3 area of the hippocampus; IDG –Inner blade

of dentate gyrus; ODG – Outer blade of dentate gyrus.

Amygdala: The amygdala was defined according to Paxinos & Watson (1998) and was identified by its almond shaped structure located deep within the temporal lobes, adjacent to the hippocampus (fig.7.E). In the basolateral nucleus of the amygdala, pyramidal neurons were analyzed.

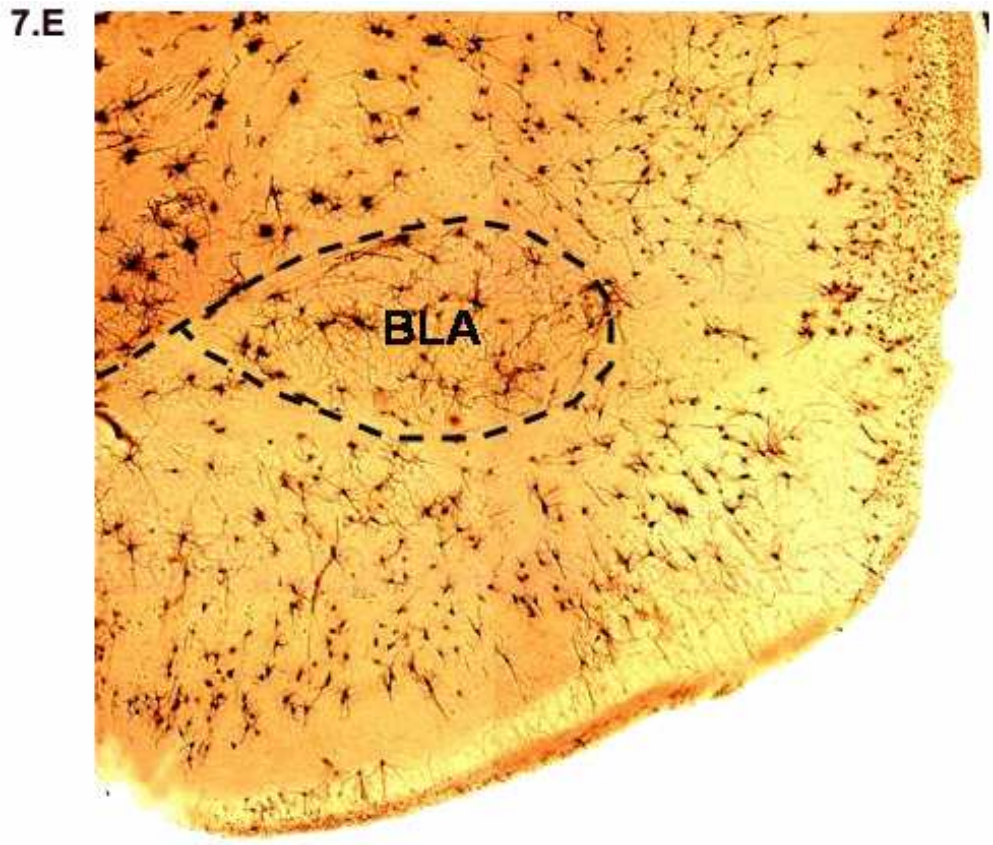


Figure 7. E Coronal section showing basolateral amygdala (BLA).

2.6 3-D Reconstruction of neurons

Pyramidal and granular neurons were reconstructed three-dimensionally using image analysis software, NEUROLUCIDA®. (MicroBrightField, Williston, VT). Neurolucida is advanced scientific software for performing neuron tracing, brain mapping, anatomical mapping, and morphometry (fig.8A). Neurolucida performs detailed morphometric analysis of neurons such as – i) trace and analyze branching neuronal

processes throughout single or serial sections; ii) 3D measurement of bifurcating and trifurcating branching processes; iii) automatic measurement of process diameters and lengths while tracing; iv) analyze spine distribution, branch complexity etc. Neurolucida overlays computer graphics over the microscopic image of the specimen. The motorized microscope stage accurately allows navigating through tissue preparation in the x, y, and z axis. Additionally, as the neuron is traced, lists and files of measurements are automatically created and data on length, diameters, boutons, spines etc. is immediately available for statistical evaluation. Tracing was performed at the magnification of 400 x by moving the motorized stage through z-axis along the entire length of each dendrite. In the pyramidal neuron, the complete apical and an average of one to two basal dendrites were drawn. In case of granular neuron, the complete dendrites were drawn. Spines were plotted in parallel on the same dendrites. In this study, thin, mushroom and stubby type spines were recorded. In this manner, the length and spines of each dendritic segment were recorded. These Neurolucida datasets were then exported to generate 3-dimensional rendering of the traced neuron using Neuroexplorer, a sophisticated Neurophysiological data analysis package, which provides the complete structural details of the neuron, including branching pattern, spine location and spine density.

8. A

Figure 8.A Photograph of the image analysis system, Neurolucida (MicroBrightField)

8.B

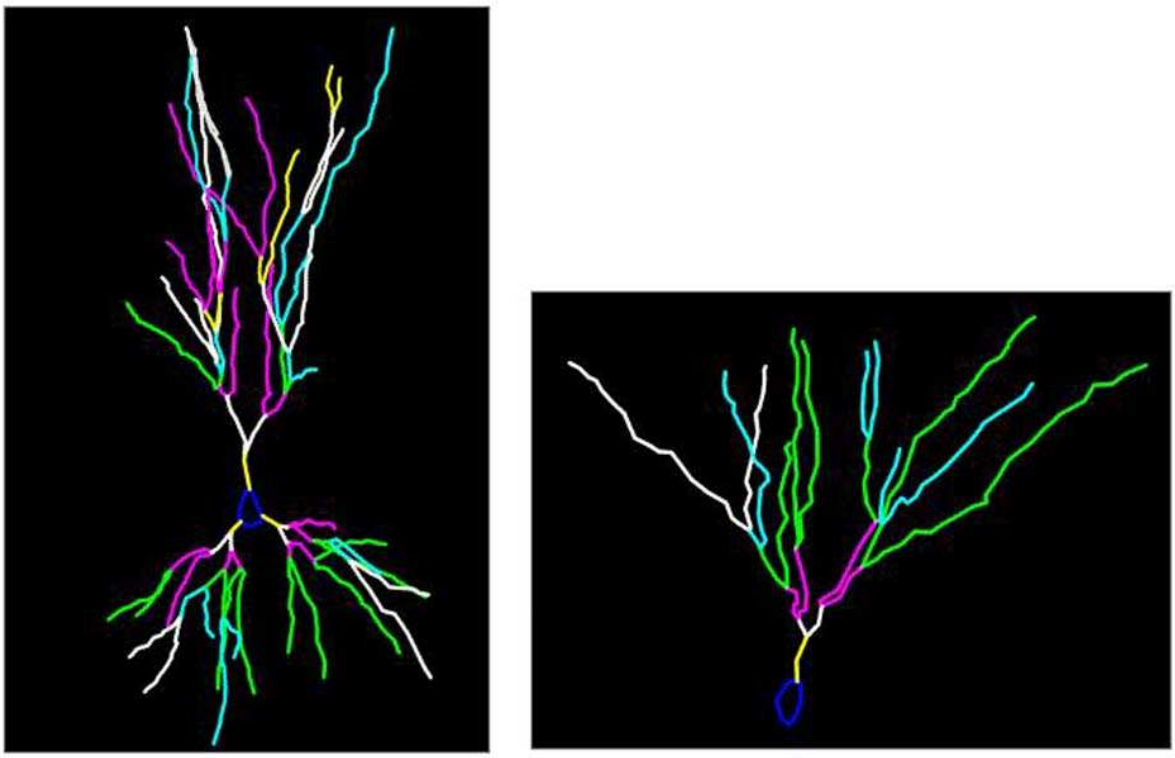


Figure 8.B Neurolucida drawings of representative pyramidal and granular neuron.

2.7 Quantitative and statistical analysis

Neurons in the left and right hemispheres were analyzed separately (n = 4 per hemisphere) and the data for each hemisphere was averaged for each animal. For each reconstructed neuron, the following parameters were quantified - i) dendritic spine density, representing the average number of spines per μm of dendritic length; ii) total dendritic length, representing the summed length of dendritic segments; iii) spine density per branch order, representing the average density of spines across different branches of the dendrite (fig.9.A); iv) Sholl´s method of segmental analysis, representing the length and complexity of dendrites at different radii from the neuronal soma (fig.9.B). Spine density (number of visible spines per μm) for each neuron was calculated by dividing the total number of spines with the total dendritic length. No attempt was made to correct for hidden spines (Feldman & Peters 1979), since the use of visible spine counts for comparison between different experimental conditions and this have been validated previously (Horner & Arbuthnott 1991).

Branch order analysis: To evaluate spine density changes within the dendritic arbors, branch order analysis was performed. For this purpose, the dendritic branches were ordered centrifugally. In the apical dendrite, the branch which emerged from the cell body was considered as first-order segment, those arising from first-order segment were considered as second-order segments until they bifurcate symmetrically into third-order segments and so on (fig.9.A). Similarly, in the basal dendrite, branches emerging from the soma were termed as first order segments. Those arising from first order segments were considered as second-order segments and so on. In the granular neurons, branches arising from the cell body were considered as first-order segments, those arising from first-order segments were considered as second-order segments and so on. Branch order analysis was performed only on those dendrites which displayed changes in the total spine density. Spine density was quantified on the following branches of the neuron**.**

- In the OFC and ACd apical dendrite, spine density was quantified on first, second, third, fourth, fifth and sixth order branches.
- In the OFC and ACd basal dendrite, spine density was quantified on first, second, third, fourth and fifth order branches.
- In the CA1 apical dendrite, spine density was quantified on first, second, third, fourth, fifth, sixth, seventh, eight, ninth and tenth order branches.
- In the granular neuron, spine density was quantified on first, second, third, fourth, fifth and sixth order branches.
- In the amygdaloid apical dendrite, spine density was quantified on first, second, third, fourth, fifth and sixth order branches.
- In the amygdaloid basal dendrite, spine density was quantified on first, second, third, fourth and fifth order branches.

Sholl´s analysis: To track down changes in the dendritic length and arborization in further detail, a three-dimensional version of the Sholl analysis (Sholl 1953) was performed. In an attempt to assess the principles of neuronal interconnections, Sholl DA had performed several studies on dendritic branching pattern and established a functional

relationship between the number of intersections per unit area and the distance from the centre of the perikaryon. A Sholl analysis estimates the amount and distribution of dendritic material by counting the dendritic length and number of dendritic intersections with an overlay of concentric rings centred on the soma. Using the soma as reference point, dendritic length and intersection was quantified at different concentric circles from the soma. Sholl analysis was only performed when there were changes in the total dendritic length/arborization. Following measurements were made:

- In OFC and ACd apical dendrite, dendritic length and intersection was quantified at 20µm, 40µm, 60µm, 80µm, 100µm, 120µm, 140µm, 160µm, 180µm, 200µm and 220 µm from the soma.
- In CA1 and CA3 apical dendrite, dendritic length and intersection was quantified at 40µm, 80µm, 120µm, 160µm, 200µm, 240µm, 280µm, 320µm and 360µm from the soma.
- In CA1 and CA3 basal dendrite, dendritic length and intersection was quantified at 20µm, 40µm, 60µm, 80µm, 100µm, 120µm, 140µm and 160 µm from the soma.
- In dentate gyrus granular neuron, dendritic length and intersection was quantified at 20µm, 40µm, 60µm, 80µm, 100µm, 120µm, 140µm, 160µm, 180µm and 200 µm from the soma.
- In amygdaloid apical dendrite, dendritic length and intersection was quantified at 20µm, 40µm, 60µm, 80µm, 100µm, 120µm, 140µm and 160µm from the soma.

Statistical analysis: Statistical significance for the effects of prenatal stress, prenatal stress + handling on spine density, dendritic length and intersection was determined by three-way analysis of variance using i) treatment one (prenatal stress Vs control) as the first factor ii) treatment two (prenatal stress + handling Vs control) as the second factor and iii) hemisphere (left Vs right) as the third factor. The effects of neonatal handling on dendritic parameters were determined by two-way analysis of variance using treatment (handling Vs control) as first factor and hemisphere (left Vs right) as the second factor. The differences between untreated controlled males and females were determined by

two-way analysis of variance using sex (male Vs female) as first factor, and hemisphere (left Vs right) as the second factor. For the statistical analysis, Sigma stat (2.0, Jandel Germany) was used.

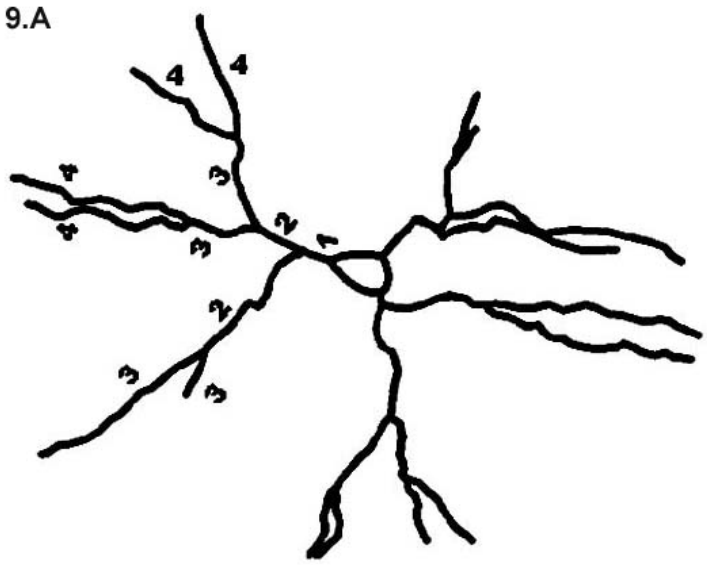


Figure 9.A An illustration of the method of quantifying spine density in different branch orders. Branch order analysis is used to evaluate changes within the dendritic arbors. The dendritic branches are ordered centrifugally. Branches arising from the cell body are termed as first order branches, those arising from first order branches are termed as second order branches and so on.

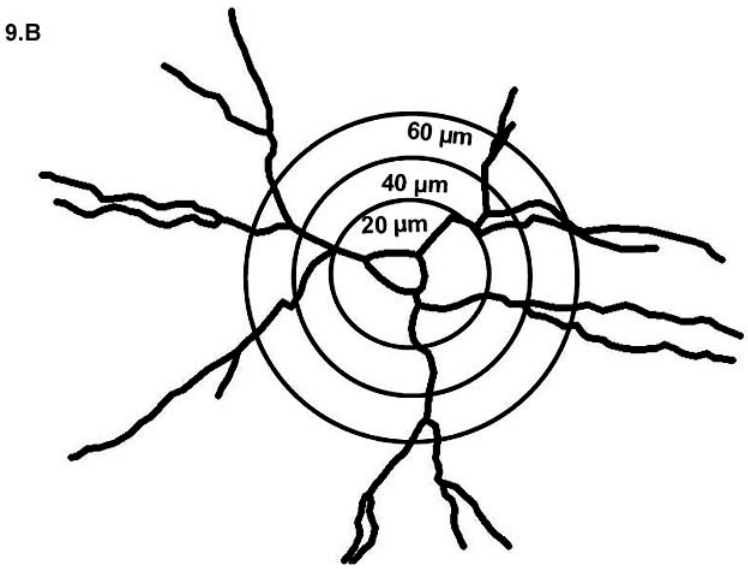


Figure 9.B An illustration of the method of quantifying dendrites. The concentric ring forms the grid for the Sholl analysis. Using the center of soma as reference point, the dendritic length and intersections are quantified at different concentric circles from the soma. The number of branches crossing the ring gives an estimate of the length of dendrite and its complexity.

3.0 RESULTS

3.1 Sex differences in the neuronal morphology of untreated control animals

3.1.1 Orbitofrontal Cortex (OFC): dendritic length & arborization is greater in the untreated control males relative to the females

Spine density

Apical dendrite: There were no effects of sex or hemisphere on the apical dendritic spine density of OFC pyramidal neurons (fig.10.A).

Basal dendrite: Like the apical dendrite, there were no effects of sex or hemisphere on the basal dendritic spine density of OFC pyramidal neurons (fig.10.B).

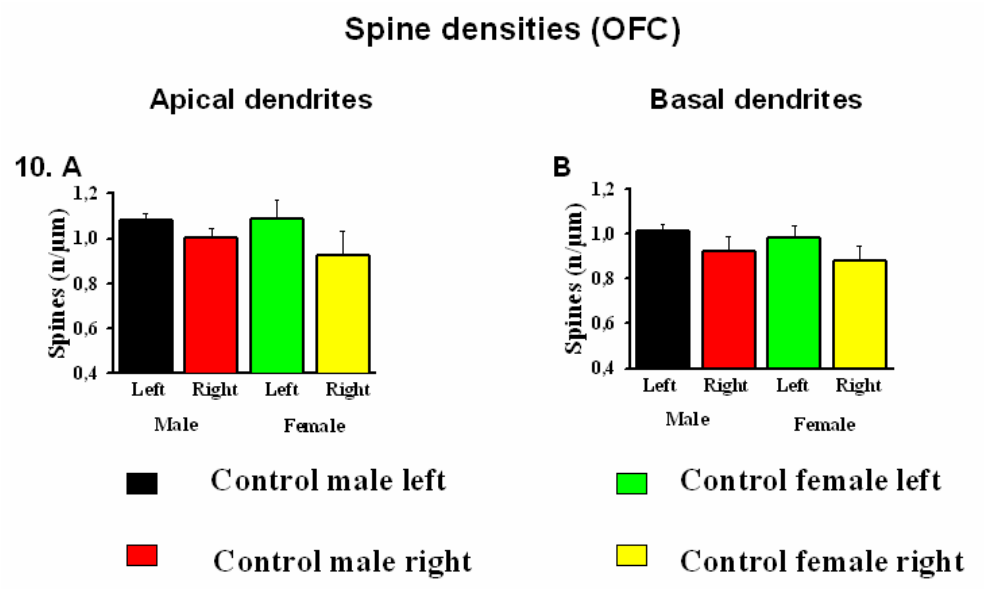


Figure 10. Histograms represent spine density of layer II/III OFC pyramidal neurons of untreated control animals. Values are given as mean ± S.E.M. A) In the apical dendrites; B) In the basal dendrites.

Dendritic length and complexity of dendritic arborization

Apical dendrite: Two-way ANOVA revealed significant effect of sex on dendritic length and arborization of OFC pyramidal neurons. The total apical dendritic length was

significantly higher in untreated controlled males (p = 0.009, upto 26%) compared to the untreated controlled females (fig.11.A). Similarly, the number of dendritic intersection was greater in untreated controlled males (p = 0.022, upto 29%) relative to the untreated controlled females (fig.11.B).

Basal dendrite: Unlike the apical dendrite, there were no effects of sex or hemisphere on dendritic length and arborization of the basal dendrite (fig.11.C & D).

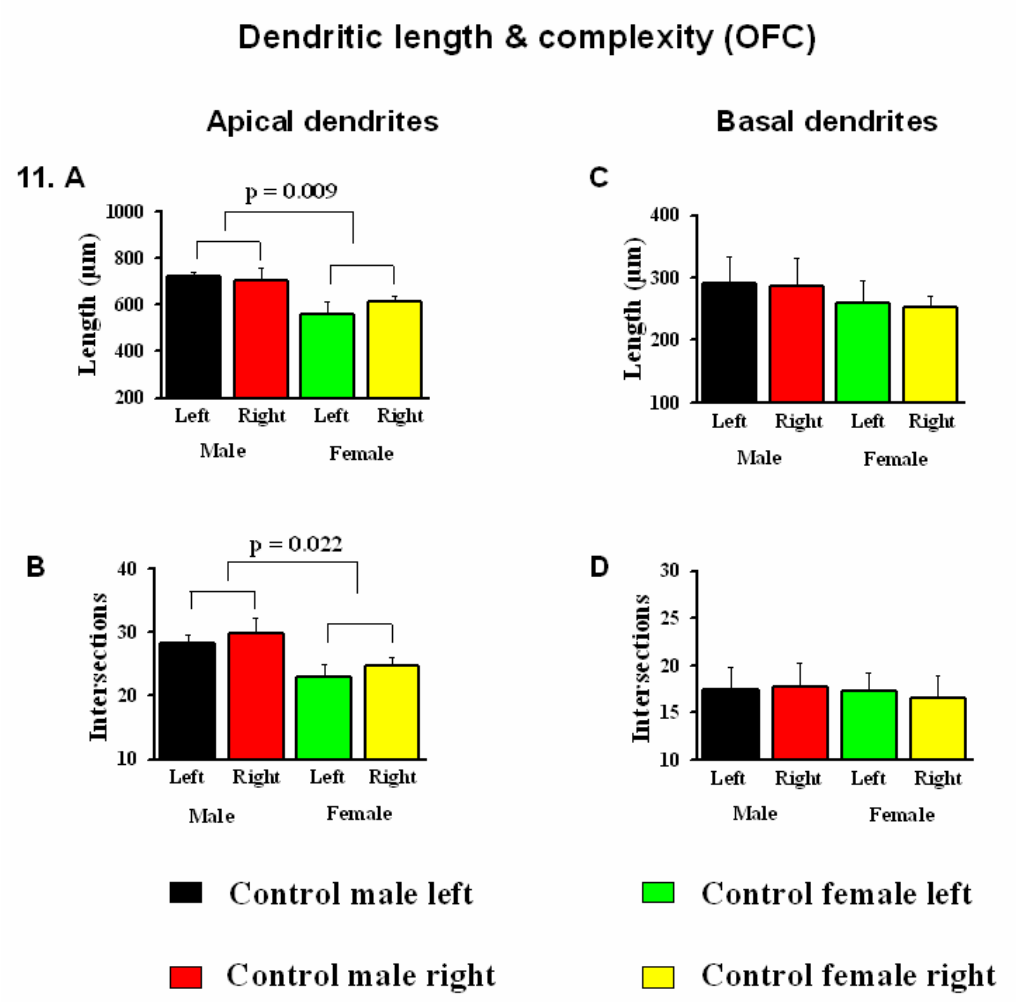


Figure 11. Histograms represent dendritic length & complexity of layer II/III OFC pyramidal neurons of untreated control animals. Values are given as mean ± S.E.M. A) Total dendritic length and B) Number of intersections in the apical dendrites; C) Total dendritic length and D) Number of intersections in the basal dendrites.

3.1.2 Anterior Cingulate Cortex (ACd)

Spine density

Apical dendrite: There were no effects of sex or hemisphere on apical dendritic spine density (fig.12.A).

Basal dendrite: Similar effects were seen on the spine density of basal dendrite (fig. 12.B).

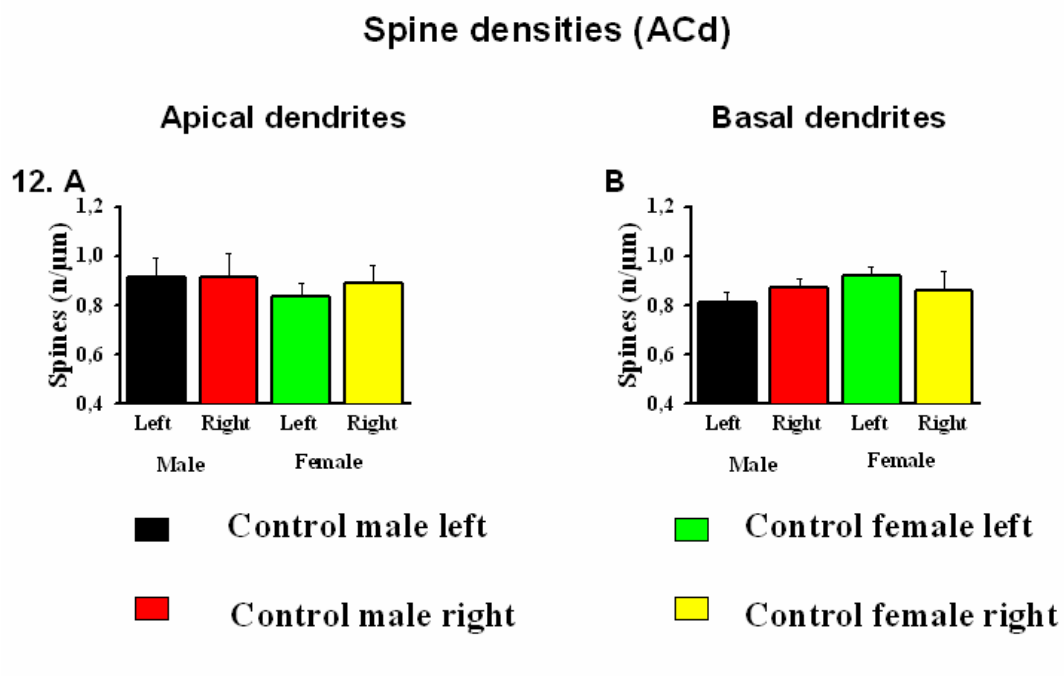


Figure 12. Histograms represent spine density of layer II/III ACd pyramidal neurons of untreated control animals. Values are given as mean ± S.E.M. A) In the apical dendrites; B) In the basal dendrites.

Dendritic length and complexity of dendritic arborization

Apical dendrite: Unlike the OFC apical dendrite, there were no effects of sex or hemisphere on apical dendritic length and arborization of ACd pyramidal neurons (fig.13.A & B).

Basal dendrite: Similar effects were observed on the basal dendritic length and arborization of ACd pyramidal neurons (fig. 13.C & D).

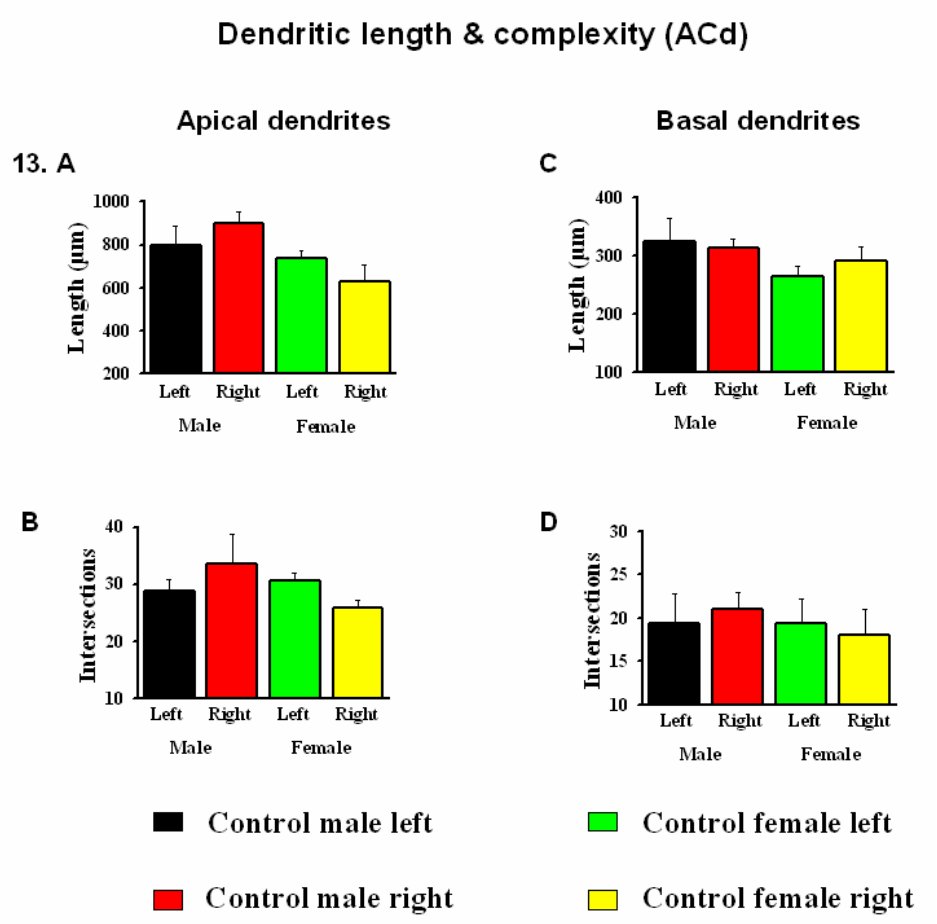


Figure 13. Histograms represent dendritic length & complexity of layer II/III ACd pyramidal neurons of untreated control animals. Values are given as mean ± S.E.M. A) Total dendritic length and B) Number of intersections in the apical dendrites; C) Total dendritic length and D) Number of intersections in the basal dendrites.

3.1.3 CA3 region

Spine density

Apical dendrite: There were no effects of sex or hemisphere on the spine density of CA3 apical dendrites (fig.14.A).

Basal dendrite: Similarly, there were no effects of sex or hemisphere on the spine density of CA3 basal dendrites (fig. 14.B).

Dendritic length and complexity of dendritic arborization

Apical dendrite: There were no effects of sex or hemisphere on the apical dendritic length and arborization of CA3 pyramidal neurons (fig.15.A & B).

Basal dendrite: Similar effects were found on the basal dendritic length and arborization of CA3 pyramidal neurons (fig. 15.C & D).

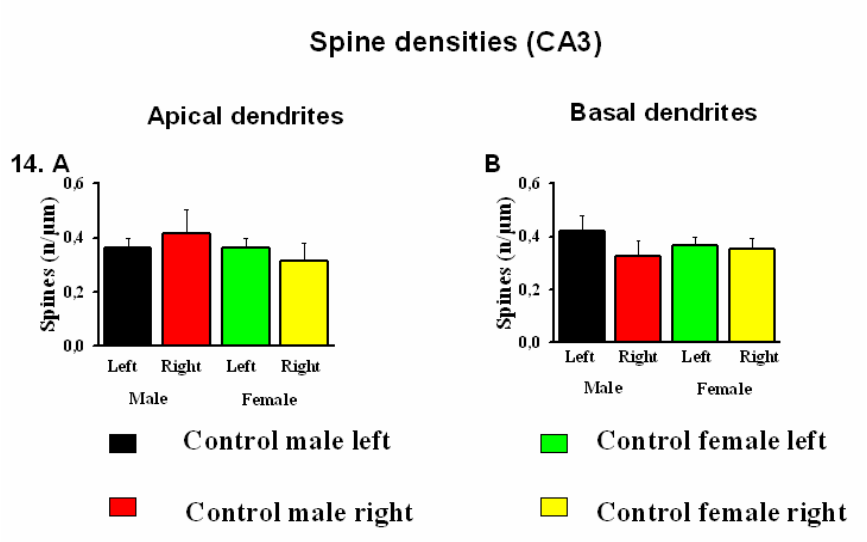


Figure 14. Histograms represent spine density of CA3 pyramidal neurons of untreated control animals. Values are given as mean ± S.E.M. A) In the apical dendrites; B) In the basal dendrites.

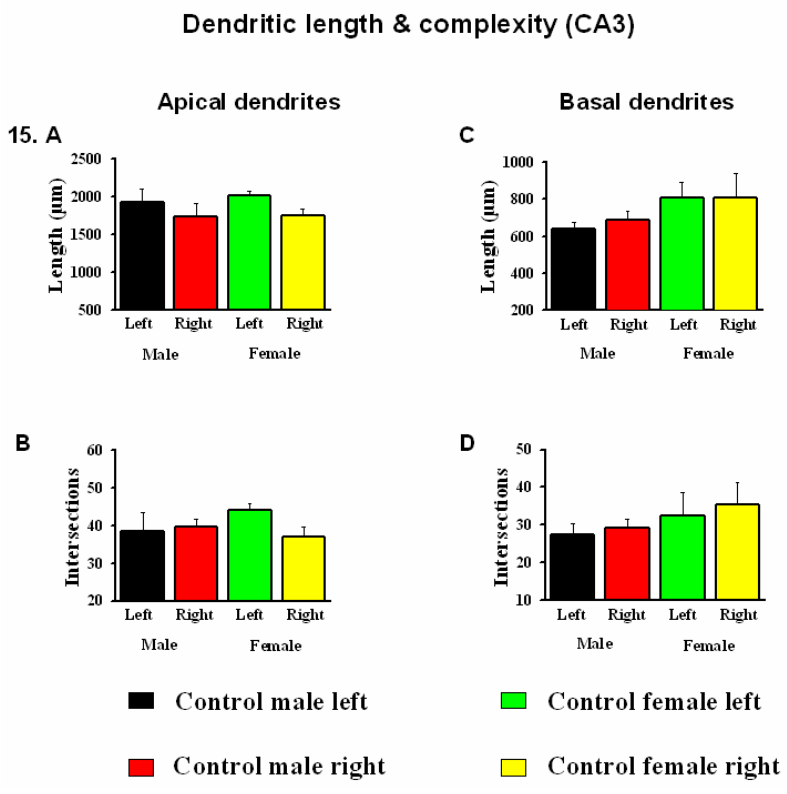


Figure 15. Histograms represent dendritic length & complexity of CA3 pyramidal neurons of untreated control animals. Values are given as mean ± S.E.M. A) Total dendritic length and B) Total number of intersections in the apical dendrites; C) Total dendritic length and D) Total number of intersections in the basal dendrites.

3.1.4 CA1 region: Untreated females show greater dendritic spine density, length & arborization compared to the untreated males

Spine density

Apical dendrite: Two-way ANOVA revealed significant effect of sex on the spine density of CA1 pyramidal neurons. The untreated controlled females displayed higher spine density ($p = < 0.001$, upto 23%) compared to the untreated controlled males in the apical dendrites (fig. 16.A).

Basal dendrite: Contrary to apical dendrites, no effects of sex or hemisphere was found on the spine density of CA1 basal dendrites (fig.16.B).

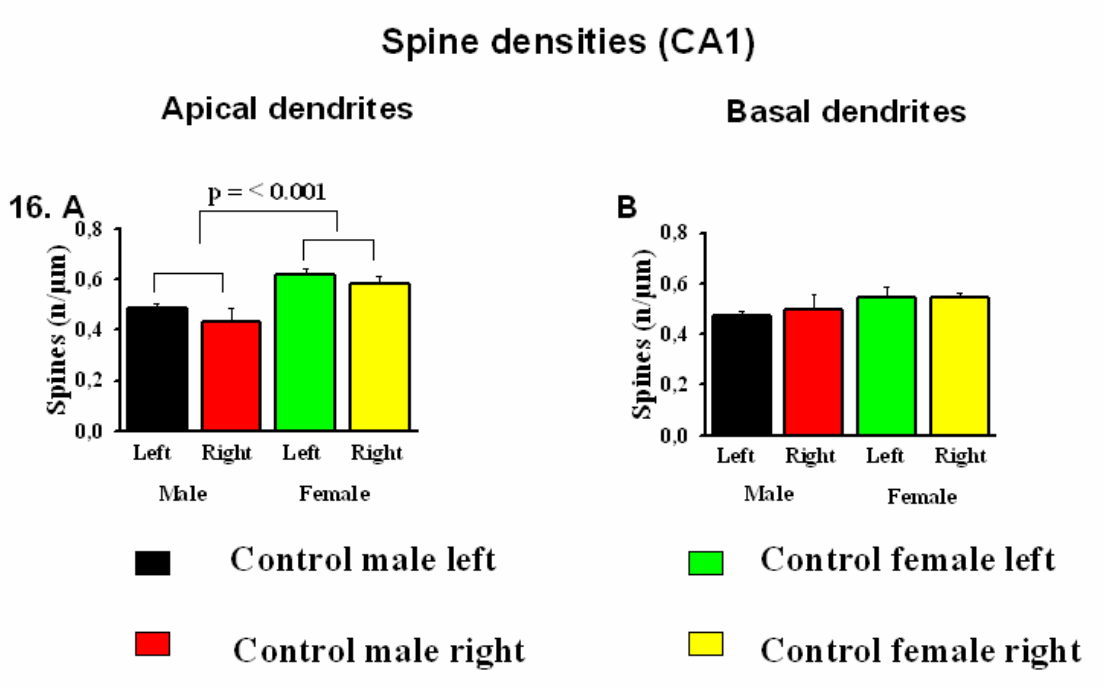


Figure 16. Histograms represent spine density of CA1 pyramidal neurons of untreated control animals. Values are given as mean ± S.E.M. A) In the apical dendrites; B) In the basal dendrites.

Dendritic length and complexity of dendritic arborization

Apical dendrite: Two-way ANOVA revealed significant effect of sex on dendritic length and arborization of CA1 pyramidal neurons. Like the spine density, the apical dendritic length was significantly higher in untreated controlled females ($p = 0.025$, upto 16%) compared to the untreated controlled males (fig.17.A). Similarly, the number of dendritic intersection was greater in untreated controlled females ($p = 0.004$, upto 21%) relative to untreated controlled males (fig.17.B) in the apical dendrites.

Basal dendrite: Unlike the apical dendrites, there were no effects of sex or hemisphere on the basal dendritic length and arborization. (fig. 17.C & D).

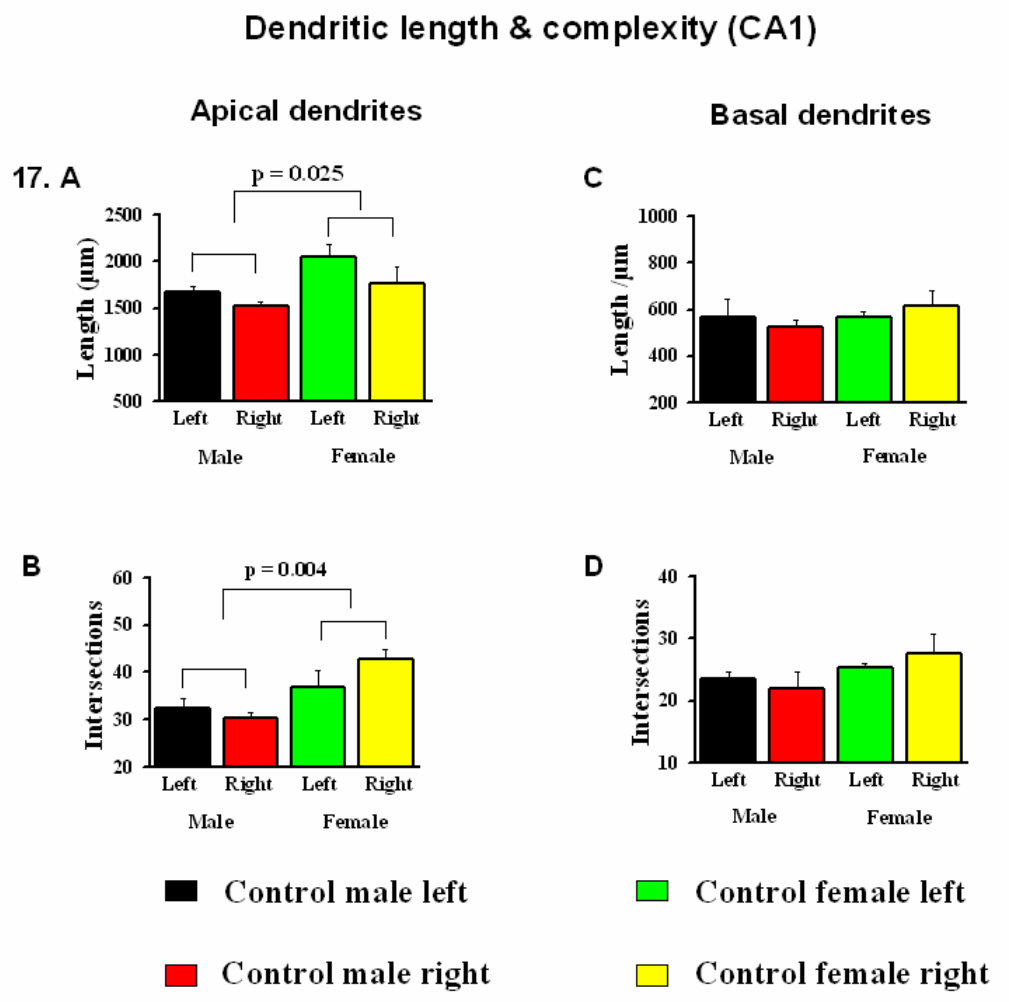


Figure 17. Histograms represent dendritic length & complexity of CA1 pyramidal neurons of untreated control animals. Values are given as mean ± S.E.M. A) Total dendritic length and B) Total number of intersections in the apical dendrites; C) Total dendritic length and D) Total number of intersections in the basal dendrites.

3.1.5 Dentate gyrus: Untreated control females show higher dendritic spine density, length & arborization relative to the untreated control males

Spine density

Outer blade of dentate gyrus: Two-way ANOVA indicated significant effect of sex on spine density. The untreated controlled females showed higher spine density ($p = <0.001$, upto 32%) compared to the untreated controlled males in the outer blade of dentate gyrus (fig.18.A).

Inner blade of dentate gyrus: There was a significant effect of sex on spine density. Like the outer blade of dentate gyrus, the untreated controlled females displayed higher spine density ($p = 0.003$, upto 33%) compared to the untreated controlled males in the inner blade of dentate gyrus (fig.18.B).

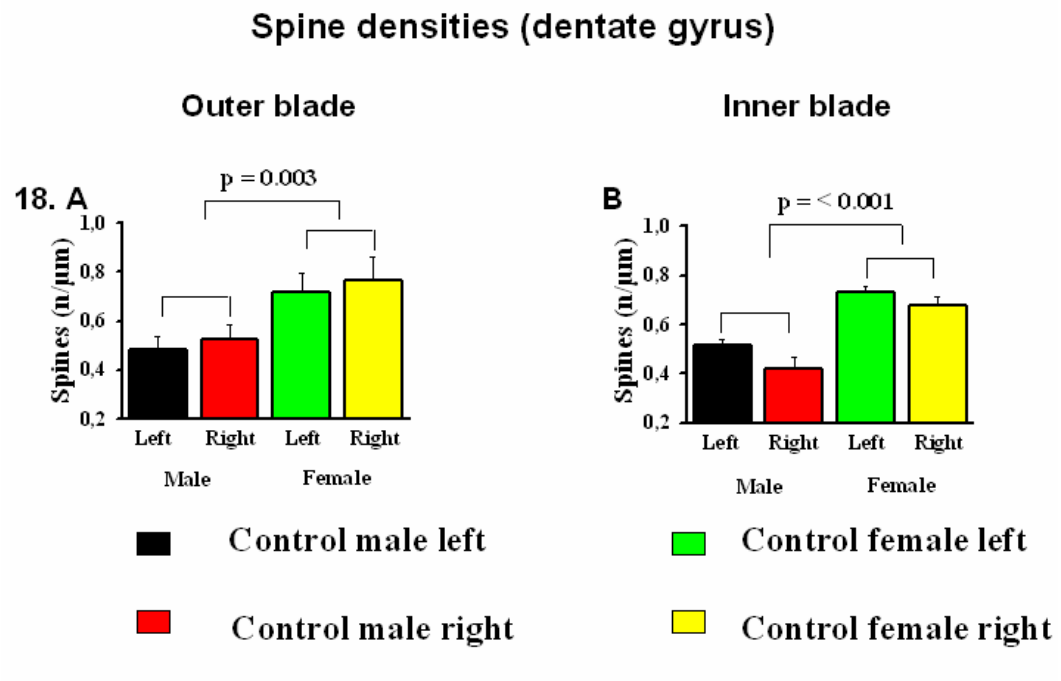


Figure 18. Histograms represent spine density of dentate gyrus granular neurons of the untreated control animals. Values are given as mean ± S.E.M. A) In the outer blade; and B) In the inner blade.

Dendritic length and complexity of dendritic arborization

Outer blade of dentate gyrus: Two-way ANOVA revealed significant effect of sex on

dendritic length and arborization. The total dendritic length was higher in untreated controlled females (p = <0.001, upto 20%) compared to the untreated controlled males (fig.19.A). In addition, the number of dendritic intersection was greater in untreated controlled females (p = <0.001, upto 19%) relative to untreated controlled males (fig.19.B) in the outer blade of dentate gyrus.

Inner blade of dentate gyrus: There was a significant effect of sex on dendritic length and arborization. Like the outer blade of dentate gyrus, the total dendritic length was higher in untreated controlled females (p = 0.003, upto 49%) compared to the untreated controlled males (fig.19.C). Similarly, the number of dendritic intersection was greater in untreated controlled females (p = 0.033, upto 35%) relative to the untreated controlled males in this layer of dentate gyrus (fig.19.D).

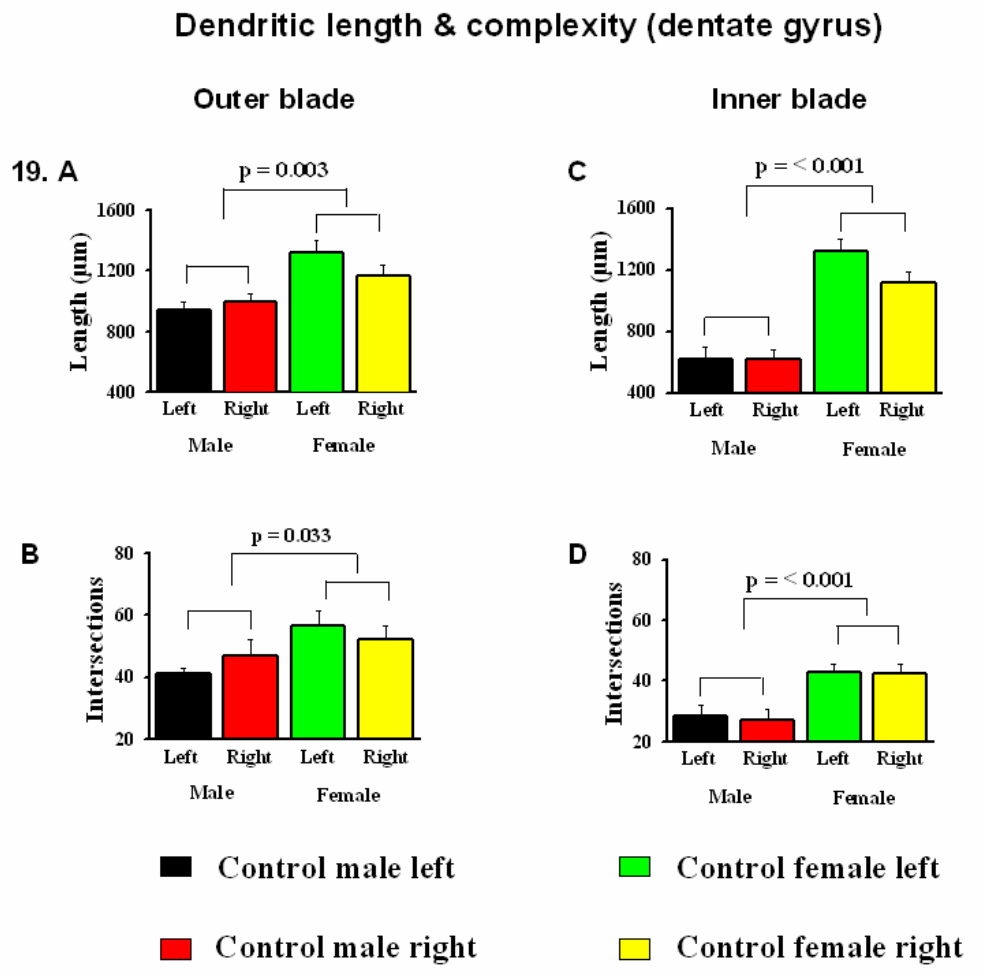


Figure 19. Histograms represent dendritic length & complexity of dentate gyrus granular neurons of the untreated control animals. Values are given as mean ± S.E.M. A) Total dendritic length and B) Total number of intersections in the outer blade; C) Total dendritic

length and D) Total number of intersections in the inner blade of dentate gyrus.

3.1.6 Basolateral amygdala: dendritic length & arborization is greater in untreated control males compared to untreated control females

Spine density

Apical dendrite: There were no effects of sex or hemisphere on the spine density of apical dendrites (fig.20.A).

Basal dendrite: Similar effects were found on the basal dendritic spine density in this region (fig.20.B).

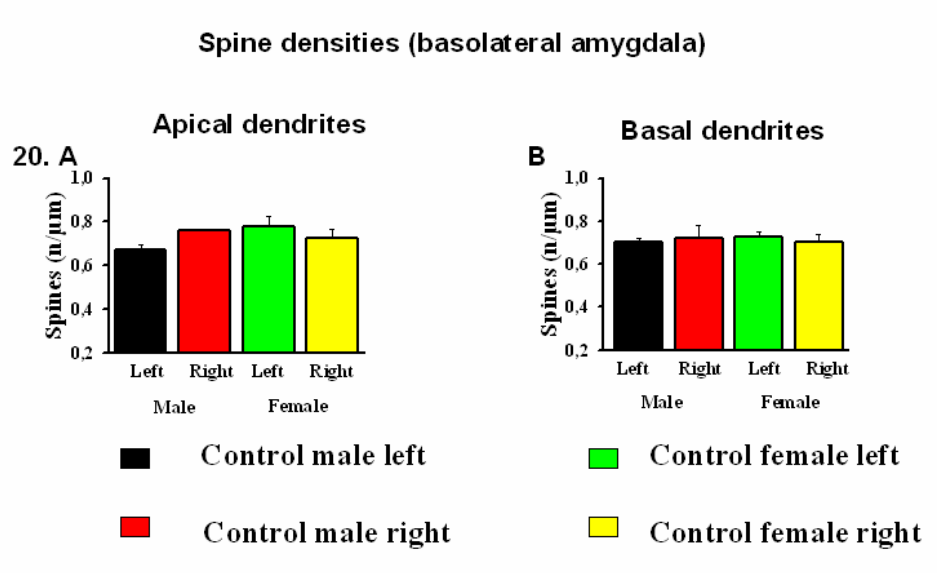


Figure 20. Histograms represent spine density of pyramidal neurons located in the basolateral amygdala of untreated control animals. Values are given as mean ± S.E.M. A) In the apical dendrites; B) In the basal dendrites.

Dendritic length and complexity of dendritic arborization

Apical dendrite: Two-way ANOVA indicated significant effect of sex on the apical dendritic length and arborization. The untreated control males displayed greater dendritic length ($p = <0.001$, upto 32%) and higher number of dendritic intersections ($p = <0.001$, upto 37%) compared to untreated control females in the apical dendrites (fig.21.A & B).

Basal dendrite: There were no effects of sex or hemisphere on dendritic length and arborization of the basal dendrite (fig.21.C & D).

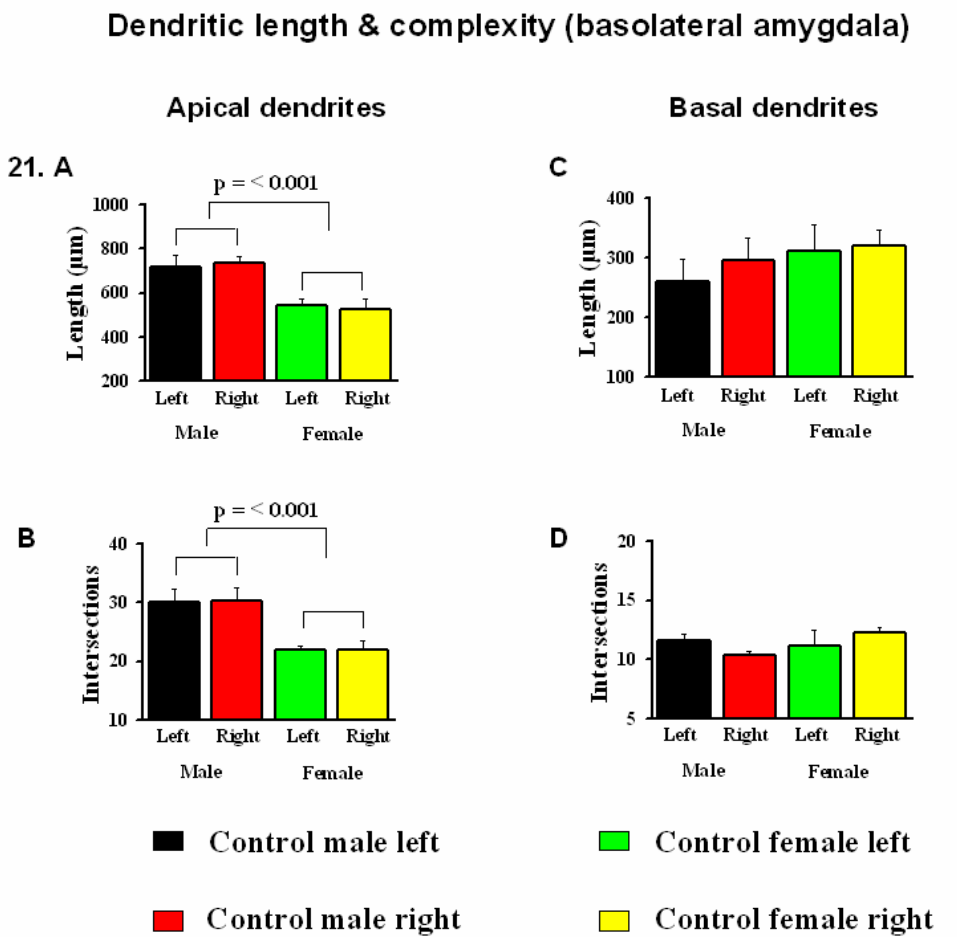


Figure 21. Histograms represent dendritic length & complexity of pyramidal neurons located in the basolateral amygdala of untreated control animals. Values are given as mean ± S.E.M. A) Total dendritic length and B) Total number of intersections on the apical dendrites; C) Total dendritic length and D) Total number of intersections on the basal dendrites.

3.2 Prenatal stress alters the development of neurons in the limbic brain areas of rodents

3.2.1 Orbitofrontal cortex: Prenatal stress reduces spine density in both sexes & alters dendritic length in a sex-specific manner

Spine density

Apical dendrite. a) Males: Three-way ANOVA indicated the main effect of prenatal stress on spine density. Prenatal stress significantly reduced spine density (p = <0.001, -

24%) in the males apical dendrite (fig.22.A). Branch order analysis indicated that the second ($p<0.05$), third ($p<0.05$), fourth ($p<0.05$) and fifth ($p<0.05$) order branches had the lowest density of spines (fig.22.B).

b) Females: Three-way ANOVA indicated significant effects of prenatal stress as well as prenatal stress + handling on spine density. In the females, prenatal stress led to lower apical spine density ($p<0.05$, - 21%) than the untreated controls (fig.22.C). Here, the third ($p<0.05$), fourth ($p<0.05$), fifth ($p<0.05$) and sixth ($p<0.05$) order branches had the lowest spine density (fig.22.D). Similarly, prenatally stressed + handled females displayed lower spine density than the untreated control females ($p<0.05$, -39%) which was found at the third ($p<0.05$), fourth ($p<0.05$), fifth ($p<0.05$) and sixth ($p<0.05$) order branches (fig.22.C & D). Additionally, prenatally stressed + handled females displayed lower spine density ($p<0.05$, -23%) compared to prenatally stressed females (fig.22.C) indicating that handling amplifies prenatal stress-evoked changes in this case rather than preventing them.

Basal dendrite. a) Males: Similar to the findings in the apical dendrite, three-way ANOVA indicated that the main effect on spine density is induced by prenatal stress. Basal dendritic spine density was reduced in the prenatally stressed males ($p<0.05$, -20%) compared to the untreated controls (fig.22.E). Lower spine density was observed at the second ($p<0.05$) and third ($p<0.05$) branch of the basal dendrite (fig.22.F).

b) Females: There was a significant effects of prenatal stress as well as prenatal stress + handling on spine density. Prenatal stress reduced spine density on the basal dendrite of females ($p<0.05$, -20%) compared to the untreated controls (fig.22.G). Spine density was reduced at the third ($p<0.05$) and fourth ($p<0.05$) branch of the dendrite (fig.22.H). Similarly, prenatal stress + handling led to lower spine density compared to the untreated controls ($p<0.05$, -36%) and to the prenatally stressed animals ($p<0.05$, -16%) (fig.22.G) indicating that handling amplifies prenatal stress-evoked effects in this case rather than preventing them. As compared to the untreated control females, prenatally stressed + handled females had lower spine density at the third ($p<0.05$) and fourth ($p<0.05$) branch of the basal dendrites (fig.22.H). As compared to prenatally stressed females, prenatally stressed + handled females had lower spine density at the third ($p<0.05$) branch of the

basal dendrites (fig.22.H).

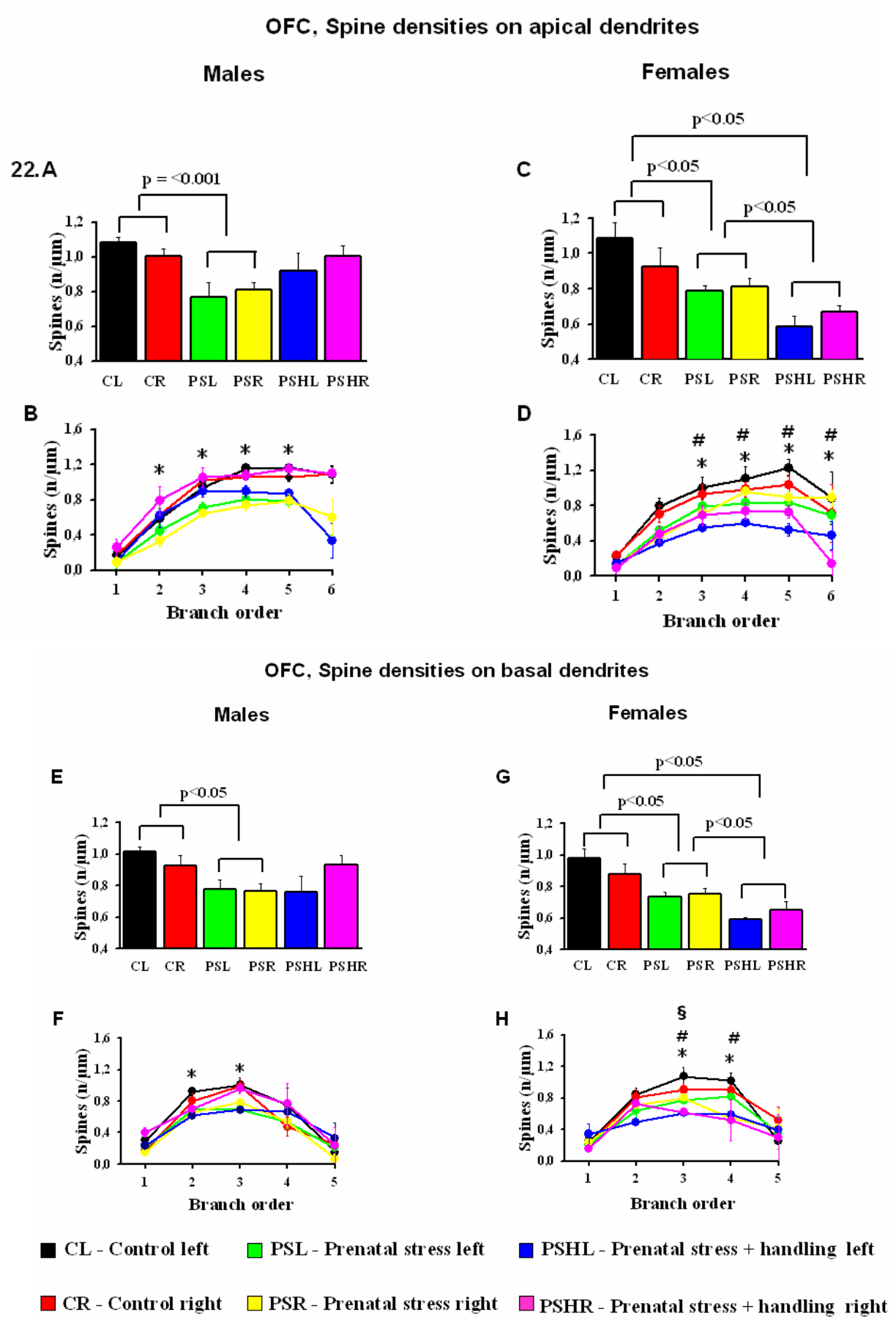


Figure 22. Histograms represent spine density of layer II/III OFC pyramidal neurons. Values are given as mean ± S.E.M. A) Total spine density in the males and C) Females apical dendrites; B) Spine density across different branch orders of the males and D) Females apical dendrites; E) Total spine density in the males and G) Females basal dendrites; F) Spine density across different branch orders of the males and H) Females basal dendrites; * indicates significant differences in prenatally stressed animals relative to untreated control animals; # indicates significant differences in prenatally stressed + handled animals relative to untreated controls. § indicates significant differences in prenatally stressed + handled animals relative to prenatally stressed animals.

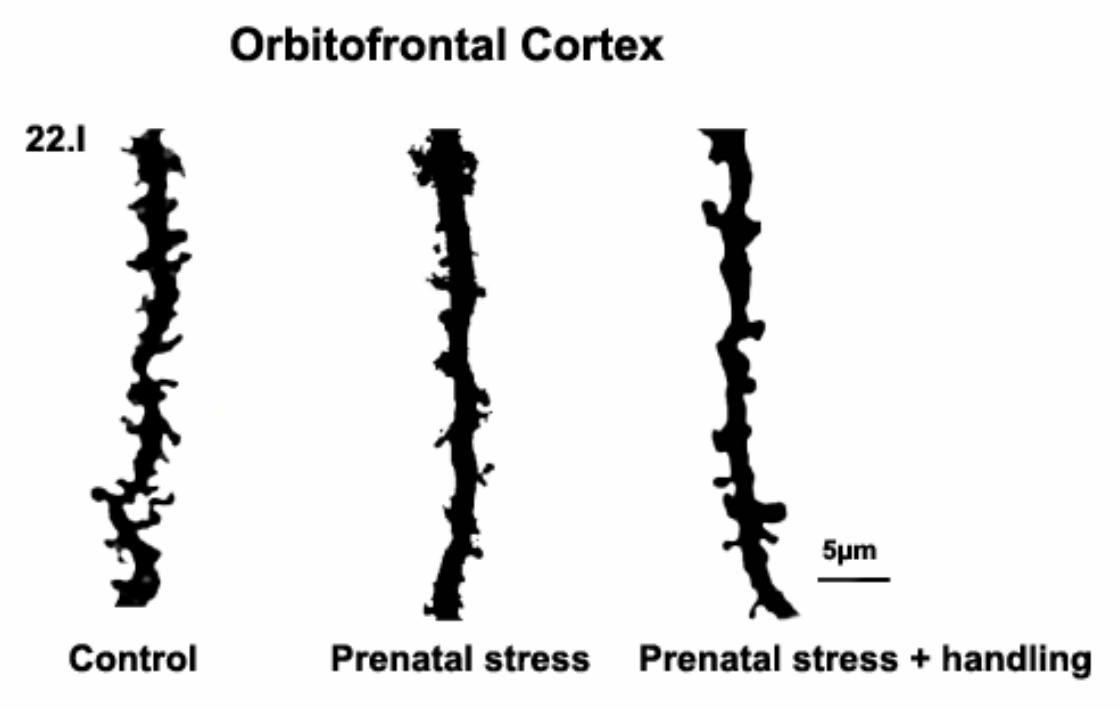


Figure 22.I Micrographs represent segments from control, prenatally stressed and prenatally stressed + handled females. The prenatally stressed females and the prenatally stressed + handled females had lower density of spines as compared to untreated controlled females in the orbitofrontal cortex.

Dendritic length and complexity of dendritic arborization

Apical dendrite. a) Males: The main effect on dendritic morphology was induced by prenatal stress. Prenatal stress led to shorter dendritic length ($p<0.05$, -26%) and lower number of dendritic intersections ($p<0.05$, -29%) compared to the untreated control males

(fig.23.A & C).

b) Females: There were no effects of treatment, hemisphere or interaction on the apical dendritic length and arborization of females (fig.23.E & F).

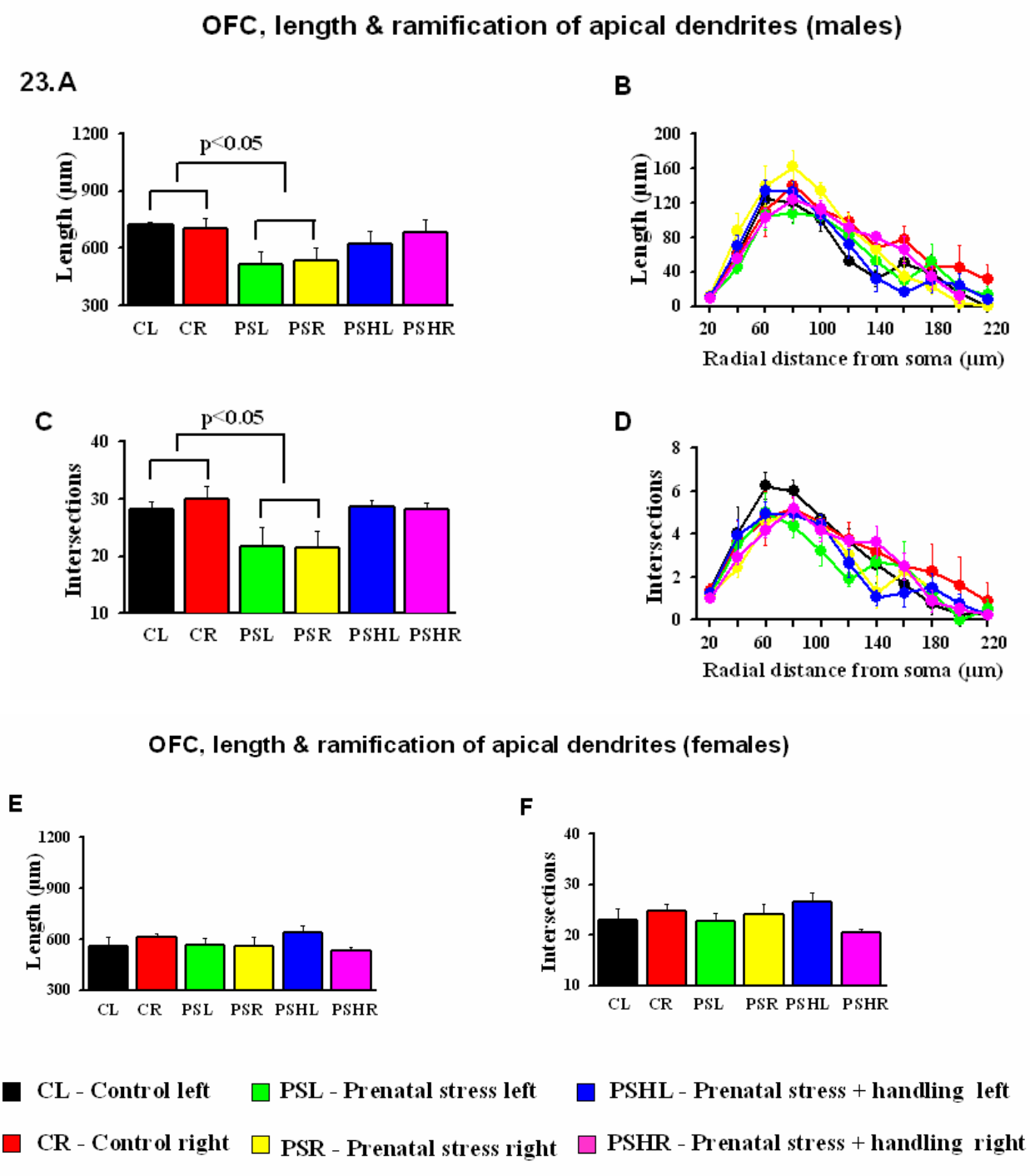


Figure 23. Histograms represent dendritic length and complexity of layer II/III OFC pyramidal neurons. Values are given as mean ± S.E.M. A) Total apical dendritic length of males and E) Females; B) Dendritic length at different concentric circle from the soma of males apical dendrites. C) Total number of apical dendritic intersections of males and F) Females; D) Number

of dendritic intersections at different concentric circles from the soma of males apical dendrites.

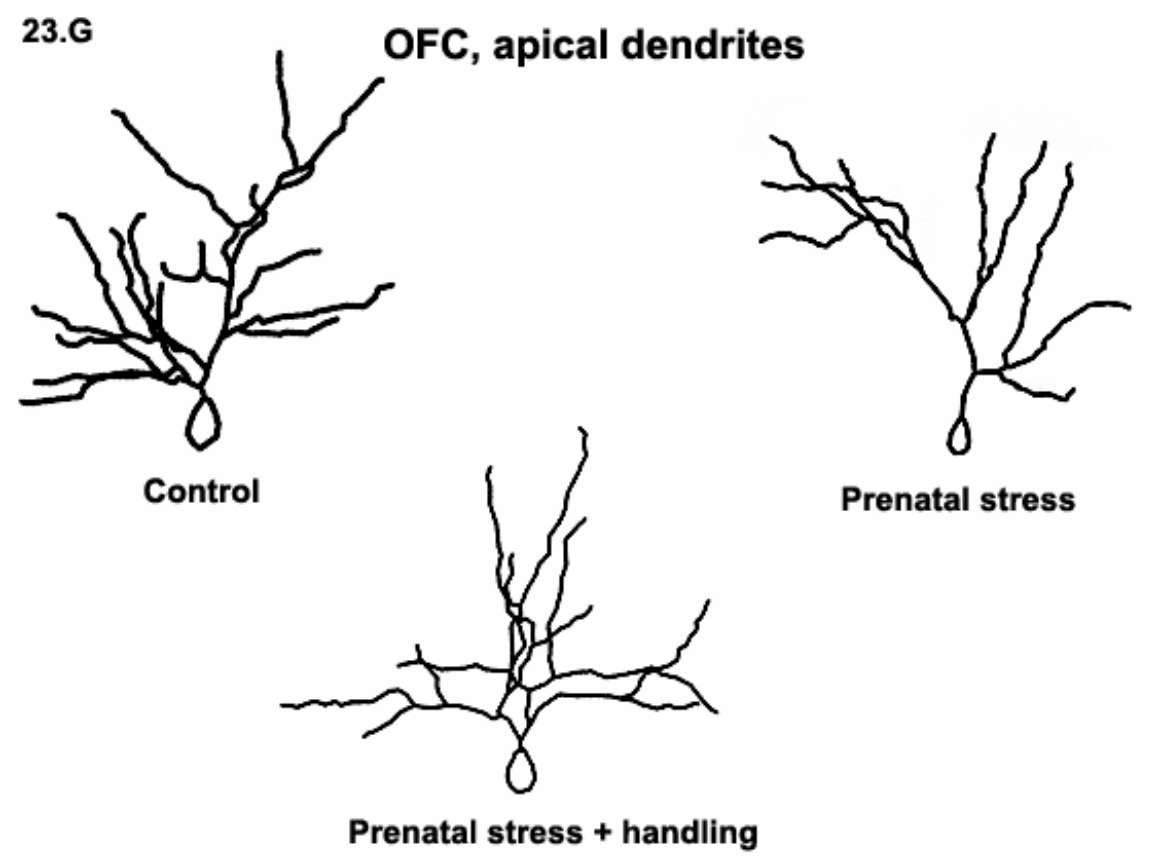


Figure 23.G Representative examples of apical dendrites from the orbitofrontal cortex of males. Prenatally stressed males had smaller dendritic length and less complex dendritic arbors compared to the untreated controls and to the prenatally stressed + handled males.

Basal dendrite. a) Males: There were no effects of treatment, hemisphere or interaction on the basal dendritic length of males (fig.24.A).

b) Females: Similarly, there were no effects of treatment, hemisphere or interaction on the basal dendritic length of females (fig.24.B).

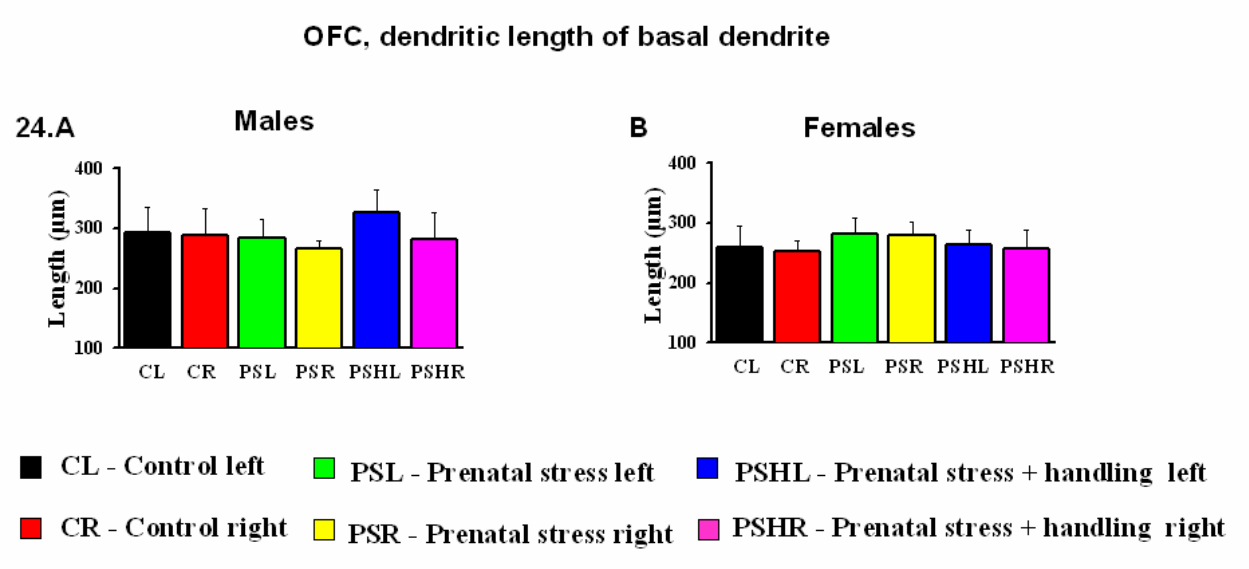


Figure 24. Histograms represent dendritic length of layer II/III OFC pyramidal neurons. A) In the males basal dendrites; B) In the females basal dendrites.

3.2.2 Anterior Cingulate Cortex: Prenatal stress reduces spine density in both sexes and alters dendritic length only in males; handling prevents/reverses the effects of prenatal stress in the males

Spine density

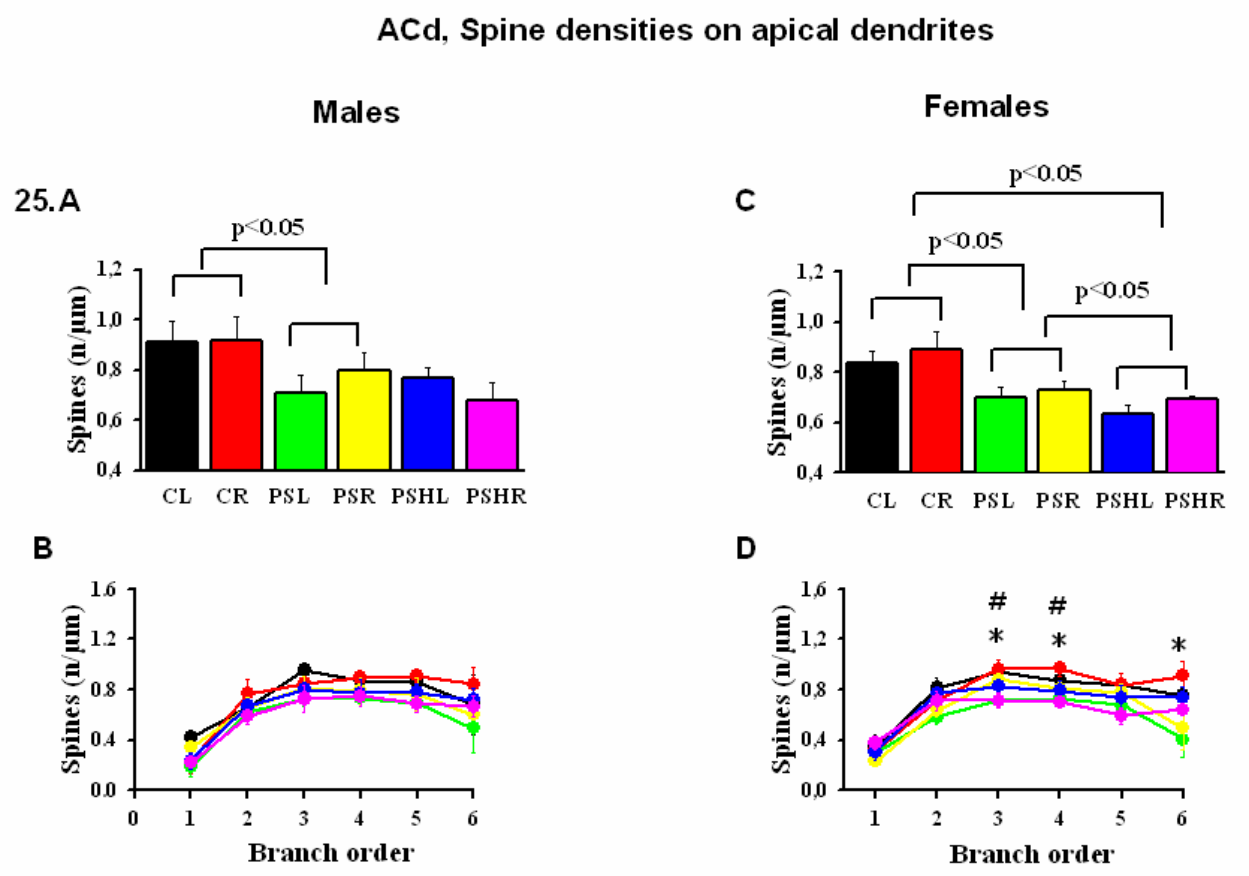
Apical dendrite. a) Males: Three-way ANOVA revealed that the main effect on spine density was induced by prenatal stress. Prenatal stress resulted in lower spine density in males ($p<0.05$, -20%) compared to the untreated controls (fig.25.A).

b) Females: The main effect on spine density was induced by prenatal stress as well as prenatal stress + handling. Like in the males, prenatal stress resulted in lower spine density in females ($p<0.05$, -17%) compared to the untreated control females (fig.25.C). Lower spine density was observed at the third ($p<0.05$), fourth ($p<0.05$) and sixth ($p<0.05$) branch of the dendrite (fig.25.D). Furthermore, prenatally stressed + handled females displayed lower spine density than the untreated controls ($p<0.05$, -16%) as well as the prenatally stressed ($p<0.05$, -9%) females (fig.25.C) indicating that the effects of prenatal stress are not normalised in this region. As compared to the untreated control females, prenatally stressed + handled females had lower spine density on the third

(p<0.05) and fourth (p<0.05) branch of the dendrite (fig.25.D).

Basal dendrite. a) Males: There were no effects of treatment, hemisphere or interaction (fig.25.E & F).

b) Females: The main effect on spine density was induced by prenatal stress and prenatal stress + handling. Prenatal stress reduced spine density in females (p<0.05, -16%) compared to the untreated control females (fig.25.G), which was seen at the third branch of the dendrite (fig.25.H). Spine density was also reduced in prenatally stressed + handled females compared to the untreated control females (p = <0.001, -16%) and the prenatally stressed (p<0.05, -16%) females (fig.25.G) indicating that the effects of prenatal stress are not normalised in this region. As compared to the untreated control females, prenatally stressed + handled females had lower spine density at the second (p<0.05) and third (p<0.05) branch of the dendrite (fig.25.H).



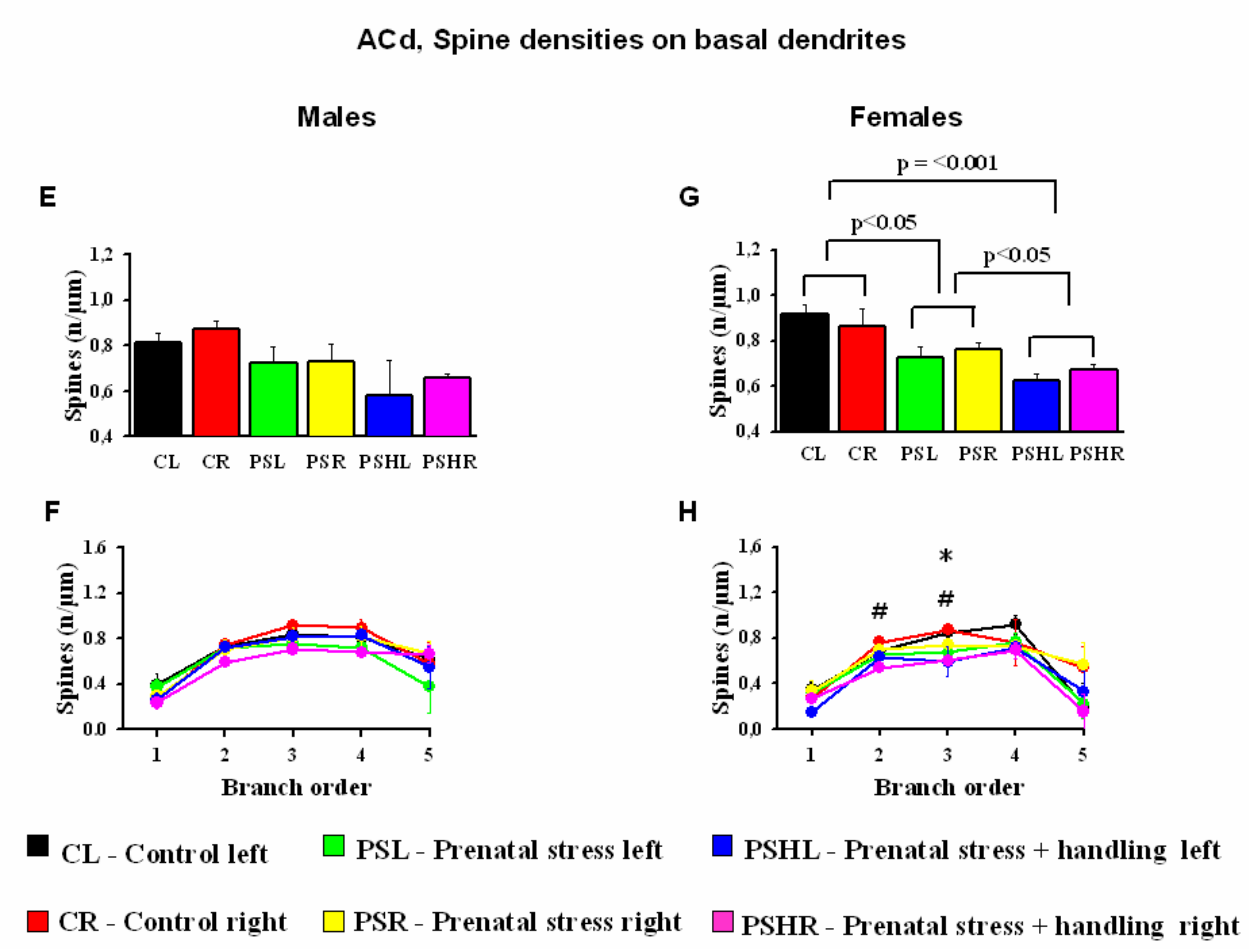


Figure 25. Histograms represent spine density of layer II/III ACd pyramidal neurons. Values are given as mean ± S.E.M. A) Total spine density in the males and C) Females apical dendrites; B) Spine density across different branch orders of the males and D) Females apical dendrites; E) Total spine density in the males and G) Females basal dendrites; F) Spine density across different branch orders of the males and H) Females basal dendrites; * indicates significant differences in prenatally stressed animals relative to untreated control animals; # indicates significant differences in prenatally stressed + handled animals relative to untreated controls.

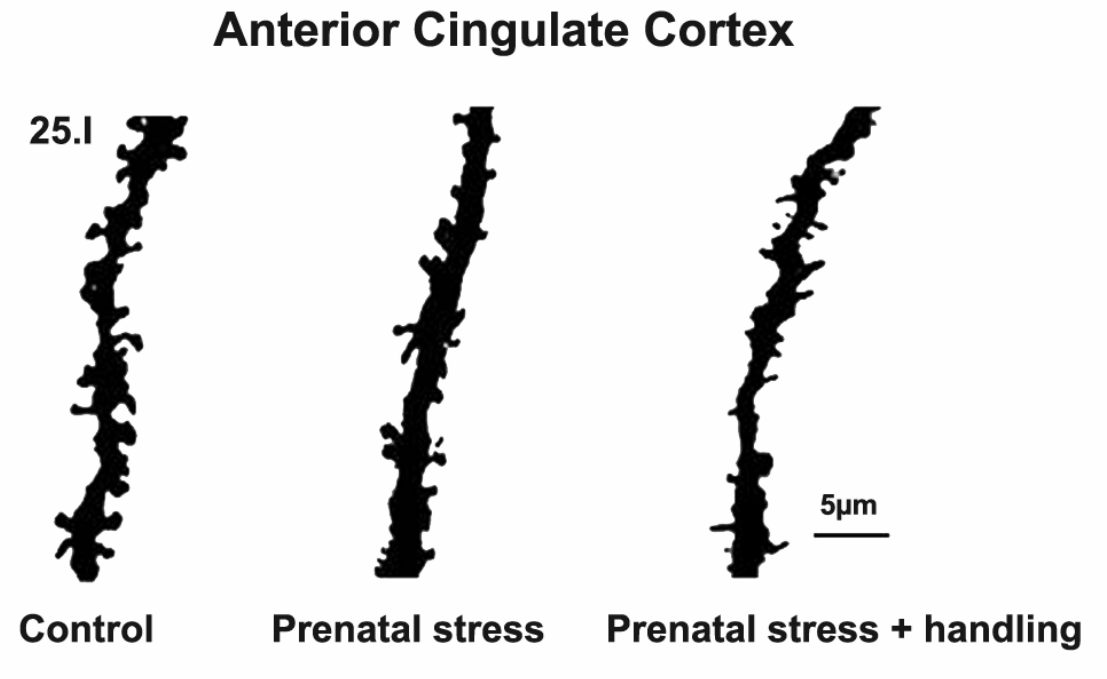


Figure 25.I Micrographs represent segments from control; prenatally stressed & the prenatally stressed + handled females. Exposure to prenatal stress and prenatal stress + handling decreased spine density compared to the untreated controls in the anterior cingulate cortex.

Dendritic length and complexity of dendritic arborization

Apical dendrite. a) Males: The main effect on dendritic morphology was induced by prenatal stress and prenatal stress + handling. Prenatal stress led to shorter dendritic length ($p<0.05$, -30%) in males (fig.26.A) which was most pronounced on the distal segments, at the distance of 160µm ($p<0.05$), 180µm ($p<0.05$) and 200µm ($p<0.05$) from the soma (fig.26.B). Handling prevented/ reversed the effects of prenatal stress in males since prenatally stressed + handled males displayed increased dendritic length ($p<0.05$, upto 23%) compared to the prenatally stressed animals (fig.26.A). Dendritic length was increased on the distal segments, at the distance of 140µm ($p<0.05$) and 160µm ($p<0.05$) from the soma (fig.26.B).

b) Females: There were no effects of treatment, hemisphere or interaction (fig.26.E & F).

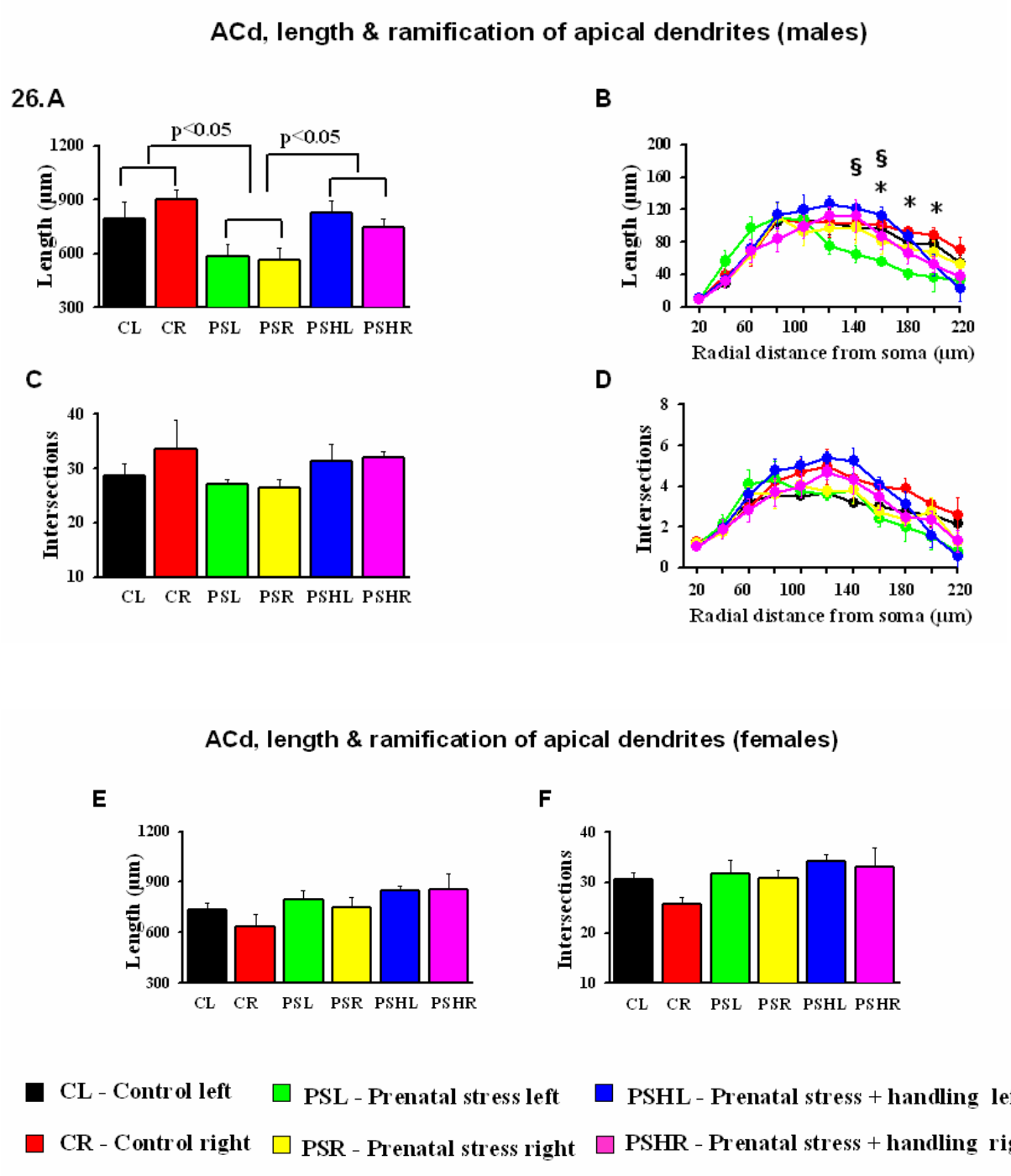


Figure 26. Histograms represent dendritic length and complexity of layer II/III ACd pyramidal neurons. Values are given as mean ± S.E.M. A) Total apical dendritic length in males and E) Females; B) Dendritic length at different concentric circle from the soma of males; C) Total number of apical dendritic intersections of males and F) Females; D) Number of dendritic intersections at different concentric circle from the soma of males. * indicates significant differences in prenatally stressed animals relative to untreated control animals; § indicates significant differences in prenatally stressed + handled animals relative to prenatally

stressed animals.

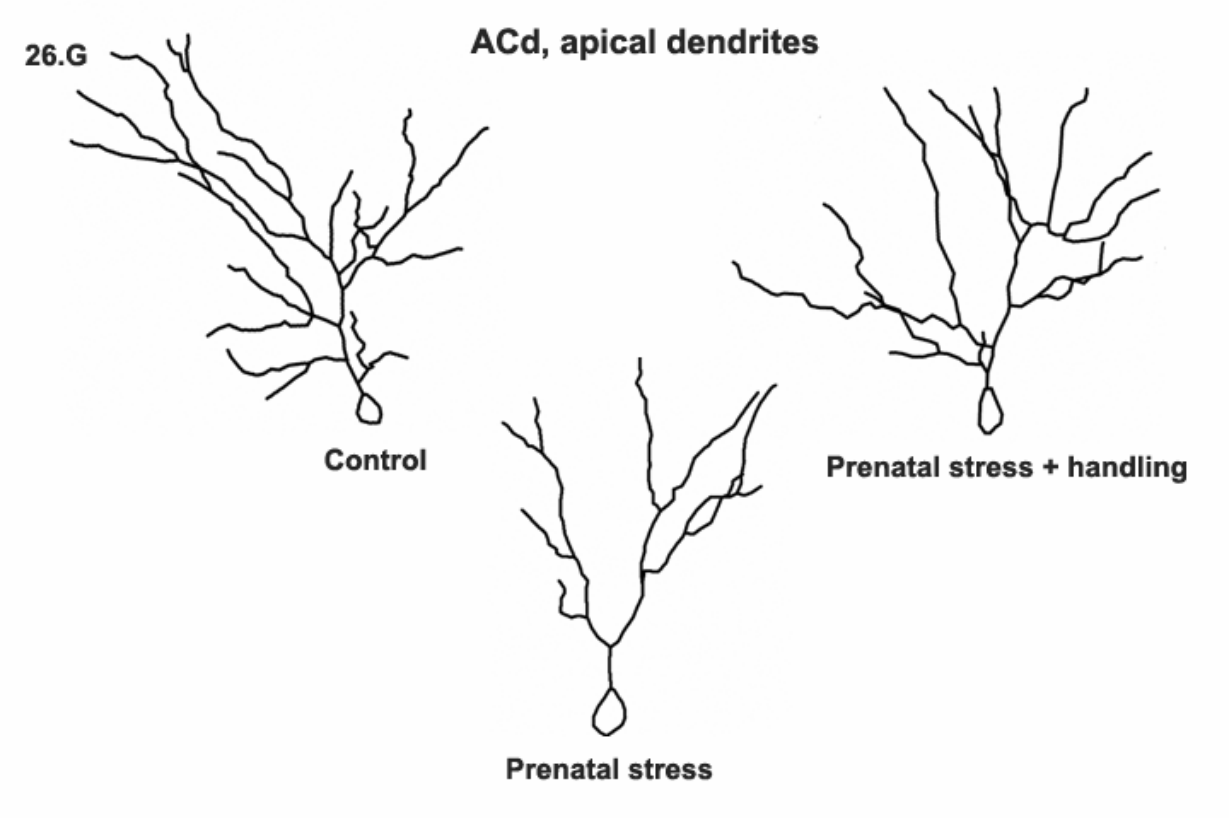


Figure 26.G Representative examples of apical dendrites of layer II/III ACd pyramidal neurons of males. Prenatally stressed males displayed shorter dendrites and less complex dendritic arbors compared to the untreated controls. Handling normalised the effects of prenatal stress since prenatally stressed + handled males were found to display increased dendritic length similar to that of controls.

Basal dendrite. a) Males: There were no effects of treatment, hemisphere or interaction on the basal dendritic length of males (fig.27.A)

b) Females: There were no effects of treatment, hemisphere or interaction on the basal dendritic length of females (fig.27.B).

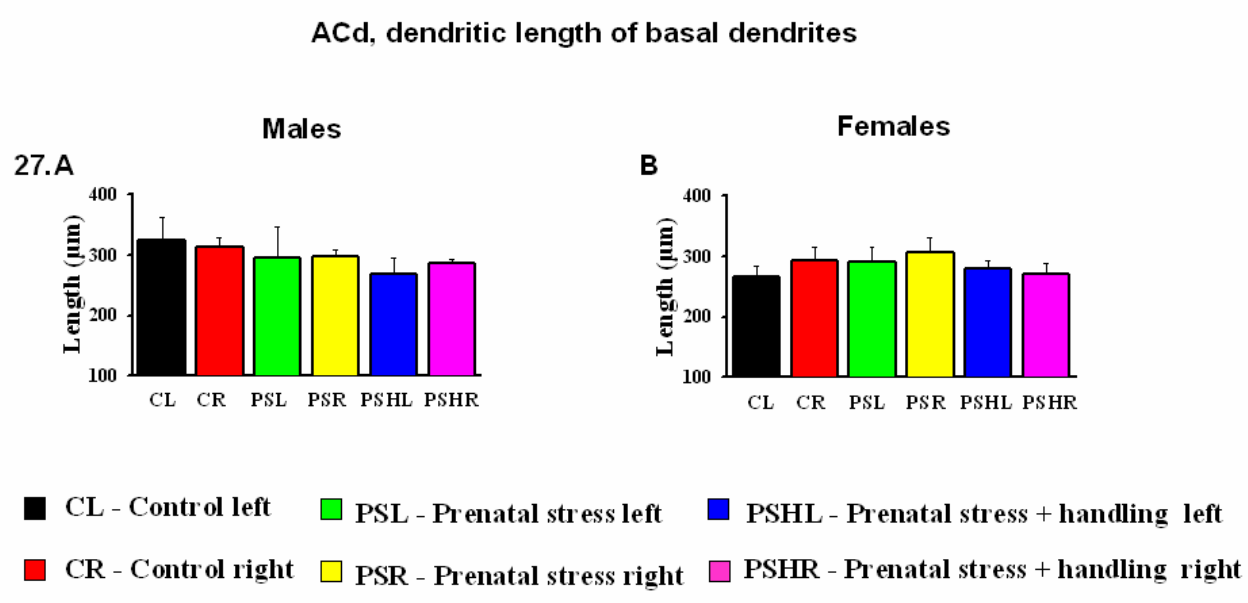


Figure 27. Histograms represent dendritic length of ACd pyramidal neurons. Values are given as mean ± S.E.M. A) Basal dendritic length of males; B) Females.

3.2.3 CA3 region: Prenatal stress causes dendritic atrophy of CA3 pyramidal neurons, handling prevents/reverses the effects of prenatal stress

Spine density

Apical dendrite. a) Males: There were no effects of treatment, hemisphere or interaction on the CA3 apical spine density of males (fig.28.A).

b) Females: Similarly, there were no effects of treatment, hemisphere or interaction on the CA3 apical spine density of females (fig.28.B).

Basal dendrite. a) Males: There were no effects of treatment, hemisphere or interaction on the CA3 basal spine density of males (fig.28.C).

b) **Females:** There were no effects of treatment, hemisphere or interaction on the CA3 basal spine density of females (fig.28.D).

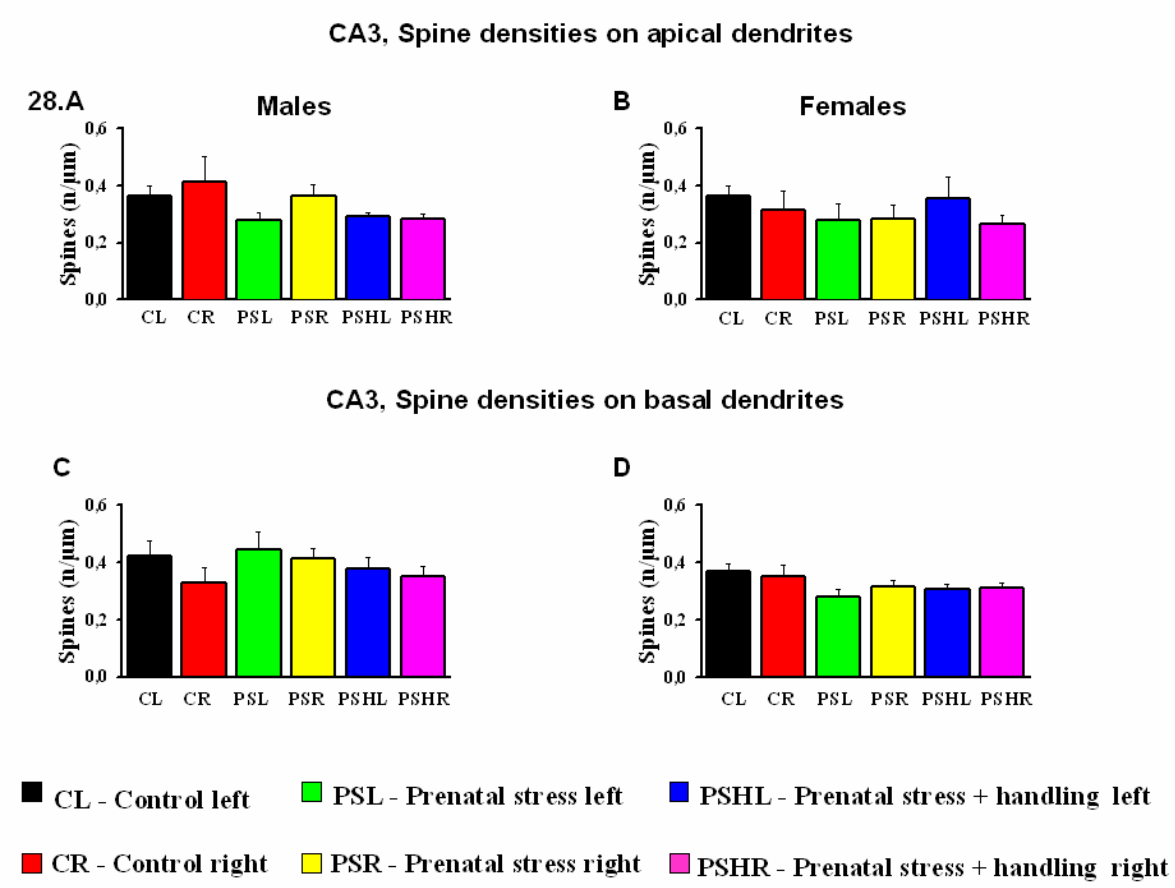


Figure 28. Histograms represent spine density of CA3 pyramidal neurons. Values are given as mean ± S.E.M. A) Total spine density on the males and B) Females apical dendrites; C) Total spine density on the males; and D) Female basal dendrites.

Dendritic length and complexity of dendritic arborization

Apical dendrite. a) Males: Three-way ANOVA revealed significant effects of prenatal stress as well as prenatal stress + handling on dendritic morphology of CA3 pyramidal neurons. Prenatally stressed males showed significant dendritic atrophy ($p<0.05$, -27%) on the apical dendrites (fig.29.A), which was most pronounced at the distance of 240µm ($p<0.05$) and 280µm ($p<0.05$) from the soma (fig.29.B). The number of dendritic intersections were also reduced in prenatally stressed males ($p<0.05$, -27%) compared to untreated control males (fig.29.C). Handling prevented/ reversed the effects of prenatal stress since prenatally stressed + handled males displayed increased dendritic length ($p<0.05$, upto 22%) compared to the prenatally stressed males (fig.29.A), observed at the distance of 120 µm ($p<0.05$) from the soma (fig.29.B).

b) Females: Three-way ANOVA revealed that the main effect on dendritic morphology was induced by prenatal stress. The apical dendrites of prenatally stressed females undergoes significant dendritic atrophy ($p<0.05$, -22%) (fig.29.E). Also, the number of dendritic intersections are lowered ($p<0.05$, -20%) due to prenatal stress in the females (fig.29.G).

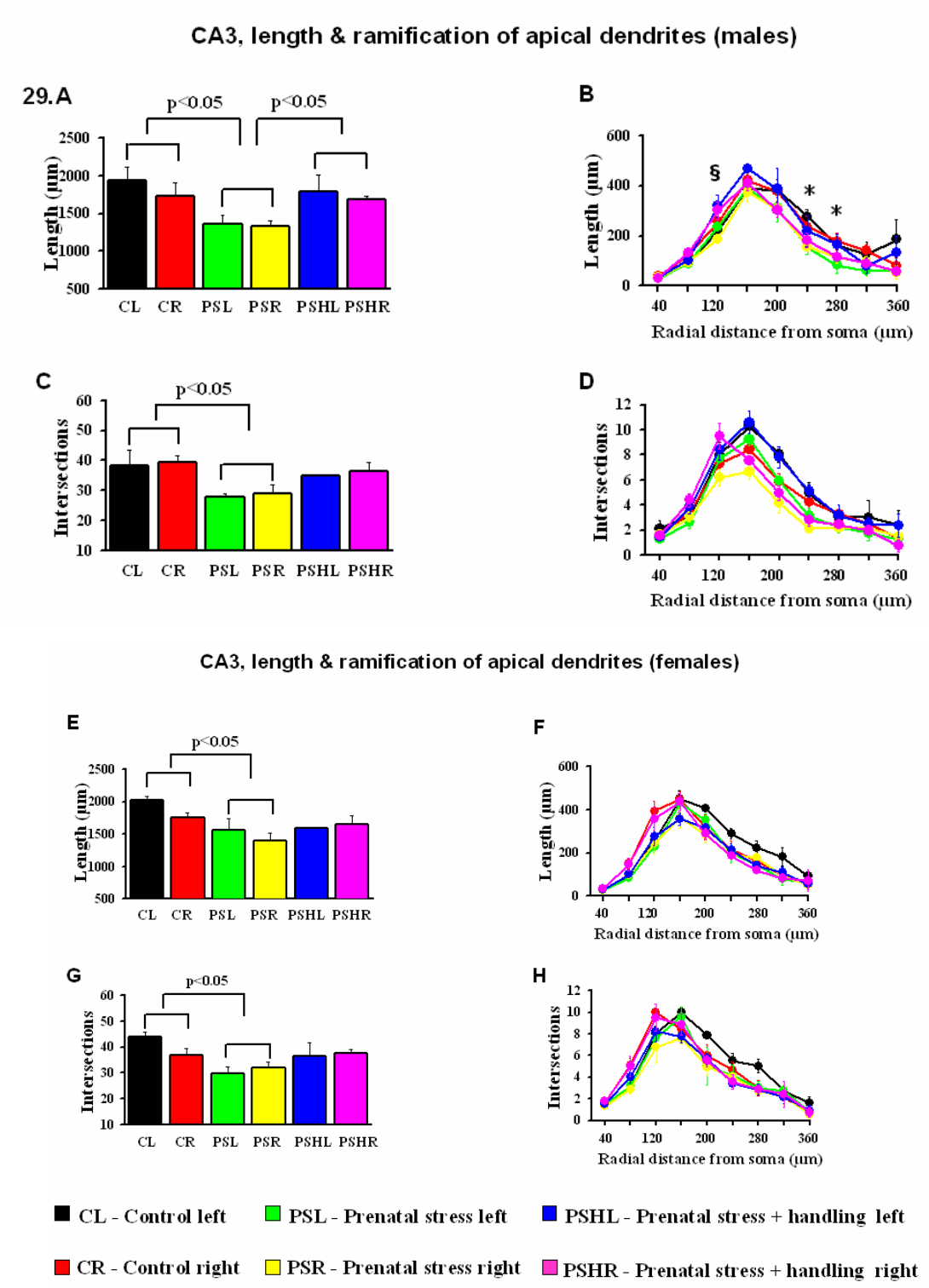


Figure 29. Histograms represent dendritic length and complexity of CA3 pyramidal neurons. Values are given as mean ± S.E.M. A) Total apical dendritic length of males and E) Females; B) Dendritic length at different concentric circles from the soma of males and F) Females apical dendrites; C) Total number of apical dendritic intersections of males and G) Females; D) Number of dendritic intersections at different concentric circles from the soma of males and H) Females apical dendrites. * indicates significant differences in prenatally stressed animals relative to untreated control animals; § indicates significant differences in prenatally stressed + handled animals relative to prenatally stressed animals.

Basal dendrite. a) Males: Three-Way ANOVA revealed significant effects of prenatal stress as well as prenatal stress + handling on dendritic length and arborization. Prenatal stress led to shorter dendritic length ($p<0.05$, -24%) and lower number of intersections ($p<0.05$, -22%) in males (fig.30.A & C). The length of basal dendrite was reduced at the radii of 100µm ($p<0.05$) and 120µm ($p<0.05$) around the soma (fig.30.B). The number of intersections were lowest at a radii of 80µm ($p<0.05$) and 100µm ($p<0.05$) around the soma (fig.30.D). In the CA3 basal dendrite, handling prevented the effects of prenatal stress since prenatally stressed + handled males displayed increased dendritic length ($p<0.05$, upto 27%) and intersections ($p<0.05$, upto 25%) compared to prenatally stressed males (fig.30.A & C) which was similar to that of untreated controls.

b) Females: Three-way ANOVA revealed that the main effect on dendritic morphology is induced by prenatal stress. Prenatal stress reduced CA3 basal dendritic length ($p<0.05$, -27%) in females (fig.30.E). Dendritic length was most significantly reduced at the distance of 160µm ($p<0.05$) from the soma (fig.30.F). As opposed to males, in females the effects of prenatal stress were not prevented/ reversed by handling (fig.30.E & G).

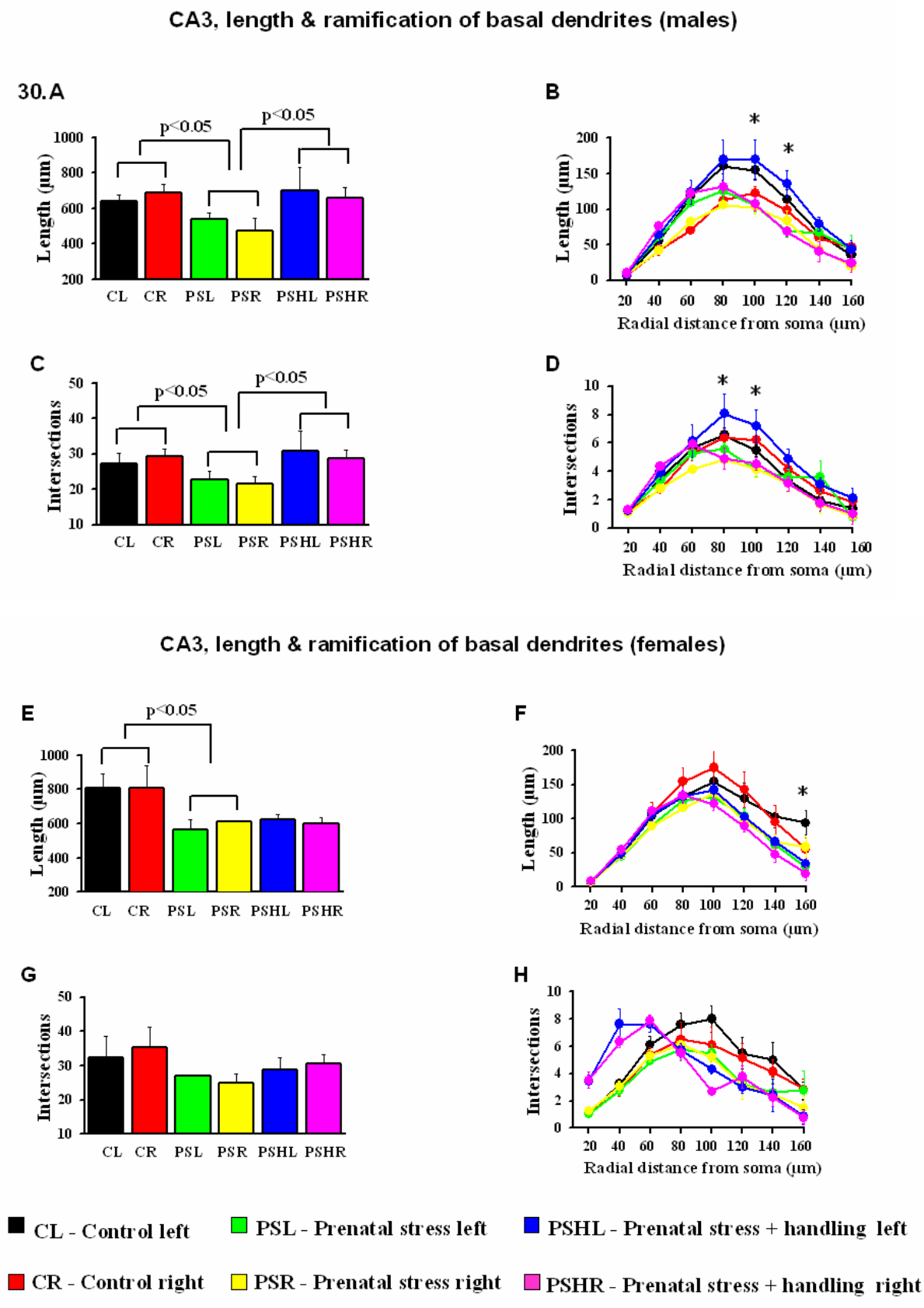


Figure 30. Histograms represent dendritic length and complexity of CA3 pyramidal neurons. Values are given as mean ± S.E.M. A) Total basal dendritic length of males and E)

Females; B) Dendritic length at different concentric circles from the soma of males and F) Females basal dendrites; C) Total number of basal dendritic intersections of males and G) Females; D) Number of dendritic intersections at different concentric circles from the soma of males and H) Females basal dendrites. * indicates significant differences in prenatally stressed animals relative to untreated control animals.

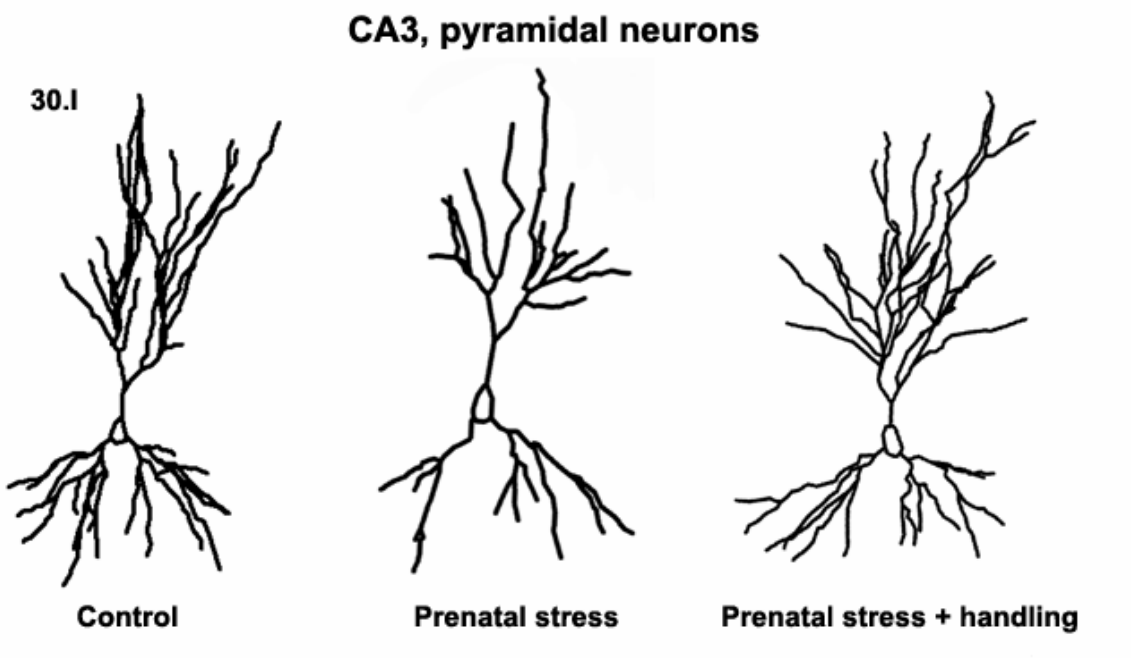


Figure 30.I Representative examples of CA3 pyramidal neurons of males. Prenatal stress caused significant dendritic atrophy evidenced by shorter dendrites and less complex dendritic arbors compared to the untreated controls. The effects of prenatal stress are normalised by handling since prenatal stress + handling increased the total dendritic length and complexity of dendrites back to normal control values.

3.2.4 CA1 region: Prenatal stress resulted in shorter dendrites and it affected spine density in a sex-specific manner

Spine density

Apical dendrite. a) Males: Three-Way ANOVA indicated significant effects of prenatal stress and prenatal stress + handling on spine density. Prenatal stress increased spine density ($p<0.05$, upto 28%) compared to the untreated controls (fig.31.A). Spine density was increased at the third ($p<0.05$), fourth ($p<0.05$), fifth ($p<0.05$), sixth ($p<0.05$) and tenth ($p<0.05$) branch of the dendrite (fig.31.B). Prenatally stressed + handled males also had higher spine density than the controls ($p<0.05$, upto 10%) which was found at the

third ($p<0.05$), fourth ($p<0.05$), fifth ($p<0.05$), sixth ($p<0.05$) and tenth ($p<0.05$) branch of the apical dendrite (fig.31.A & B) indicating that the effects of prenatal stress are not normalised by handling in this region.

b) Females: There were no effects of treatment, hemisphere or interaction (fig.31.C).

Basal dendrite. a) Males: There were no effects of treatment or hemisphere or interaction (fig.31.E). **b) Females:** There were no effects of treatment or hemisphere or interaction (fig.31.F).

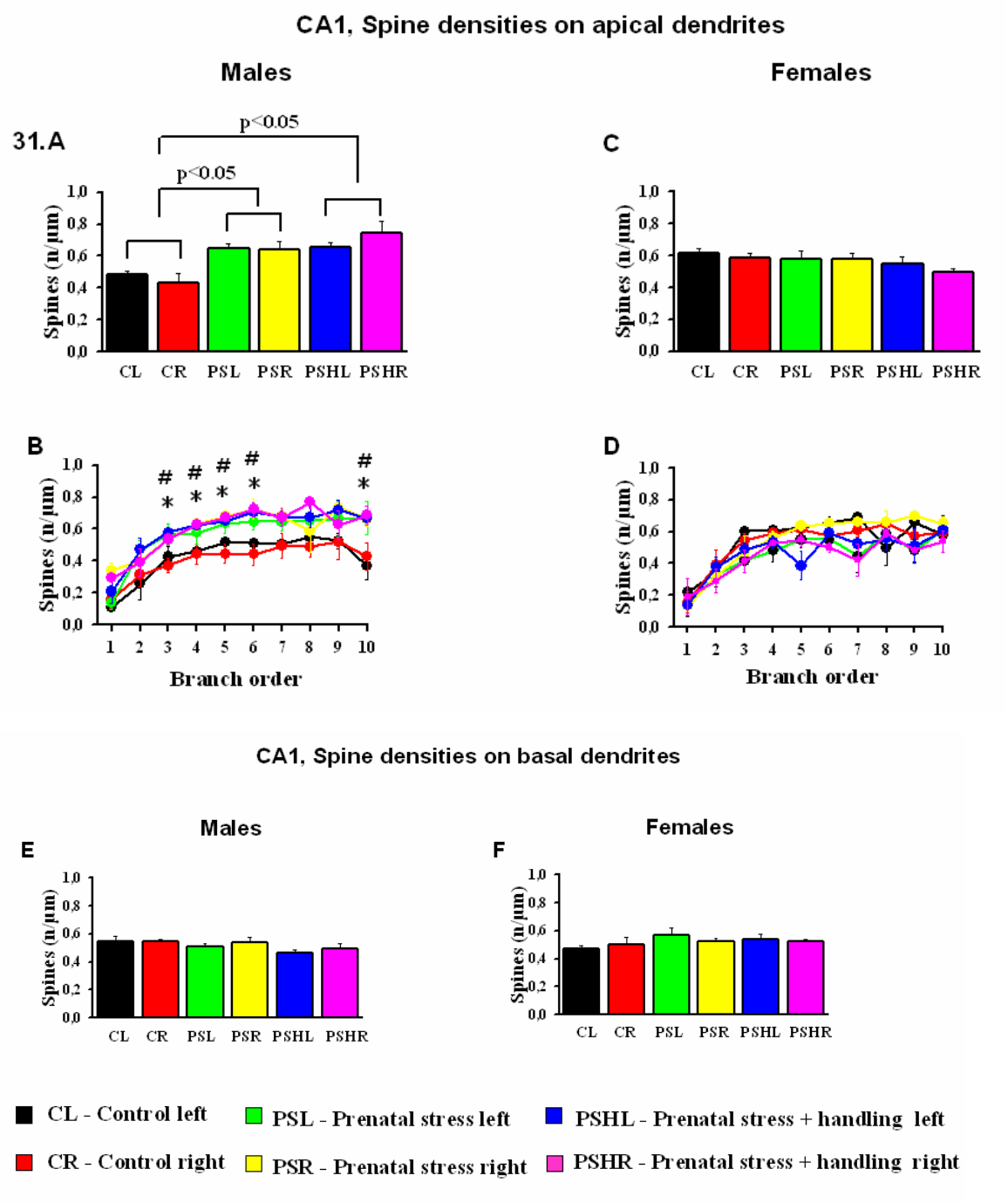


Figure 31. Histograms represent spine density of CA1 pyramidal neurons. Values are given as mean ± S.E.M. A) Total spine density in the apical dendrites of males and C) Females; B) Spine density across different branch orders of males and D) Females apical dendrites; E) Total spine density in the basal dendrites of males and F) Females; * indicates significant differences in prenatally stressed animals relative to untreated controls; # indicates significant differences in prenatally stressed + handled animals relative to untreated controls.

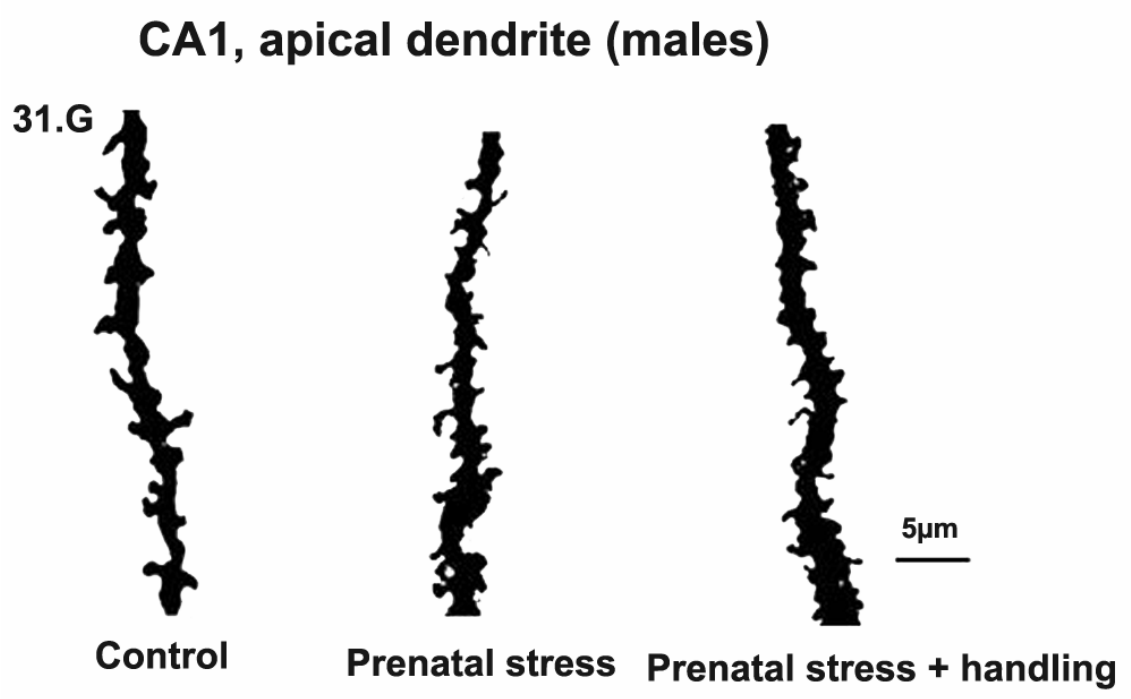


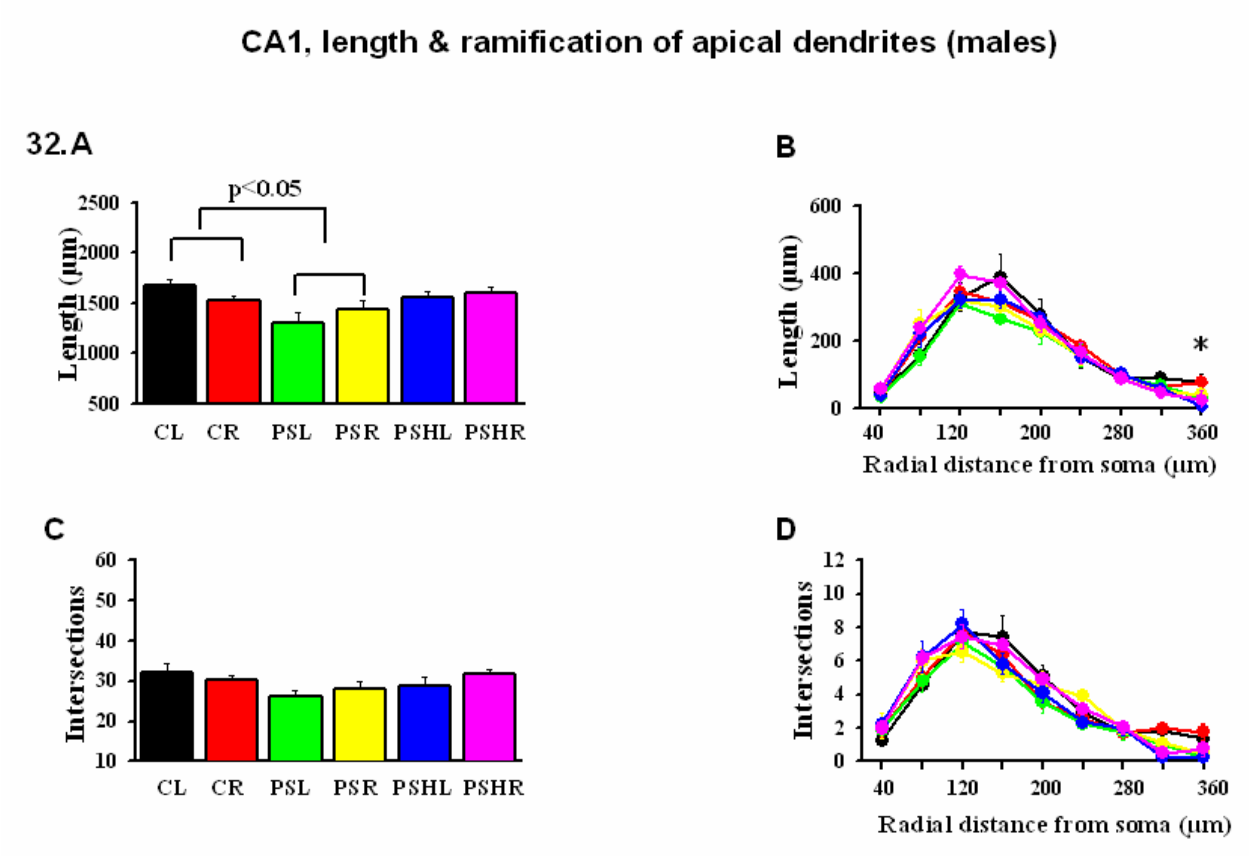
Figure 31.G Micrographs represent segments from untreated controls; prenatally stressed and the prenatally stressed + handled animals. Prenatally stressed as well as the prenatally stressed + handled males displayed increased spine density in the CA1 pyramidal neurons as compared to untreated controls.

Dendritic length and complexity of dendritic arborization

Apical dendrite. a) Males: Three-Way ANOVA indicated the main effect of prenatal stress on dendritic morphology. In the CA1 region, prenatally stressed males displayed smaller apical dendritic length than the controls ($p<0.05$, -14%) which was most evident at the distance of 360µm ($p<0.05$) from the soma (fig. 32.A & B).

b) Females: Three-Way ANOVA revealed significant effects of prenatal stress as well as

prenatal stress + handling on dendritic length and arborization. Like the males, the apical dendritic length was significantly reduced ($p<0.05$, -26%) in the prenatally stressed females compared to the untreated controlled females (fig.32.E). The apical dendritic length was reduced at the distance of 200µm ($p<0.05$) and 240 µm ($p<0.05$) from the soma (fig. 32.F). Similarly, the number of dendritic intersection was reduced due to prenatal stress ($p<0.05$, -31%) which was found at the distance of 160µm ($p<0.05$), 200µm ($p<0.05$), 240µm ($p<0.05$), 280µm ($p<0.05$) and 320 ($p<0.05$) from the soma (fig.32.G & H). Furthermore, prenatally stressed + handled females also displayed shorter dendritic length ($p<0.05$, -29%) and lower number of dendritic intersections ($p<0.05$, -27%) than the untreated control females (fig.32.E & G). Prenatally stressed + handled females had smaller apical dendritic length at the distance of 240µm ($p<0.05$) from the soma (fig.32.F). The number of dendritic intersections were reduced at the distance of 160µm ($p<0.05$) and 200µm ($p<0.05$) from the soma (fig.32.H) indicating that handling did not normalise the effects of prenatal stress in the CA1 region of the females.



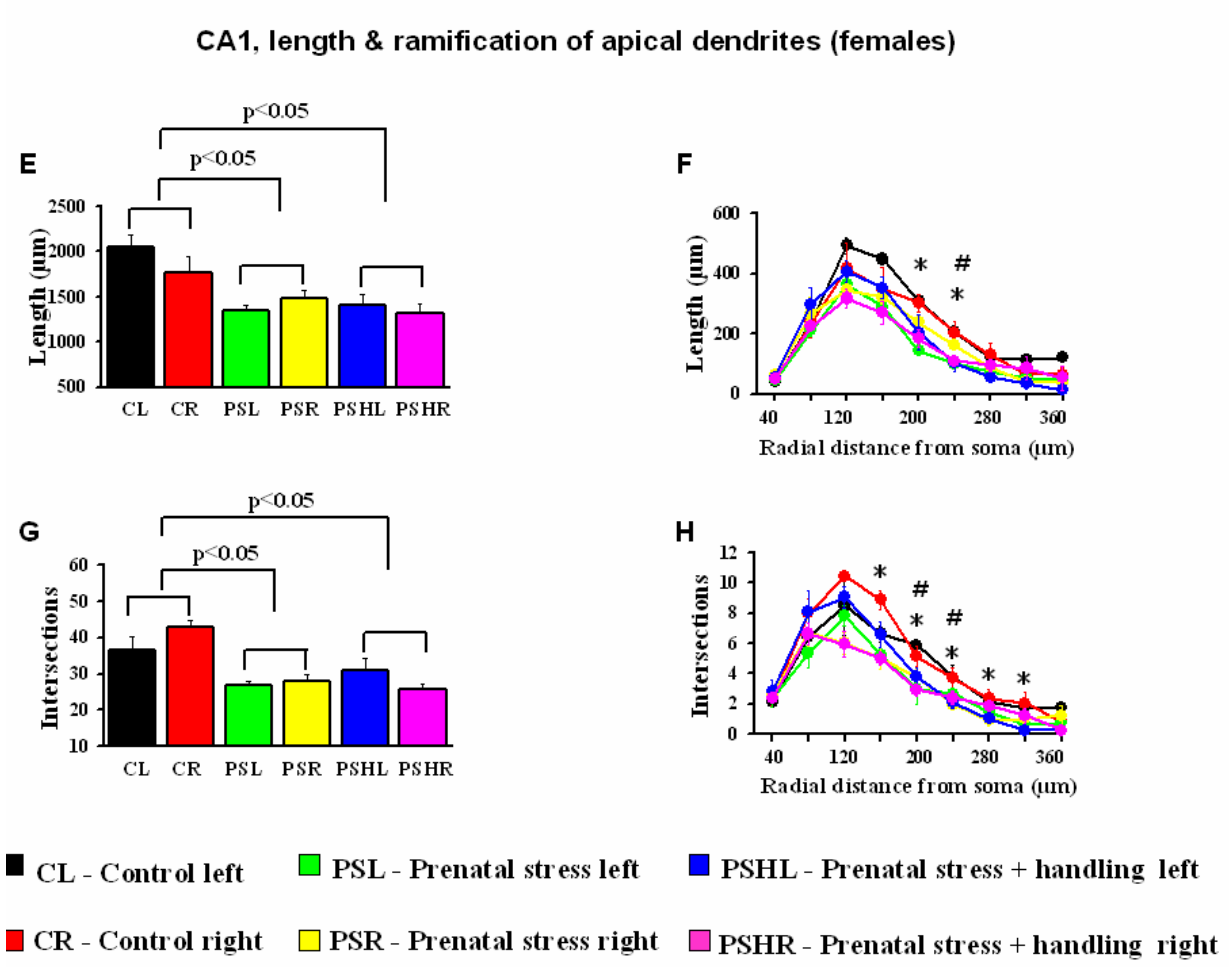


Figure 32. Histograms represent dendritic length and complexity of CA1 pyramidal neurons. Values are given as mean ± S.E.M. A) Total apical dendritic length of males and E) Females; B) Apical dendritic length at different concentric circles from the soma of males and F) Females; C) Total number of apical dendritic intersections of males and G) Females; D) Number of apical dendritic intersections at different concentric circles from the soma of males and H) Females. * indicates significant differences in prenatally stressed animals relative to untreated controls; # indicates significant differences in prenatally stressed + handled animals relative to untreated controls.

Basal dendrite. a) Males: There were no effects of treatment, hemisphere or interaction on the basal dendritic length of males (fig.33.A & B).

b) Females: Three-Way ANOVA revealed the significant effects of prenatal stress and prenatal stress + handling on basal dendritic morphology. Unlike the males, prenatal stress led to shorter basal dendrites ($p<0.05$, -22%) in females (fig.33.C). The dendritic

length was reduced at the distance of 140µm ($p<0.05$) from the soma (fig.33.D). The number of intersection was also reduced after prenatal stress ($p<0.05$, -23%) which was most pronounced at the distance of 100µm ($p<0.05$), 120µm ($p<0.05$) and 140µm ($p<0.05$) from the soma (fig.33.E & F). Furthermore, prenatal stress + handling led to shorter basal dendrites ($p<0.05$, -21%) and lower number of dendritic intersections ($p<0.05$, -23) in females compared to the untreated controls (fig.33.C & E), indicating that handling did not normalise prenatal stress effects in this region. Here, the dendritic length was reduced at the distance of 80µm ($p<0.05$), 100µm ($p<0.05$), 120µm ($p<0.05$) and 140µm ($p<0.05$) from the soma (fig.33.D). The number of dendritic intersections were lowest at the distance of 100µm ($p<0.05$) from the soma (fig.33.F).

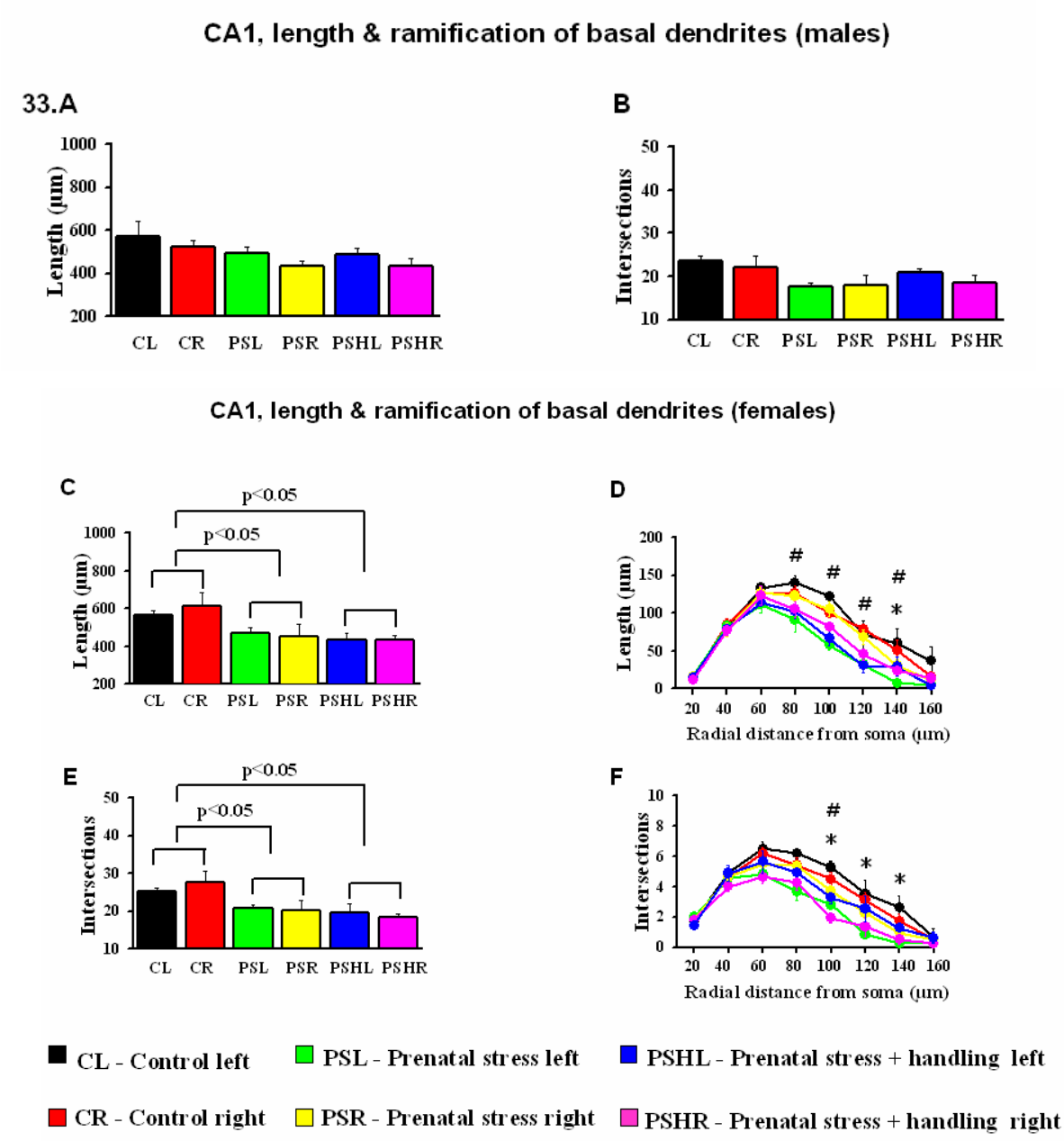


Figure 33. Histograms represent dendritic length and complexity of CA1 pyramidal neurons. Values are given as mean ± S.E.M. A) Total basal dendritic length of males and C) Females; B) Total number of basal dendritic intersections of males and E) Females; D) Dendritic length at different concentric circles from the soma of females; F) Number of basal dendritic intersections at different concentric circles from the soma of females. * indicates significant differences in prenatally stressed animals relative to untreated control animals; # indicates significant differences in prenatally stressed + handled animals relative to untreated control animals.

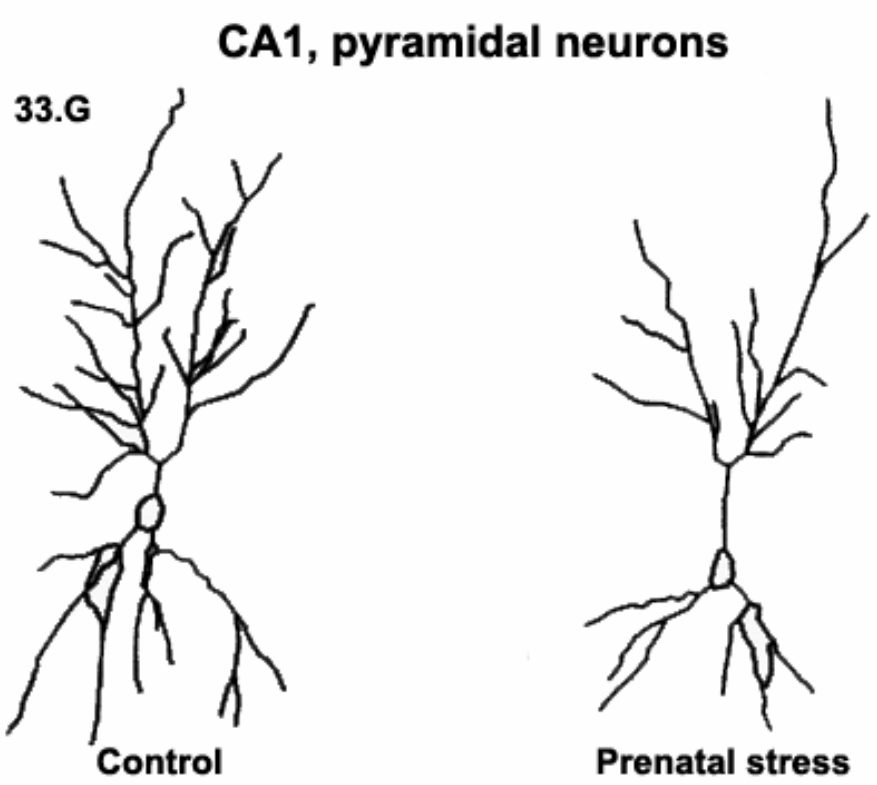


Figure 33.G Representative examples of CA1 pyramidal neurons in female rats. Prenatal stress induced shorter dendrites and lower complex dendritic arbors compared to the untreated controls.

3.2.5 Dentate gyrus: Sexually dimorphic effects of prenatal stress on spine density and dendritic length of granular neurons

Spine density

Outer blade of dentate gyrus. a) Males: There were no effects of treatment, hemisphere

or interaction (fig.34.A)

b) Females: Three-Way ANOVA revealed significant effects of prenatal stress and prenatal stress + handling on spine density. Unlike in males, the spine density was significantly decreased in females ($p<0.05$, -17%) after prenatal stress which could be clearly seen at the second branch ($p<0.05$) of the dendrite (fig.34.C & D). Furthermore, prenatal stress + handling led to lower spine density in females as compared to the untreated control females ($p<0.05$, -44%) as well as the prenatally stressed females ($p<0.05$, -33%) (fig.34.C). indicating that it did not normalise prenatal stress effects in this region. As compared to the untreated controls, prenatally stressed + handled females had lower spine density at the second ($p<0.05$), third ($p<0.05$), fourth ($p<0.05$) and fifth ($p<0.05$) branch of the granular neurons (fig.34.D).

Inner blade of dentate gyrus. a) Males: Three-Way ANOVA revealed significant effects of prenatal stress and prenatal stress + handling on spine density. Prenatal stress enhanced spine density in males compared to the untreated controls ($p<0.05$, upto 23%) which was found at the third ($p<0.05$) and fifth ($p<0.05$) branch of the granular neurons (fig.34.E & F). Conversely, prenatal stress + handling leads to lower spine density ($p<0.05$, -37%) relative to prenatally stressed animals in this region (fig.34.E).

b) Females: Three-Way ANOVA revealed significant effects of prenatal stress as well as prenatal stress + handling on spine density. Contrary to males, prenatal stress decreased spine density ($p<0.05$, -25%) in the females (fig.34.G). In the females, the spine density was reduced at the fifth ($p<0.05$) branch of the neuron (fig.34.H). Furthermore, prenatal stress + handling led to lower spine density in the females as compared to the untreated control females ($p<0.05$, -24%) and the prenatally stressed females ($p<0.05$, -33%) (fig.34.G), indicating that handling did not normalise prenatal stress effects in this region. As compared to untreated controls, the prenatally stressed + handled females had lower spine density at first ($p<0.05$), second ($p<0.05$) and third ($p<0.05$) branch of the dendrite (fig.34.H). As compared to the prenatally stressed females, prenatally stressed + handled females had lower spine density at the third ($p<0.05$) branch of the granular neurons (fig.34.H).

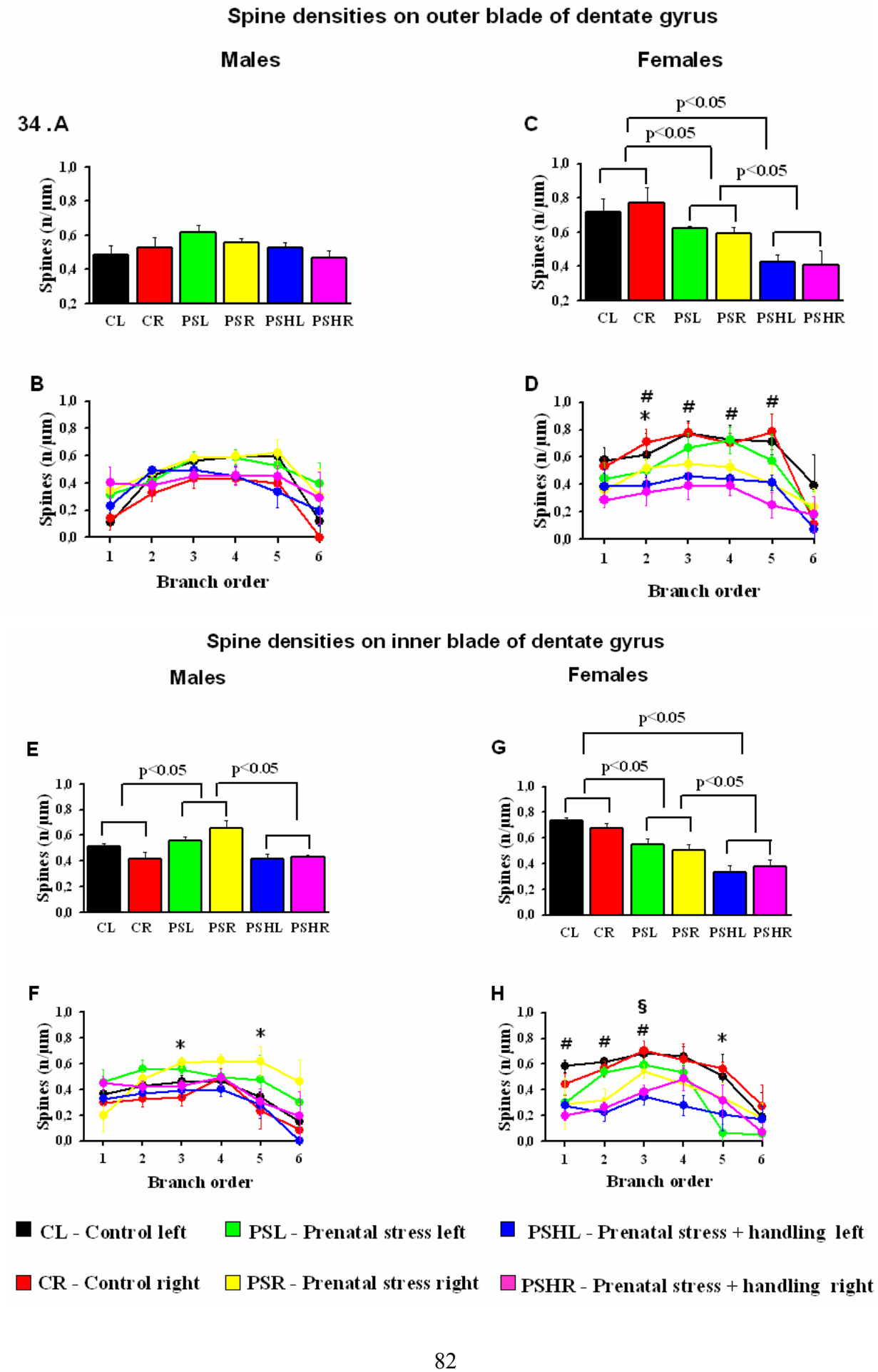

Spine densities on outer blade of dentate gyrus
Males
Females
34 .A
C
p<0.05
p<0.05
p<0.05
Spines (n/µm)
1,0
0,8
0,6
0,4
0,2
CL CR PSL PSR PSHL PSHR
B
D
#
*
Branch order
Spine densities on inner blade of dentate gyrus
Males
Females
E
G
F
H
§
CL - Control left
PSL - Prenatal stress left
PSHL - Prenatal stress + handling left
CR - Control right
PSR - Prenatal stress right
PSHR - Prenatal stress + handling right

Figure 34. Histograms represent spine density of granular neurons located in dentate gyrus. Values are given as mean ± S.E.M. A) Total spine density in the males; and C) Females outer blade of dentate gyrus; B) Spine density across different branch orders of the males; and D) Females outer blade of dentate gyrus; E) Total spine density in the males and G) Females inner blade of dentate gyrus; F) Spine density across different branch orders of the males and H) Females inner blade of dentate gyrus. * indicates significant differences in prenatally stressed animals relative to untreated controls; # indicates significant differences in prenatally stressed + handled animals relative to untreated controls; § indicates significant differences in prenatally stressed + handled animals relative to prenatally stressed animals.

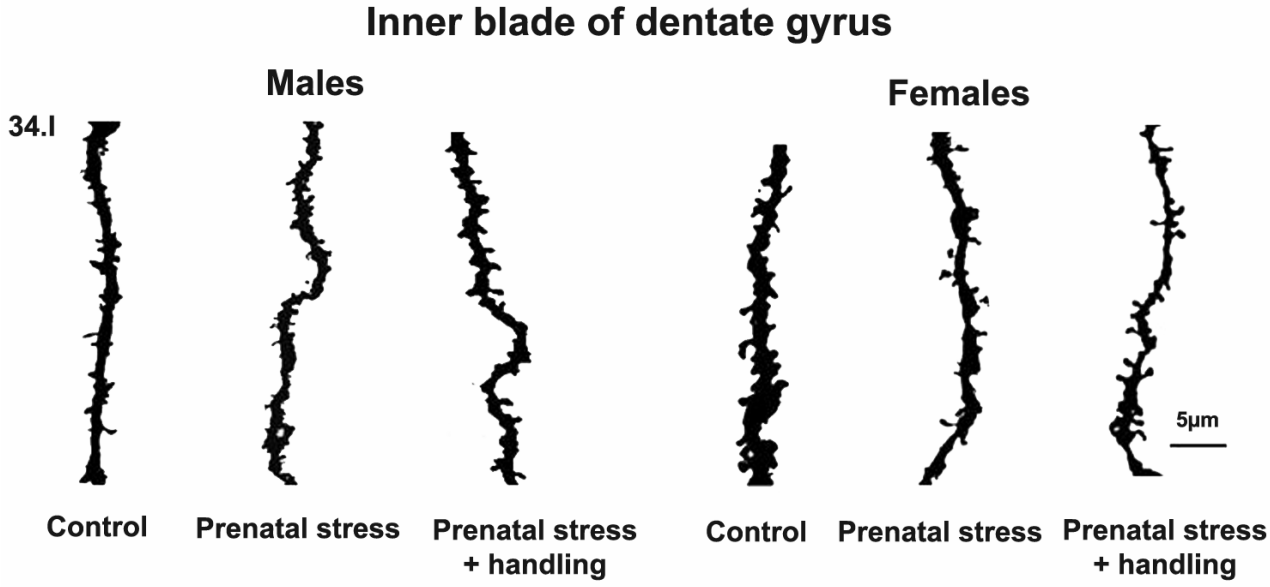


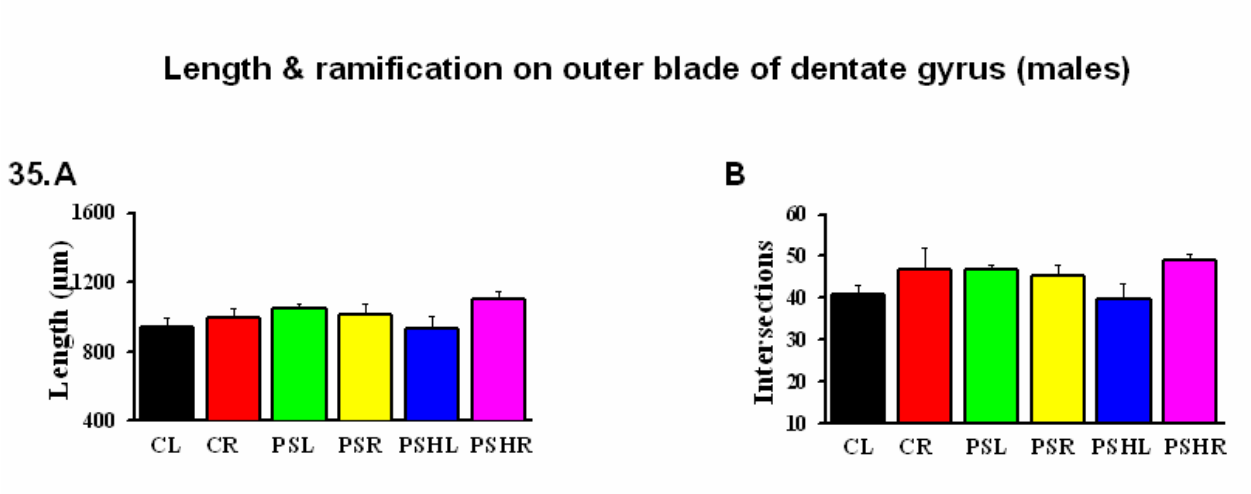
Figure 34.I Micrographs represent segments from the granular neurons located in the inner blade of dentate gyrus. The males and females react in the opposite directions in response to prenatal stress. Stress elevates spine density in prenatally stressed males but decreased spine density in prenatally stressed females as compared to the untreated controls. Unlike prenatal stress, the spine density is decreased both in the males as well as in the females due to prenatal stress + handling.

Dendritic length and complexity of dendritic arborization

Outer blade of dentate gyrus. a) Males: There were no effects of treatment, hemisphere or interaction (fig.35.A & B).

b) Females: Three-Way ANOVA revealed significant effects of prenatal stress as well as

prenatal stress + handling on dendritic development. Dendrites of the granular neurons of prenatally stressed females were shorter ($p<0.05$, -18%) with lower number of intersections ($p<0.05$, -17%) compared to the untreated control females (fig.35.C & E). Dendritic length was reduced at the distance of 200µm ($p<0.05$) from the soma (fig.35.D). The number of intersections were lowest at the distance of 180µm ($p<0.05$) and 200µm ($p<0.05$) from the soma (fig.35.F). Prenatal stress + handling also caused shorter dendritic length ($p<0.05$, -28%) and lower intersections ($p<0.05$, -27%) than the untreated controls (fig.35.C & E), indicating that handling did not prevent/ reverse prenatal stress-induced changes in the dendritic morphology but in contrast, it amplifies the prenatal stress-evoked dendritic alterations. Dendritic length was reduced at the distance of 120µm ($p<0.05$), 140µm ($p<0.05$), 160µm ($p<0.05$) and 180µm ($p<0.05$) from the soma (fig.35.D). Number of dendritic intersections were lowest at the distance of 80µm ($p<0.05$), 100µm ($p<0.05$), 120µm ($p<0.05$) 140µm ($p<0.05$) and 160µm ($p<0.05$) from the soma (fig.35.F).



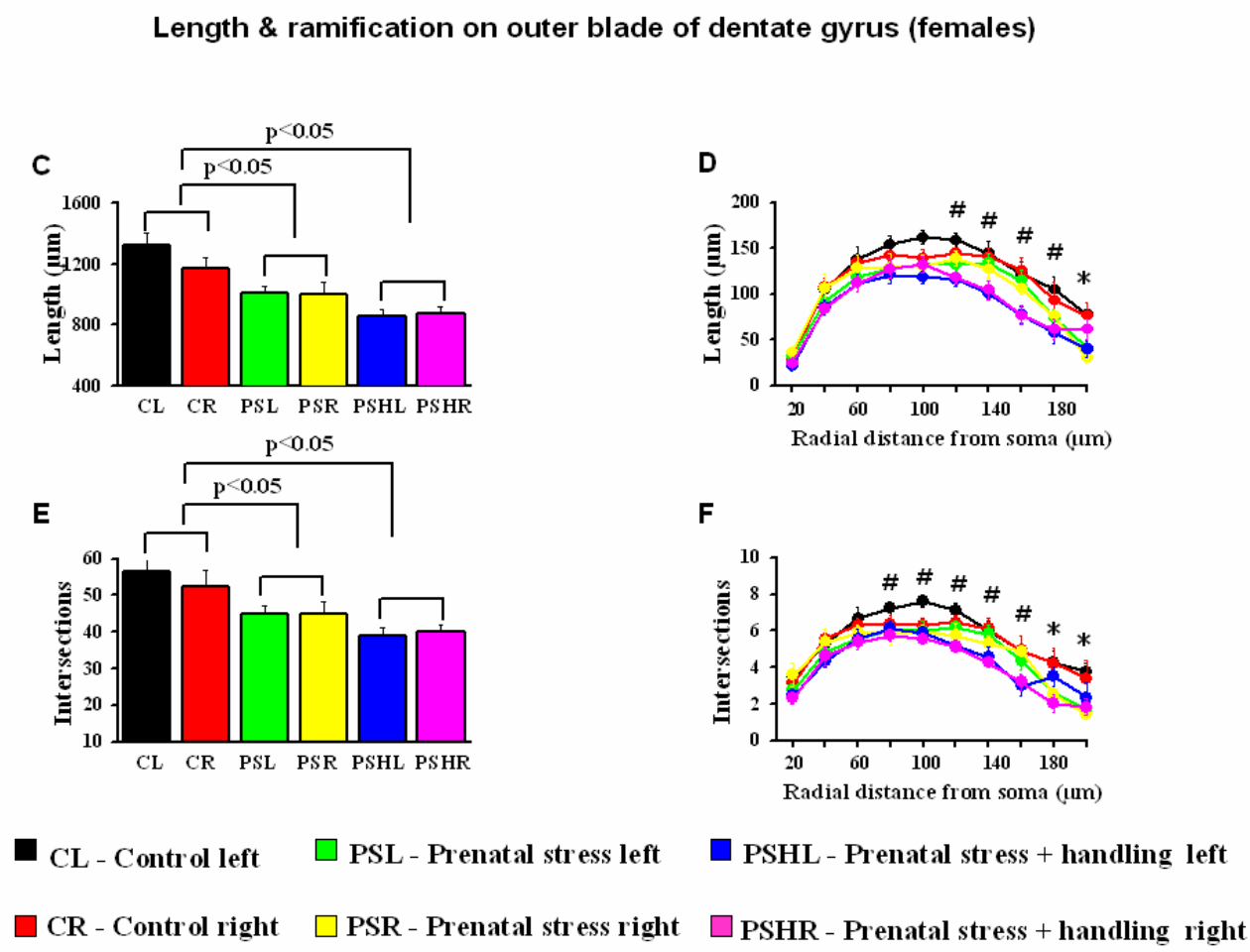
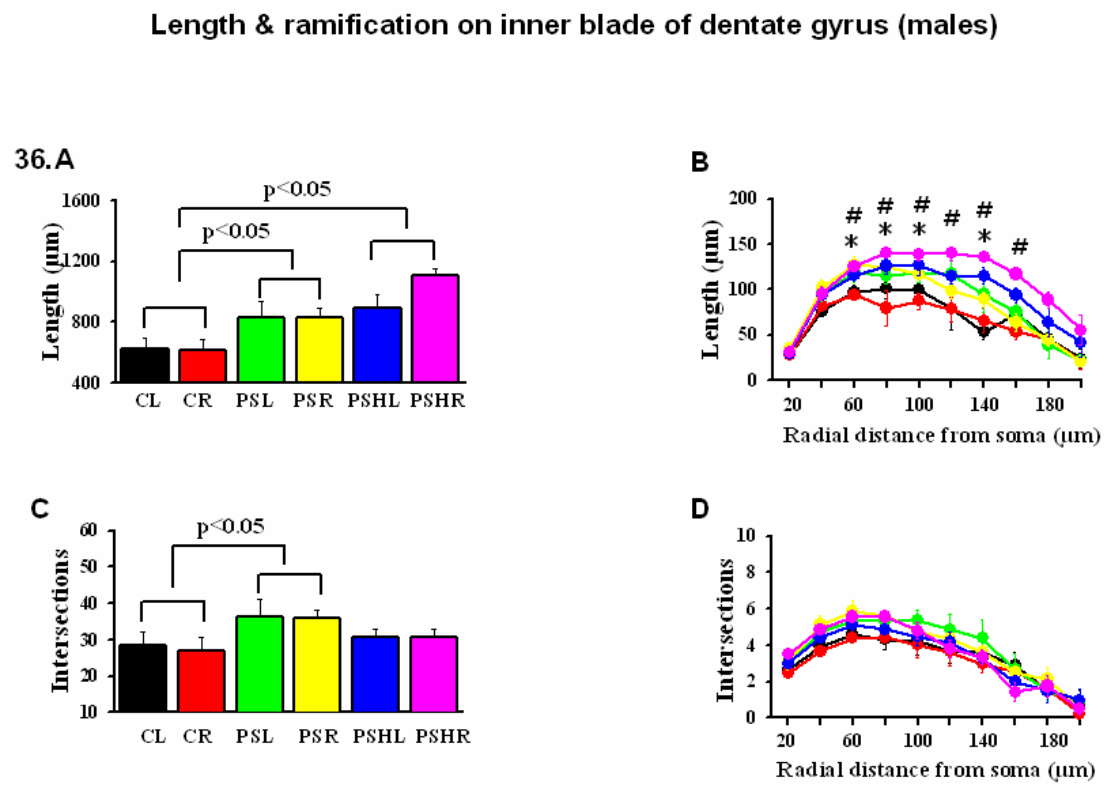


Figure 35. Histograms represent dendritic length and complexity of granular neurons located in the outer blade of dentate gyrus. Values are given as mean ± S.E.M. A) Total dendritic length of males and C) Females; B) Total number of dendritic intersections of males and E) Females; D) Dendritic length at different concentric circles from the soma of females; F) Number of dendritic intersections at different concentric circles from the soma of females. * indicates significant differences in prenatally stressed animals relative to untreated control animals; # indicates significant differences in prenatally stressed + handled animals relative to untreated controls.

Inner blade of dentate gyrus. a) Males: Three-Way ANOVA revealed significant effects of prenatal stress and prenatal stress + handling on dendritic development. Prenatally stressed males displayed longer dendritic length ($p<0.05$, upto 25%) and higher number of intersections ($p<0.05$, upto 23%) than the untreated control males (fig.36.A & C). Dendritic length was increased at the distance of 60µm ($p<0.05$), 80µm ($p<0.05$), 100µm ($p<0.05$) and 140µm ($p<0.05$) from the soma (fig.36.B). Similarly,

prenatal stress + handling also resulted in elongated dendrites as compared to the untreated control males ($p<0.05$, upto 37%) (fig.36.A), indicating that handling did not prevent/ reverse prenatal stress-induced changes in this region. Here, the dendritic length was increased at the distance of 60µm ($p<0.05$), 80µm ($p<0.05$), 100µm ($p<0.05$), 120µm ($p<0.05$), 140µm ($p<0.05$) and 160µm ($p<0.05$) from the soma (fig.36.B).

b) Females: Three-way ANOVA revealed significant effects of prenatal stress as well as prenatal stress + handling on dendritic development. However, in contrast to the males, in the females, prenatal stress reduced total dendritic length ($p<0.05$, -17%) and the number of intersections ($p<0.05$, -21%) compared to the untreated control females (fig.36.E & G). The number of dendritic intersections were lowest at the distance of 100µm ($p<0.05$) and 140µm ($p<0.05$) from the soma (fig.36.H). Similarly, prenatal stress + handling also resulted in shorter dendrites compared to the untreated control females ($p<0.05$, -28%), indicating that handling amplifies the stress-induced dendritic changes rather than reversing them (fig.36.E). The number of dendritic intersections were also reduced ($p<0.05$, -21%) in these animals which was found at the distance of 100µm ($p<0.05$), 120µm ($p<0.05$), 140µm ($p<0.05$), 160µm ($p<0.05$) and 180µm ($p<0.05$) from the soma (fig.36.G & H).



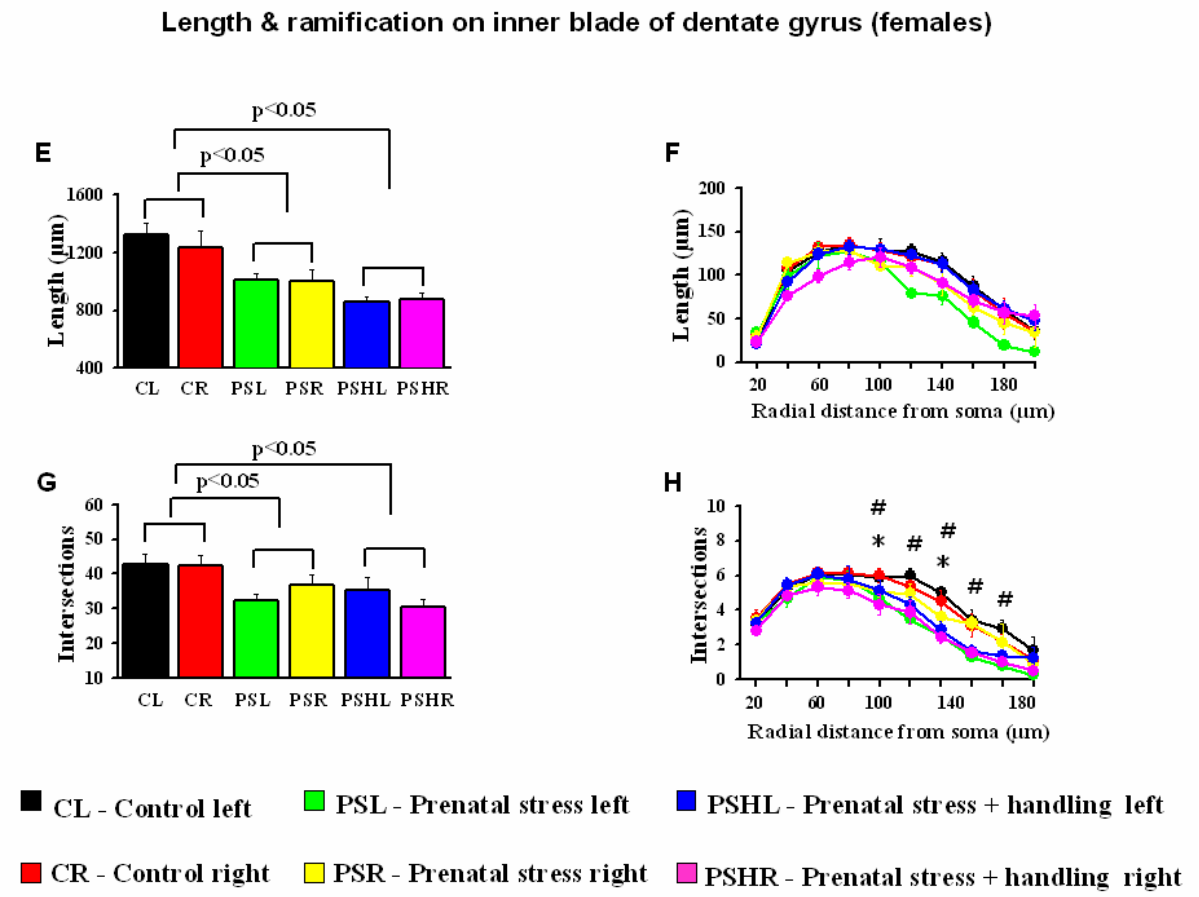


Figure 36. Histograms represent dendritic length and complexity of granular neurons located in the inner blade of dentate gyrus. Values are given as mean ± S.E.M. A) Total dendritic length of males and E) Females; B) Dendritic length at different concentric circles from the soma of males and F) Females; C) Total number of dendritic intersections of males and G) Females; D) Number of intersections at different concentric circles from the soma of males and H) Females. * indicates significant differences in prenatally stressed animals relative to untreated controls; # indicates significant differences in prenatally stressed + handled animals relative to untreated controls.

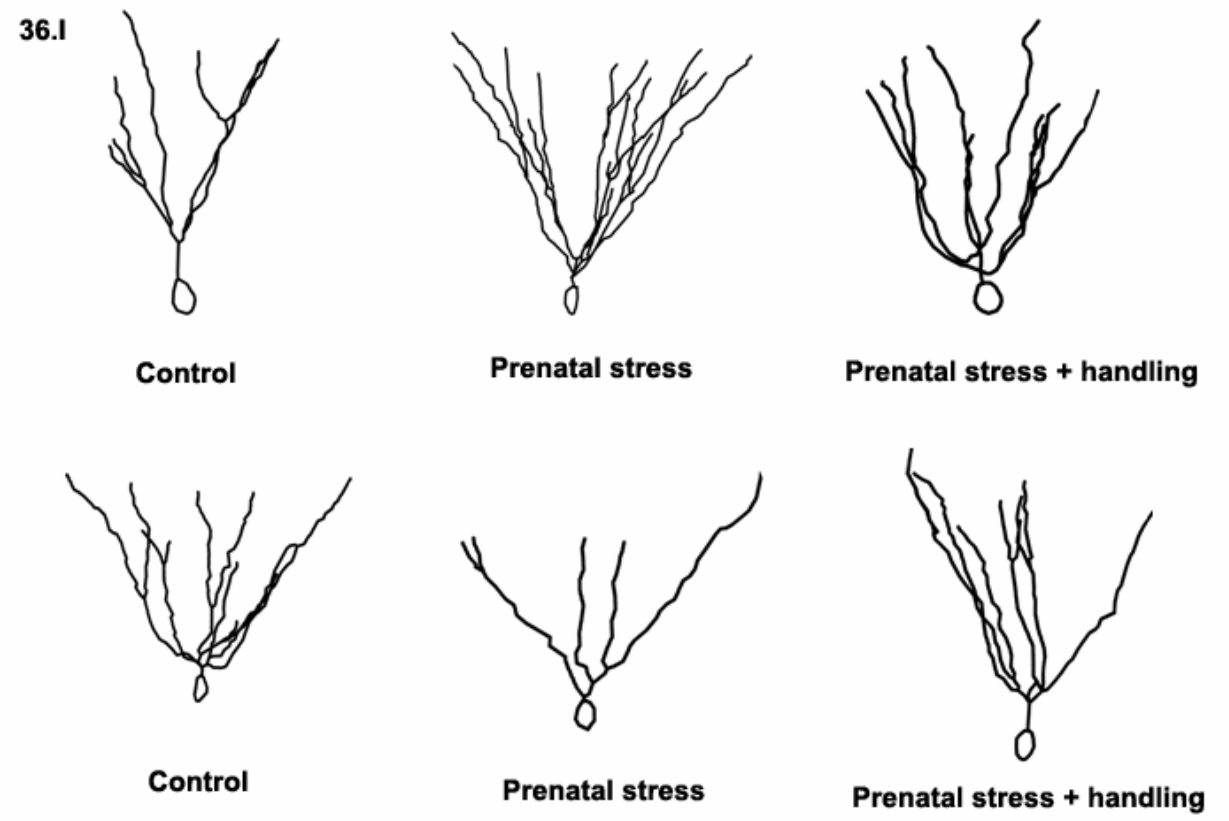


Figure 36.I Representative examples of granular neurons demonstrating the opposite effects of prenatal stress. In males, prenatal stress increased the total dendritic length and complexity of neurons whereas in females prenatal stress decreased the total dendritic length and complexity of neurons in the inner blade of dentate gyrus. Prenatally stressed + handled males also displayed increased dendritic length and complexity whereas the females showed shorter dendrites and less complex arbors relative to untreated controls. The top three neurons are from the males and the bottom three neurons are from the females.

3.2.6 Basolateral amygdala: Prenatal stress decreased dendritic length; handling prevents/ reverses the effects of prenatal stress in males

Spine density

Apical dendrite. a) Males: Three-Way ANOVA indicated that the main effect on spine density was induced by prenatal stress + handling. Prenatal stress + handling reduced spine density as compared to the untreated control males ($p = <0.001$, - 48%) and the prenatally stressed males ($p = <0.001$, - 46%) (fig.37.A). As compared to the untreated control males, prenatally stressed + handled males had lower spine density at third ($p =$

<0.001), fourth (p = <0.001) and sixth (p = <0.001) branch of the dendrite. As compared to prenatally stressed males, prenatally stressed + handled males had lower spine density at the third (p = <0.001) and fourth (p = <0.001) branch of the dendrite (fig.37.B).

b) Females: Similar to males, three-Way ANOVA revealed the main effect of prenatal stress + handling. Prenatal stress + handling resulted in reduced spine density compared to the untreated control females (p = <0.001, - 42%) and prenatally stressed females (p = <0.001, - 43%) (fig.37.C). As compared to the untreated control females, prenatally stressed + handled females had lower spine density on the third (p = <0.001), fourth (p = <0.001) and fifth (p = <0.001) branch of the dendrite. As compared to the prenatally stressed females, prenatally stressed + handled females had lower spine densities on the second (p = <0.001), third (p = <0.001), fourth (p = <0.001) and fifth (p = <0.001) branch of the dendrite (fig.37.D).

Basal dendrite. a) Males: Three-Way ANOVA revealed that the main effect on spine density was induced by prenatal stress + handling. Prenatal stress + handling reduced the spine density in these animals as compared to the untreated controls (p = <0.001, - 49%) and the prenatally stressed males (p = <0.001, - 41%) (fig.37.E). As compared to the untreated control males, prenatally stressed + handled males had fewer spine density at the second (p = <0.001), third (p = <0.001) and fourth (p = <0.001) branch of the dendrite (fig.37.F). As compared to the prenatally stressed males, prenatally stressed + handled males had fewer spine density at second (p = <0.001) and third (p = <0.001) branch of the dendrite (fig.37.F).

b) Females: Three-Way ANOVA revealed that the main effect on spine density was induced by prenatal stress + handling. Lower spine density was observed in the prenatally stressed + handled females compared to the untreated control females (p = <0.001, - 43%) and the prenatally stressed females (p = <0.001, - 47%) (fig.37.G). As compared to the untreated control females, prenatally stressed + handled females had lower spine density at the first (p = <0.001), second (p = <0.001), fourth (p = <0.001) and fifth (p = <0.001) branch of the dendrite (fig.37.H). As compared to the prenatally stressed females, prenatally stressed + handled females had lower spine density at the first (p = <0.001), second (p = <0.001) and third (p = <0.001) branch of the dendrite (fig.37.H).

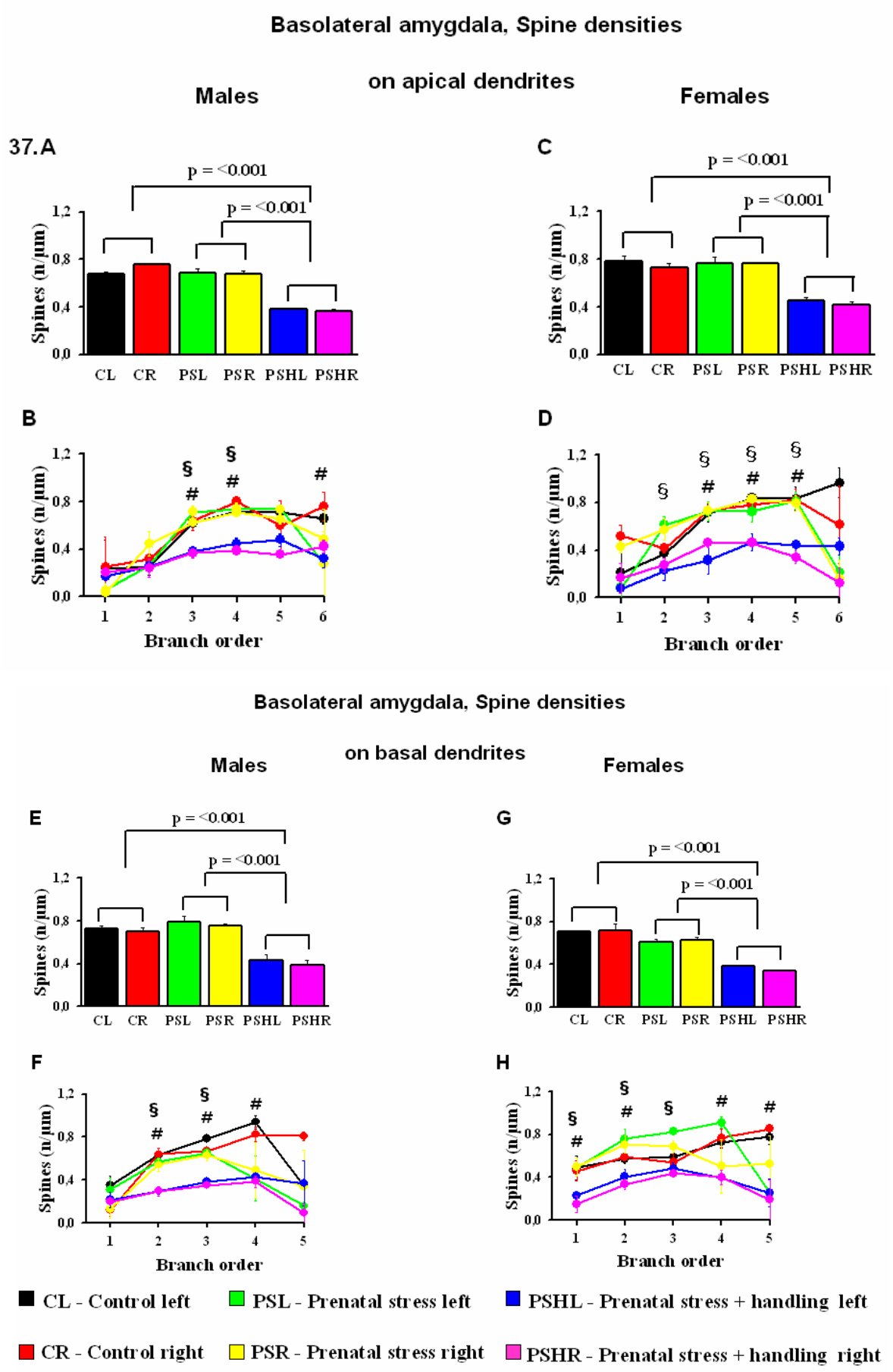


Figure 37. Histograms represent spine density of pyramidal neurons located in basolateral

nucleus of the amygdala. Values are given as mean ± S.E.M. A) Total spine density on the males and C) Females apical dendrites; B) Spine density across different branch orders of the males and D) Females apical dendrites; E) Total spine density on the males and G) Females basal dendrites; F) Spine density across different branch orders of the males and H) Females basal dendrites. # indicates significant differences in prenatally stressed + handled animals relative to untreated controls; § indicates significant differences in prenatally stressed + handled animals relative to prenatally stressed animals.

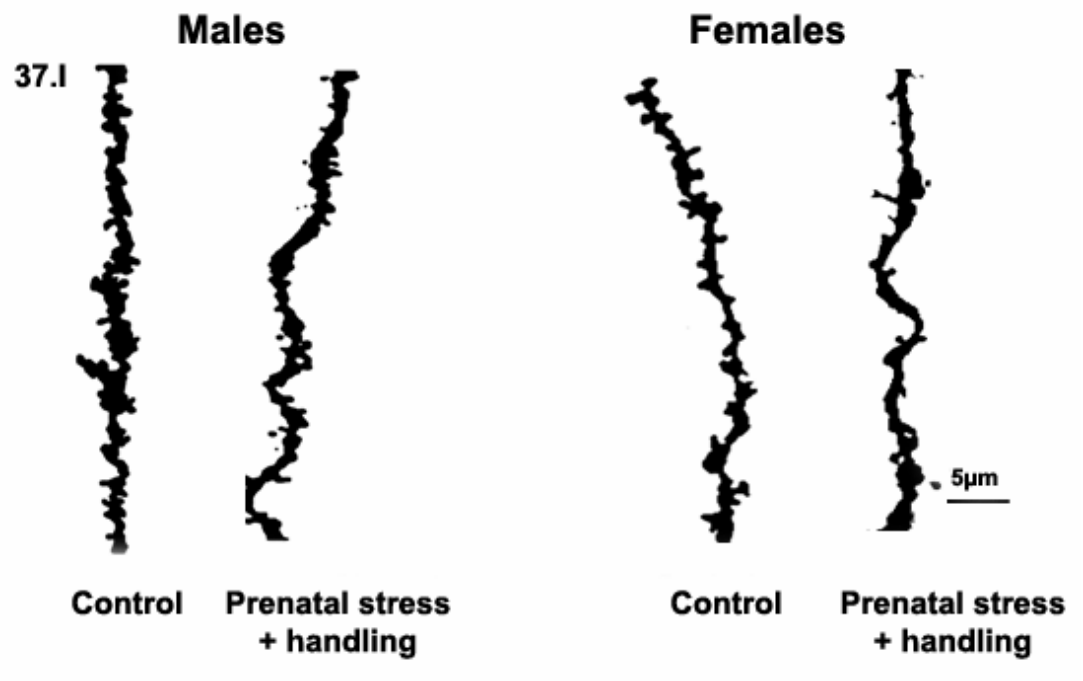


Figure 37.I Micrographs represent segments from untreated control and prenatally stressed + handled animals. Prenatal stress + handling significantly reduced spine density in the males as well as the females compared to untreated controls in the basolateral amygdala.

Dendritic length and complexity of dendritic arborization

Apical dendrite. a) Males: Three-Way ANOVA indicated significant effects of prenatal stress as well as prenatal stress + handling on dendritic development. Prenatal stress causes atrophy of amygdaloid apical dendrite ($p<0.05$, - 27%) in males (fig.38.A). Apical dendrite underwent most significant reduction at the distance of 80µm ($p<0.05$), 100µm ($p<0.05$), 120µm ($p<0.05$) and 160µm ($p<0.05$) from the soma (fig.38.B). The number of dendritic intersections were also reduced in the prenatally stressed males as compared to the untreated controls ($p<0.05$, -29%) (fig.38.C). Prenatal stress + handling prevents/

reverses the effects of prenatal stress in this region since prenatally stressed + handled males showed increased dendritic length ($p<0.05$, upto 23%) and number of intersections ($p<0.05$, upto 27%) compared to the prenatally stressed males (fig.38.A & C). Dendritic length was increased at the distance of 120µm ($p<0.05$), 140µm ($p<0.05$) and 160µm ($p<0.05$) from the soma (fig.38.B). Number of intersections were increased at the distance of 140µm ($p<0.05$) and 160µm ($p<0.05$) from the soma (fig.38.D).

b) Females: There were no effects of treatment or hemisphere or interaction (fig.38.E & F).

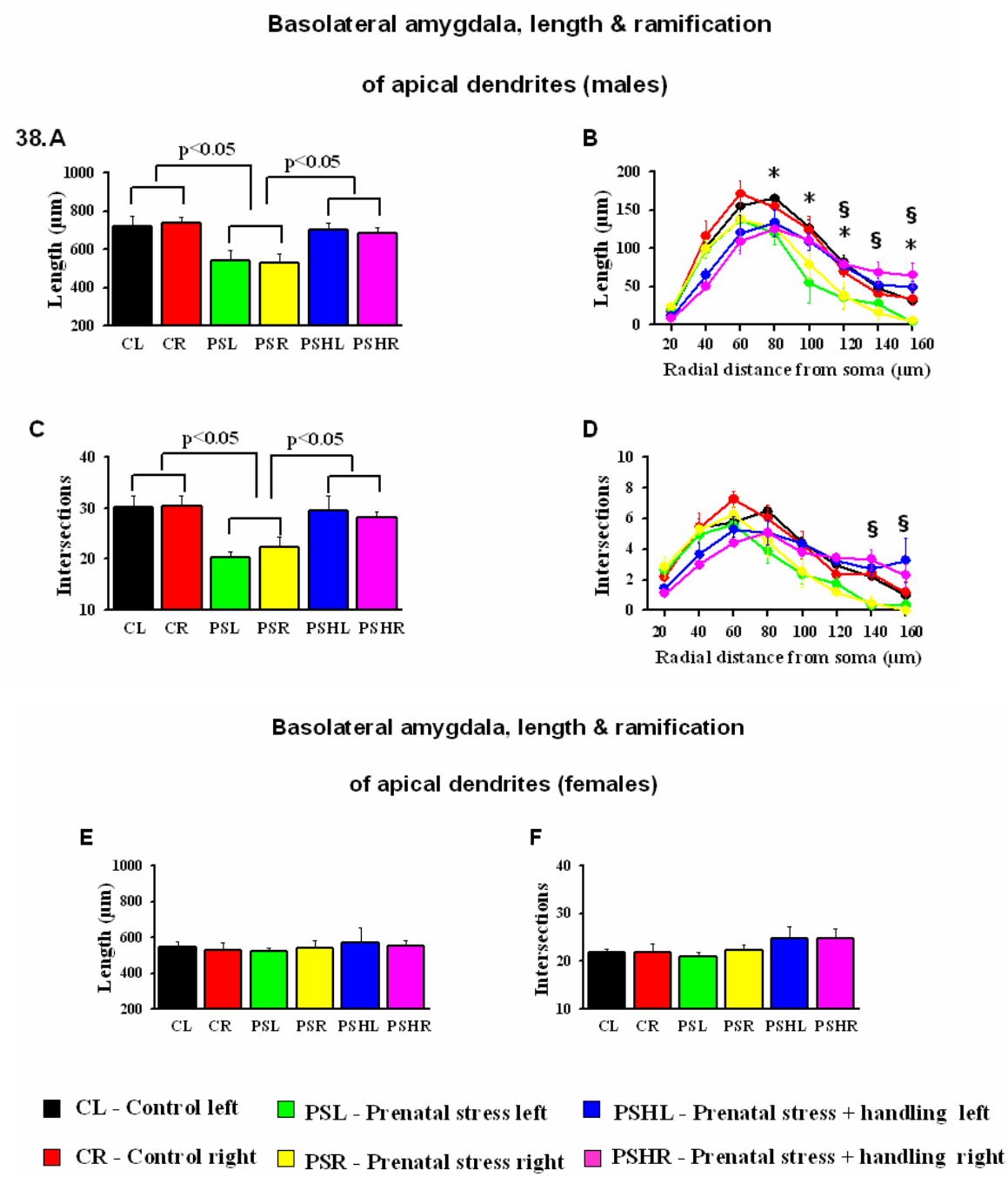


Figure 38. Histograms represent dendritic length and complexity of pyramidal neurons located in basolateral nucleus of the amygdala. Values are given as mean ± S.E.M. A) Total apical dendritic length of males and E) Females; B) Dendritic length at different concentric circles from the soma of males; C) Total number of apical dendritic intersections of males and F) Females; D) Number of intersections at different concentric circles from the soma of males. * indicates significant differences in prenatally stressed animals relative to untreated controls; § indicates significant differences in prenatally stressed + handled animals relative to prenatally stressed animals.

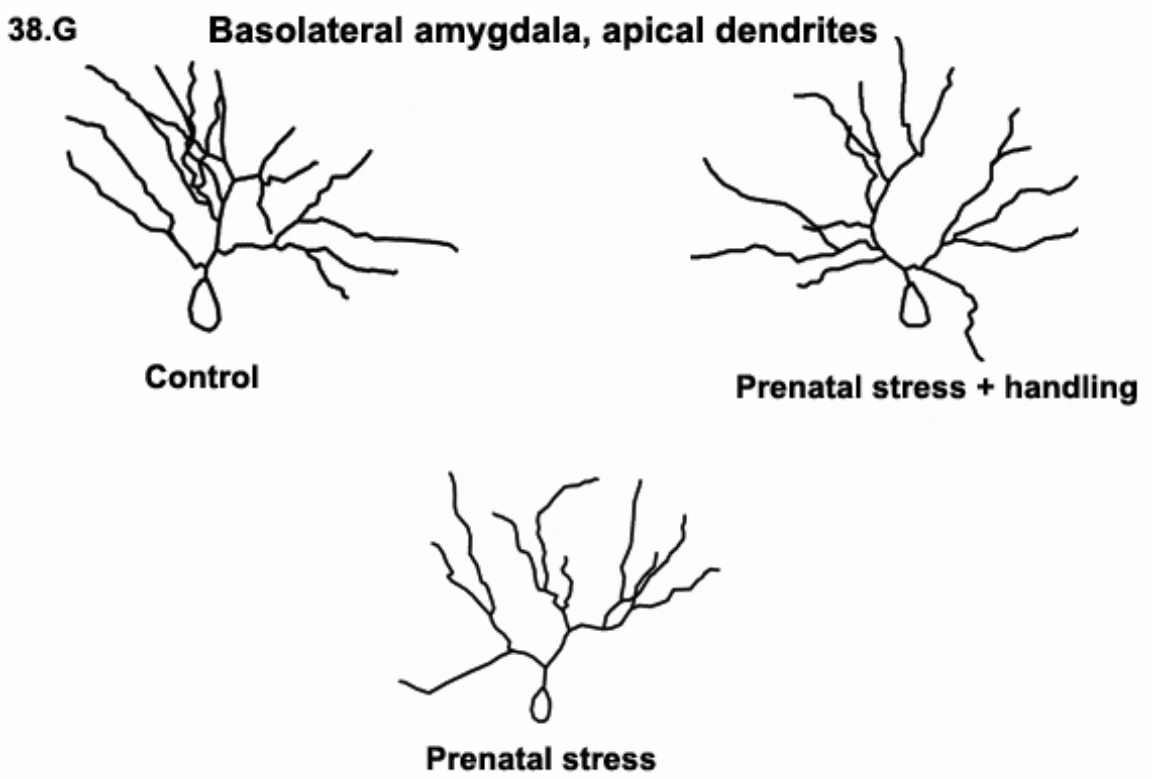


Figure 38.G Representative examples of apical dendrites from basolateral nucleus of the amygdala of males. Prenatal stress caused shorter dendrites compared to the untreated controls. Neonatal handling normalises the effects of prenatal stress since prenatal stress + handling increased the total dendritic length and complexity of neurons back to normal values.

Basal dendrite. a) Males: There were no effects of treatment or hemisphere or interaction on the basal dendritic length of males (fig.39.A)

b) Females: There were no effects of treatment or hemisphere or interaction on the basal dendritic length of females (fig.39.B).

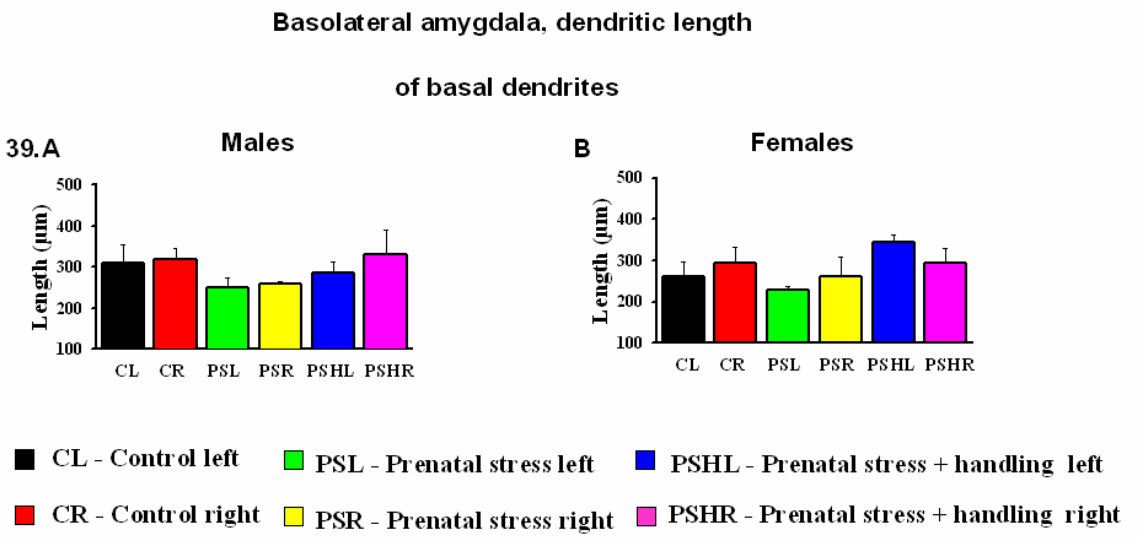


Figure 39. Histograms represent dendritic length of pyramidal neurons located in basolateral nucleus of the amygdala. Values are given as mean ± S.E.M. A) Basal dendritic length of males and E) Females.

40.A Effect of Prenatal Stress on Dendritic Spine Density

Investigated Areas	Males		Females	
	Dendritic Spines			
	Apical	Basal	Apical	Basal
Orbitofrontal Cortex	↓	↓	↓	↓
Anterior Cingulate Cortex	↓	-	↓	↓
Area CA3	-	-	-	-
Area CA1	↑	-	-	-
Outer blade (dentate gyrus)	-		↓	
Inner blade (dentate gyrus)	↑		↓	
Basolateral Amygdala	-	-	-	-

Figure 40.A Summary of the effects of prenatal stress on spine density in the limbic system of male and female rats. Red arrows indicate a decrease in spine density. Pink arrows indicate increased spine density in response to prenatal stress.

40.B Effect of Prenatal Stress on Dendritic Length

Investigated Areas	Males		Females	
	Dendritic Length			
	Apical	Basal	Apical	Basal
Orbitofrontal Cortex	↓	-	-	-
Anterior Cingulate Cortex	↓	-	-	-
Area CA3	↓	↓	↓	↓
Area CA1	↓	-	↓	↓
Outer blade (dentate gyrus)	-		↓	
Inner blade (dentate gyrus)	↑		↓	
Basolateral Amygdala	↓	-	-	-

Figure 40.B Summary of the effects of prenatal stress on apical and basal dendritic length in the limbic system of male and female rats. Red arrows indicate decreased dendritic length. Pink arrow indicates increased dendritic length in response to prenatal stress.

40.C Effect of Prenatal Stress and Handling on Dendritic Morphology

Areas	Males				Females			
	Dendritic Spines		Dendritic Length		Dendritic Spines		Dendritic Length	
	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal
OFC	-	↓	↑	-	↓	↓	-	-
ACd	↓	↓	↑	-	↓	↓	-	-
CA3	-	-	↑	↑	-	-	↓	↓
CA1	↑	-	↑	-	-	-	↓	↓
ODG	-		-		↓		↓	
IDG	↓		↑		↓		↓	
BLA	↓	↓	↑	-	↓	↓	-	-

Figure 40.C Summary of the effects of prenatal stress + handling in different areas of the limbic system. In this table you will find that handling prevented prenatal stress-induced neuronal alterations in a sex, dendrite and region-specific manner. Here, the blue arrows indicate significant reversal. Pink arrow indicates a tendency towards reversal. Red arrows indicate either a decrease or increase in dendritic parameters. Abbreviations; OFC - Orbitofrontal cortex; ACd - Anterior cingulate cortex; CA3; CA1 area of the hippocampus; ODG - Outer blade of dentate gyrus; IDG - Inner blade of dentate gyrus; BLA - Basolateral nucleus of amygdala.

3.3 Neonatal handling alters dendritic & synaptic development in the limbic brain areas of rodents

3.3.1 Orbitofrontal Cortex: Spine density is reduced due to handling

Spine density

Apical dendrite. a) Males: Two-Way ANOVA indicated significant effect of handling on spine density. Spine density was reduced due to handling ($p = <0.001$, - 22%). Branch order analysis showed that the third ($p = 0.013$), fourth ($p = <0.001$), fifth ($p = 0.016$) and sixth ($p = 0.023$) order branches had the lowest density of spines (fig.41.A & B).

b) Females: Two-Way ANOVA showed significant effect of handling. Handling reduced the density of spines in the females ($p = 0.008$, - 30%) which was mostly seen at the second ($p = 0.001$), third ($p =0.040$), fourth ($p = 0.031$) and fifth ($p = 0.006$) branch of the dendrite (fig.41.C & D).

Basal dendrite. a) Males: Two-Way ANOVA revealed significant effect of handling. Handled males displayed significantly lower spine density than the controlled males ($p = <0.001$, - 19%) which was mostly seen at the second ($p = 0.013$) and third ($p = 0.013$) order branches (fig.41.E & F).

b) Females: Two-Way ANOVA indicated significant effect of handling on spine density. The spine density on the basal dendrite was reduced in the handled females ($p = 0.010$, - 19%) which was seen at the third ($p = 0.019$) order branches (fig.41.G& H).

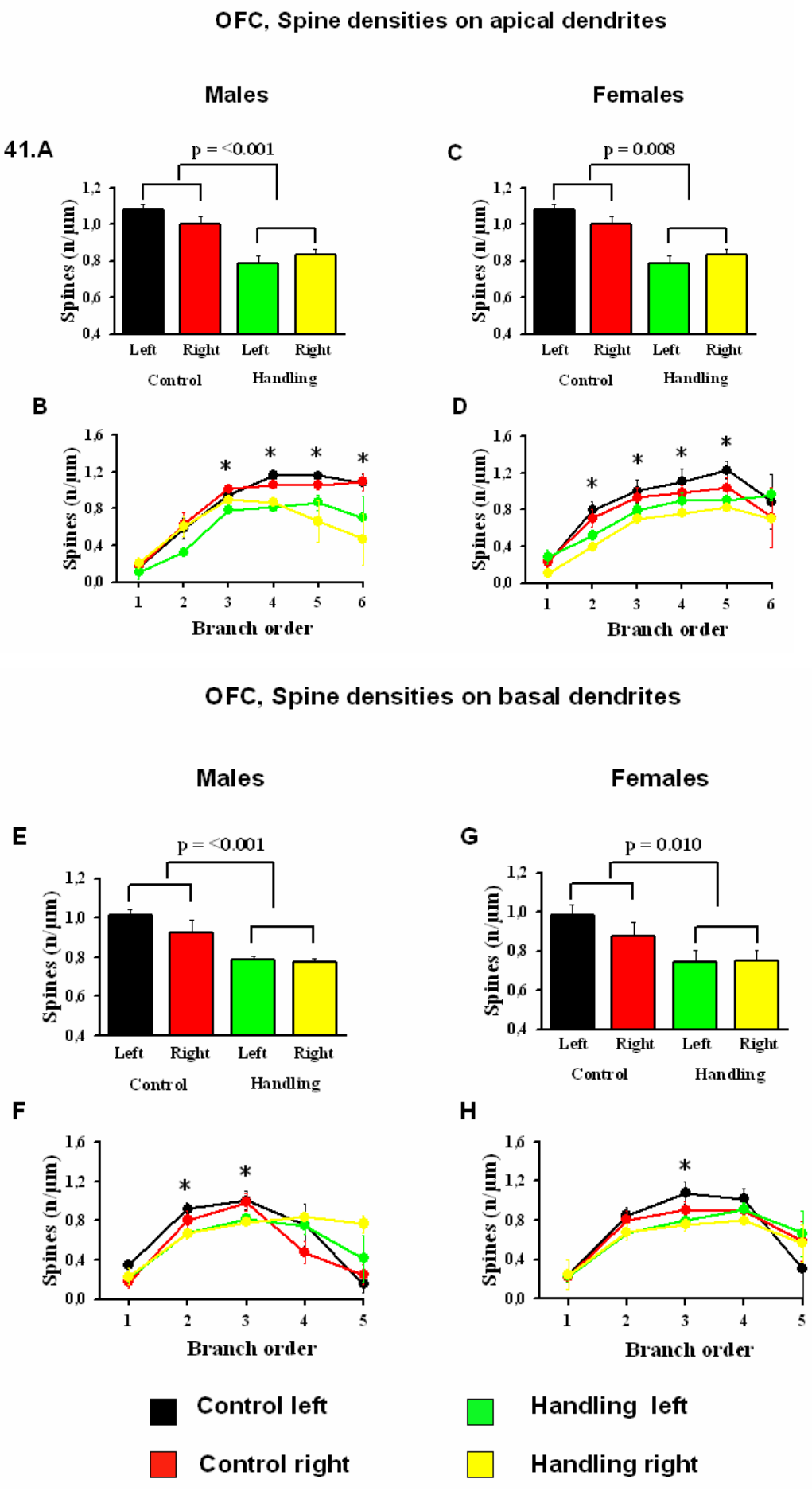

OFC, Spine densities on apical dendrites
Males
Females
41.A
p = <0.001
p = 0.008
C
Spines (n/µm)
Left
Right
Left
Right
Control
Handling
B
D
Branch order
OFC, Spine densities on basal dendrites
Males
Females
E
p = <0.001
p = 0.010
G
F
H
Control left
Handling left
Control right
Handling right

Figure 41. Histograms represent spine density of layer II/III OFC pyramidal neurons. Values are given as mean ± S.E.M. A) Total spine density in the apical dendrite of males and C) Females; B) Spine density across different branch orders of the males and D) Females apical dendrites; E) Total spine density in the basal dendrite of males and G) Females; F) Spine density across different branch orders of the males and H) Females basal dendrites; * indicates significant differences between untreated controlled and handled animals.

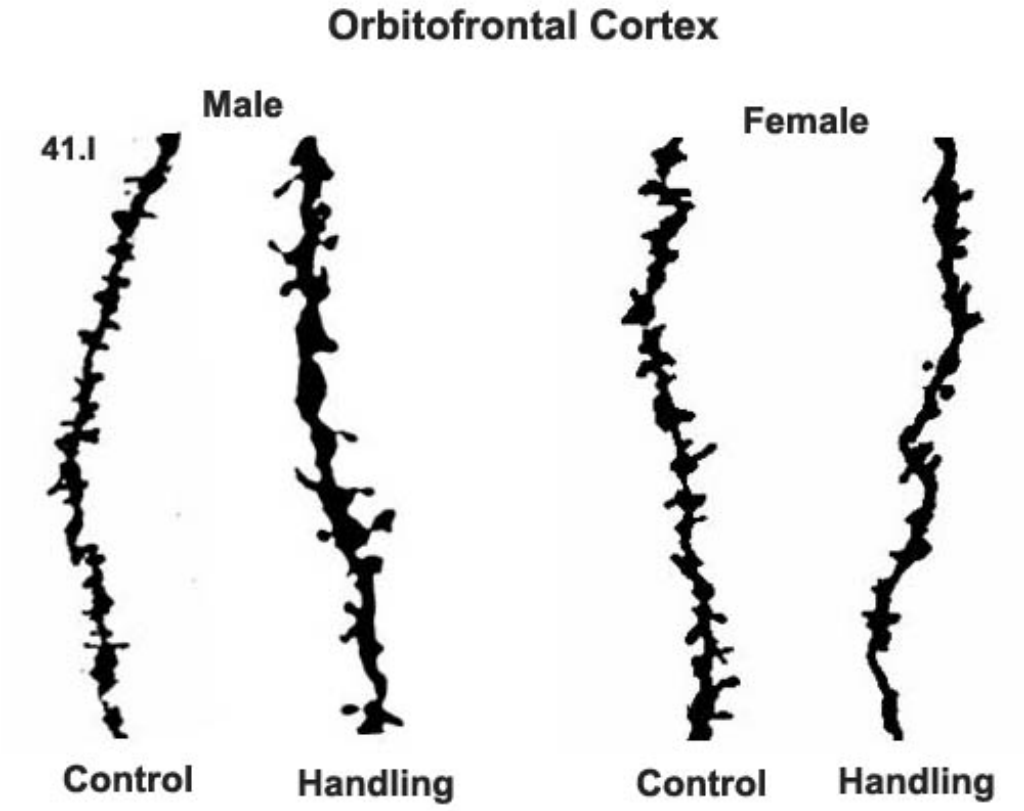


Figure 41.I Micrographs represents segments from control and handled animals. Spine density is reduced in the handled animals compared to the untreated controls.

Dendritic length and complexity of dendritic arborization

Apical dendrite. a) Males: There were no effects of handling or hemisphere or interaction (fig.42.A)

b) Females: There were no effects of handling or hemisphere or interaction (fig.42.B).

Basal dendrite. a) Males: There were no effects of handling or hemisphere or interaction

(fig.42.C)

b) Females: There were no effects of handling or hemisphere or interaction (fig.42.D).

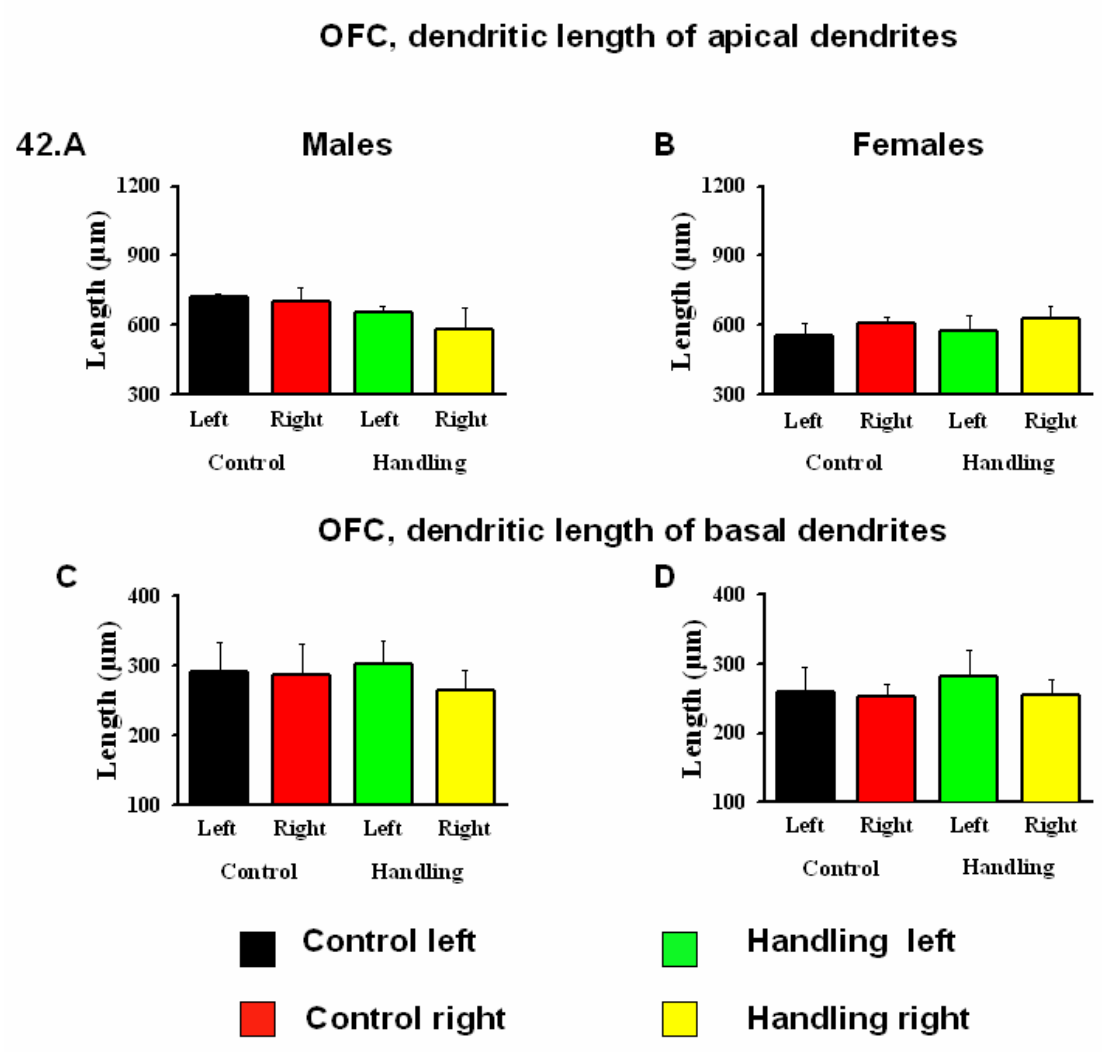


Figure 42. Histograms represent dendritic length of layer II/III OFC pyramidal neurons. Values are given as mean ± S.E.M. A) Total apical dendritic length of males and B) Females; C) Basal dendritic length of males and D) Females.

3.3.2 Anterior Cingulate Cortex: Spine density is reduced in the apical dendrites of both sexes, basal spine density is reduced only in females

Spine density

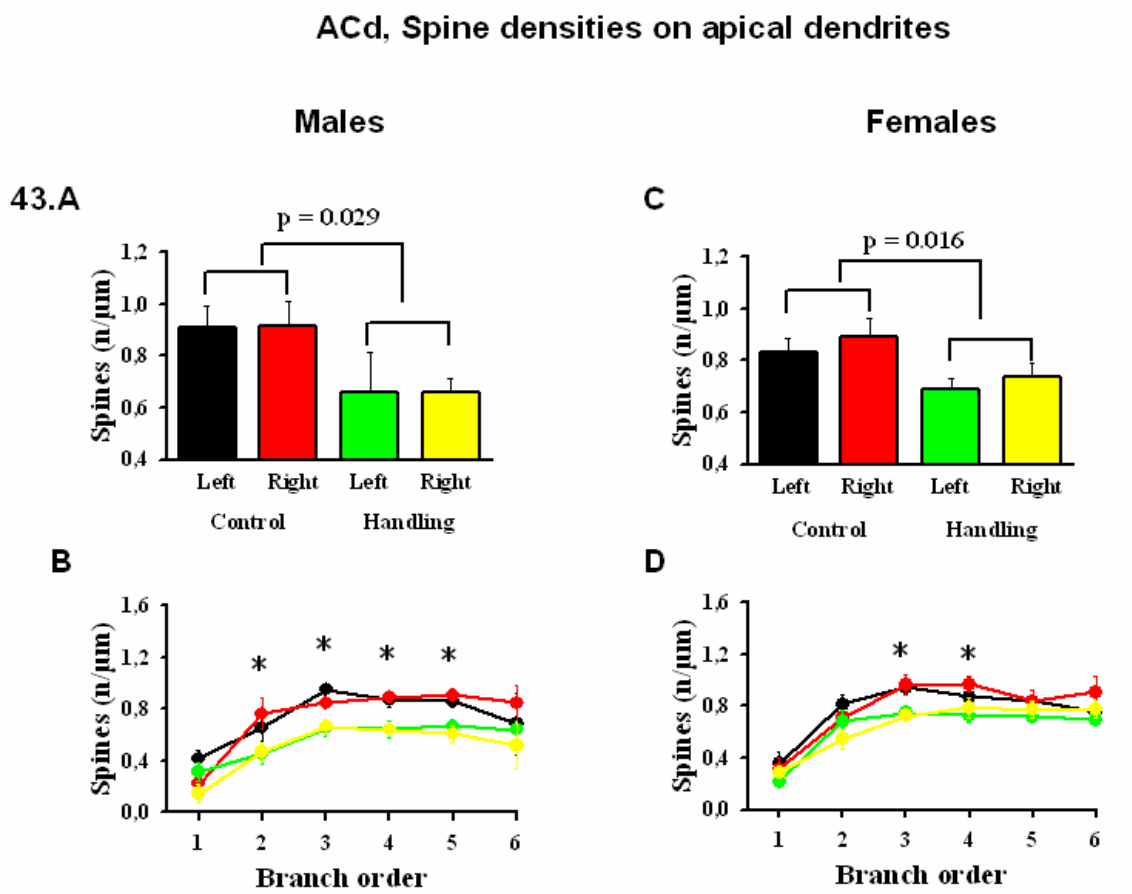
Apical dendrite. a) Males: There was a significant effect of handling on spine density. The apical dendritic spine density was reduced ($p = 0.029$, - 19%) in the handled males as

compared to the untreated control males (fig.43.A). Lower density of spines were observed on the second (p = 0.018), third (p = <0.001), fourth (p = <0.001) and fifth (p = <0.001) branch of the apical dendrites (fig.43.B).

b) Females: Two-Way ANOVA indicated significant effect of handling. Apical dendritic spine density was significantly reduced in the handled females as compared to the control females (p = 0.016, - 21%) which was mostly seen at the third (p = 0.003) and fourth (p = 0.031) branch of the dendrite (fig.43.C & D).

Basal dendrite. a) Males: There were no effects of handling or hemisphere or interaction (fig.43.E).

b) Females: Two-Way ANOVA indicated significant effect of handling. The spine density on the basal dendrite was significantly reduced in the handled females as compared to the controlled females (p = 0.013, - 23%). There were no effects of hemisphere or interaction (fig.43.G & H).



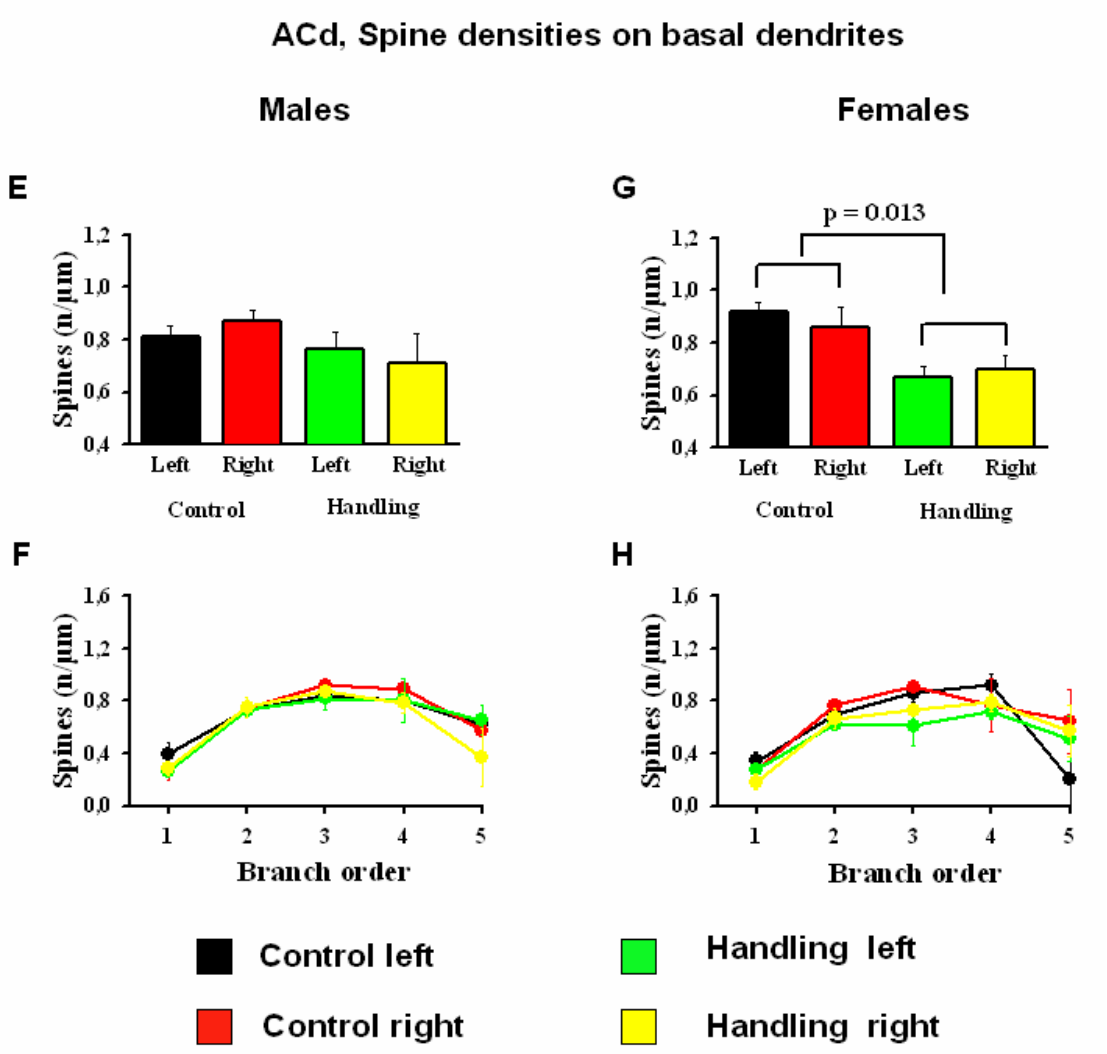


Figure 43. Histograms represent spine density of layer II/III ACd pyramidal neurons. Values are given as mean ± S.E.M. A) Total spine density in the apical dendrite of males and C) Females; B) Spine density across different branch orders of the males and D) Females apical dendrites; E) Total spine density in the basal dendrite of males and G) Females; F) Spine density across different branch orders of the males and H) Females basal dendrites; * indicates significant differences between controlled and handled animals.

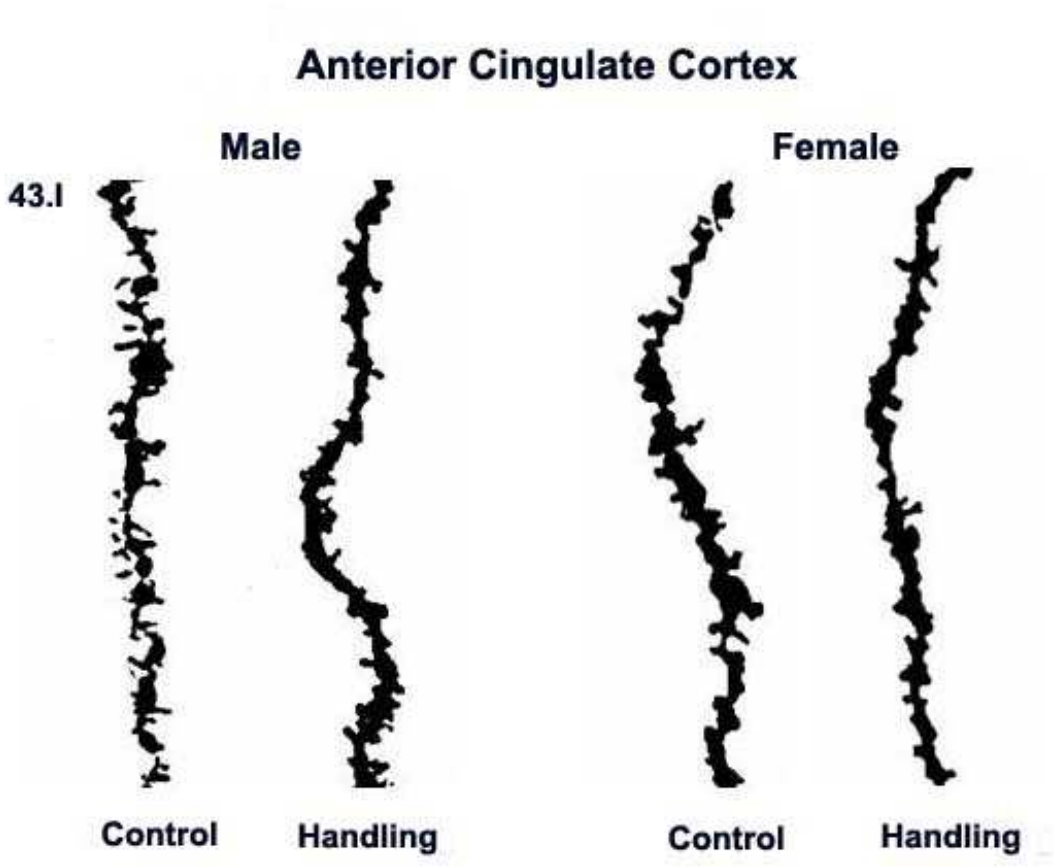


Figure 43.I Micrographs represents segments from control and handled animals. Spine density is reduced in the handled animals compared to the untreated controls.

Dendritic length and complexity of dendritic arborization

Apical dendrite. a) Males: There were no effects of handling or hemisphere or interaction (fig.44.A).

b) Females: There were no effects of handling or hemisphere or interaction (fig.44.B).

Basal dendrite. a) Males: There were no effects of handling or hemisphere or interaction on the basal dendritic length of males (fig.44.C).

b) Females: There were no effects of handling or hemisphere or interaction on the basal dendritic length of females (fig.44.D).

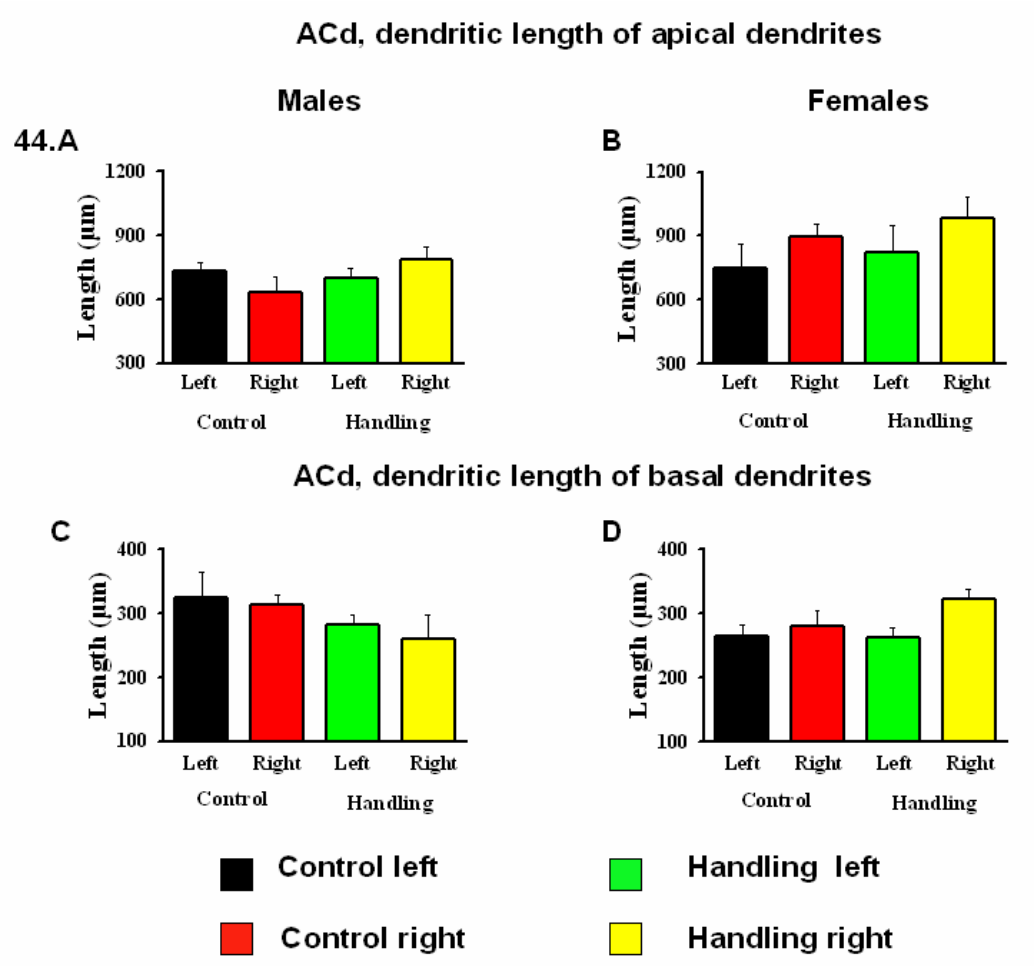


Figure 44. Histograms represent dendritic length of layer II/III ACd pyramidal neurons. Values are given as mean ± S.E.M. A) Total apical dendritic length of males and B) Females; C) Basal dendritic length of males and D) Females.

3.3.3 CA3 region: Handling alters dendritic length and complexity in a sex-specific manner

Spine density

Apical dendrite. a) Males: There were no effects of handling or hemisphere or interaction (fig.45.A).

b) Females: There were no effects of handling or hemisphere or interaction (fig.45.B).

Basal dendrite. a) Males: There were no effects of handling or hemisphere or

interaction (fig.45.C).

b) Females: There were no effects of handling or hemisphere or interaction (fig.45.D).

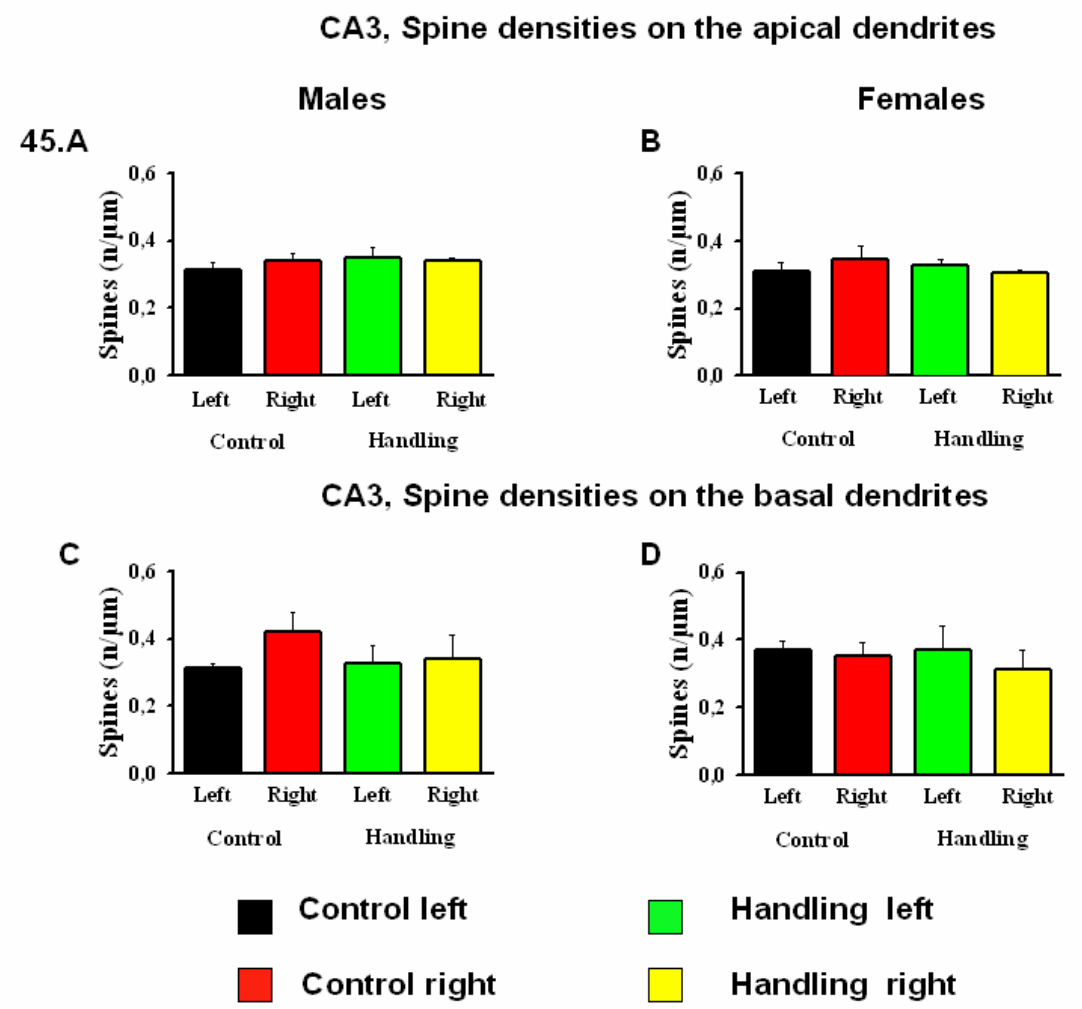


Figure 45. Histograms represent spine density of CA3 pyramidal neurons. Values are given as mean ± S.E.M. A) Total spine density in the males and B) Females apical dendrites; C) Total spine density in the males and D) Females basal dendrites.

Dendritic length and complexity of dendritic arborization

Apical dendrite. a) Males: Two-Way ANOVA indicated significant effect of handling on dendritic morphology. Handling reduced the total apical dendritic length ($p = 0.005$, - 25%) and intersections ($p = 0.010$, - 24%) in males (fig.46.A & C). Dendritic length was most significantly reduced on the distal segments, at the distance of 200µm ($p = 0.004$), 240µm ($p = 0.007$), 280µm ($p = 0.012$), 320µm ($p = 0.018$) and 360µm ($p = 0.042$) from

the soma (fig.46.B). The number of dendritic intersections were lowest at the distance of 160µm (p = 0.005), 200µm (p = 0.018), 240µm (p = 0.005) and 280µm (p = 0.040) from the soma (fig.46.D).

b) Females: There were no effects of handling or hemisphere or interaction (fig.46.E & F).

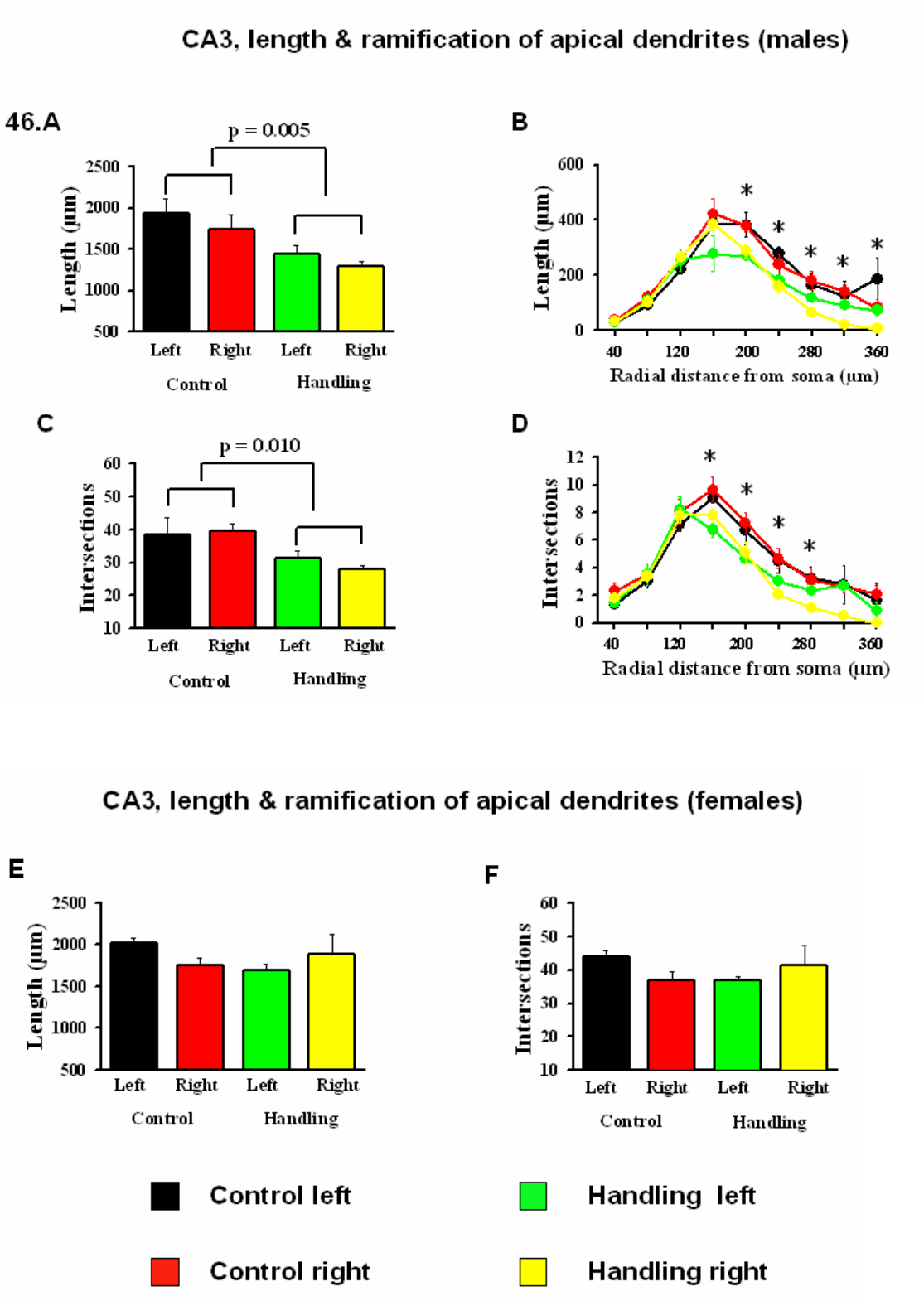
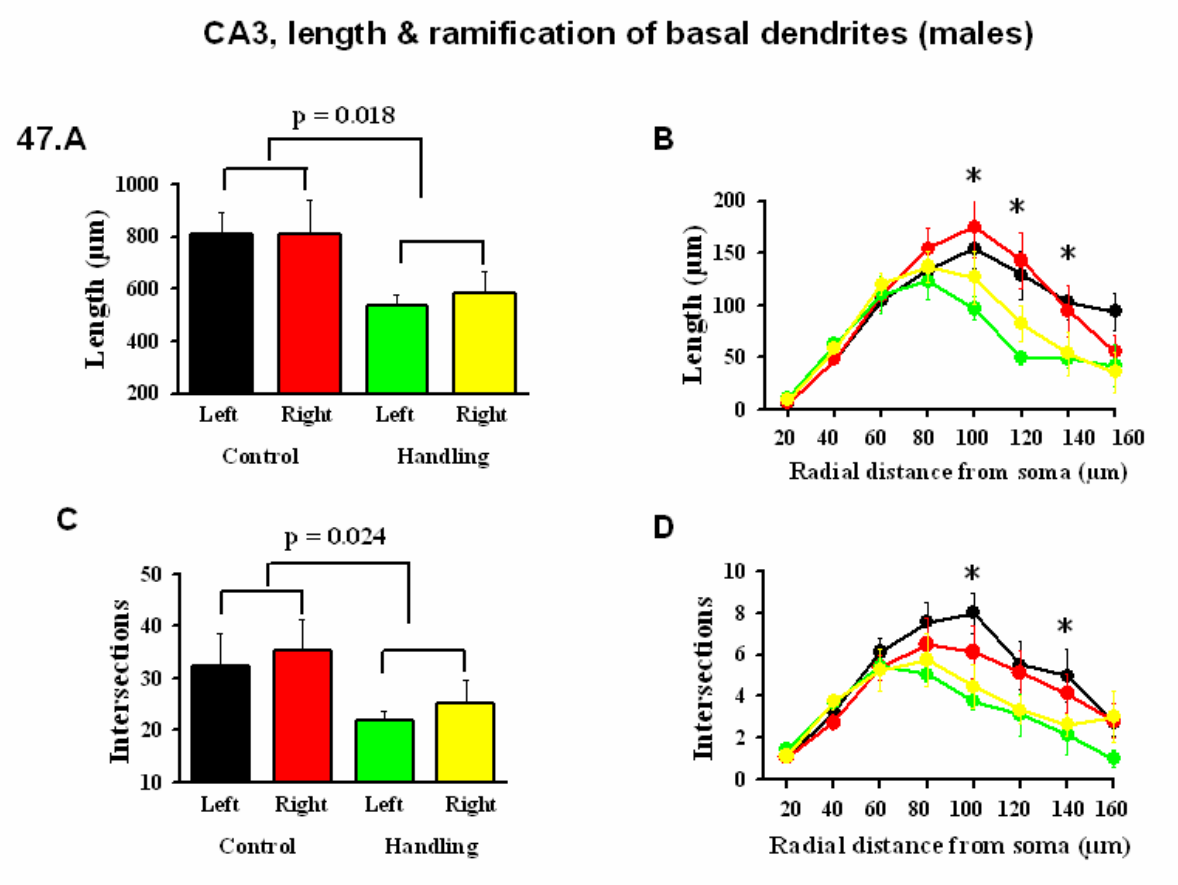


Figure 46. Histograms represent dendritic length and complexity of CA3 pyramidal neurons. Values are given as mean ± S.E.M. A) Total apical dendritic length of males and E) Females; B) Dendritic length at different concentric circles from the soma of males; C) Total number of apical dendritic intersections of males and F) Females; D) Number of dendritic intersections at different concentric circles from the soma of males. * indicates significant differences between controlled and handled animals.

Basal dendrite. a) Males: Two-Way ANOVA indicated significant effect of handling on basal dendritic morphology. Handling reduced CA3 basal dendritic length (p = 0.018, -15%) (fig.47.A), which was seen at the distance of 100µm (p = 0.035), 120µm (p = 0.004) and 140 µm (p = 0.025) from the soma (fig.47.B). The number of intersection was also reduced due to handling (p = 0.024, -11%) which was seen at 100 µm (p = 0.010) and 140µm radii (p = 0.042) from the soma (fig.47.C & D).

b) Females: There were no effects of handling or hemisphere or interaction (fig.47.E & F).



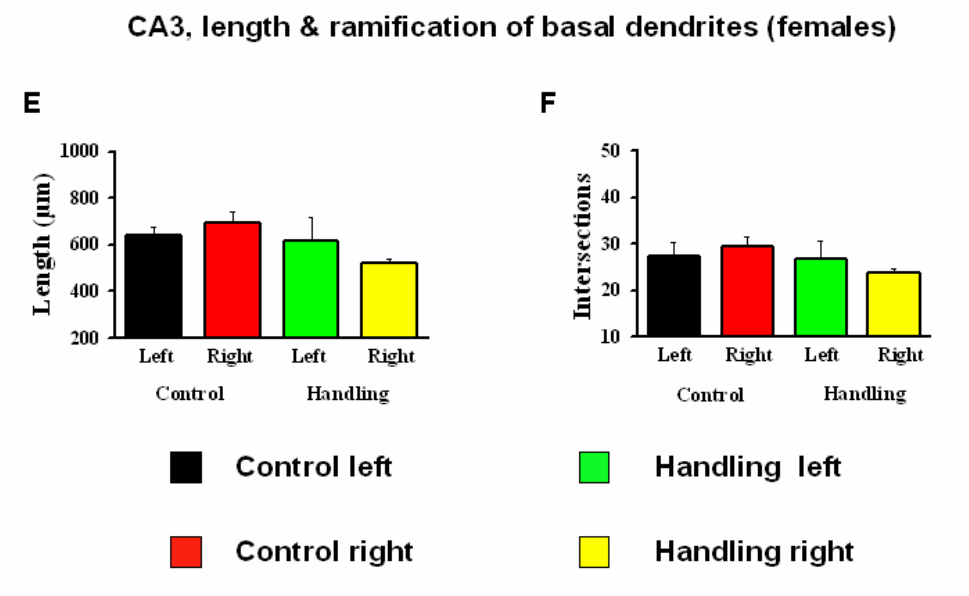


Figure 47. Histograms represent dendritic length and complexity of CA3 pyramidal neurons. Values are given as mean ± S.E.M. A) Total basal dendritic length of males and E) Females; B) Dendritic length at different concentric circles from the soma of males; C) Total number of basal dendritic intersections of males and F) Females; D) Number of dendritic intersections at different concentric circles from the soma of males. * indicates significant differences between controlled and handled animals.

3.3.4 CA1 region: Handling elevates spine density in males but not in the females

Spine density

Apical dendrite. a) Males: Two-Way ANOVA revealed significant effect of handling on spine density. Handling elevates the density of spines in the CA1 apical dendrites ($p = 0.002$, upto 25%) compared to the control males (fig.48.A). Spine density was elevated on the fourth ($p = 0.030$), fifth ($p = 0.004$), sixth ($p = 0.002$) and seventh ($p = 0.010$) branch of the dendrite (fig.48.B). There were no effects of hemisphere or interaction.

b) Females: There were no effects of handling or hemisphere or interaction (fig.48.C).

Basal dendrite. a) Males: There were no effects of handling or hemisphere or interaction (fig.48.E).

b) Females: There were no effects of handling or hemisphere or interaction (fig.48.F).

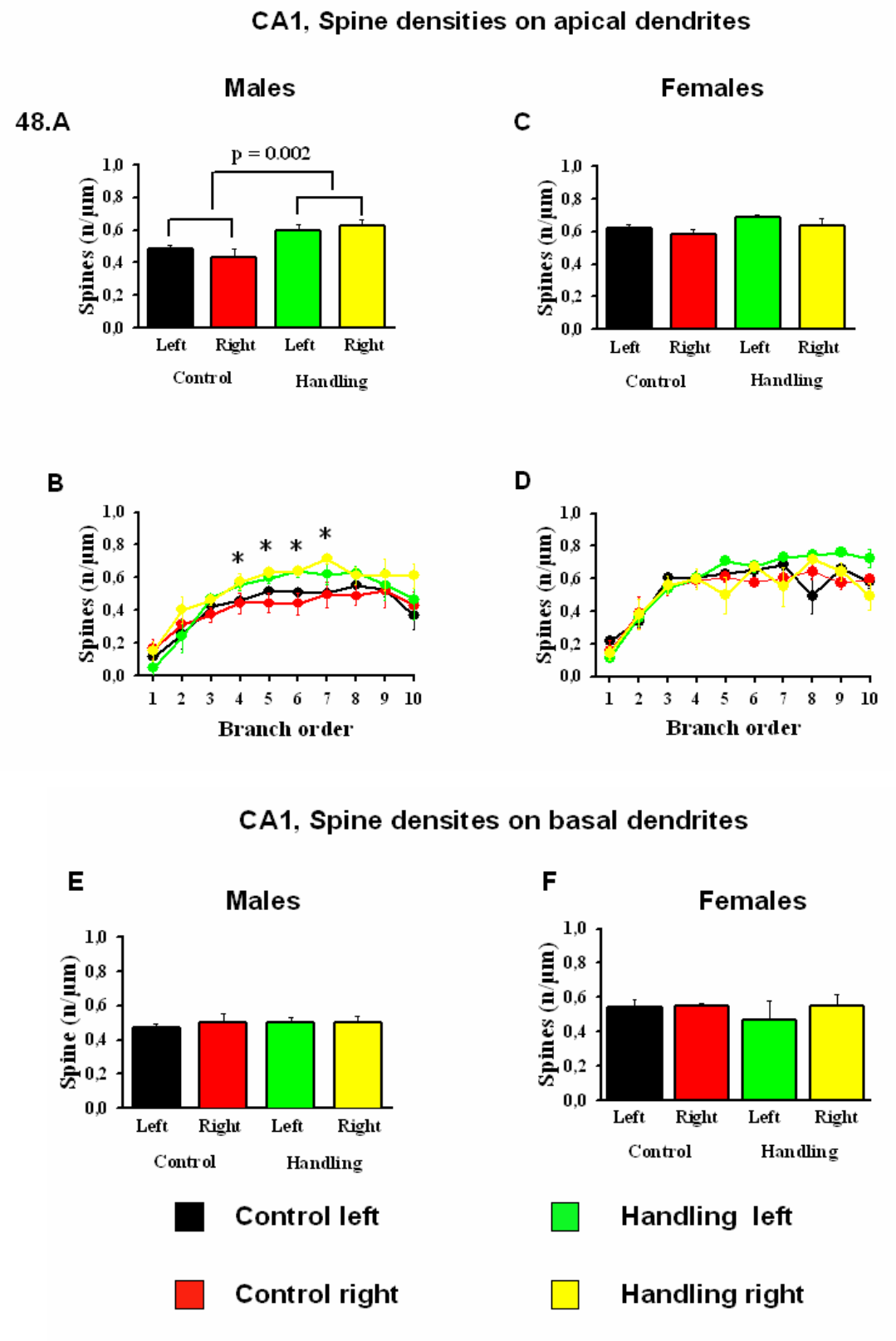


Figure 48. Histograms represent spine density of CA1 pyramidal neurons. Values are given as mean ± S.E.M. A) Total spine density on the apical dendrite of males and C) Females; B)

Spine density across different branch orders of males and D) Females apical dendrites; E) Total spine density on the basal dendrite of males and F) Females; * indicates significant differences between controlled and handled animals.

Figure 48.G Micrographs represent segments from control and handled animals. Spine density is enhanced in the handled animals as compared to the controls in the CA1 apical dendrites.

Dendritic length and complexity of dendritic arborization

Apical dendrite. a) Males: There were no effects of handling or hemisphere or interaction (fig.49.A).

b) Females: There were no effects of handling or hemisphere or interaction (fig.49.B).

Basal dendrite. a) Males: There were no effects of handling or hemisphere or interaction (fig.49.C).

b) Females - There were no effects of handling or hemisphere or interaction (fig.49.D).

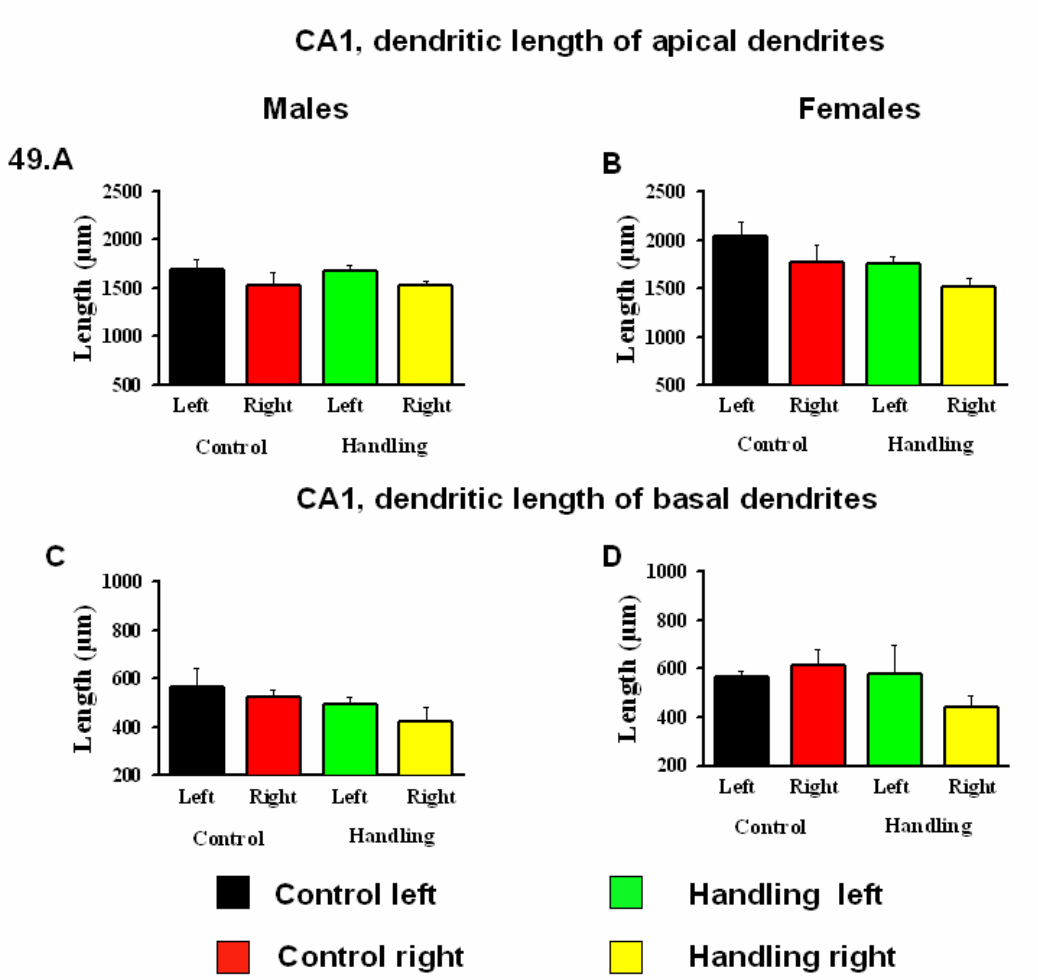


Figure 49. Histograms represent dendritic length and complexity of CA1 pyramidal neurons. Values are given as mean ± S.E.M. A) Total apical dendritic length of males and B) Females; C) Total basal dendritic length of males and D) Females.

3.3.5 Dentate gyrus: Handling reduced spine density in females but not in the males

Spine density

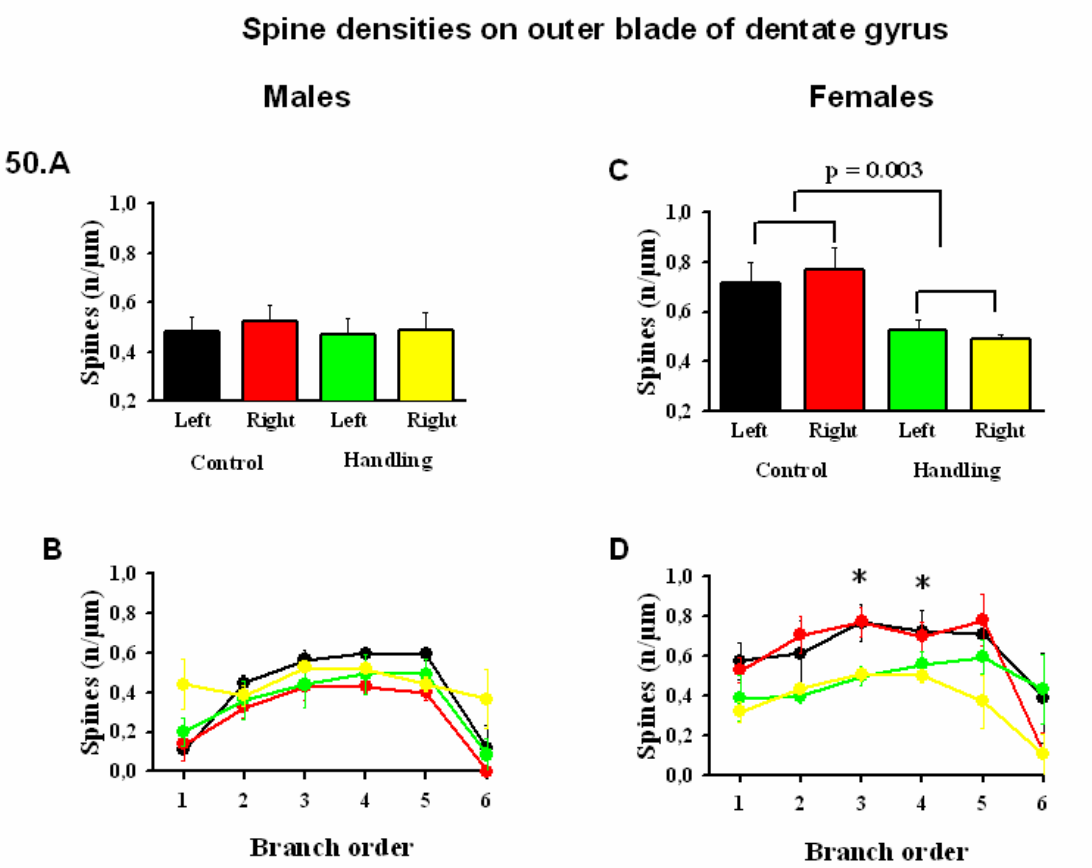
Outer blade of dentate gyrus. a) Males: There were no effects of handling or hemisphere or interaction (fig.50.A).

b) Females: Two-Way ANOVA indicated an effect of handling. Handled females displayed lower spine density ($p = 0.003$, - 32%) than the untreated control females (fig.50.C). The spine density was reduced at the third ($p = 0.002$) and fourth ($p = 0.027$) branch of the granular neurons (fig.50.D). There were no effects of hemisphere or

interaction.

Inner blade of dentate gyrus. a) Males: There were no effects of handling or hemisphere or interaction (fig.50.E).

b) Females: Two-Way ANOVA revealed an effect of handling and there were no effects of hemisphere or interaction. Handled females displayed lower spine density (p = <0.001, -37%) than the controlled females (fig.50.G). Lower density of spines were observed at the first (p = 0.001), second (p = <0.001) and third (p = <0.001) branch of the granular neurons (fig.50.H).



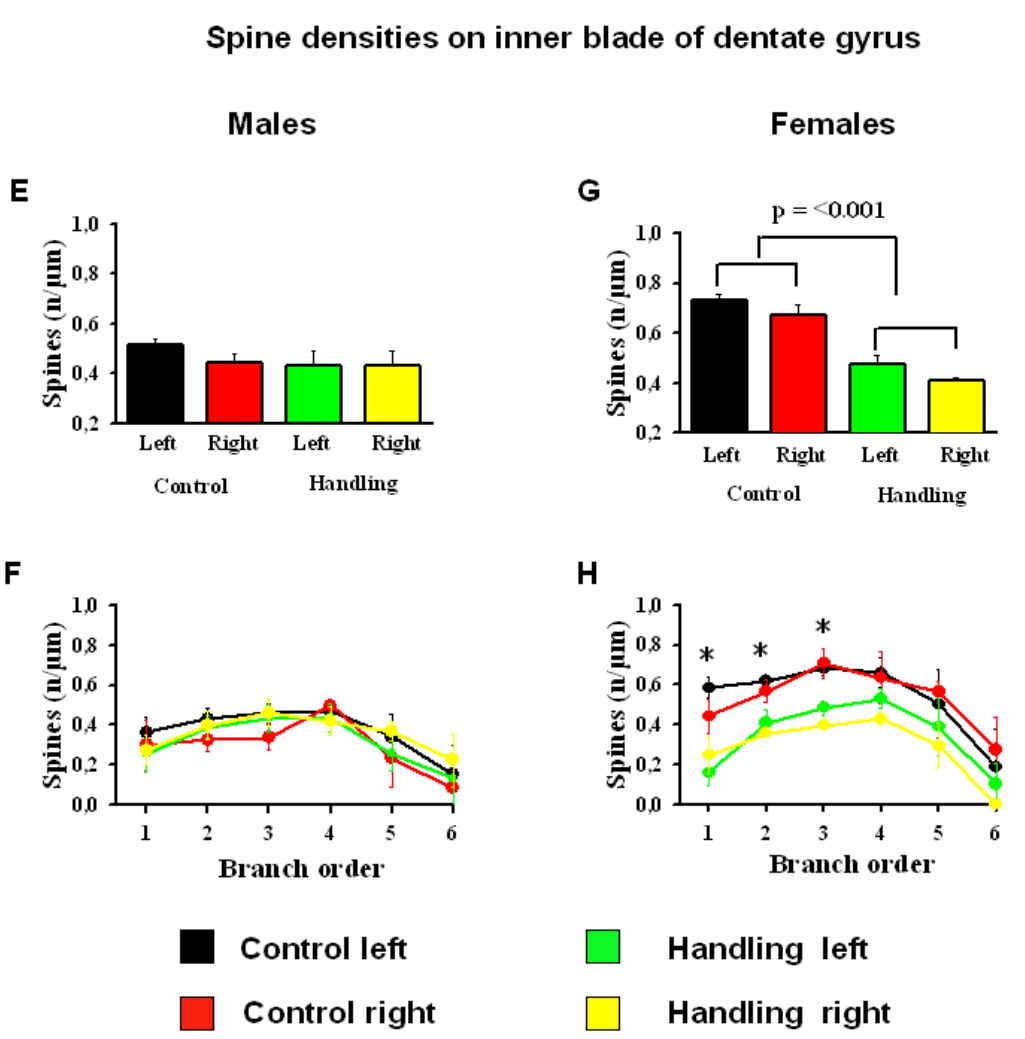


Figure 50. Histograms represent spine density of granular neurons located in the dentate gyrus. Values are given as mean ± S.E.M. A) Total spine density on the outer blade of dentate gyrus of males and C) Females; B) Spine density across different branch orders of the males and D) Females outer blade of dentate gyrus; E) Total spine density on the inner blade of dentate gyrus of males and G) Females; F) Spine density across different branch orders of the males and H) Females inner blade of dentate gyrus; * indicates significant differences between controlled and handled animals.

Dendritic length and complexity of dendritic arborization

Outer blade of dentate gyrus. a) Males: There were no effects of handling or hemisphere or interaction (fig.51.A & B).

b) Females: Two-Way ANOVA revealed significant effect of handling on dendritic morphology. Handled females displayed smaller dendritic length (p = 0.005, - 20%) than

the control females which was seen at the distance of 200µm (p = 0.026) from the soma (fig.51.C & D). The number of dendritic intersection was also reduced in the handled females as compared to the untreated controls (p = 0.010, - 21%), which was found at the distance of 120µm (p = 0.008), 140µm (p = 0.009), 160 µm (p = 0.008) and 180µm (p = 0.043) from the soma (fig.51.E & F). There were no effects of hemisphere or interaction.

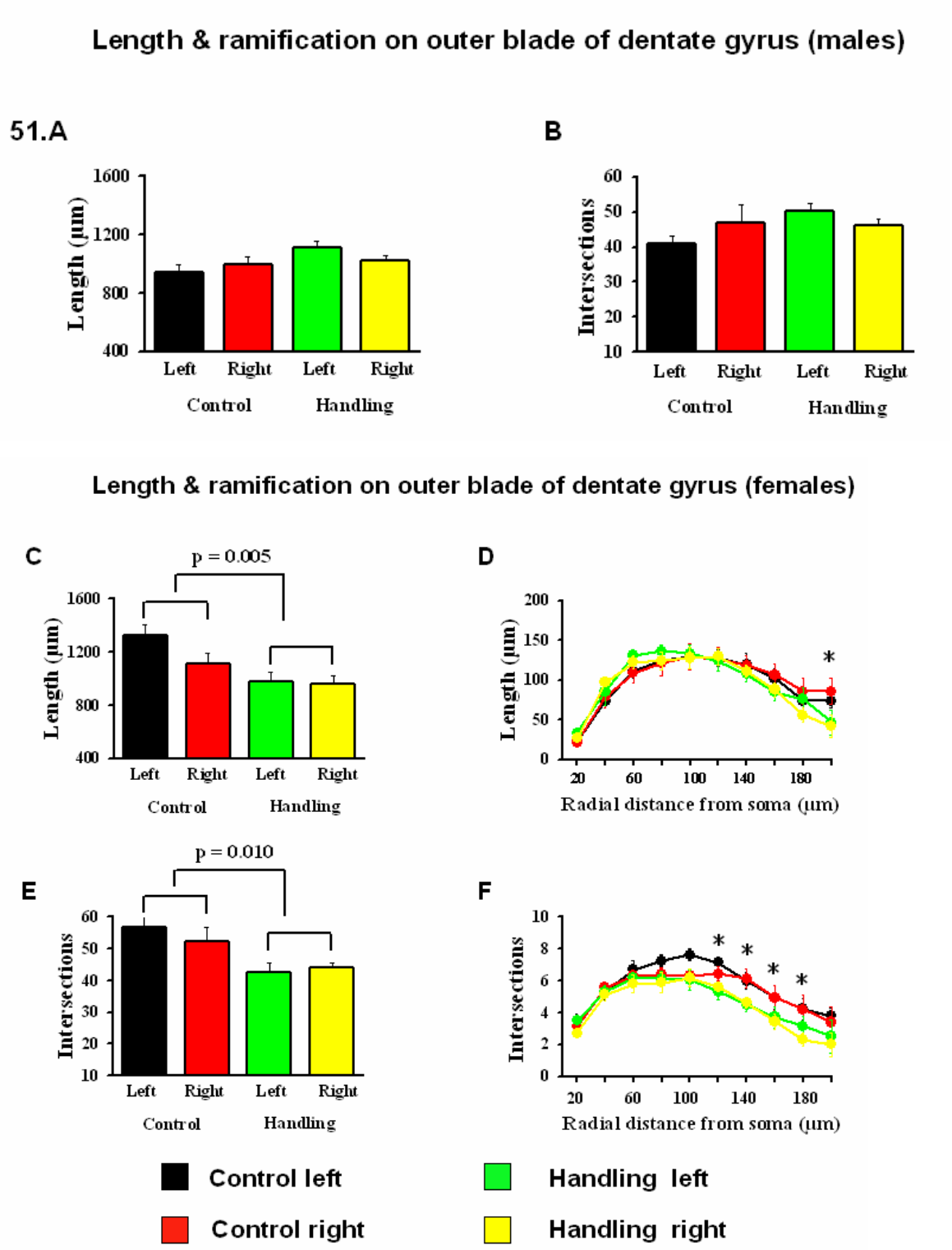


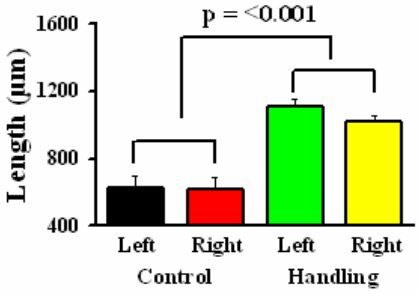
Figure 51. Histograms represent dendritic length and complexity of granular neurons located in the outer blade of dentate gyrus. Values are given as mean ± S.E.M. A) Total dendritic length of males and C) Females; B) Total number of dendritic intersections of males and E) Females; D) Dendritic length at different concentric circles from the soma of females; F) Number of intersections at different concentric circles from the soma of females. * indicates significant differences between controlled and handled animals.

Inner blade of dentate gyrus. a) Males: Two-Way ANOVA revealed significant effect of handling on dendritic morphology and there were no effects of hemisphere or interaction. Handling increases the total dendritic length ($p = < 0.001$, upto 42%) and number of intersections ($p = 0.016$, upto 26%) in the males (fig.52.A & C). Dendritic length was increased at the distance of 60µm ($p = 0.001$), 80µm ($p = 0.002$), 100µm ($p = <0.001$), 120µm ($p = 0.002$) and 140µm ($p = <0.001$) from the soma (fig.52.B). The number of dendritic intersection was increased at the distance of 20µm ($p = 0.046$), 40µm ($p = <0.001$), 60µm ($p = 0.007$), 80µm ($p = 0.007$) and 100µm ($p = 0.050$) from the soma (fig.52.D).

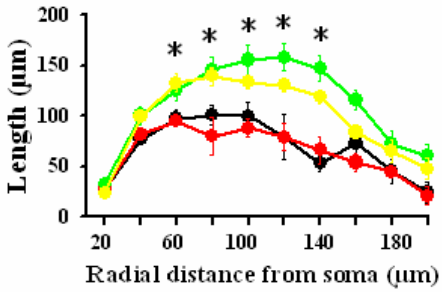
b) Females: There was an effect of handling but no effects of hemisphere or interaction. Unlike males, handling decreases the total dendritic length ($p = 0.005$, - 20%) and number of intersections ($p = 0.002$, - 22%) in the females (fig.52.E & G). The number of dendritic intersections were lowest at the distance of 120µm ($p = <0.001$), 140µm ($p = 0.005$), and 160µm ($p = 0.048$) from the soma (fig.52.H).

Length & ramification on inner blade of dentate gyrus (males)

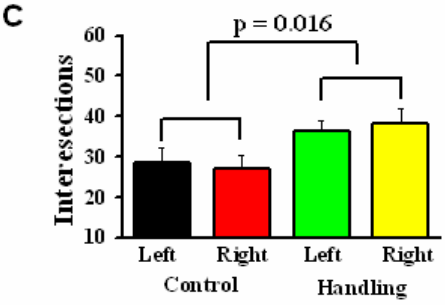
52.A



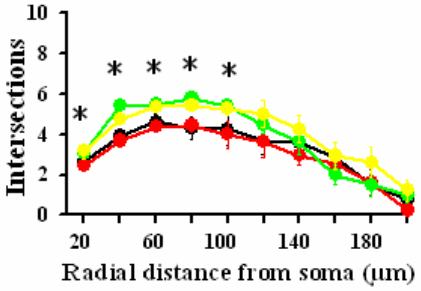
B



C

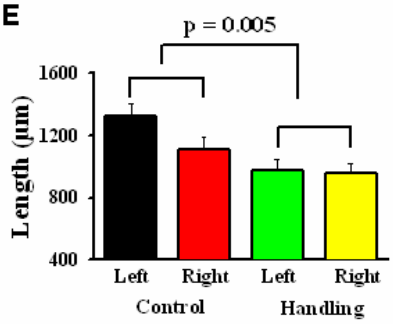


D

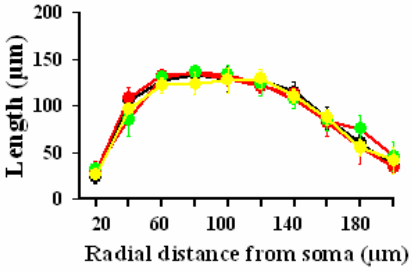


Length & ramification on inner blade of dentate gyrus (females)

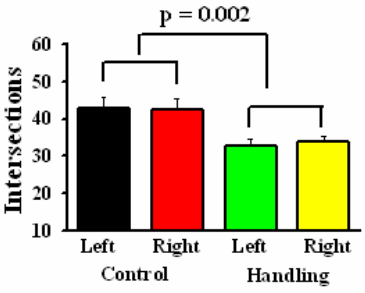
E



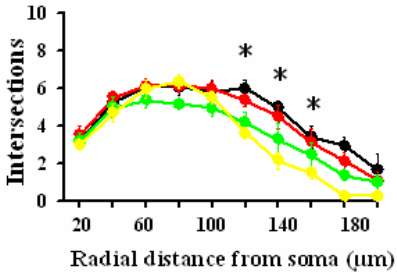
F



G



H



Control left

Handling left

Control right

Handling right

Figure 52. Histograms represent dendritic length and complexity of the granular neurons located in the inner blade of dentate gyrus. Values are given as mean ± S.E.M. A) Total dendritic length of males and E) Females; B) Dendritic length at different concentric circles from the soma of males and F) Females; C) Total number of dendritic intersections of males and G) Females; D) Number of intersections at different concentric circles from the soma of males and H) Females. * indicates significant differences between controlled and handled animals.

3.3.6 Basolateral amygdala: Handling reduced spine density in both sexes but reduced dendritic length/complexity only in the males

Spine density

Apical dendrite. a) Males: Two-Way ANOVA indicated an effect of handling. Handled males displayed significantly lower spine density than the controls ($p = <0.001$, -58%). Here, the spine density was reduced at the third ($p = 0.001$), fourth ($p = < 0.001$) fifth ($p = 0.035$) and sixth ($p = < 0.001$) branch of the dendrite (fig.53.A & B).

b) Females: There was an effect of handling but no effects of hemisphere or interaction. Handled females displayed lower spine density than the controlled females ($p = <0.001$,-57%), which was found at the second ($p = 0.004$), third ($p = < 0.001$), fourth ($p = < 0.001$), fifth ($p = < 0.001$) and sixth ($p = 0.049$) branch of the dendrite (fig.53.C & D).

Basal dendrite. a) Males: There was an effect of handling and no effects of hemisphere or interaction. Handled males displayed significantly lower density of spines ($p = <0.001$, - 54%) than the controls (fig.53.E). Spine density was reduced at the first ($p = < 0.001$), third ($p = 0.046$), fourth ($p = < 0.001$) and fifth ($p = 0.005$) branch of the dendrite (fig.53.F).

b) Females: Two-way ANOVA indicated significant effect of handling and no effects of hemisphere or interaction. Handled females had lower spine density than the controls ($p = < 0.001$, - 59%), which was most evident at the second ($p = < 0.001$) and third ($p = < 0.001$) branch of the dendrite (fig.53.G& H).

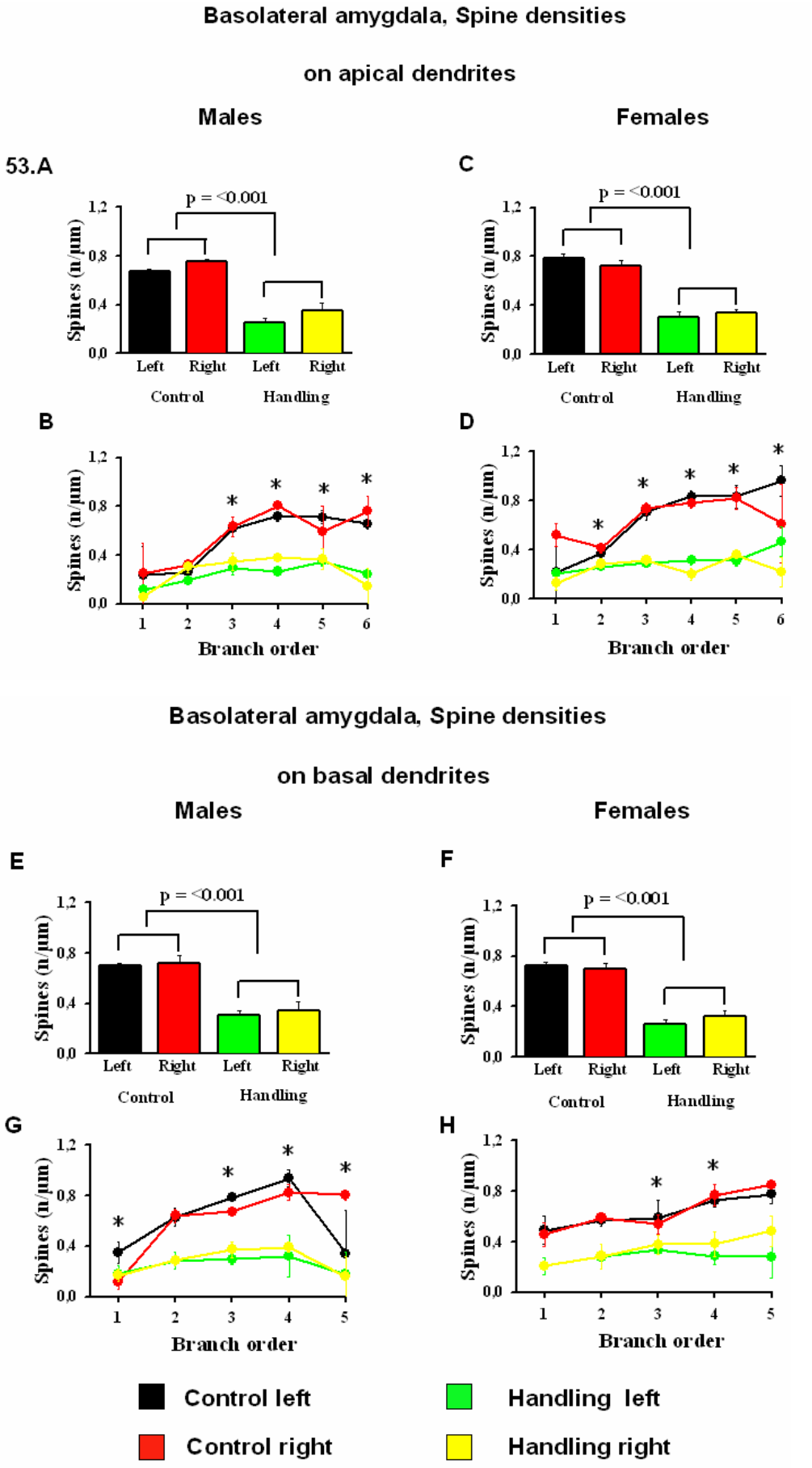
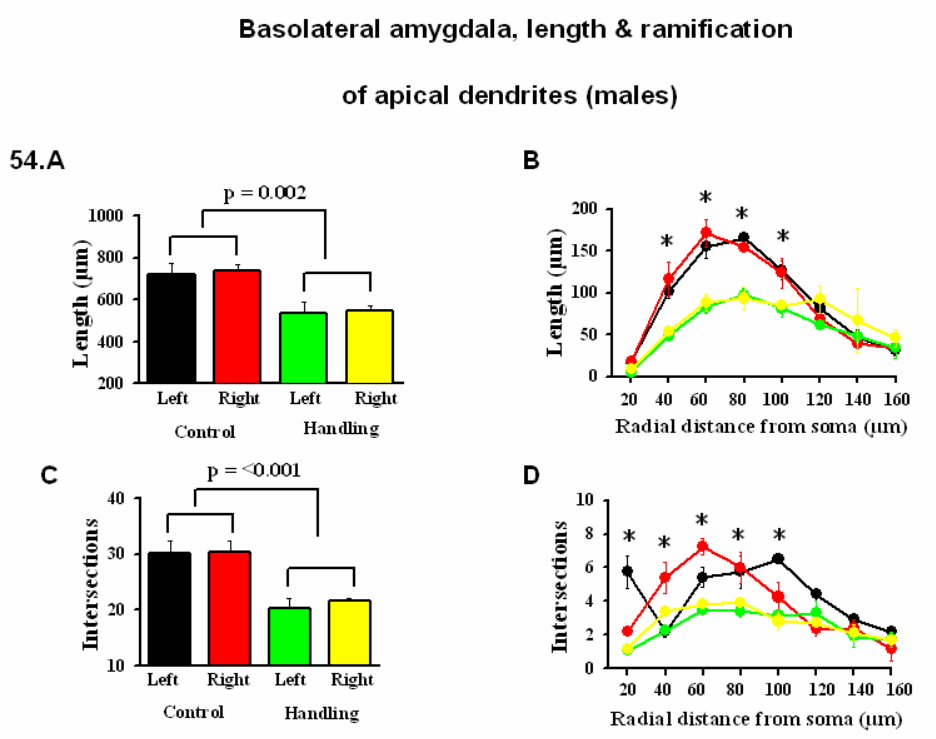

Basolateral amygdala, Spine densities
on apical dendrites
Males
Females
53.A
C
p = <0.001
Spines (n/µm)
1,2
0,8
0,4
0,0
Left
Right
Left
Right
Control
Handling
B
D
*
Branch order
1
2
3
4
5
6
Basolateral amygdala, Spine densities
on basal dendrites
Males
Females
E
F
G
H
Control left
Control right
Handling left
Handling right

Figure 53. Histograms represent spine density of pyramidal neurons located in the basolateral amygdala. Values are given as mean ± S.E.M. A) Total spine density in the males and C) Females apical dendrites; B) Spine density across different branch orders of the males and D) Females apical dendrites; E) Total spine density in the males and F) Females basal dendrites; G) Spine density across different branch orders of the males and H) Females basal dendrites. * indicates significant differences between controlled and handled animals.

Dendritic length and complexity of dendritic arborization

Apical dendrite. a) Males: Two-Way ANOVA revealed an effect of handling and there were no effects of hemisphere or interaction. Handled males displayed lower dendritic length (p = 0.002, - 25%) compared to the controls (fig.54.A). Dendritic length was reduced at the distance of 40µm (p = < 0.001), 60µm (p = < 0.001), 80µm (p = < 0.001) and 100 µm (p = 0.007) from the soma (fig.54.B). The number of dendritic intersections were also reduced due to handling (p = <0.001, - 25%) which was found at the distance of 20µm (p = 0.001), 40µm (p = < 0.001), 60µm (p = < 0.001), 80µm (p = 0.001) and 100µm (p = 0.041) from the soma (fig.54.C & D).

b) Females: There were no effects of handling or hemisphere or interaction (fig.54.E & F).



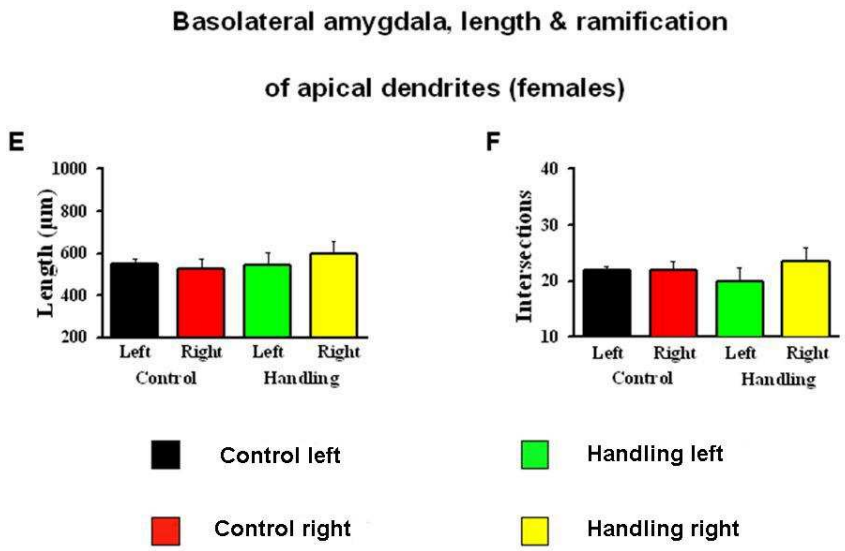


Figure 54. Histograms represent dendritic length and complexity of pyramidal neurons located in the basolateral amygdala. Values are given as mean ± S.E.M. A) Total dendritic length in the males and E) Females apical dendrites; B) Dendritic length at different concentric circles from the soma of males; C) Total number of dendritic intersections in the males and F) Females apical dendrites; D) Number of intersections at different concentric circles from the soma of males. * indicates significant differences between controlled and handled animals.

Basal dendrite. a) Males: There were no effects of handling or hemisphere or interaction (fig.55.A). **b) Females:** There were no effects of handling or hemisphere or interaction (fig.55.B).

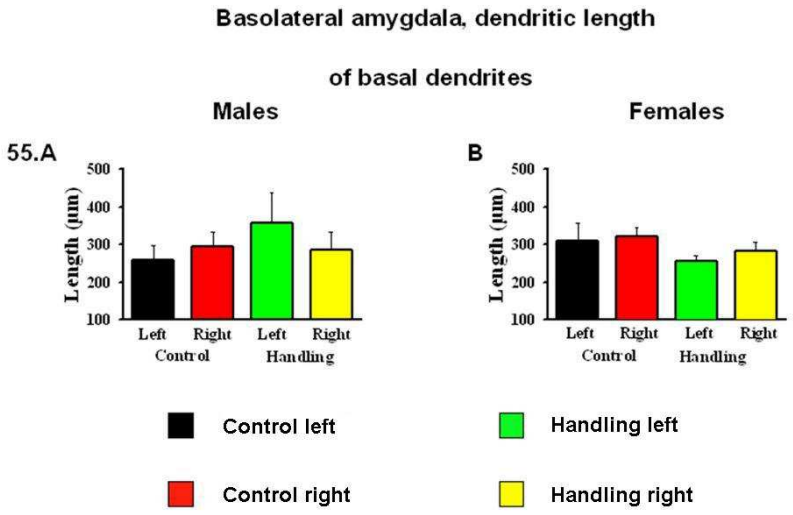


Figure 55. Histograms represent dendritic length of pyramidal neurons located in the

basolateral amygdala. Values are given as mean ± S.E.M. A) Basal dendritic length of the males; and B) Females.

56.A Effect of Neonatal Handling on Dendritic Spine Density

Investigated Areas	Males		Females	
	Dendritic Spines			
	Apical	Basal	Apical	Basal
Orbitofrontal Cortex	↓	↓	↓	↓
Anterior Cingulate Cortex	↓	-	↓	↓
Area CA3	-	-	-	-
Area CA1	↑	-	-	-
Outer blade (dentate gyrus)	-		↓	
Inner blade (dentate gyrus)	-		↓	
Basolateral Amygdala	↓	↓	↓	↓

Figure 56.A Summary of the effects of neonatal handling on spine density in the limbic system of male and female rats. Red arrows indicate a decrease in spine density. Pink arrow indicates increased spine density in response to neonatal handling.

56.B Effect of Neonatal Handling on Dendritic Length

Investigated Areas	Males		Females	
	Dendritic Length			
	Apical	Basal	Apical	Basal
Orbitofrontal Cortex	-	-	-	-
Anterior Cingulate Cortex	-	-	-	-
Area CA3	↓	↓	-	↓
Area CA1	-	-	-	-
Outer blade (dentate gyrus)	-		↓	
Inner blade (dentate gyrus)	↑		↓	
Basolateral Amygdala	↓		-	-

Figure 56.B Summary of the effects of neonatal handling on apical and basal dendritic

length in the limbic system of male and female rats. Red arrows indicate decreased dendritic length. Pink arrow indicates increased dendritic length due to neonatal handling.

4.0 DISCUSSION

There are 4 novel results of this study – i) Significant sex differences in the neuronal morphology of untreated control animals; ii) Exposure to prenatal stress interferes with the development of neurons in the rodent limbic system; iii) Prenatal stress differentially alters the morphology of neurons in the brains of males and females; iv) prenatal stress-induced neuronal alterations are prevented and/or reversed by neonatal handling in a sex, dendrite and region-specific manner.

4.1 Sex differences in the neuronal morphology of untreated control animals

The results of the present study show considerable sex differences in the morphologic characteristics of pyramidal and granular neurons of untreated control animals. In the orbitofrontal cortex, dendritic length and arborization is greater in the untreated control males compared to the females. This is in agreement with the previous study, in which fundamental differences in the organization of dendrites in the prefrontal cortex of male and female rats have been described (Kolb & Stewart 1991). Sex differences are also observed in the dentate gyrus and CA1 area of the hippocampus, with untreated control females having higher spine density and greater dendritic length as well as arborization compared to untreated control males. The presence of sexual dimorphism in the hippocampal formation is in line with findings in rats and mice, in which sex differences have been described in the anatomy of dentate gyrus (Madeira et al 1991, Tabibnia et al 1999, Wimer & Wimer 1985), morphology (Gould et al 1990a, Juraska 1990) and number of hippocampal neurons (Wimer & Wimer 1985) as well as long-term potentiation of the perforant pathway (Maren et al 1994, Maren 1995). Similarly, sex

differences have been described in the neuroanatomy of amygdala. In particular, the prepubertal male rats were reported to have more excitatory synapses in the medial amygdala compared to the female rats (Cooke & Woolley 2005). Sex differences have also been shown in the organization of dendritic branches in the basolateral nucleus (Akhmadeev & Kalimullina 2005), volume (Hines et al 1992) and synaptic number (Nishizuka & Arai 1983) of medial amygdala in the rodents. My study, which illustrates that the untreated control male's displays greater apical dendritic length and arborization in the basolateral nucleus relative to the untreated control females, provides further evidence that the cytoarchitecture of amygdala is sexually dimorphic. This study also revealed that the sex differences in spine density and dendritic morphology are dendrite-specific. The fact that sex differences are observed only in the apical dendrite and not in the basal dendrite reflects yet unreported sex differences in the innervation of these two dendritic compartments. Finally, not all limbic regions displayed sex differences in the neuronal morphology. For instance, in the prefrontal cortex and in the hippocampal formation, there were no sex differences in the neuronal morphology of anterior cingulate cortex and CA3 as opposed to orbitofrontal cortex, CA1 and dentate gyrus suggesting that there may be regional differences.

It is well accepted that the males and the females behave differently. For example, women are superior in certain verbal skills and men perform better with navigational related tasks (Lewin et al 2001). In a domain of memory, sex differences have been reported in episodic memory with women typically outperforming men (Herlitz et al 1997). Evidence have been accumulating that sexually dimorphic behaviors are due to sexually dimorphic structures in the brain region. For instance, a study by Jacobs et al (1993) demonstrated a co-relation between extensive dendritic arbors in the Wernicke´s area and superior verbal abilities of women relative to men. Animal research has also shown that hippocampus-dependent forms of learning are sexually dimorphic with males exhibiting better capacity than the females (Galea et al 1996, Roof 1993, Williams et al 1990). My study, which shows considerable sex differences in the neuronal morphology of untreated control animals, might contribute to or underlie the sexually dimorphic behaviors of rodents.

4.2 Exposure to prenatal stress and handling interferes with synaptic and dendritic development in the rodent limbic system

The results of this study provide the first evidence that exposure of pregnant mothers to stressors significantly interferes with the development of neuronal morphology and the establishment of synaptic circuits in the offspring. In addition, this study also indicated that handling during the early weeks alters neuronal and synaptic development in the limbic system of male and female rats.

4.2.1 Dendritic spine density is decreased due to prenatal stress and neonatal handling

A variety of studies in different functional pathways have demonstrated that dendritic spines, specialized postsynaptic structures receiving mainly excitatory input, are sensitive to experiences which occur during development, including periodic stress episodes or drugs (Bock & Braun 1999a.b, Goldin et al 2001, Harris & Kater 1994, Koch & Zador 1993, Lieshoff & Bischof 2003, Segal 2002, Segal 2005, Stewart et al 2005, Trachtenberg et al 2002). Analogous to experience-driven maturation of sensory and motor systems (Rosenzweig & Bennett 1996, Turner & Greenough 1985), the synaptic development of limbic cortical regions is sensitive towards environmental conditions, in particular to emotional experience. For instance, quantitative neuromorphological studies have demonstrated that both positive as well as negative emotional experiences significantly alter dendritic spine number and distribution, and size and morphology in neonate birds and rodents (Bock & Braun 1998, Helmeke et al 2001 a.b, Poeggel et al 2003, Wallhausser & Scheich 1987). The present study supports the growing body of evidence indicating that dendritic spines are modulated by stressful experiences. The data presented here illustrates that exposure to prenatal stress decreased dendritic spine density in the orbitofrontal and anterior cingulate cortices of the male and female rats, relative to the untreated controls. In addition, spine densities are also reduced in the orbitofrontal cortex, anterior cingulate cortex and basolateral amygdala of neonatally

handled animals. The finding of reduced spine density in the prefrontal cortex is consistent with the study by Radley et al (2006), in which decreased dendritic spine density was described in the prefrontal pyramidal neurons of rodents after stress exposure.

It has been shown that the magnitude and direction of experience-induced changes of neuronal structure strongly depends on the time window during which stress is encountered, indicating that this modulation is related to the maturity of the sensory and limbic brain circuits and also to the attendant hormone systems at the time stress is induced (Bock et al 2005). Three-week-old rats, which were stressed at a relatively immature stage of brain development (days 1-3 after birth), showed reduced spine densities on layer II/III pyramidal neurons of the anterior cingulate cortex; whereas the same stress paradigm given on postnatal days 14-16 resulted in increased spine densities (Bock et al 2005). Interestingly, the spine reduction found in the present study after exposure to stress at the last trimester of gestation is of similar magnitude to that found in the rats that were stressed during postnatal days 1-3. Based on the concept that neuronal development is regulated by the complexity of incoming and converging patterned synchronous neuronal activity, it can be assumed that the magnitude and direction of the described synaptic changes is likely to be determined by preexisting synaptic connections, reflecting the functional maturity of the sensory and modulatory input systems at the time when stressful experience is encountered. However, it is quite clear that before and a few days after birth, at the time of stress exposure, the neuron types analyzed in these studies were all relatively immature (Van Eden & Uyling 1985, Zhang 2004). This raises the intriguing question, how does a neuron, which has not yet developed its final network connections, "remember" these early emotional events and gradually, days and weeks thereafter, adapt its morphology and presumably its synaptic connections? The future challenge will be to identify possible hormonal or intracellular "tags" which are triggered by pre- or postnatal emotional experience and which appear to persist for days or weeks to guide neural development.

4.2.2 Alterations of dendritic length/complexity in the offspring of mothers exposed to stress during pregnancy and in neonatally handled rats

The present study provides evidence that stress experienced by a mother during pregnancy modulates dendritic growth in the offspring. There was a significant reduction in total dendritic length in the prefrontal cortex and basolateral amygdala of prenatally stressed males, and in the CA1 and CA3 hippocampal areas of both sexes, compared to untreated controls. In addition, neonatally handled male rats displayed shorter dendritic length in the CA3 region of the hippocampus and in the basolateral amygdala. This finding of reduced dendritic length is consistent with previous studies, in which decreased dendritic length has been described in the hippocampal formation and prefrontal cortex after stress exposure (Brown et al 2005, Cook & Wellman 2004, Magarinos et al 1996, Radley et al 2004, Watanabe et al 1992a). However, the finding of reduced dendritic length in the basolateral amygdala contrasts with the morphological changes described after stress exposure. For instance, Vyas et al (2002) have shown that chronic stress resulted in enhanced dendritic length and arborization in the basolateral nucleus of rodents, whereas this study indicates that the total dendritic length is reduced due to prenatal stress exposure. The opposite effects of stress on amygdaloid neuronal morphology could be attributable to differences in the time period when stress was encountered. The present study also revealed that not only the total dendritic length but also the complexity of dendritic ramification patterns can be altered by stress exposure. The reductions in the total dendritic length were accompanied by reduced complexity of dendritic arborization in several limbic regions.

This study further indicated that prenatal stress alters the dendritic morphology of neurons in a qualitatively different manner in relation to dendritic region. For instance, in the orbitofrontal cortex, anterior cingulate cortex, CA1 as well as in the basolateral amygdala of males, apical dendrite undergoes pronounced changes due to prenatal stress, whereas the basal dendrite of prenatally stressed males did not differ from that of unstressed controls in most cases. Similar effects were seen in the CA1 and basolateral amygdala of neonatally handled males. There are some studies which have also shown

that stress causes significant changes in the apical dendrites but failed to alter dendritic morphology of the basal dendrites (Brown et al 2005, Cook & Wellman 2004, Magarinos et al 1996, Radley et al 2004, Watanabe et al 1992a). The fact that in the perinatally-stressed brain dendritic retardation is restricted to the apical dendrites and not seen on the basal dendrites suggest a role of differential afferent input activity and different expression of transmitter receptors in these two dendritic compartments. Further studies are needed to test this hypothesis and to identify the role of such input specific synaptic plasticity in these areas.

The present study further indicated that stress-induced dendritic changes are located at a specific portion of the apical and basal arbor. For example, branch order analysis and sholl analysis indicated that the alterations in dendritic spines, length and arborization were mostly found at the middle and distal portion of the apical and basal arbor and not in the proximal part of the dendritic tree. Because the pyramidal neurons are known to segregate their inputs, the observation that the changes are located at the specific portion of the dendrite seems to have important functional consequences. Studies have shown that the pyramidal neurons of the prefrontal cortex segregate inputs with afferent from the hippocampal CA3 area and thalamus tending to cluster on distal dendrites (Groenewegen 1988, Swanson & Cowan 1977), and synapses of local cortical circuits tending to cluster on the proximal portion of the apical and basal arbor (Scheibel & Scheibel 1970). In the prefrontal cortex, dendritic changes at the distal portion of the apical and basal dendrites are likely to be mediated by decreases in afferent from the hippocampus, which is known to cluster at the distal portion of the dendrite. It is possibe that the atrophy of distal dendrites of layer II/III pyramidal neurons is a result of loss of input from CA3 pyramidal neurons.

4.3 Prenatal stress and neonatal handling alters the morphology of neurons in a sex-specific manner

The present study revealed that prenatal stress alters spine density and dendritic morphology of pyramidal and granular neurons in a sex-specific manner. In the

prefrontal cortex and the basolateral amygdala, prenatal stress resulted in shorter dendrites and less complex dendritic arbors in males but not in the females. In addition, in the inner blade of dentate gyrus, the morphology of granular neurons is altered in opposite directions in the male and female rats. There is increasing evidence supporting the hypothesis of sex-specific effects after prenatal stress exposure. Dysregulation of the HPA axis response to stressors are substantially more marked in PS females than in males (Weinstock 2005). This could explain why a reduction of the number of granule cells in the hippocampus was observed only in PS female rats but not in males (Schmitz et al 2002). Prenatal stress also induces a number of sex dependent differences in behavior. The development of learned helplessness in the forced swim test (Alonso et al 1991), anhedonia (Keshet & Weinstock 1995) and impairment in a step-through passive avoidance test were only seen in female offspring (Gué et al 2004). On the other hand, an increased level of latent inhibition only occurred in PS males (Bethus et al 2005). The reason for these sex differences are not entirely clear but may be related to the disparity in foetal hormonal levels in response to maternal stress on days 18 and 19 of gestation, which in turn could result from a difference in the rate of development of the hypothalamic pituitary adrenal axis in males and females (Ohkawa et al 1991).

Similar to prenatal stress, neonatal handling was found to alter spine density and dendritic morphology in a sex-specific manner. Previous studies suggest that neonatal handling differentially influence the male and the female rats. In particular, sex differences have been described on stress reactivity (Park et al 2003), exploratory activity (Weinberg et al 1978) as well as in the concentration and turnover of monoamines (Papaioannou et al 2002, Panagiotaropoulos et al 2004). The striking sex differences in response to neonatal handling on dendritic morphology provide a morphological substrate for the sex-specific neurochemical and behavioral changes.

This study illustrates that prenatal stress-induced dendritic alterations are more pronounced in males relative to females. For instance, in the prefrontal cortex and basolateral amygdala, stressed males displayed shorter dendrites and less complex dendritic arbors compared to untreated control males, whereas the females did not show

any such changes. This suggests that there may be "protective effects" in female rats that prevent alterations in dendritic morphology in response to stress. Previous studies have demonstrated that females have higher levels of corticosterone-binding globulin (CBG) relative to males (Galea et al 1997, Mataradze et al 1992). Higher CBG levels on the other hand have been shown to decrease glucocorticoid receptor activation in rats (Dhabhar et al 1995). Therefore, it can be assumed that the higher CBG levels may be one of the factors in the "protective effects" seen in the females. Further studies are needed to explore the mechanisms which "protect" the female brain from dendritic alterations after prenatal stress exposure.

Factors that shape the development of the neuroanatomical sex differences in response to stressors are not clearly known, although it is suggested that gonadal hormones may be involved. A number of postnatal developmental studies have demonstrated sex differences and the effects of gonadal hormones on pyramidal cell structure in the prefrontal cortex. For instance, in the anterior cingulate cortex, pyramidal cell arborization in layer II/III is greater in males compared to females, and in contrast the orbital prefrontal region's dendritic arborization is greater in the females, suggesting a fundamental difference in the organization of the prefrontal regions in males and females (Kolb & Stewart 1991, Kolb et al 1998). These structural differences are presumably related to the presence of gonadal hormones during postnatal development, since neonatal gonadectomy or treatment with testosterone has been shown to influence cortical morphology and neuronal structure (Stewart & Kolb 1988, 1994). The influence of gonadal hormones on pyramidal cell structure has also been demonstrated for the hippocampus. Exposure to estrogen either exogenously or endogenously during proestrous leads to alterations of spine densities in the CA1 area of the rat hippocampus, which are paralleled by differences of synapses formed on dendritic spines (Gould et al 1990, Woolley et al 1990b, Woolley & McEwen 1992). In an extension of these studies Shors and colleagues demonstrated that the females in proestrous have greater spine densities than the males. Additionally, this sex difference in spine density is affected in opposite directions by stressful experience (Shors et al 2001).

Previous studies have indicated that the stress levels of corticosterone and the degree of NMDA receptor activation regulate dendritic alterations after stress exposure (Magarinos & McEwen 1995). On the other hand, sex differences in the central NMDA receptor function (Maren et al 1994) as well as in the corticosterone response to stressors have been described (Galea et al 1997). Therefore, the sex differences in NMDA receptor function and corticosterone might contribute to sex differences in the neuronal morphology. Further studies are needed to explore the role of the NMDA receptor and corticosterone antagonist on sex differences in dendritic morphology caused due to prenatal stress.

What are the functional consequences of observed sex differences in the neuronal morphology in response to stress? Previous studies indicate substantial sex differences in stress-induced learning and memory impairments in the rodents. For example, exposure to acute stress impairs spatial learning in males but not in the female rats (Conrad et al 2004). Similarly, sex differences have been described in the Y-maze performance with females showing enhanced performance relative to male rats after chronic stress exposure (Conrad et al 2003). The observed sex differences in the neuroanatomy might contribute to or underlie the behavioral impairments observed after stress exposure.

Clinical studies have demonstrated that nearly all neurodevelopmental diseases are either more common or severe in one gender than the other. Depression, for example appears to be twice as common in women as in men (Noble 2005). Similarly, brain-imaging studies have shown that the prevalence of post-traumatic stress disorder as well as anxiety disorder is higher in females relative to males (Cloitre et al 2004, Landheim et al 2003). On the other hand, males are more susceptible to suffer from schizophrenia (Iacono & Beiser 1992), bipolar disorder (Scully et al 2002) and attention-deficit-disorder compared to females (Hartung et al 2002). It is important to note that there are well-documented sex differences in the induction of depression and schizophrenic-like features in the rodents after exposure to prenatal stress. For example, latent inhibition, which has frequently been proposed as an animal model of schizophrenia, is impaired due to prenatal stress in the males but not in the prenatally stressed females (Bethus et al 2005, Shalev & Weiner

2001). Likewise, *in utero* stress resulted in depressive-like behavior in rats, which was more marked in females than the males (Weinstock 2001a). Sex differences in the neuronal morphology in response to stressful stimuli may have important implications towards understanding the sexually dimorphic behavioral and neurological disorders.

4.4 Prenatal stress-induced neuronal alterations are prevented and/or reversed by neonatal handling in a sex and region-specific manner

This study indicated that prenatal stress-induced dendritic and synaptic alterations are prevented and/or reversed by neonatal handling. The results of this study support the growing body of evidence indicating that it is possible to prevent and/or reverse prenatal stress-induced alterations by manipulation during the neonatal period. It has been shown previously that neonatal handling prevented and/or reversed the behavioral abnormalities caused due to prenatal stress. For instance, exposure of pregnant rats to random light and noise stress resulted in enhanced emotionality and timidity whereas handling was reported to reduce behavioral reactivity in the rat offspring (Wakshlak & Weinstock 1990). Similarly, prenatal stress was found to cause higher anxiety but handling was shown to reduce anxiety in the rodents (Vallee et al 1997). The present study also revealed that neonatal handling prevented and/or reversed the effects of prenatal stress in a qualitatively different manner in relation to region, sex and the dendrites. For instance, in the dentate gyrus, prenatal stress-induced structural alterations are prevented or reversed in the inner blade but not in the outer blade. Likewise, in the anterior cingulate cortex and in the CA1 area, the structural alterations are prevented only in the apical dendrite and not in the basal dendrite. These findings indicate that different regions and dendritic compartments possess a specific structural plasticity. It is important to note that the reversal was evident only in the males. The fact that in females handling failed to prevent stress-induced structural alterations indicates that the females are more susceptible to the damages caused by prenatal stress relative to males.

4.5 Possible functional relevance of the structural alterations caused by adverse prenatal and postnatal experiences

One of the most intriguing questions in behavioral neuroscience is how the brain does, modifies its function throughout one's lifetime. Increasing basic, clinical and epidemiological evidence supports the notion that there is a strong relationship between brain plasticity and behavioral change. The study of brain and behavioral correlation is based on the assumptions that changes in the structural properties of brain will reflect changes in brain function. Furthermore, it is assumed that the most likely place to identify neural changes associated with behavior is at the synapse. Various factors such as experiences, exposure to stressors, hormones and drugs have been found to significantly alter synaptic organization of brain in variety of species. Evidence that these changes are functionally meaningful is difficult to collect; nevertheless there is now little doubt that changes in synaptic organization are correlated with changes in behavior.

Dendrites are the primary site of synaptic connectivity between neurons and are considered an important indicator of the functional capacity of the neuronal network (Jan & Jan 2001). It has been shown that the proper growth and branching of dendrite is crucial for nervous system function. There is a direct relationship between dendritic geometry and synaptic input, and the geometry of dendrites i.e its overall shape, branching pattern and complexity, determines the number of synaptic input a neuron can process. Neurons with single or fewer dendrites are innervated by fewer numbers of axons and neurons that have extensive dendritic arborization receive larger number of inputs. Stress-induced decreases in the dendritic length and complexity of neurons are most likely to result in a massive loss of synaptic input in the neuronal circuitry of prenatally stressed and neonatally handled animals, which might in turn, can alter signal processing in these circuits.

The present study also showed that there is a significant spine loss in the prefrontal cortex and basolateral amygdala in response to prenatal stress as well as to neonatal handling. Spines are the sites of excitatory input in the neocortex. Studies have shown that 95% of

all cortical excitatory synapses are made into pyramidal neurons, with each spine head receiving one excitatory terminal (Harris & Stevens 1988, Spacek & Hartmann 1983). Changes in a spine number may therefore represent an index of total excitatory input. Stress-induced decreases in the spine densities in the prefrontal cortex and amygdala are likely to reduce the total number of excitatory synapses in these areas.

At the system network level, the questions arise as to which afferents could be affected by these environmental changes, and in what way the observed dendritic and synaptic alterations could affect the functioning of the limbic system? Dendritic spines of prefrontal pyramidal neurons are thought to be the target of thalamic fibers (Krettek & Price 1977), callosal fibers (Carr & Sesack 1998), and fibers from the basolateral amygdala (Bacon et al 1996), as well as fibers arising from hippocampus (Carr & Sesack 1996). An altered balance between synapses and different neurotransmitters systems (Braun et al 2000, Poeggel et al 1999, 2000) can change the output characteristics of the pyramidal neurons into their limbic projecting area, including the amygdala (Aggleton et al 1980), hippocampus (Joyce 1993), and mediodorsal nucleus of the thalamus (Kuroda et al 1998) etc. Thus, it is possible that stress-induced decreases in the spine density of OFC and ACd pyramidal neurons might influence the output characteristics of the prefrontal cortex to these regions.

Finally, this study showed that prenatal stress as well as neonatal handling alters spine density and dendritic morphology of pyramidal and granular neurons in a region-specific manner. For example, in the hippocampal formation, the spine density is altered in CA1 and dentate gyrus; however there were no changes in the spine density of CA3 pyramidal neurons in response to prenatal stress. Similarly, neonatal handling alters dendritic morphology in the CA3, dentate gyrus and amygdala whereas it failed to alter dendritic morphology in the CA1 and prefrontal cortex. There is direct evidence that the distribution of receptor system varies across region, strata and cellular compartments (Kraemer et al 1995, Kohler et al 1991, Tommim & Millington 1998, Zilles et al 1991, Zilles et al 1993). This segregation might underlie the specific plasticity exhibited by neurons in different limbic regions. Studies have also shown that there are substantial

regional and laminar differences in the distribution of nerve growth factor (Mufson et al 1994), which suggests that the different degree of plasticity of different neurons and regions might also be due to differences in the local source of trophic support.

Several studies, including this one, provide evidence that the development of corticostriatal and corticolimbic pathways is permanently modified in response to emotional experience. Are these stress-induced synaptic and dendritic changes caused by or perhaps related to increased levels of stress hormones, neurotransmitters or peptides? It is well known that exposure of the mother to psychological stress increases the levels of circulating glucocorticoids (Maccari et al 2003), and the raised plasma levels of maternal glucocorticoids can alter the development of the HPA axis and of central monoaminergic systems in the unborn or neonate animals (Berger et al 2002, Fride & Weinstock 1987, Matthews 2002, Muneoka et al 1997, Takahashi et al 1992). Glucocorticoids can easily cross placental and blood-brain barrier and can directly affect the developing fetus. Additionally, other stress hormones such as adrenocorticotropin, ß-endorphin and corticotropin-releasing hormone may also mediate these effects since it has been shown that prenatal stress increases the levels of these hormones, which in turn can significantly alter brain functions and behavior (Weinstock 1997).

In addition to the postulated direct effect of maternal stress hormones on the developing foetus, a change in maternal behavior, resulting from the dam´s exposure to chronic stress during the last week of pregnancy, could also have contributed to these anatomical findings. Indeed, it has been shown that prenatal stress affects mother-infant interactions (Moore & Power 1986). In this context it is of particular interest that these changes might be sex-dependent. Prenatal stress was shown to eliminate the differential maternal attention to male offspring. In contrast to unstressed families, where male pups typically received more maternal licking than their female siblings, prenatally stressed male and female pups received similar levels of maternal licking (Power & Moore 1986). Cross fostering experiments may help to reveal the contribution of maternal behaviors to the observed structural changes. However, these important control experiments will be complex, since there is now evidence that cross-fostering itself induces a number of

behavioral and physiological alterations (Bartolomucci et al 2004). Further cross fostering studies should be undertaken to determine how changes in maternal behavior mediate these structural changes after prenatal stress.

Neurotransmitters are important signal guiding development and have been implicated in the maturation of dendritic processes and the refinement of synaptic circuits in the cortex (Herlenius and Lagercrantz 2004). Exposure to stress both pre- and postnatal alters the levels of dopamine, serotonin and glutamate in different forebrain regions of the rodents (Berger et al 2002, Boldon et al 1990). Stress-induced morphological changes could be mediated by alterations in the dopaminergic, glutamatergic and serotonergic neurotransmission. The disruption of neurotrophin system might also be involved in causing these structural alterations since it has been shown that neurotrophins are important for the survival of neurons as well as its development and maturation and that exposure to stress significantly reduced the levels of neurotrophins in several cortical areas of the rodents (Fumagalli et al 2004, Fumagalli et al 2005). Furthermore, the NMDA receptor complex may also be involved in causing alterations in dendritic morphology since there is now convincing evidence that changes of spine synapses induced by early emotional learning or postnatal stress exposure are NMDA-receptor dependent (Bock & Braun 1999b, Shors et al 2004). In addition, the activity-dependent development of neuronal connectivity in sensory systems and the development of excitatory circuitry and the formation of new spines after LTP in hippocampal slices have been shown to be regulated by NMDA-receptor activation (Engert & Bonhoeffer 1999, Goodman & Shatz 1993, Luthi et al 2001). Furthermore, a study by Kinnunen et al (2003) indicated that a repeatedly applied, variable prenatal stress paradigm, which was very similar to that applied in this study, influenced the response of the HPA axis to acute stress in the adult male offspring. These alterations in the HPA axis were coupled to changes in gene expression in the frontal cortex. In particular, genes that are associated with the NMDA receptor/postsynaptic complex and NMDA receptor NRI and NR2A subunits were affected.

What could be the functional and behavioral consequences of the structural changes observed after prenatal stress? In addition to the present results, gestational stress was reported to induce a loss of cerebral asymmetry in dopamine turnover in the prefrontal cortex (Fride & Weinstock 1989). Prenatal stress also reduced cortical N-acetylaspartic acid levels indicating neuronal damage or cell loss (Poland et al 1999) and the density of hippocampal synapses (Hayashi et al 1998), while causing expansion of the lateral nucleus of the amygdala (Salm et al 2004). These structural changes along with the dendritic alterations observed in my study represent the neurological substrate for the behavioral changes which were observed after gestational stress in rats. On the behavioral level, hyperanxiety and fear of novelty, depressive-like behavior and alterations in prepulse and latent inhibition, consistent with that seen in humans with schizophrenia (Koenig et al 2002, Weinstock 2001a.b) have been reported in PS animals.

Likewise, disruptions of normal mother-infant interactions in the form of neonatal handling induce persistent structural and neurochemical alterations. In particular, neonatal handling have been shown to induce- i) Increases in 5-HT turnover in the hippocampus and frontal cortex of neonatal rat (Smythe et al 1994); ii) Increases in stress-induced dopamine content in the nucleus accumbens of mice (Anisman et al 1998); iii) Decreases in neutral endopeptidase activity in the amygdala (Irazusta et al 1999); iv) Increase in the binding of GABA receptors (Boldon et al 1990); v) Increase in the magnitude of hippocampal long-term potentiation (Wilson et al 1986); vi) Increase in hippocampal nerve growth factor (NGF) mRNA (Mohammed et al 1990); vii) Enhancement of NADPH-diaphorase-positive neurons (Vaid et al 1997); viii) Alterations in brain serotonergic and dopaminergic system (Papaioannou et al 2002); and ix) Lower number of C-Fos positive cell nuclei in different cortical region (Abraham & Kovacs 2000). Neonatally handled animals have also been shown to synthesize and secrete less corticotropin-releasing hormone (CRH), adrenocorticotropin hormone (ACTH) and corticosterone in response to variety of stressors (Meaney et al 1989, Viau et al 1993) and to exhibit permanent increase in hippocampal type II, glucocorticoid receptor binding at adulthood (Meaney et al 1989). Neonatal handling-induced dendritic alterations provide a structural basis for the behavioral changes observed in these animals. At the behavioral

level, neonatal handling has been found to variously influence anxiety and fearfulness as well as learning and memory in the rodents (Anisman et al 1998, Vallee et al 1997).

The prefrontal cortex, hippocampus and amygdala mediate many of the behaviors that are altered by stressors or chronic corticosterone administration. The hippocampus-dependent forms of learning and memory are severely impaired by exposure to pre- as well as postnatal stressors (Conrad et al 1996, Lemaire et al 2000, Luine et al 1994, Szuran et al 2000). The prefrontal cortex is implicated in the intregation of cognitive as well as emotionally relevant information (Dalley et al 2004, Fuster 2001) and it has been shown that stress as well as chronic elevation of glucocorticoid induces cognitive deficits in the rodents (Bowman et al 2004, Dachir et el 1993). Similarly, the amygdala plays an important role in the regulation of fear and anxiety and studies has shown that exposure to stress can influence anxiety (Fride & Weinstock 1988, Patin et al 2005, Vallee et al 1997) as well as fearful behaviors in the rodents (Dickerson et al 2005, Ward et al 2000). Stress-induced morphological alterations have important implications for the functioning of limbic system and the behaviors mediated by it.

The pathophysiological characteristics of schizophrenia appear to involve altered synaptic connectivity in the prefrontal cortex as well as in the hippocampal formation. Previous studies have demonstrated that the spine density is reduced in the PFC pyramidal neurons of schizophrenics (Glantz & Lewis 2000). Similarly, a study by Kolomeets et al (2005) has shown that the size and dendritic arborization of CA3 pyramidal neurons are altered in schizophrenia. On the other hand, stress has been shown to play a pivotal role in the pathogenesis of schizophrenia (Ventura et al 1989). Stress-induced alterations in the spine density and dendritic morphology of neurons in the prefrontal cortex and hippocampal formation have implications for the etiology of schizophrenia.

Stress and dysfunction of the limbic system is hypothesized to play an important role in disorders such as depression (Frodl et al 2003, Lange & Irle 2004, Lai et al 2000), schizophrenia (Molina et al 2005, Ragland et al 2004), and post-traumatic-stress disorder (Lindauer et al 2004) etc. The morphological sensitivity of the limbic system in response

to stress has important implications for the etiology of these disorders. It is possible that the stress-induced decreases in the spine density, dendritic length and complexity of neurons in the prefrontal cortex, hippocampus and amygdala could significantly contribute to the observed reduction in the volume of limbic system in patients with PTSD and depression.

5.0 CONCLUSION

- My study clearly shows that stress *in utero* alters dendritic and synaptic development in the limbic brain areas of the rodents. Prenatal stress-induced neuronal changes can be characterized by decreases in dendritic length, amount of dendritic arborization as well as the density of dendritic spines in most cases. This study supports the hypothesis that stress experienced by a mother during pregnancy can significantly alter brain morphology and thereby the development of the offspring.

- My study shows substantial sex difference in response to prenatal stress. Prenatal stress was found to alter pyramidal and granular neuronal morphology of the prefrontal cortex, hippocampal formation and the basolateral amygdala in a sex-specific manner supporting the growing body of evidence that the effects of prenatal stress differ between the sexes.

- Present study indicates that the effects of prenatal stress can be prevented and/or reversed by neonatal handling in males. Therefore, in males the effects of prenatal stress are not permanent and can be prevented and/or reversed via manipulation during the neonatal period. Furthermore, handling prevented and/or reversed the effects of prenatal stress in a highly selective manner and is region as well as dendrite specific.

- Finally, my study shows that the disruption of mother-infant bond in the form of neonatal handling alters neuronal morphology in different areas of the limbic system, which significantly differed between the sexes.

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7.0 APPENDICES

Zusammenfassung

Die Art und Weise, in der Erfahrung die sich entwickelnden neuronalen Schaltkreise formt, ist eine der faszinierendsten Fragen der Entwicklungsneurobiologie. Es mehren sich die Beweise dafür, dass epigenetische Faktoren die Hirnentwicklung und das Verhalten viel stärker beeinflussen als bisher angenommen. Die zellulären Mechanismus, die solchen erfahrungsgesteuerten Entwicklungsänderungen zu Grunde liegen, sind jedoch bei weitem noch nicht geklärt. Die gegenwärtige Studie präsentiert die ersten Hinweise darauf, dass Stress *in uteri*

einen epigenetischen Faktor darstellt, der die Entwicklung der neuronal Morphologie im limbischen System von Nagern beträchtlich zu stören vermag.

In den letzten Jahren hat die Psychobiologie eine zunehmend größere Bedeutung für das Verstehen abnormalen Verhaltens erlangt. Man ist sich in zunehmendem Maße bewusst, dass verschiedene Formen pathologischen Verhaltens bei Menschen das Ergebnis von widrigen oder traumatischen Erfahrungen wie z.B. frühzeitig im Leben auftretenden Stresses sein könnten. Es wurde beispielsweise gezeigt, dass der Stress, den eine Mutter während der Schwangerschaft erfährt, einen prädisponierenden Risikofaktor für die Entwicklung von Störungen wie Hyperaktivitätsaufmerksamkeitsdefizit, Schizophrenie, Depression oder Autismus in der Nachkommenschaft darstellt. Gleichermaßen gibt es eine beträchtliche Zahl von Befunden, die besagen, dass die Trennung des Säuglings von der Mutter während der ersten Wochen ihres Lebens zu Verhaltensproblemen beim Erwachsenen führen kann. Wir postulieren in unserer Arbeitshypothese, dass Stress während kritischer Entwicklungsperioden die Entwicklung der neuronalen Morphologie sowie den Aufbau und die Verbesserung synaptischer Verschaltungen stört. Gestützt auf diese Hypothese war das Ziel dieser Studie, den Einfluss pränatalen Stresses und des Berührens der Neugeborenen auf die Entwicklung von Neuronen und ihrer synaptischen Netzwerke im limbischen System von Nagern nachzuweisen. Das limbische System ist ein Ziel für Hormone, die in die Stressantwort involviert sind, ebenso ist es an zahlreichen psychiatrischen und Verhaltensstörungen beteiligt, die durch Stress ausgelöst oder verschlimmert werden. Daher kann die Untersuchung der Auswirkungen von Stress auf das limbischen System wichtige Implikationen für die Ursachen und die Verhinderung von Störungen haben, die auf seiner Dysfunktion beruhen.

Die quantitative Analyse offenbart ausgeprägte Änderungen in der dendritischen Morphologie der pyramidalen und granularen Neurone als Reaktion auf pränatalen Stress. Ausgelöst durch pränatalen Stress zeigte sich eine signifikant geringere Spinedichte in den orbitofrontalen und anterioren cingulären Kortizes gegenüber unbehandelten Tieren. Zusätzlich war eine signifikante Reduktion in der absoluten Dendritenlänge and der Verzweigung in den orbitofrontalen und anterioren cingulären Kortizes der männlichen Tiere, CA1 and CA3 hippocampalen Bereichen von beiden Geschlechtern, sowie im basolateralen Nukleus der männlichen Tiere zu verzeichnen. Die Vorliegende Untersuchung liefert auch den Beweis, daß der Effekt des pränatalen Stresses sexuell-dimorph ist. Die neuronale Morphologie der männlichen und weiblichen Tiere wird unterschiedlich durch pränatalen Stress geändert. Diese Studie zeigt weiter, dass die neuronalen

Änderungen, die durch pränatalen Stress verursacht werden, durch pränatale Behandlung Neugeborener verhindert oder aufgehoben werden. Neugeborenenbehandlung verhindert Stress-induzierte neuronale Änderungen in einem Geschlecht, Region und in einer dendritischen-spezifischen art und Weise. Vorliegende Untersuchung zeigte auch, dass die Trennung der Kinder von der Mutter während der frühen Wochen des Lebens bedeutende Änderungen in der Spinedichte und in der dendritischen Morphologie der pyramidalen und granularen Neurone verursachte, die sich deutlich zwischen den Geschlechtern unterscheiden. Schließlich deckte diese Studie auf, dass es beträchtliche Geschlechtsunterschiede bezüglich der neuronalen Morphologie der unbehandelten Kontrollierten gibt.

Die Ergebnisse dieser Studie zeigen ein neuroanatomisches Substrat für das Verhaltensdefizit, das in pränatalen gestresst Tieren beschrieben wird. Die in dieser Arbeit gezeigten Ergebnisse können helfen zelluläre Mechanismen zu verstehen, durch die Stress sowohl während der Embryonalentwicklung als auch frühen Kindheit negativen Einfluss auf die Entwicklung der neuronalen Netzwerke des limbischen Systems nehmen kann.

Selbständigkeitserklärung

Erklärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation mit dem Thema:

“The impact of prenatal stress and neonatal handling on the neuronal development of the rodent limbic system”

selbständig verfasst, nicht schon als Dissertation verwendet und die benutzten Hilfsmittel und Quellen vollständig angegeben habe.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr.rer.nat.) an anderen Einrichtungen eingereicht habe.

Wilhelm-Bode Str. 27, Braunschweig, 28.06.2006.

Meena Sriti Murmu

8.0 CURRICULUM VITAE

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Educational Background

- **2003 - 2006** — Ph.d in neuroscience
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  Developmental Neurobiology
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- **2000 - 2002** — Master of Science (Zoology)
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  Presidency College (India)

- **1997 - 2000** — Bachelor of Science (Zoology, Chemistry & Botany)
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Ph.D Project

2003 - 2006

Project: **"The impact of prenatal stress and neonatal handling on the neuronal development of the rodent limbic system".**

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Seminar Presentations

2004

Poster presented at the 37th Annual Meeting of the International Society for Developmental Psychobiology (ISDP) Aix-en-Provence (France). Topic- "Gestational stress affects the development of dendritic spines in the rodent prefrontal cortex".

2004

Poster presented at the 13th Annual Meeting of the Israel Society for Neuroscience (ISFN), Eilat (Israel). Topic- "Gestational stress alters spine density in the orbitofrontal and anterior cingulate cortex of rats".

2005

Poster presented at the 35th Annual Meeting of the Society for Neuroscience (SFN), Washington (USA). Topic- "Gender-specific effects of prenatal stress on the dendritic morphology of hippocampal neuron".

2005

Oral presentation at the 38th Annual Meeting of the International Society for Developmental Psychobiology (ISDP), Washington (USA) by the supervisor. Topic- "Gender-specific effects of prenatal stress on the dendritic morphology of hippocampal neuron".

Publications

- Meena Sriti Murmu, Shiri Solomon, Marta Weinstock, Katharina Braun and Jörg Bock (2006) **"Changes of spine density and dendritic complexity in the prefrontal cortex in offspring of mothers exposed to stress during pregnancy".** *Eur.J. Neurosci.,* **24**:1477-1487.

- Meena Sriti Murmu, Shiri Solomon, Marta Weinstock, Katharina Braun and Jörg Bock**. "Prenatal stress alters the morphology of pyramidal and granular neurons in a sex-specific manner"** (In preparation).

- Meena Sriti Murmu, Shiri Solomon, Marta Weinstock, Katharina Braun and Jörg Bock. **"Effects of prenatal stress are prevented and/or reversed by neonatal handling: reversal is region and sex-specific"** (In preparation).