“Refinement of neuronal networks in the rodent prefrontal cortex and hippocampus: Critical impact of early and late social experiences”

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Dedicated to my honorable

Mom (Mrs. Mariam Soren)

And

Dad (Mr. Dominic Murmu)

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Reena Prity Murmu

ABSTRACT

The process of weaning programmes the physiological and neurobehavioural development of various animal species and is thus a critical formative period for adult behaviour. The neural substrates which may underlie these behavioural changes are largely unknown. This study for the first time show that the timing as well as the amount of social contact with family members significantly interferes with the refinement of prefrontal cortical and hippocampal synaptic networks.

Studies have quite intensively investigated the critical importance of emotional experience (for instance, time of weaning) at behavioural level. In addition, studies have provided compelling evidence that during development environmental factors (such as social or isolated environment) dynamically modify animal's behaviour and brain development. Nevertheless, the impact of these two different developmental time windows of emotional experience have never been systematically studied and the neural mechanism remains unknown. In this study, I tested our working hypothesis that during late childhood the neuronal networks in various limbic areas such as the prefrontal cortex and hippocampus are reorganized in response to the timing and the extent of social interaction with the mother and the siblings. This was done by investigating the impact of time point of weaning (21 vs. 30), of social environments (social vs. isolation) and in addition, by investigating the *interactions* between these two factors on the neuronal morphology in the prefrontal cortex and hippocampus. The prefrontal cortex and the hippocampus are part of the limbic system which are involved in emotional self-regulation and the expression of emotional behaviours. Since, the time of weaning appears to be a major emotional challenge during late childhood it can be assumed that this transition to independence should involve major structural changes in limbic areas such as the prefrontal cortex and hippocampus, which are involved in emotional behaviours.

The findings of this study demonstrate that emotional experience (i.e. the extent of social experience with mother and the siblings) induces dendritic and synaptic reorganization, which occurs in a highly temporal, regional and dendrite-specific manner. The development of spine density was particularly sensitive to the amount of preweaning social experience, as the animals that spent less time with their mother i.e. early weaned demonstrated elevated spine densities in their dendrites of anterior cingulate cortex (ACd) and hippocampus. The dendritic length and complexity of apical dendrites in the prefrontal area, anterior cingulate cortex (ACd) and

orbitofrontal cortex (OFC) and in the CA3 area of hippocampus displayed only the *interactions* between the factors time of weaning x postweaning social environment and not the effect of time of weaning and social conditions alone. In the anterior cingulate cortex and in CA3 area, the most socially deprived group (early weaning and isolated postweaning i.e. EWI) displayed longer and more complex apical dendrites compared to other animals. In contrary, in orbitofrontal cortex these animals displayed reduction in apical dendritic length and complexity. In all these regions, the length and the complexity of basal dendrites remained unaffected by either treatment. In dentate gyrus, the dendritic length and complexity of granular neurons, mainly in the infra pyramidal layer of dentate gyrus displayed not only *interactions* between time of weaning x postweaning social environment but also the influence of time of weaning per se, and of social conditions alone. Similar to that in ACd and CA3, in dentate gyrus the animals with fewest social experience or EWI demonstrated longer and more complex infra and supra granular dendrites compared to other animals. Taken together, the findings demonstrate that the timing as well as the amount of social contact with family members significantly affects the refinement of prefrontal cortical and hippocampal synaptic networks which as an integral part of the limbic system are essential for emotional and cognitive behaviour.

In summary, the findings of this study may provide the neurobiological substrate for the behavioural changes induced by different emotional experiences.

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1.0 Introduction

Genes can predispose an organism to exhibit certain characters or traits but the quality of its development is determined by environmental factors. Neuroscientific studies have provided compelling evidences that during development environmental factors continuously and dynamically modify animal's behaviour and brain development (Hubel and Wiesel, 1970; Juraska, 1984; Kolb and Whishaw, 1998). The central nervous system (CNS) of mammals is not mature at birth and, although in most brain regions neurogenesis is complete, the fine detail of neuron shape and connections are established in early postnatal period. The initial development of the central nervous system involves the production and differentiation of neurons, the formation of axonal pathways, and the elaboration of vast numbers of synapses. The circuits that emerge from these processes mediate an increasingly complex array of behaviours in humans as well as in non-human species. Subsequent experience during postnatal life continues to shape neural circuits, the related behavioural repertoires, and ultimately cognitive abilities. These changes are most pronounced during developmental windows in early life called "critical periods". Classical studies on the visual system have shown that there is a critical stage for the building of synaptic contacts. If the input from one eye to the visual cortex is transitorily eliminated by covering the eye during the first week of life, the afferents from that eye are enabled to establish the correct number of synapses when the eye is opened again (Hubel and Wiesel, 1970; Blakemore et al., 1978). Not only dramatic changes such as total removal of visual input but also milder deprivation, obtained by reducing the degree of complexity of the environment during the early stages of life, affects the development of the visual system. For instance, rats reared in an isolated environment have reduced dendritic material in their visual cortical neurons compared to rats reared in enriched environments (Greenough and Volkmar, 1973; Juraska, 1984). Comparable to visual cortices, a critical period for the development of the neocortex is the early postnatal life, when the majority of synapses are formed and when much of synaptic remodeling takes place (Huttenlocher, 1984, 1990, 1994; Greenough et al., 1987). A variety of studies indicate that initial synaptic connections

are overabundant and lack precision and that this preliminary wiring pattern is refined by experience-evoked activity through selective elimination and stabilization of synapses and dendritic arborizations (Blue and Parnavelas, 1983; Huttenlocher, 1984; Rakic et al., 1986; Warton and McCart, 1989; Huttenlocher and Dabholkar, 1997).

Over the past 40 years an extensive literature has accumulated demonstrating that the structure of cortical neuron is influenced by various types of sensory, motor and learning experience (Greenough et al., 1987; Greenough and Chang, 1989; Kolb and Whishaw, 1998). Similar to the experience-dependent maturation of sensory cortices, evidence is accumulating that the prefrontal cortical regions and hippocampus, which are tightly connected to the limbic system, are even more dramatically shaped by early experience. The major trigger of synaptic refinement in these limbic areas appears to be social and emotional experience, upon which the synaptic wiring patterns of the associative cortical regions are continuously modified (Helmeke et al., 2001a, 2001b; Ovtscharoff and Braun, 2001, Poeggel et al., 2003a, 2003b; Bock et al., 2005; Ovtscharoff et al., 2006).

1.1 Weaning: the process of separation and individuation of the young mammal

The term weaning is derived from the Anglo-Saxon word *"wenian"* which means "to become accustomed to something different". One of the most important emotional challenges in mammals during late childhood is the time of weaning (Bateson and Young, 1981, Bateson et al., 1990; Cook, 1999). Since weanling pups become nutritionally and bio-behaviourally independent from their mothers and show quite striking behavioural transformations (Cramer et al., 1990; Thiels et al., 1990), it is not surprising that the process of weaning programmes the development of a number of physiological and neurobehavioural mechanism (Bateson et al., 1990; Smith, 1991; Nakamura et al., 2003; Kanari et al., 2005). It has been argued that weaning is not merely a transition from suckling to independent ingestion, but a unique, critical, irreversible behavioural metamorphosis determining adult affective and social behaviour (Cook, 1999; Kanari et al., 2005). A weanling rat pup actively involves itself into self-

grooming, play-fighting and feeding behaviours and shows gradual decline in suckling behaviours (Thiels et al., 1990). In a study on the behavioural development in dogs, Scott and Fuller, 1965 found that this period, during which puppies stop suckling but continue to live with their parents is crucial for their social and behavioural development. Not only in dogs, but also in cats and rodents, this crucial period has been shown to affect social behaviours (Bateson and Young, 1981; Thiels et al., 1990). Weaning-induced physiological changes involve subsequent increase in plasma, urinary cortisol levels as well as temporary reductions in weight and growth of the weanlings (Ichimura et al., 1987; Hay et al., 2001).

Generally, both farm and laboratory practices result in a more abrupt weaning than would be experienced in the absence of human management, making it of interest to elucidate what effects such practices have on subsequent adult behaviour and physiology. Abrupt weaning is implicated in premature interruption of the mother and the infant bond which is considered to be crucially formative for adjustment and social competence of infant in adulthood and is extensively studied in animal models (Janus, 1987a, 1987b; Fahlke et al., 1997; Cook, 1999). Studies have provided compelling evidence that environmental stimuli, provided by the mother, play a critical role in subsequent neural, endocrine, and behavioural development in nonhuman species such as rats (Hofer, 1994) and perhaps also in humans (Kuhn and Schanberg, 1998) and that maternal separation induces a number of behavioural abnormalities in rats such as anxiety, depression, altered cognition, increased activity and fearfulness in response to novelty (Hofer et al., 1989; Matthews et al., 1996; Kehoe et al., 1998; Caldji et al., 2000; Lehmann et al., 2000; Huot et al., 2001; Kalinichev et al., 2002). In humans, maternal separation or early deprivation is regarded as an animal model of child neglect/trauma that is associated with subsequent depression and anxiety in children (Brown et al., 1987; Heim and Nemeroff, 2001). In addition to modifying pup's behaviour, maternal separation interferes with physiological, structural and functional maturations of pups resulting in changes in their homeostatic and hormonal secretion (Kuhn et al., 1978; Hofer, 1984; Kuhn et al., 1990; Ladd et al., 1996, 2000, 2005; Plotsky et al., 2005); alterations in their neuronal structures such as the dendritic length, spine and synaptic densities (Helmeke et al., 2001a, 2001b; Ovtscharoff and Braun, 2001); alterations in their cortical neurotransmitter systems such as the dopaminergic,

serotonergic and GABA-ergic innervations (Crespi et al., 1992; Braun et al., 2000; Caldji et al., 2000) and reductions in resistance of pups to lethal disorders such as cancer (LaBarba and White, 1971).

The mother-infant interactions even at weaning appear important to the development of a wide variety of physiological features (Cramer et al., 1990; Thiels et al., 1990; Hofer, 1994; Van Oers et al., 1998). Learning in infant around the time of natural weaning appears important (Alberts, 1994) and studies in rodents have shown that the process of weaning interacts with emotional experiences encountered during later life which continuously shape adolescent and adult emotional behaviours (Laughlin and Zanella, 2002; Kikusui et al., 2004; Souza and Zanella, 2004; Poletto et al., 2006a, 2006b). In addition, a number of behavioural studies in rats, mice and other animals have shown that early weaning induces a wide range of behavioural changes. For instance, early weaned mammals show increased emotionality in response to novel environment, increased fearfulness, enhanced separation-induced vocalization and escape behaviours (Janus, 1987a, 1987b; Gardner et al., 2001; Orgeur et al., 2001; Tuchscherer et al., 2004). Furthermore, early weaning was shown to hamper social interaction in juvenile animals (Terranova and Laviola, 2001) and induce long-lasting anxiety and aggression in rat and mouse pups (Nakamura et al., 2003; Kikusui et al., 2004; Kanari et al., 2005).

Although on the behavioural level, the critical importance of emotional experience during specific sensitive time windows (for instance, time of weaning) has been investigated quite intensively, the underlying neuronal and synaptic adaptations of limbic cortical regions such as the prefrontal cortex and hippocampus in response to different neonatal emotional experiences still require more detailed investigation. Pharmacological studies have revealed an involvement of the neurohormones oxytocin and vasopressin in the process of weaning-induced behavioural transition (Kavushansky and Leshem, 2004). Biochemical studies in brain homogenates have shown that weaning interferes with the development of opioid receptors (Kelly et al., 1998; Terranova and Laviola, 2001). Nevertheless, the impact of the social interaction between the young rats and their mothers on neuronal development in limbic cortical regions such as the prefrontal cortex and hippocampus which are involved in the regulation of emotional behaviours is yet to be determined. Therefore, in this study I

have investigated the impact of social interaction between young rats and their mother on the neuronal development in pups.

1.2 Social environments: factors that dynamically shape adult behaviour and physiology

In social species such as mammals, interactions with conspecifics are essential to ensure normal neurological and physiological maturations and these interactions are provided by environments to which the animals are exposed during their development. An extensive literature has accumulated demonstrating that the prenatal and the postnatal environment of an animal play a critical role in shaping the behaviour and the physiology of an animal in its subsequent life (Einon and Morgan, 1977; Weinstock, 2001). Exposure to different social environments is reported to have differential effects on animal's behaviour and brain. For example, laboratory animals ranging from rats to cats and monkeys are placed in complex environments versus living in standard laboratory cages; there are large changes in length of dendrites and number of synapses throughout the primary visual and somatosensory cortex (Greenough and Volkmar, 1973; Beaulieu and Colonnier, 1987). Moreover, these animals have a greater number of astrocytes and a greater volume of capillaries per nerve cell (that provides greater supply of blood to the brain) compared to the caged animals, regardless of whether the caged animal lived alone or with companions (Black et al., 1987). On the other hand, laboratory animals reared in a deprived environment display decreased brain size, cortical thickness, dendritic tree complexity and neuron numbers in sensory and motor areas (Rosenzweig and Bennett, 1978, 1996; Herrmann and Bischof, 1986; Rollenhagen and Bischof, 1991).

Rearing rats in social isolation from weaning is a non-pharmacologic and nonlesion manipulation that is characterized by isolation of a rat from its counterparts at a socialization period that is critical for the development of its future social and adult behaviours (Einon and Morgan, 1977; Hol et al., 1999). There is compelling evidence demonstrating that social isolation in rats is implicated in the genesis of a number of psychological disorders; for instance, hyperactivity, anxiety, aggression, learning and

cognitive deficits, spatial working memory errors, enhanced response to novelty, greater tendencies towards preservation and an altered locomotor response to psycostimulants (Sahakian et al., 1975; Gentsch et al., 1988; Jones et al., 1991, 1992; Domeney and Feldon, 1998; Del Arco et al., 2004).

Epidemiological studies have revealed social isolation to induce acceleration of the development and growth of either transplanted or chemically induced tumors, enhancement of small intestinal sensitivity to chemotherapy and exacerbation of the autoimmune diseases in rats (Weinberg and Emerman, 1989; Liu and Wang, 2005; Chida et al., 2006). Moreover, isolation rearing has been proposed as a developmental animal model for certain neuropsychiatric disorders: mainly, schizophrenia, as both socially isolated animals and schizophrenic patients exhibit sensorimotor gating deficits, evidenced by a disrupted prepulse inhibition or the inability to filter responses to incoming sensory information (Geyer et al., 1993; Wilkinson et al., 1994; Bakshi and Geyer, 1999).

In addition, socially isolated rodents display an increased or decreased response of hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (Sanchez et al., 1998; Weiss at al., 2004). Furthermore, social isolation alters the dopaminergic and serotonergic system of the brain which is comparable to that seen in Attention Deficit Hyperactivity Disorder (ADHD) and schizophrenia (Jones et al., 1992; Bickerdike et al., 1993; Fulford and Marsden, 1998; Hall et al., 1998; Braun et al., 2000; Poeggel et al., 2003a). At neural level, social isolation reduces the amount of N-acetyl aspartate (a marker for neuronal loss and cellular dysfunction), impairs the firing rates of pyramidal cells in the prefrontal areas (Harte et al., 2004; Peters and O'Donnell, 2005), reduces the synaptic content, neurogenesis, synaptic plasticity in hippocampus (Varty et al., 1999; Lu et al., 2003), and induces morphological alterations in pyramidal and granular neurons of prefrontal cortex and hippocampus (Poeggel et al., 2003; Silva-Gomez et al., 2003). A few recent studies have provided evidence that social isolation interacts with emotional experience such as weaning; when social isolation (even for a very brief period) is carried out in early weaned animals they show impairment in emotional behaviour as well as in tasks that are regulated by the prefrontal cortex and hippocampus such as learning, memory and cognition (Laughlin and Zanella, 2002; Souza and Zanella, 2004).

Studies have extensively investigated the impact of social environments such as, social isolation and have demonstrated that it had enduring effects on animal's behaviour, physiology as well as neuronal architecture. In addition, some studies have investigated the impact of social isolation in combination with other environmental conditions such as the time of weaning at behavioural level. Nevertheless, the synaptic adaptations of limbic cortical regions such as the prefrontal cortex and hippocampus are yet to be determined. This study investigates the effects of social environments in combination with time of weaning on the neuronal development in rats.

1.3 The Limbic system: the emotional centre of the brain

In the 1850s, Paul Broca had originally used the term "limbic lobe" to refer to the parts of cerebral cortex that formed a rim (*Limbus* = rim, in Latin) around the corpus callosum on the medial face of the hemisphere. For most of the century, the scientific community believed the "limbic lobe" to be primarily cortical until it became evident from studies that many other major nuclei were also limbic. Functional studies by Heinrich Klüver and Paul Bucy in 1937, 1938 and 1939 began to elucidate complex emotional and motivational process associated with limbic lobe that became the forefront in limbic biology.

By inducing bilateral temporal lobectomy in monkeys Klüver and Bucy reported marked changes in their emotional behaviour; for instance, psychic blindness, diminished anger, fear, oral tendencies and altered sexual behaviour. After their pioneering studies it became evident that the medial temporal lobe (MT) was a crucial component of the limbic system. It was, however, James Papez in 1937, who provided an anatomical presumption of the limbic system and proposed that specific brain regions of the medial temporal lobe are devoted to emotional experience and expression. Papez argued that hypothalamus influences the expression of emotion and it is known that the emotions reach consciousness and that emotion can be effected by higher cognitive functions. He showed that the cingulate cortex and the hypothalamus are interconnected

via projection from the mammillary body to the anterior nucleus of dorsal thalamus, which projects in turn to cingulate gyrus. The cingulate gyrus projects to the hippocampus and the hippocampus projects to the hypothalamus via a large fibre bundle called fornix. Papez suggested that these pathways provided the connections necessary for cortical control of emotional expression, and they became known as the “Papez circuit” (Figure-1B).

Over time, the circuitry initially described by James Papez has been revised by Paul D. MacLean and others and is shown to include parts such as the orbital and medial prefrontal cortex, ventral parts of basal ganglia, the mediodorsal nucleus of thalamus, and a large nuclear mass in the temporal lobe anterior to hippocampus called the “amygdala”. This set of structures, together with the hippocampus, hypothalamus and cingulate gyrus, is generally referred to as the “limbic system” (Figure-1A, 1B). All these structures interconnect intensively and none of them is solely responsible for any specific emotional state. However, some contribute more than others to this or that kind of emotion.

1.A

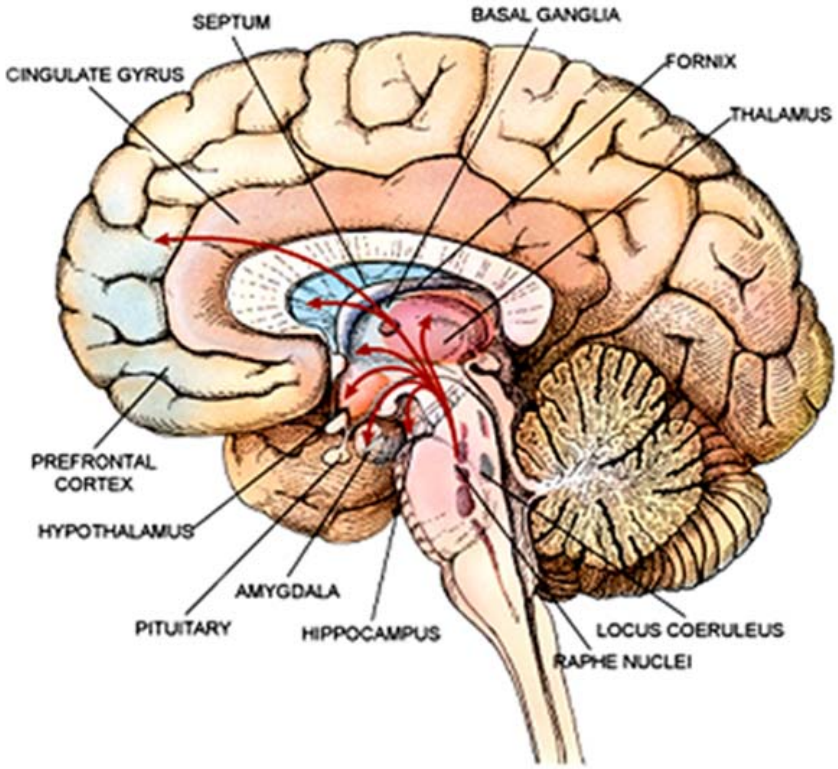


Figure 1. A) The major structures of the limbic system. The structures described both by James Papez and Paul D. MacLean is shown in the figure. Figure adapted from an educational website, (www.humanityinunity.org/HIU/teaching).

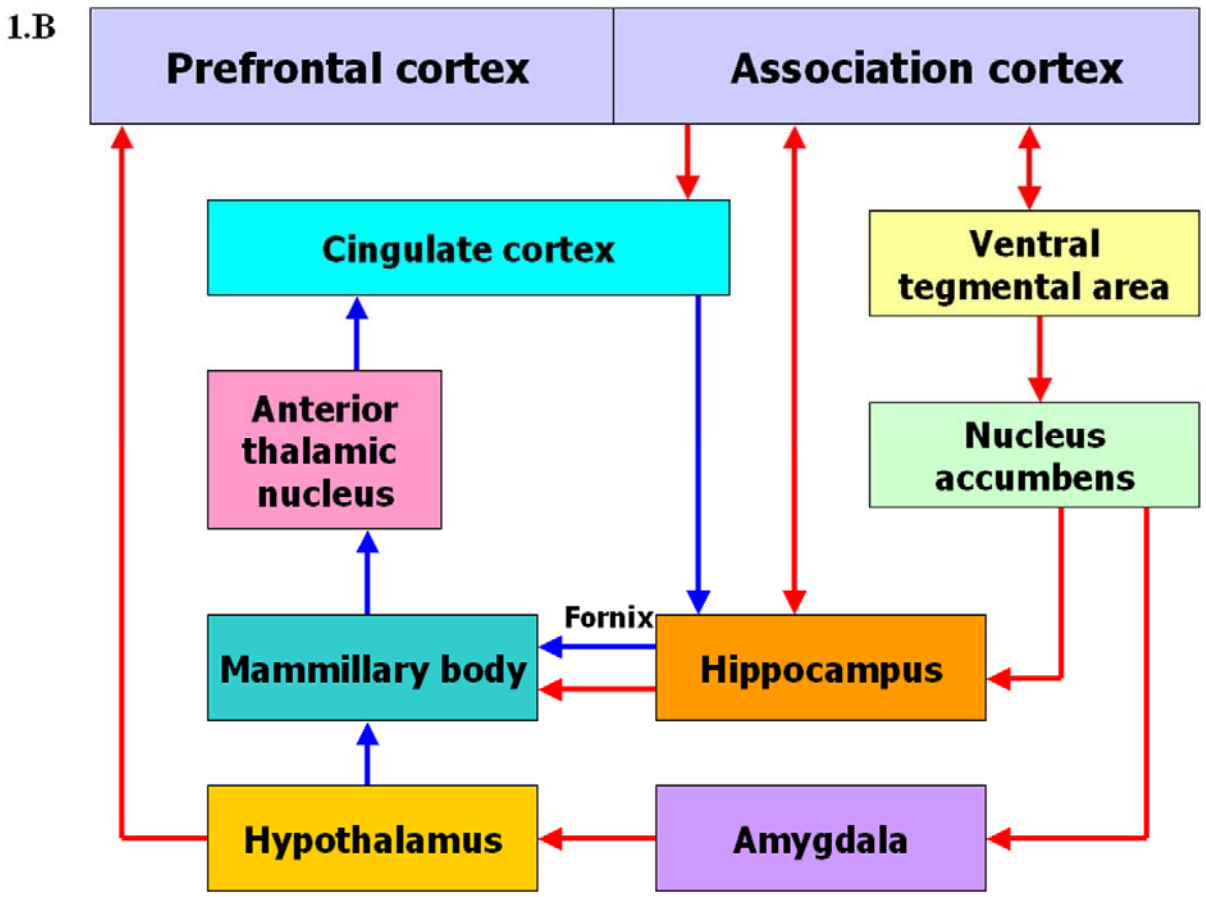


Figure 1.B) A neural circuit for emotion proposed by James Papez and extended by Paul D. MacLean. The circuit originally proposed by Papez is indicated by blue arrows; more recently described connections are indicated by red arrows. Reciprocal connections are indicated by double-headed arrows. Single-headed arrows indicate non-reciprocal connections (Adapted from Eric R. Kandel, 1982).

1.3.1 Limbic system part I: The prefrontal cortex (PFC)

For a long time after Brodmann's studies, the prefrontal cortex was considered unique to the primate species and called the "frontal granular cortex" (Brodmann, 1909).

Subsequently, years later, a series of studies revealed that not only primates but also rats possessed a prefrontal cortex that very much resembled the prefrontal cortex of primates on the basis of functional analogy and a projection from the mediodorsal nucleus of thalamus that characterizes the prefrontal cortex in nonprimate species (Rose and Woolsey, 1948; Akert, 1964; Krettek and Price, 1977; Uylings and Van Eden, 1990; Fuster, 1997; Groenewegen and Uylings, 2000; Uylings et al., 2003).

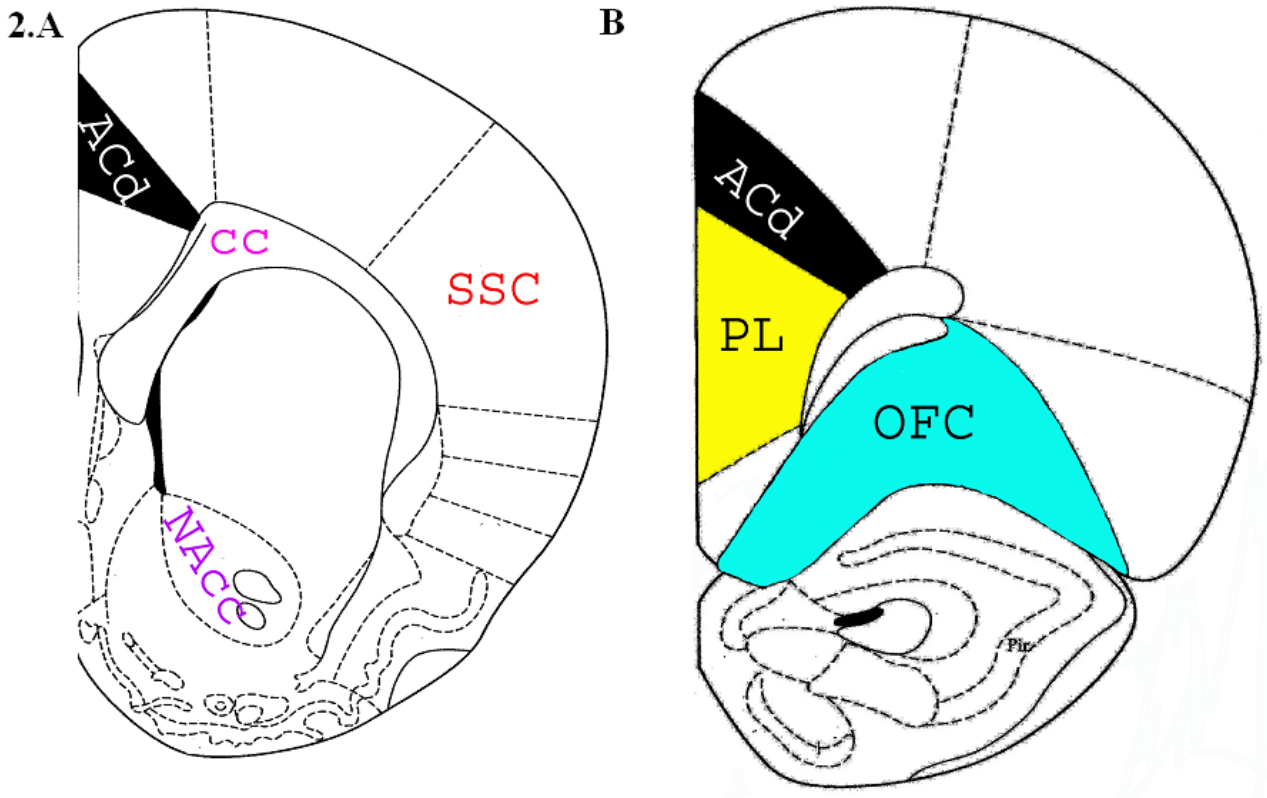


Figure 2. (A-B) Schematic diagram of a coronal section through the rat prefrontal cortex (modified from Paxinos and Watson, 1998). (A) Bregma 1.70 mm (B) Bregma 3.70 mm. Prefrontal areas that I have analysed in my study include the ACd = dorsal anterior cingulate cortex and OFC = orbitofrontal cortex. cc = Corpus Callosum, NAcc = Nucleus accumbens, PL = prelimbic cortex, Pir = Piriform cortex and SSC = Somatosensory cortex.

1.3.2 Anatomy and connections of the prefrontal cortex

The prefrontal cortex is the association cortex of the frontal lobe. It comprises areas 8-13, 24, 32, 46, and 47 according to the cytoarchitectonic map of Brodmann (1909). In rats, it is generally divided into three topologically different regions. First, a medially located cortical region, the medial prefrontal cortex (mPFC), which 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 further subdivided into four distinct areas, the medial precentral area (PrCm), dorsal anterior cingulate area (ACd), prelimbic area (PrL) and the infralimbic area (IL) (Figure- 2A, B and 7A). Second, a ventrally located cortical region that is termed the orbital prefrontal cortex (OFC) and that lies in part dorsal to the caudal end of the olfactory bulb in the dorsal bank of the rhinal sulcus (Figure – 2B and 7A). Third, a laterally located cortical region, the lateral or sulcal prefrontal cortex that in rats is located in the anterior part of rhinal sulcus (Krettek and Price, 1977a; Sarter and Markowitsch, 1983, 1984; Groenewegen, 1988; Heidbreder and Groenewegen, 2003). All prefrontal areas (the medial, the lateral, as well as the orbital) have a prominent architectonic feature in common, viz. they lack the small granular cell layer IV. It is this layer (layer IV) that receives the main thalamic projection in the visual and primary sensory cortices (Herkenham, 1980). In the rat prefrontal cortex, the fibres from the mediodorsal nucleus terminate mainly in layer III.

Neural information from various sensory areas and cerebral cortex reaches the prefrontal cortex through the thalamic mediodorsal nucleus in layer 3 of cerebral cortex. Neural information processed by the prefrontal cortex is relayed by populations of projection pyramidal neurons situated in deep layer 5 and 6 of the cortex.

Connections of medial prefrontal cortex (mPFC): The medial prefrontal cortex consisting of anterior cingulate cortex (ACd) receives input via the mediodorsal nucleus of thalamus (Krettek and Price, 1977a; Ray and Price, 1993). The ACd is connected to various limbic structures such as the basolateral nucleus of amygdala, the lateral hypothalamus, hippocampus and enthorhinal cortex (Krettek and Price, 1977b; McDonald, 1991; Carmichael and Price, 1995a). It also sends projections to basal ganglial structures such as striatum, nucleus accumbens and ventral tegmental area

(McDonald, 1991; Carr and Sesack, 2000). It receives projections from brain stem structures-the periaqueductal grey matter, parabrachial nucleus, nucleus of solitary tract, motor nucleus of vagus, parts of reticular formation, and spinal cord (Floyd et al., 2000). The medial prefrontal cortex also receives auditory, visual, olfactory, gustatory, somatosensory and motor input from corresponding sensory and motor cortices (Amaral, 1987; Cavada and Goldman-Rakic, 1989b).

Connections of the orbitofrontal cortex (OFC): The OFC receives direct connection from the primary taste cortex, primary olfactory cortex and pyriform cortex (Barbas, 1993; Baylis et al., 1995). Visual informations reach the OFC from the inferior temporal cortex, the cortex in the superior temporal sulcus and the temporal pole (Barbas, 1988, 1993). The OFC receives auditory inputs and somatosensory inputs from somatosensory cortical areas 1, 2 and SII in the frontal and pericentral operculum, and from insula (Barbas, 1988; Carmichael and Price, 1995b). The OFC receives strong input from amygdala (Carmichael and Price, 1995a; Schoenbaum et al., 1998). It also receives inputs via the mediodorsal nucleus of thalamus (Krettek and Price, 1977a). It sends projections to inferior temporal cortex, enthorhinal cortex, cingulate cortex, preoptic regions, lateral hypothalamus, ventral tegmental area and to caudate nucleus (Petrides and Pandya, 1984; Pandya and Yeterian, 1996; Ongur and Price, 2000; Rolls, 2004).

Each of the major prefrontal regions – the medial, orbital and lateral- are extensively connected with each other suggesting that they participate in concert in central executive functions (Jacobson and Trojanowski, 1977a, 1977b; Pandya and Yeterian, 1990). Some of the corticocortical connectivity of the PFC are interhemispheric, and almost all of it is reciprocal and topologically organized (Figure-2C) (Cavada and Goldman-Rakic, 1989a, 1989b; Pandya and Yeterian, 1990).

With respect to the transmitter system, the PFC receives cholinergic innervations from the magnocellular nucleus of the basal forebrain and from laterodorsal tegmental nucleus (Gaykema et al., 1991; Ghashghaei and Barbas, 2001). The noradrenergic fibres from the locus coeruleus and the serotonergic fibres from the dorsal and median raphe nuclei also projects to the prefrontal cortex (Jodo and Aston-Jones, 1997; Hajos et al., 1998; Jankowski and Sesack, 2004). The ventral tegmental area (VTA) is the origin of major dopaminergic input to the prefrontal cortex (Kalsbeek et al., 1989). The

histaminergic neurons in the tuberomammillary hypothalamic region also have connections with prefrontal cortex (Wouterlood et al., 1987).

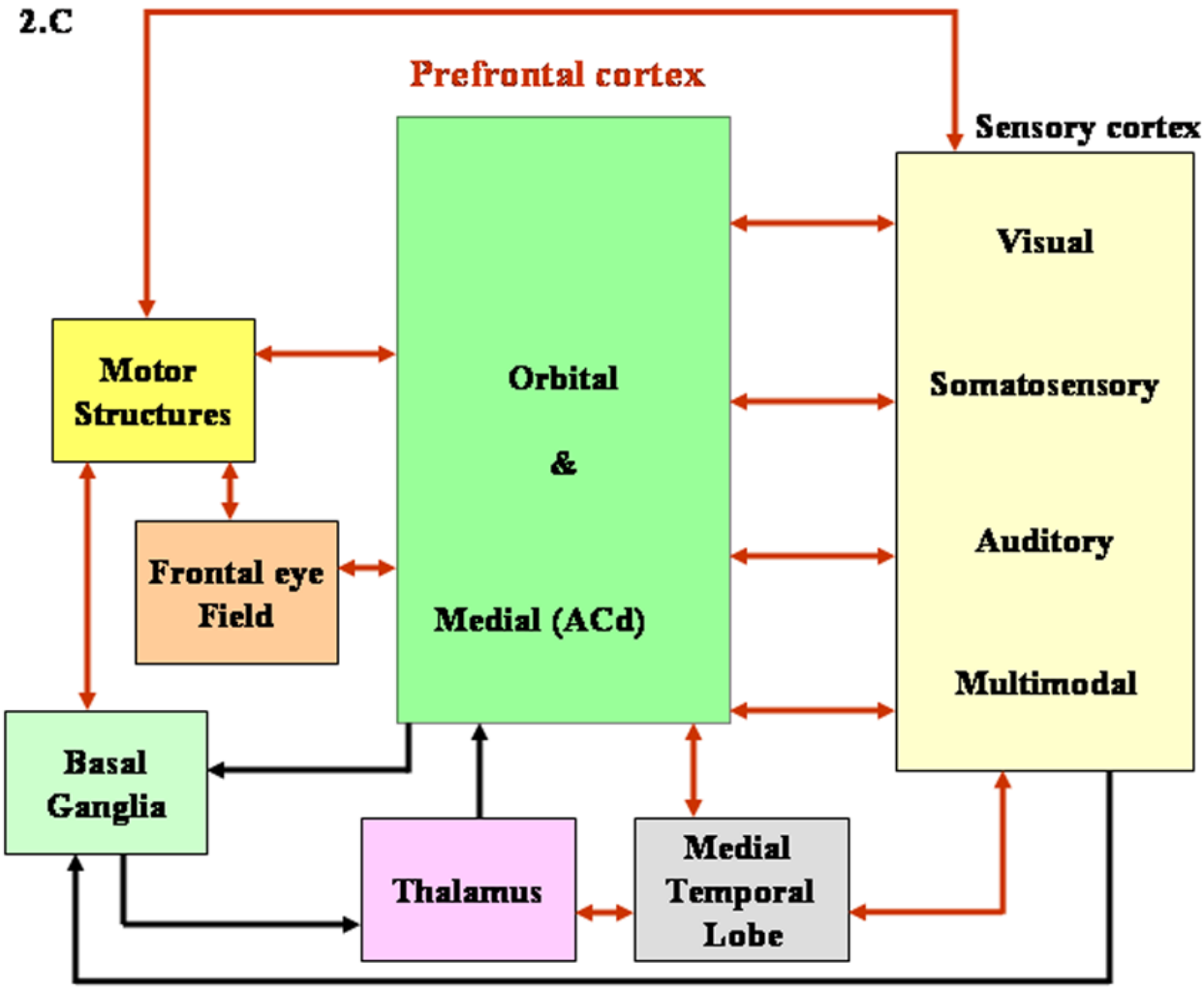


Figure 2.C) Some of the extrinsic and intrinsic connections of the prefrontal cortex (PFC). ACd = dorsal anterior cingulate cortex. Limbic structures such as, hippocampus, amygdala and hypothalamus are located in the medial temporal lobe. Reciprocal connections are indicated by double-headed red arrows; simple black arrows indicate non-reciprocal connections (Adapted from Miller and Cohen, 2001. Annu Rev Neurosci. 24:167-202).

1.3.3 Neuropsychology and plasticity of the prefrontal cortex (PFC)

First, I will very briefly describe the most general functions of primate and rodent prefrontal cortex and then describe in detail those functions that are regulated by the anterior cingulate and the orbital prefrontal areas. It is generally accepted that the prefrontal cortex is involved in different aspects of executive control that guides behaviour, thought, and affect using working memory such as regulation of attention, planning, impulse control, mental flexibility, and the initiation and monitoring of action, including self-monitoring. Lesion to the PFC produced symptoms such as forgetfulness, distractibility, impulsivity, preservation, locomotor hyperactivity and disorganization (Goldman-Rakic, 1995; Barbas, 1995, 2000; Godefroy and Rousseaux, 1996; Fuster, 1997; Miller, 2000, Wallis et al., 2001; Aron et al., 2004). However, there seems to be division of labor among distinct prefrontal cortices in processing information underlying cognition, emotion and memory.

1.3.3(A) Functions of medial prefrontal/anterior cingulate cortex (mPFC/ACd)

The medial prefrontal cortex as a whole is implicated in attentional processes, working memory and behavioural flexibility (Fuster, 1997) The dorsal anterior cingulate of medial prefrontal area has been hypothesized to have specific roles in emotional and cognitive processing (Carter et al., 1995; Bush et al., 1998, 1999; Luu et al., 2000; Kerns et al., 2004). Studies have demonstrated that the anterior cingulate area has a specific role in different aspects of emotions such as processing emotions or emotion-related stimuli (Morris et al., 1999; Hadland et al., 2003; Rolls et al., 2003; Ueda et al., 2003), emotional decision making, emotional experience (Bush et al., 2000; Damasio et al., 2000), expression of emotions (Neafsey, 1990), emotional self regulation (Posner and Rothbart, 1998; Davidson, 2000; Levesque et al., 2004) and emotional behaviour (Morgan et al., 1993; Hornak et al., 2003; Sinha et al., 2004).

In humans, lesion of anterior cingulate cortex for the treatment of the affective disorders produces striking personality changes, including lack of emotional liability (Phan et al., 2002). Imaging studies have shown that the anterior cingulate area has been activated during studies of emotional processing in normal healthy volunteers as well as in symptom provocation studies in a number of psychiatric disorders such as anxiety,

simple phobia and obsessive-compulsive disorders (Phan et al., 2002). It has also been activated repeatedly by induced sadness/happiness in normal subjects and in individuals with major depression (Phan et al., 2002; Pelletier et al., 2003; Killgore and Yurgelun-Todd, 2004). In rodents and monkeys, selective lesions of the ACd can increase conditioned fear response, impair their performance in various tasks requiring attention such as shuttle avoidance task and single-trial random-foraging task (Uylings et al., 2003). Recent anatomical studies in rodents have indicated that the neuronal structures such as the dendritic spines and dendrites in medial prefrontal cortex in general and of anterior cingulate cortex in particular are dramatically shaped by various emotional experience such as brief and chronic maternal separation, social isolation and prenatal stress (Helmeke et al., 2001a, 2001b, Silva-Gomez et al., 2003; Bock et al., 2005; Murmu et al., 2006). Moreover, other types of negative or neutral experiences such as stress or injection of corticosterone also induces major alterations in neuronal structures of medial prefrontal cortex (Wellman, 2001; Seib and Wellman, 2003; Cook and Wellman, 2004; Radley et al., 2004; Brown et al., 2005). Experience-induced changes in the neurochemistry and the synapses of medial PFC are also described (Braun et al., 2000; Ovtscharoff and Braun, 2001; Poeggel et al., 2003).

1.3.3(B) Functions of orbitofrontal cortex (OFC)

Ever since Harlow (1848) described the famous case of Phineas Gage, it has been known that lesions of orbital prefrontal cortex often induce dramatic changes of personality (Damasio et al., 1994; Fuster, 1997). The orbital prefrontal cortex through its connection with amygdala (Davis, 1992; Le Doux, 1993) plays an important role in emotional reactions to faces and objects. Patients with OFC damage show impairment in the identification of facial emotional expression and display inappropriate social behaviour (Hornak et al., 1996). In addition, studies have indicated that the orbitofrontal cortex is implicated in the regulation of emotional behaviour (Quirk et al., 2000).

Both in animals and humans, orbitofrontal cortex lesion interrupts emotional responses necessary for communicating with their conspecifics and consequently disrupts their social interactions. For instance, studies have demonstrated that damage to orbitofrontal cortex in primates produces emotional changes (e.g decreased aggression to human or to stimuli that are associated with fear such as a snake) (Butter and Snyder, 1972, De Bruin et al., 1983; Bechara et al., 1994, Hornak et al., 1996, 2003).

Furthermore, a very important function of orbitofrontal cortex is the evaluation of emotion-related reinforcement contingencies (in other words, representing rewards and punishers) and in learning stimulus-reinforcement associations (Thorpe et al., 1983; Gaffan et al., 1993; Critchley and Rolls, 1996; Rolls et al., 1996, 2003; Baxter et al., 2000; O'Doherty et al., 2001). Animals with orbitofrontal lesion are impaired at tasks (e.g. Go/NoGo task, NoGo trials) which involve learning about which stimuli are rewarding and which are not and especially in altering behaviour when reinforcement contingencies change (Butter, 1969; Iversen and Mishkin, 1970; Jones and Mishkin, 1972). In addition, damage to orbitofrontal cortex also leads to severe disorders of attention, hyperkinesis and deficits in processing of olfactory and gustatory information (Rolls, 2004). In humans, OFC damage can lead to euphoria, irresponsibility and lack of affect (Kolb and Whishaw, 1998). A recent study by Murmu et al., (2006) has demonstrated that emotional experiences such as prenatal stress can significantly alter the dendritic spines and the length and complexity of pyramidal neurons in orbitofrontal cortex of the offspring.

1.4 Limbic system – part II: The hippocampus/hippocampal formation

Hippocampus derives its name due to its visual resemblance to a seahorse (*hippocampus = sea horse in Greek*) (Figure- 3A). Located in the medial temporal lobe (MT) the hippocampus plays a key role in learning, memory and navigation (Krebs et al., 1989; Jarrard, 1993; Clark and Squire, 1998; Eichenbaum et al., 1999; Maguire et al., 2000). Lesion to hippocampus in humans produces anterograde amnesia (Milner, 1966). Hippocampus, as an integral part of the limbic system, also plays an important role in some aspects of emotion (Sinha et al., 2004). In addition, in rats the hippocampus is involved in the regulation of neuroendocrine response to stress. As a target for adrenal steroids it constitutes the major negative feedback regulation of the stress via the hypothalamic-pituitary-adrenal (HPA) axis thereby, providing a model for studying

neurobiological consequences of stress (Sapolsky et al., 1991; McEwen and Sapolsky, 1995).

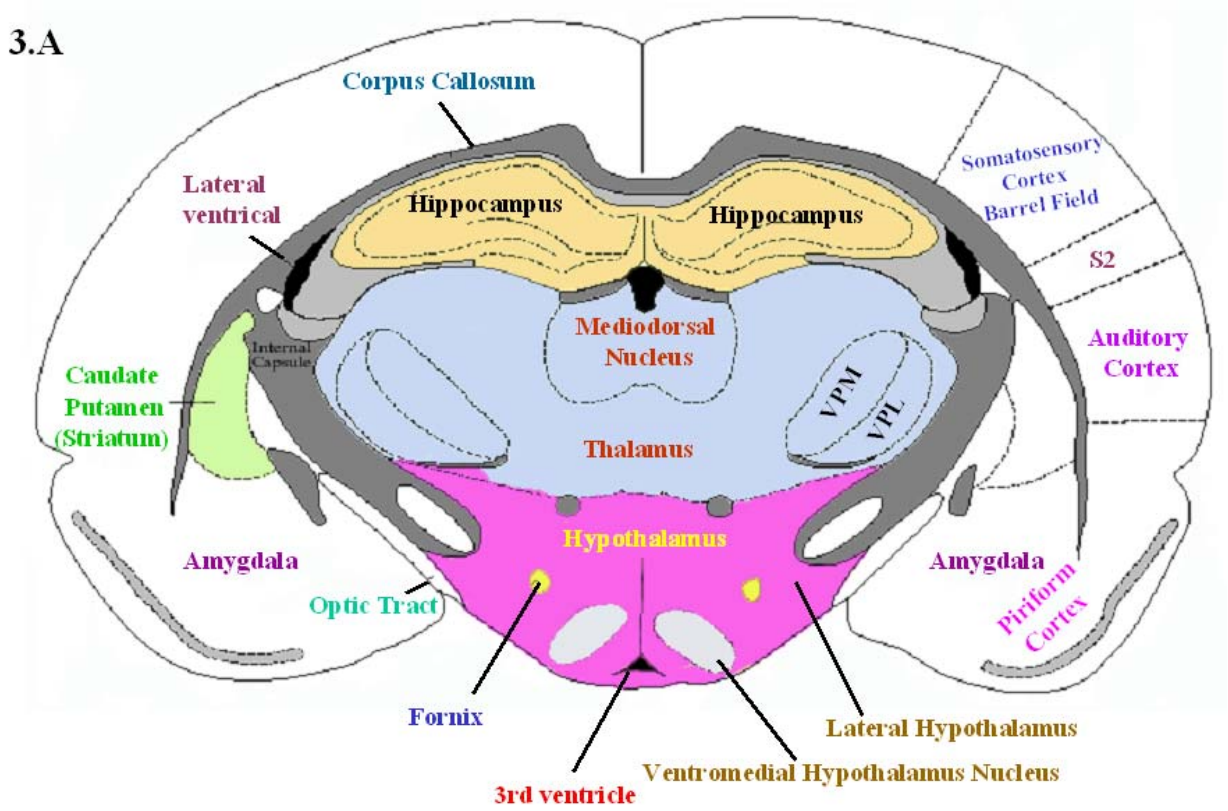


Figure 3.A) Schematic diagram of a coronal section through the rat hippocampus (modified from Paxinos and Watson, 1998). Bregma –3.14 mm. S2 = secondary somatosensory cortex, VPL = Ventral posterolateral thalamic nucleus and VPM = Ventral posteromedial thalamic nucleus.

1.4.1 Anatomy and connections of the hippocampal formation

The hippocampal formation comprises four relatively simple cortical regions. These include the dentate gyrus, the hippocampus proper (which can be divided into three sub-fields, namely CA1, CA2 and CA3), the subicular complex (which can also be divided into three subdivisions: the subiculum, presubiculum and parasubiculum) and the enthorhinal cortex which, in rodents, is generally divided into medial and lateral

subdivisions. The hippocampal formation receives input via thalamus (Dolleman-Van Der Weel and Witter, 1996) and direct hippocampal projections terminate in distinct limbic structures such as the orbital, cingulate, and dorsolateral prefrontal cortex, amygdala, hypothalamus, nucleus accumbens and ventral tegmental area (Swanson, 1981; Kohler et al., 1985; Mello et al., 1992; Gasbarri et al., 1994; Verwer et al., 1997). The hippocampal formation is also extensively connected to non-limbic structures such as the septum, olfactory, occipital and parietal cortex (Haberly and Price, 1978; Linke et al., 1995; Insausti et al., 1997).

Information is acquired in the hippocampus through processing in one or more of the polymodal association cortices such as frontal-parieto-occipital-temporal cortices that synthesize visual, auditory and somatic information. From there the information is conveyed in series of parahippocampal and perihinal cortices and then to the enthorhinal cortex (Caballero-Bleda and Witter, 1994) (Figure-3B). The axons of the enthorhinal cortex, known as the perforant path, make connections with granular cells of the dentate gyrus (Hjorth-Simonsen and Jeune, 1972; Zimmer and Hjorth-Simonsen, 1975). The axons of granular cells of dentate gyrus through the mossy fibre pathway project in turn to CA3 area of hippocampus proper (Frotscher, 1985). From there the axons of CA3 divide into two branches; one branch forms the commissural fibres that projects to the contralateral hippocampus via the corpus callosum. The other branch forms the Schaffer collateral pathways that make connections with the neurons in area CA1 (Amaral and Witter, 1989). Lastly, the axon of CA1 projects to the neuron of the subiculum and finally back to the enthorhinal cortex (Figure-3B). Thus, in processing information to the hippocampus, the enthorhinal cortex has dual functions. First, it is the main input to the hippocampus via the perforant pathway; second, the enthorhinal cortex is also the major output of the hippocampus.

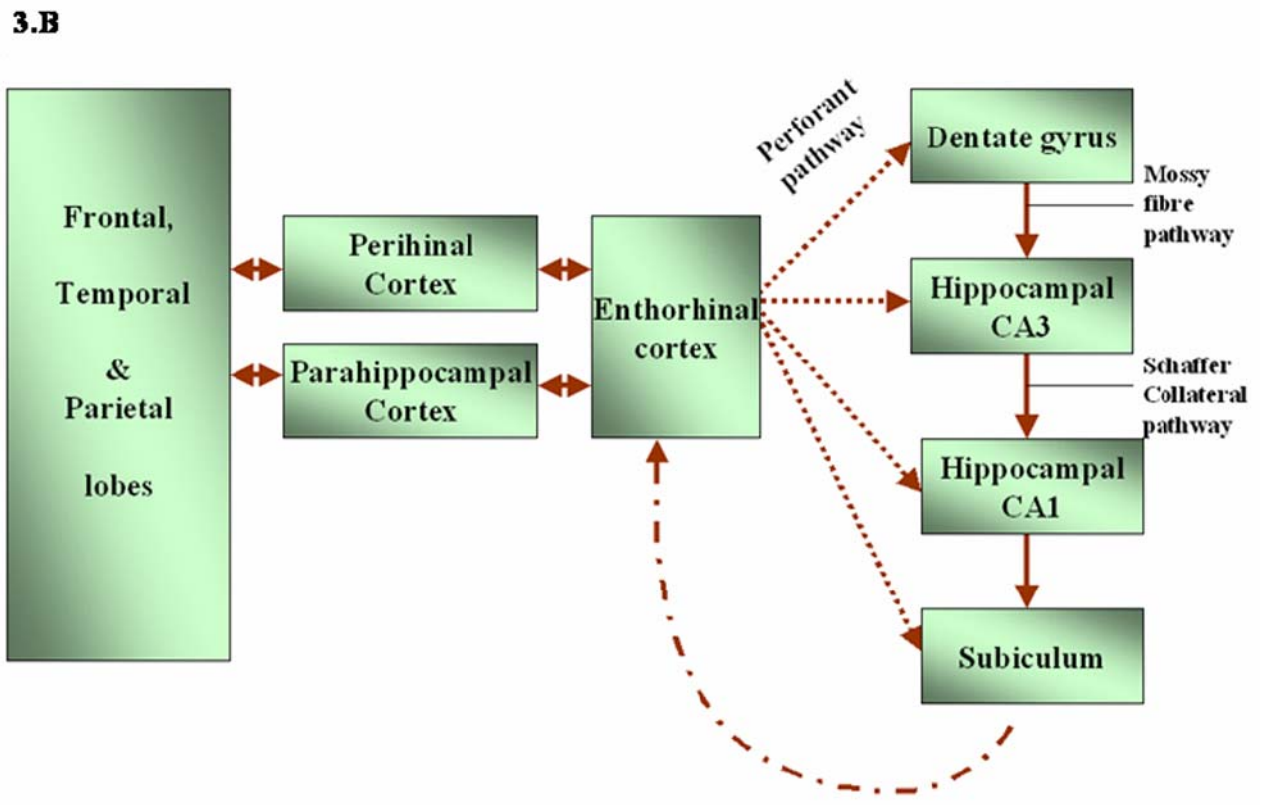


Figure 3.B) The input and output pathways of hippocampal formation (Adapted from Eric R. Kandel, 1982, Fundamentals of Neuroscience). Double-headed arrows in the figure indicate reciprocal connections. Single-headed arrows indicate non-reciprocal connections.

1.4.2 Plasticity of the hippocampus

The hippocampus is highly plastic and undergoes considerable change following different kinds of experiences. In fact, most of the hippocampal synapse has plastic properties, which may play a role in learning processes (Bliss and Lomo, 1973; Moser et al., 1994; Andersen et al., 1996). Experiences of all kinds, positive or negative, can cause structural changes in the hippocampus. For instance, species of birds that store large number of food items and use an accurate, long-lasting spatial memory to retrieve their stores, have comparatively large hippocampal volume than species that are nonstorers (Krebs et al., 1989; Clayton and Krebs, 1995). Similarly, humans with high dependence on hippocampal-related navigational tasks (e.g. London taxi drivers) have relatively large hippocampal volume compared to those that do not (Maguire et al.,

2000). In addition, neutral or positive experiences such as voluntary physical activity, exercise and running enhance neurogenesis, learning and long-term potentiation (LTP) in hippocampi of laboratory animals (Van Praag et al., 1999). Environmental conditions also modulate hippocampal plasticity. For instance, rodents and primates reared in enriched environments display enhanced neurogenesis in the dentate gyrus of hippocampus (Kozorovitskiy et al., 2005; Leggio et al., 2005). Similarly, animals reared in deprived or social isolation show alterations in the morphology and the neurochemistry of hippocampus (Varty et al., 1999; Bartesaghi and Serrai, 2001; Del-Bel et al., 2002; Poeggel et al., 2003).

The human hippocampus undergoes atrophy in the aftermath of traumatic stress, recurrent depressive illness, schizophrenia, Cushing's syndrome as well as in some aging individuals (Starkman et al., 1992, 1999; Golomb et al., 1994; Bremner et al., 1995, Lupien et al., 1998; Kalisch et al., 2006). The hippocampal formation is also vulnerable to damage from seizures, ischemia and head trauma (McEwen, 2001). Recently, a growing body of evidence indicates that the rodent hippocampus is highly vulnerable to stress and that any kind of stress, chronic or acute can cause abnormalities in hippocampal structures and hippocampal-dependent behaviours. For instance, stress is implicated in impaired performance on hippocampal-related tasks, significant atrophy and loss of the hippocampal neurons and hippocampal cell death (Watanabe et al., 1992; Magarinos and McEwen, 1995; Magarinos et al., 1996; Lambert et al., 1998; McKittrick et al., 2000; Sousa et al., 2000; Westenbroek et al., 2004).

1.5 Dendritic spines – structure and plasticity

The human brain contains at least 100 billion neurons; hence, highly sophisticated and efficient mechanisms are needed to enable communication among this astronomical number of elements. This is made possible by the huge number of contacts known as "synapse" that one neuron makes with the other. Some synapses, particularly the excitatory ones, often form at small appendages known as "dendritic spines" that are present in large numbers on the dendrites.

Dendritic spines were discovered by Santiago Ramon y Cajal in 1888, who, applying the relatively novel Golgi method, noticed that Purkinje cell dendrites were covered with espinas or small thorns (Spanish: *"espinas" = thorns*) (Cajal, 1888). In 1891, he put spines in the spotlight of the nervous system by proposing that spines not only served to connect the axons and dendrites, but also were the site of long-term, stable memory in CNS neurons. Sixty years later, the recently developed technique of electron microscopy (EM) enabled investigators to explore the structure of dendritic spines in detail. Studies by Gray, in 1959, proved Cajal's hypothesis correct by showing that spines were indeed the site of synaptic contact. Now, it is known that more than 95% of the excitatory synapse onto the principal neuron (Pyramidal and stellate) in the cerebral cortex occur at dendritic spines (Gray, 1959).

Dendritic spines generally consist of a head (up to a micron in length) attached to a dendrite via a stalk or a neck and can take a variety of shapes. Traditionally, based on the ultrastructural analysis of cerebral cortex (Peters and Kaiserman-Abramof, 1970), spines have been classified into four types: filopodia-like spines, thin spines, stubby spines and mushroom-like spines. Dendritic spines are regarded as biochemical compartment because of the unique calcium compartments that they contain (Wickens, 1988; Koch and Zador, 1993; Yuste et al., 2000). Besides, the spines possess voltage-gated calcium channels, glutamate-activated channels, primarily of NMDA type and calcium stores (Segal, 1995; Yuste and Denk, 1995; Korkotian and Segal, 1999; Kovalchuk et al., 2000). Dendritic spines are characterized by the absence of intracellular organelles such as mitochondria, microtubules or ribosomes and by the presence of a specialized form of smooth endoplasmic reticulum termed the "spine apparatus" (Fifkova et al., 1983, Fifkova, 1985). Although spines lack neurofilaments they contain dense network of actin filament (Jones and Powell, 1969; Blomberg et al., 1977; Crick, 1982; Fifkova and Delay, 1982; Matus et al., 1982).

The dendritic spines were traditionally assumed to be relatively stable structures; however, this idea was challenged in 1982 by Crick who proposed that spines are motile structures that move in response to synaptic stimulation (Crick, 1982; Fischer et al., 1998; Dunaevsky et al., 1999; Lendvai et al., 2000). The mechanism responsible for this

motility is actin-dependent and is developmentally regulated (Blomberg et al., 1977; Fifkova and Delay, 1982; Fischer et al., 1998; Dunaevsky et al., 1999).

A number of experimental and behavioural conditions have been associated with changes in spine morphology, number and density. Studies have demonstrated that spines are highly plastic structures and changes in spine number and morphology are associated with changes in neuronal activity and experience. For example, light deprivation in mice causes reversible reductions in the number of spines (Globus and Scheibel, 1967; Valverde, 1967, 1971). Similarly, increase in spine density occurs after visual stimulation (Parnavelas et al., 1973). A recent *in vivo* study indicates that dendritic spines appear and disappear in neocortex following novel sensory experience such as whisker trimming (Holtmaat et al., 2006). In addition, sensory deprivation caused significant alteration in the motility of spines in the barrel and the visual cortex *in vivo* (Lendvai et al., 2000; Majewska and Sur, 2003; Konur and Yuste, 2004). Other environmental manipulations such as rearing animals in complex environments enhance spine density in cortical neurons (Globus et al., 1973; Kozorovitskiy et al., 2005). Similarly, rearing animals in a deprived environment consisting of parental separation and social isolation significantly alters spine density (Connor and Diamond, 1982; Rollenhagen and Bischof, 1991; Silva-Gomez et al., 2003).

Events such as space flight also cause alteration of the spine morphology (Belichenko and Krasnov, 1991). A reduction in the size of the spine has also been reported after the first orientation flight in honeybees (Brandon and Coss, 1982). In birds, spine morphological plasticity is observed during postnatal development (Rausch and Scheich, 1982) and imprinting (Bradley and Horn, 1979; Bock and Braun, 1999b). Various learning and training tasks are implicated in changes in spine morphology and number (Moser et al., 1997; O'Malley et al., 1998, 2000; Knafo et al., 2001; Leuner and Shors, 2004). A single learning event in the life of a young chick has been shown to produce rapid and marked changes in spine density in selective regions of its brain (Lowndes and Stewart, 1994). Some mammals, such as squirrels, have been documented to lose 40% of their spines during hibernation and to recover them in a few hours after arousal from hibernation (Popov et al., 1992; Popov and Bocharova, 1992).

Activity related changes in dendritic spines have been extensively reported by studies involving stimulation protocols associated with long-term potentiation (LTP),

which is regarded as a cellular mechanism for learning and memory. LTP induced by tetanic stimulation produces a spectrum of effects on dendritic spines, ranging from changes in spine dimensions, synaptic contact area and shape, spine head bifurcation, to the formation of novel spines or disappearance of existing ones (Lee et al., 1980; Trommald et al., 1996; Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999).

Considerable changes in spine morphology and number occur after psychostimulant sensitisation (Robinson and Kolb, 1997, 1999; Li et al., 2003). Changes in spine form and number have also been observed *in vitro*. In dissociated cultures as well as in brain slices, pyramidal neurons have increased spine densities compared to those found *in vivo* (Papa et al., 1995; Boyer et al., 1998; Kirov et al., 1999). Pharmacological manipulations also influence spine morphology and number. For example, stimulation of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor is needed for the maintenance of spines, whereas blocking AMPA receptors reduces the number of spines (McKinney et al., 1999). Alternatively, synaptic blockade with Mg^{++} and low CA^{++} increases both spine number and size (Kirov and Harris, 1999). This is also found in cultured neurons disinhibited with bicuculline (Papa and Segal, 1996) and after stimulation of internal calcium release (Korkotian and Segal, 1999).

Moreover, changes in dendritic spine morphology also occur with development and aging (Purpura, 1974). Studies have indicated that spines are overproduced and later reduced in number during development and aging (Cajal, 1904). Indeed in the process of aging, cortical neurons lose up to 50% of their dendritic spines (Feldman and Dowd, 1975; Scheibel et al., 1975). Even during the estrous cycle of some mammals, large number of spines are produced in the hippocampus and later eliminated in substantial numbers (Woolley et al., 1990; Woolley and McEwen, 1993). Spine densities are reported to be altered following stressful experience (Sunanda et al., 1995). Many diseases, such as mental retardation, dementia, Down's syndrome, irradiation, malnutrition, fragile X syndrome and epilepsy can produce abnormalities in spine morphology (Marin-Padilla, 1972; Purpura, 1974; Mehraein et al., 1975; Brizzee et al., 1980; Salas, 1980; Wisniewski et al., 1991; Multani et al., 1994). Moreover, alterations

in dendritic spine densities have also been reported in certain neuropsychiatric diseases such as schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000).

1.6 Aim of the dissertation

In the mammals, the mother-infant relationship is terminated at weaning. However, age of weaning can vary considerably in relation to physical development and varies by species, strain, or particular circumstance, thereby determining the extent and duration of the mother-infant relationships (Janus, 1987a, 1987b; Fahlke et al., 1997; Cook, 1999; Zimmerberg and Weston, 2002). Few studies have investigated the impact of emotional experience such as the time of weaning at behavioural level. After weaning, social rearing conditions have clearer effects on affects. Post-weaning social isolation is widely reported to influence adult behaviour and the physiology (introduction. 1.2). Hence, both age of weaning and rearing conditions are important determinants of adult behaviour. Yet they have rarely been systematically studied. Some studies have examined the interactive effects of weaning and social rearing conditions at behavioural level; nevertheless, the underlying neuronal and synaptic adaptations (or morphological adaptations) in the limbic cortical regions such as the prefrontal anterior cingulate cortex (ACd), orbitofrontal cortex (OFC), hippocampal CA3, infra (IDG) and supra pyramidal layers of dentate gyrus (SDG) remains to be investigated. The limbic structures such as the prefrontal cortex and the hippocampus are involved exclusively in modulating emotional behaviours in animals (described in detail in introduction). Since, the time of weaning appears to be a major emotional challenge during late childhood it can be assumed that this transition to independency should involve major structural changes in limbic areas such as the prefrontal cortex and hippocampus, which are involved in emotional behaviours. Therefore, it would be interesting to speculate what effects such emotional experience may have on the neuronal development in above mentioned limbic areas. Therefore, the aim of my study was two-fold:

1) To determine in young rats the impact of the time spent with their mother on the development of neurons in their limbic PFC and hippocampus. This was achieved by comparing the time of weaning in experimental animals.
2) To examine the impact of duration and extent of interaction between young rats with the mother and the siblings on the development of neurons in prefrontal cortex and hippocampus, which was tested by determining the effects of postweaning social environments in addition to examining the effect of time of weaning.

2.0 Materials and methods

The experiments were approved by the University of Haifa Committee on Animal Experimentation. Male Wistar rats (Figure- 4A) were born in the laboratory of Prof. Micah Leshem (Department of Psychology, University of Haifa, Israel). They were raised with their dams in large polycarbonate cages (56 x 35 x 19 cm high) with wood-flake bedding. Litters were culled to 10 pups within 24h of birth and then left undisturbed until weaning which was by removal of the dam from the home cage. Subsequently, the rats were raised in groups; 5-6 juveniles per cage. Light in the animal rooms were on 07-19h, and the temperature maintained at 22$\pm$2°C.

4.A

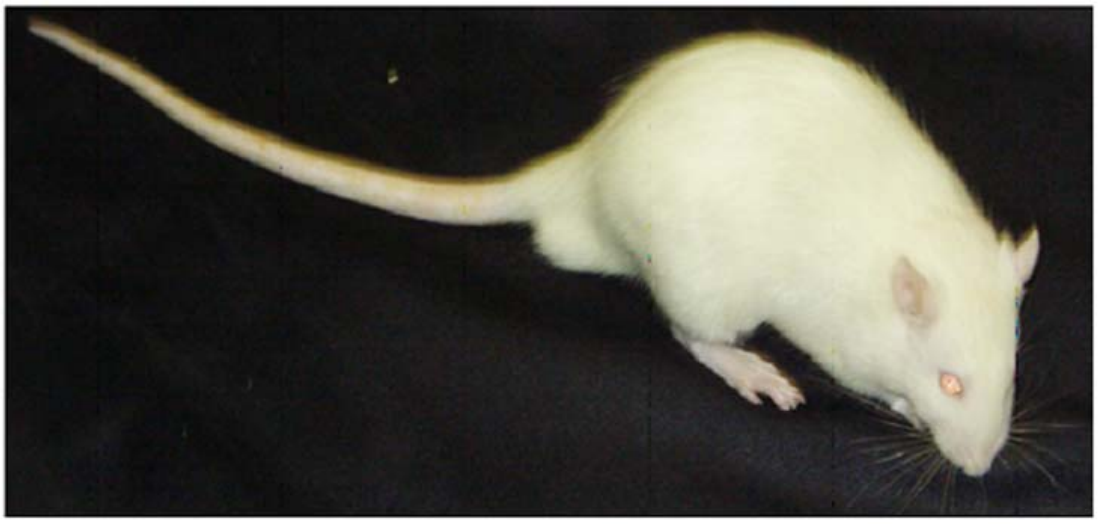


Figure 4.A) Photograph of the experimental animal-Wistar rat.

2.1 Experimental animals and groups

Four litters were weaned at 21 days of age and four at 30 days of age. After weaning, rats from half of each litter were allocated to individual cages in the rat colony. The other half of the litter was raised in groups, 4-5 juveniles in each cage; so, that weaning age was compared between litters whilst rearing condition was a within litter comparison. Wild and domesticated rat pups reared in semi-natural environments in a laboratory make their first departure from the natal nest between postnatal days 16 and 19 (Alberts, 2005). In the Norway rat, weaning occurs naturally across postnatal days 14 to 34 and is a transient process. It is common laboratory practice to wean a litter (i.e. to separate offspring from the dam) at postnatal day 21. Thus, the two groups termed "early weaning" in this study represent the time point for common laboratory weaning.

In this study, there were four experimental groups:

a) **Early weaned/social group (EWS)**: are group of rats that were weaned at postnatal day 21 and raised until adulthood (14 weeks) in groups of 4-5 siblings (Figure 4B)
b) **Late weaned/social group (LWS)**: refers to group to rats that were weaned at postnatal day 30 and raised until adulthood in groups of 4-5 juveniles (Figure 4B).
c) **Early weaned/isolated group (EWI)**: represents group of rats that were weaned at postnatal day 21 and thereafter subjected to chronic social isolation until adulthood (Figure 4B).
d) **Late weaned/isolated group (LWI)**: are group of rats that were weaned at postnatal day 30 and thereafter subjected to chronic social isolation until adulthood (Figure 4B).

At adulthood (14 weeks) the rats were decapitated and their brains were rapidly removed and immersed in 50 ml of Golgi-Cox solution for 14 days.

4.B

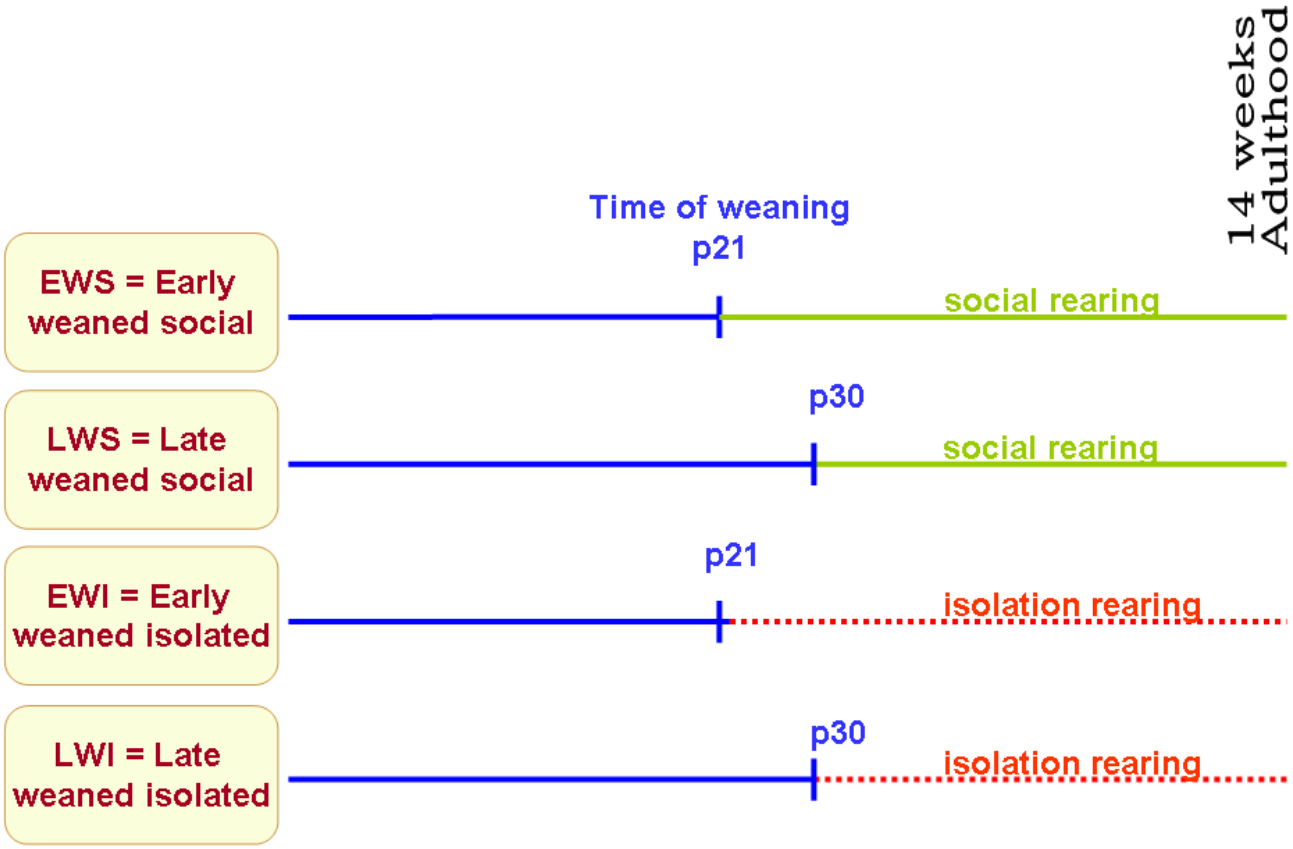


Figure 4 B. Schematic representations of the experimental groups.

2.2 The Golgi-Cox method

The Golgi method was discovered as a coincident by Golgi in 1873. During the silver impregnation of the innermost membrane of pia mater with silver nitrate ($AgNO_3$) and potassium dichromate ($K_2Cr_2O_7$); Golgi detected occasional nerve cells that had stained dark brown due to the precipitation of silver chromate (Ag_2CrO_4). Potassium dichromate or Muller's fluid was widely used at that time as a fixative to harden the nervous tissue. Golgi then proceeded with silvering the previously chromated material and found that mercury could be used efficiently to intensify the staining (Golgi, 1879). In this way, the original Golgi method was discovered which consisted of treatment of the nervous tissues with potassium dichromate ($K_2Cr_2O_7$) for several months; then in

solution of mercuric chloride ($HgCl_2$) that lead to the deposition of silver chromate (Ag_2CrO_4) in the cells. The silver is selective tending to impregnate a few cells completely which then became blackened when silver is reduced.

A significant improvement of the Golgi method was made by Cox in 1890, 1891 who introduced the use of mixture of potassium dichromate ($K_2Cr_2O_7$) and mercuric chloride ($HgCl_2$) with potassium chromate (K_2CrO_4) added to moderate the acid reaction of the solution. This method was known as the Golgi-Cox method. The Golgi-Cox method generated such an excellent impregnation of the neuronal cells that pioneer like Santiago Ramon y Cajal (1909) used this method in his studies of the hippocampus and obtained better neuronal impregnation with this method than with the original Golgi technique.

2.3 Principles of the Golgi-Cox method

The original technique created by Golgi can be categorized into two groups: a) those leading to deposition of silver chromate are referred to as “Golgi methods” (Golgi, 1873). b) Those producing deposit of metallic mercury and complex oxides of mercury is called “Golgi-Cox” method (Golgi, 1879, 1891; Cox, 1890, 1891). The Golgi-Cox method is one of the simplest of the complex and time consuming Golgi methods for demonstrating the relationships of dendrites and axons to the nerve cell bodies. For this reason, it is regarded as an outstanding method for the morphological analysis of the nervous system. There are few special characteristics that make the Golgi-Cox method different from other methods of metallic staining:

- Only a few nerve cells are stained out of a large number of neurons present in the brain; thus, allowing for complete studies of individual cells and their processes
- In a few cells that take the stain, the metallic deposit is massive and includes the soma as well as the ramifying processes
- Cell bodies and dendritic processes are stained dark brown against a light yellowish or transparent background

- The unstained structures remain undetectable as they form a transparent background against which the stained cells stand out clearly
- There are no staining gradations. The structures that are visible are either completely opaque to light or perfectly transparent

Although the Golgi-Cox method of staining does not reveal details of the internal structures of the nerve cells, it does provide, very importantly, a unique view of the entire nerve cell and its processes. Example of Golgi-Cox impregnated neurons is demonstrated in figure 5A.

2.4 Impregnation of neurons by the Golgi-Cox method

After decapitating the animals at adulthood (14 weeks) the brains were rapidly removed and immersed in 50 ml of Golgi-Cox solution for 14 days. Thereafter, the brains were dehydrated and embedded in 8% celloidin. Serial transverse sections, 150 µm were collected, mounted on glass slides and processed using a Golgi-Cox protocol modified by Glaser and Van der Loos, 1981 (the Golgi-Cox protocol is attached in appendices).

5.A

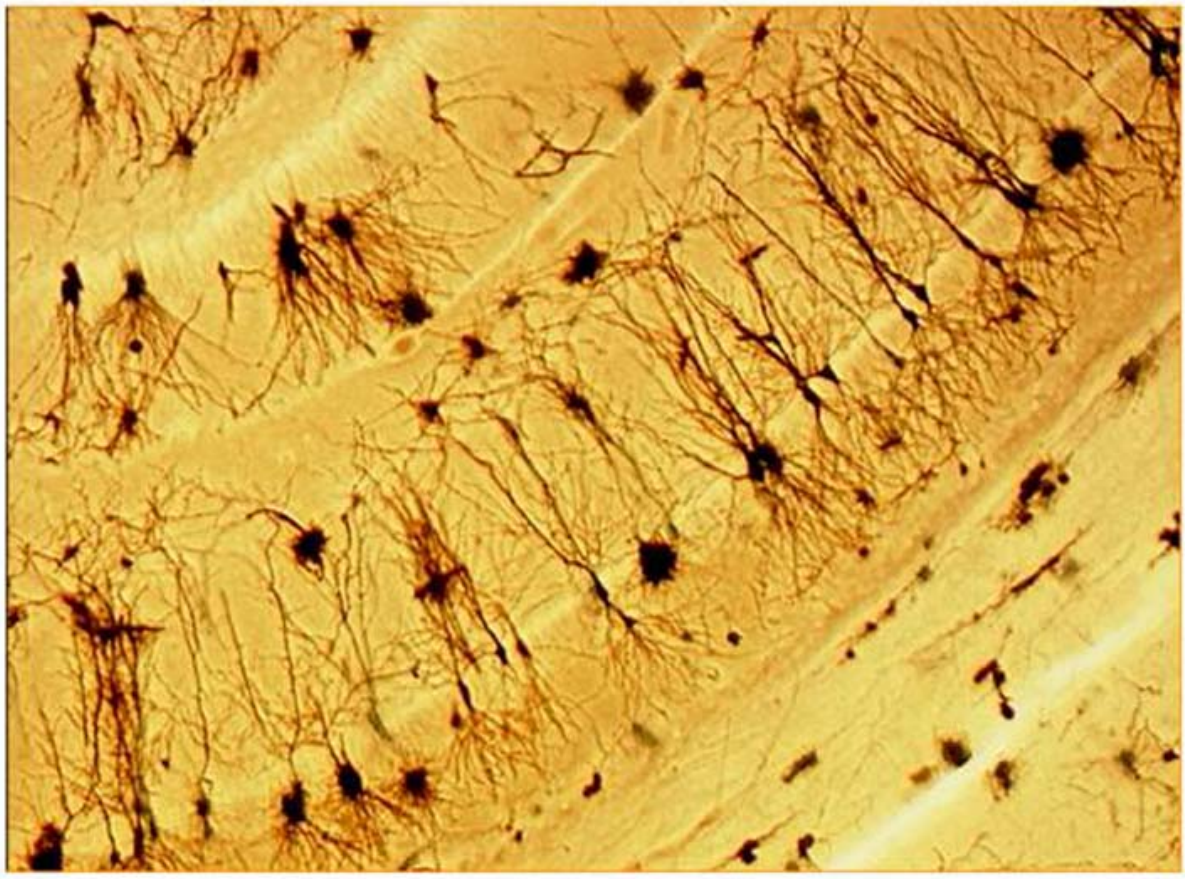


Figure 5.A) Low power micrograph demonstrating Golgi-Cox impregnated neurons from the hippocampus of a Wistar rat. A clear and unique view of an entire nerve cell and its processes are evident by the Golgi-Cox technique.

2.5 Morphological analysis of the Golgi-stained neurons

The neurons were reconstructed at a final magnification of 1000 X, using a computer based neuron tracing system or the image analysis system (NEUROLUCIDA®; MicroBrightField, Williston, VT); equipped with an Olympus BX51 microscope (Figure-6A). Neurolucida is an advanced scientific software for performing a number of morphometric analysis; for instance, 3-D reconstruction of the neurons. The neurons are traced in a computer controlled motorized X-Y-Z axis. The tracing of neurons in Neurolucida allows for a 3-D measurement of bifurcating and

trifurcating branching processes (Figure-6A). The length and the diameter of the structures (e.g. neurons) are measured automatically while they are being traced. Similarly, structures such as the dendritic spines can be marked and the density measurements are generated automatically by Neurolucida's Neuroexplorer software (Figure 6C). In principle, Neurolucida features sophisticated tools for complete morphometric analysis of the neurons allowing for a 3-D reconstruction of the neurons from serial or single sections. Moreover, high quality graphics of the reconstructed neurons can also be obtained from Neurolucida (Figure-6 B).

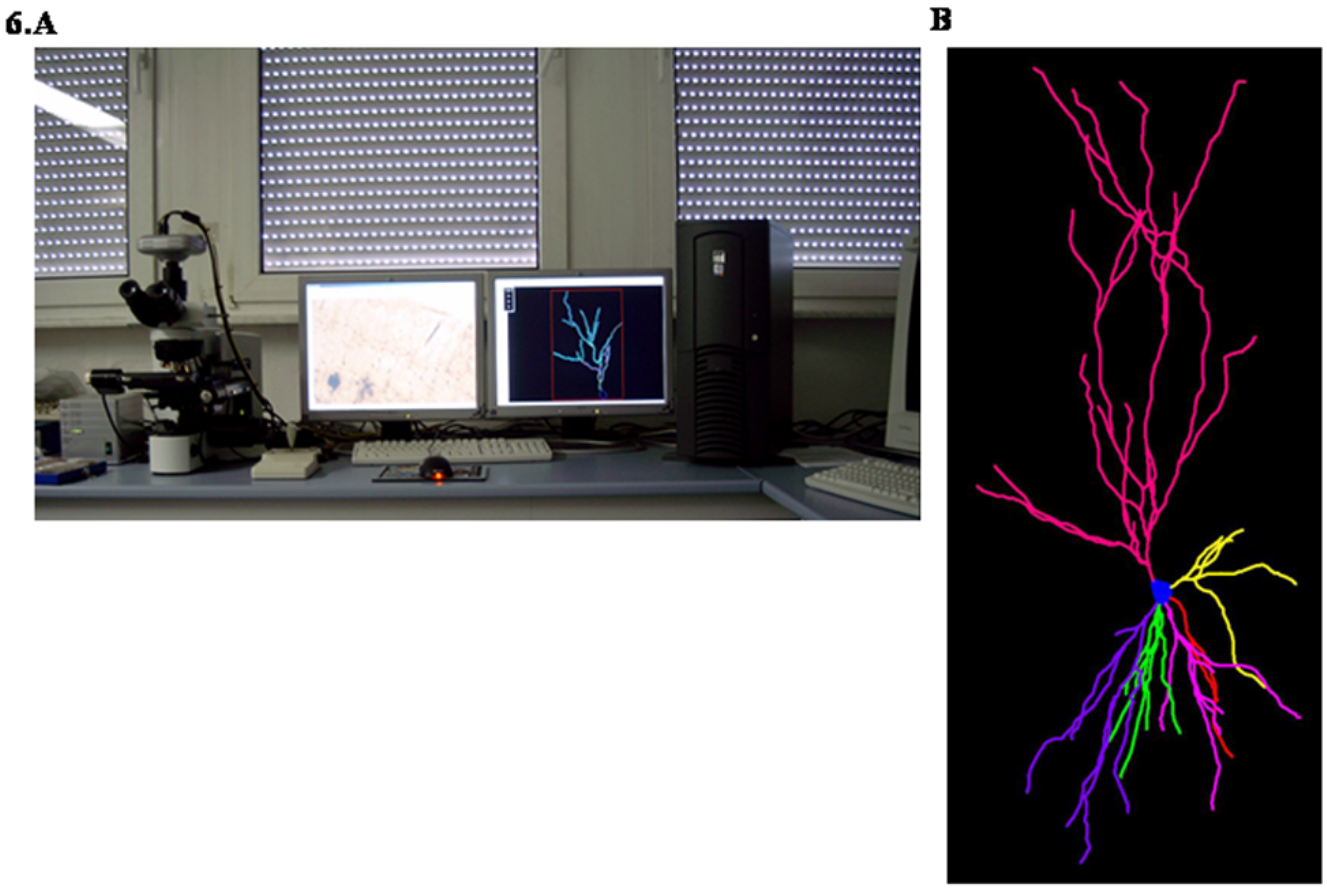


Figure 6.A) Images of (NEUROLUCIDA®; MicroBrightField, Williston, VT); equipped with an Olympus BX51 microscope B) Example of a 3-dimensionally reconstructed pyramidal neuron in Neurolucida.

6.C

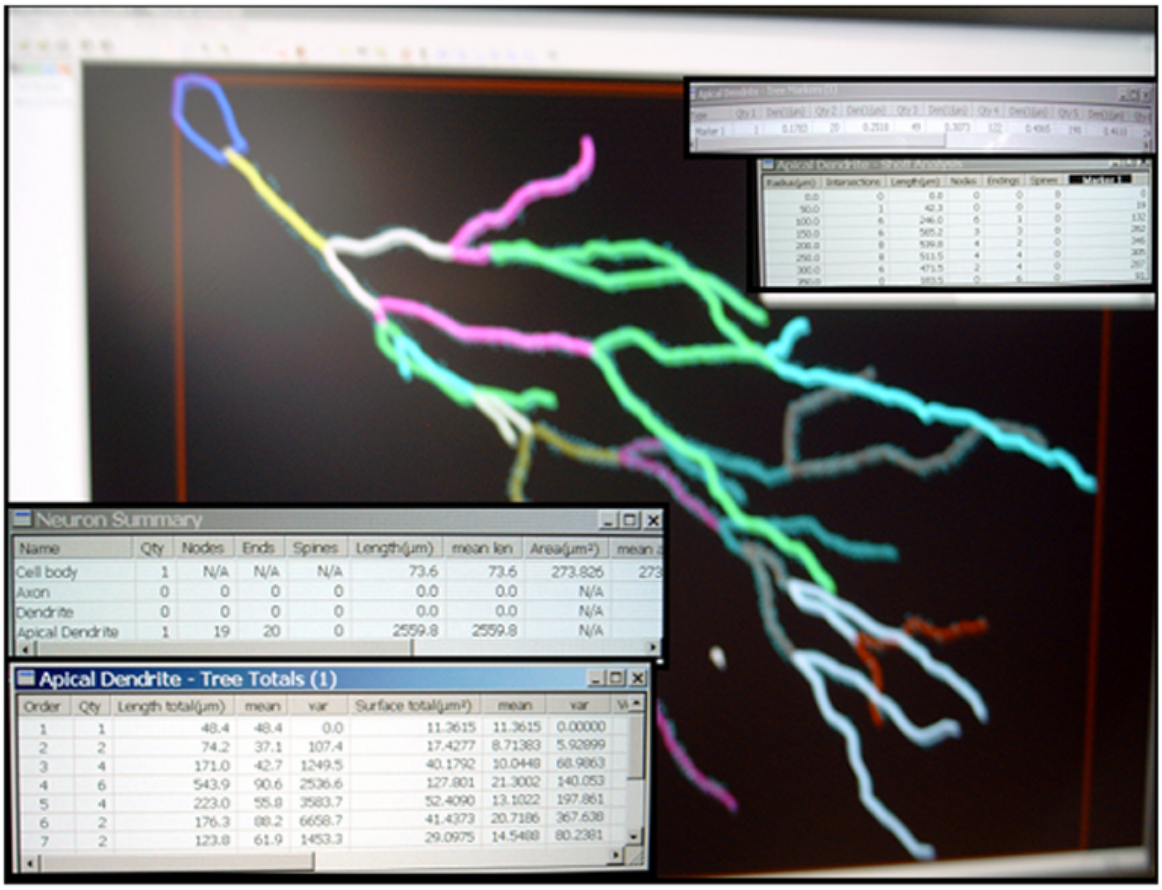


Figure 6.C) Neuroexplorer of (NEUROLUCIDA®; MicroBrightField, Williston, VT); which, quantifies automatically the diameter and length of 3-D reconstructed structures such as the neurons.

2.6 Brain areas analysed

The Golgi-impregnated neurons from following brain areas were included in my analysis:

- Two prefrontal cortical brain areas such as layer II/III dorsal anterior cingulate cortex (ACd) (Figure-7A, B) and layer II/III orbitofrontal cortex (OFC) (Figure-7A, D) were chosen for my study. The reason for choosing layer II/III of ACd and OFC is that it is this layer viz. the layer II/III in prefrontal cortex of rats that receives the main thalamic input. The rat prefrontal cortex lacks the granular cell

layer IV which typically receives the thalamic input in visual and primary sensory cortices (Herkenham, 1980). The ACd was defined according to the commonly used nomenclature of Krettek and Price, (1977). This area corresponds to the Cg1 of Paxinos and Watson, (1998) or Zilles and Wree, (1995). The Cg1 area of medial prefrontal cortex is readily identified by its position on the medial wall of rostral cortex and its location dorsal to infralimbic cortex (Figure 7A); which is markedly thinner than Cg1 area and has fewer, less well-defined layers (Zilles and Wree, 1995). Within Cg1 layer II/III is readily identifiable in Golgi-stained materials based on its cytoarchitecture. Its position is immediately ventral to the relatively cell-poor layer I and immediately dorsal to layer V. In addition, layer II/III of ACd can be readily differentiated in Golgi-stained materials by its greater cell packing density and smaller somata of the pyramidal cells compared to layer V (Cajal, 1995; Zilles and Wree, 1995). The orbitofrontal cortex of PFC is a ventrally located cortical region that was identified readily by its position dorsal to the caudal end of the olfactory bulb (Paxinos and Watson, 1998). The prefrontal area termed OFC included the ventral orbital cortex (VO) and lateral orbital cortex (LO) (Paxinos and Watson, 1998) (Figure-7A); but, since this staining did not allow to distinguish the boundaries between these two sub-regions; neurons from both sub-regions were taken for the analysis.

- Three regions of the hippocampal formations such as the infra pyramidal layer of dentate gyrus (IDG), the supra pyramidal layer of dentate gyrus (SDG) and the field CA3 of hippocampus proper (Figure-7F, 7G) were included in my study. The hippocampus was readily identifiable by its characteristic *"horn of Ammon"* shape; within which, each of the sub-areas of hippocampal formation, the CA3, the infra and the supra pyramidal layer of dentate gyrus occupied a distinct position (Figure- 7F, 7G).

2.7 Neuron types used for 3-D reconstruction

There were two types of neurons that I used for the morphological analysis:

- **Pyramidal neurons** - the pyramidal neurons were readily identified by their characteristic triangular soma shape, presence of numerous dendritic spines and a distinct primary apical dendrite originating from the apex of the cell body or soma as the main dendrite and extending towards the pial surface; 2-4 secondary and tertiary basal dendrites originating from the base of the cell body and extending towards the deeper layers. In my analysis for the prefrontal cortex, I had chosen a particular, morphologically characterized pyramidal neuron type; which has its soma located near the layer II/III border. Representative examples of the analysed pyramidal neuron of layer II/III dorsal anterior cingulate cortex (ACd) and orbitofrontal cortex (OFC) are illustrated in Figure- 7C and 7E. The apical dendrites of these neurons typically start 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. The pyramidal neurons in CA3 area of hippocampus were readily identified by their triangular cell body; apical dendrite originating from the apex of the cell body as the main dendrite and 2-4 secondary and tertiary basal dendrites originating from the base of the cell body. Representative examples of pyramidal neurons from CA3 are illustrated in Figure- 7G, 7I and 7K.

- **Granular neurons** – the granular neurons obtain their name from their small size (*granule = small*). The granular neurons that I used in my study were characterized by their triangular or multipolar shaped somata; two to four primary dendrites that arise from the soma and bifurcate once or more to produce an extensive dendritic arborization and by the presence of numerous thorny excrescences or spines on their dendrites. (Figure-7H and 7J).

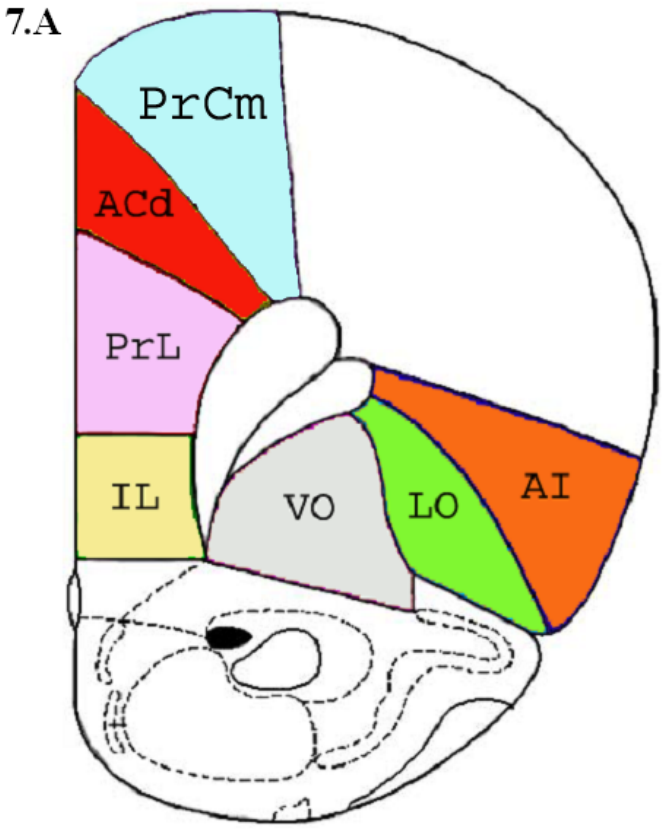
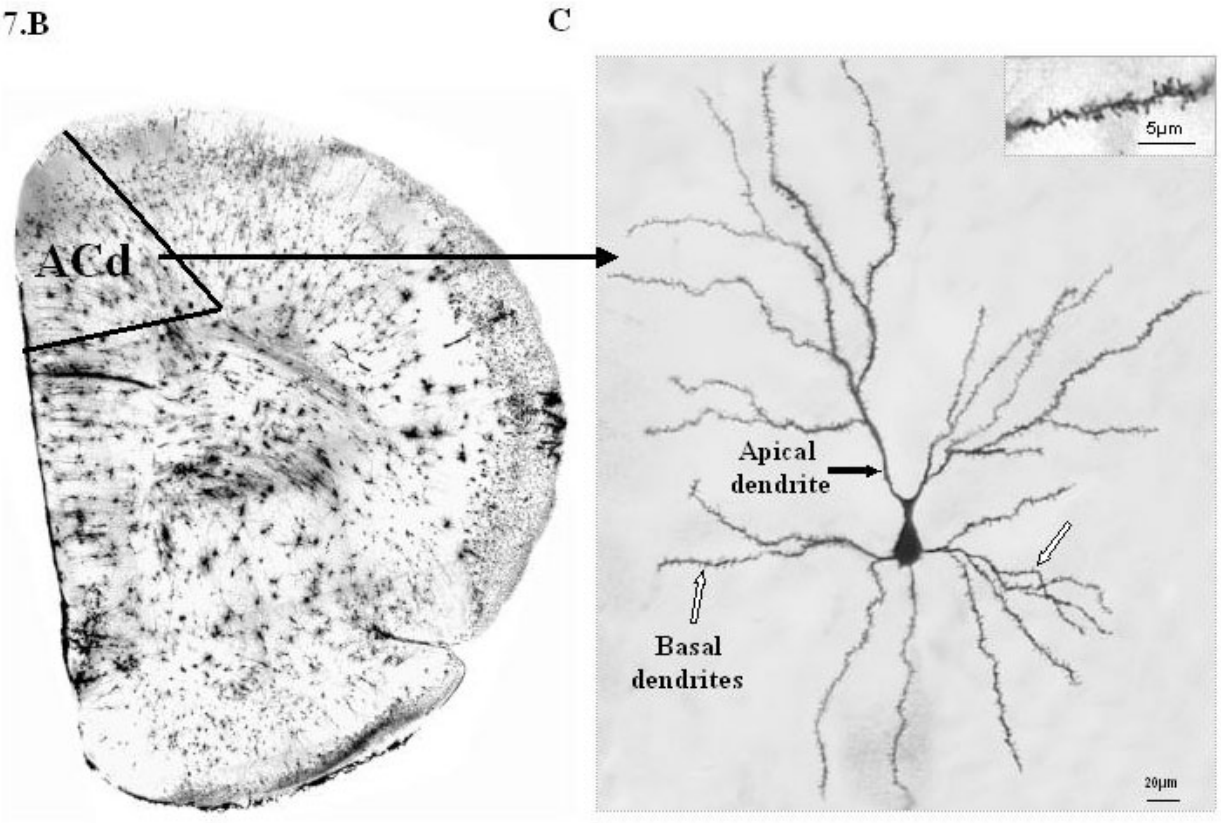


Figure 7 A. Schematic diagram of a coronal section through the rat Prefrontal cortex (modified from Paxinos and Watson, 1998). Bregma 3.20 mm (B) Golgi-Cox impregnated forebrain hemisphere of a Wistar rat. The margins outline the pregenual ACd, in which the neurons were analysed (C) Representative Golgi-Cox impregnated pyramidal neurons located in layer II/III of the dorsal anterior cingulate cortex (ACd). The inset shows a dendritic segment of an apical branch AI = agranular insular cortex, IL = infralimbic cortex, LO = lateral orbital cortex, PrCm = precentral medial, PrL = prelimbic cortex, VO = ventral orbital cortex.



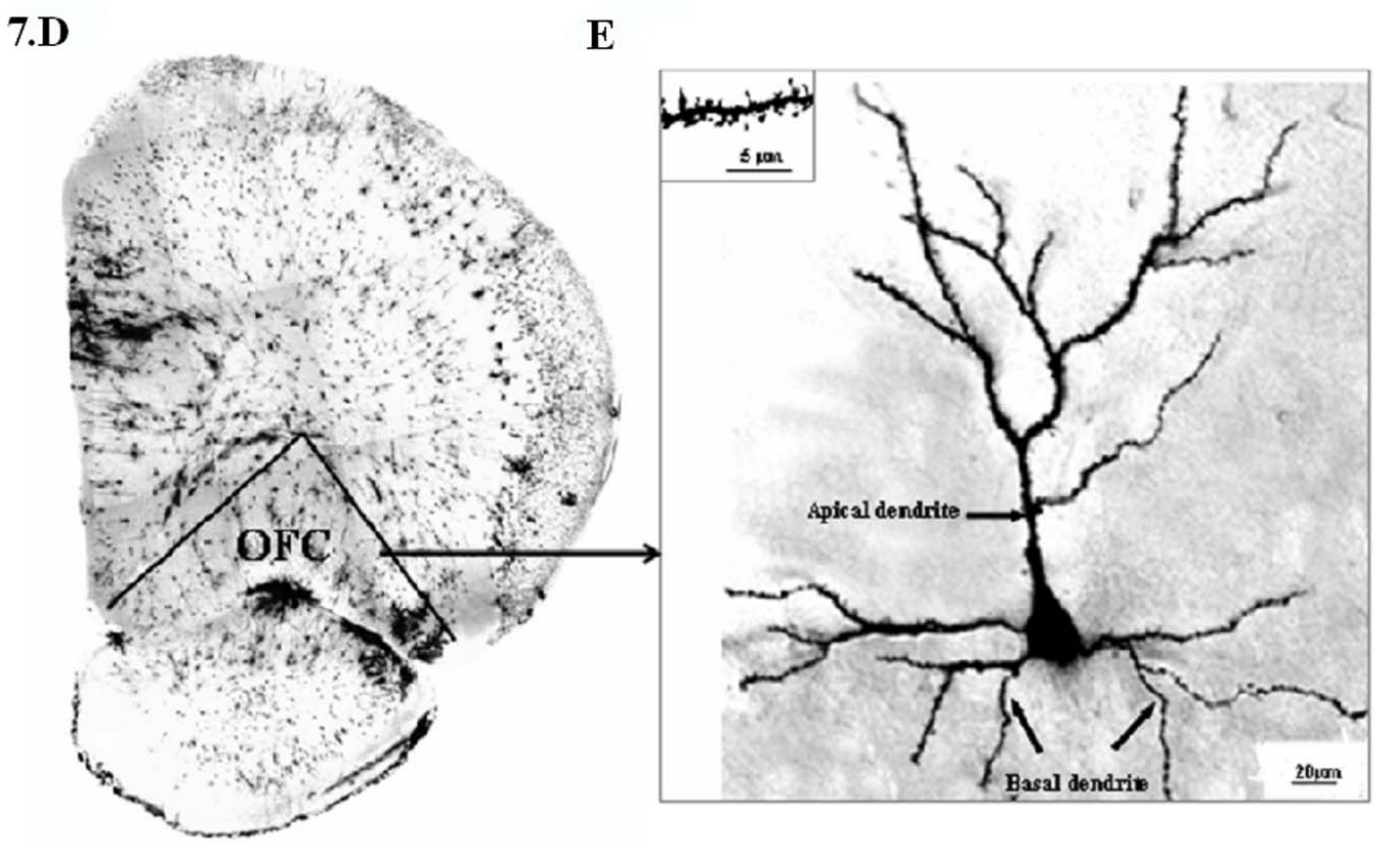
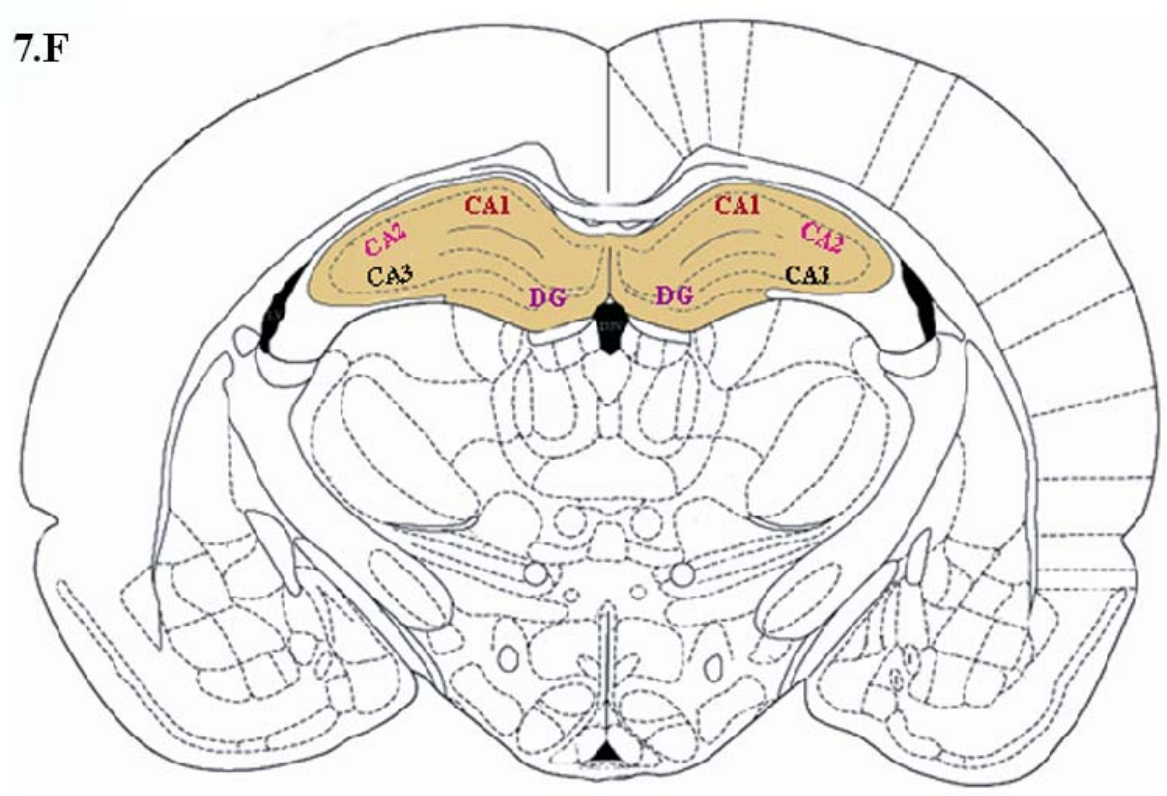


Figure 7.D) Golgi-Cox impregnated forebrain hemisphere of a Wistar rat. The margins outline the prefrontal area, orbitofrontal cortex (OFC) in which the neurons were analysed (E) Representative Golgi-Cox impregnated pyramidal neuron from layer II/III orbitofrontal cortex. The inset shows a dendritic segment of an apical branch.

7.F



7.G

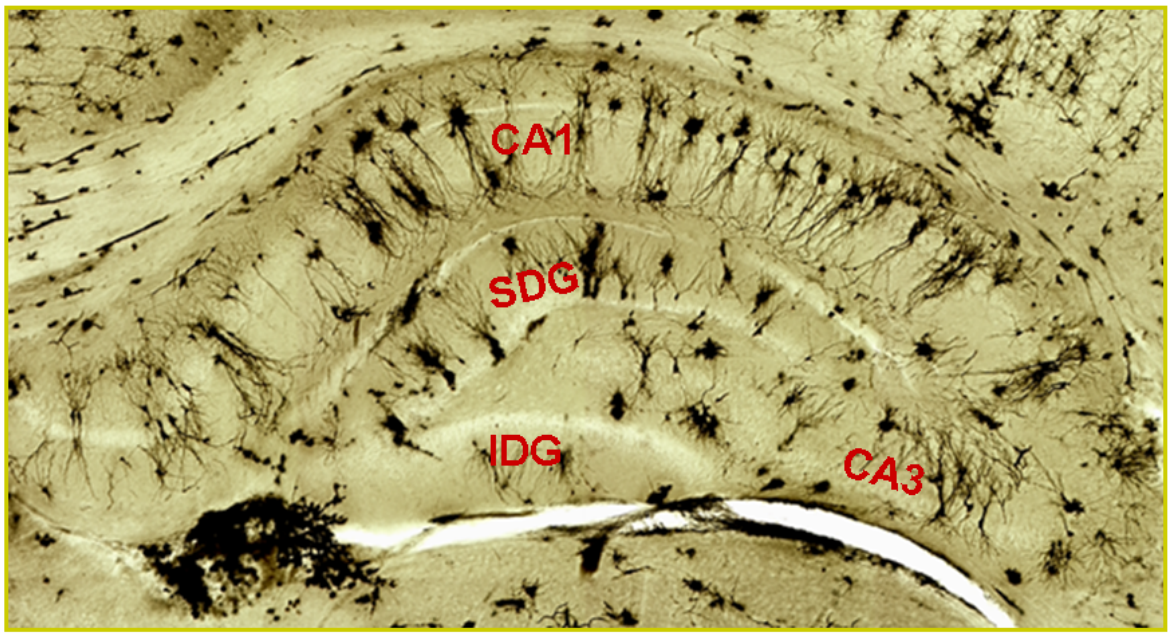


Figure 7.F) Schematic diagram of a coronal section through the rat hippocampus (modified from Paxinos and Watson, 1998). Bregma –3.14 mm (G) Golgi-Cox impregnated midbrain hemisphere of a Wistar rat in which different hippocampal areas are labelled. CA1 =

field CA1 of hippocampus, CA2 = field CA2 of hippocampus, CA3 = field CA3 of hippocampus, DG = dentate gyrus, IDG = infra pyramidal layer of dentate gyrus, SDG = supra pyramidal layer of dentate gyrus.

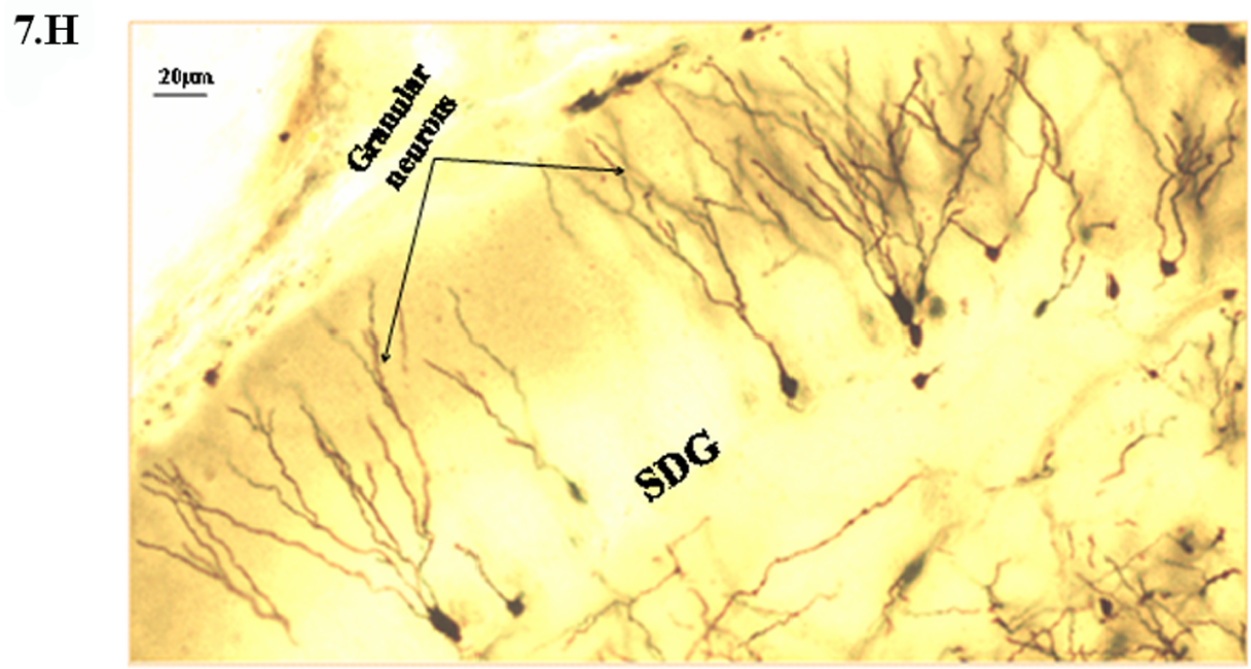
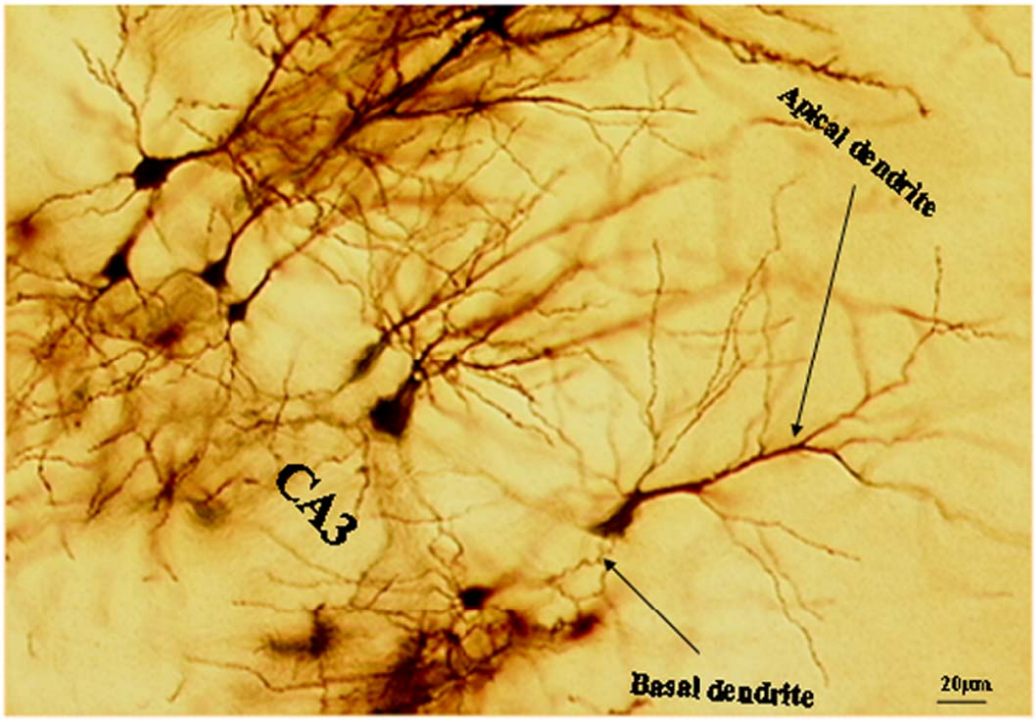
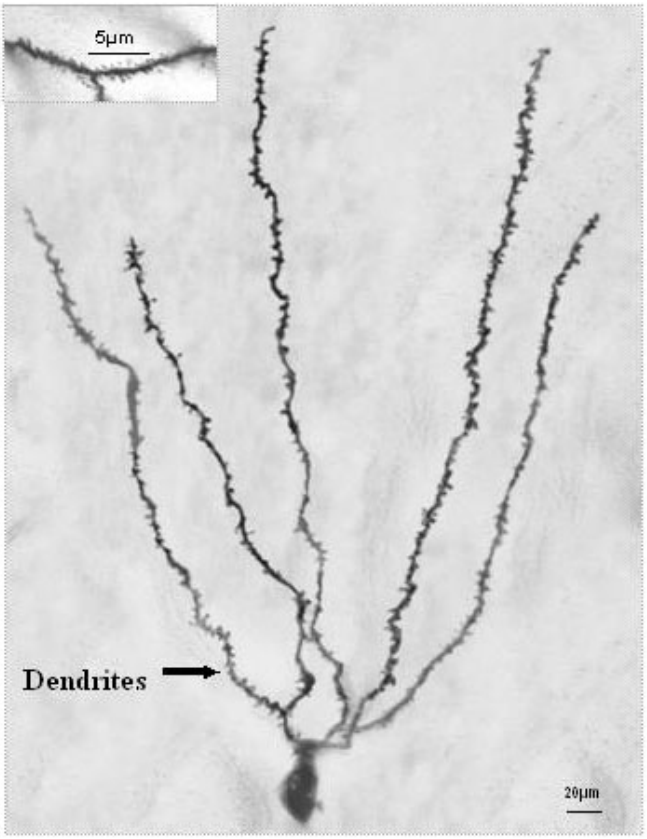


Figure 7.H) Photomicrograph of Golgi-Cox impregnated granular neurons from the supra pyramidal layer of dentate gyrus of a Wistar rat. SDG = Supra pyramidal layer of dentate gyrus. The granular neurons of the dentate gyrus contain 2-4 primary dendrites originating from the cell body or somata. Magnifications 20 X.

7.I


Apical dendrite
CA3
Basal dendrite
20µm

7.J


5µm
Dendrites
20µm

K

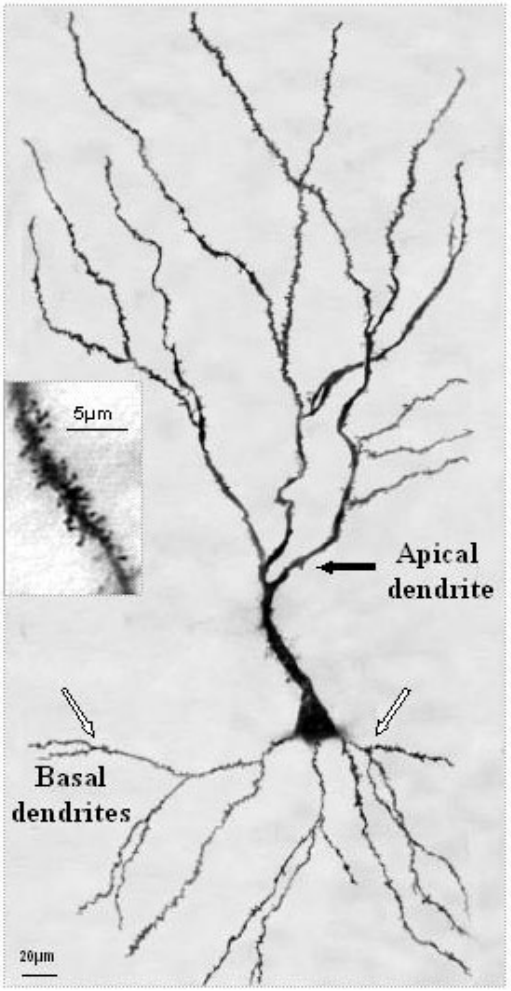

5µm
Apical dendrite
Basal dendrites
20µm

Figure 7.I) Photomicrograph of Golgi-Cox impregnated pyramidal neurons from CA3 area (hippocampus) of a Wistar rat. The pyramidal neurons of area CA3 contain distinct apical dendrite originating from the apex and 2-3 basilar dendrites originating from the base of the cell body or somata. Magnification 20 X. J) Representative Golgi-Cox impregnated granular neuron from infra pyramidal layer of dentate gyrus (IDG). The inset shows a segment of a dendritic branch. K) Representative Golgi-Cox impregnated pyramidal neuron from CA3 area of hippocampus. The inset shows a dendritic segment of an apical branch.

2.8 Selection of the neurons for 3-D reconstruction

The following criteria's were taken for the selection of neurons for 3-D reconstruction:

- Intact neurons whose cell bodies or somas were located in the center of the 150 µm sections were selected to minimize the number of truncated branches (because relatively thick sections, 150 µm were taken through prefrontal cortex and hippocampus, the apical and basilar dendrites of almost all neurons were completely contained within a single section (Figure 7C, E, H, I, J and K). In all animal groups, complete impregnation of numerous neurons were apparent
- In prefrontal cortex, the soma of the neurons had to be located within layer II/III and within the boundaries of anterior cingulate cortex (ACd) and orbitofrontal cortex (OFC)
- Neurons that exhibited complete staining of their dendritic trees within the 150 µm section evidenced by well-defined endings
- Neurons that displayed intact primary, secondary and tertiary branches
- Neurons that did not show overlap with other branches that would obscure visualization of the dendritic spines
- Neurons that were relatively isolated from neighbouring impregnated cells

Representative examples of the analysed neurons are illustrated in Figure 7C, E, H, I, J and K.

2.9 Measurements

For each reconstructed neuron the following morphometric parameters were quantified using Neuroexplorer software (NEUROLUCIDA®; MicroBrightField, Williston, VT) (Figure-6C).

- **Spine frequencies/densities** – Spine frequencies or densities represent number of visible spines per µm which was obtained for each neuron by dividing the total number of spines counted through the total dendritic length. The results were expressed as mean spine frequencies per total neuron. All types of dendritic protrusions including mushroom, thin, and stubby were considered as spines if they were in direct continuity with the dendritic shaft (Figure- 7C, E, J and K). An attempt to correct for hidden spines (Feldman and Peters, 1979) was not made since the use of visible spines counts for comparison between different experimental conditions has been validated previously (Horner and Arbuthnott, 1991)
- **Spine densities per branch order** – represents the average number of spines per branch order of a dendrite. The branches of the dendritic trees were numbered in a centrifugal nomenclature system (Uylings et al., 1986) starting from their originating point in the cell body to the point where the branching ends and spines were counted on every branching order. Therefore, dendritic branching arising from the soma are the first branch or branch order 1 until they bifurcate into second branch or branch order 2 which branches into third branch or branch order 3 and so on (Figure-8A)
- **Total dendritic length** – refers to summed length of the dendrites for a given neuron. The length of the dendritic tree is estimated by Neurolucida while tracing the entire neuron

- **Dendritic branching patterns and complexity** – refers to number of crossings at a given neuron. In addition to calculating the summed dendritic length, the dendritic length and complexity was quantified by a three-dimensional version of Sholl analysis (Sholl, 1953)

2.10 3-D version of Sholl analysis

Sholl in 1953, 1956 and 1959 in an attempt to assess the principles of neuronal interconnections performed several studies on the dendritic branching pattern and established functional relationships between the number of intersections per unit area and the distance from the center of the soma. The Sholl's method of analysis estimates the amount and distribution of dendritic material by counting the number of intersections of dendrites with an overlay of concentric rings centered on the soma. Hence, this method is known as the Sholl's method of segmental analysis or 3-D version of Sholl analysis that assess the differences in the amount and location of dendritic material in a three-dimensional way providing information about the complexity of a neuron.

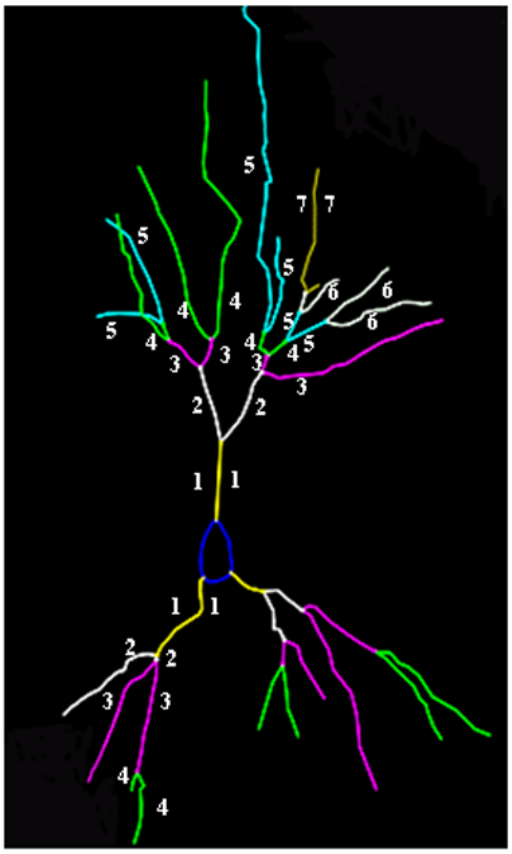
In order to find out the complexity of a neuron and track down the changes in dendritic length and branching in greater detail, I performed Sholl's method of segmental analysis with the help of Neuroexplorer software (NEUROLUCIDA®; MicroBrightField, Williston, VT) (Figure-6C). Using the center of the soma as a reference point, I measured dendritic length and intersections at different radial distances relative to the soma (Figure-8B, C, and D). The neurons from different brain areas varied considerably in their respective lengths. For example, the pyramidal neurons from CA3 area of hippocampus are much longer than the prefrontal pyramidal neurons (compare Figure-7I and 7K with 7C and 7E) and the granular neurons are generally smaller than the prefrontal and hippocampal pyramidal neurons (compare figure 7H and 7J with 7C, 7E and 7K). Keeping this in mind, I measured dendritic length and intersections at different radial distances depending on the neuronal size:

- For the apical and basilar dendrite of pyramidal neuron of prefrontal anterior cingulate (ACd) and orbitofrontal cortex (OFC) the dendritic length and intersections were measured at distances 20 µm relative to the soma. For example, for an apical dendrite measuring 280 µm, dendritic length and intersections were measured at radial distances 20 µm, 40 µm, 60 µm, 80 µm, 100 µm, 120µm, 140 µm, 160 µm, 180 µm, 200 µm, 220 µm, 240 µm, 260 µm and 280 µm of the dendrite (Figure 8B)

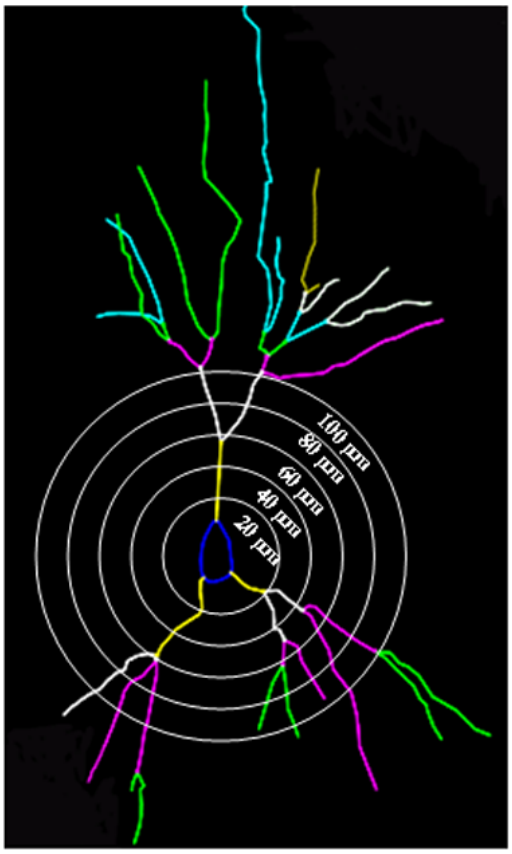
- For the granular neurons of the infra (IDG) and supra pyramidal layer of dentate gyrus (SDG) the dendritic length and intersections were measured at radial distances 40 µm relative to soma (Figure 8C)

- For the CA3 sub-region of the hippocampus, dendritic length and intersections of apical dendrite were measured at 50 µm and basal dendrite at 30 µm relative to soma. (Figure 8D)

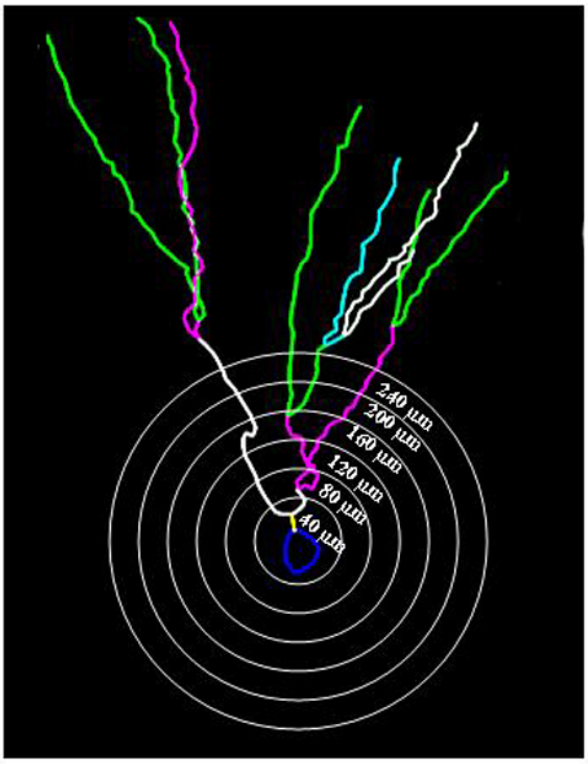
8.A



B


100 µm
80 µm
60 µm
40 µm
20 µm

8.C


240 µm
200 µm
160 µm
120 µm
80 µm
40 µm

D

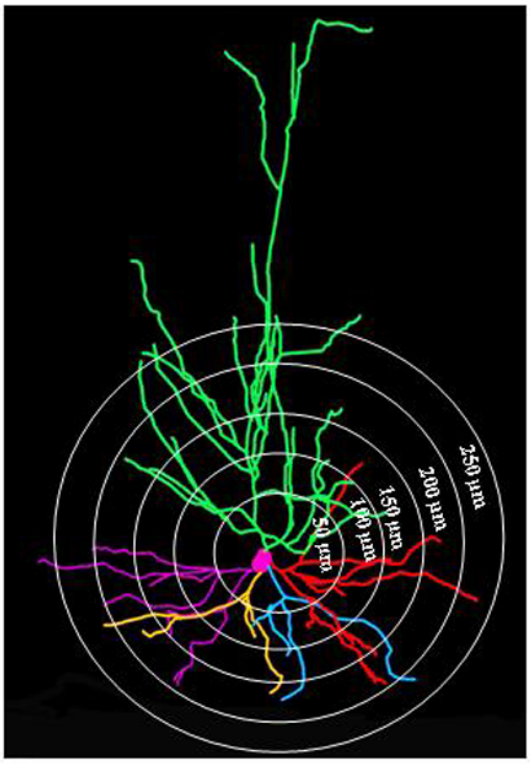

250 µm
200 µm
150 µm
100 µm
50 µm

Figure 8. Three-dimensionally reconstructed neurons (A) reconstructed neuron representing hypothetical model for branch order analysis. The branches or dendrites of a given neuron are numbered consequently in a centrifugal manner ranging from 1-7 (Uylings et al., 1986). (B) Three-dimensionally reconstructed pyramidal neuron from the prefrontal cortex demonstrating hypothetical model for Sholl analysis that measures dendritic length and complexities at concentric rings overlying the soma. For the pyramidal neurons of ACd, dendritic length and complexities were measured at radial distances 20 µm relative to soma. (C) 3-D reconstructed granular neuron from dentate gyrus in which the dendritic length and complexities were measured at 40 µm relative to soma. (D) 3-D reconstructed pyramidal neuron from CA3 area of hippocampus in which the apical dendritic length and complexities were measured at 50 µm relative to soma.

2.11 Statistical analysis

Statistical analysis was carried out using SIGMA-STAT 2.0 software (Jandel Scientific, Erkrath, Germany). Data's such as the mean spine frequencies, mean spine frequencies per branch order, total dendritic length, complexity and branching of the dendrite were calculated for each experimental animal. For statistical analysis data of the individual neurons were averaged for each animal, which was used as the unit of analysis. 4-5 animals per groups were used in this study. 6 neurons per animal, including 3 neurons per hemisphere were analysed. The neurons from left and right hemisphere were analysed separately; since no hemispheric difference was observed the values from both hemispheres were pooled for statistical analysis. Difference between the histological parameters were tested by applying the Two-Way Analysis of Variance (Two-Way ANOVA) using weaning (early x late) as the first factor and treatment (social x isolation) (treatment = rearing conditions) as the second factor. If an *interaction* between the two factors was detected a post-hoc test was performed using the Student-Newman-Keuls test for pairwise comparison between groups. The data for radial distances or Sholl analysis were compared by mixed-design ANOVA using radial distances from soma as within subject factor and weaning and treatment (or rearing) as

between subject factors using SPSS, 13.0 for windows. Group data were further analysed by post-hoc (Bonferroni) and t-tests.

Values were represented as mean $\pm$ S.E.M. A probability level of $P \leq 0.05$ was considered to be statistically significant. All measurements were performed blind by me without knowing the experimental conditions of the animal. All experimental subjects were coded and the code was only broken at the time of statistical analysis.

3.0 Results

3.1 Effects of time point of weaning and social environments on the morphology of pyramidal neurons in prefrontal cortex (PFC)

3.2 Dorsal anterior cingulate cortex (ACd):

A. Spine frequencies:

Apical dendrite - For the apical dendritic spine density the main effect was found to be the time point of weaning ($F_{(1, 12)} = 12.01$; $P = 0.005$; early versus late), whereas no effect of postnatal rearing environments ($F_{(1, 12)} = 0.10$; $P = 0.753$) or *interactions* between the two factors were found ($F_{(1, 12)} = 1.64$; $P = 0.225$). Two-way ANOVA revealed that the animals weaned early (EWS + EWI) displayed higher density of spines (+13%) on their apical dendrite compared to late weaned animals (LWS + LWI); ($P < 0.05$; Figure- 9A, 9B, 9C; table- 23A, 24A and B). Branch order analysis revealed that the elevated spine density was particularly evident on the 5th order dendritic branches ($P < 0.05$; Figure- 9D).

Basal dendrite - Similar to the findings for the apical dendrite, the spine density on basal dendrite was affected only by weaning time ($F_{(1, 12)} = 7.51$; $P = 0.018$) and neither by postweaning environment ($F_{(1, 12)} = 2.72$; $P = 0.125$) nor by an *interaction* between

the two factors ($F_{(1, 12)}$ = 2.81; P = 0.120). The animals weaned early (EWS + EWI) displayed higher density of spines (+17%) on their basal dendrite compared to late weaned animals (LWS + LWI), (P <0.05; Figure- 9E, 9F, 9G; table 23A, 24A and B). Branch order analysis did not reveal any difference in spine distribution across the basal dendrite (Figure –9H).

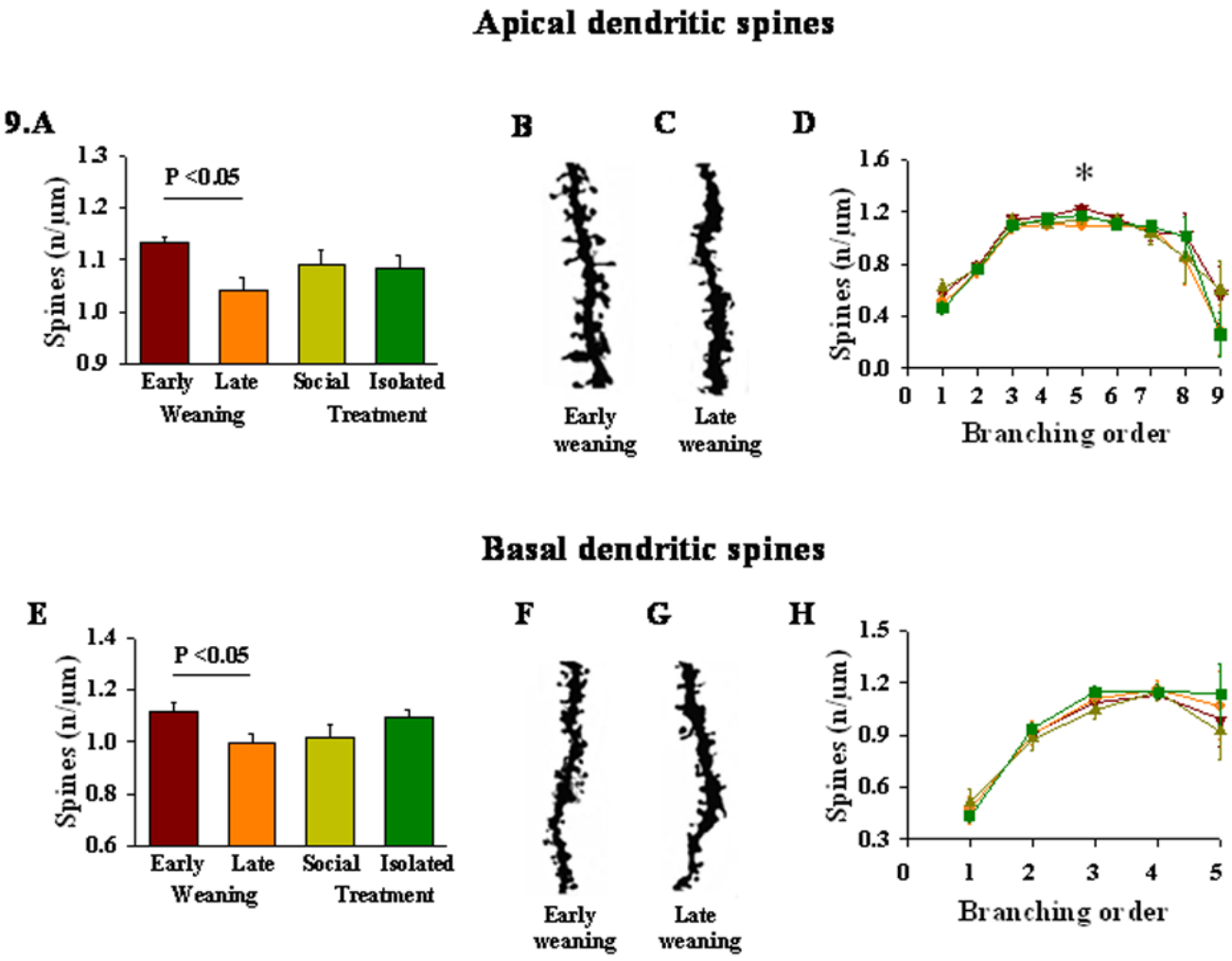


Figure 9. Analysis of spine frequencies of pyramidal neurons located in layer II/III anterior cingulate cortex (ACd). (A) Apical dendritic tree. (D) Branch order analysis of apical dendritic tree. At branch order 5th, Early weaned > late weaned (P <0.05). (E) Basal dendritic tree. (H) Branch order analysis of basal dendritic tree. Values are given as mean ± S.E.M. Statistical comparisons by Two-Way Analysis of Variance (A, D, E, and H). (B, C, F, and G) Representative images of Golgi-Cox impregnated segments. (B, C) Segments from apical

dendrites of early weaned (B) and late weaned (C) animals. (F, G) Segments from basal dendrites of early weaned (F) and late weaned (G) animals.

B. Dendritic length and complexity:

A) Total dendritic length and complexity:

Apical dendrite – The apical dendritic length of ACd pyramidal neuron was not affected by weaning time ($F_{(1, 12)} = 3.14$; $P = 0.102$) and by postweaning environments ($F_{(1, 12)} = 4.12$; $P = 0.065$). Similarly, the apical dendritic complexity was not affected by weaning time ($F_{(1, 12)} = 2.76$; $P = 0.123$) and by postweaning environments ($F_{(1, 12)} = 1.95$; $P = 0.188$). However, an *interaction* between the factors weaning time x postweaning environments reorganized the apical dendritic length ($F_{(1, 12)} = 11.09$; $P = 0.006$) and complexity ($F_{(1, 12)} = 6.04$; $P = 0.030$). Post-hoc Student-Newman-Keuls test revealed that the animals with fewest social experiences (i.e. early weaned from their dam and isolated postweaning or EWI) displayed elongated and complex apical dendrites. Compared to early weaned socials (EWS), EWI animals displayed longer (+32%; $P < 0.05$) and more complex (+27%; $P < 0.05$) apical dendrites, and compared to late weaned isolated (LWI) animals, EWI displayed longer (+31%; $P < 0.05$) and more complex (+29%; $P < 0.05$) apical dendrites (Figure- 10A, C, 11A-D; table 23B, C, 24E and F).

B) Apical dendritic length and complexity per radial distance from soma:

Sholl analysis for apical dendritic length revealed only the main effect of radial distances from soma ($\boldsymbol{F}_{(14,168)} = 32.81$; $\boldsymbol{P} = 0.000$) and not the effect of weaning ($\boldsymbol{F}_{(1,12)} = 3.03$; $\boldsymbol{P} = 0.107$) or rearing ($\boldsymbol{F}_{(1,12)} = 0.12$; $\boldsymbol{P} = 0.737$) and no *interactions* between weaning x rearing x radial distances from soma ($\boldsymbol{F}_{(14,168)} = 0.37$; $\boldsymbol{P} = 0.983$) and between weaning x rearing ($\boldsymbol{F}_{(1,12)} = 0.37$; $\boldsymbol{P} = 0.554$) were found. t-test revealed that the elongated apical dendrites were confined to radial distances of 80-160 µm relative to the soma ($P < 0.05$; Figure-10B).

Sholl analysis for apical dendritic complexity revealed only the main effect of radial distances from soma ($\boldsymbol{F}_{(13,156)} = 22.55$; $\boldsymbol{P} = 0.000$) and not the effect of weaning ($\boldsymbol{F}_{(1,12)} = 2.97$; $\boldsymbol{P} = 0.111$) or rearing ($\boldsymbol{F}_{(1,12)} = 0.56$; $\boldsymbol{P} = 0.467$) and no *interactions*

between weaning x rearing x radial distances from soma ($\boldsymbol{F}_{(13,156)} = 1.39$; $\boldsymbol{P} = 0.170$) and between weaning x rearing ($\boldsymbol{F}_{(1,12)} = 0.65$; $\boldsymbol{P} = 0.437$) were found. t-test revealed that the complex apical dendrites were confined to radial distances of 80-140 µm relative to the soma ($P < 0.05$; Figure-10D).

Basal dendrite - Contrary to apical dendrite, the basal dendritic length of ACd pyramidal neuron was not significantly affected by the time point of weaning ($\boldsymbol{F}_{(1, 12)} = 1.42$; $\boldsymbol{P} = 0.256$) or postweaning rearing conditions ($\boldsymbol{F}_{(1, 12)} = 0.16$; $\boldsymbol{P} = 0.700$) and no *interactions* between the two factors were detected ($\boldsymbol{F}_{(1, 12)} = 1.74$; $\boldsymbol{P} = 0.211$). Similarly the basal dendritic complexity was not significantly affected by the time point of weaning ($\boldsymbol{F}_{(1, 12)} = 1.42$; $\boldsymbol{P} = 0.257$) or postweaning rearing conditions ($\boldsymbol{F}_{(1, 12)} = 0.55$; $\boldsymbol{P} = 0.471$) and no *interactions* between the two factors were detected ($\boldsymbol{F}_{(1, 12)} = 0.43$; $\boldsymbol{P} = 0.522$; table 23B, C, 24E and F). Since, no effect of any experimental factor was observed on the total dendritic length and complexity of ACd basal dendrite, the Sholl analysis for basal dendritic length and complexity per radial distances from soma was not performed.

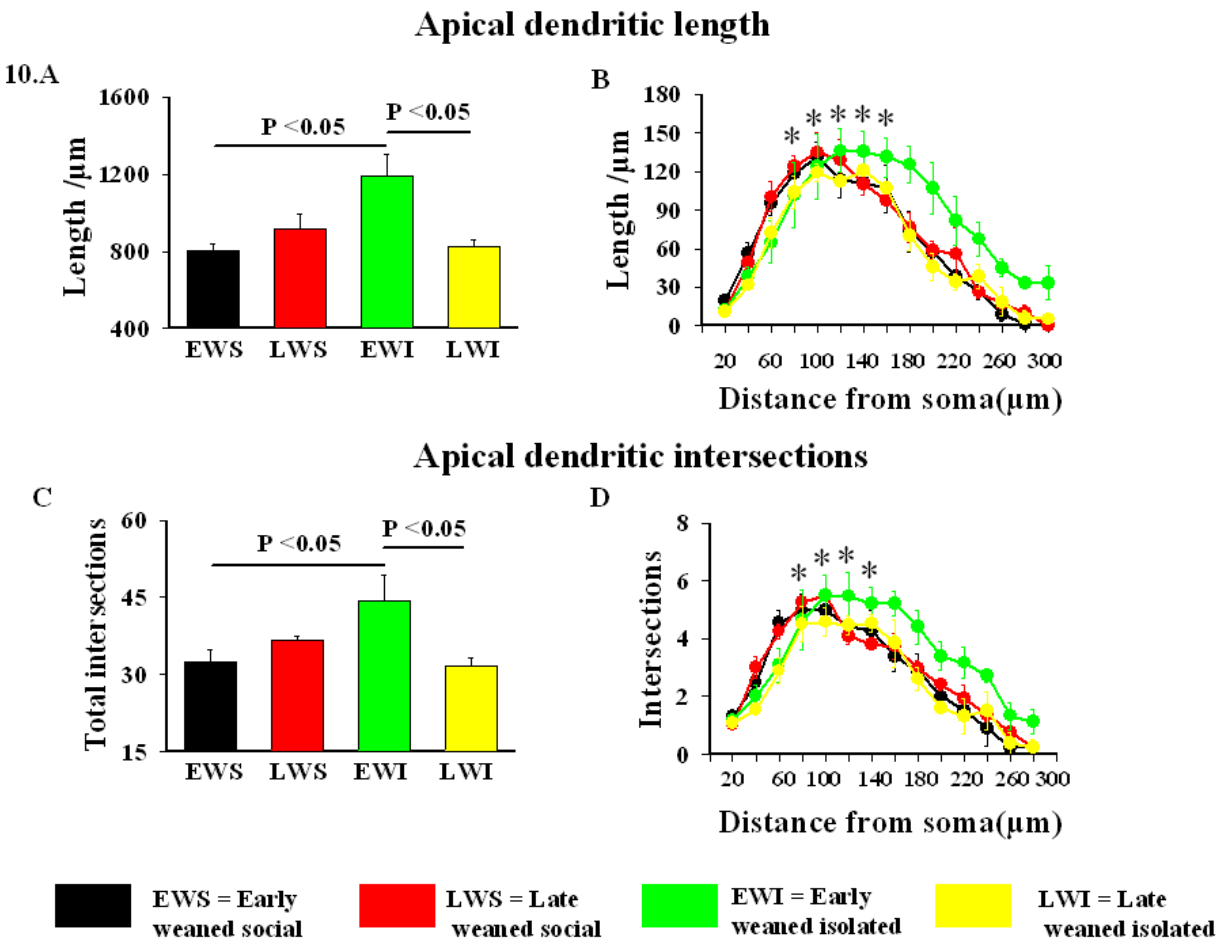


Figure 10. Analysis of apical dendritic length (A, B) and complexity (C, D) of pyramidal neurons located in layer II/III anterior cingulate cortex (ACd). (A-C) Statistical comparisons by Two-Way Analysis of Variance followed by post-hoc Student-Newman-Keuls (A) Mean of total dendritic length. (B-D) Statistical comparisons by mixed-design ANOVA and t-test. (B) Sholl analysis for dendritic length per 20 µm relative to soma. (*) indicates elongated dendrites at radial distances 80-160 µm relative to soma (***P*** < 0.05). (C) Mean intersection numbers (total dendritic complexity). (D) Sholl analysis for dendritic intersections per 20 µm relative to soma. (*) indicates higher complexity of dendrites at radial distances 80-140 µm relative to soma (***P*** < 0.05). Values are given as mean ± S.E.M.

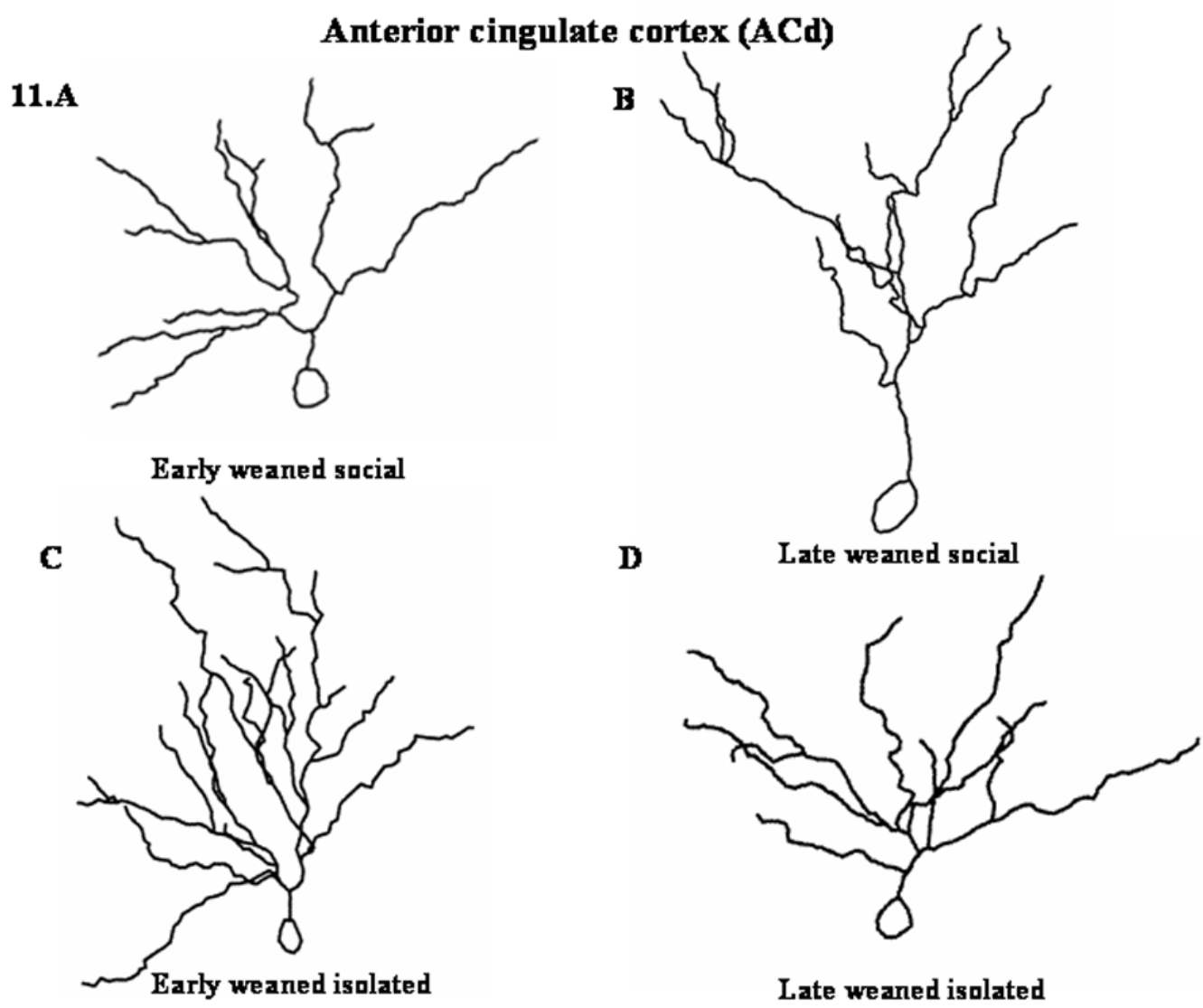


Figure 11. Representative reconstructions of apical dendrite from layer II/III anterior cingulate cortex (ACd). (A) Apical dendrite of Early weaned social animal (EWS). (B) Apical dendrite of Late weaned social animal (LWS). (C) Apical dendrite of Early weaned isolated animal (EWI) demonstrating longer and more complex apical dendrites compared to other groups. (D) Apical dendrite of late weaned isolated animals (LWI).

3.3 Orbitofrontal cortex (OFC):

A. Spine frequencies:

Apical dendrite - In contrary to the findings for ACd, the apical dendritic spine density of orbitofrontal cortical pyramidal neurons did not modify in response to weaning time ($\boldsymbol{F}_{(1, 16)} = 0.50$; $\boldsymbol{P} = 0.488$) or postweaning rearing environments ($\boldsymbol{F}_{(1, 16)} = 0.09$; $\boldsymbol{P} = 0.770$) and no *interactions* between the two factors were found ($\boldsymbol{F}_{(1, 16)} = 1.34$; $\boldsymbol{P} = 0.264$; datas shown in table 23A, 24A and B).

Basal dendrite – Similar to the findings for the apical dendrite, the basal dendritic spine density of pyramidal neurons in orbitofrontal cortex did not modify in response to weaning time ($\boldsymbol{F}_{(1, 16)} = 0.003$; $\boldsymbol{P} = 0.955$), or postweaning rearing environments ($\boldsymbol{F}_{(1, 16)} = 0.44$; $\boldsymbol{P} = 0.518$) and no *interactions* between the two factors were found ($\boldsymbol{F}_{(1, 16)} = 1.65$; $\boldsymbol{P} = 0.217$; datas shown in table 23A, 24A and B).

B. Dendritic length and complexity:

A) Total dendritic length and complexity:

Apical dendrite – Similar to the findings for the ACd, the apical dendritic lengths of OFC pyramidal neurons were not affected by weaning time ($\boldsymbol{F}_{(1, 16)} = 2.44$; $\boldsymbol{P} = 0.139$) and by postweaning environments ($\boldsymbol{F}_{(1, 16)} = 1.72$; $\boldsymbol{P} = 0.209$). Similarly, the apical dendritic complexities of OFC pyramidal neurons were not affected by weaning time ($\boldsymbol{F}_{(1, 16)} = 0.54$; $\boldsymbol{P} = 0.473$) and by postweaning environments ($\boldsymbol{F}_{(1, 16)} = 1.62$; $\boldsymbol{P} = 0.221$). However, an *interaction* between the factors weaning time x postweaning environments reorganized the apical dendritic length ($\boldsymbol{F}_{(1, 16)} = 9.58$; $\boldsymbol{P} = 0.007$) and complexity ($\boldsymbol{F}_{(1, 16)} = 15.53$; $\boldsymbol{P} = 0.001$) of orbitofrontal cortical apical dendrite in a way dissimilar to that observed in the apical dendrite of anterior cingulate cortex. Post-hoc test revealed that the apical dendritic length and complexity was increased in animals with short preweaning but longer postweaning social experience i.e. early weaned social animals (EWS). Compared to late weaned social (LWS), EWS animals displayed longer (+33%;

P <0.05) and more complex (+31%; ***P*** <0.05) apical dendrites and compared to early weaned isolated (EWI) animals, EWS animals displayed longer (+32%; ***P*** <0.05) and more complex (+35%; ***P*** <0.05) apical dendrites (Figure-12A, 12C, 13A-D; table 23B, C, 24E and F).

B) Apical dendritic length and complexity per radial distance from soma:

Sholl analysis for apical dendritic length revealed the main effect of radial distances from soma ($F_{(14,224)}$ = 154.65; ***P*** = 0.000) and rearing ($F_{(1, 16)}$ = 5.31; ***P*** = 0.035) but not the effect of weaning ($F_{(1, 16)}$ = 0.16; ***P*** = 0.693). In addition, for apical dendritic length *interactions* between the factors weaning x rearing ($F_{(1, 16)}$ = 15.44; ***P*** = 0.001) and between weaning x rearing x radial distances from soma ($F_{(14,224)}$ = 2.20; ***P*** = 0.008) were found. Post-hoc comparisons revealed that the early weaned social animals had longer dendrites at radial distances of 100-300 µm from soma compared to early weaned isolated and elongated dendrites at radial distances of 180-300 µm compared to late weaned social animals (***P*** < 0.05; Figures- 12B).

Sholl analysis for apical dendritic complexity revealed only the main effect of radial distances from soma ($F_{(13,208)}$ = 106.66; ***P*** = 0.000) and not the effect of weaning ($F_{(1, 16)}$ = 0.76; ***P*** = 0.396) or rearing ($F_{(1, 16)}$ = 1.24; ***P*** = 0.281). In addition, for apical dendritic complexity there was no *interactions* between weaning x rearing x radial distances from soma ($F_{(13,208)}$ = 1.35; ***P*** = 0.187) but an *interaction* between the factors weaning x rearing ($F_{(1, 16)}$ = 6.16; ***P*** = 0.025) was observed. Post-hoc comparisons revealed that early weaned social animals had enhanced apical dendritic complexity at radial distances of 120, 140, 160, 200, 220, 240 and 260 µm from the soma (***P*** < 0.05; 12D).

Basal dendrite – Contrary to the apical dendrite, the basal dendritic length of OFC pyramidal neurons was not significantly affected by the experimental factors; time point of weaning ($F_{(1, 16)}$ = 1.36; ***P*** = 0.261), postweaning rearing conditions ($F_{(1, 16)}$ = 0.65; ***P*** = 0.431) and no *interactions* between the two factors were detected ($F_{(1, 16)}$ = 2.35; ***P*** = 0.145). Similarly, the basal dendritic complexity was not significantly affected by the time point of weaning ($F_{(1, 16)}$ = 1.22; ***P*** = 0.286), postweaning rearing conditions ($F_{(1, 16)}$ = 0.05; ***P*** = 0.826), and by *interactions* between the two factors ($F_{(1, 16)}$ = 2.57; ***P*** =

0.129); (table 23B, C, 24E and F). Since, no effect of any experimental factor was observed on the total dendritic length and complexity of OFC basal dendrite, the Sholl analysis for basal dendritic length and complexity per radial distances from soma was not performed.

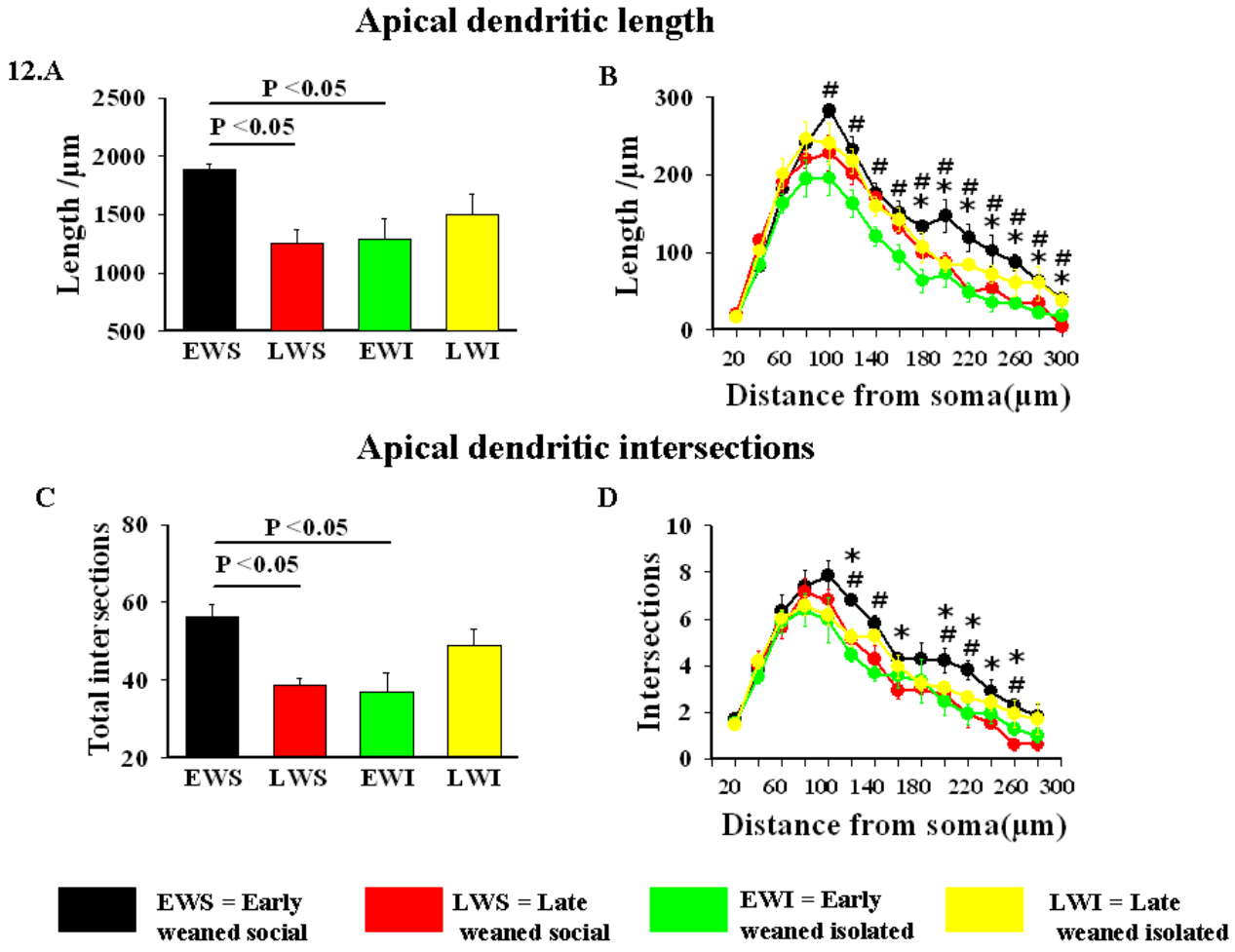


Figure 12. Analysis of apical dendritic length (A, B) and complexity (C, D) of pyramidal neurons located in layer II/III orbitofrontal cortex (OFC). (A-C) Statistical comparisons by Two-Way Analysis of Variance followed by post-hoc Student-Newman-Keuls test. (A) Mean of total dendritic length. (B-D) Statistical comparisons by mixed-design ANOVA, post-hoc comparisons and t-test. (B) Sholl analysis for dendritic length per 20 µm relative to soma. (C) Mean intersection numbers (total dendritic complexity). (D) Sholl analysis for dendritic intersections per 20 µm relative to soma. (*) indicates difference between Early weaned

social (EWS) and Late weaned social animals (LWS). (#) indicates difference between Early weaned social (EWS) and Early weaned isolated animals (EWI). Values are given as mean ± S.E.M.

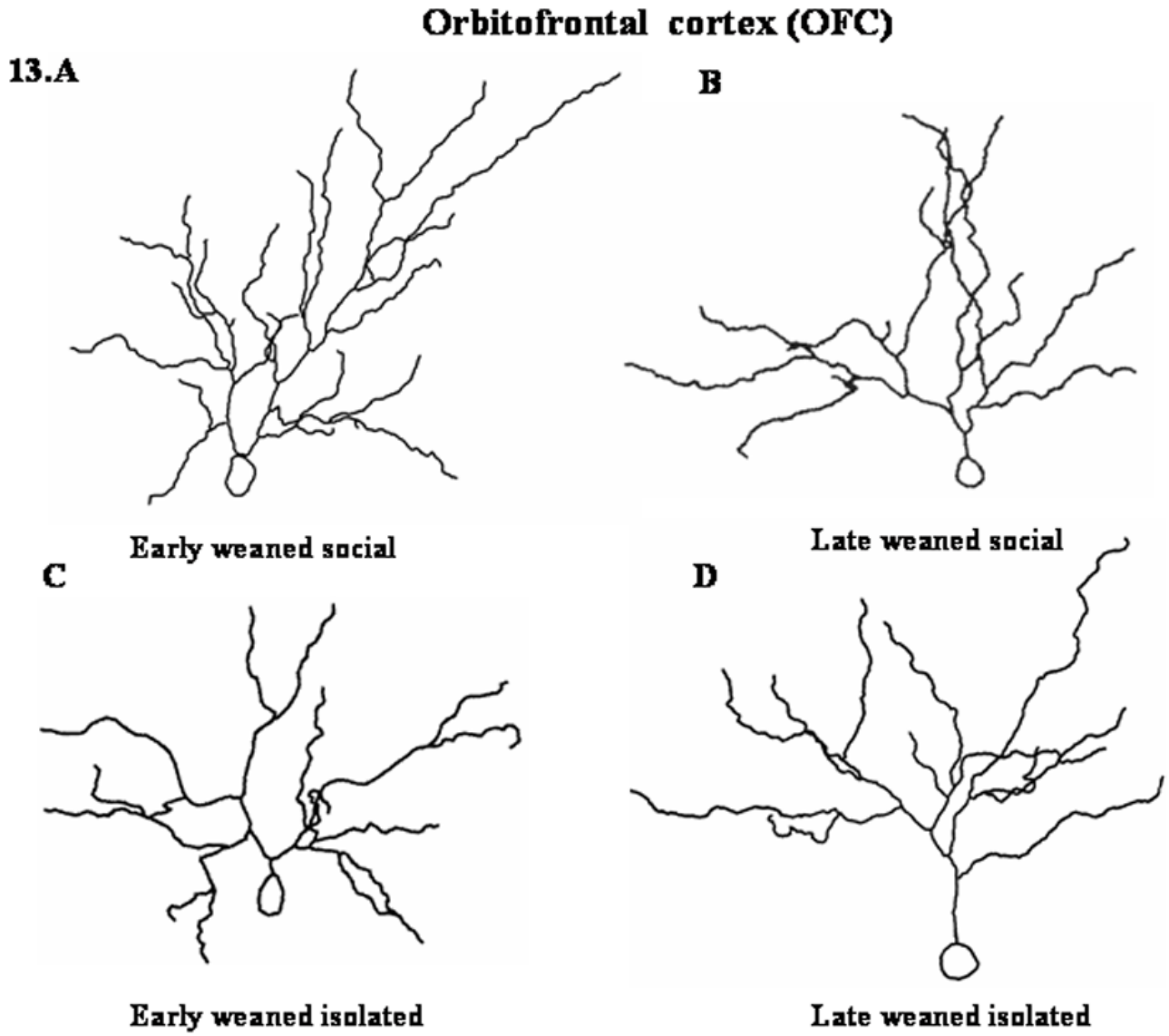


Figure 13. Representative reconstructions of apical dendrite from layer II/III orbitofrontal cortex (OFC). (A) Apical dendrite of Early weaned social animal (EWS) demonstrating longer and complex dendrites. (B) Apical dendrite of Late weaned social animal (LWS). (C) Apical dendrite of Early weaned isolated animal (EWI). (D) Apical dendrite of late weaned isolated animals (LWI).

3.4 Effects of time point of weaning and social environments on the morphology of pyramidal and granular neurons in hippocampus

3.5 Infra pyramidal layer of dentate gyrus (IDG):

A. Spine frequencies: For the spine density of infra granular neurons, the main effect was found to be the time point of weaning ($F_{(1, 16)}$ = 19.73; P = <0.001; early versus late), whereas no effect of the postnatal rearing environment ($F_{(1, 16)}$ = 4.32; P = 0.054) and no *interactions* between the two factors were found ($F_{(1, 16)}$ = 1.57; P = 0.228). The animals weaned early (EWS + EWI) displayed higher densities of spines (+25%) on their infra granular neurons compared to late weaned animals (LWS + LWI) (P <0.05; Figure- 14A, B, C; table 23A, 24C and D). Branch order analysis revealed that the elevated spine densities were particularly evident on mid-distal dendritic branch orders 4th and 5th (P <0.05; Figure- 14D).

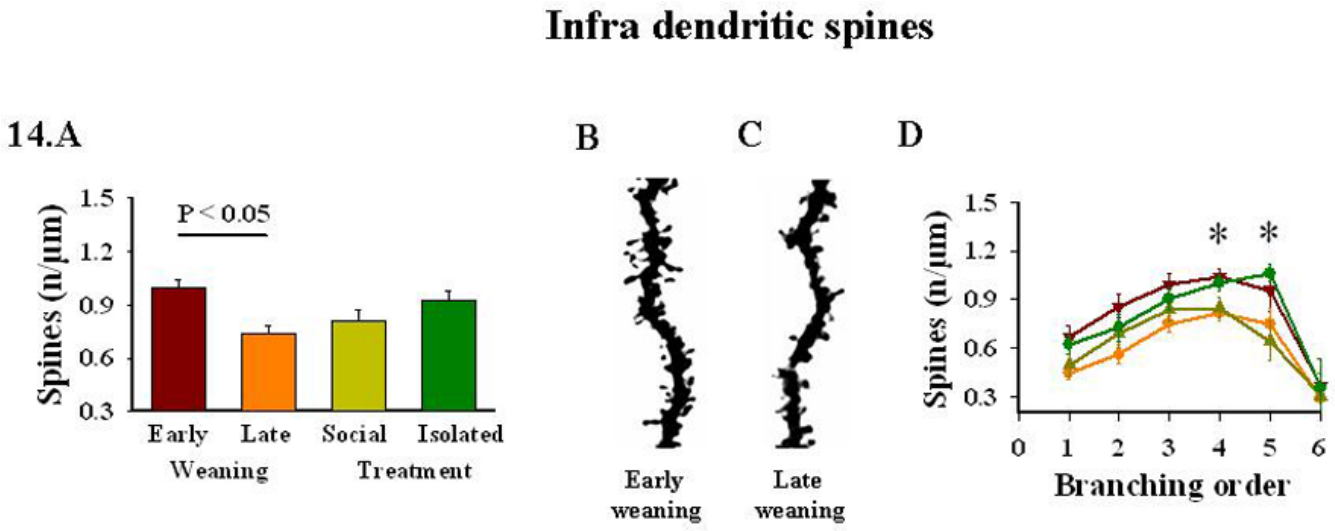


Figure 14. Analysis of spine frequencies of granular neurons located in the infra pyramidal layer of dentate gyrus (A) Infra granular dendritic tree. (D) Branch order analysis of infra granular dendrite. At branch order 4th and 5th, Early weaned > late weaned (P < 0.05). Values are given as mean ± S.E.M. Statistical comparisons by Two-Way Analysis of Variance

(A, D). (B, C) Representative images of Golgi-Cox impregnated segments. Segments from infra granular dendrites of early weaned (B) and late weaned (C) animals.

B. Dendritic length and complexity:

A) *Total dendritic length and complexity:*

The dendritic length of granular neurons located in the infra pyramidal layer of dentate gyrus, was affected by the weaning time ($F_{(2, 14)} = 5.64$; $P = 0.016$), by postweaning environments ($F_{(1, 14)} = 8.36$; $P = 0.012$) and in addition by an *interaction* between the factors weaning time x postweaning environments ($F_{(2, 14)} = 5.05$; $P = 0.022$). The dendritic complexity was affected only by weaning time ($F_{(1, 16)} = 12.72$; $P = 0.003$) and by *interactions* ($F_{(1, 16)} = 8.06$; $P = 0.012$) but not by postweaning environments ($F_{(1, 16)} = 4.32$; $P = 0.054$). The animals weaned early (EWS + EWI) demonstrated longer dendritic length (+19%) and higher dendritic complexity (+24%) compared to animals weaned later (LWS + LWI) ($P < 0.05$; Figure – 15A, D). Moreover, the animals that were reared in isolation (EWI + LWI) postweaning also demonstrated longer (+19%) dendrites compared to animals that were reared socially postweaning (EWS + LWS) ($P < 0.05$; Figure – 15A). The post-hoc analysis demonstrated that the animals with fewest social experiences i.e. early weaned from their dam and isolated postweaning (EWI) displayed elongated and complex infra granular dendrites compared to all other groups. For instance, compared to early weaned social (EWS) animals, these animals displayed longer (+34%; $P < 0.05$) and more complex dendrites, (+33%; $P < 0.05$); compared to late weaned isolated (LWI) animals, EWI animals displayed longer (+34%; $P < 0.05$) and more complex dendrites (+36%; $P < 0.05$) and compared to late weaned social (LWS) animals, EWI animals displayed longer (+32%; $P < 0.05$) and more complex dendrites (+37%; $P < 0.05$); (Figure- 15B, E, 16A-D; table 23B, C, 24G and H).

B) Dendritic length and complexity per radial distance from soma:

Sholl analysis for infra dendritic length revealed only the main effect of radial distances from soma ($F_{(7,112)} = 72.95$; $P = 0.000$) and rearing ($F_{(1,16)} = 5.97$; $P = 0.026$) but not the effect of weaning ($F_{(1,16)} = 3.62$; $P = 0.075$) and no *interactions* between

weaning x rearing ($F_{(1,16)}$ = 4.41; P = 0.052) and between weaning x rearing x radial distances from soma ($F_{(7,112)}$ = 2.86; P = 0.110) were found. However, an *interaction* between radial distances from soma x rearing ($F_{(7,112)}$ = 4.84; P = 0.004) was found for infra granular dendritic length. Post-hoc test revealed that the elongated dendrites were confined to mid-distal dendritic segments at radial distances of 160-320 µm related to the soma, in the early weaned and socially isolated animals (EWI) compared to other groups (P <0.05; Figure- 15C)

Sholl analysis for dendritic complexity revealed the main effect of radial distances from soma ($F_{(6,96)}$ = 43.47; P = 0.000), weaning ($F_{(1,16)}$ = 6.42; P = 0.022) and rearing ($F_{(1,16)}$ = 7.82; P = 0.013), but no *interactions* between weaning x rearing x radial distances from soma ($F_{(6,96)}$ = 1.39; P = 0.227) and between weaning x rearing ($F_{(1,16)}$ = 1.43; P = 0.249) were detected. However, an *interaction* between radial distances from soma x rearing ($F_{(6, 96)}$ = 3.75; P = 0.012) was found for infra granular dendritic complexity. Post-hoc test revealed that the enhanced dendritic complexity was found at radial distances of 120-280 µm from the soma in the early weaned and socially isolated animals (EWI) compared to other groups (P <0.05; Figure- 15 F).

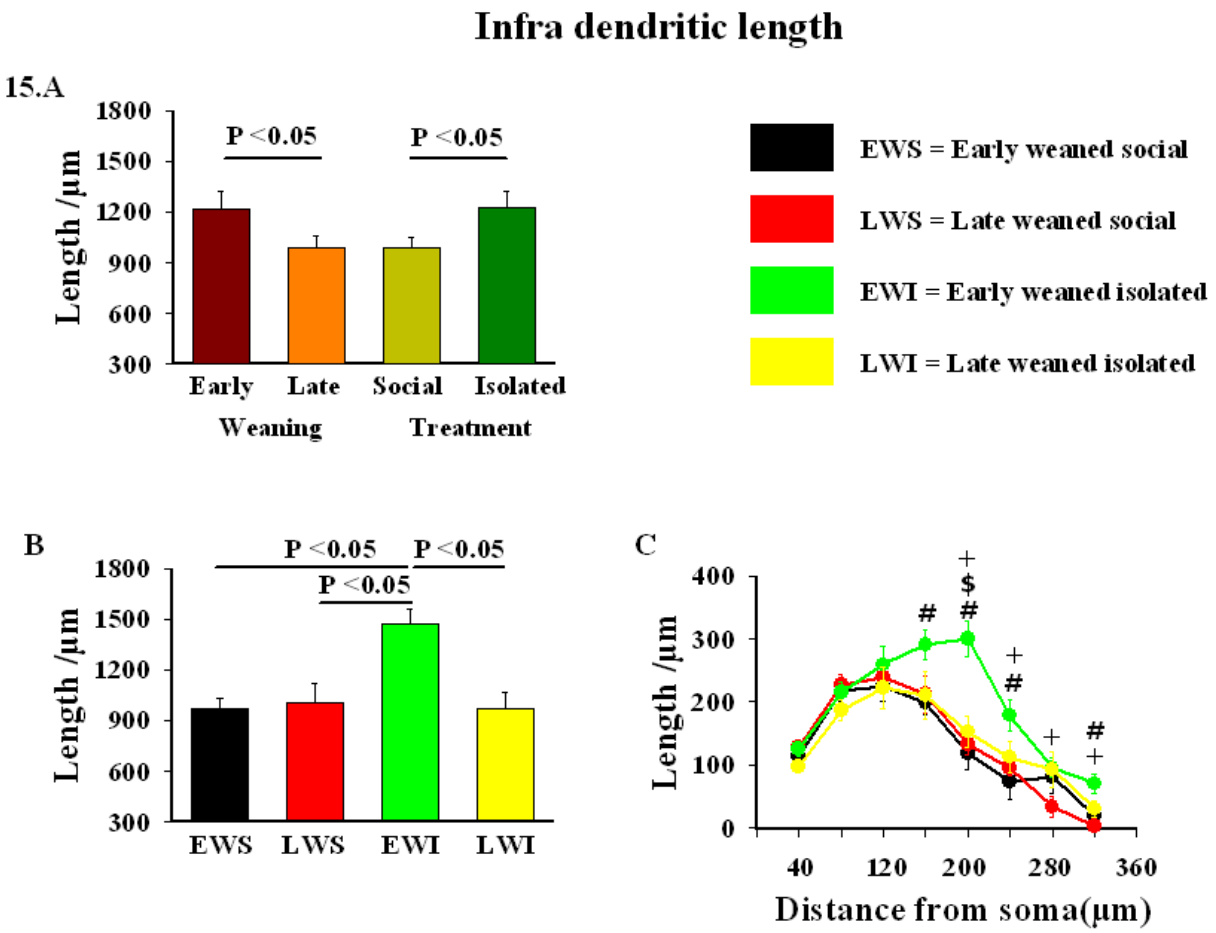


Figure 15. Analysis of dendritic length (A, B, C) of granular neurons located in the infra pyramidal layer of dentate gyrus. (A) Mean of total dendritic length statistically compared by Two-Way ANOVA. (B) Mean of total dendritic length statistically compared by post-hoc Student-Newman-Keuls test. (C) Sholl analysis for dendritic length per 40 µm relative to soma; statistically compared by mixed-design ANOVA, post-hoc comparisons and t-test. **(#)** indicates difference between Early weaned isolated (EWI) and Early weaned social animals (EWS). **($)** indicates difference between Early weaned isolated (EWI) and Late weaned isolated animals (LWI). **(+)** indicates difference between Early weaned isolated (EWI) and Late weaned social animals (LWS). Values are given as mean ± S.E.M.

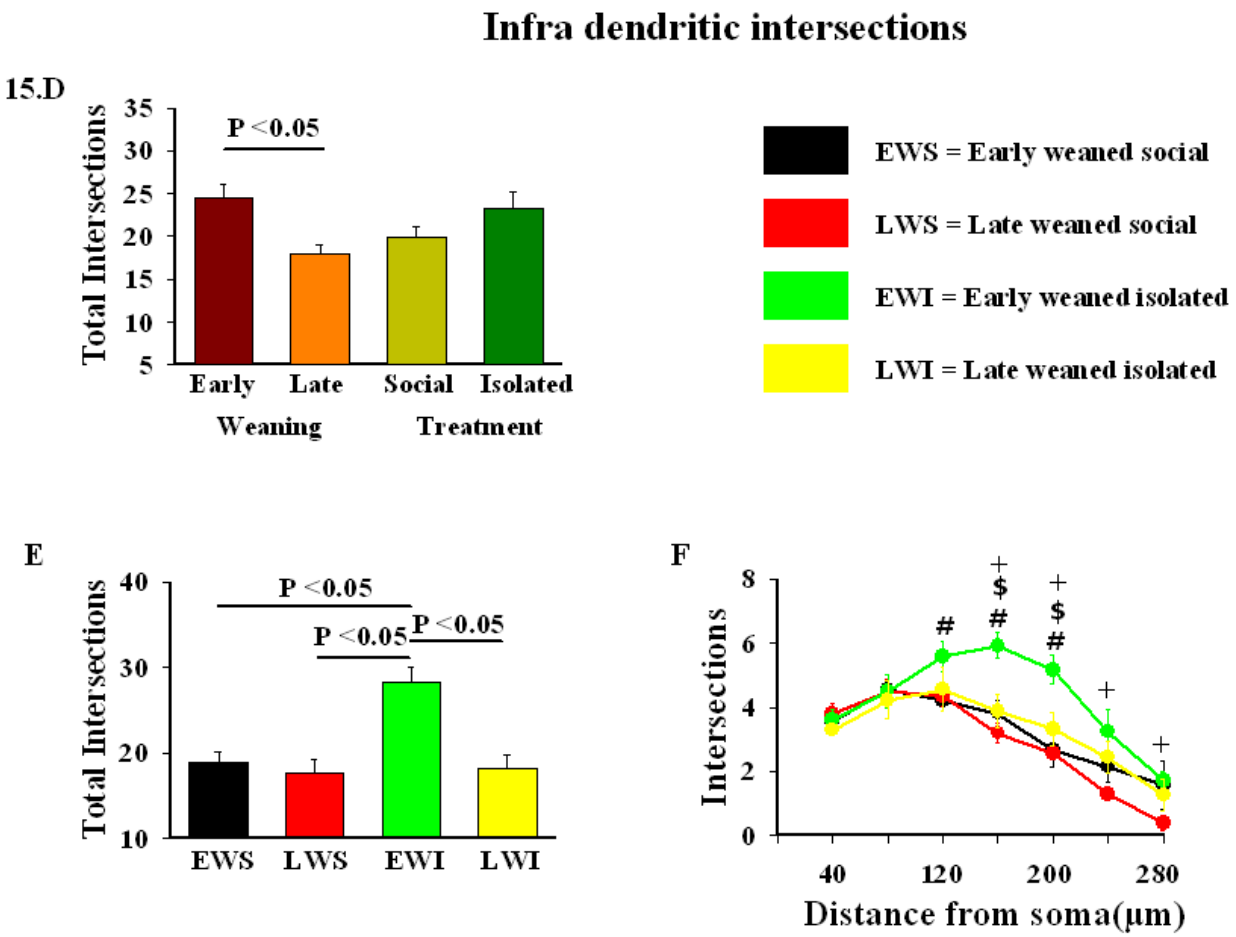
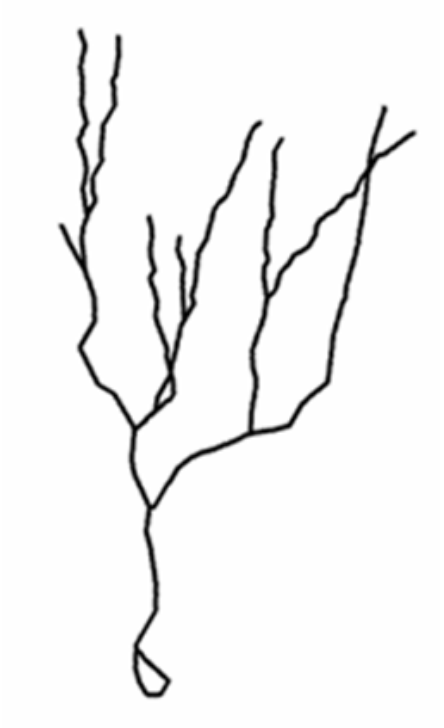


Figure 15. Analysis of dendritic complexity (D, E, F) of granular neurons located in the infra pyramidal layer of dentate gyrus. (D) Mean intersection numbers (total dendritic complexity) statistically compared by Two-Way Analysis of Variance (E) Mean intersection numbers (total dendritic complexity) statistically compared by post-hoc Student-Newman-Keuls test (F) Sholl analysis for dendritic intersections per 40 µm relative to soma; statistically compared by mixed-design ANOVA, post-hoc comparisons and t-test. (#) indicates difference between Early weaned isolated (EWI) and Early weaned social animals (EWS). **($)** indicates difference between Early weaned isolated (EWI) and Late weaned isolated animals (LWI). (+) indicates difference between Early weaned isolated (EWI) and Late weaned social animals (LWS). Values are given as mean ± S.E.M.

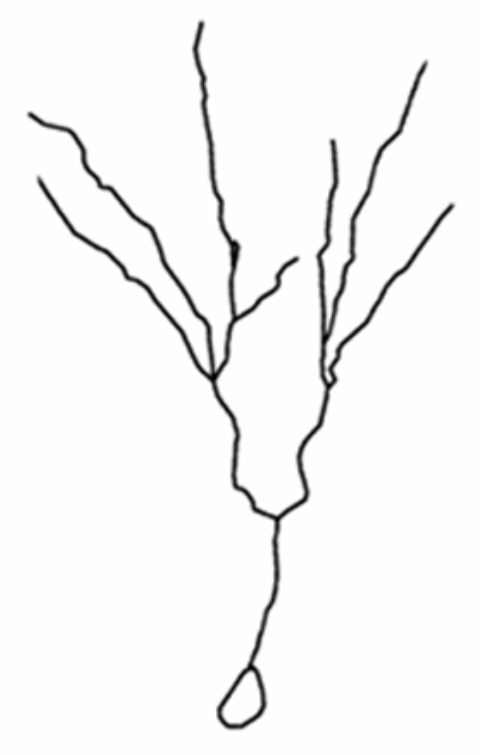
Infra granular neuron

16.A



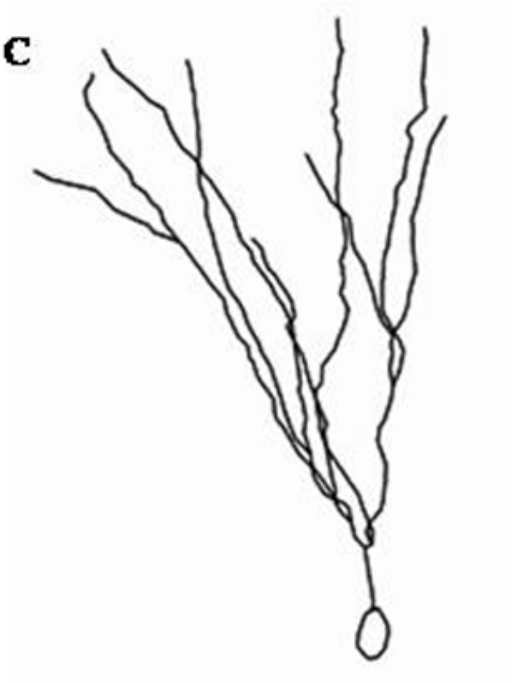
Early weaned social

B



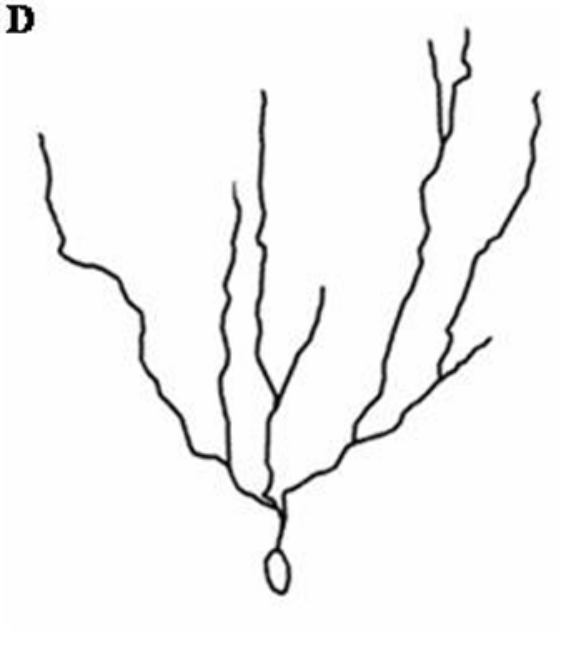
Late weaned social

C



Early weaned isolated

D



Late weaned isolated

Figure 16. Representative reconstructions of granular neurons from the infra pyramidal layer of dentate gyrus. (A) Granular neuron of Early weaned social animal (EWS). (B) Granular neuron of Late weaned social animal (LWS). (C) Granular neuron of Early weaned isolated animal (EWI) demonstrating longer and complex dendrite compared to other group. (D) Granular neuron of Late weaned isolated animal (LWI).

3.6 Supra pyramidal layer of dentate gyrus (SDG):

A. Spine frequencies: Similar to the effects in the infra pyramidal layer of dentate gyrus, the spine density on supra granular neurons demonstrated main effect of weaning time ($F_{(1, 16)} = 6.99$; $P = 0.018$; early versus late), and no effect of postnatal rearing environments ($F_{(1, 16)} = 4.21$; $P = 0.057$) and no *interactions* between the two factors ($F_{(1, 16)} = 0.27$; $P = 0.608$). The animals weaned early (EWS + EWI) displayed higher densities of spines (+14%) on their supra granular neurons compared to late weaned animals (LWS + LWI); (P <0.05; Figure- 17A, B and C; table 23A, 24C and D). Branch order analysis revealed that the elevated spine density was particularly evident on mid-distal dendritic branch orders 4th and 5th (P <0.05; Figure- 17D).

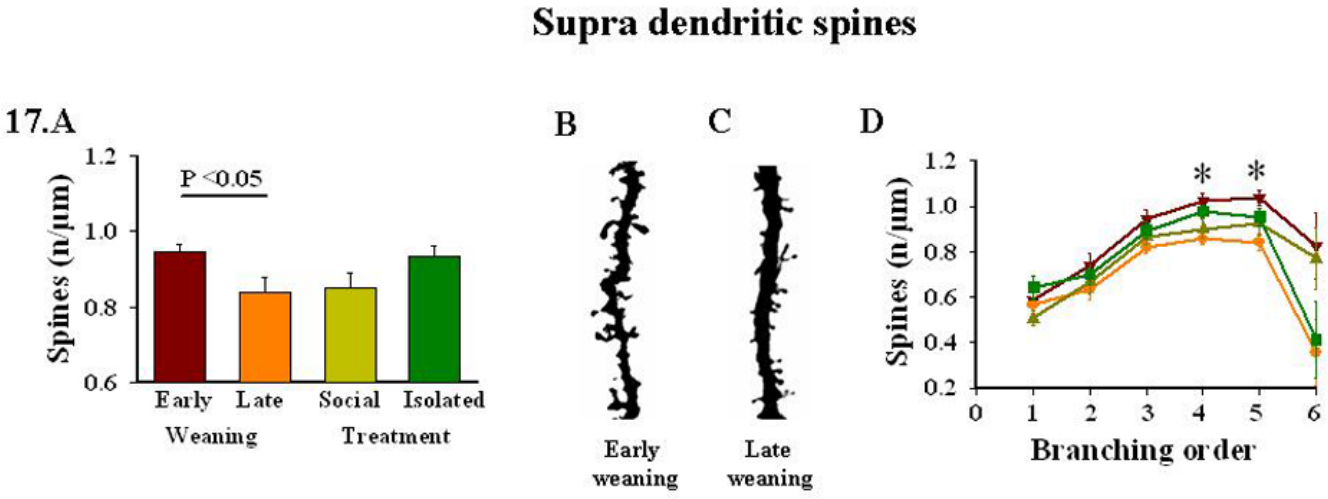


Figure 17. Analysis of spine frequencies of granular neurons located in the supra pyramidal layer of dentate gyrus. (A) Supra granular dendritic tree. (D) Branch order analysis of supra granular dendrite. Values are given as mean ± S.E.M. Statistical comparisons

by Two-Way Analysis of Variance (A, D). (B, C) Representative images of Golgi-Cox impregnated segments. Segments from supra granular dendrites of early weaned (B) and late weaned (C) animals.

B. Dendritic length and complexity:

A) Total dendritic length and complexity:

The dendritic length of supra granular neurons was affected only by weaning time ($F_{(1, 16)}$ = 20.91; P = <0.001) and by *interactions* between the factors weaning time x postweaning environments ($F_{(1, 16)}$ = 5.20; P = 0.037). No effect of postweaning rearing environments was observed ($F_{(1,16)}$ = 0.85; P = 0.370). Similarly, the dendritic complexity was affected only by weaning time ($F_{(1,16)}$ = 4.75; P = 0.045) and by *interactions* between the factors weaning time x postweaning environments ($F_{(1,16)}$ = 7.06; P = 0.017) and no effect of postweaning rearing environments was observed ($F_{(1,16)}$ = 1.67; P = 0.215). Two-Way Anova revealed that animals weaned early (EWS + EWI) demonstrated longer (+29%) and complex (+18%) dendrites compared to animals weaned later (LWS + LWI) (P <0.05; Figure -18A, D). Similar to that in the infra dentate gyrus, a post-hoc analysis revealed that the animals with fewest social experience i.e. early weaned from their dam and isolated postweaning (EWI) displayed elongated and complex supra granular dendrites compared to all other experimental groups. For e.g. compared to early weaned socials (EWS), EWI rats displayed longer (+18%; P <0.05) and more complex dendrites (+28%; P <0.05); compared to late weaned isolated (LWI) animals, EWI animals displayed elongated (+39%; P <0.05) and more complex dendrites (+34%; P <0.05) and compared to late weaned social (LWS) animals, EWI animals displayed longer (+31%; P <0.05) and more complex supra granular dendrites (+24%; P <0.05); (Figure- 18B, E, 19A-D; table 23B, C, 24G and H).

B) Dendritic length and complexity per radial distance from soma:

Sholl analysis for supra granular dendritic length revealed only the main effect of radial distances from soma ($F_{(8,128)}$ = 89.30; P = 0.000) and not the effect of weaning ($F_{(1,16)}$ = 2.05; P = 0.172) or rearing ($F_{(1,16)}$ = 0.38; P = 0.546) and no *interactions* between weaning x rearing x radial distances from soma ($F_{(8,128)}$ = 0.46; P = 0.885) and

between weaning x rearing ($F_{(1,16)}$ = 0.94; P = 0.347) were found. t-test revealed that the elongated dendrites were confined to radial distances of 80-200 µm relative to the soma (P < 0.05; Figure-18C).

Sholl analysis for dendritic complexity revealed only the main effect of radial distances from soma ($F_{(7,112)}$ = 70.94; P = 0.000) and not the effect of weaning ($F_{(1,16)}$ = 3.25; P = 0.090) or rearing ($F_{(1,16)}$ = 0.74; P = 0.403) and no *interactions* between weaning x rearing x radial distances from soma ($F_{(7,112)}$ = 0.82; P = 0.572) and between weaning x rearing ($F_{(1,16)}$ = 4.31; P = 0.054) were found. t-test revealed that the complex dendrites were confined to radial distances of 80-160 µm relative to the soma (P < 0.05; Figure-18F).

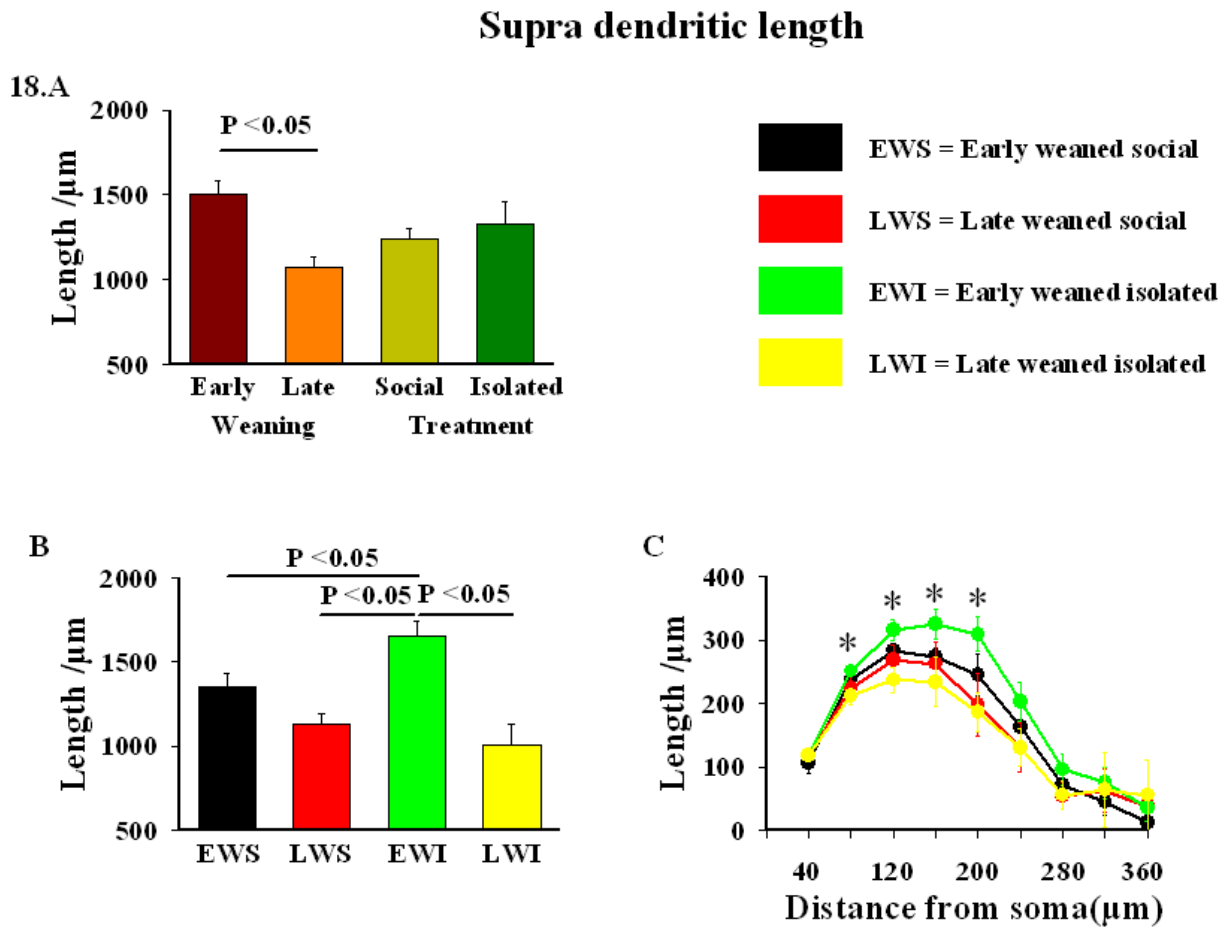


Figure 18. Analysis of dendritic length (A, B, C) of granular neurons located in the supra pyramidal layer of dentate gyrus. (A) Mean of total dendritic length statistically compared by

Two-Way ANOVA. (B) Mean of total dendritic length statistically compared by post-hoc Student-Newman-Keuls test. (C) Sholl analysis for dendritic length per 40 µm relative to soma; statistically compared by mixed-design ANOVA and t-test. (*) indicates elongated dendrites at radial distances 80-200 µm relative to the soma (***P*** < 0.05). Values are given as mean ± S.E.M.

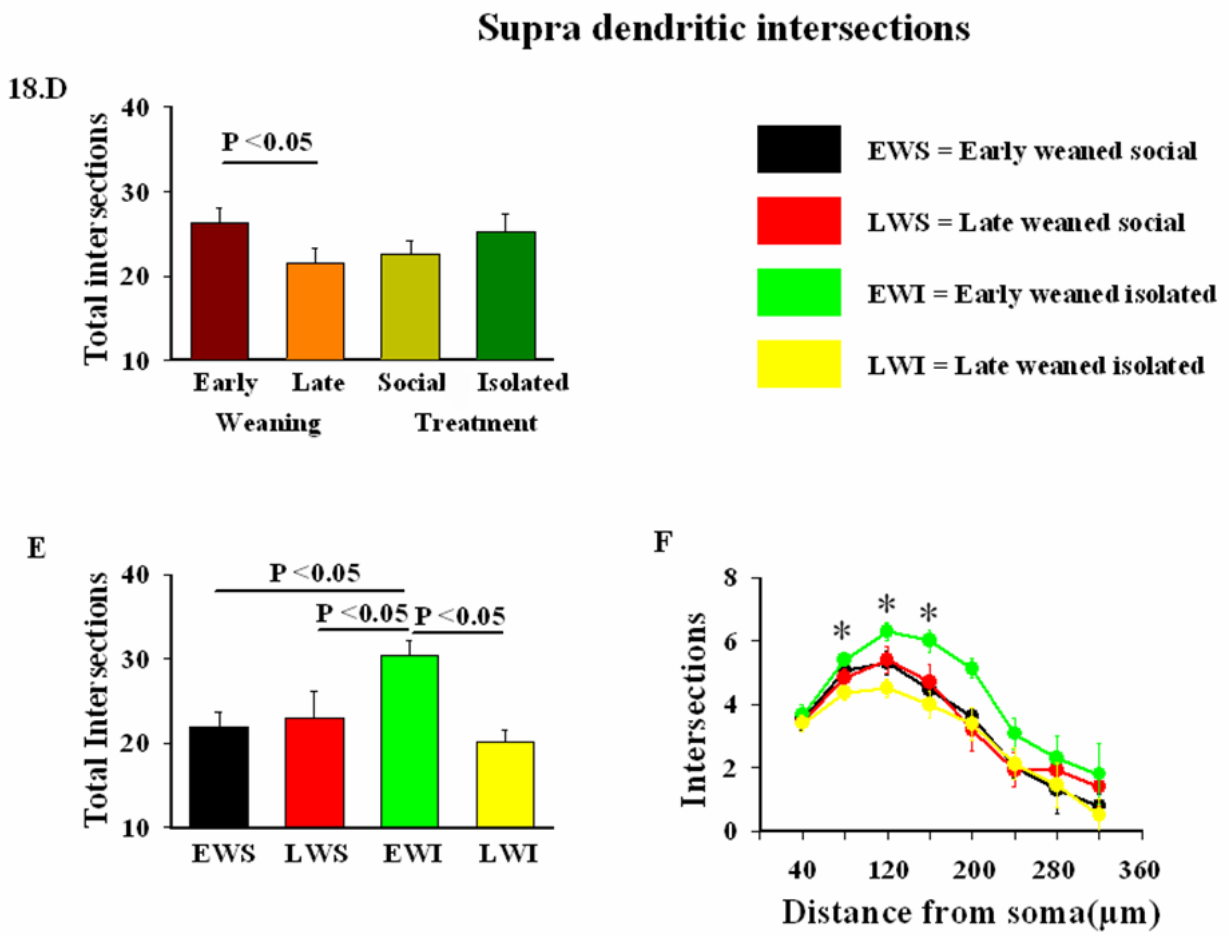


Figure 18. Analysis of dendritic complexity (D, E, F) of granular neurons located in the supra pyramidal layer of dentate gyrus. (D) Mean intersection numbers (total dendritic complexity) statistically compared by Two-Way Analysis of Variance (E) Mean intersection numbers (total dendritic complexity) statistically compared by post-hoc Student-Newman-Keuls test. (F) Sholl analysis for dendritic intersections per 40 µm relative to soma; statistically

compared by mixed-design ANOVA and t-test. $(*)$ indicates higher complexity of dendrites at radial distances (80-160µm) from the soma ($\boldsymbol{P} < 0.05$). Values are given as mean $\pm$ S.E.M.

Supra granular neuron

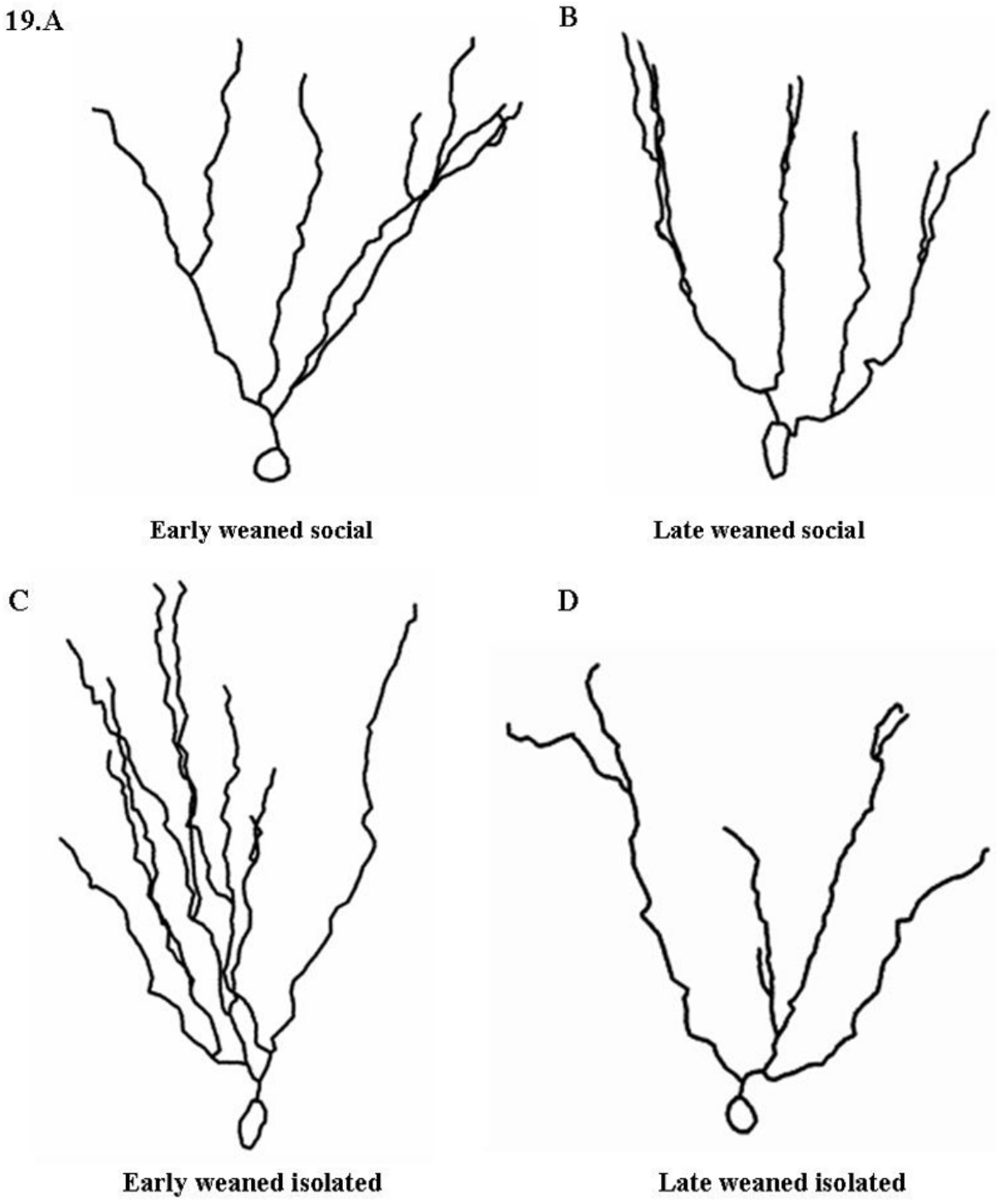


Figure 19. Representative reconstructions of granular neurons from the supra pyramidal layer of dentate gyrus (A) Granular neuron of Early weaned social animal (EWS). (B) Granular neuron of Late weaned social animal (LWS). (C) Granular neuron of Early weaned isolated animal (EWI) demonstrating longer and complex dendrite compared to other group. (D) Granular neuron of Late weaned isolated animal (LWI).

3.7 Hippocampal CA3 area:

A. Spine frequencies:

Apical dendrite – For the spine density of CA3 apical dendrite the main effect was found to be only the time point of weaning ($F_{(1,16)} = 8.83$; $P = 0.009$; early versus late), and not the postweaning rearing conditions ($F_{(1,16)} = 0.37$; $P = 0.551$) or the *interaction* between the factors ($F_{(1,16)} = 1.85$; $P = 0.193$). Two-Way Anova revealed that the animals weaned early (EWS + EWI) displayed higher densities of spines (+17%) on their CA3 apical dendrite compared to late weaned animals (LWS + LWI) ($P < 0.05$; Figure- 20A, B, C; table 23A, 24C and D). Branch order analysis revealed that the elevated spine density was particularly evident on middle dendritic branch orders 5^{th} and 6^{th} ($P < 0.05$; Figure- 20D).

Basal dendrite – Similar to apical dendrite, the spine density on CA3 basal dendrite displayed main effect of time point of weaning ($F_{(1,16)} = 5.71$; $P = 0.030$; early versus late), no effect of postweaning rearing conditions ($F_{(1,16)} = 0.48$; $P = 0.498$) and no *interaction* between the factors ($F_{(1,16)} = 3.53$; $P = 0.078$). Two-Way Anova revealed that the animals weaned early (EWS + EWI) displayed higher densities of spines (+23%) on their CA3 basal dendrite compared to late weaned animals (LWS + LWI) ($P < 0.05$; Figure- 20E, F, G; table 23A, 24C and D). Branch order analysis revealed that the elevated spine density was particularly evident on middle dendritic branch order 5^{th} ($P < 0.05$; Figure- 20H).

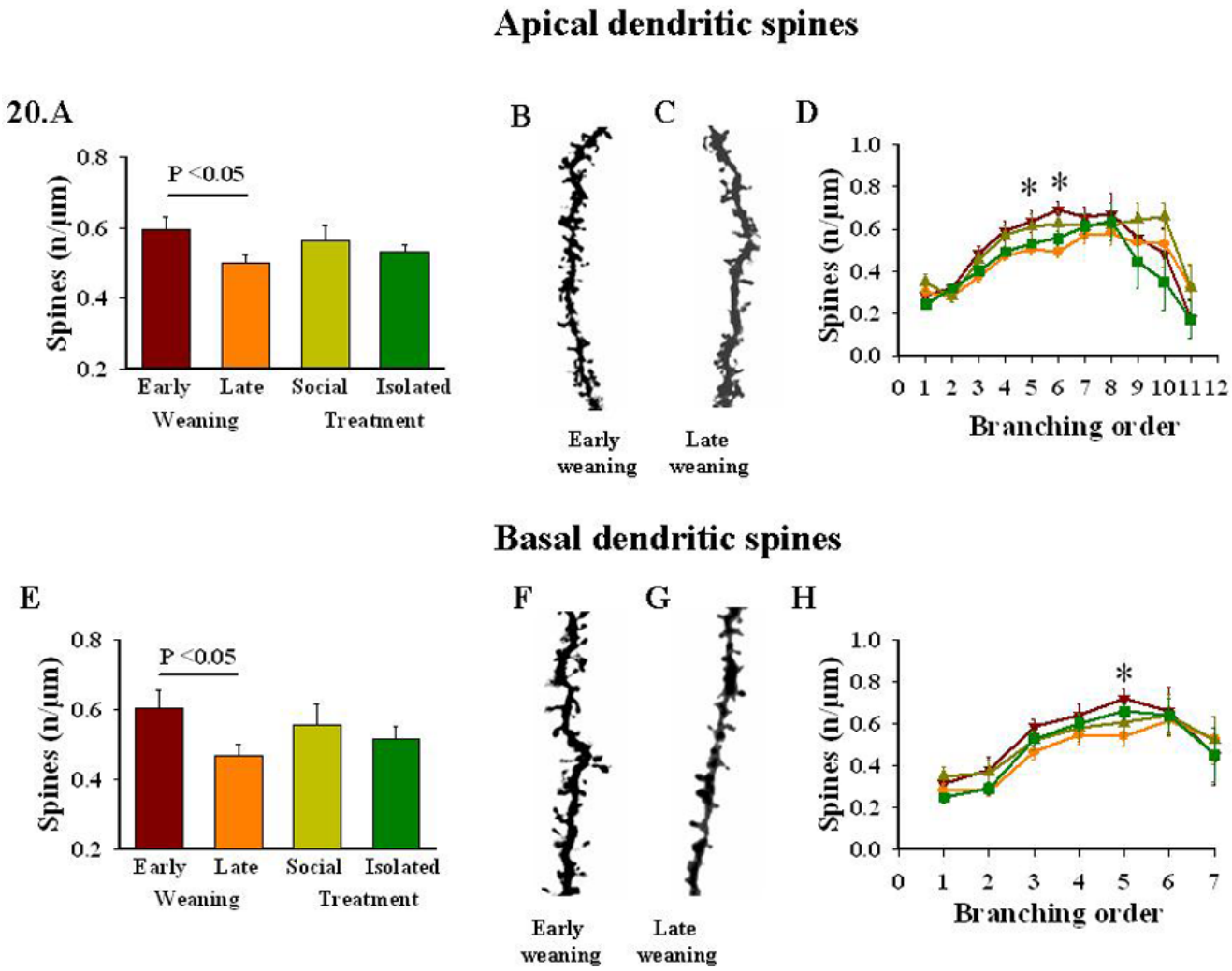


Figure 20. Analysis of spine frequencies of pyramidal neurons located in CA3 area of hippocampus (A) Apical dendrite. (D) Branch order analysis of apical dendrite. At branch order 5th and 6th, Early weaned > late weaned (***P*** <0.05). (E) Basal dendrite. (H) Branch order analysis of basal dendritic tree. At branch order 5th, Early weaned > late weaned (***P*** < 0.05). Values are given as mean $\pm$ S.E.M. Statistical comparisons by Two-Way Analysis of Variance (A, D, E, and H). (B, C, F, and G) Representative images of Golgi-Cox impregnated segments (B, C) Segments from apical dendrites of early weaned (B) and late weaned (C) animals. (F, G) Segments from basal dendrites of early weaned (F) and late weaned (G) animals.

B. Dendritic length and complexity:

A) Total dendritic length and complexity:

Apical dendrite - Just like the apical dendrite of prefrontal cortex, the apical dendritic length of CA3 pyramidal neurons was not affected by weaning time ($F_{(1,16)} = 1.67$; $P = 0.215$) and by postweaning rearing environments ($F_{(1,16)} = 2.24$; $P = 0.154$). Similarly, the apical dendritic complexity of CA3 pyramidal neurons was not affected by weaning time ($F_{(1,16)} = 1.32$; $P = 0.267$) and by postweaning rearing environments ($F_{(1,16)} = 0.14$; $P = 0.716$). However, an *interaction* between the factors weaning time x postweaning environments reorganized the apical dendritic length ($F_{(1,16)} = 18.42$; $P = <0.001$) and complexity ($F_{(1,16)} = 21.76$; $P = <0.001$) of CA3 pyramidal neuron. Post-hoc analysis revealed that the animals with fewest social experiences i.e. early weaned from their dam and isolated postweaning (EWI) displayed elongated and complex apical dendrites compared to other groups. Compared to early weaned socials (EWS) animals, EWI animals displayed longer (+21%; $P <0.05$) and more complex (+34%; $P <0.05$) apical dendrites; compared to late weaned isolated (LWI) animals, EWI animals displayed longer (+25%; $P <0.05$) and more complex (+27%; $P <0.05$) apical dendrites, (Figure- 21A, C, 22A-D). In addition, the late weaned social (LWS) animals displayed longer (+36%; $P <0.05$) and more complex (+41%; $P <0.05$) apical dendrites compared to early weaned social animals (EWS) and longer (+39%; $P <0.05$) and more complex (+35%; $P <0.05$) apical dendrites compared to late weaned isolated animals (LWI); (Figure 21A,C, 22A-D; table 23 B, C, 24G and H).

B) Apical dendritic length and complexity per radial distance from soma:

Sholl analysis for apical dendritic length revealed only the main effect of radial distances from soma ($F_{(10,160)} = 98.57$; $P = 0.000$) and not the effect of weaning ($F_{(1,16)} = 0.15$; $P = 0.705$) or rearing ($F_{(1,16)} = 4.03$; $P = 0.062$). However, an *interaction* between weaning x rearing x radial distances from soma ($F_{(10,160)} = 5.38$; $P = 0.002$) and between weaning x rearing ($F_{(1,16)} = 10.46$; $P = 0.005$) was detected for apical dendritic length. Post-hoc test revealed that elongated apical dendrites were confined to proximal-middle dendritic segments at radial distances of 150-350 µm relative to soma ($P <0.05$; Figure- 21B).

Sholl analysis for apical dendritic complexity revealed only the main effect of radial distances from soma ($F_{(9,144)} = 69.47$; $P = 0.000$) and not the effect of weaning ($F_{(1,16)} = 0.05$; $P = 0.822$) or rearing ($F_{(1,16)} = 2.88$; $P = 0.109$). However, an *interaction* between weaning x rearing x radial distances from soma ($F_{(9,144)} = 4.72$; $P = 0.003$) and between weaning x rearing ($F_{(1,16)} = 7.48$; $P = 0.015$) were found. Post-hoc test revealed that enhanced apical dendritic complexity was found at radial distances of 150-200 µm from the soma ($P < 0.05$; Figure- 21D).

Basal dendrite - Contrary to apical dendrite, the basal dendritic length of CA3 pyramidal neuron was not significantly affected by the time point of weaning ($F_{(1,16)} = 1.29$; $P = 0.273$) or postweaning rearing conditions ($F_{(1,16)} = 1.46$; $P = 0.245$), and no *interactions* between the two factors were detected ($F_{(1,16)} = 0.37$; $P = 0.553$). Similarly, the basal dendritic complexity was not significantly affected by the time point of weaning ($F_{(1,16)} = 2.16$; $P = 0.161$) or postweaning rearing conditions ($F_{(1,16)} = 0.31$; $P = 0.584$), and no *interactions* between the two factors were detected ($F_{(1,16)} = 0.007$; $P = 0.932$; table 23B, C, 24G and H). Since, no effect of any experimental factor was observed on the total dendritic length and complexity of CA3 basal dendrite, the Sholl analysis for basal dendritic length and complexity per radial distances from soma was not performed.

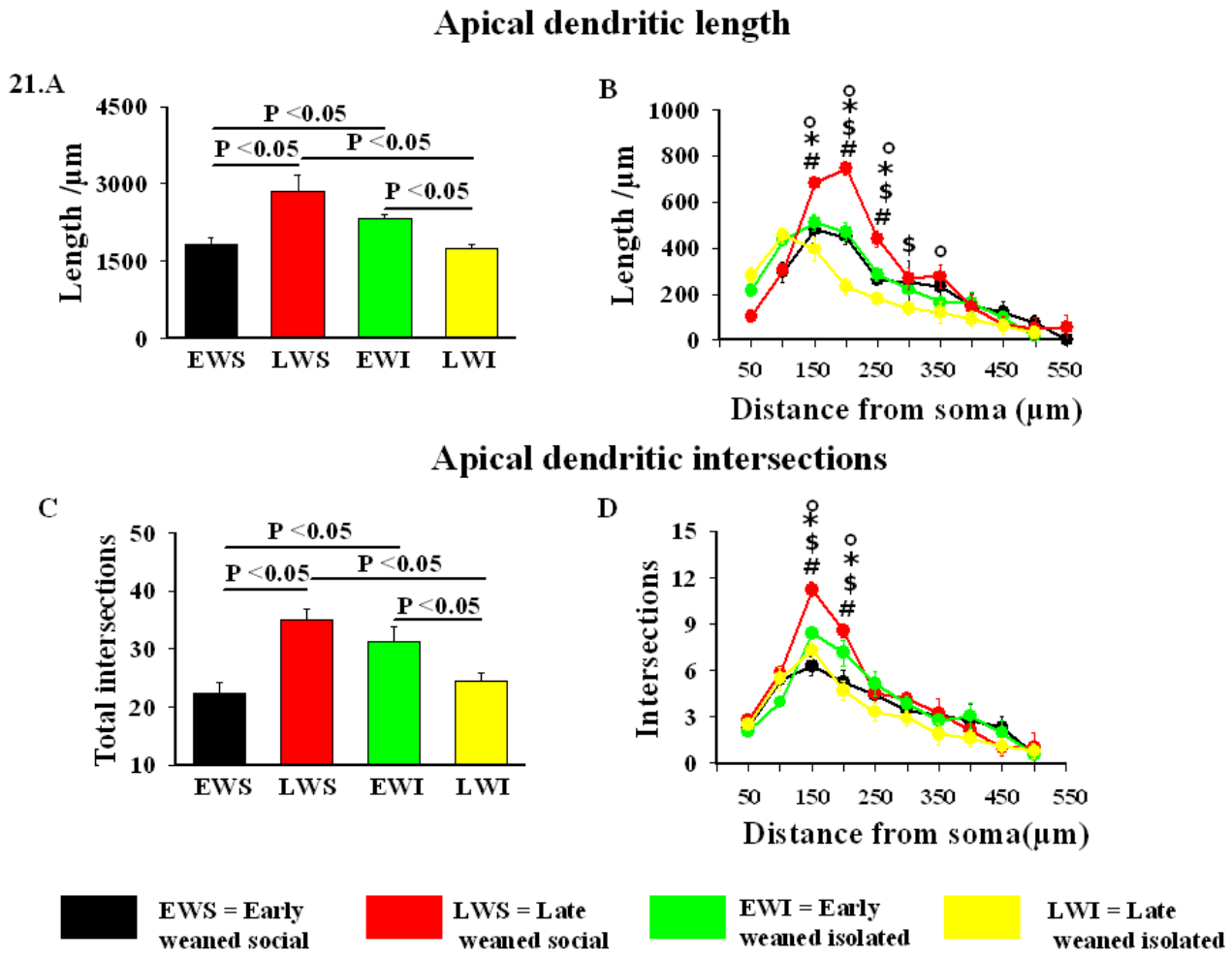


Figure 21. Analysis of apical dendritic length (A, B) and complexity (C, D) of pyramidal neurons located in field CA3 of hippocampus. (A-C) Statistical comparisons by Two-Way Analysis of Variance and by post-hoc Student-Newman-Keuls test. (A) Mean of total dendritic length. (B-D) Statistical comparisons by mixed-design ANOVA, post-hoc comparisons and t-test. (B) Sholl analysis for dendritic length per 50 µm relative to soma. (C) Mean intersection numbers (total dendritic complexity). (D) Sholl analysis for dendritic intersections per 50 µm relative to soma. (#) indicates difference between Early weaned social (EWS) and Early weaned isolated animals (EWI). ($) indicates difference between Early weaned isolated (EWI) and Late weaned isolated animals (LWI). (*) indicates difference between Early weaned social (EWS) and Late weaned social animals (LWS). (°) indicates difference between Late weaned isolated (LWI) and Late weaned social animals (LWS). Values are given as mean ± S.E.M.

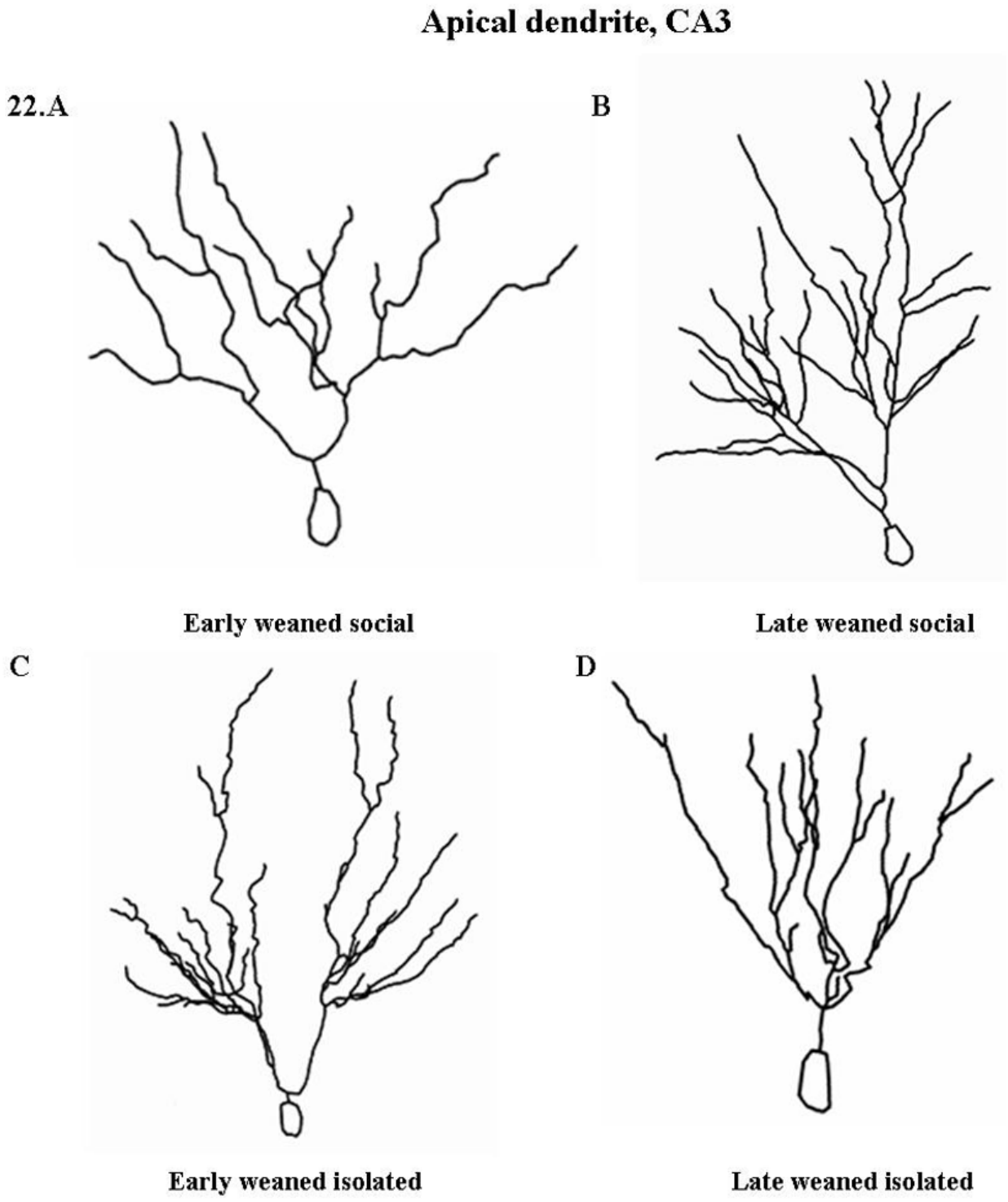


Figure 22. Representative reconstructions of apical dendrite from field CA3 of hippocampus (A) Apical dendrite of Early weaned social animal (EWS). (B) Apical dendrite of Late weaned social animal (LWS) demonstrating longest and most complex dendrite. (C) Apical dendrite of Early weaned isolated animal (EWI) demonstrating longer and complex dendrite compared to EWS and LWI. (D) Apical dendrite of Late weaned isolated animal (LWI).

3.8 Tables representing summary of results

23.A

Experimental Factors	Spine density changes in PFC & hippocampus							
	ACd		OFC		CA3		IDG	SDG
	Apical dendrite	Basal dendrite	Apical dendrite	Basal dendrite	Apical dendrite	Basal dendrite	Granular neuron	Granular neuron
Time of weaning (early vs late)	↑ **(Early weaned)**	↑ **(Early weaned)**	No effect	No effect	↑ **(Early weaned)**	↑ **(Early weaned)**	↑ **(Early weaned)**	↑ **(Early weaned)**
Postweaning environment (social vs isolated)	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Interactions (Time of weaning x social environment)	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect

B

Experimental Factors	Changes in dendritic length in PFC & hippocampus							
	ACd		OFC		CA3		IDG	SDG
	Apical dendrite	Basal dendrite	Apical dendrite	Basal dendrite	Apical dendrite	Basal dendrite	Granular neuron	Granular neuron
Time of weaning (early vs late)	No effect	No effect	No effect	No effect	No effect	No effect	↑ **(Early weaned)**	↑ **(Early weaned)**
Postweaning environment (social vs isolated)	No effect	No effect	No effect	No effect	No effect	No effect	↑ **(isolated animals)**	No effect
Interactions (Time of weaning x social environment)	**Effect EWI >EWS & LWI**	No effect	**Effect EWS >EWI & LWS**	No effect	**Effect EWI >EWS & LWI LWS >EWS & LWI**	No effect	**Effect EWI >EWS, LWI & LWS**	**Effect EWI >EWS, LWI & LWS**

C

Experimental Factors	Changes in dendritic complexity in PFC & hippocampus							
	ACd		OFC		CA3		IDG	SDG
	Apical dendrite	Basal dendrite	Apical dendrite	Basal dendrite	Apical dendrite	Basal dendrite	Granular neuron	Granular neuron
Time of weaning (early vs late)	No effect	No effect	No effect	No effect	No effect	No effect	↑ **(Early weaned)**	↑ **(Early weaned)**
Postweaning environment (social vs isolated)	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Interactions (Time of weaning x social environment)	**Effect EWI >EWS & LWI**	No effect	**Effect EWS >EWI & LWS**	No effect	**Effect EWI >EWS & LWI LWS >EWS & LWI**	No effect	**Effect EWI >EWS, LWI & LWS**	**Effect EWI >EWS, LWI & LWS**

Figure 23. Tables summarizing the results, viz. the effects of time of weaning, postweaning environments (social vs isolation) and interactions between the factors on prefrontal and hippocampal neurons. ↑ indicate subsequent increase in spine density, dendritic length and complexity. (A) Spine density changes in prefrontal cortex and hippocampus (B) Changes in dendritic length in prefrontal cortex and hippocampus. (C) Changes in dendritic complexity in prefrontal cortex and hippocampus. ACd = anterior cingulate cortex, CA3 = field CA3 of hippocampus, IDG = infra pyramidal layer of dentate gyrus, OFC = orbitofrontal cortex, PFC = prefrontal cortex and SDG = supra pyramidal layer of dentate gyrus.

4.0 Discussion

The results of my study support the hypothesis that the extent of social experience during pre- and postweaning periods is an essential factor for the reorganization of neuronal networks in limbic areas such as the prefrontal cortex and hippocampus. Dendritic spines are postsynaptic structures that are the site of more than 90% of the excitatory synapses with every spine head receiving at least one excitatory terminal

(Spacek and Hartmann, 1983; Harris and Stevens, 1989; Peters et al., 1991). Moreover, spines do not only serve to connect the axons and dendrites, but also are the site of long-term, stable memory in CNS neurons (Cajal, 1888). A number of experimental and behavioural conditions have demonstrated that dendritic spines are highly plastic structures that undergo marked alterations in their number and morphology following exposure to various stimuli such as sensory stimulation, kindling, pharmacological treatments, chronic stress, learning, emotional experience and psychological disorders and in most cases such changes have been reported to most likely accompany and modify behavioural development (Gould et al., 1990; Moser et al., 1994; Garey et al., 1998; Robinson et al., 2002; Poeggel et al., 2003; Radley et al., 2006).

A major emotional challenge during late childhood is the time of weaning; therefore, it can be assumed that this transition to independence should involve major structural changes in prefrontal cortex and hippocampus, which are involved in emotional self- regulation and the expression of emotional behaviours. In wild or in natural environments, weaning occurs transiently and gradually with the rats weaning between postnatal days 14 to 34. In contrary to this, it is a common laboratory practice to wean a litter at postnatal day 21 which may be implicated in behavioural as well as structural alterations in the pup. The quantitative neuromorphological analysis in relation to early social experience revealed that in certain areas of the limbic system such as the prefrontal anterior cingulate cortex (ACd), the field CA3 of hippocampus, the infra pyramidal layer (IDG) and the supra pyramidal layer of dentate gyrus (SDG), the development of spine density is particularly sensitive to the amount of preweaning social experience, whereas spine density in the prefrontal area orbitofrontal cortex (OFC) is not. Irrespective of the postweaning social environment, the density of spines was elevated (+13-25%) in the dendrites of ACd, CA3, infra and supra pyramidal layers of early weaned rats who experienced shorter social contact with their mothers.

One of the fundamental phenomena of brain development is the overproduction and subsequent reduction in the amount of synapses that occur between childhood and puberty and cause remodeling of synaptic connectivity. In recent years, many studies have investigated the temporal course of changes in synaptic density in primates,

humans and other mammals, revealing that at the early stages of fetal development, synaptic density rises at a constant rate, until a peak is attained (at 2-3 years of age in humans). Then, after a relatively short period of stable synaptic density (until the age of 5 years in humans), an elimination process begins; synapses are being constantly removed, yielding a marked decrease in synaptic density. This process proceeds until puberty, when synaptic density stabilizes at adult levels which are maintained until old age. The phenomenon of synaptic over-growth and pruning was found in humans (Huttenlocher, 1979; 1990; Huttenlocher et al., 1982) as well as in other mammals such as monkeys (Zecevic and Rakic, 1991; Bourgeois and Rakic, 1993; Bourgeois et al., 1994), rats (Takacs and Hamori, 1994) and cats (Innocenti, 1995). It is observed throughout widespread brain regions including cortical areas e.g. visual (Huttenlocher et al., 1982; Bourgeois and Rakic, 1993), motor and associative areas (Huttenlocher, 1979), cerebellum (Takacs and Hamori, 1994), projection fibres between hemisphere (Innocenti, 1995) and the dentate gyrus (Eckenhoff and Rakic, 1991).

The process of synaptic proliferation and elimination or pruning correlates with experience-dependent activity (Stryker and Harris, 1986; Roe et al., 1990). Synaptic proliferation is demonstrated in various animal species as a result of visual experience, enriched environment, training, physical exercise and learning (Globus et al., 1973; Greenough et al., 1985; Moser et al., 1997; Van Praag et al., 1999; Kozorovitskiy et al., 2005; Mehta and Sernagor, 2006). Likewise, synaptic pruning represents anatomical substrate underlying synaptic reorganization in events such as juvenile emotional learning viz. filial imprinting (Bock and Braun, 1998, 1999a 1999b). Assuming that experience-driven synaptic reorganization involves both, proliferation as well as pruning of synapses (Rakic et al., 1986; Huttenlocher and Dabholkar, 1997), and given the fact that developmental pruning is not a passive but active, experience-dependent event (Wallhausser and Scheich, 1987; Bock and Braun, 1998, 1999, Andersen and Teicher, 2004; Segal, 2005), the elevated spine density observed in the dendrites of anterior cingulate cortex (ACd), field CA3 of hippocampus, the infra and the supra pyramidal layers of dentate gyrus of animals impoverished of their dams at a relatively early age, could be the result of suppressed pruning due to the lack of appropriate social stimulations. Though this interpretation requires further analysis the results might

indicate that unlike in the sensory and motor cortical regions, where increased input activity provided by enriched sensory or motor environments appears to interfere with the proliferative synaptic events (Greenough et al., 1990; Purves, 1994, Kolb et al., 1998), in the higher associative prefrontal and hippocampal regions the input activity provided by social stimulations might induce the selective pruning of synaptic connections. In this context, it can be assumed that disruption of the developmental pruning of synapses through inappropriate social stimulations (such as emotional stress, neglectful conditions or maternal deprivation) might lead to increased density of spines as in the case of early weaned rats. However, this assumption requires further analysis. In order to determine if the increased spine density observed in the prefrontal cortex and hippocampus of early weaned rats are caused by suppressed pruning provided by inappropriate social stimulation i.e. early separation from the dams, it would be helpful to compare the data with a "natural weaning group" (a group representing the ones in the wild or in a laboratory something like a "self-weaning" group).

Dendrites are the major recipient structures of synaptic connectivity. Adult cortical neurons receive approximately 15,000 synaptic inputs (Huttenlocher, 1994), and the extent and pattern of dendritic branching determines the range and scope of synaptic inputs a neuron can process and integrate. In general, the growth and refinement of dendrites and axons is modulated by sensory and motor/physical experience, and - as I have discovered in the present study – also by social experience. Furthermore, postnatal reorganization of dendritic trees is achieved by two principal events, the shortening of dendritic segments and retraction of dendritic arbours and the elongation of dendritic segments and growth of new ramifications. With this respect it is interesting that in the two prefrontal regions (ACd and OFC), the experience-evoked dendritic reorganization was affected in the opposite direction, and that in both prefrontal regions only the apical, not the basal, dendrites were altered.

Concerning the ACd, the animals with the most impoverished pre- and postweaning social experience (i.e. early weaned and socially isolated rats (EWI), displayed elongated (+31-32%) and more complex (+27-29%) apical dendrite compared to early weaned socials (EWS) and late weaned socially isolated animals (LWI),

suggesting that in this prefrontal area an enriched social environment might drive these neurons to develop or reorganize their dendritic trees towards a smaller and more compact dendritic extension. Enriched rearing involves rearing animals in a socially and physically stimuli-rich environment that is reported to enhance behavioural performance such as learning, and modify brain development; for instance, it causes enlargement of cortical volume and increases dendritic branching in sensory and motor brain areas (Diamond et al., 1972; Volkmar and Greenough, 1972; Greenough and Volkmar, 1973; Kolb et al., 2003; Leggio et al., 2005). Enrichment rearing which enhances dendritic branching in many brain areas failed to increase the dendritic length and branching of medial prefrontal neurons (which includes the ACd, Kolb et al., 2003). Based on this finding, it can be assumed that in the ACd of prefrontal cortex an enriched social environment might cause these neurons to develop their dendritic trees towards smaller and more compact dendritic extension and isolation rearing the opposite because isolation rearing is reported to have the opposite effects on animal's behaviour and brain as contrasted with enriched rearing (Diamond et al., 1972; Volkmar and Greenough, 1972; Gardner et al., 1975; Van Waas and Soffie, 1996; Varty et al., 2000). In relation to spine plasticity, the sensitive period for dendritic reorganization in the ACd appears to occur slightly later in development. However, as opposed to the changes in spine density, which were primarily affected by the early preweaning social environment, dendritic refinement appears to be primarily modulated by the *interaction* of preweaning and postweaning social experience. In contrast to the ACd, neurons in the OFC of animals with the most impoverished social experience i.e. early weaned and socially isolated rats (EWI) displayed a -32% reduction of the apical dendritic length and -35% reduced apical dendritic complexity compared to the early weaned social rats (EWS). Thus, unlike the pyramidal neurons of the ACd, the OFC neurons developed simpler dendrites in response to the pre- and postweaning impoverished social environment, as animals reared in impoverished environments display atrophy of dendrites and dendritic branching (Diamond et al., 1972; Silva-Gomez et al., 2003). However, in the OFC the extent of the preweaning social experience also appears to play a critical role in dendritic development, as revealed by the EWS animals, which displayed the longest (+32-33%) and most complex (+31-35%) apical dendrites compared to groups, EWI and LWS.

The effects of time of weaning and social environments on dendritic morphology across different hippocampal areas were quite similar. The granular dendritic length and complexity of mainly the infra and partly the supra pyramidal layer of dentate gyrus was affected by preweaning and postweaning environments as well as by *interactions* between the factors. The animals that had spent less time with their dams (early weaned = EWS + EWI) demonstrated longer (+19-29%) and more complex (+18-24%) infra and supra granular dendrites compared to animals that had stayed with their mother for a longer period (late weaned, such as LWS + LWI). Similarly, the isolated animals (EWI + LWI) displayed longer (+19%) granular dendrites compared to socials (EWS + LWS) but only in the infra pyramidal layer of dentate gyrus. Moreover, a post-hoc test comparing individual groups revealed just the same as for the prefrontal anterior cingulate cortex (ACd); in all three hippocampal areas the animals with the fewest social experience, early weaned and socially isolated rats (EWI), displayed elongated and complex dendrites compared to other experimental groups; for instance, in the infra and supra pyramidal layer of dentate gyrus these animals displayed elongated (+32-34%) and more complex (+33-37%) infra granular dendrite and elongated (+18-39%) as well as more complex (+24-34%) supra granular dendrite compared to other experimental animals. Similarly, in the field CA3 of hippocampus, these animals (EWI) displayed (+21-25%) longer and (+27-34%) complex apical dendrite, suggesting that in all these hippocampal areas an enriched social environment might drive these neurons to develop or reorganize their dendritic trees towards a smaller and more compact dendritic extension. However, in CA3 quite similar to that observed in OFC, the extent of the preweaning social experience also appears to play a critical role in dendritic development, as revealed by the late weaned social animals (LWS), which displayed the longest (+36-39%) and most complex apical dendrites (+35-41%). In summary, it can be assumed that the effect of early and late social experience on dendritic reorganization in prefrontal cortex and hippocampus is highly variable which might suggest that the extent of experience-driven network refinement depends on the developmental time window of the respective cortical region.

In CA3 area of hippocampus, just like in the prefrontal ACd, in relation to spine plasticity the sensitive period for dendritic reorganization appears to occur slightly later in development. Nevertheless, as opposed to the changes in spine density, which were primarily affected by the early preweaning social environment, dendritic refinement in CA3 area of hippocampus (similar to the prefrontal cortex) appears to be primarily modulated by the *interactions* of preweaning and postweaning social experience. In contrast, dendritic refinements in infra and supra pyramidal layers of dentate gyrus are modulated both by preweaning and postweaning experiences as well as by *interactions* between these factors. The dentate gyrus is highly vulnerable to early and late social and emotional experiences. A number of studies have demonstrated that the morphology and the number of dentate granular neurons are remarkably affected by the complexity of environmental conditions during the early stages of life (Fiala et al., 1978; Juraska et al., 1985; Kempermann et al., 1997; Bartesaghi and Serrai, 2001; Poeggel et al., 2003).

Similar to that observed in the pyramidal neurons of ACd and OFC, the dendritic length and complexity of basilar dendrite of CA3 pyramidal neurons remained unchanged. This dendrite specificity suggests a specific impact of as yet unidentified experience-induced input activity on the apical dendrite or on a given apical dendritic segment. Moreover, the restricted effects observed on specific parts of the dendrite (e.g. by Sholl analysis and by branch order analysis) may be explained by the fact that the inputs on a given neuron might be segregated. It is evident from the literature that some neurons, mostly pyramidal, segregate their input. For instance, in piriform cortex, the distal part of the apical dendrite of layer III pyramidal neurons receive extrinsic input, while more proximal portions of the apical dendrite, as well as the basilar dendrites, receive intrinsic inputs (Price, 1973). While the segregation of inputs to pyramidal neurons in the neocortex is not that straightforward, pyramidal neurons in the medial PFC, to which the ACd belongs, nonetheless tends to segregate inputs, with extracortical afferents (for instance from the mediodorsal nucleus of the thalamus and hippocampal CA3) tending to cluster on distal dendrites (Swanson and Cowan, 1977; Groenewegen, 1988) and synapse of local cortical circuits tending to cluster on proximal portions of the apical and basilar dendrite (Scheibel and Scheibel, 1970). Difference in the innervation patterns might contribute to the differential spatial effects

along the dendritic arbors. In addition, there are studies demonstrating that the distribution of receptor system varies across regions, strata and cellular compartments (Kohler et al., 1991; Zilles et al., 1991, 1993; Kraemer et al., 1995). This segregation might contribute to the specific plasticity exhibited by the neurons in different limbic areas. Furthermore, there are substantial regional and laminar differences in the distribution of nerve growth factor (Mufson et al., 1994) which suggests that the variation in plasticity between different neurons and regions might be due to difference in the local support of trophic factors.

On the synaptic level, spine density of layer II/III pyramidal neurons of the orbitofrontal cortex appear to be less sensitive towards socio-emotional environmental conditions than those in the prefrontal ACd and the hippocampus. With respect to dendritic reorganization neurons in the two prefrontal regions modify their dendrites in different or even opposite directions in response to socio-emotional stimulation. This region specificity is probably determined by various factors, such as the specific developmental profiles (i.e. different rates of maturation) as well as the functional role of a given cortical region in processing social and emotional stimuli. The ACd and OFC are both parts of the prefrontal cortex, but they are functionally dissociable (Kolb, 1984, 1990). Lesions to the medial and orbital regions in rats produce different behavioural syndromes (Kolb, 1984, 1990); lesions to the medial PFC produce deficits in attentional as well as working memory task, whereas lesions to the OFC produce deficits in olfactory and taste discrimination tasks, as well as deficits in discrimination of stimuli when the value of that stimulus has changed. Comparing the developmental profiles of the two prefrontal areas, these studies have also indicated a different time course of functional maturation for the medial PFC and the OFC (Kolb and Nonneman, 1976; Kolb, 1984), with the orbital PFC maturing slightly earlier than the medial PFC. The cytoarchitecture in the anterior cingulate cortex is developing up to postnatal day 18 (i.e. 3 days prior to my early weaning group), with the cortical layers attaining their adult proportional width around postnatal day 24 (i.e. 3 days after my early weaning and 6 days prior to my late weaning group). Furthermore, cortical volume in the medial prefrontal cortex (which includes the ACd) increases between postnatal day 6-24 (i.e. includes the early weaning phase in our experiment), after which a reduction in volume

occurs until postnatal day 30 (i.e. includes the late weaning phase in our experiment), when adulthood levels are reached (Van Eden and Uylings, 1985a, 1985b). Since the volume of the cortex is predominantly determined by the extension of the dendritic trees, the rapid volumetric decrease between postnatal days 21 and 30 indicates a developmental time window of dendritic retraction, which might be sensitive to environmental factors, including social and emotional experience. Assuming that, similar to the pruning of dendritic spines, the pruning or retraction of dendritic branches is also experience- and activity-dependent, experience-induced dendritic retraction might have been suppressed due to the paucity of appropriate (social, emotional) input activity during the preweaning and also the postweaning period in the early weaned and socially isolated animals (EWI), as they displayed increased dendritic length and complexity compared to other animals at a developmental phase that involves regression in the cytoarchitecture and the volume of that cortical region, in this case the anterior cingulate cortex (ACd) of the medial prefrontal cortex.

Compared to the developmental profiles in the ACd, the cortical layers in the orbital PFC reach their adult proportional width already around postnatal day 14 (Van Eden and Uylings, 1985a, 1985b); however, compared to the ACd, the increase in volume in the orbital PFC is less rapid and persists until the maximum volume is attained around day 30, when it is 80% larger than the adult volume (Van Eden and Uylings, 1985a, 1985b). Thus, cortical “shrinkage” which starts around the time of late weaning phase represents a time window of pronounced dendritic and synaptic refinement, which appears to be sensitive towards the complexity of the social environment, as revealed by the comparisons of postweaning impoverished or social environments. In addition, the cytoarchitectural and volumetric developmental profiles appear to correspond with the differential rates of the development of thalamocortical connections, which in the OFC precedes that in the medial PFC (Corwin et al., 1983).

Comparing the developmental profiles in hippocampus, the rapid neuronal growth occurs in different subareas early in development around p10. In late postnatal periods the maturations takes place with the neurons attaining an adult-like appearance mostly by postnatal day 15 (in CA3) and 20 (in dentate gyrus). After that period, the neuronal

growth ceases and it gradually begins to decline, indicating that the synaptic and dendritic structures in these areas undergo developmental pruning (Minkwitz, 1976a, and 1976b). Thus, in hippocampus the period after postnatal day 15 (in CA3) and postnatal day 20 (in dentate gyrus) might represent a developmental time window of dendritic retraction, which might be sensitive to environmental factors, including social and emotional stimulations. Assuming that the pruning or retraction of dendritic branches is also experience- and activity-dependent, experience-induced dendritic retraction in hippocampus might have been suppressed due to the lack of appropriate (social, emotional) input activity during the preweaning and also the postweaning period in the EWI animals, as they displayed increased granular and apical dendritic length and complexity compared to other animals. However, in CA3 in contrast to dentate gyrus, the late weaned social animals (LWS) also demonstrated longest and most complex apical dendritic length and complexity suggesting that in CA3 the extent of social experience preweaning is modulatory to the development of dendritic structures in this hippocampal subregion. In contrast with the dentate gyrus, the neuronal growth and maturations in CA3 occurs earlier where the adult cytoarchitectonial and neuronal pattern is seen already by postnatal day 15 (Wenzel et al., 1981; Perez-Delgado et al., 1994). Therefore, in CA3 the extent of social experience in early periods (i.e. preweaning periods) may appear modulatory in shaping the synaptic and dendritic structures of neurons located in this area. In contrary, the development of granular cells appears to lag behind pyramidal neurons of hippocampus that continues to postnatal day 20 and even further (Ribak et al., 1985). Therefore, the dendritic structures of granular neurons might be modulated by social stimulations provided early as well in late postnatal life. Hence, the dendritic length and complexity of granular neurons are modified by preweaning and postweaning environments as well as by *interactions* between the factors.

The neurons in the limbic prefrontal cortex and hippocampus demonstrate high degree of plasticity. They show changes in their morphology and structures following various experimental manipulations such as: exposure to drugs (Robinson et al., 2002); manipulation of gonadal hormones (Woolley et al., 1990; Woolley and McEwen, 1993; Forgie and Kolb, 2003); in response to environmental complexity and deprivation

(Juraska et al., 1985; Bartesaghi and Serrai, 2001; Silva-Gomez et al., 2003); in emotional and learning experience (Moser et al., 1994; Helmeke et al., 2001; Ovtscharoff and Braun, 2001; Poeggel et al., 2003; Bock et al., 2005) and in response to nerve growth factor (Kolb and Whishaw, 1998). The experience-induced dendritic and synaptic refinement which I observed in the prefrontal cortex and hippocampus are not in all aspects similar to what has been observed in sensory and motor cortical regions in response to environmental stimulations, such as enrichment or impoverishment. For instance, increased synaptic densities were also found after exposure to enriched environmental conditions, e.g. in the occipital cortex (Volkmar and Greenough, 1972; Turner and Greenough, 1985), medial preoptic area (Sanchez-Toscano et al., 1991) and in the striatum (Comery et al., 1995, 1996) of rats. Similarly, animals raised in an enriched environment displayed enhanced dendritic branching in the parietal cortex, hippocampus as well as in the prefrontal cortex (Juraska et al., 1985; Kozorovitskiy et al., 2005; Leggio et al., 2005). In contrast, environmental impoverishment or deprivation generally resulted in smaller dendrites and fewer spine densities in various limbic areas such as, hippocampus and medial prefrontal cortex (Silva-Gomez et al., 2003). The findings of these studies contradict my results. This discrepancy might be due to several methodological differences such as different rat strains, different brain areas in which the neurons were analysed and perhaps different cell types that are used in these studies. Nevertheless, other studies have demonstrated that impoverished environment after weaning led to an increase of asymmetric synapses (spine and shaft) in the motor cortex of cats (Beaulieu and Colonnier, 1989) and an increase in symmetrical synapses per neuron in the cat visual cortex (Beaulieu and Colonnier, 1987). In addition, an increase in mean synaptic density after postweaning impoverished environment is also reported in rats (Diamond et al., 1975), which may be similar to my findings of increased spine density in the dendrites of prefrontal ACd, hippocampal CA3 area and the infra and supra pyramidal layers of dentate gyrus.

Morphological studies provide further evidence that the neurons in prefrontal cortex and hippocampus demonstrate plasticity in response to other kinds of stimulations; for example, stress that was reported to cause atrophy of dendritic arbours and reduction in spine density in prefrontal cortex and hippocampus (Magarinos et al.,

1996; Cook and Wellman, 2004; Brown et al., 2005; Radley et al., 2006). In addition, enhanced spine density and dendritic arborization in prefrontal pyramidal neurons following psychostimulant administration and reduced dendritic arborization in hippocampus following malnutrition are reported (Andrade et al., 1996; Robinson and Kolb, 1999; Robinson et al., 2002). Reductions in dendritic arbours and spines are also reported in various disorders such as in schizophrenic and epileptic brains (Isokawa and Levesque, 1991; Garey et al., 1998; Broadbelt et al., 2002). These seemingly inconsistent results indicate that the mechanisms underlying experience-driven postnatal refinement of neuronal networks in the cortex are more complex than previously appreciated. My experiments addressed this question more specifically by not only testing the impact of two different developmental time windows of experience-related neuronal plasticity, but, in addition, also revealed *interactions* between neonatal and postweaning social experience. This study confirmed that neuronal development in certain limbic areas such as the prefrontal cortex and hippocampus are particularly sensitive towards emotional stimulation. The prefrontal ACd, OFC and hippocampus are integrated in synaptic circuits of the limbic system (Figure 1A, B) and thereby involved in processing, regulating, and experiencing emotions and emotionally-related behaviours (Bush et al., 2000; Hornak et al., 2003; Sinha et al., 2004). In contrast to the sensory systems, where synaptic changes have only been reported after relatively severe and chronic long-term deprivation of visual (Valverde, 1967), acoustic (Perier et al., 1986) and somatosensory (Bryan and Riesen, 1989; Kossut, 1998) stimulation, synaptic development of the limbic cortex appears to be sensitive towards comparably mild (brief and subtle) environmental changes. Even unspecific emotional experiences such as, daily handling and daily saline injections or drug administration were found to induce significantly elevated spine density in prefrontal cortical neurons (Dawirs et al., 1991; Helmeke et al., 2001a; Seib and Wellman, 2003). Moreover, in many organisms for instance; humans, the brainstem is almost fully functional at birth whereas the limbic system is more plastic, “experience-expectant” and slower to mature and develop. Hence, unlike some parts of the brain (e.g. brainstem) which is more "hard wired" and initially under direct genetic and reflexive sensory control, the limbic system requires considerable social, emotional, perceptual, and cognitive stimulation during the juvenile period in order to develop normally. If the limbic system is denied sufficient social,

maternal, and emotional “experience-expectant” stimulation early in development (for example, if the animals are exposed to abnormal, neglectful, or abusive environment) the limbic structures undergo alteration in synaptic connectivity, may establish aberrant interconnections, and cease to function normally (Henriksen et al., 1978; Helmeke et al., 2001a, 2001b; Ovtscharoff and Braun, 2001; Poeggel et al., 2003; Bock et al., 2005; Murmu et al., 2006).

Alterations in neuronal network patterns are likely to have consequences in the functioning of areas and the pathways in which they are embedded. The results of my study demonstrate that the extent of neonatal social experience, determined by the time of weaning, together with the social experience encountered after weaning, are critical determinants which significantly affect the development of neurons thereby affecting the functional neural networks in limbic prefrontal and hippocampal regions. At system network level, it might be interesting to elucidate which circuits of the prefrontal cortex and hippocampus are altered by these experiences and how this might affect the functioning of the limbic system. Dendritic spines of prefrontal cortical pyramidal neurons are the target of thalamic (Krettek and Price, 1977a), callosal, associational fibres (Pandya and Yeterian, 1996), and the fibres arising from basolateral amygdala, the hippocampus and the infralimbic cortex (McDonald, 1991; Carmichael and Price, 1995a; Carr and Sesack, 2000). Alterations in synaptic structures and dendritic arborization in prefrontal cortex might change the output characteristics of the prefrontal pyramidal neurons into their limbic projections areas including the nucleus accumbens (Brog et al., 1993), mediodorsal thalamic nucleus (Kuroda et al., 1998), via the enthorhinal cortex to ventral striatum and the hippocampus (Joyce, 1993) and to the amygdala (Aggleton et al., 1980). In hippocampus, dendritic spines of granular neuron in dentate gyrus and CA3 pyramidal neurons receive input from various polymodal associational areas that synthesize sensory information through processing in enthorhinal cortex (Hjorth-Simonsen and Jeune, 1972; Caballero-Bleda and Witter, 1994). These neurons also receive inputs from various limbic structures such as the thalamus (Dolleman-Van Der Weel and Witter, 1996), prefrontal cortex (Swanson, 1981; Verwer et al., 1997), amygdala, hypothalamus (Kohler et al., 1985; Mello et al., 1992), ventral tegmental area (VTA) and nucleus accumbens (Gasbarri et al., 1994).

Alterations in spine density and dendritic arborization in hippocampal areas might effect the output characteristics of granular and pyramidal neurons located in dentate gyrus and CA3 into their limbic projection areas such as orbital, medial and dorsolateral prefrontal cortex, amygdala, hypothalamus, nucleus accumbens and VTA as well as into their non-limbic projection areas including septum, olfactory, parietal and occipital cortex (Swanson and Cowan, 1977; Gasbarri et al., 1994; Insausti et al., 1997). Furthermore, the prefrontal-hippocampal connections are implicated in learning, memory and cognition (Marquis et al., 2006; Wang and Cai, 2006). If hippocampal-prefrontal connections are affected, the outcome may be change in behaviours that are regulated by these areas and pathways.

Behavioural studies in rodents and other mammals indicate that early weaning is able to induce alterations in adult emotional behaviour (Janus, 1987a, 1987b; Gardner et al., 2001; Orgeur et al., 2001; Terranova and Laviola, 2001; Nakamura et al., 2003; Kikusui et al., 2004; Tuchscherer et al., 2004; Weiss et al., 2004; Kanari et al., 2005). Likewise, an extensive literature has accumulated demonstrating that socially isolated rats exhibit many behavioural disorders such as anxiety, hyperactivity and cognitive deficits that involve the limbic prefrontal areas (Jones et al., 1991, 1992; Domeney and Feldon, 1998; Del Arco et al., 2004; Weiss at al., 2004) which in turn is mediated by altered mesocortical dopaminergic pathways (pathways connecting the prefrontal areas to the reward center i.e. the ventral tegmental area), as well as serotonergic and noradrenergic systems in prefrontal cortex (Crespi et al., 1992; Jones et al., 1992; Bickerdike et al., 1993 ; Fulford and Marsden, 1998; Hall et al., 1998; Braun et al.,2000; Del-Bel et al., 2002; Preece at al., 2004). A few very recent studies have provided evidence that early weaned animals when socially isolated (even for a very brief period), show impaired learning, memory as well as cognitive deficits, functions that are modulated by the prefrontal cortex and hippocampus (Laughlin and Zanella, 2002; Souza and Zanella, 2004). Early weaning, together with social isolation, leads to increased alcohol consumption in juvenile rats (Rockman et al., 1987; Fahlke et al., 1997). Modifications at cellular level in early weaned and socially isolated animals are also reported. For instance, reduced expression of glucocorticoid and mineralocorticoid receptors in the frontal cortex and hippocampus are described in young mammals

subjected to early weaning and social isolation (Poletto et al., 2006a). These receptors provide the negative feedback mechanism for corticosterone and are abundantly present in prefrontal cortex and hippocampus (Gerlach and McEwen, 1972; Meaney and Aitken, 1985; Reul and de Kloet, 1985). Another study by Poletto et al., (2006b) has reported in the early weaned and socially isolated animals, reduction in the expression of plasticity-related genes (e.g. diazepham binding inhibitor, DBI); those that are associated with neuronal function, structure and protection. The behaviour test conducted on my experimental animals suggests that the early weaned animals were most active in the open field test (Ferdman et al., 2007), and the late weaned and group housed were the most explorative. The animals that were weaned early and isolated (EWI) were found to be hyperactive and anxious compared to other experimental animals (Ferdman et al., 2007). Activity in the open field is used as a measure of anxiety. Rats and mice tend to avoid brightly illuminated, novel, open spaces, so the open field environment acts as an anxiogenic stimulus and allows for measurement of anxiety-induced locomotor activity and exploratory behaviours.

The cellular and molecular mechanisms underlying the neuronal changes in my experimental animals are yet to be determined and could be the matter of future investigations. It is likely that a variety of neurochemical changes that occur during different developmental phases may interfere with the proliferation and pruning of synaptic contacts (Bourgeois and Rakic, 1993; Granger et al., 1995). Although it is well known that the shape and growth of a neuron is modulated through both nervous and hormonal mechanism, much less is known about the mechanism by which environment might affect neuronal morphology. However, the release of hormones, neurotransmitters, growth factors, and the inductions of immediate early genes or transcriptional factors could be part of the molecular machinery that underlies these synaptic changes (Cirulli et al., 1998, 2000; Bock et al., 2005). Some studies have demonstrated an age-dependent sensitivity of the brain pathways to stress hormones such as cortisol or corticosterone (in rats) (Kanitz et al., 1998; Kaufman et al., 2000; Plotsky et al., 2001). During weaning, the responsiveness of hypothalamic-pituitary-adrenal system is heightened and the early weaned rats are reported to show altered level of corticosterone in their sensory cortex (Cook, 1999). Most of the studies

analyzing stress (or other environmental effects on neurons) have demonstrated that stress and chronic corticosterone administration induces significant alteration in the dendritic spine density and dendritic morphology of prefrontal and hippocampal neurons (Woolley et al., 1990; Magarinos and McEwen, 1995; Wellman, 2001; Radley et al., 2004), and the administration of corticosterone antagonist e.g. tianeptine prevented the subsequent corticosterone or stress-induced alterations in the dendritic morphology (Watanabe et al., 1992; Magarinos et al., 1999). Thus, it can be assumed that the stress hormone corticosterone, (in addition to other factors), is involved in the regulation of synaptic composition and dendritic morphology in the limbic system. Other mechanisms which are widely reported to cause alterations in some neurons (mostly hippocampal) are the afferent/efferent constituting excitatory neurotransmitters such as the glutamate and serotonin (5-HT). The excitatory amino acid glutamate constitutes more than 90% of excitatory neurotransmission in the brain and is implicated in stress-induced dendritic alterations in the hippocampus (Magarinos and McEwen, 1995; Magarinos et al., 1999). Moreover, studies have reported that the blockade of NMDA (a receptor for glutamate) by its antagonist (phenytoin and CGP 43487), prevented stress-induced atrophy of the CA3 apical dendrites (Watanabe et al., 1992; Magarinos and McEwen, 1995). In addition, the learning-induced pruning of dendritic spine density was prevented by blocking the activity and expression of NMDA receptor suggesting that this receptor of glutamate might be involved in experience-dependent pruning of dendritic spine density and synapses (Yen et al., 1993, 1995; Bock et al., 1996; Bock and Braun, 1999a). Additionally, the 5-HT (serotonin) system is reported to be clearly involved in mediating stress effects in hippocampus (Fujino et al., 2002). Thus, the alterations in the dendritic morphology observed in my study might be due to alterations at hormonal level, particularly the corticosterone system (which is also altered by early weaning and social isolation), as well as due to alterations at other afferent/efferent systems that are also reported to have altered by social isolation or other environmental manipulations (described in previous paragraphs). Moreover, alterations in one system might affect the functioning of the other system or systems related to it (Lowy et al., 1993; Rahman and Neuman, 1993).

In order to determine if the synaptic and dendritic refinements observed in my study are beneficial or detrimental to animal's ability to cope with their environment in later life, firstly it would be helpful to provide an equivalent to the "natural weaning group" (like the ones in the wild), and compare the data between these animals and finally the morphological changes need to be correlated to behavioural and cognitive capacities. In the wild, a gradual and lengthening separation at the time of weaning seems likely (Barnett, 1975) and, as such, a normal weaning group may be closest to the animal's weaning in wild. However, in the laboratory or in an enclosed cage situation it is clearly difficult for a mother to separate herself for any length of time from her pups, particularly when they are older than 21 days of age. As such, there must be something like a "self-weaning" group which may show little similarity to the "natural weaning" group or groups in the wild. Continuation of this study would include determining the behavioural and morphological consequences in the "self-weaning" group.

In this study, it was noteworthy that the 9-day difference between early and late weaned rats still caused the synaptic and dendritic refinement in the animal which persisted even in adulthood i.e. ~90 days later. Some studies have demonstrated that with repeated social interactions or stress, dependent upon weaning age, rearing conditions and timing, the different behavioural and neural changes may dissipate (Vale and Montgomery, 1997); may be reversed (Einon and Morgan, 1977; Niesink and van Ree, 1982; Radley et al., 2005); or may persist and even influence morbidity (Arakawa, 2003; Kikusui et al., 2004). My findings are based on manipulations which are similar to routine laboratory weaning ages (21 and 30 days) and housing practices (individual or group cages); yet their impact upon synaptic and dendritic parameters were remarkable. Hence, management practices at weaning may also impact research results obtained from adult animals, given the influence of time of weaning on a number of morphological and behavioural parameters. This suggests the importance of such management practices for broad areas of animal-related research.

5.0 Conclusions

My study provided a vivid answer to our hypothesis that was designed to determine if the synaptic and dendritic refinements in the prefrontal cortex and hippocampus in our experimental animals are dependent on the time and the extent of social contact with their family members. The results indicate that the refinement of neuronal networks in rodent higher associative limbic areas such as the prefrontal cortex and hippocampus occur in a highly temporal, regional and dendrite-specific manner. The preweaning social experience or the times spent with their dams appeared important in modulating the postsynaptic structures such as the dendritic spines (the density of which was dramatically increased in animals separated from their dams at a relatively early age compared to those that stayed with their dams for longer period).

In contrast with synaptic refinement, the dendritic refinement of granular neurons was modulated both by the pre- and postweaning social environments as well as by *interactions* and the dendritic refinement of pyramidal neurons (mainly, of apical dendrite) was modulated only by *interactions* between the two factors. This study demonstrated that the animals with fewest social experience i.e. the ones that were separated early from their dams and in addition were subjected to social isolation postweaning displayed elongated and more complex apical dendrites in the prefrontal area, ACd and field CA3 of hippocampus (with few exceptions as in case of OFC). In addition, these rats also displayed elongated and more complex granular dendrites in infra and supra pyramidal layers of dentate gyrus. In consequence, various or all aspects of limbic system based functions or behaviours such as the social, emotional and cognitive functioning may undergo alterations.

The experience-dependent synaptic and dendritic refinements in the limbic areas might correlate to developmental time windows for the given cortical region and in addition might also represent the underlying neuronal mechanism for behavioural modifications caused by different experimental manipulations. For instance, the enhanced spine density observed in the early weaned animals might correlate to increased activity observed in these animals in the open field test (Ferdman et al., 2007).

Similarly, the changes in dendritic arborization such as the longer and complex dendrites in early weaned isolated animals (EWI) might correlate to the hyperactive and anxious behaviour demonstrated by these animals in the behaviour test (Ferdman et al., 2007). There is an extensive literature demonstrating that the shape, growth, proliferation and pruning of synapse and dendrites are modulated by activity and experience.

6.0 References

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7.0 Appendices

7.1 Protocol for Golgi – Cox Staining

Step I: Preparation of the Golgi-Cox solution

Three different aqueous solutions are prepared in distilled water:

- Solution A: 5% solution of Potassiumdichromate ($K_2Cr_2O_7$)
- Solution B: 5% solution of Mercuric chloride ($HgCl_2$)
- Solution C: 5% solution of Potassiumchromate (K_2CrO_4)

From the above solutions two new solutions are prepared:

- Solution D: solution A + solution B mixed in a ratio of (1:1)
- Solution E: solution C + distilled water mixed in a ratio of (1:2:5)

From these solutions the actual Golgi-Cox solution is prepared. In a beaker 1000 ml of solution D is mixed with 1400 ml of solution E while stirring continously. This solution is stored in a glass stoppered 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

The brains are dehydrated and embedded in celloidin following these steps:

- Washed in distilled water, 3 x 2 minutes.
- Slowly dehydrated in the refrigrator (4-6°C) as follows:
- 50% alcohol – 4 hours; 70% alcohol-overnight; 80% alcohol-overnight; 96% alcohol-overnight; 96% alcohol-overnight.
- Brains are then embedded at the dark room temperature in:

  100% alcohol–Diethylether (1:1) - 2 hours

  2% Celloidin-3 days

  4% Celloidin-3 days

  8 % Celloidin- 3 days

The brains are inserted in 9% celloidinmass and placed in the evaporating glass. This is kept overnight in the exitator with open faucet. Subsequently, a second glass with closed faucet containing phosporous 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. Phosporous pentaoxide is

removed and choloroform is placed in the bowl so that the celloidin polymerizes in 1-2 days. Then the celloidin block is cut into a desired form and is placed in 70% alcohol at 4°C for 2 days, which hardens the celloidin. This block can be kept in the refrigator for long time. For cutting, the celloidin block containing the brain is fixed into a wodden block with the help of adhesives. While cutting, the knief should be continously moistened with 70% alcohol to prevent the slices from getting dried.

III. Staining

- 150µm coronal sections were cut at the level of prefrontal cortex and hippocampus using a sliding microtome.
- Tissue sections were collected serially and immersed in 70 % alcohol until all the necessary sections were obtained.
- Washing in distilled water, 3 x 1 minutes.
- Alkalinised with ammonia (NH_3:H_20, 1:1) - 1 x 60 minutes.
- 0,5 % phenyldiamine solution (Merck, Germany) - 0.5 gm phenyldiamine in 100 ml distilled water – 1 x 1 minutes.
- 0,5 % phenyldiamine solution (Merck, Germany) – 1 x 4 minutes. This treatment intensifies the colour of the staining.
- Washing in distilled water, 2 x 2 minutes.
- 1 % dektol (Kodak, Germany) - 0.5 gm dektol in 50 ml distilled water - 1 x 2 minutes. This treatment stablizes the tissue impregnation.
- Washing in distilled water, 2 x 1 minutes.
- 5 % tetenal (Kodak, Germany) - 2.5 gm tetenal in 50 ml distilled water – 1 x 10-15 minutes. This is important for fixing the tissue impregnation.
- Washing 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 coverslipped for further microscopic observation. The slides were coded with numericals which was only broken after the analysis was completed.

7.2 Tables with mean values and S.E.M

24.A

Experimental groups	Spine density/µm (Prefrontal cortex) Mean ± S.E.M			
	Apical dendrite (ACd)	Basal dendrite (ACd)	Apical dendrite (OFC)	Basal dendrite (OFC)
Early weaned	1.15 ± 0.01	1.11 ± 0.03	0.94 ± 0.03	0.91 ± 0.03
Late weaned	1.01 ± 0.02	0.93 ± 0.03	0.91 ± 0.04	0.92 ± 0.05
Social	1.09 ± 0.03	1.02 ± 0.05	0.93 ± 0.03	0.90 ± 0.04
Isolated	1.08 ± 0.02	1.09 ± 0.03	0.92 ± 0.03	0.93 ± 0.03

B

Experimental groups	Spine density/µm(Prefrontal cortex) Mean ± S.E.M			
	Apical dendrite (ACd)	Basal dendrite (ACd)	Apical dendrite (OFC)	Basal dendrite (OFC)
EWS	1.16 ± 0.01	1.12 ± 0.06	0.97 ± 0.02	0.93 ± 0.03
LWS	1.03 ± 0.02	0.92 ± 0.03	0.89 ± 0.06	0.86 ± 0.09
EWI	1.14 ± 0.02	1.12 ± 0.04	0.91 ± 0.04	0.90 ± 0.05
LWI	0.99 ± 0.04	0.94 ± 0.05	0.93 ± 0.04	0.97 ± 0.04

C

Experimental groups	Spine density/µm (Hippocampus) Mean ± S.E.M			
	Apical dendrite (CA3)	Basal dendrite (CA3)	Dendrite (IDG)	Dendrite (SDG)
Early weaned	0.60 ± 0.04	0.61 ± 0.05	0.99 ± 0.05	0.94 ± 0.03
Late weaned	0.50 ± 0.03	0.47 ± 0.03	0.74 ± 0.04	0.81 ± 0.04
Social	0.57 ± 0.04	0.56 ± 0.06	0.81 ± 0.07	0.85 ± 0.04
Isolated	0.53 ± 0.02	0.52 ± 0.03	0.93 ± 0.05	0.90 ± 0.04

D

Experimental groups	Spine density/µm (Hippocampus) Mean ± S.E.M			
	Apical dendrite (CA3)	Basal dendrite (CA3)	Dendrite (IDG)	Dendrite (SDG)
EWS	0.64 ± 0.07	0.68 ± 0.09	0.97 ± 0.07	0.92 ± 0.02
LWS	0.49 ± 0.04	0.43 ± 0.02	0.65 ± 0.03	0.79 ± 0.06
EWI	0.55 ± 0.01	0.53 ± 0.04	1.02 ± 0.07	0.98 ± 0.03
LWI	0.51 ± 0.04	0.50 ± 0.06	0.84 ± 0.05	0.89 ± 0.04

E

Experimental groups	Dendritic length/µm(Prefrontal cortex) Mean ± S.E.M			
	Apical dendrite (ACd)	Basal dendrite (ACd)	Apical dendrite (OFC)	Basal dendrite (OFC)
Early weaned	998.10 ± 91.07	394.33 ± 63.37	1590.41 ± 129.04	641.65 ± 46.20
Late weaned	870.29 ± 42.68	481.90 ± 36.68	1379.23 ± 106.17	564.86 ± 49.52
Social	860.10 ± 43.55	423.61 ± 47.57	1573.74 ± 120.14	629.86 ± 47.39
Isolated	1007.38 ± 88.63	452.63 ± 59.88	1395.90 ± 119.20	576.65 ± 50.12
	Dendritic complexity/µm (Prefrontal cortex) Mean ± S.E.M			
Early weaned	38.35 ± 3.42	15.54 ± 2.39	46.57 ± 4.30	19.95 ± 1.50
Late weaned	33.23 ± 1.36	19.68 ± 1.20	43.80 ± 2.75	17.79 ± 1.31
Social	33.64 ± 1.48	16.55 ± 1.62	47.58 ± 3.37	19.09 ± 1.25
Isolated	37.94 ± 3.45	18.67 ± 2.33	42.80 ± 3.71	18.65 ± 1.63

F

Experimental groups	Dendritic length/µm (Prefrontal cortex) Mean ± S.E.M			
	Apical dendrite (ACd)	Basal dendrite (ACd)	Apical dendrite (OFC)	Basal dendrite (OFC)
EWS	804.75 ± 35.80	331.33 ± 58.64	1889.31 ± 48.18	617.73 ± 58.72
LWS	917.24 ± 73.89	515.89 ± 38.01	1258.17 ± 113.32	641.99 ± 81.13
EWI	1191.44 ± 111.82	457.34 ± 112.50	1291.51 ± 167.15	665.57 ± 76.61
LWI	823.33 ± 39.65	447.91 ± 63.74	1500.29 ± 174.79	487.72 ± 38.46
	Dendritic complexity/µm (Prefrontal cortex) Mean ± S.E.M			
EWS	32.41 ± 2.36	13.17 ± 1.70	56.36 ± 3.21	18.60 ± 1.82
LWS	34.87 ± 1.91	19.94 ± 1.31	38.79 ± 1.45	19.58 ± 1.89
EWI	44.29 ± 5.06	17.92 ± 4.47	36.78 ± 4.98	21.30 ± 2.42
LWI	31.60 ± 1.79	19.43 ± 2.23	48.82 ± 4.39	16.00 ± 1.61

G

Experimental groups	Dendritic length/µm (Hippocampus) Mean ± S.E.M			
	Apical dendrite (CA3)	Basal dendrite (CA3)	Dendrite (IDG)	Dendrite (SDG)
Early weaned	2071.19 + 109.56	860.98 + 63.01	1220.43 + 98.97	1500.14 + 78.13
Late weaned	2296.33 + 239.48	958.13 + 57.22	989.80 + 68.22	1068.70 + 69.28
Social	2339.72 + 234.32	857.95 + 70.97	988.13 + 59.14	1240.88 + 62.67
Isolated	2027.81 + 108.91	961.16 + 46.26	1222.10 + 104.23	1327.95 + 129.99
	Dendritic complexity/µm(Hippocampus) Mean ± S.E.M			
Early weaned	28.11 + 2.15	20.37 + 1.42	23.57 + 1.89	26.23 + 1.81
Late weaned	31.13 + 3.16	23.82 + 1.72	17.92 + 1.07	21.56 + 1.74
Social	30.11 + 3.44	21.44 + 1.14	18.28 + 1.00	22.51 + 1.72
Isolated	29.13 + 1.81	22.75 + 2.06	23.21 + 2.03	25.28 + 2.03

H

Experimental groups	Dendritic length/µm (Hippocampus) Mean ± S.E.M			
	Apical dendrite (CA3)	Basal dendrite (CA3)	Dendrite (IDG)	Dendrite (SDG)
EWS	1823.64 ± 133.91	783.45 ± 97.27	967.83 ± 63.44	1349.05 ± 88.15
LWS	2855.79 ± 309.79	932.45 ± 102.11	1008.43 ± 107.27	1132.71 ± 63.67
EWI	2318.74 ± 73.80	938.52 ± 73.46	1473.03 ± 90.28	1651.23 ± 91.01
LWI	1736.87 ± 74.84	983.80 ± 63.06	971.18 ± 96.22	1004.67 ± 124.48
	Dendritic complexity/µm(Hippocampus) Mean ± S.E.M			
EWS	22.45 ± 1.74	19.82 ± 1.77	18.86 ± 1.35	22 ± 1.70
LWS	37.76 ± 4.56	23.06 ± 1.18	17.71 ± 1.60	23.02 ± 3.21
EWI	33.76 ± 1.34	20.93 ± 2.41	28.29 ± 1.77	30.46 ± 1.70
LWI	24.50 ± 1.47	24.56 ± 3.41	18.13 ± 1.60	20.10 ± 1.51

Figure 24. Tables representing the mean values ± S.E.M of experimental animals and groups (A) Spine density/µm in prefrontal cortex by Two-Way ANOVA. (B) Spine density/µm of prefrontal neurons representing mean values from individual groups; EWS = early weaned social, LWS = late weaned social, EWI = early weaned isolated and LWI = late weaned isolated. (C) Spine density/µm of hippocampal neurons by Two-Way ANOVA (D) Spine density/µm of hippocampal neurons representing mean values from individual groups (E) Dendritic length and complexity/µm of prefrontal neurons by Two-Way ANOVA. (F) Dendritic length and complexity/µm of prefrontal neurons representing mean values from individual groups. (G) Dendritic length and complexity/µm of hippocampal neurons by Two-Way ANOVA. (H) Dendritic length and complexity/µm of hippocampal neurons representing mean values from individual groups. ACd = anterior cingulate cortex, CA3 = field CA3 of hippocampus, IDG = infra pyramidal layer of dentate gyrus, OFC = orbitofrontal cortex, PFC = prefrontal cortex, SDG = supra pyramidal layer of dentate gyrus.

7.3 Abbreviations

ACd	Dorsal anterior cingulate cortex
AI	Agranular insular cortex.
CA1	Field CA1 of hippocampus
CA2	Field CA2 of hippocampus
CA3	Field CA3 of hippocampus
cc	Corpus Callosum
IDG	Infra pyramidal layer of dentate gyrus
IL	Infralimbic cortex
LO	Lateral orbital cortex
mPFC	Medial prefrontal cortex
MT	Medial temporal lobe
NAcc	Nucleus accumbens
OFC	Orbitofrontal cortex
PFC	Prefrontal cortex
PrL	Prelimbic cortex
PrCm	Precentral medial
S2	Secondary somatosensory cortex
SDG	Supra pyramidal layer of dentate gyrus
SSC	Somatosensory cortex
VPL	Ventral posterolateral thalamic nucleus
VPM	Ventral posteromedial thalamic nucleus
VO	Ventral orbital cortex

7.4 Zusammenfassung

Der Prozess der Entwöhnung von der Muttermilch beeinflusst die physiologische und neurologische Entwicklung zahlreicher Spezies und ist somit eine kritische Periode der Prägung für das spätere Verhalten im Erwachsenenalter. Die diesen Verhaltensänderungen zugrunde liegenden neuronalen Mechanismen sind weitgehend unbekannt.

Zahlreiche Studien belegen einen bedeutenden Einfluss emotionaler Erfahrungen (wie etwa in der Zeit der Entwöhnung) auf spätere Verhaltensweisen. Darüber hinaus ist bekannt, dass sich Umwelteinflüsse entwicklungsabhängig sowohl auf das Verhalten als auch auf die Gehirnentwicklung maßgeblich auswirken können. Die Bedeutung des Entwöhnungszeitpunktes und des darauf folgenden sozialen Kontaktes für die emotionale Entwicklung ist bis jetzt jedoch ebenso wenig bekannt, wie die zugrunde liegenden neuronalen Mechanismen.

In dieser Arbeit wurde die These untersucht, ob es während der späten Kindheit, in Abhängigkeit vom Zeitpunkt der Entwöhnung und dem Ausmaß des nachfolgenden sozialen Kontaktes, zu Umstrukturierungen in den neuronalen Netzwerken verschiedener limbischer Areale, wie dem Präfrontalen Kortex und dem Hippokampus kommt. Hierzu wurde der Einfluss sowohl von später (30 Tage nach der Geburt) als auch früher (21) Entwöhnung auf die Morphologie von Neuronen des Präfrontalen Kortex und Hippokampus, sowie eine Beeinflussung durch anschließende soziale Isolation oder weiteren Kontakt zu Mutter und Wurfgeschwistern, untersucht. Darüber hinaus wurde eine Kombination der Parameter Zeit der Entwöhnung und anschließender sozialer Interaktion untersucht.

Präfrontaler Kortex und Hippokampus sind Teil des limbischen Systems, welches von entscheidender Bedeutung für emotionales Verhalten ist. Da es sich bei der Entwöhnung um eine wichtige Phase der emotionalen Prägung für das Erwachsenenalter handelt, wird angenommen, dass der Übergang zu einer größeren Selbständigkeit von strukturellen Veränderungen an den Nervenzellen dieser Hirnregionen begleitet wird.

Die Ergebnisse dieser Arbeit zeigen, dass emotionale Erfahrungen (wie etwa mit Mutter und Wurfgeschwistern) eine in hohem Maße zeitlich und regionsspezifisch determinierte dendritische und synaptische Reorganisation zur Folge haben. Die Entwicklung der Spine-

Dichte war insbesondere durch das Ausmaß an sozialem Kontakt nach der Entwöhnung beeinflusst. Tiere mit dem kürzesten Kontakt zur Mutter, zeigten dabei erhöhte Spine-Zahlen im anterioren cingulären Kortex (ACd) und Hippokampus. Dendritische Länge und Komplexität von apikalen Dendriten im Präfrontalen Kortex, anteriorer cingulärer Kortex (ACd) und orbitofrontaler Kortex (OFC) und in der CA3 Region des Hippokampus zeigten ein Zusammenspiel der Faktoren Zeit der Entwöhnung und anschließendem sozialen Kontakt, jedoch keine Beeinflussung durch einen dieser Faktoren allein. Die am stärksten sozial isolierte Gruppe (frühe Entwöhnung und anschließende soziale Isolation), zeigte im Vergleich zu anderen Tieren eine Erhöhung der dendritischen Länge und Komplexität im ACd und in der CA3 Region. Im Gegensatz hierzu, zeigte sich im orbitofrontaler Kortex (OFC) eine Verminderung der dendritischen Länge und Komplexität der apikalen Dendriten. Eine Beeinflussung der basalen Dendriten war in keinem der Fälle festzustellen.

Im *Gyrus dentatus* waren Länge und Komplexität der Dendriten der Körnerzellen, insbesondere in der infra-pyramidalen Schicht, sowohl durch eine Kombination der Parameter Zeit der Entwöhnung und anschließendem sozialen Kontakt, als auch durch jeden dieser Parameter allein beeinflusst. Ähnlich wie im ACd und in der CA3 Region wiesen auch im *Gyrus dentatus* die Tiere mit der geringsten sozialen Interaktion, im Vergleich zu anderen Tieren, längere und komplexere Dendriten auf.

Zusammengenommen zeigen diese Ergebnisse erstmals, dass sowohl die zeitliche Koordination als auch die Quantität sozialer Interaktion mit Familienmitgliedern signifikante Auswirkungen auf die Feinabstimmung neuronaler Netzwerke im Präfrontalen Kortex und Hippokampus hat, die beide als Teil des limbischen Systems, wesentlichen Anteil an emotionalem und kognitivem Verhalten haben.

Im Rahmen dieser Arbeit war es möglich, Veränderungen auf der zellulären Ebene von Neuronen mit unterschiedlichen emotionalen Erfahrungen zu korrelieren.

7.5 Selbständigkeitserklärung

Erklärung

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

“Refinement of neuronal structures in the rodent prefrontal cortex and hippocampus: Critical impact of early and late social experiences”

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

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.

Braunschweig, 26.06.2007.
Reena Prity Murmu

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Publications:

Ferdman N, **Murmu RP,** Bock J, Braun K, Leshem M. (2007). Weaning age, social isolation, and gender, interact to determine adult explorative and social behaviour and dendritic and spine morphology in prefrontal cortex of rats (Behav Brain Res. 180(2):174-82).

Bock J, **Murmu RP** Leshem M, Ferdman N, Braun K. (2007). “Refinement of dendritic and synaptic networks in the rodent anterior cingulate and orbitofrontal cortex: Critical impact of early and late social experience (article in press; Dev Neurobiol).

Murmu RP, Bock J, Leshem M, Ferdmann N, Braun K. (2007). “Refinement of dendritic and synaptic networks in the rodent hippocampus: Critical impact of early and late social experience (in preparation).