Postnatal Development of the Primate Prefrontal Cortex (areas 9 and 46): Maturation of Pyramidal Neurons and Serotonergic Innervation in Layer 3.

THESIS SUBMITTED FOR Ph.D.

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The dorsolateral prefrontal cortex (DLPFC) of the monkey brain exhibits a unique combination of intraareal and interareal connections, as well as distinctive neuronal properties, which correlate with diverse cognitive and mnemonic behaviours. Many of these attributes are found in the homologous region of the human brain. This thesis describes and compares the developmental time courses of two important components of the cortical circuitry in the monkey DLPFC: pyramidal neurons providing extensive, elaborately-patterned intrinsic connections in the superficial layers and the serotonin (5-HT) innervation in the same region. We hope that the results from these studies will contribute to a model of the human DLPFC; aiding the understanding of complex interactions within the DLPFC during postnatal development. The layer 3 pyramidal neurons providing intrinsic lattice connectivity within layers 1-3, are also the sources and targets for efferent and afferent corticocortical connections between the DLPFC and the rest of the brain. The postnatal time course of maturation for layer 3 pyramidal neurons was quantified by means of the changes in the relative density of their dendritic spines, which represent the postsynaptic sites of excitatory (asymmetric) synapses. We compared the developmental sequence for dendritic spines to the time courses of maturation for other components of layer 3 circuitry, e.g. asymmetric synapses, inhibitory parvalbumin-immunoreactive (PV-IR) cartridges, dopaminergic (DAergic) axons and we comment on possible implications for observed temporal similarities or differences between them. Layer 3 is also the site of a dense 5-HTergic axon innervation, which originates from the mid-brain raphe nuclei and comprises 2 classes of axon fibres ("thick" and "thin"). We examined the time-scale of postnatal development for 5-HTergic axons in layer 3 and attempted to find temporal correlation’s between 5-HT axon maturation and pyramidal neuron dendritic spine development, as well as with previously examined layer 3 components, including DAergic axons and 5-HT tissue concentration. Findings included the demonstration of concurrent time courses of maturation for dendritic spines and PV-IR cartridges; synchrony between rates of spine and excitatory synapse production in the first 2 postnatal months and a subsequent plateau phase, but different time periods of decline (from 1.5 and 3.0 years, respectively) and complex relationships between the stages of spine maturation and changes in density of DAergic axons coinciding with the rise (birth-2 months) and fall (1.5-4.6 years) of spine numbers. We also demonstrated temporal interrelations between 5-HTergic axon development and that of other neuropil components, including modulatory interaction between the density of 5-HTergic axons and dendritic spines as well as strong reciprocal correlation’s between the 5-HTergic and DAergic axon innervations.
2.0: Introduction.

2.1: The prefrontal cortex in the primate brain.

The prefrontal cortex (PFC) of the primate brain is an anatomically and physiologically heterogeneous region, which occupies 29% of the entire cerebral cortex in humans (Brodmann, 1909; see updated map Figure 1A) and 11.5% of the total in non-human primates such as the macaque monkey (Brodmann, 1905, 1912; see updated map Figure 1B). The large size of the PFC, together with the profound functional deficits observed in patients with surgically or disease-induced prefrontal lesions, demonstrate the PFC to be an extremely important cognitively-related region of the human cerebral cortex. In the monkey brain, one major sub-region of the PFC, the dorsolateral (DL)PFC (area 46; see Figure 1B) has been well characterised as being the locus for working memory function as has been observed for normal human subjects, as demonstrated by performance in delayed response tasks, which are analogous to aspects of human decision making (see section 2.3).

The monkey PFC is composed of many cytoarchitectonically distinguishable areas or sub-regions, which have been defined by various schemes of nomenclature, each differing slightly in their cytoarchitectonic criteria (Brodmann, 1909; Vogt and Vogt, 1919; Walker, 1940; von Bonin and Bailey, 1947, see Figure 2). More recently the anatomical divisions have been recognised to possess their own functionally distinct physiological properties (reviewed by Fuster, 1989 and Goldman-Rakic, 1987a). In addition, continued anatomical research has led to further subdivision of the Walker-defined areas (Barbas and Pandya, 1989; Preuss and Goldman-Rakic, 1991; Carmichael and Price, 1994; Petrides and Pandya, 1994; Pandya and Yeterian, 1996a; see Figure 1B).

Cytoarchitectonically, the monkey PFC shows a significant diversity of structure in adjacent areas; dorsal areas tend to be granular (i.e. showing a distinct layer 4 of densely packed small cells, in Nissl-stained tissue) but the ventral areas are agranular in structure (i.e. layer 4 is not so distinct in Nissl-stained tissue). In addition, even areas within this dorsal-ventral dichotomy show subtle differences; such that caudal areas in both locations seem to be less differentiated, i.e. not so clear in their overall laminar organisation, whilst areas more rostrally located, are the most differentiated in terms of the structure and clarity of their laminae. In the mediolateral axis of the PFC, the dorsolateral areas are more differentiated than the dorsomedial and the ventrolateral areas are more differentiated than the ventromedial areas (Barbas and Pandya, 1989; Pandya and Yeterian, 1990; Barbas and Pandya, 1991; Barbas, 1992). The existence of a continuum of increasingly distinctive laminar structure progressing from the caudal to rostral PFC areas and from the medially to the laterally
Figure 1. Lateral views of the frontal lobe, based on maps by Petrides and Pandya (1994).

(A) The human brain and (B) The Macaque monkey brain, illustrating the striking similarities of the cytoarchitectonic organisation within these two primate species (based on the observation of cellular and laminar organisation in nissl-stained sections. Rostral is left and caudal is right in both diagrams. AS = arcuate sulcus. IFS = inferior frontal sulcus. LOS = lateral orbital sulcus. MFS = middle frontal sulcus. MOS = medial orbital sulcus. PS = principal sulcus. SFS = superior frontal sulcus.
located PFC areas and from the ventral to dorsal PFC areas shows the dorsolateral PFC to be the most differentiated sub-region of the PFC and the ventromedial PFC to be the least differentiated sub-region.

With respect to this thesis, where areas 9 and 46 were examined, the cytoarchitectonic structure of these areas has previously been described to be very similar in many aspects (reviewed in Barbas, 1992). There is not a clear-cut border between the 2 areas, but rather there is evidence of a gradual change in the laminar architecture between the 2 areas, both in the human and monkey prefrontal cortex (Petrides and Pandya, 1994). For this reason, to all intents and purposes areas 9 and 46 are often viewed together as a single region - the dorsal prefrontal cortex - with lateral (areas 9/46) and medial (area 9) components. We have taken this view into account in this first study of the thesis, such that spine density measures from individual pyramidal neurons in areas 9 and 46 were pooled within each animal to obtain an overall mean value of relative spine density over postnatal development for the dorsal PFC.

2.2: Concurrent development: Same maturational time course within all cortical areas.

The "concurrent hypothesis" of development is supported by studies in the macaque monkey which have examined the time courses for maturation of synaptic density (Rakic et al, 1986; reviewed in Rakic et al, 1994); callosal axons (LaMantia and Rakic, 1990); neurotransmitter tissue concentration (Brown and Goldman, 1977; Goldman-Rakic and Brown, 1982). There is also evidence for this global synchrony of brain maturation, from studies of functional development in the monkey, as measured by levels of performance in behavioural tasks evaluating either sensory, motor or cognitive abilities individually or in combination (reviewed in Goldman-Rakic, 1987b).

The studies of synaptogenesis (Rakic et al, 1986; Granger et al, 1995) have examined the primary visual, primary motor, primary somatosensory, dorsolateral prefrontal and cingulate cortices in detailed quantitative EM analyses of the time course for the attainment of mature synaptic numbers in all cortical layers using measures of synaptic density per unit volume and per unit area (for total, asymmetric and symmetric synapses). A concurrent time course of synaptogenesis has been demonstrated for asymmetric (excitatory) synapses in cortical layers 1-6 (most clearly for layers 1-3) within all the above cortical regions.

Generally for every layer of each area, the time course for asymmetric synapse development was as follows: synaptic density began to rise in the late prenatal period and continued to increase in postnatal life until 2 months of age when levels reached a peak or
"overshoot" (except in layer 6). The peak levels of synaptic density were consolidated between 2 months and 3.0 years of age (puberty), thereafter a gradual decline in the numbers of synapses was observed throughout adult life (oldest animal in this study was 20 years of age). Levels of total synaptic density followed a similar time course to that for asymmetric synapses in all regions examined (at least 80% of all cortical synapses are excitatory), whilst the overall numbers of symmetric (probably inhibitory) synapses did not change significantly in any region across the entire period of pre- and postnatal development.

Observations of concurrent maturation were also found for the tissue concentrations of monoaminergic neurotransmitters in biochemical analyses (Brown and Goldman, 1977; Goldman-Rakic and Brown, 1982) of various cortical regions including primary visual, primary motor, primary somatosensory, parietal, premotor, orbital and dorsolateral prefrontal cortices. The levels of dopamine, serotonin and noradrenaline in each region were observed to increase dramatically within the first 5 to 8 months of life, with some variation between individual regions in the exact rate of development, as well as more considerable differences in the changes in the concentrations of these neurotransmitters between 8 months and 3 years of age (onset of puberty).

Evidence from numerous behavioural studies in both monkeys and humans (reviewed in Goldman-Rakic, 1987b) has indicated that a synchronous rather than a sequential pattern of cortical maturation occurs in primates, with emergence of diverse "critical" sensory, motor and cognitive functions during the early postnatal period when levels of synaptic density are at their highest (2-4 months in monkeys; 8 months-2 years in human infants).

In monkeys, some of these observations have included the appearance of visual cortex-dependent functions such as visual-tracking of small stimuli, visually-guided reaching and differential recognition of facial features (Mendelson et al, 1983; Boothe et al, 1985) at between 6-8 weeks after birth. It is also around this time (i.e. 2 months of age) when visual object discrimination ability - characteristic of medial temporal lobe memory systems - first becomes evident in monkeys (Goldman et al, 1974; Bachevalier and Mishkin, 1984). Infant monkeys as young as 10 weeks old, were able to demonstrate adult-like abilities of the determination of size and texture in a tactile discrimination task (Carlson, 1984) indicative of a certain degree of primary somatosensory cortex maturation. Finally, the monkey DLPFC first exhibits signs of its most distinctive function at between 2-4 months of age, with regard to working memory ability in the delayed response task, object retrieval task and Piaget's A-not-B hiding task (Harlow et al, 1964; Diamond and Goldman-Rakic, 1986, 1989).

In humans, comparable tests of cortical functional development have shown
that the development of visual acuity as subserved by the primary visual cortex exhibits a "critical period" of plasticity within the first 2 years of life (reviewed in Garey, 1984). Emergence of functions such as visuospatially guided hand orientation and visually controlled preparation for grasping of objects have been demonstrated by infants of 4.5-8.5 months and 9-13 months respectively. In the first task, rapid learning was seen in the ability to adjust hand posture to the orientation of an object on the basis of visual inspection (von Hofsten and Fazel-Zandy, 1984) and in the second task, significant improvement in the ability to modify distance between thumb and fingers prior to contact with an object rather than in reaction to it (von Hofsten and Ronnqvist, 1988), was evident during the above period of development. In the case of medial temporal lobe and hippocampal functions, the ability to perform well in a visual-paired comparison task, by comparative recognition of novel objects, is present prior to 4-6 months in human infants (Overman et al, 1993) and also the ability to perform on single- and multiple-object discrimination is present by at least 12 months of age (Overman et al, 1992) if not as early as 5-6 months of age (Diamond, 1995). There is also evidence for some degree of recognition-memory emergence in the early postnatal period, as measured by successful performance in a reaching task-with-delay in infants as young as 6 months of age (Diamond et al, 1994). DLPFC-specific functions, as measured by the delayed response task and Piaget's A-not-B task appear to emerge between 7.5 and 12 months of age in human infants (Diamond and Doar, 1989; Diamond and Goldman-Rakic, 1989).

2.3: Hierarchical development: Different rates and periods of maturation within each cortical area.

The "hierarchical theory" of cortical maturation, which has been more traditionally accepted, is that different regions of the primate brain progressively mature, both anatomically and functionally at different rates during the course of postnatal life in the manner of a hierarchically-organised sequence. Observations which would support such a theory have been observed for levels of myelination in post-mortem brains (human: Flechsig, 1920; Yakovlev and Lecours, 1967; monkey: Gibson, 1970; Quencer et al, 1980; Quencer, 1982; reviewed in Greenfield, 1991), myelination in MRI studies of humans (infants: Girard et al, 1991; Nomura et al, 1994; adolescents and adults: Nomura et al, 1994) and grey matter volume with MRI (children and adults: Jernigan et al, 1991; children and adolescents: Reiss et al, 1996).

There have been studies of synaptic density in various cortical regions (in human: Huttenlocher, 1979; Huttenlocher et al, 1982, 1993), synaptically-related proteins (in rat:
Alvarez-Bolado, 1996). Also there is supporting evidence from the maturational time course of neurotransmitter innervations (cat: Vu and Törk, 1992; monkey: Foote and Morrison, 1984 and present observations), calcium-binding protein-labelled (PV-IR) interneurons (Condé et al, 1996), a neurochemical subtype of pyramidal neurons (Kostovic et al, 1988), axonal projection pathways (Kostovic et al, 1983; LaMantia and Rakic, 1994) and cellular metabolic enzymes (Farkas-Bargeton and Diebler, 1978). Studies of levels of glucose metabolism in humans (infants: Chugani and Phelps, 1986; Chugani et al, 1987; adolescents and adults: Chiron et al, 1992) and monkeys (infants: Kennedy et al, 1982; adolescents: Kennedy et al, 1978; and of all ages: Jacobs et al, 1995; reviewed in Raleigh et al, 1996b; Distler et al, 1996) have also shown there to be different rates of energy-usage at a given age in different cortical regions.


In primates, the more posteriorly-situated primary sensory areas, such as primary visual, auditory and somatosensory cortices are thought to reach their adult state in many aspects, both anatomically and functionally, much earlier, i.e. within the 6 months of postnatal life in monkeys (over the first 3-4 years in humans), than the more anteriorly-located higher association areas, e.g. DLPFC, ventrolateral PFC and anterior cingulate cortices which are thought to reach maturity at later ages. In the case of the DLPFC, the final stage of functional and anatomical maturity is thought to occur at around the time of puberty (3 years of age) in monkeys (anatomically: Andersen et al, 1995; Rosenberg and Lewis, 1995; present thesis studies; functionally: Goldman and Alexander, 1977; Alexander, 1982; Kubota, 1993, 1994) and in humans at around 16-18 years of age (Uylings et al, 1995; reviewed in Kostovic, 1990; Kostovic et al, 1995).

By contrast, the orbitofrontal cortex is known to mature at a much earlier time, at around 7 months-1 year of age in monkeys (functionally: Goldman, 1972; Raleigh et al, 1996a). In the case of the primary motor cortex, the maturation of its functional properties (monkey: Lawrence and Hopkins, 1976; Armand et al, 1994; Olivier et al, 1997; Human: Muller et al, 1994) and anatomical projections (monkey: Galea and Darian-Smith, 1995; Armand et al, 1994; human: Amunts et al, 1995) are found to be achieved by some time
point intermediate to those for the primary sensory and most association regions. This idea has been given credence by the observation of functional and anatomical maturation of corticospinal projections from the hand region of primary motor cortex to the spinal cord by around 8-11 months of age in monkeys (Armand et al, 1994; Galea and Darian-Smith, 1995). In humans, the conductance velocity of afferent axonal pathways between median nerve fibres in the hand and the primary motor cortex mature between 5-7 years of age (Muller et al 1994), while the functional properties of the efferent pathway from the primary motor cortex to the nerves of the hand show a slightly longer time course of postnatal maturation, i.e. by around 10 years of age (Muller et al, 1994).

3.0: Anatomical connections of the DLPFC (area 46) in the macaque monkey.

3.1: Introduction: Afferent and efferent connections.

The PFC as a whole receives inputs from cortical and subcortical areas which demonstrate a particular involvement in every possible sensory modality and sub-modality. These afferent projections converge on distinct areas of the PFC in accordance with their particular class of sensory information. We were concerned in this thesis with 2 areas of the DLPFC (areas 9 and 46; see Figure 1B and Figures 3A and B). The functional roles of area 9 are not well understood, although it is thought to be involved in sensorimotor integration and is known to be heavily interconnected with somatosensory, auditory, cingulate and extrastriate visual areas (Barbas, 1992). More is known regarding the functional and anatomical structure of area 46, which is primarily appears to receive visuospatially related information. It in addition receives auditory, somatosensory and object-related visual input. Most of the extensive corticocortical afferent inputs converging on area 46, originate from pyramidal neurons in the posterior parietal cortex (Petrides and Pandya, 1984; Barbas and Mesulam, 1985; Barbas, 1988; Cavada and Goldman-Rakic, 1989b) and some sectors of the superior temporal association cortex (Barbas and Mesulam, 1985; Barbas, 1988; Seltzer and Pandya, 1989a).

These two heterogeneous cortical regions are known to consist respectively, of multiple visual-, visuomotor- or somatosensory-related (parietal) and visual- or polysensory-related (temporal) areas. These regions are linked by numerous reciprocal interconnections not only with the DLPFC (area 46) and the extrastriate visual regions (Morel and Bullier, 1990; Baizer et al, 1991). They are also mutually interconnected by way of pathways between their respective areas (Seltzer and Pandya, 1984; Neal et al, 1990; Seltzer and Pandya, 1994).

In addition to the primary reciprocal pathways between the DLPFC, the posterior
parietal region, the cortex of the superior temporal gyrus and the upper bank of the STS, there are numerous other PFC connections (Alexander et al, 1986, 1990; Pandya and Yeterian 1990; Barbas and Pandya, 1991). These other PFC interconnections fall into five major classes of pathways according to their primary functional specificity: those involving general motor control functions (i.e. cingulate motor areas; frontal and supplementary eye fields; premotor cortex and primary motor cortex).

Secondly, there are interconnections with subcortical and thalamic areas with a important motor component specifically concerned with spatially-guided behaviour, involving the lateral, dorsal and medial subdivisions of the pulvinar, the parvocellular division of the mediodorsal nucleus, the central portion of the striatum (caudate nucleus and putamen) and other basal ganglia nuclei e.g. the globus pallidus; claustrum as well as the dentate nucleus of the cerebellum.

The third major group of areas interconnected with the PFC are the medial temporal lobe areas with limbic, associative and procedural memory-related roles (amygdala; hippocampus; parahippocampal, perirhinal and entorhinal cortices).

The PFC is also interconnected with the superior temporal polysensory (TPO), concerned with multiple modality integration. Lastly, there are extrathalamic neurotransmitter pathway projections from the various mid-brain nuclei to the PFC which are also reciprocated by feedback projections from the PFC: the locus coeruleus (noradrenaline, NA); the ventral tegmental area (dopamine, DA); the dorsal and median raphe (serotonin, 5-HT) and the lateral septum (acetylcholine, ACh).

Study 1 of the thesis examines the time course of maturation of a population of pyramidal neurons in mid-layer 3 of dorsal areas 9 and 46 and in study 2 the postnatal development of 5-HTergic axons which innervate this same layer in ventral area 46. Since many of the corticocortical efferent projection pathways within the macaque monkey brain arise from pyramidal neurons in this layer and also many afferents terminate in layer 3, normally as part of a columnar distribution through all layers, it is of importance to describe in summary the complexity and diversity of these various afferent (incoming) and efferent (outgoing) projections. It is these extensive interconnections with other cortical (including other prefrontal areas) and subcortical regions which are an essential factor enabling the DLPFC to play a crucial integrative role in many aspects of cognitive and mnemonic processing (see Goldman-Rakic, 1987a; Fuster, 1989; Barbas, 1992; Goldman-Rakic, 1995b; Petrides, 1996 for reviews).
Figure 2 Lateral views of the macaque monkey brain illustrating the various early cytoarchitectonic schemes of the prefrontal cortex, (based on observations of cellular and laminar arrangements in nissl-stained sections) devised by different investigators. Walker’s map (C) was the most widely used of these schemes until its recent revision by Petrides and Pandya, (1994). (A), (B) and (D) are orientated rostral (left) and caudal (right), whereas (C) is in the opposite orientation showing only the frontal lobe.
Figure 3. The subdivisions of the lateral prefrontal cortex in the macaque monkey brain. (A) A lateral view of the frontal lobe, showing the most recent cytoarchitectonic subdivisions of the dorsolateral and ventrolateral prefrontal cortex as seen on the lateral surface. (Preuss and Goldman-Rakic, 1991; Petrides and Pandya, 1994). (B) A lateral view of the frontal lobe, showing the borders of the various areas hidden within the depths of the arcuate sulcus (AS) and principal sulcus (PS). (Petrides and Pandya, 1994). LOS = lateral orbital sulcus, MOS = medial orbital sulcus.
Afferent terminations of projections from posterior parietal regions to the PS region (using anterograde tracing methods)

Petrides and Pandya (1994)
Efferent cell origins of projections from posterior parietal cortex to PS region (using retrograde tracing methods)
Petrides and Pandya (1994)

**Figure 5**

**Lateral View**
- Middle/caudal IPL (areas 7a and 7b; Kawamura and Naito, 1984)
- Caudal ventral IPS (Barbas and Mesulam, 1985)
- Caudal/area 7/ventral IPS (Kawamura and Naito, 1984), Caudal ventral IPS (Barbas and Mesulam, 1985), area 7a, PG/posterior ventral IPS/area PE (Neal et al, 1990)

**View within the depths of the principal sulcus**
- Middle/caudal IPL (areas 7a and 7b; Kawamura and Naito, 1984)
- Caudal ventral IPS (Barbas and Mesulam, 1985), Caudal and Ventral IPL (Pandya and Yeterian, 1996b)
- Ventral IPS/area POa-e (Barbas, 1988), ventral IPS/area 7b, PF (Neal et al, 1990)
- Rostral IPL/Ventral IPS (Pandya and Yeterian, 1996b)

**Lateral View**

- Ventral IPS (Pandya and Kuypers, 1969)
- Dorsal/ventral IPS (Pandya et al, 1971)

**A**

- Area 10
- Area 46
- Area 9/46d
- Area 9/46v
- Area 8Ad
- Area 8Av

**View within the depths of the principal sulcus**

**B**

- Upper (dorsal) bank
- Dorsal/ventral IPS (Pandya et al, 1971)
- Area 9/46d
- Area 8Ad
- Area 8Av
- Area 9/46v

- Lower (ventral) bank
- Caudal/middle ventral IPS and ventral IPL (Pandya et al, 1971)
- Mid ventral IPL (Pandya et al, 1971)
Efferent cell origins of projections from the PS region to posterior parietal areas (using retrograde tracing methods). Petrides and Pandya (1994)

Lateral View

- Rostral area 7a (CS; Andersen et al, 1990)
- Area 7m (Cavada and Goldman-Rakic, 1989b)
- Caudal area 7a (Cavada and Goldman-Rakic, 1989b)
- Caudal LIPv + LIPd (Schall et al, 1995)
- Rostral area 7a (CS; Andersen et al, 1990)

View within the depths of the principal sulcus

- Area 7m (Cavada and Goldman-Rakic, 1989b)
- Rostral area 7a (CS; Andersen et al, 1990)
- Caudal LIPv + LIPd (Schall et al, 1995)
- Area 7a, PG (CS)/ventral IPS (Neal et al, 1990)
- Caudal and middle LIPd + LIPv (Schall et al, 1995)
- Area 7IP (Cavada and Goldman-Rakic, 1989b)
- Rostral area 7a (CS; Andersen et al, 1990)
- Caudal area 7a (Cavada and Goldman-Rakic, 1989b)
- Area 7b (Cavada and Goldman-Rakic, 1989b)
- Area 7a (Neal et al, 1990), caudal and middle LIPd + LIPv (Schall et al, 1995)
- Area 7b (Neal et al, 1990), rostral area 7a (CS; Andersen et al, 1990), caudal and middle LIPd + LIPv (Schall et al, 1995)
Afferent terminations of projections from superior temporal cortex to the PS region (using anterograde tracing methods)
Petrides and Pandya (1994)
Afferent terminations of projections from superior temporal cortex to the PS region (using anterograde tracing methods)

Petrides and Pandya (1994)

Lateral View

View within the depths of the principal sulcus

Caudal ventral STG (areas Tpt/ Pa-Alt; Chavis and Pandya, 1976)

Middle STG (rostral area Ts3; Petrides and Pandya, 1988)

Middle STG (Jones and Powell, 1970)

Rostral STG (area Ts2; Chavis and Pandya, 1976)

Middle dorsal STS (middle area TPO; Seltzer and Pandya, 1989a)

Caudal STG (caudal Pa-Alt/Tpt; Petrides and Pandya, 1988)

Middle STG (areas dorsal Ts3/Pa-Alt; Petrides and Pandya, 1988)

Caudal dorsal STS (caudal TPO/ TAA; Seltzer and Pandya, 1989a)

Middle/caudal STG (Pandya and Kuypers, 1969)

Caudal STG (areas Tpt; Petrides and Pandya, 1988)
Efferent cell origins of projections from superior temporal cortex to PS region (using retrograde tracing methods).
Petrides and Pandya (1994)

A

Lateral View

Caudal ventral STG (Pandya et al, 1971)

Rostral/middle dorsal STS (Pandya et al, 1971)

Area 10
Area 46

Area 9/46d
Area 9/46v
Area 8Ad
Area 8Av

Anterior insula (anterior Ig; Mufson and Mesulam, 1982)
Rostral/ middle STS (Kuypers et al, 1965)

Rostral STG/rostral dorsal STS (Pandya et al, 1971)
Rostral/middle dorsal STS (Pandya et al, 1971)
Caudal ventral STG (Pandya et al, 1971)

View within the depths of the principal sulcus

Rostral STG/rostral dorsal STS (Pandya et al, 1971)
Rostral/middle dorsal STS (Pandya et al, 1971)
Caudal ventral STG (Pandya et al, 1971)

Rostral/middle STS (both banks); rostral/middle/caudal ventral STS and caudal dorsal STS (Pandya et al, 1971)

B

Upper (dorsal) bank

Rostral dorsal/ventral STS (Pandya et al, 1971)

Rostral STG/rostral dorsal STS (Pandya et al, 1971)
Rostral/middle dorsal STS (Pandya et al, 1971)
Caudal ventral STG (Pandya et al, 1971)

Lower (ventral) bank

Rostral dorsal/ventral STS (Pandya et al, 1971)
Anterior insula (anterior Ig; Mufson and Mesulam, 1982)
Rostral/middle dorsal/ventral STS (Pandya et al 1971)
Efferent cell origins of projections from the PS region to the superior temporal cortex (using retrograde tracing methods).
Petrides and Pandya (1994)

Figure 11

Lateral View

Rostral dorsal STS (rostral areas TPO/PGa; Seltzer and Pandya, 1989a),
Middle STS (middle area TPO; Seltzer and Pandya, 1989a)

View within the depths of the principal sulcus

Middle/caudal dorsal STS (caudal areas TPO; Seltzer and Pandya, 1989a),
Caudal dorsal STS (caudal area TPO/PGa caudal Tpt/caudal MT (OAa; Seltzer and Pandya, 1989a).

Anterior insula (anterior Ia-p/anterior Idg; Mufson and Mesulam, 1982)
Middle dorsal STS (middle TPO; Seltzer and Pandya, 1989a)

Upper (dorsal) bank

Middle/caudal dorsal STS (caudal areas TPO; Seltzer and Pandya, 1989a),
Caudal dorsal STS (caudal area TPO/PGa caudal Tpt/caudal MT (OAa; Seltzer and Pandya, 1989a).

Midanterior insula (middle Idg; Mufson and Mesulam, 1982)
Middle dorsal STS (middle TPO; Seltzer and Pandya, 1989a)

Lower (ventral) bank

Anterior insula (anterior Ia-p/anterior Idg; Mufson and Mesulam, 1982), Posterior insula (Ig; Mufson and Mesulam, 1982)
Figure 12 Lateral views of the macaque monkey brain illustrating the connections of dorsal and ventral portions of area 46 (on the surface of the lateral convexity) with other prefrontal cortical areas. Afferent projections of (A) dorsal area 46 and (B) ventral area 46 are shown in simplified form based on the data of Barbas and Pandya (1989). Figures (A) and (B) from Pandya and Yeterian (1990).
Afferent terminations of projections from the thalamic and pulvinar nuclei to the PS region (using anterograde tracing methods) Petrides and Pandya (1994)

**Figure 13**

Lateral View

- Lateral mediodorsal nucleus (dorsal half MDpc; Giguere and Goldman-Rakic, 1988)
- Medial pulvinar (medial PM; Trojanowski and Jacobsen, 1976)
- Peripheral mediodorsal nucleus (dorsolateral MDpc; Giguere and Goldman-Rakic, 1988)

**View within the depths of the principal sulcus**

- Lateral mediodorsal nucleus (dorsal half MDpc; Giguere and Goldman-Rakic, 1988)
- Peripheral mediodorsal nucleus (dorsolateral MDpc/MDmp; Giguere and Goldman-Rakic, 1988)
- Peripheral mediodorsal nucleus (dorsolateral MDpc; Giguere and Goldman-Rakic, 1988)
- Medial pulvinar (medial PM; Trojanowski and Jacobsen, 1976)
- Area 9/46d
- Area 8Ad
- Area 9/46v
- Area 8Av
- Central/lateral PM (Romanski et al 1997)
- Area 10
- Area 46
- Area 8Ad
- Area 8Av
- Area 9/46v

**A**

Upper (dorsal) bank

- Medial pulvinar (medial PM; Trojanowski and Jacobsen, 1976)
- Central/lateral PM (Romanski et al 1997)
- Lateral mediodorsal nucleus (dorsal half MDpc; Giguere and Goldman-Rakic, 1988)

**B**

Lower (ventral) bank

- Medial pulvinar (medial PM; Trojanowski and Jacobsen, 1976)
- Central/lateral PM (Romanski et al 1997)
- Lateral mediodorsal nucleus (dorsal half MDpc; Giguere and Goldman-Rakic, 1988)
Efferent cell origins of projections from the thalamic and pulvinar nuclei to the PS region (using retrograde tracing methods)

Petrides and Pandya (1994)

**Figure 14**

**Lateral View**
- Mediodorsal nucleus (dorsolateral half MDpc), dorsal portion of medial pulvinar (PM), border of ventral anterior nucleus (VAmc, dorsal), ventral anterior nucleus (VA, ventral) and anteroventral nucleus (AV dorsal half); Goldman-Rakic and Porrino (1985).
- Medial pulvinar (medial PM; Jacobsen, 1975; Trojanowski and Jacobsen, 1976; Asanuma, 1985) Mediodorsal nucleus (MDmf and MDpc; Asanuma, 1985).
- Mediodorsal nucleus (ventral medial half MDmf and MDpc); central portion of medial pulvinar (PM), ventral anterior nucleus (VAmc, dorsomedial half); border of anteroventral nucleus (AV, ventral) and anteromedial nucleus (AM, dorsal); Goldman-Rakic and Porrino, 1985.

**View within the depths of the principal sulcus**
- Mediodorsal nucleus (MDpc, dorsolateral half), dorsal portion of medial pulvinar (PM), border of VAmc (dorsal), VA (ventral) and AV (dorsal half); Goldman-Rakic and Porrino, 1985.
- Medial pulvinar (PM, medial); Jacobsen (1975); Trojanowski and Jacobsen (1976); Asanuma et al (1985). Mediodorsal nucleus (MDmf and MDpc; Asanuma et al, 1985).
- Mediodorsal nucleus (MDpc, ventromedial half and MDmf, medial), central portion of medial pulvinar (PM), VAmc (dorsomedial half), border of AV (ventral) and AM (dorsal); Goldman-Rakic and Porrino (1985).
- Central portion of medial pulvinar (PM), rostral lateral pulvinar (PL) and inferior pulvinar (PI); Romanski et al (1997).

Lateral/medial mediodorsal nucleus (MDpc; Siwek and Pandya, 1991)

View within the depths of the principal sulcus
Efferent cell origins of projections from the PS region to the thalamus and pulvinar nucleus (using retrograde tracing methods). Petrides and Pandya (1994)

Lateral View

Lateral mediodorsal nucleus (dorsal half MDpc; Giguere and Goldman-Rakic, 1988)

Central/lateral PM (Romanski et al, 1997)

View within the depths of the principal sulcus

Lateral mediodorsal nucleus (dorsal half MDpc; Giguere and Goldman-Rakic, 1988)

Central/lateral PM (Romanski et al, 1997)
4.0: Functional and behavioural studies of the primate DLPFC.

4.1: Introduction: Functional roles of the PFC and those unique to the DLPFC (areas 9 and 46).

The functional picture that emerges from the wealth of non-human primate and human neuropsychological, neuroanatomical and neurophysiological data, is one of a massive convergence of information occurring throughout the PFC, originating from all sensory modalities. Thus one broad major role for the PFC is in the interrelation and sorting of sensory information - using a process of selective attention to those stimuli relevant at that point in time.

The PFC appears to be able to suppress or juggle more than one item of information in "short-term" or working memory so that each can be rapidly evaluated and either acted upon, ignored or conveyed for storage into "long-term" memory. This is achieved via the many pathways connecting the prefrontal cortex indirectly with the various nuclei of the hippocampus and other medial temporal lobe structures, where declarative or long term memories are thought to be located in a distributed manner, i.e. information is rendered into its most basic constituent parts, each being coded for by the activity of cells in different regions or sub-regions, perhaps coding for a wide range of attributes in a specific sensory modality or for a single attribute across all modalities.

The PFC is also implicated in humans, with playing roles in abstract thought, lexical formulation and speech, decision making, motor behaviour, organisational planning and emotional association. On the evidence of PET and fMRI studies in humans, the DLPFC in particular can probably lay claim to being significantly involved in the "higher association" processing, merging and mnemonic encoding of information in the visuospatial, visuomotor, somatosensory, speech and auditory domains. Many of these attributes, some perhaps uniquely human, have been examined in as direct a way as possible in the macaque monkey, using indirect methods, such as electrophysiology, lesion studies, behavioural psychological testing and various combinations of two or all three of these approaches.

Recent PET and functional magnetic resonance imaging (fMRI) studies in normal human subjects have shown the importance of the DLPFC in many higher order mnemonic (memory-related), executive (goal- and planning-related), cognitive (thought-processing) and motor-related functions. Several recent PET imaging studies in normal volunteers have examined the effects of administrating various neurotransmitter antagonists or agonists (specific for particular receptor subtypes, especially those for the neurotransmitters serotonin and dopamine) on the performance of tasks particularly sensitive to prefrontal cortical...
Figure 17 A cartoon illustrating the basic sequence of events in the spatial delayed response task as commonly used in neuropsychological and neurophysiological studies in non-human primates. Figure from Friedman and Goldman-Rakic (1988).

Figure 18 A cartoon illustrating the basic sequence of events in the spatial delayed alternation task as commonly used in neuropsychological and neurophysiological studies in non-human primates. Figure from Friedman and Goldman-Rakic (1988).
function such as the Wisconsin card-sorting task (Mattay et al, 1996) and sub- and supra-span auditory-verbal memory tasks (Grasby et al, 1992, 1995).

The thesis concentrates on areas 9 and 46 of the DLPFC in the macaque monkey. These areas together represent the best overall characterised region of the PFC so far, both in terms of functional roles and anatomical organisation. This region receives its major cortical inputs from areas within the posterior parietal and superior temporal visual association regions. It has been found that several forms of cellular activity occur in the DLPFC during the performance of visuospatial working memory tasks in non-human primates (reviewed in Goldman-Rakic, 1987a; Fuster, 1989 and Funahashi and Kubota, 1994); each pattern of neuronal activity appears to be specifically related to particular stages of the sequence of task behaviour. Pronounced deficits are observed in the performance of these working memory tasks when portions of the DLPFC have been lesioned in monkeys (Petrides, 1991b, 1995; reviewed in Passingham, 1993) particularly following lesions located in and around the principal sulcus (area 46) where the form of cellular activity is most strongly temporally related to the various stages of delayed response (DR) task behaviour (Fuster and Alexander, 1971; Kubota and Niki, 1971; Fuster, 1973; Niki, 1974a). See Figure 17.

It has been possible to examine the mechanism by which these cells implement a response to a stimulus in area 46 by means of a revised version of the classic delayed response task; this uses eye movements rather than manual reaching movements - the oculomotor delayed response (ODR; reviewed in Goldman-Rakic et al, 1990 and Goldman-Rakic, 1996). A certain population of the prefrontal cells recorded, has been found to possess a so-called "memory field" (Funahashi et al, 1989), which is analogous to the receptive field of a cell in visual cortex, except the area 46 cells code for the memory of the spatial location of a visual cue, rather than for the sensory perception of the position as represented by the angle subtended by the stimulus on the retina.

The complexity of the structure and function of the PFC in the monkey is reflected by the diversity and number of hypotheses that have been proposed to describe the various functional roles of this region (reviewed in Fuster, 1989; Goldman-Rakic, 1987a; Passingham, 1993). The breadth and development of ideas and discoveries in this field are briefly reviewed, before some specific examples are examined in more detail, to illustrate the range of functions attributable to the DLPFC and its interactions - via extensive reciprocal anatomical connections - with multiple cortical and sub-cortical areas elsewhere in the primate brain.

The functional and behavioural aspects of the DLPFC, especially the PS region (area 46) are of fundamental importance when considering the organisation of the anatomically
unique lattice pattern of intrinsic circuitry in the superficial layers of this region. Also, equally important are the extensive connections of the DLPFC with the rest of the brain, via reciprocal corticocortical and cortico-subcortical pathways which individually are thought to represent overlapping sectors of the large and extensive multiple distributed networks of projections between areas of similar functional-relations.

It is thought that mnemonically distinct but temporally-overlapping patterns of behaviour may be achieved via the topographically arranged sub-level pathways of these different sectors being recruited as required, in a kaleidoscope of constantly changing permutations over time, with swapping and switching of information occurring such that it travels along the widest/fastest available sub-pathways between a variety of dedicated networks each enabling the integrative recording from sensory modality to mnemonic template to motor action and vice versa with variations on all the intermediate stages in between.

4.2: Methodological advances and diverse studies of the DLPFC in the adult non-human primate.

By the early 1970's, a large body of literature had accumulated of studies both in monkeys and in humans, which had examined the relationship between the removal or permanent inactivity of a particular PFC area or region and the resulting changes in behavioural and psychological performance on delayed response tasks in monkeys and on the Wisconsin Card Sorting Test in humans. Many studies were concerned specifically with examining the DLPFC (area 46; cortex within and immediately around the principal sulcus region), believed to be the site of spatial memory (Milner, 1964; Mishkin, 1964; Goldman and Rosvold, 1970; Goldman et al, 1971; Butters et al, 1972; Passingham, 1972a,b; Goldman-Rakic, 1987a; Fuster, 1989). In human studies, experimenters were only able to test patients with large, uncontrolled, variably located lesions of disease origin or resulting from surgical treatment for neurological disorders, comparing their task performances with those of normal subjects. In studies using experimental monkeys, relatively circumscribed, surgical lesions could be made and it was thus possible to compare the post-surgical results of these monkeys on certain tasks, compared to their pre-surgical ability (i.e. acting as their own control). The results from these behavioural studies strongly supported the case that lesions of the equivalent DLPFC regions in monkeys and humans could result in comparable deficits on analogous tests of spatial memory.

Despite the large numbers of investigations that had been conducted on the
neuropsychological aspects of the PS region in the monkey DLPFC, there still remained many unanswered questions even after nearly 40 years of research and many groups held equally compelling evidence to explain the differences which arose despite identical lesions and testing with the same performance tasks. One aspect of functional specificity which was clearly reinforced over many different studies was the general idea of the PS as the functional locus of the "spatial engram" (Butters et al., 1972), i.e. the anatomical location of visuospatial memory, essential to the successful completion of a delayed response task.

Neurophysiological recordings carried out in the intact normal monkey DLPFC have related the various behavioural stages in delay task performance to the simultaneous changes occurring in the activity of single cells during the performance of these tasks (Fuster and Alexander, 1971; Kubota and Niki, 1971; Fuster, 1973). In these electrophysiological studies, awake behaving monkeys with an implanted electrode - directed into the PS region of the DLPFC - were required to perform visually-guided manual delay tasks such as spatial delayed alternation (see Figure 18; Kubota and Niki, 1971) and spatial delayed response (Fuster, 1973; Fuster and Alexander, 1971). Continuous recording of single unit neuronal activity before, during and after the delay stage of the task, for each trial, enabled a comparison of that activity (based on mean data for many neurons) with the distinct stages of the task performance.

The task stages were divided into: presentation of the stimulus, delay period and active response (i.e. reaching for the food reward). The various alterations in neuronal activity during the course of each trial appeared to reflect the temporal sequence and duration of these behavioural subdivisions. It was demonstrated that during a delayed alternation task some units were more active after the delay period (i.e. just before the response was made) than during it and others were more active during the delay period than just before the onset of the response (Kubota and Niki, 1971). Alternatively, during the performance of the delayed response task, DLPFC neurons could be classed into 6 different types, on the basis of the temporal relation of their activity to the delay period of the trial (Fuster, 1973; Fuster and Alexander, 1971). In either case, the anatomical location of the recorded types of neurons appeared to show no clear relation to their particular pattern of electrical activity during the performance of the task. Instead all types of units were apparently randomly distributed throughout the extent of the sampled region of the PS.

Neurophysiological and neuropsychological studies of delayed response task performance were not solely restricted to the DLPFC alone; areas known to be interconnected with the DLPFC, such as the posterior parietal cortex (Fuster, 1973) and
the medial dorsal (MD) nucleus of the thalamus (Fuster and Alexander, 1973) were also examined for evidence of task-related neuronal activity or lesion-induced deficits, during task performance.

Both these regions were found to show temporally-related activity - comparable to that found in the DLPFC - with respect to the delay period of the spatial delayed response task. These observations provided further support for the view that the mutual anatomical connections linking these widely spaced regions, facilitate the essential integration of sensory and mnemonic information from the various processing levels in the brain, as a necessary component of the complex organised motor behaviour needed to correctly accomplish the task.

From the early 1970's to the early 1980's, many of the experimental studies carried out were still based on the use of the traditional lesioning methods (either surgical ablation or reversible cooling) (Passingham, 1978; Kojima et al, 1982; reviewed in Fuster, 1989). It was only with the advent of more sophisticated investigative techniques (beginning in the early 1980's), that significantly new discoveries were made concerning the specific relations between the anatomical organisation of the DLPFC and its range of functional attributes, along with their eventual in vivo visualisation. These included more complex computer-based testing apparatus (leading to less chance of experimenter-induced bias), more specific lesioning made possible by knowledge acquired from the more detailed pathway tracing of the anatomical connections between the PFC and a host of other cortical and thalamic areas (see review section 3.0 on the anatomical connections of the DLPFC).

Gradually with the development of more sophisticated behavioural single unit recording techniques, the neurophysiological study of monkey DLPFC diversified to include the examination of more sophisticated neuronal properties such as visuokinetic activity during delayed visual discrimination, i.e. delayed response with visual light cues (Kubota et al, 1974), direction of response activity in the delay period i.e. directed towards left or right (Niki, 1974a), absolute versus relative direction of response in the delay period i.e. the literal location of left and right response keys versus their relation to each other (Niki, 1974b) during the performance of the spatial delayed alternation task.

The same paradigm was examined for the spatial delayed response task as well (Niki, 1974c) and overall for both tasks about 5-6% of DLPFC cells recorded showed this response-direction dependent activity during the delay period, hinting at a possible likelihood of cells exhibiting this property during the cue period (light spot stimulus) also. The relative importance of the light stimulus or key pressing response towards the cellular
activity during the delay period in a visually guided key pressing task was also investigated (Sakai, 1974) and it was found that increased cellular activity prior to the light cue were indicative of the animal's "expectancy".

Some studies concentrated upon the properties of prefrontal neurons in and around the principal sulcus (area 46) in "timing" behaviour, i.e. changes in neuronal activity relative to the events during the performance of a long latency task (Niki and Watanabe, 1979). Whilst others investigated cellular activity in the DLPFC during the performance of a task which tests the relative significance of the stimulus to the animal, beyond its physical attributes, e.g. using the delayed conditional discrimination task paradigm (Watanabe, 1981). The increasing numbers of physiological studies, combined with the development of new varieties of behavioural tasks, was bringing to light, new and diverse properties of DLPFC neurons and allowing the formulation of new theories on how the DLPFC could initiate and functionally interact in structured behaviour via its connections with a multitude of cortical and thalamic areas (reviewed in Kubota, 1978).

From the middle 1980's until the present day, the use of lesioning methods has continued and the results obtained by this basic, but ambiguous method still warrant attention. The results of lesion studies in monkeys can still be highly informative when they are augmented by more specific studies involving the pharmacological manipulation of neurotransmitter systems in the DLPFC and the subsequent effects on psychological tests of spatial memory and other paradigms. The development of the measurement of local cerebral glucose uptake (LCGU) - using the 2-[14C]-deoxyglucose (2DG) technique in monkey brain slices (Kennedy et al, 1978) enabled the functional contributions of a variety of cortical (in particular the DLPFC) and subcortical regions in the performance of spatially-related tasks to be evaluated on a quantitative scale (reviewed in Goldman-Rakic and Friedman, 1991). This technique is based on the hypothesis that an increase in the levels of energy usage during the performance of a sequence of task behaviour is a reflection of raised neuronal activity and hence an increased demand for metabolic precursors, i.e. radio-labelled glucose, which can be measured by rapidly perfusing the animal immediately following the end of the task and visualising the levels of 2DG present prior to sacrifice, with autoradiography.

The catecholaminergic neurotransmitter dopamine (see Neurotransmitter review section for more detail) has long been associated with an important role in normal DLPFC function and its deficit or overabundance (as reflected by receptor density) has been hypothesised to be a major factor in the cognitive dysfunction observed in some neuropsychiatric disorders such as Parkinson's disease and schizophrenia,
respectively. The initial identification of DA as the crucial neurotransmitter in cognitive/motor-related mechanisms in the DLPFC came from a biochemical/behavioural study (Brozoski et al, 1979) which examined the cognitive (i.e. DLPFC) effects of administering various neurotoxins specific to either DA, NA or 5-HT in monkeys, by subsequently testing them on the spatial delayed alternation task. It was found that only the depletion of DA produced a substantial deficit on task performance, leading to the premise that DA was particularly important in normal DLPFC cognitive functioning.

Following this significant finding, studies using more sensitive techniques to measure the specificity of DAergic mechanisms in DLPFC function i.e. at the cellular or receptor level, were used to examine the exact locus of involvement of dopamine in the performance of cognitively-related tasks, e.g. the oculomotor version of the delayed response task (ODR). Firstly, the effects of DA at the cellular level have been examined in the DLPFC (Sawaguchi, 1987b; Sawaguchi et al, 1986, 1990a,b) during the performance of various delay tasks and more recently with the development of specific antagonists, at the level of the selective effects modulated by specific DAergic receptor subtypes (Arnsten et al, 1994, 1995; Sawaguchi and Goldman-Rakic, 1991, 1994; Williams and Goldman-Rakic, 1995).

Recent investigations, using a combination of anatomy, neurophysiology and experimental psychology, have emphasised a role for the DLPFC (area 46 in particular) in the cellular and circuit basis of working memory function in the monkey (Goldman-Rakic, 1995b; Goldman-Rakic et al, 1990a). Area 46 has emerged to be an integral part of a functionally distributed network (Goldman-Rakic, 1988) made up of multiple interconnected cortical and sub-cortical areas (also including posterior parietal cortex, cingulate cortex, the striatum and various thalamic nuclei) as determined by dual anterograde tracing studies (Selemon and Goldman-Rakic, 1988). Others using predominantly neurophysiology and neuropsychology have found DLPFC (area 46) to be essential for the temporal structuring (integration or organisation) of behaviour (reviewed in Fuster, 1985a, and 1989), imbuing the DLPFC with the roles of "mediator of cross-temporal contingencies" (reviewed in Fuster, 1985b) and the "bridging of temporal gaps in the perception-action cycle" (reviewed in Fuster, 1990b).

The employment of new tracer substances has enabled the further unravelling of the anatomical connectivity of the DLPFC with a complex network of other cortical areas, including its immediate neighbours in the rest of the PFC. The intensive neurophysiological study of many behavioural tasks in awake behaving monkeys has led to the reappraisal of how the DLPFC might be uniquely functionally and anatomically organised to be able to meet the demands of the roles which it seems to be involved in (Passingham, 1993). There has
been a recent attempt to further clarify the role of areas 9 and 46 in object and spatial memory processing, by making separate as well as combined lesions of these 2 adjacent areas in adult monkeys (Petrides, 1991a,b). The subsequent testing of the animals on a delayed-matching-to-sample task has revealed there to be an unexpected performance deficit, which may suggest that the DLPFC is not strictly confined to the processing of visuospatial information as traditionally thought (Petrides, 1995). Instead, perhaps the mid-DLPFC region, where lesions produce this non-spatial deficit most profoundly - in addition to possessing a spatially-coding subset of neurons - could also be specialised for particular non-spatial aspects of visual processing involving memory for self-ordered behaviour, guided from within the PFC on the basis of an internal representation rather like the hypothesis of "on-line" working memory (Goldman-Rakic, 1987a), only not restricted to the spatial domain, but more diversely including mnemonic-coding for the colour, form and shape as well as for the spatial location or relative position of stimuli (Petrides, 1996).

There is then good reason to suppose that the DLPFC may be sub-divisible into a variety of functional and anatomically distinct sub-domains whereby various neuronal attributes of spatial and object-related memory coding are differentially distributed, but as a whole the DLPFC may integrate these diverse processing modules into a single memory "trace" which could form the basic unit of memory and cognition in the same way as the receptive fields of different neurons in V1 (area 17) code for a selective variety of diverse stimulus properties.

The applications of the 2DG technique were further extended by its modification for combined use with two radioactive labels $[^{14}\text{C}]$ and $[^{3}\text{H}]$ (Friedman et al, 1987), enabling the simultaneous observation of colocalised metabolic activation in 2 cortical regions, measured sequentially for a distinct experimental condition, in the brain slices of the same animal (Friedman et al, 1989). This was then applied to the functional examination of the distributed cortical circuitry thought to be involved in a working memory role, the most prominent of these being the principal sulcus region of the DLPFC (area 46) and the posterior parietal areas (7a, 7b, 7ip and 7m). These 2 cortical regions as well as being heavily reciprocally connected anatomically (Cavada and Goldman-Rakic, 1989), are found to be very closely functionally related, as revealed by their concurrent metabolic activation during the performance of a spatial DR task in monkeys (Friedman and Goldman, 1994).

The approach of localised pharmacological manipulation of the DLPFC in monkeys, involving the application of neurotransmitter-specific receptor antagonists or agonists (e.g. for neurotransmitters: GABA, DA, NA) has made it possible to potentially differentiate
the distinct functional contributions of individual receptor subtypes: GABA_Ar (Sawaguchi et al, 1988), D_1- and D_2-DAergic (Sawaguchi and Goldman-Rakic, 1991, 1994), a_2r-adrenergic (Cai et al, 1993; Li and Mei, 1994) to the biochemical processes necessary for the successful performance of certain behavioural tasks, as indicated by the deficits when their normal receptor function is disrupted. These studies have brought about a much clearer understanding of the wide range of modulatory events occurring within the complex cortical circuitry of the DLPFC which are an essential component in the shaping of the unique functional roles of this particular cortical region.

Many approaches, both old and new are currently being employed to probe the detailed anatomical, neurophysiological and pharmacological aspects of the DLPFC and the areas with which it is intimately connected, i.e. posterior parietal cortex. So far, no one general theory can be said to account for the organisation of the diverse functional attributes which have been found to be exhibited by the various behaviourally-related neuron populations which are arranged in an as yet unknown manner within the complex anatomical framework of the DLPFC.

5.0: The cortical pyramidal neuron.
5.1: Introduction: Postnatal development of the dorsolateral prefrontal cortex.

The DLPFC is known to reach functional maturation at quite a late stage in postnatal development in monkeys and humans. It is generally agreed that the mature adult state is reached shortly after puberty, i.e. at around 3.0 years of age in macaque monkeys and at about 16.0-18.0 years of age in humans. Between birth and the achievement of the mature adult state, many alterations are thought to occur in the circuitry of the DLPFC. This hypothesis is supported by the changes observed in synaptic density for dendritic spines of layer 3 pyramidal neurons in humans which are found to rise rapidly in the early neonatal period to a peak value at 1.0 year of age, which is maintained until around 7.0 years postnatal, when the first sign of a drop in synaptic numbers becomes evident (Huttenlocher, 1979, 1984; reviewed in Huttenlocher, 1993). Thereafter, a gradual decline in the numbers of synapses continues until around 16.0 years of age (i.e. puberty), when stable adult-like levels of synapses are found for layer 3 pyramidal neurons.

The first part of this thesis examines the postnatal development of monkey DLPFC (areas 9 and 46) and shows that a comparable time course is observed for early changes in layer 3 pyramidal neuron dendritic spine density during the equivalent postnatal period to that in the human DLPFC (Anderson et al, 1993; Classey et al, 1994; Anderson et al, 1995), when
an apparently stable level of adult spines is achieved by around 3.0 years of age (i.e. puberty).

The process of pyramidal neuron excitatory synapse overproduction in layers 2 and 3 (most spines are contacted by the lateral projections of the same pyramidal neuron population) and their subsequent attrition at puberty serves as an indicator of the degree of functional maturation of the intrinsic lattice connections in these layers. Since this lattice organisation would appear to be an essential anatomical component in the framework providing the basis for the unique functional attributes exhibited by the DLPFC, it is of particular importance to be able to indirectly establish the time course of its probable functional development, using the timing of its primary postsynaptic points of interaction, i.e. dendritic spines, as a reflection of the degree of overall intrinsic lattice development. By understanding the normal process of postnatal anatomical maturation for layer 3 in the monkey DLPFC, it may be possible to extrapolate this to the timing of events presumed to occur during the normal postnatal development of DLPFC anatomy and function in humans.

The time course of pyramidal neuron maturation is compared with that of a subset of local circuit neurons in layer 3, the chandelier neurons, which form multiple inhibitory synapses with the axon initial segments of these pyramidal neurons. As well, the temporal maturation of the DAergic and 5-HTergic innervations (see Study 2) of the same layer of the monkey DLPFC are compared for the degree of synchrony and reciprocity in the timing of the various stages of their postnatal development.

Layer 3 in the DLPFC is the site of an rich dopaminergic (DAergic) innervation originating from the ventral tegmental nucleus in the mid-brain. This DAergic innervation shows dynamic changes in its density during postnatal development, finally resulting in a lower level of innervation in adulthood than is found in either the infant or adolescent animal. These DAergic axons are known to form synaptic interactions with the spines and dendritic shafts of pyramidal neurons (Goldman-Rakic et al, 1989) and GABAergic interneurons (Smiley and Goldman-Rakic, 1993).

Also forming contacts with pyramidal neurons are the characteristic axon terminal arrangements of several GABAergic inhibitory interneurons. The varicosities of one particular class of these local circuit neurons, the chandelier neuron, are observed to form cartridge arrangements in synaptic contact with the axon initial segments of pyramidal neurons (Somogyi et al, 1982; Williams et al, 1992; Lund and Lewis, 1993). They can be distinguished by their intense levels of parvalbumin-immunoreactivity PV-IR during certain periods of postnatal development (Anderson et al, 1995). The time course of development of layer 3 pyramidal neuron dendritic spines will be compared with the development of the
DAergic innervation (Rosenberg and Lewis, 1995) and PV-IR axon cartridges with which they are known to closely interact in the developing and adult primate (Anderson et al, 1995).

These various components of the cortical circuitry in layer 3 of the adult monkey DLPFC are spatially closely related and in some cases exhibit synaptic associations. It is therefore likely that there is interaction via both synaptic and non-synaptic neurochemical and electrophysiological mechanisms during postnatal development. If there is found to be temporal coincidence in the relative time courses of maturation for these components during postnatal development it may assist in our understanding of how complex cellular interactions occur during the development of the normal primate. From the evidence available, it is probable that in the DLPFC the mature adult circuitry is attained by a gradual maturational process featuring a combination of simultaneous and consecutive acquisition and removal of synaptic or other components, until the adult state is achieved.

The first study in the thesis focuses on the development of a particular pyramidal neuron population located in mid-layer 3 of the monkey DLPFC (areas 9 and 46). Layer 3 - in particular its middle and deep portions - is known to be the site of pyramidal neurons whose axon collateral's provide a complex, stripe-like excitatory intrinsic lattice network of intraareal interconnections (Levitt et al, 1993; Kritzer and Goldman-Rakic, 1995; Pucak et al, 1996). Any given point in cortex appears to connect reciprocally to a series of other points arrayed in a discontinuous stripe-like fashion. This relationship can be thought of as a combination of divergence, i.e. in the case of pyramidal neuron axon collateral’s projecting intrinsically to many other pyramidal neurons. Conversely, there is a convergence of intraareal projections, i.e. with a single pyramidal neuron receiving intrinsic innervation from the axon collateral’s of multiple pyramidal neurons (Kritzer and Goldman-Rakic, 1993; Pucak et al, 1996).

This particular organisation of excitatory intrinsic connectivity in layers 1-3 of the DLPFC, is unique to the prefrontal cortex in the monkey (Levitt et al, 1993; Kritzer and Goldman-Rakic, 1995; Pucak et al, 1996) and in humans (Kritzer and Goldman-Rakic, 1993; Kritzer, 1994) and may make an important contribution to the many neuronal properties and functions known to be specific to this cortical region. These wide lateral intralaminar projections spread in a tangential fashion throughout layers 1-3 and less extensively in layer 5 (Levitt et al, 1993; Pucak et al, 1996).

The discontinuous stripe-like organisation of intrinsically-projecting pyramidal neurons was also observed to be comparable in dimensions to that of the similarly stripe-like arrangement of the afferent termination’s of association projections to other PFC areas.
(e.g. areas 10 and 24), as well as to distant portions of areas 9 and 46 (Pucak et al, 1996). These association connections were also found to be reciprocal in nature (Pucak et al, 1996), with areas 9 and 46 being targeted by association projections from pyramidal neurons in some of the same PFC sectors (i.e. areas 9, 10, 24 and 46) which were found to receive association projections from the DLPFC.

The important roles which pyramidal neurons are hypothesised fulfil in their furnishment of both reciprocal intrinsic and extrinsic excitatory projections in adult primates, as well as the developmental changes in their morphological characteristics (e.g. dendritic spines, excitatory synapses and axonal termination’s), merit a comprehensive examination of this most numerous class of cortical neurons.

5.2: Morphology of cortical pyramidal neurons.

Pyramidal neurons represent the single most ubiquitous neuronal population in the mammalian cerebral cortex, making up approximately three-quarters of the total neuronal complement and fulfilling a role as the primary mediators of both local and long-distance excitatory communication within the cortex. Their axon varicosities are known to terminate onto both other pyramidal neurons and non-pyramidal (mainly GABAergic) neurons (Hendry et al, 1983c; Kisvarday et al 1986; McGuire et al, 1991). Although at first sight, cortical pyramidal neurons in different cortical regions may appear to be almost identical in their morphology, they are in fact an extremely heterogeneous neuronal class in terms of their laminar location, dendritic tree arrangements, numerical complement of dendritic spines, axon trajectories, projection targets and sources of incoming afferent inputs; as well as they can differ markedly in their biochemical content - structural proteins, enzymes and neurotransmitter molecules - and possible functional attributes (reviewed in Feldman, 1984 and DeFelipe and Farinas, 1992).

The basic structure of a pyramidal neuron consists of a pyramidal shaped cell body (soma) with a single prominent apical dendrite most commonly found to extend towards the pial surface and a set of shorter basal dendrites which emanate from the soma (or cell body). Pyramidal neurons are absent from layer 1 and only rarely found in layer 4 of most cortical regions. The apical dendritic trunk passes upwards through the cortical neuropil - with oblique branches arising along its length and sometimes it bifurcates into 2 secondary trunks away from the cell body. The apical dendrite terminates in a tuft of terminal branches within a more superficial layer - generally layer 1 in the case of layer 3 pyramidal cells. In addition to these primary distinguishing features the basal dendritic tree generally emanates from
the lower half of the soma and the dendrites radiate outwards, exhibiting an extensive degree of (first, second, third order etc) branching along the way, within a hemispheric field - although quite often in individual pyramidal cells there are basal dendrites travelling in all directions from the cell body.

5.3: Types of projections formed by cortical pyramidal neurons.

Cortical pyramidal neurons can exhibit 3 basic types of connectivity: (i) long interareal association (intrahemispheric) or callosal (interhemispheric) projections, see Figure 19, (ii) local or longer intraareal intrinsic projections and (iii) subcortical projections. Individual pyramidal neurons may contribute to all three or only one or two of these 3 types of projection patterns in the primate brain. We shall concentrate on the first 2 types of projection which are mainly (>70%) provided by layer 3 pyramidal neurons - the subject of the first study in this thesis - whereas sub-cortical projections primarily arise from pyramidal neurons in layers 5 and 6.

5.4: Organisation of excitatory intrinsic connections in the primate cerebral cortex.

Pyramidal neuron projections distributed within the same area and even the same layer as that of the projecting cells are known as intrinsic, intrareal connections and derive from laterally spreading collateral branches emerging from the primary axon in close proximity to the cell soma. Collateral branches are known to terminate on the dendritic shafts (5%) and spines (75%) of other pyramidal neurons and the smooth dendritic shafts (20%) of interneurons in the primary visual cortex (McGuire et al, 1991). In the DLPFC (Melchitzky et al, 1995, 1996), a large proportion of the synaptic targets of intrinsic pyramidal neuron collateral's are dendritic spines (95%) of other pyramidal neurons, while only 5% are smooth dendritic shafts of interneurons.

5.5(A): Pyramidal neuron excitatory intrinsic lattice circuitry in the monkey DLPFC.

The intraareal intrinsic connectivity of pyramidal neurons has been examined in areas 9 and 46 of the adult monkey DLPFC using small, focal (200-400 μm wide) injections of biocytin in layers 2-6 and mapping the location of the resultant local labelling of anterograde axon fibres and terminals within these same areas (Levitt et al, 1993). A stripe-like pattern of clustered axon fibres and terminals was observed in layers 1-3, surrounding the injection site. Narrow bands of terminal labelling (200-400 μm wide; see Figure 20A), were seen to be interspersed by regular, similarly sized, label-free bands surrounding the injection site. Overall,
Figure 19 The organisation of callosal (from a contralateral area 46 injection) cortical afferent terminations in the PS region (area 46) of the DLPFC in the monkey. (A) shows an opened-up view of the inner surface of the PS (i.e. ventral bank = left, dorsal bank = right, A = anterior, P = posterior), with the pattern of afferent terminations within layer 4 plotted on the surface as a flat tangential map constructed from serial coronal sections through the PS region. (B) shows an enlarged version of the same tangential map of afferent terminations as seen in (A) allowing a greater appreciation of the overall stripe-like pattern, with cross-junctions, blind-endings and Y-shaped overlaps. The rostrocaudal extent of the reconstruction in proportion to the rest of the cortex is indicated on the small lateral view of the macaque monkey brain (top) in (A). Figure (A) from Goldman-Rakic (1981a) and figure (B) from Goldman-Rakic (1984).
these labelled domains occupied an elongate slab of cortex (2-4 mm in length) tangentially parallel with the pial surface and oriented such that the long axis of the labelled terminal fields was approximately at right angles to the nearby principal sulcus. These stripes extended in a parallel vertical arrangement through and within layers 1-3 of areas 9 and 46, maintaining their discrete separated lattice-like organisation in all 3 dimensions in tangential reconstruction’s of serial coronal sections.

The terminal fields of pyramidal neuron axons furnishing the superficial layer intrinsic lattice connectivity within the DLPFC cover a larger area with clusters of terminals distributed in the form of discontinuous elongated stripes (Levitt et al, 1993; Pucak et al, 1996). These stripes are almost identical in size for the distribution of both the terminal fields (Levitt et al, 1993; Lund et al, 1993; Pucak et al, 1996; see Figure 21B) and the projecting neurons of origin within layer 3 of areas 9 and 46 (Kritzer and Goldman-Rakic, 1995; Pucak et al, 1996; see Figure 21A). These stripe domains of anterogradely-labelled axon terminals or retrogradely-labelled pyramidal cells run tangentially across layers 2 and 3 of both areas 9 and 46 with no obvious disruption in their organisation at the border region between these 2 areas.

The dimensions of stripe-like arrangements of intrinsically-connected pyramidal cells and their axon collateral’s and terminals in the DLPFC have been described in some detail (Levitt et al, 1993; Kritzer and Goldman-Rakic, 1995; Pucak et al, 1996). It has been observed that the horizontal (tangential) distribution of neurons or terminals fields within the superficial layers (less clear in layer 5) shows a distinct preference for extending further along the mediolateral axis than the anteroposterior axis (anisotropy or asymmetry), for example sometimes producing a ratio of 7 mm : 700 μm, respectively for the most distant clusters of intrinsically-labelled layer 3 pyramidal cells (Kritzer and Goldman-Rakic, 1995; Pucak et al, 1996).

Tracer injections placed in layers 2 and 3a resulted in a pattern of labelled cell or terminal clusters with a lateral extent that exhibited a minor difference of a few hundred mm between the medial and lateral extent of labelling on either side of the injection locus. By contrast layer 3c injections could produce very significant asymmetry in the mediolateral distribution of labelled cells with for example a lateral extent of 7 mm and a medial extent of 3 mm for the discontinuous, tangentially-oriented elongated patch domains of labelled neurons (Kritzer and Goldman-Rakic, 1995). Less extensive connections spread from the injection sites placed in layer 5 of the DLPFC with recurrent axon collateral’s ascending into the superficial layers (Levitt et al, 1993; Pucak et al, 1996).
Figure 20 Tangential organisation of intrinsic lattice circuitry in the monkey DLFPC (areas 9 and 46). (A) shows a flat-map reconstruction from serial coronal sections of anterogradely-labelled axon/terminal clusters distributed in area 9 (on dorsal surface) near the border with area 46. (B) shows a flat-map reconstruction from serial coronal sections of retrogradely-labelled neurons distributed in area 9 (on dorsolateral surface) near the border with area 46. Note the roughly orthogonal orientation of stripes as they near the principal sulcus (A) or the 9/46 border (B). In both (A) and (B), the asterisk represents the injection site in area 9, following an injection of in (A) biocytin, 450 μm core size and in (B) cholera toxin subunit-B, 400 μm core size. N.B. slightly different scales of (A) and (B). Figure (A) from Levitt et al (1993) and figure (B) from Pucak et al (1996).
Figure 21 Schematic drawings (N.B. not to exactly the same scale) showing the comparative dimensions of intrinsic lattice circuitry in the monkey DLFPC (areas 9 and 46). (A) Retrogradely-labelled pyramidal neurons originating from an injection site in layer 3c, give rise to intrinsic lattice circuitry. These intrinsically-projecting pyramidal neurons are arranged in clusters within layer 3c (deep layer 3) in the coronal plane and form a discontinuous pattern of parallel stripes across the DLPFC in the tangential plane (mediolaterally). (B) Anterogradely-labelled axons and terminals resulting from an injection site in layers 3b/3c furnished by intrinsically-projecting pyramidal neurons in the same layer. These intrinsically-derived axons and terminals form ascending columns which pass through layers 2 and 3 and form terminal tufts in layer 1. They also form a discontinuous pattern of parallel stripes across the DLPFC in the tangential plane (mediolaterally). Figure (A) from Kritzer and Goldman-Rakic (1995). Figure (B) from Levitt et al (1993).
Figure 22 A schematic diagram showing the scale of intrinsic lattice circuitry (A-D) in the monkey DLPFC, with a comparison of the distribution of terminations and cell origins for short-range callosal projections in the same region (E, G and H) and long-distance associational pathways in the same (E and F) and other PFC regions (I and J). The lengths of the bars illustrate the widths of stripe (dense distribution of terminals or cells) and interstripe (sparse distribution of terminals or cells) compartments in each case. Also shown are the mean centre-to-centre distances between stripes, where available, which represent the maximal separation between stripe-centres at their narrowest point in the coronal, pial-to-white matter or tangential axes, where appropriate. Figure based on an original diagram in Levitt et al (1993) while the data was compiled from the literature (references indicated above).
5.5(B): Comparison of intrinsic pyramidal neuron axon/terminal domains and cell body domains in the DLPFC.

The dimensions of clusters made up of retrogradely-labelled intrinsically-projecting pyramidal neurons in areas 9 and 46 (200-600 μm wide, 5-7 mm long; Kritzer and Goldman-Rakic, 1995) were observed to be of the same order of magnitude, in width and length as those of the pyramidal neuron intrinsic terminal clusters (100-600 μm wide, 3.5-8.0 mm long; Levitt et al, 1993).

In the most recent study of pyramidal neuron intrinsic lattice connections (Pucak et al, 1996), elongated stripes composed of clusters of either anterogradely-labelled axon fibres/terminals or retrogradely-labelled pyramidal neurons, resulting from separate injections of different tracers, were observed to be of comparable size to those observed in the 2 previous studies (Levitt et al, 1993; Kritzer and Goldman-Rakic, 1995). In the tangential plane i.e. parallel to the pial surface, the lateral extent of the overall alternating pattern of labelled and unlabelled stripe domains was of the order of (1.0-7.4 mm for anterograde, 0.8-6.0 mm for retrograde) on either side of the injection site, with between (9-17 for anterograde, 9-21 for retrograde) stripes resulting from a single injection case (see Figure 20B). Both types of stripes, composed of either anterogradely-labelled axons/terminals or retrogradely-labelled neurons were found to be oriented in all directions surrounding an injection site, but were predominantly aligned length-ways along the mediolateral plane of the cortex, as previously observed (Levitt et al, 1993; Kritzer and Goldman-Rakic, 1995).

Specifically, using the tracer BDA Pucak et al (1996) found that the mean width of stripes composed of clusters of anterogradely-labelled intrinsic pyramidal neuron axons/terminals through layers 1-3 in the DLPFC (247.9 μm; see Figure 22) was found to compare favourably with the previous finding of 267 μm for the width of axon/terminal stripes labelled with biocytin in the DLPFC (Levitt et al, 1993; see Figure 22). Also, in the same study (Pucak et al, 1996), the width of stripes composed of clusters of CtB retrogradely-labelled intrinsic pyramidal neurons (309.1 μm; see Figure 22) was within the range of that found by Kritzer and Goldman-Rakic (1995), i.e. between 200-600 μm in width (see Figure 22).

5.6: Maturation of the corticocortical projections of the monkey DLPFC.

Since study 1 of the thesis concerns the postnatal development of dendritic spines located on layer 3 pyramidal neurons in the DLPFC, we shall review the maturational time
course of the associational and callosal connections of layer 3 pyramidal neurons in the monkey DLPFC. In the adult monkey, these extrinsic projections are known to contact the dendritic spines present on the dendrites of pyramidal neurons; these same pyramidal cells in turn sending efferents to other cortical areas.

As a consequence of the primarily layer 3 location of their input and output, these interareal connections are likely to be in close association with the intrinsic lattice connections of layers 1-3 in the DLPFC. Therefore, the developmental time course of afferent termination’s to and efferent neuronal projections from the DLPFC, are presumed to have a significant impact on the maturation of the intrinsic circuitry within this region.

The well-defined columnar arrangements of afferent terminal input found in the dorsolateral PFC and in other cortical association regions were first demonstrated by making injections of radio-labelled amino acids into the appropriate areas. Radio-labelled terminals and fibres were visualised by autoradiography for each of the homotopic regions in the hemisphere contralateral to the injection site (Goldman and Nauta, 1977). Terminal labelling was found to be distributed in a periodic fashion, composed of vertically-oriented stripes or columns: 200-500 μm wide, with terminals and fibres present throughout layers 1 to 6 in all areas. This form of afferent distribution was observable in monkeys of all ages examined, i.e. 4 days through to 5.5 months. This columnar organisation of labelled afferents was most clearly seen in neonatal animals, while the clarity of their distribution was observed to decrease and become more diffuse with increasing age.

Very few studies have been carried out which investigate the time course of formation of connections between area 46 and other cortical areas during pre- and postnatal development and whether the immature organisation of corticocortical connectivity varies greatly from that of the adult pattern of connections. It is of particular importance to discover how the maturation of DLPFC corticocortical afferents and efferents is related to the functional maturation of the region and the complex sensory-integrative behaviours it mediates. Those studies conducted, have been restricted to autoradiographic tracing with large injections of radioactive amino acids placed into the PS of foetal or young infant monkeys, with examination of the resulting arrangement of terminal labelling in the contralateral PS (Goldman-Rakic, 1981; reviewed in Goldman-Rakic, 1982 and Goldman-Rakic, 1987b).

5.7: The maturation of layer 3 pyramidal neuron morphology in the monkey DLPFC.

The unique organisation of the complex intrinsic stripe-like lattice circuitry
which has been shown to exist in the superficial layers of the primate DLPFC (areas 9 and 46), is thought to be the major distinguishing component of an elaborate anatomical and neurochemical milieu mediating the diverse functional properties exhibited by this cortical region. It is important to establish the time course for the anatomical and functional development of this intrinsic lattice organisation, to be able to determine whether it is coincident with or distinct from that of other cortical regions. This knowledge would assist in answering the questions of how large-scale distributed networks of areas each with their own form of intrinsic circuitry, may provide substrates for the gradual emergence of particular aspects of functional behaviour at different stages of development and if these go awry, e.g. as may occur in the schizophrenic brain, how the formation of abnormal circuitry may bring about the diverse symptomatology observed in this disease.

The postnatal changes occurring for pyramidal neurons in the primary visual cortex (area 17) in the macaque monkey have been examined in some detail and they appear to show a close relation in their time course to the emergence of the mature functional properties of this sensory cortical region in primates. The numbers of dendritic spines on pyramidal and stellate neurons in layers 3 and 4, respectively, of the macaque monkey striate cortex (area 17), have been observed to change substantially during postnatal development (Boothe et al, 1979). The number of asymmetric synapses in all layers of area 17 have also been observed to show an identical time course (Bourgeois and Rakic, 1993). A rapid rise in spine numbers occurs between birth and 8 weeks of age (during which the spine number doubles) followed by a plateau phase of stable levels between 8 weeks and 36 weeks and then a gradual decrease in spine numbers occurs throughout puberty and into adulthood.

These changes have been found to closely coincide with the various stages of critical events involved in the maturation of visual function. These events have been documented in neurophysiological, anatomical and behavioural studies and primarily consist of the final stages of ocular dominance column segregation in area 17 (LeVay et al, 1980). There appears to be a correlation between the sharp rise in the numbers of dendritic spines and the timing of the critical or "sensitive" period of visual plasticity, i.e. the early stage of the neonatal period during which monocularly-deprived monkeys still demonstrate the ability to recover their normal functional and anatomical ocular dominance properties when the deprived eye is reopened or they show additional changes when the conditions for the deprived and normal eyes are reversed (Lund et al, 1991).

Changes in spine numbers have been found to vary for different neuron populations according to their laminar environment (Mates and Lund, 1983a,b). More
specifically, these differences have also been observed for neurons of the same class targeted by different types of afferent input in adjoining layers (Lund and Holbach, 1991). Even different portions of the dendritic tree of a single pyramidal neuron may exhibit variation in the maturational time course of spine populations during postnatal development, depending on their particular laminar surroundings (Boothe et al, 1979).

Changes in synaptic density for all cortical layers, have been quantitatively analysed in several different areas of the monkey cerebral cortex, for animals ranging in age from the early prenatal period through postnatal development and into the period of middle adulthood (Rakic et al, 1986). Samples were made from the visual, motor, somatosensory and prefrontal cortices. The shape of the time course of synaptogenesis was found to be identical for all these functionally distinct areas and in all but the primary visual cortex (area 17) - where values reached maximum earlier in development than for any of the other regions - the absolute densities of synapses (per 100 μm² of neuropil) were also the same for all areas sampled. For both these parameters, there also appeared to be no significant differences between the values for the individual layers of any given area, apart from area 17.

This type of analysis has also been carried out in specific layer-by-layer study of the DLFPC (area 46; Bourgeois et al, 1994). In this study, measures of absolute number, density and proportion of synapses per unit area and per unit volume of neuropil were acquired for each layer of each area in monkeys of ages ranging from E (embryonic day) 47 through to 20.0 years of age. The densities were also described in terms of the 2 main types of synaptic thickening's, i.e. asymmetric (excitatory, Gray's type 1; Gray, 1959) or symmetric (inhibitory, Gray's type 2; Gray, 1959) and the proportion of synapses situated on dendritic spines or shafts across the time-scales of prenatal and postnatal development. Synaptogenesis was divided into 5 distinct periods or phases (in synapses per μm² of neuropil; see Figure 23C), the "pre-cortical" phase (between E47-E78) was characterised by the presence of synapses both above and below the cortical plate and the "early cortical" period (E78-E104) was distinguished by a low-level accumulation of synapses within the cortical plate itself. Thirdly, the "rapid" phase (E104-2 months postnatal) was marked by a dramatic increase in mean synaptic density (per μm²) over this period which reached a peak at around the end of the second postnatal month. The peak "rate" of synaptic acquisition was at E165 just prior to birth, when levels of synapses equalled that found in the adult animal. The fourth "plateau" period of synaptogenesis occurred between 2 months and 3.0 years of age, when the high
Figure 23 Histograms showing the timescale of changes in the density of synaptic contacts (per 100 μm² area) of synaptic contacts in the neuropil of (A) layers 2-3, (B) layer 6 and (C) all cortical layers in area 46 of the monkey DLPFC. Pooled values for all types of synapses = filled circles. Histograms showing the timescale of changes in the density (per 100 μm³ volume) of synaptic contacts in the neuropil of (D) layers 2-3, (E) layer 6 and (F) and all cortical layers, in area 46 of the monkey DLPFC. Axospinous synapses = open squares, axodendritic synapses = crosses. In (A)-(F), B = birth, DAC = days after conception and P = puberty. Figures from Bourgeois et al (1994).
mean synaptic density (per μm²) attained in the previous phase appeared to be maintained at
the same constant level throughout this period. Finally, the last "declining" phase of synaptic
development was observed between 3.0 years and 20.0 years of age, in which there was a
slow and gradual, but sustained reduction in the density of synapses throughout adulthood.

The overall finding was that the superficial layers 1-3 (see Figure 23A), all exhibited
the same overall time course of maturation, magnitude of total synaptic density and were
synchronous in terms of their rates and patterns of total synaptic accumulation and reduction.
The excessive level ("overshoot" in the initial portion of the rapid phase) of mean synaptic
density (per μm²) observed in the superficial layers was found to be of a lesser magnitude in
layers 4 and 5 and was not observed in layer 6 (see Figure 23B). The authors considered
these results to be unsurprising since almost three-quarters of all cortical synapses in area 46
were located in the superficial layers, together with the majority of corticocortically projecting
cells and incoming afferent terminals and so these layers were concluded to make a
considerable contribution to the shape of the overall cortical synapse distribution. When the
total synaptic density was expressed in terms of number per 100 μm² of neuropil (taking into
account the length of synaptic profiles in section thickness; see Figure 23D-F) it still retained
the same pattern and time-scale of events as that seen for the area per 100 μm² of neuropil
(see Figure 23A-C).

The classification of synapses, at various developmental stages, into distinct classes
on the basis of their target locus, i.e. either dendritic spines, shafts or cell somata, varied
according to the particular time point sampled, across both prenatal and postnatal
development. The 3 kinds of synapses defined by their postsynaptic targets were found to
represent between: 25-40% (axodendritic), 60-75% (axospinous) and < 1% (axosomatic) of
total synapses (see Figure 23F), across the entire range of ages examined. The earliest
synapses to appear in the prenatal monkey were identified as those contacting dendritic shafts,
increasing gradually in number between E60 and 2 months of age, they showed no further
change in their density throughout adolescence or adult life. A constant population of
synapses would therefore appear to be sustained on neuronal dendritic shafts, with no
evidence of either overproduction, plateau or depletion during the animal’s life-span. It has
been proposed (Bourgeois et al, 1994) that this stability in numbers may be the result of the
conversion of many of these synapses to the axospinous type during the "rapid" phase of
synaptogenesis.

Axospinous synapses were first observed approximately 2-4 weeks following the
appearance of axodendritic synapses, i.e. at between E78-E89 and gradually accumulated
Figure 24 Histograms showing the percentage representation of asymmetric and symmetric synapses within the overall numbers, respectively of (A) all, (B) axospinous and (C) axodendritic classes of cortical synapses, pooled for all cortical layers during the course of pre- and postnatal development, in area 46 of the monkey DLPFC. Asymmetric synapses = filled triangles and symmetric synapses = open triangles. In (A)-(C), B = birth, DAC = days after conception, MAB = months after birth, P = puberty. Figure from Bourgeois et al (1994).
from thereon. Between E104 and E149, the early part of the "rapid" phase of synaptic acquisition began. The levels of axospinous synapses accelerated postnatally, attaining a peak by the end of the second postnatal month a level which was maintained until 3 years of age and beyond, as the onset of puberty occurred. From then on, through puberty and into adulthood, the numbers of these synapses slowly, but consistently declined, eventually reaching 50% of their peak value as seen at the beginning of adolescence. Synapses terminating on dendritic spines make up most of the total synaptic complement in area 46 at all stages of development and as such are mainly responsible for the dynamic changes in synaptic density that are exhibited by this region during synaptogenesis.

Further subdivision of synapses in area 46 into those of either the symmetric or asymmetric type in the adult monkey, revealed the former to account for 20% and the latter to represent 80% of the total synaptic complement (see Figure 24A). The same values were also found at early, middle and late stages of prenatal development (E78-E149), it could be assumed that these proportions remain unchanging across the life of the animal, reflecting consistent proportional rates of production for each synaptic class at all stages of development. The respective contribution of each type of synaptic morphology to each of the 2 major types of postsynaptic contact, was such that approximately 60-80% of axospinous synapses consisted of the asymmetric class while < 10% were of the symmetric type (see Figure 24B), the rest were unclassified due to their being obliquely sectioned. In the case of synapses terminating onto dendritic shafts, this population split roughly into equal subpopulations of the symmetric and asymmetric forms (see Figure 24C). In both cases, either for synapses contacting dendritic spines or shafts, all layers of area 46 individually exhibited an identical proportion and pattern of distribution for symmetric and asymmetric forms of synapses in both prenatal and adult stages of development and these were comparable in relative levels to the overall proportions found for the pooled values for all layers.

5.8: Summary.

The cortical pyramidal neurons of the DLPFC are not a homogeneous class of cell they may be distinguished on an individual basis by their dendritic morphology (including spine number), local or long-distance axonal projection targets, synaptic inputs, developmental time course, neurochemical signature and functional properties. It appears that pyramidal neurons are as heterogeneous in their anatomical, biochemical and functional
properties as the morphologically and neurochemically diverse (reviewed in DeFelipe, 1993) cortical non-pyramidal class of neurons.

In terms of postnatal development of pyramid neuron circuitry dendritic spines, which are each the postsynaptic half of an asymmetric synapse show dynamic changes in density within all cortical layers of the DLPFC during the postnatal period. An examination of the time course of maturation in the density of these markers of excitatory synapses on individual pyramid neurons of layer 3, where intrinsic and extrinsic projections converge and diverge, will provide important information regarding the anatomical and functional development of the intraareal and interareal cortical circuitry furnished by these neurons.

6.0: Neurotransmitter Systems.

6.1: Introduction: Organisation of neurotransmitters in the monkey PFC.

The functional heterogeneity of the PFC is best illustrated by the diversity and specificity of the distribution patterns of various neurotransmitters across its constituent areas, as measured by immunohistochemical staining of their synthetic enzymes or the transmitter molecules themselves. All the major modulatory neurotransmitters are represented throughout the PFC, albeit to varying degrees, whereas in the primary sensory cortical areas these neurotransmitters may either be almost totally absent or alternatively they may predominate. For example, in monkey primary visual cortex (V1), the 5-HTergic and noradrenergic (NAergic) innervations are strongest, the cholinergic input is moderate, whilst dopaminergic axons are virtually absent. By contrast, in the DLPFC, axons of the 5-HTergic, NAergic, AChergic and DAergic systems are all well represented within the 6 cortical laminae, with their own distinctive patterns of density distribution and fibre orientation within the various layers.

All 4 neurotransmitters achieve many of their functional effects via their binding to various receptor subtypes specific for each neurochemical. These receptors are distinguishable by their differences in molecular structure, cortical areal, laminar and cellular location (somatic, dendritic, axonal, i.e. pre- or postsynaptic locus) and in their mechanisms of action (i.e. G-protein linked, phosphoinositide-linked etc). In this way the 4 major modulatory neurotransmitters are able to exert a whole host of diverse effects on various components (even distinct portions of the same neuron may express different subtypes of the same or another neurotransmitter receptor) of the cortical circuitry in the DLPFC. In various studies of cortical areas, the density distribution of a particular neurotransmitter receptor subtype has been observed not to coincide exactly with that of the axon innervation which conveys
the neurotransmitter itself. This so-called "mismatching" of neurotransmitter release sites and receptors seems to hold true for the DLPFC and for 5-HT and DA specifically.

Neurotransmitter receptors can be located either pre- or postsynaptically; a significant proportion, if not the vast majority, of modulatory neurotransmitter-containing axons appear not to form synaptic contacts in the monkey prefrontal cortex (Smiley and Goldman-Rakic, 1993, 1996; Mrzljak et al, 1995) and so are not restricted to the confines of localised "classical" synaptic neurotransmission. It has been hypothesised that these terminals release their transmitter by a diffuse "volume transmission" mechanism (reviewed in Descarries et al, 1991). In order for this latter mechanism of neurotransmitter release to be effective and to justify the heterogeneous anatomy of the various neurotransmitter systems, it would be highly dependent on there being a variety of different receptor subtypes and diverse locations for them on different classes of cells and axons (even on the terminal axons of other transmitter systems). By this means they would be able to exert specific effects and enable temporally coherent action, perhaps at the level of a population of neurons in a certain layer in each area. On the basis of the low synaptic frequency for their axons (Smiley and Goldman-Rakic, 1993, 1996) both the 5-HTergic and DAergic systems appear to be prime candidates for this form of neurotransmission in the DLPFC.

There is significant evidence to implicate DA and its receptors with an important modulatory role in the mechanisms of cognitive functions such as working memory in the primate DLPFC, in monkeys (Sawaguchi and Goldman-Rakic, 1991; Williams and Goldman-Rakic, 1995). There is also evidence in visual cortex that 5-HT can exert a profound modulatory influence on sensory processing and although its effects have not been examined to the same extent or in the same detail as dopamine, it may well emerge to be making as important a contribution as DA in the mechanisms of DLPFC function. Preliminary pharmacological studies have been conducted in the primate DLPFC and many PET studies have been carried using specifically designed ligands to localise the various 5-HT receptor subtypes in the human brain; it has been found that as in the monkey (for 5-HT\textsubscript{1} and 5-HT\textsubscript{2} receptors) they have distinctive distribution patterns in different layers and areas, including in the DLPFC. There is also much evidence to suggest that serotonin and dopamine may act reciprocally towards each other, either directly via their partial antagonistic effects on each others receptors or indirectly via their binding to their own receptors which may be localised on the same cellular components and might have opposing fine regulatory control over the same functional mechanism, e.g. working memory in the monkey DLPFC.

In addition, 5-HT and its receptors have been well characterised with an
important modulatory role in the development of circuitry in several areas of the cerebral
cortex in many mammalian species (Bennett-Clarke et al, 1995; Matsukawa et al, 1995; Rörig

It is of interest, where possible to compare the developmental time courses for
different neurotransmitters in the primate DLPFC, in order to explore the possible parallels
which can be drawn between the permanent modulatory/regulatory interactions which they
show in the adult monkey and human DLPFC. In this way it might be possible to relate the
pattern and time course of axon development to the transient or cycling nature of the
neurotransmitters, with their potentially trophic or neuromodulatory roles during the
prolonged developmental maturation of the unique cortical circuitry and functional attributes
of the primate DLPFC.

6.2: Dopamine (DA).

6.2(A): Introduction.

Dopamine is a catecholaminergic neurotransmitter, localised within an afferent system
of projections originating from several subcortical nuclei, namely the substantia nigra (SN;
pars reticulata and pars compacta subdivisions) and the ventral tegmental area (VTA) of the
mid-brain. These nuclei contain large numbers of DA synthesising neurons which send out
long distance axon projections to innervate various regions within the cerebral cortex.
Dopamine is produced via a synthetic biochemical pathway common to all catecholamines
(noradrenaline and adrenaline), its major rate limiting synthetic enzyme being tyrosine
hydroxylase (TH).

The DLPFC (areas 9 and 46) is thought to receive the bulk of its extrinsic DAergic
axon projections from the ipsilateral anterior two-thirds of the VTA and in addition to receive
only a very sparse input from the ipsilateral medial third of the pars compacta (pc) subdivision
of the SN (Porrino and Goldman-Rakic, 1982). A recent study using a combination of
retrograde tracer injections in area 46 and double-labelling with a TH-specific antibody in the
SN-VTA region (Goldman-Rakic and Williams, 1995), has revealed that not all prefrontally-
projecting DAergic neurons are restricted to the VTA as previously thought; showing that the
DLPFC receives a significant DAergic input via the direct projection it receives from the
lateral-medial extent of the dorsal SNpc as well as from the VTA (2/3 of double-labelled cells
distributed equally through both nuclei) and also from the dorsal retrorubral area (1/3 of
double-labelled cells).
The DAergic innervation of the DLPFC has been characterised at both light and electron microscope level and is probably the best understood of all neurotransmitters, in terms of its actions and interactions with other components of the cortical circuitry. DAergic axon terminals have been shown to synapse with the dendritic spines of pyramidal neurons in the DLPFC (a subject of study in this thesis); often they are accompanied by unlabelled axon terminals forming asymmetric (excitatory) synapses onto the same spines. These inputs are presumed to be corticocortical or thalamocortical afferent projections as well as intracortical connections, which are thought to use glutamate as their modulatory neurotransmitter. The localisation of glutamate immunoreactivity in the PFC has not yet been examined, but it is reasonable to assume that glutamate plays an important role in corticocortical, intracortical and thalamocortical neurotransmission to and from the DLPFC. The possible interaction of DAergic afferents with major excitatory inputs to pyramidal neurons is thought to be an integral part of the cellular mechanism for modulating their internal cellular activity levels; perhaps placing DA in a position to control and mediate the interactions between multiple cortical areas. In addition, the cellular and sub-cellular locations of various subtypes of DAergic receptors have been determined in the DLPFC and it is thought that their specific distribution enables the DAergic system to have a powerful influence over both the intrinsic, corticocortical and corticothalamic connectivity of the DLPFC in the primate brain. Dopamine is also known to be subject to complex modulatory interactions with other neurotransmitters, e.g. serotonin (studied in this thesis), acetylcholine, neuropeptides such as CCK and excitatory amino acids such as glutamate and aspartate via the synergistic actions of some of their respective receptor subtypes.

The postnatal maturational time course of the DAergic innervation in layer 3 of the monkey DLPFC (Rosenberg and Lewis, 1994, 1995; see Spine study Discussion) is hypothesised to correlate with certain stages in the postnatal development of spines located on the dendrites of layer 3 pyramidal neurons (Anderson et al, 1995 and this thesis). In this thesis we also examine a similar hypothesis, for the possible relationship of the postnatal time course of development of the 5-HTergic innervation in layer 3 of the monkey DLPFC, to developmental of connections to layer 3 pyramidal neurons, as well as other components of the neuropil.

6.2(B): Distribution of TH-labelled and DAergic axons.
6.2(B).(i): TH-labelling.

Studies using an antibody against tyrosine hydroxylase (TH), the synthetic
enzyme of dopamine production, have found the DLPFC (area 46) to have an intermediate to sparsely distributed TH axon innervation in terms of the overall density of the innervation across the entire cerebral cortex, primary motor cortex having the greatest density (Lewis et al, 1987, 1988). Although TH-immunoreactivity is found to be present in all layers, layers 1 - superficial 3 and layers 5 and 6 show the densest TH-axon innervation in area 46, producing a characteristic bilaminar pattern (Lewis et al, 1987). Area 46 on the dorsal and lateral surface is relatively densely innervated whilst the density of TH-axons is much lower in both banks of the principal sulcus (PS) and lower still in the fundus of the PS (Lewis et al, 1988). By contrast, area 9 is described as being heavily innervated in terms of the overall TH-axon distribution in the cerebral cortex, the medial portion, 9M being more densely innervated than the lateral portion, 9L (Lewis et al, 1988). A bilaminar innervation pattern of TH-axons is seen in area 9, as in area 46 (Lewis et al, 1987, 1988).

6.2(B).(ii): DA-labelling.

In the last few years antibodies to the dopamine molecule itself have been developed and with one such antibody a similar distribution of dopaminergic (DAergic) axons has been seen in areas 9 and 46, to that observed with the TH-antibody, i.e. a bilaminar innervation pattern. However, DAergic axons were described as being more densely distributed in the dorsal as opposed to the ventral bank of the PS in area 46, mainly due to a greater innervation of layer 3 (Williams and Goldman-Rakic, 1993).

6.2(C): Distribution of DAergic or TH-labelled varicosities and their postsynaptic targets.

In an electron microscopic (EM) study of the PFC (Goldman-Rakic et al, 1989), DAergic and TH-labelled synapses have been found in large numbers in the dorsal bank of the PS, their density distribution is equivalent to that of the bilaminar organisation of presynaptic axons as seen in light microscopic studies of DAergic immunoreactive axons. Symmetric synaptic contacts are observed to be made by TH-labelled varicosities with the cell bodies, dendrites and spines of Golgi-impregnated pyramidal neurons (N.B. contacts between DAergic varicosities and pyramidal neurons are not readily visualised in Golgi material). Synapses between DAergic axons and dendritic spines as seen in EM material, are normally accompanied by an asymmetric unlabelled bouton (indicative of glutamatergic input) contacting the same spine in a so called synaptic "triad" or complex (Goldman-Rakic et al, 1989).
A serial section EM study in monkey prefrontal cortex in the dorsal bank of the PS (Smiley and Goldman-Rakic, 1993) has shown that on average, 39% of DAergic varicosities through all layers form synapses (40% in layer 3). The mean overall distribution of synapses is as follows: 2% on somata; 34% on dendritic spines and 64% on dendritic shafts (0% on somata; 50% on spines and 50% on shafts in layer 3). The identity of all observed postsynaptic dendrites is: 42% spiny (pyramidal) dendrites; 29% smooth (interneuron) dendrites and 42% are of uncertain dendritic identity (of the 2 dendritic shafts receiving DAergic synapses in layer 3, both were of uncertain origin).

In layers 1-3 of monkey DLPFC (area 9), the synaptic incidence of TH-labelled varicosities on GABA-labelled dendrites is found to account for 44% of all TH-labelled synaptic contacts; 56% of synapses are formed with unlabelled dendrites (Sesack et al, 1995a). Also in area 9, a negative result has shown that although some TH-labelled boutons synapse onto the GABA-labelled dendrites of some local circuit neurons, the GABAergic, calretinin-positive (CalR) subclass are exempt from this synaptic input (Sesack et al, 1995b). A mean 26% of total TH-labelled varicosities were said to be closely associated with GABA-labelled postsynaptic dendrites but only 13% of the total number of TH-labelled varicosities formed actual synaptic contacts; 12% of the total TH-labelled varicosities were observed to be closely associated with CalR-positive dendrites, but no synaptic contacts were observed.

A more recent preliminary EM-level immunohistochemical study by the same group (Lewis et al, 1996) has identified at least one of the calcium-binding protein (CBP) subtypes of GABAergic interneurons which form the postsynaptic targets of TH-labelled axon terminals. Using a double-labelling EM procedure with antibodies specific for the CBP parvalbumin (PV) and tyrosine hydroxylase (TH) to label DAergic axon terminals in monkey DLPFC (area 9), it was observed that TH-labelled varicosities were apposed to PV-immunoreactive dendrites. About 33% of these contacts were found to represent symmetric synapses, but many TH-labelled varicosities contacted unlabelled dendrites in close proximity to PV-IR structures. It is likely that DAergic axons form synaptic contacts on one or other or both of either wide arbour (basket) neurons or chandelier neurons in the monkey DLPFC. These EM-level observations of DAergic/GABAergic circuitry interaction suggest that DAergic afferents are in a position to indirectly modulate the levels of inhibitory control exhibited by PV-IR interneurons which are known to target pyramidal neurons in the DLPFC.
6.3: Gamma-aminobutyric acid (GABAergic) and/or calcium-binding-protein-positive local circuit interneurons in monkey DLPFC.

6.3(A): Introduction.

Non-pyramidal cells of the cerebral cortex, can be broadly divided into those that are GABAergic i.e. they express immunoreactivity for the inhibitory neurotransmitter GABA, presumably reflecting their use of it to carry out their modulatory functions, and those that are non-GABAergic, i.e. they do not appear to use GABA as their neurotransmitter - or it is present at levels below the threshold of immunocytochemical detectability - and instead contain one or more of various neuropeptide molecules. Other populations of non-pyramidal cells have been found to express both GABAergic and neuropeptide molecules. In recent years, interest has grown regarding the morphological diversity and local circuitry of inhibitory GABA-containing interneurons in the cerebral cortex of the primate brain (Somogyi et al, 1981,1983; DeFelipe and Jones, 1985; reviewed in Jones and Hendry, 1984; Peters, 1984; Somogyi and Cowey, 1984).

Of the 13 classes of local circuit interneuron found in areas 9 and 46 (Lund and Lewis, 1993), we shall be examining only those which either have their cell bodies and axons in layer 3 or whose cell bodies are outside layer 3 but whose axon arbours pass through layer 3 (sometimes furnishing lateral projections) and terminate in other layers.

These generally inhibitory cells have been further subdivided into subpopulations of GABAergic interneurons, on the basis of their content of one or more of 3 distinct calcium binding proteins - CBP; calbindin (CalB), calretinin (CalR) and parvalbumin (PV) - as well as in some cases one of numerous neuropeptide molecules (see later in this review). The distribution and morphology of these interneurons has been described most notably for the primary visual cortex (V1) of the monkey brain (Hendry et al, 1989; Van Brederode et al, 1990; reviewed in Jones et al, 1994), but some studies have also been conducted which examine the biochemistry and morphology of these cells in the PFC (Lewis and Lund, 1990; Williams et al, 1992; Lund and Lewis, 1993, Condé et al, 1994; Gabbott and Bacon, 1996a,b).

The study by Lund and Lewis (1993) identified the Golgi-impregnated morphological characteristics of 13 distinct classes of local circuit interneurons in the DLPFC (areas 9 and 46). This catalogue was then used to identify the observed morphologies of CBP-specific (calbindin, CalB-IR; calretinin, CalR-IR and parvalbumin, PV-IR) or neuropeptide-specific (CCK and prosomatostatin) interneurons in the same region, thus providing further subclasses of interneurons on the basis of their biochemical heterogeneity. No significant
differences were observed between interneurons in area 9 or area 46, so both their populations were pooled together.

Three well described inhibitory neuronal subtypes (based on their distinct morphologies) present throughout all regions of the cerebral cortex, are chandelier, large basket and double-bouquet neurons (Jones and Hendry, 1984; Peters, 1984; Somogyi and Cowey, 1984). In the DLPFC, they are found throughout the depth of the cortex from layers 2 to 6 (Williams et al, 1992; Lund and Lewis, 1993; Condé et al, 1994).

6.3(B): Chandelier neurons.

Lewis and Lund (1990) have examined the morphology of chandelier neurons and their content of parvalbumin in areas 9 and 46 of monkey DLPFC. Using the Golgi-rapid technique they identified chandelier neurons throughout layers 2-5, as possessing small cell bodies of 12-15 μm diameter, from which emerged fine vertically arranged dendrites. These dendrites were found to be arrayed in columnar-shaped arbours (50-75 μm wide, 200-300 μm length), sometimes spreading into layer 1. Cells in layers 2-3, were observed to give rise to axons, either from the base of their soma or sometimes from a proximal dendritic segment, which spread beyond the dendritic field in deep layer 3, but which did not enter layer 1. The axon's thickness varied along its extent, but upon reaching the vertical cartridge arrangements of varicosities, unique to chandelier neurons, the intervaricose segments between these columns of varicosities were often very fine.

Axon cartridges were found in all layers except layer 1, many were found to be comprised of the axon contributions from several chandelier neurons, since the numbers of varicosities traced in cartridges arising from the axons of single neurons were fewer than for those free cartridges seen to be associated with individual pyramidal neurons in the neuropil as had been previously shown for the chandelier neurons in other cortical regions by Somogyi et al (1982). The varicosities contained within axon cartridges were occasionally observed to contact the axon initial segments (AIS) in Golgi-impregnated pyramidal cells but in immunohistochemical preparations (with an antibody specific for PV) of tissue they could be observed to enclose the shadows of un-impregnated pyramidal cell AIS's and somata. In Golgi preparations, axon cartridges were most commonly seen in layers 2-4, but were also present in layers 5-6.

Using an antibody against parvalbumin (PV), Williams et al (1992) and Lund and Lewis (1993) observed PV-immunoreactive (PV-IR) vertical arrangements of densely
labelled boutons (sometimes connected with intervaricose segments) of 1-2.5 μm width and 15-40 μm length. In adult monkeys, these PV-IR cartridges were mostly seen in deep layer 2-superficial 3, were present at a lower density in superficial layer 2 and deep layer 3 and even less commonly observed in the other cortical layers (absent in layers 1 and 6). It was assumed from the similarities between the Golgi and the immunohistochemical observations that the PV-IR processes were chandelier neuron axon cartridges. However, PV-IR cannot be regarded as a wholly reliable marker of chandelier neurons and their axon cartridges in the monkey DLPFC, due to the absence of labelling in the superficial and deep layers seen in the adult monkey; however these neurons may be clearly seen in all layers in Golgi preparations of both infant and adult material. In addition PV-IR is present in other GABA cell subtypes such as basket neurons, in areas 9 and 46 (Lewis and Lund, 1990).

PV-IR chandelier neurons are known to form symmetric synaptic contacts onto the axon initial segments (AIS, DeFelipe et al, 1989: motor cortex, area 4; Williams et al, 1992: DLPFC, area 46) of pyramidal neurons in monkey cerebral cortex. It has been noted that the number of termination’s made by GABAergic chandelier neuron axon terminals contacting the AIS's of pyramidal neurons in monkey somatosensory (area 3b) and motor (area 4) cortices (DeFelipe et al, 1985), varies widely perhaps in relation to the inhibitory regulation of particular functional properties exhibited by those pyramidal neurons, with a sub-population of large callosally-projecting pyramidal cells in layer 3 receiving an especially dense innervation compared to their associational and/or intrinsic projecting neighbours. This observation has also been made for commissural-projecting pyramidal neurons in layer 3 of the primate supplementary motor area (Williams et al, 1990), where these neurons were seen to be densely innervated by PV-IR varicosities and it is presumed that the same is true for callosally-projecting pyramidal neurons of the PFC (area 46).

The time course of development for PV-IR axon cartridges - representing the terminal inhibitory inputs of a sub-population of GABAergic chandelier neurons - will be compared - (in section 13) with the rate of development for their postsynaptic targets, the pyramidal neurons of layer 3, which are known to receive inhibitory synaptic inputs onto their AIS's from these PV-IR cartridges.

6.3(C): Basket neurons.

Another morphologically distinct PV-IR cell class is evident in the DLPFC, namely the wide arbour "basket" neuron. PV-IR wide arbour "basket" neurons are known to form symmetric synaptic contacts with the proximal dendrites, axon hillocks and the cell
bodies of pyramidal neurons in the DLPFC (area 46) as well as other cortical regions (DeFelipe et al, 1985; Hendry et al, 1989; Williams et al, 1992. As in the case of GABAergic chandelier neuron axon terminals contacting AIS's, GABAergic basket cell axon terminals have been observed to vary in the number of contacts which they make with the axon hillocks of layer 3 and 5 pyramidal neurons, in line with the particular class (callosal, associational, intrinsic) of their projection trajectory (DeFelipe et al, 1985: somatosensory and motor cortices). Similar observations have been made in the primate supplementary motor cortex for PV-IR synaptic varicosities (Williams et al, 1990).

It has been proposed that the dimensions and shapes of the axon arbours of wide arbour "basket" cells may ideally suit them to the role of selectively mediating the inhibition of sub-populations of pyramidal neurons in layer 3 (Lund et al, 1993) which furnish excitatory intrinsic lattice connections, which in the DLPFC are organised in a discontinuous elongated stripe-like pattern across the surface of the cortex in the tangential plane (Levitt et al, 1993; Kritzer and Goldman-Rakic, 1995; Pucak et al, 1996). The width of an average "basket" neuron is at least 3 times greater (900-1000 µm) than the largest of pyramidal neuron basal dendritic field diameters (mean 355 µm) in the DLPFC (Lund et al, 1993). It is thought that these parameters enable the cell body of a "basket" neuron situated closely to that of a given pyramidal neuron to be in a position to influence - via its elongated axonal arbour - only the pyramidal cells in the surrounding mediolateral portion of cortex (see Figure 25). This would still permit the local axon collateral's of the pyramidal neuron - which arise horizontally in layer 3, but arbourise in the plane orthogonal (i.e. vertically) to that of the "basket" cell axon - to excite other pyramidal neurons within layers 2-3 located in the nearest parallel stripe domains situated on either side of the intrinsically-projecting pyramidal neuron.

6.3(D): Distribution of interneurons containing calcium-binding proteins (CBP's).

Condé et al (1994) have examined the morphology and the laminar distribution of neurons expressing each of 3 CBP's (calbindin, CalB, calretinin, CalR and parvalbumin, PV) in areas 9 and 46 of the monkey DLPFC. The laminar distribution of CalR-IR neurons spread through all cortical layers, but most were concentrated in layers 2 and 3 and they were rarely seen in layer 6. For CalB-IR neurons the expression was again present in all layers, predominantly in layers 2 and 3, but also with some concentration in layers 5 and 6. PV-IR neurons were present throughout layers 2-6 (although layer 2 was sparsely populated)
Figure 25 A model of working memory circuitry in deep layer 3 of the monkey DLPFC (area 46). The schematic diagram (coronal section) shows a population of reciprocally-linked (via their axon collaterals; bottom of figure) intrinsically-projecting pyramidal neurons (centre) which may be the best candidates for the neuronal loci of "memory" fields specific for the different portions of visual space as shown in neurophysiological studies (Goldman-Rakic et al., 1990). These neurons may interconnect on the basis of their mnemonic-coding for like-orientations visual space. Also shown are the "basket" neurons which may be reciprocally-connected with these intrinsic pyramidal neurons and may modulate their coordinated activity in the lateral axis (see text for extra details) while the intrinsic pyramidal neurons and their axon terminations are arranged in clusters which are organised in vertical stripes in the tangential and mediolateral planes. Figure from Goldman-Rakic (1995, 1996).
and highest numbers were seen in layer 4 and the adjacent parts of deep layer 3 and upper I.

Quantitative analysis of the laminar distribution of CalB, CalR and PV immunoreactive neurons supported the qualitative observations, with no significant differences in the laminar level of expression of each CBP between areas 9 and 46. Within each region, the overall number of CalR-IR neurons was consistently almost twice that of the CalB-IR and PV-IR neurons, although care should be taken in comparing the expression of different antibodies (there may be as yet unknown differences in antibody penetration and sensitivity for these CBP's). In layers 2-3, the percentage of CalB-IR and CalR-IR neurons was significantly greater than that of PV-IR neurons, in areas 9 and 46 (being 58.5%: 61.9%: 49.1% for area 9 and 68.3%: 66.7%: 49.1% for area 46, respectively).

For each CBP (except maybe PV) the density of labelled neurons was invariant across PFC regions, perhaps providing a consistent framework of intrinsic local neuron circuitry for the functional role of each CBP in the PFC. This observation is contrasts with the representation of neuropeptides - which are all GABAergic (Hendry et al, 1984) and are also often co-localised with CBP's in other brain regions (DeFelipe et al, 1990: a few large CalB-IR/tachykinin-IR unidentified cells in primary auditory).

The synaptic relationship of the majority of local circuit neurons with pyramidal neurons in the DLPFC, remains so far unexamined at EM level. It is possible that particular subpopulations of pyramidal neurons (on the basis of morphology, efferent targets and biochemical heterogeneity) may be preferentially innervated by particular sub-classes of local circuit neurons. It is also evident that certain CBP-defined subclasses of interneuron in the DLPFC may also contact other interneurons as well as interacting with pyramidal cells (Gabbott and Bacon, 1996a).

It appears that the general activity of a layer 3 pyramidal neuron is regulated by synaptic contacts from a range of local circuit neurons, each targeting different portions of the pyramidal neuron dendritic tree, soma or axon initial segment. Each interneuron has its own discrete distribution pattern of synaptic innervation interacting with other cells (both pyramidal and non-pyramidal types), a distinctive biochemical identity (usually as a co-transmitter with GABA) and axonal arbour architecture, with differing and specific functional roles subserved by particular neuronal subclasses.
7.0: Anatomy and functions of the serotonergic neurotransmitter system in the macaque monkey.

7.1: Introduction: Serotonin and the prefrontal cortex.

The second part of the thesis examines the development of the serotonergic (5-HTergic) innervation in mid-layer 3 of the monkey DLPFC (area 46) which originates predominantly from cells in the dorsal and median raphe nuclei in the brainstem (Porrino and Goldman-Rakic, 1982; Wilson and Molliver, 1991b). The existence of this dual origin for the 5-HTergic axon innervation has been thought to account for the observation of two morphologically distinct subpopulations of 5-HTergic axons with differential laminar distributions throughout the cerebral cortex, including the DLPFC (Kosofsky et al., 1984; Wilson and Molliver, 1991a). The premise for the existence of these two types of 5-HTergic axon projections, perhaps fulfilling distinct functional modulatory roles, has been based mainly on observations originally made in rodents (Kosofsky and Molliver, 1987; Mamounas and Molliver, 1988) and cats (Mulligan and Törk, 1988, 1993). However, this differentiation of two axon morphologies is not always clearly seen in monkeys (see Smiley and Goldman-Rakic, 1996, as well as present observations), although they have been seen in post-mortem human neocortex (Kosofsky and Kowall, 1989; Hornung and Celio, 1992).

Although the three-dimensional anatomical arrangement of the 5-HTergic axon innervation has not been examined in detail in the monkey cerebral cortex, a study employing small injections of 5,7-dihydroxytryptamine to ablate localised portions of the cortical 5-HT axon population produced restricted spherical regions of 5-HT denervation in the cortex (Wilson and Molliver, 1986). This result demonstrated that the 5-HTergic innervation of the monkey cerebral cortex is unlikely to be arranged such that individual dorsal or median raphe axons follow a tangential course through the anteroposterior extent of the cortical axis of the brain, which is observed for the rat cortical serotonergic (Lidov and Molliver, 1982) and noradrenergic (Morrison et al., 1978, 1981) axon innervations, or like the noradrenergic (Morrison et al., 1982) innervation in the monkey neocortex. Instead it is more likely that the 5-HT innervation of the primate cerebral cortex is arranged in a discretely topographic fashion with single 5-HT parent axons from raphe neuron groups projecting only as far as their target region before ramifying and branching intrinsically, with very little evidence of extrinsic collateralisation of long distance projections to more than one area from a single cell group.

The synaptic associations of 5-HTergic axons in the monkey prefrontal cortex have not been examined in great detail, but from preliminary observations it appears that only
about a fifth of all 5-HTergic varicosities form synaptic contacts, and most of these (approx. 90%) are associated with the smooth dendritic shafts of interneurons in area 46 of prefrontal (Smiley and Goldman-Rakic, 1996) and in primary visual cortex (De Lima et al., 1988), whilst only about 10% of 5-HTergic varicosities contact pyramidal neuron dendrites. The similarity of the synaptic frequencies in these 2 different regions is unexpected, since there is a much greater overall density of 5-HTergic innervation in area 17 than in area 46. A much higher synaptic frequency has been determined for the varicosities of 5-HTergic axons in non-primate species such as the rat, where at least 50% of [3H]-5-HT-bound (visualised by autoradiography) varicosities sampled in a serial-section EM analysis, were demonstrated to form asymmetrical synaptic contacts with the spines and shafts of pyramidal neuron dendrites in visual cortex (Parnavelas et al., 1985).

Serotonin (5-hydroxytryptamine or 5-HT) is one of the 4 major monoaminergic neurotransmitters found in the mammalian brain, along with acetylcholine, dopamine and noradrenaline. Each of them demonstrate distinctly individual innervation patterns, both across different areas and within the boundaries of individual areas of the cerebral cortex.

Although most research investigating the anatomy, physiology and pharmacology of the serotonergic (5-HTergic) system has been carried out in the rat, a sizeable literature has now accumulated on the equivalent system in the non-human primate, in particular the old world monkey genus *Macaca* (reviewed in Foote and Morrison, 1987a). It is this system in the macaque monkey that will provide the focus for the present review.

We shall review the literature up to the present on the anatomical architecture of the cerebral cortical 5-HTergic system, concentrating on the morphology and distribution of the extensive 5-HT axon innervation in the monkey neocortex. We shall focus particularly on the principal sulcus (area 46) region of the DLPFC, as examined in this thesis. The importance of the 5-HTergic input to the normal functioning of these cortical areas will be examined, especially in area 46. Possible functional roles for 5-HT during the development of the cerebral cortex in primates will also be discussed.

7.2: Different serotonergic projections from the raphe nuclei each exhibiting a distinctive axonal morphology for their innervation of the cerebral cortex.

Having established the evidence for the existence of 2 distinct pathways from the DRN and the MRN, to thalamic/basal ganglia structures and the neocortex respectively in the monkey; it is now appropriate to describe studies which have characterised the regionally-specific terminal innervation of these projections in the monkey cerebral cortex.
Studies carried out have examined whether any differences in morphology could be related to differences in the raphe nuclei of origin, or whether different cell types within both raphe nuclei are the source of the heterogeneous cortical 5-HT innervation.

The organisation of a dual 5-HTergic innervation of the cerebral cortex has been hypothesised in primates (Wilson et al, 1989; Wilson and Molliver, 1991b), i.e. an extensively distributed pathway of fine-calibre axons, originating from the DRN and a sparsely distributed pathway of coarse-calibre axons, stemming from the MRN. This hypothesis was inferred from a combination of observations in the monkey, where 5-HT immunohistochemistry was used to label 2 morphologically distinct populations of 5-HTergic axons in the neocortex, whose projection origins in the raphe nuclei were known from a retrograde tracing study (Wilson and Molliver, 1991b) as has also been seen in the rat (Kosofsky and Molliver, 1987). Also, in the monkey neocortex, administration of amphetamine derivatives appears to eliminate the fine axon population (60-90% of the total axon population, compared to controls) but not the beaded axons (Wilson et al, 1989), as previously seen in the rat (Mamounas and Molliver, 1988 and Mamounas et al, 1991). All this evidence has enabled a conclusion to be reached, that the same arrangement of 2 differential 5-HT projection pathways exist to the neocortex, in the 2 species, which share comparable degrees of innervation pattern and functional roles and may be anatomically represented by the same 2 groups of morphologically distinctive axon terminals (Wilson and Molliver, 1991b). Compared to the rat, however primates including humans exhibit a greater degree of regional heterogeneity in the organisation of their cortical 5-HTergic innervation, with widely varying axon fibre densities in one area relative to another.

7.3: The 5-HTergic innervation of the DLPFC.

The earliest study to examine the presence of 5-HT in the DLPFC of the adult monkey, used biochemical analysis of the gross tissue content of 5-HT and its catabolic breakdown product 5-hydroxyindoleacetic acid (5-HIAA) and also measured the in vivo synthesis rate of its synthetic precursor 5-hydroxytryptophan (5-HTP; Brown, et al, 1979). The tissue level of 5-HT in the DLPFC were regarded as low compared to that of other cortical areas examined (namely somatosensory, anterior inferotemporal and superior temporal cortices). The same was true of the levels of 5-HIAA - primary 5-HT metabolite - although in this case levels increased steadily along a rostro-caudal gradient from the DLPFC to the occipital cortex. The estimates of in vivo synthesis rate of 5-HTP in DLPFC, were only measurable in monkeys pre-treated with an aromatic amino acid decarboxylase inhibitor
NSD 1015. Samples of tissue taken at 15, 30 and 45 minutes following the administration of the inhibitor, showed a linear increase in accumulation of 5-HTP with time. It was concluded that the 30 minute measure would be an accurate reflection of rate of 5-HT synthesis. The rate of 5-HTP accumulation for the DLPFC was low compared to that of the parietal and occipital cortices and the brainstem; rate constant of 0.41/hour for DLPFC and 0.66/hour for parietal cortex. In terms of overall cortical distribution, 5-HTP synthesis rates were equivalent for the PFC (including DLPFC and orbital PFC), premotor and primary motor cortices but much higher in somatosensory, parietal and visual cortices.

Up until the early 1990's the immunohistochemical staining of 5-HTergic axons in the adult cynomolgus monkey PFC had only been described in brief (Lewis, 1990; Lewis et al, 1985). In these studies, using a 5-HT specific antibody (raised in rabbits or guinea pigs) the overall PFC 5-HTergic innervation, was described as being homogeneous and of moderate density; concentrated in layers 1 and 4, with some areas e.g. area 9, where the infragranular layers were slightly denser than the supragranular layers. In addition, 5-HT "basket" arrangements of 5-HTergic axons were observed and 5-HTergic axons were described as being morphologically heterogeneous. More extensive observations were reported by Wilson and Molliver (1991a), where the distribution of 5-HTergic axons in the DLPFC of the adult monkey (cynomolgus and rhesus) was examined using a rabbit 5-HT specific antibody. The 5-HTergic innervation of DLPFC was described as being moderate in density relative to other cortical areas, with the ventral bank of the principal sulcus (area 46) being slightly more densely innervated than the dorsal bank, especially in layer 1.

In the cynomolgus monkey, 5-HTergic axons were quite evenly distributed throughout all cortical layers. While the same pattern was generally true for the rhesus monkey, it was noted that there was a sparseness in the innervation of layer 3, as characterised by a clustering or patchiness in the distribution of 5-HTergic axons. In both species there were similar differences in the orientation of 5-HTergic axons in different layers through the cortical depth. In layer 1, tangentially directed axons were pre-eminent, whilst in layers 2 and 3, 5-HTergic axons were generally radial in their trajectory. In layers 4-6, most 5-HTergic axons were obliquely arranged in a tortuous fashion, although some long tangential axons were also seen, mainly in layer 6. In layer 1, 5-HTergic axons were mainly of the large type, whilst the 5-HTergic axons in layer 2-6 were predominantly of the fine type.
7.4: Localisation of 5-HTergic axons to particular neuronal subtypes (synaptic and non-synaptic relations).

Recently, an estimation of the synaptic incidence of 5-HTergic axon terminals on putative interneurons or pyramidal cells was carried out for the adult monkey DLPFC (area 46) using a rabbit anti-5-HT antibody (Smiley and Goldman-Rakic, 1996). The light-microscopic observations in one monkey, revealed a complex network of 5-HTergic axons, which was particularly dense in layers 1 and 4, as seen in previous studies of adult primate DLPFC (rhesus and cynomolgus monkey: Wilson and Molliver, 1991a and marmoset: Hornung et al, 1990). Serotonergic axons were described as showing a diverse range of morphologies, both in terms of calibre and the shape, size and presence of terminals. Entire axons or even the portions of a given axon could vary between non-varicose and smooth, varicose, with small terminals (thin axon type) or highly varicose with large terminals (thick axon type). Many axons demonstrated this range of variability in their appearance and were thus hard to classify strictly into one or other type of 5-HTergic axon and were thought of as an intermediate class of serotonergic axon. Layers 3-6 were observed to be populated mainly by non-varicose, smooth axons, although they were also seen in layers 1-2 as well.

The authors also observed basket arrangements in the superficial cortical layers, as seen previously in primate DLPFC (rhesus monkey: Wilson et al, 1989), but they were very rarely encountered, with between 1-3 being seen in any one section of the PS.

At EM level the appearance of 5-HTergic axons in the DLPFC of 2 monkeys was equally heterogeneous, most being less than 0.2 μm in diameter (these were too fine to be included in the analysis of synaptic frequency) and others, which were less in number, ranged from 0.2 μm up to 1 μm in calibre (these were surveyed for synaptic interactions). The thinner axons were characterised by their content of synaptic vesicles, these were mainly of the clear type, but occasionally dense core vesicles were also seen, along with microtubules. Within the thicker axons, microtubules were the main components, with clear synaptic vesicles being less common and sometimes absent for long stretches of serial-sectioned axon. This larger type of axon was most prevalent in layer 1 and could exhibit vesicle-containing protuberances or varicosities which formed synaptic contacts; dense core vesicles were also encountered in these axons.

Typical varicosities exhibited by either thick or thin type 5-HTergic axons were distinguished by their content of many clear synaptic vesicles, which could vary in shape depending on the perfusion fixation used for that animal (either glutaraldehyde alone or a glutaraldehyde and paraformaldehyde mixture), being more spherical in the latter
case and more aspherical in the former. Thus overall, 70% of varicosities were described as containing dense core vesicles; some varicosities only possessed 1-2 vesicles, while others contained between 10-20 vesicles. In the animal perfused with the mixed fixation medium, dense core vesicles were found in 85% of varicosities, whilst in the glutaraldehyde-only perfused animal, they were found in only 60% of varicosities. These 2 types of synaptic vesicles were prominent features of 5-HTergic axon morphology, but there did not appear to be any relation between the 2 morphologically distinct types of axon and their proportional content of vesicles. This was supported by the observation that dense core vesicles were present in both large and small numbers within both axon types, but for any one axon the densities were comparable for each individual varicosity, across the range of different sized varicosities.

The synaptic frequency for 5-HTergic axon varicosities was much less than that previously observed for other neurotransmitter innervations in the primate DLPFC. Only 23% (48/213) of serotonergic varicosities analysed forming unequivocal synapses in layers 1, 3 and 5 of area 46, compared to 39% (59/153) for dopaminergic varicosities in all layers (Smiley and Goldman-Rakic, 1993) and 44% (44/100) for cholinergic (as represented by ChAT labelling) varicosities also in all cortical layers (Mrzljak et al, 1995). This low frequency of 5-HTergic synapses was observed, despite the fact that most varicosities contained large numbers of synaptic vesicles, hinting that unconventional forms of 5-HTergic synapses may exist, which are not detectable by present methods. This was supported by the occasional appearance of faint membrane darkening’s at associations between 5-HTergic varicosities and cellular processes, but these were not pronounced enough to be regarded as synapses in the normal sense. Observed 5-HTergic synapses were asymmetric (Gray's type 1) in form, with prominent postsynaptic densities and were quite small in terms of this synaptic class, being detectable through the course of between only 2-5 (mean = 2.83) serial sections (mean width = 70 nm) or 0.21 μm in diameter at their thickest part. These dimensions are much smaller than those for other asymmetric synapses - such as glutamatergic synapses formed between corticothalamic or corticocortical afferents and pyramidal neuron dendritic spines - in area 46 of the monkey DLPFC.

The postsynaptic targets of 5-HTergic axon synapses have also been characterised by complete serial-section analysis and found to be almost exclusively dendritic shafts. Dendritic shafts were classified as previously described (Smiley and Goldman-Rakic, 1993) into 3 main types smooth, interneuron (S), pyramidal (P) and those of uncertain classification (U). In the 3 layers examined, 48% (23/48) of all labelled 5-HTergic varicosities which formed
Synapses in area 46 were most commonly found to contact the S-type of postsynaptic process, 8% (4/48) contacted the P-type processes and over two-fifth's i.e. 42% (20/48) of all synapse-forming 5-HTergic varicosities contacted processes of the U-type. The observation of the latter population resulted from half not being distinguishable into either S- or P-type processes and the other half being too fine to trace through sufficient numbers of serial-sections.

For layer 3, where we examined in this thesis, the maturational time course of 5-HTergic axons across development, the percentage of synapses formed by 5-HTergic varicosities (Smiley and Goldman-Rakic, 1996) was of the order of 21%, with all postsynaptic targets having the characteristics of S-type, interneuron dendrites and none having the features of P-type, pyramidal neuron spiny dendrites. Overall for the synaptic contacts that were identifiable in layers 1, 3 and 5 in area 46 of the monkey DLPFC (Smiley and Goldman-Rakic, 1996), over 85% of 5-HTergic synapses were found to contact the dendrites of interneurons and only 15% of postsynaptic targets were made with the dendrites of presumed pyramidal neurons.

7.5: The postnatal development of the serotonergic innervation of the cerebral cortex: Maturation of layer 3.

The second part of the thesis examines the postnatal maturation of the serotonergic (5-HTergic) innervation of layer 3 in area 46 of the monkey DLPFC. In the adult monkey, the 5-HTergic innervation, originating from the mid-brain raphe nuclei, is known to be distributed throughout the cerebral cortex as well as sub-cortical regions. It is most densely distributed within the primary visual cortex (area 17) of the macaque monkey, and exhibits an extremely varied areal and laminar pattern of innervation, both within and across different cortical areas, including DLPFC (areas 9 and 46), (Morrison et al, 1984; Foote and Morrison, 1987a; Wilson and Molliver, 1991a). Biochemical studies have revealed that the tissue concentration of 5-HT achieves its adult levels by about 5 months postnatally in the DLPFC, compared to between 2-5 months of age in area 17 (Goldman-Rakic and Brown, 1982).

It has been demonstrated from pharmacological and neurophysiological studies in the adult nervous system, that serotonin has a primarily, inhibitory modulatory role (although unequivocal excitatory-type properties have also been observed for 5-HT in various neural systems) in the functioning of various cortical and thalamic areas. Serotonin has also been also been found to show evidence of an important modulatory role during the early stages of both pre- and post-natal development in diverse brain regions in several species, e.g.
during the early cortical period in the cat primary visual cortex, both 5-HT$_1$ and 5-HT$_2$ receptors are known to be mutually essential for the plasticity which facilitates the postnatal rearrangement of ocular dominance columns (Gu and Singer, 1995). In the layer 4 barrel fields of the developing rat primary somatosensory cortex (S-I), normal serotonergic innervation of the vibrissae-specific patches are necessary for the correct timing of the pattern formation of thalamic inputs to these compartments (i.e. 5-HT might be playing a trophic role; Blue et al, 1991) and for the attainment of normal patch size, as dictated by the ingrowth of these thalamocortical afferents (Bennett-Clarke et al, 1994, 1995). The thalamocortical and 5-HT innervations to the barrel fields may in fact mutually influence each other’s maturation, during early postnatal development (Rhoades et al, 1990).

Additional evidence which supports the crucial involvement of the 5-HT system in the development of cortical circuitry comes from the observation that 5-HT$_{1B}$ receptors are specifically and transiently expressed on thalamocortical axon terminals in S-I and primary visual cortex in the developing rat (Bennett-Clarke et al, 1993), coinciding with the time course of a dense 5-HT axon innervation in these areas (D’Amato et al, 1987). It is therefore possible that serotonin in conjunction with its various receptor subtypes might play an important role in the functional plasticity of DLPFC circuitry during early development. It might be involved in functional development e.g. increased levels of DR task performance and/or in anatomical maturation e.g. formation of the intrinsic lattice arrangement of lateral connections in the superficial layers. Disruption of the maturational process of the growth of the 5-HT innervation within the DLPFC, might therefore perhaps adversely affect functional performance in adolescent and adult humans, which may possibly occur in the case of the schizophrenia.

The rationale for studying the postnatal development of the 5-HT innervation in layer 3 of area 46, was to examine how the maturation of numbers of putative 5-HT release sites (i.e. 5-HTergic axon varicosities) might be temporally related to the development of excitatory postsynaptic inputs (i.e. dendritic spines; see first thesis study) between pyramidal neurons providing intrinsic lattices in this region. Since the dorsal and median raphe nuclei in the mid-brain have been shown to provide separate inputs in the form of fine axons with small varicosities and coarse axons with large varicosities, respectively in the DLPFC of the macaque monkey (Wilson and Molliver, 1991a,b), these two systems might be expected to exhibit different developmental time courses as well. So the thesis also examines the issue of the possibly differential developmental aspects of these 2 components of the 5-HT system. The developmental time course of the 5-HTergic innervation could then be expected
perhaps to relate to other developmental events which are known to take place in other
cortical systems which also contribute to the intrinsic circuitry of the DLPFC. The different
systems for which time course was compared to that of 5-HTergic axons included, the
DAergic axon innervation (Rosenberg and Lewis, 1995), 5-HT and DA neurotransmitter
tissue concentrations (Goldman-Rakic and Brown, 1981, 1982), the parvalbumin-
immunoreactivity present within axon cartridges of inhibitory local circuit chandelier neurons
(Anderson et al, 1995; Condé et al, 1996) and levels of excitatory (asymmetric) synapses
(Bourgeois et al, 1994).

So far, to our knowledge, no qualitative or quantitative studies have previously
examined the prenatal or postnatal time course of serotonergic axon development in the
monkey DLPFC - or in any cortical area, except for a qualitative study within the primary
visual cortex (area 17) carried out by Foote and Morrison (1984). It was our intention to fill
this major gap in the knowledge regarding the maturational time course of the 5-HTergic
system during the postnatal period that we carried out an immunohistochemical investigation,
which forms study 2 of this thesis. Although immunohistochemical studies of 5-HT
development have not been conducted in the monkey PFC, there has been some examination
using biochemical methods, of the gross endogenous tissue content of 5-HT and its synthetic
and catabolic precursor and by-product in the PFC from birth to the onset of adulthood
(Goldman-Rakic and Brown, 1982) and in ageing monkeys (Goldman-Rakic and Brown,

In the first study (Goldman-Rakic and Brown, 1982), the brains of new-born
monkeys up to the age of 3.0 years of age, were examined for 5-HT tissue content, the levels
of the metabolic precursor of 5-HT (5-hydroxytryptophan, 5-HTP) and the catabolic by­
product of 5-HT degradation (5-hydroxyindoleacetic acid, 5-HIAA) in the PFC. The "adult"
value for tissue levels of 5-HT in the PFC as a whole was attained as early as 5 months of
age, whilst for the other cortical regions sampled the mean age was 2 months (i.e. for the
occipital, parietal, somatosensory and motor cortices). The maximal statistically significant
difference in the 5-HT levels between different ages was observed between the new-born
monkeys and those of 2.0-3.0 years of age. However, in the PFC, the eventual adult value
was not achieved simply through a process of uninterrupted growth in the 5-HT tissue levels
across postnatal development, since a significant decrease in 5-HT was observed to occur
between the ages of 5 and 8 months. The value for the 8-month-old animals was significantly
lower than that for the 2-month-old and 3.0-year-old animals, whilst the 5-HT levels of the 5
month old animals were not significantly different from those of the 3.0-year-old's.
Following this drop in 5-HT concentration between 5 months and 8 months of age, the tissue levels of 5-HT were observed to rise again, between 8 months and 3 years of age to the same high levels that they had initially shown at 5 months of age. This pattern of postnatal maturation for 5-HT tissue content was similarly observed for DA tissue content in the PFC, with a parallel rise and fall in 5-HT and DA concentrations being observed between 2 and 18 months of age. The tissue content of 5-HTP in the PFC, over the same period of postnatal development was found to remain relatively stable, with no significant differences between the levels present at any age between birth and 3.0 years of age.

In a study of an older age group of animals, Goldman-Rakic and Brown (1981) used the same methods of biochemical analysis to determine the gross tissue content of 5-HT and 5-HTP in the PFC of monkeys between 2.0 and 18.0 years of age. The striking observation for 5-HT and 5-HTP content, was that there were no significant differences in either the tissue levels of 5-HT or the levels of its synthetic precursor, 5-HTP, during either the late pubertal period or throughout adult life in the monkey PFC. Thus, levels of 5-HT were identical for animals of 2.0 years of age and for those of 18.0 years of age, with no divergence in 5-HT levels for the animals of ages in between. In addition, levels of 5-HT synthesis as quantified by 5-HTP tissue content were also invariant over the same age range. So 5-HT activity in the monkey PFC, as measured by the tissue content and levels of synthetic precursor, would appear to remain constant throughout adult life and would seem not to be affected in the process of normal ageing, as measured in this study.

### 7.6: Functional roles of 5-HT in the cerebral cortex.

Serotonin has been attributed with significant involvement in numerous aspects of brain functioning in most major mammalian species including a significant role as a developmental signal or trophic factor in both pre- and postnatal development (Lauder and Krebs, 1978; reviewed in Whitaker-Azmitia et al, 1996). E.g. an intact serotonin innervation appears to be necessary for the correct somatotopic patterning of thalamocortical axons in the rat primary somatosensory cortex (Bennett-Clarke et al, 1994a); the activities of certain 5-HT receptor subtypes (5-HT\textsubscript{1C} and 5-HT\textsubscript{2C}) have been shown by \textit{in vitro} and \textit{in vivo} electrophysiological and pharmacological studies to be essential in the facilitation of the developmental plasticity necessary for the formation of ocular dominance columns in layer 4 of the kitten visual cortex (Wang et al, 1994; Gu et al, 1995; Gu and Singer, 1995) and hypothesised to be invoked through the medium of increased intracellular Ca\textsuperscript{2+} levels in turn achieved via a 5-HT-induced (binding at 5-HT\textsubscript{2} receptors in layer 4) opening of L-type
Ca\textsuperscript{2+} voltage-gated channels and/or the activation of NMDA receptors (Kojic et al, 1996).

The maturation of GABAergic cortical neurons has been shown to be dependent on the activity state of particular 5-HT receptor subtypes, as shown by \textit{in vitro} studies in neonatal rat slices (Köstner and Hornung, 1995). There is also evidence from chemical lesioning of the 5-HT innervation, that 5-HT fulfils a modulatory role which is necessary for the partial developmental refinement of excessive callosal and claustral projections to areas 17 and 18 of the kitten visual cortex (Turlejski and Djavadian, 1996). In addition, serotonin has been shown in the same way as dopamine (Rörig et al, 1995a, 1995b) to have an important role as a regulator of gap junction coupling between layer 2/3 pyramidal neurons during early postnatal development as observed by the technique of \textit{in vitro} dye coupling in slices of the neonatal rat prefrontal, frontal and somatosensory cortices (Rörig et al, 1995a, 1996).

Several studies in the rat hippocampus (Haring et al, 1995; Ogawa et al, 1995; Yan et al, 1995) and cerebral cortex (Matsukawa et al, 1995; Varela et al, 1995; Whitaker-Azmitia et al, 1995) have shown 5-HT to be important as a modulator of synaptogenesis along with either noradrenaline (Matsukawa et al, 1995) or acetylcholine (Ogawa et al, 1995). \textit{In vitro} slice preparations of the rat visual cortex have demonstrated that 5-HT inhibits the induction of long-term potentiation in layers 2/3, implying a role for 5-HT in the modulation of synaptic plasticity in early development (Edagawa et al, 1996). In rat hippocampal cell cultures, 5-HT activity as modulated via the somatodendritic 5-HT\textsubscript{1A} receptor, appears to be a major regulating factor (along with the neuronal extension factor: S100\(\beta\) and the hormone corticosterone, via its receptors) in the levels of expression of the synaptic vesicle protein: synaptophysin, particularly on surface of the neuronal cell body (Nishi et al, 1996).

In adult animals, serotonin has also been found to play an important part in the maintenance of several functional systems (reviewed in Jacobs and Azmitia, 1992), including the sleep-wake cycle, states of anxiety, attention, appetite and arousal (mediated by thalamic and brainstem nuclei) as well as being integral to the regulation of cognitive and mnemonic or limbic-associative functions (mediated through the higher association cortices: e.g. the dorsolateral prefrontal and orbitofrontal cortices, respectively).

Experimental studies have been conducted in vervet monkeys (\textit{Cercopithecus aethiops}, of both sexes, differing in their social status. In these groups it was observed that changes in social behaviour were evident for subordinate adult males who had received pharmacological manipulation of their brain 5-HT levels (reviewed in Raleigh et al, 1996a). The brain serotonin levels of these animals were found to alter chronically (over a period of 4 weeks), after treatment with tryptophan (5-HT precursor) or fluoxetine (5-HT re-uptake...
inhibitor) which increased their serotonin production and functioning, resulting in their exhibition of dominant behaviour (Raleigh et al, 1991). When these previously subordinate males were treated with fenfluramine (non-specific 5-HT agonist in acute doses, but an antagonist in chronic doses) or cyproheptadine (5-HT$_2$ antagonist) over a period of time, they did not become dominant. This difference of effect may have been due to the inhibitory actions of these chemicals acting as 5-HT$_1$ antagonists, especially in the case of fenfluramine (Muckseler and Diksic, 1996) which caused a significant decline in the animal's levels of 5-HT synthesis over time and reduced 5-HT-dependent behavioural functioning. A study in the rat (Muckseler and Diksic, 1996) has shown that fenfluramine acts at 5-HTergic terminals by increasing the rate of 5-HT release in acute doses, but with more chronic administration, the 5-HTergic neurons in the raphe nuclei - the source of 5-HT - become depleted of the neurotransmitter.

It has also been shown that an animal's social status is reflected by its levels of whole blood serotonin (WBS), with dominant monkeys having much higher levels of WBS (almost 2x greater) than subordinate animals, although this could change for an individual when its social status is reversed (Raleigh et al, 1984). Status-linked differences have also been found between animals in terms of their ability to become behaviourally affected by the administration of either tryptophan (5-HT precursor) or the 5-HT re-uptake inhibitor, fluoxetine or the 5-HT agonist, quipazine (Raleigh et al, 1985). In each case, dominant males exhibit significantly increased levels of WBS, coupled with an increase in the number of affiliative actions and decreased aggressive behaviour, than the subservient males undergoing the same treatment. These diverse behavioural and physiological observations were attributed to differences between the 5-HT systems of the 2 groups of animals, resulting in differential effects of increased 5-HT synthesis, blocking of 5-HT re-uptake sites and occupation of 5-HT$_2$A receptor sites by the actions of tryptophan, fluoxetine and quipazine, respectively. One hypothesis proposes that 5-HT$_2$A receptors might be present in higher numbers or have a increased binding-affinity for 5-HT in dominant animals.

To test this hypothesis, the levels of 5-HT$_2$A receptors (using $[^3]$H-ketanserin as a binding-ligand) were compared in various brain regions for dominant and subordinate adult males. No significant differences in the densities or affinities of 5-HT$_2$A binding sites were found for the 2 groups of animals, despite their behaviourally disparate social states (Brammer et al, 1987).

A long-term study was conducted for a group of adult male vervet monkeys whose levels of 5-HT system activity were analysed in terms of 5-HT$_2$A receptor density in the
same brain regions as the Brammer et al (1987) study, to see if there was a correlation between 5-HT$_2A$ receptor levels and the behavioural attributes of dominance or subordination for individual animals over their life-span until sacrifice (Raleigh et al, 1996a). Significantly higher mean levels of 5-HT$_2A$ receptors were found in the posterior orbitofrontal, medial prefrontal and the amygdala of animals who very seldom displayed unprovoked destructive or aggressive behaviour and vice versa. Whilst animals who demonstrated pronounced, prosocial behaviour were significantly more likely to exhibit higher levels of 5-HT$_2A$ receptors in the posterior orbitofrontal and temporal pole cortices as well as in the hippocampus compared to monkeys who didn't engage in positive behaviour patterns. Thirdly, co-operative behaviour among animals was correlated with higher values for 5-HT$_2A$ receptor binding in the posterior orbitofrontal and temporal pole cortices than animals which didn't exhibit these attributes. It is likely that the consistently increased levels of 5-HT$_2A$ receptors in the posterior orbitofrontal cortex of dominant animals plays an important contribution along with other cortical and subcortical regions and somehow confers them with an increased ability to show mutually beneficial behaviour in social engagements with other vervet monkeys.

7.7: Summary.

The monoaminergic neurotransmitter serotonin with its varied distribution both as a free transmitter in the neuropil or within its extensive network of axonal innervation in the cerebral cortex is likely to play many roles in normal cortical functioning as well as being vulnerable in disease conditions such as schizophrenia, autism and depression. The diversity of physiological and pharmacological interactions with other components of the cortical circuitry, throughout the mammalian neocortex in several species (rat, cat and monkey) may be easily attributed to the existence of the complex array of 5-HT receptor families and subtypes. These different receptors appear to be able to act as effector mechanisms in various ways depending on their cellular location (i.e. presynaptic effects at axon terminals or on en passant varicosities or postsynaptic effects at cell bodies and dendrites) enabling 5-HT to trigger-off second messenger systems and so put into operation cascades of events which ultimately bring about large-scale changes in the behavioural state of the animal, either cognitive, emotional or attentional in their nature.
8.0: Study 1. A quantitative Golgi study of the postnatal maturation of mid-layer 3 pyramidal neuron basal dendritic spines in the monkey DLPFC (areas 9 and 46).

8.1: Introduction.

We have seen from the functional and behavioural review (section 4.0) that the structural and biochemical integrity of the DLPFC in the monkey, is essential to the correct performance of important cognitive behaviours. In humans, the disruption of the DLPFC region (by head injury or disease processes) is commonly results in a profoundly cognitive dysfunction, e.g. such as that seen in schizophrenia, while motor and affective systems seem to remain relatively unaffected. This mentally-debilitating disease, usually has its onset during late adolescence (18.0 years of age) and has been considered to be a neurodevelopmental disorder, whereby the DLPFC is a major focus for the disturbance of working memory function, a hypothesis suggested by many of the symptoms exhibited in such patients. In this first study of the thesis, using the macaque monkey DLPFC as a model for the equivalent region in humans, we shall attempt to define the time course of normal development for a crucial population of pyramidal neurons situated in the mid-portion of cortical layer 3.

The review of pyramidal neurons (section 5.0) has shown that the superficial layers (2-3) of the DLPFC in the macaque monkey are known to contain large numbers of pyramidal neurons, which participate in extensive intrinsic intrareal connections. Many of these same pyramidal cells also project extrinsically to other cortical areas, either callosally to the other hemisphere or associationally within the same hemisphere. These pyramidal neurons projecting intrinsically within the DLPFC are known to possess axons with an extensive horizontal spread, which form abundant periodically-spaced collateral branches that are directed vertically upwards and possess profusely arbourising terminal branches throughout the superficial layers (1-3). This intrinsic excitatory lattice circuitry, is organised so that any given local cluster of pyramidal neurons (150-300 \( \mu \text{m} \) in width) possesses a projection field which is arranged mediolaterally as a sequence of stripe-like terminal-fields observable in tangential sections of the superficial layers following small (200-300 \( \mu \text{m} \) wide) micro-injections of anterograde tracer in layers 2/3.

The cell bodies of pyramidal neurons which furnish connectivity to individual points injected with retrograde tracer are also themselves arranged in the same regularly-spaced organisation of stripes in layers 2/3 across the cortex and the cell bodies, dendritic shafts and spines of these cells are known to be the targets of these intrinsic projections from other pyramidal neurons in the same layer. An analysis of the numbers of dendritic spines on pyramidal neurons in mid-layer 3 is likely to sample many of those pyramidal neurons...
which participate in the intrinsic lattice circuitry and an examination of the changes in relative spine complement should provide an indication of the time course of maturation for the intrinsic lattice circuitry as a whole.

Postnatal development from birth until adulthood, in all regions of the primate (including human) cerebral cortex is characterised by substantial changes in the numbers of asymmetric (excitatory) synapses which are primarily associated with the dendrites and spines of pyramidal neurons. The time course for these changes is characteristically one of initially rapid overproduction of synapses during infancy, a plateau phase when levels of synapses remain maximal until puberty - or sometimes slightly earlier in adolescence - then a period of gradual decline in numbers is observed to occur on through into adulthood. Dendritic spines could be considered to be a useful marker for the analysis of changes in the relative density of excitatory synapses on any population of pyramidal neurons at the light microscope level. While electron microscopic studies have examined the detailed changes which occur for the densities of asymmetric synapses during development, within all cortical layers in several cortical regions; the technical limitations of sampling synapses in the neuropil, rather than being able to identify the specific type of synapses associated with the dendrites of pyramidal neurons located in that same layer, inevitably means sampling populations of spines belonging to pyramidal neurons in deeper layers. For example, the sampling of synapses in layer 3 would necessarily also include spines on the apical dendrites of cells in layers 5 and 6, which very often pass through the superficial layers. It is known that individual variants of the same neuronal class, within a layer or sub-layers may exhibit slightly different time courses of development for their synaptic components, e.g. as seen for the spiny stellate cells in layers 4Cα and 4Cβ of the primary visual cortex (area 17).

The unique intrinsic excitatory lattice structure of connections within the superficial layers of the DLPFC has been hypothesised to represent an anatomical substrate essential for the particular kind of functional organisation exhibited by this cortical region. From observations in behavioural, neurophysiological and pharmacological studies, the DLPFC has been consistently implicated in the guidance of goal-directed behaviour, the temporal organisation of diverse sensory inputs and their integration with programmes of motor responses. Also, it as been found by cell recordings in the DLPFC, that neurons within area 46 appear to provide an internalised representation of the outside world, termed visuospatial working memory, whereby the mnemonic "trace" of an object's location in space (memory field) is retained "on-line" during a delay period. Ensembles of neurons have been found which share a specificity of memory-coding for a particular angle or position of a
remembered stimulus, while groups of other neurons are omnidirectional and code for the memory traces of all orientations equally. By assessing the relative changes during postnatal development for the postsynaptic sites of excitatory synapses in layer 3, presumed to be part of the intrinsic lattice connections found in the superficial layers of the DLPFC, we hoped to be able to attain a quantitative measurement, representative of the degree of functional maturation achieved by the DLPFC at each developmental stage, as implied by the levels of anatomical refinement characterised by changes in spine density.

The evaluation of the functional maturation of a region as inferred from the levels of anatomical refinement of the underlying cortical circuitry in a particular layer during postnatal development has been studied previously in primary visual cortex. The circuitry of area 17 in the monkey has been found to show changes in the density of dendritic spines and synapses in layer 3, which are in general agreement with the observations for the emergence of functional development in this region. In area 17, functional plasticity of neurons coding for both eyes is demonstrated during the early period of rapid spine acquisition on spine-bearing neurons. The disappearance of spines correlates with the final formation of alternating ocular dominance columns containing cells coding for each eye. While the emergence of the characteristically patchy distribution of clusters of terminals making up the intrinsic excitatory circuitry located in the superficial layers of this region is present prenatally, function in the superficial layers is initially plastic in the postnatal period, then matures during adolescence losing plasticity in step with spine loss.

Therefore, in the DLPFC, the maturation of intrinsic lattice circuitry, i.e. the levels of pyramidal neuron dendritic spines, should correlate with the emergence (rapid phase of synaptogenesis or spine growth) and the ability to learn to be able to correctly perform in the spatial delayed response or delayed alternation tasks. Correspondingly, the refinement (declining phase of synaptogenesis or spine loss) should correlate with the loss of the ability to significantly modify behavioural organisation in these tasks. The exceptions to the latter, are found in the cases of traumatic head injury or disease processes, when reorganisation of circuitry can occur in one region to compensate for loss of function in another. Repeated testing on these tasks provides an indication of the growth in "buffer" capacity of working memory over time, as represented by increasing the length of the delay period during which an animal can retain a "memory trace" and still provide the correct response for the reward at the end of the delay. The length of delay period over which an animal can sustain working memory function, can vary between animals according to the length of prior training received on the task or may be due to acquired genetic factors. However, the times of emergence
and refinement of this function would be expected to be dictated by the degree of maturation of the underlying cortical circuitry, particularly the intrinsic lattice connections of the superficial layers, furnished by the spine-bearing pyramidal neurons, whose development we have examined in study 1 of this thesis.

This study has been previously published as Golgi study 2 in a paper addressing the development of excitatory and inhibitory inputs onto layer 3 pyramidal neurons in prefrontal cortical areas 9 and 46 of the macaque monkey (see Anderson et al, 1995). It has also been presented in abstract form (Anderson et al, 1993, Classey et al, 1994, Anderson et al, 1995). The following Materials and Methods (8.2) and Results (9.0) sections are concerned with work undertaken by J. Classey, while the contribution by S. Anderson will be described separately in Results section 10.0.

8.2: Materials and Methods.

8.2(A). Animals.

Ten rhesus monkeys (Macaca mulatta) of both sexes and of an age range from 4 days postnatal to 7.0 years were used in this investigation (see Table 1); four of the male animals had been previously castrated, three at 14 months and one at birth (as part of another study). Animals were deeply anaesthetised with ketamine hydrochloride (25 mg/kg, intramuscularly) and pentobarbital sodium (30 mg/kg, intraperitoneally) and perfused transcardially with cold 4% paraformaldehyde in phosphate buffer (n = 6) as described previously (Lewis and Lund, 1990) or with 4% paraformaldehyde and 0.2% glutaraldehyde in phosphate buffer (n = 4). Immediately after perfusion, the brain was removed, sliced into 2-5-mm thick coronal blocks and placed in fixative. Tissue from brains perfused with the 4% paraformaldehyde/0.2% glutaraldehyde mixture were transferred to 4% paraformaldehyde several weeks before Golgi processing.

8.2(B): Golgi Study.

For each monkey, 2 mm blocks of PFC were prepared by the Golgi rapid technique (see Lund, 1973) and 90 μm coronal sections were subsequently cut for analysis. The basal dendritic trees of mid-layer 3 pyramidal neurons, in Walker's areas 9 and 46 (Walker, 1940; see Figure 26) of the dorsal lateral prefrontal cortex (DLPFC) were reconstructed and their
Table 1. Rhesus monkeys used in the 2 studies

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Age(^1)</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>Golgi study</th>
<th>5-HT study</th>
</tr>
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<tbody>
<tr>
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<td>F</td>
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<td>X</td>
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<tr>
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<td>M</td>
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<td>X</td>
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<tr>
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<tr>
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<td>M</td>
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<td></td>
<td>X</td>
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<td>M</td>
<td>N.A.(^5)</td>
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<td>X</td>
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<tr>
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<td>X</td>
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<td>X</td>
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</table>

\(^1\)d, Days; m, months; yr, years. \(^2\)Castrated at 14 months (60 weeks).
\(^3\)Castrated neonatally \(^4\)Undergone GnRH treatment. \(^5\)N.A., not available.
Figure 26 (A) A lateral view of the rhesus macaque monkey (Macaca mulatta) brain, with the shaded regions showing the locations of areas 9 and 46 (Walker, 1940; DLPFC). (B) A representative coronal section through the PFC, with the hatching indicating the limits of the sampling region within areas 9 and 46, from which mid-layer 3 pyramidal neurons from each animal were selected in the analysis of dendritic spine density, apical dendritic length and the degree of basal dendritic branching. The cross-hatched region in the dorsal bank of the PS, represents the sampling region for the PV-IR chandelier neuron axon cartridges (from Andersen et al, 1995; as compared with spine density in the Discussion section of the Spine study in the thesis). (Figure from Andersen et al, 1995). Abbreviations: AS = arcuate sulcus, CS = central sulcus, CiS = cingulate sulcus and PS = principal sulcus.
dendritic spines counted. Only pyramidal neurons located between the dorsal bank of the cingulate sulcus and the ventral bank of the principal sulcus were considered. The 10 animals used in the study were split into 5 age groups: < 4 weeks (n = 2), 10 weeks (n = 2), 1.5 years (n = 1), 2.6 years (n = 3) and > 4.5 years (n = 2).

Since the laminar boundaries in depth of areas 9 and 46 could not be easily determined directly in Golgi-impregnated material and the method of impregnating tissue blocks prevented alternate section Nissl-staining, a less direct method of establishing laminar borders was adopted. A scheme was devised, whereby the initial 30% of the cortical depth from the pial surface was hypothetically split into 3 bands of equal width (Figure 27B). Laminar divisions were then determined from Nissl-stained sections of blocks contiguous with the Golgi-reacted ones and from homologous portions of the contralateral hemisphere of each animal. It was found that the first 10% of cortical depth was equivalent to layer 1 and the next 6% to layer 2. Thus the first 10% band in the Golgi-tissue was regarded as layer 1, the second 10% band as layer 2 and upper layer 3 and the third 10% band as lower and mid-layer 3. (see Figure 28).

The population of pyramidal neurons chosen for this study were defined by the following criteria:

1. Ten layer 3 pyramidal neurons per animal from areas 9 and 46 were selected, on the basis that at least 3 of their basal dendrites came to a natural end within the extent of the 90 μm thickness of the section.
2. Each cell soma was located in mid-layer 3 (see Figure 27A).
3. The cell had a basal dendritic tree that was restricted to layer 3 (see Figure 27C).

The basal dendritic trees of each pyramidal neuron were then drawn using a 50x oil immersion objective lens and a camera lucida (drawing tube). A Sholl ring analysis was then carried out on the drawing (Sholl, 1953; see Figure 27D) whereby a transparency of 10 concentric circles spaced 20 μm apart, was placed over each drawing, centred on the cell soma. This enabled an estimation to be made of the degree of dendritic branching and the width of the dendritic arbour for each pyramidal neuron, by counting the number of dendritic intersections with each ring and estimating the distance from the cell soma to the last ring making contact with each dendrite. For each neuron 3 basal dendrites were then selected that were at least 40 degrees apart and continued to a natural ending within the confines of the tissue section. The dendritic spine density for each of these basal dendrites was then
Figure 27 Golgi studies in monkey DLPFC. (A) Bright field photomontage showing a Golgi-impregnated mid-layer 3 pyramidal neuron in the 72-day-old animal. (B) The same neuron as reconstructed by the Eutectics neuron tracing system in Golgi study 1 (Andersen et al., 1995). Three equal bands each representing 10% of the total cortical depth are indicated (as used in spine density analysis), with the laminae included within these bands: sIII = superficial layer 3, mIII = mid-layer 3. (C) A portion of the reconstructed mid-layer 3 pyramidal neuron from (A) and (B) showing the various dendritic fields for which spine densities were measured in Golgi studies 1 and 2 in Andersen et al. (1995) and study 1 of the thesis. (1) the main apical dendritic trunk and 2 side branches entering layer 1, (2) an apical oblique dendritic branch, (3) a basal dendrite (thesis study 1) and (4) indicates the total length of the apical dendritic tree. (D) For each mid-layer 3 pyramidal neuron, in study 1 of the thesis, 3 basal dendrites were analysed for spine density (per µm) as well as for the number of basal dendritic branch intersections (20 µm apart) made by the entire basal dendritic tree, using the Sholl ring method. (Figure from Andersen et al., 1995). Scale bars: (A-C) = 100 µm and (D) = 100 µm.
determined under a 100x oil objective lens by the counting spines contained within 10 µm segments every 20 µm along the dendrite, starting from the cell soma.

The objective of this investigation was to ascertain whether there were differences in the relative density of dendritic spines on mid-layer 3 pyramidal neuron basal dendrites confined to layer 3, across different age groups. There was no attempt made to determine the absolute numbers of spines on each dendrite across postnatal development. Since, equivalent portions of each dendrite were examined for every pyramidal neuron in each animal, no correction factors were applied for spines hidden behind dendritic shafts. While uncorrected spine measures obviously underestimate the absolute spine density of a dendritic population (Feldman and Peters, 1979), it has been found that both corrected and uncorrected spine counting methods provide equally good surveys of relative spine density when similar dendritic populations are being examined (Horner and Arbuthnott, 1991) as in the present study.

The rationale for conducting 2 separate studies of layer 3 pyramidal neurons in Golgi material (that by S. Anderson will be reported separately in the Results section 10.0) was due to the fact that only rarely were there found to be intact (i.e. both apical and basal dendritic trees) single pyramidal neurons in any one 90 µm thick Golgi-section. This was mainly due to the angle of cut being somewhat off that of the pia-to-white matter plane of the pyramidal neurons. It was decided to conduct separate analyses of the 2 portions of the dendritic tree of pyramidal cells and to compare the studies in terms of the relative time course for observed changes in spine density for apical and basal dendrites. To further ensure that the pyramidal neurons selected for analysis, were comparable in both cases, a second sample of basal dendritic measures were made by S. Anderson using the Eutectics method (see Results section 10.0). The use of data from two independent investigators employing different methods reduced the chances of observer and method bias in the results. It was also useful to be able to examine whether the same sequence of maturation occurred for different portions of the mid-layer 3 pyramidal neuron dendritic tree.

8.2(C). Statistical analyses.

The statistical significance of variations in Sholl ring measurements between animals of different ages was examined using a least-squares analysis of linear regression. The statistical significance of variations in relative spine density during postnatal development, was assessed using the non-parametric Kruskal-Wallis test. Animals were grouped by age and the measures were compared between age groups using the Bonferroni correction.
Figure 28 A bar plot illustrating the location by depth (as percentage of cortical pia-to-white matter distance), for each animal in age, for the cell bodies of pyramidal neurons in mid-layer 3 of areas 9 and 46. As used in apical, basal and apical oblique dendritic spine density analysis (from Golgi study 1 in Andersen et al, 1995). The plot shows that there was minimal variation between the locations of pyramidal neurons for different ages. The same rationale for selecting pyramidal neurons was adopted in the present Golgi study (study 2 in Andersen et al, 1995), with the cell bodies of pyramidal neurons, whose basal dendrite spines were counted, being located at between 31-33% of cortical depth.
Figure 29  Examples of the different morphologies observed for Golgi-impregnated mid-layer 3 pyramidal neurons in areas 9 and 46 (DLPFC) used in the spine density and Sholl branching analyses, at different ages during postnatal development.
9.0 Results.

Study 1: A quantitative Golgi study of the postnatal maturation of mid-layer 3 pyramidal neuron basal dendritic spines in the monkey DLPFC (areas 9 and 46).

This study forms the basis of Golgi study 2 in Anderson et al (1995), the contribution to that paper made by J. Classey.

9.1(A): Qualitative observations of mid-layer 3 pyramidal neurons.

As described in the Methods section 8.2, mid-layer 3 pyramidal neurons were defined as having their cell soma located within the middle third of layer 3 in areas 9 and 46, their apical dendrite ascending into layer 1 and their basal dendritic tree confined to layer 3 and not extending into layer 4 (see Figure 27). Although, all mid-layer 3 pyramidal neurons examined in this study conformed to these basic criteria, there was a degree of morphological variation between individual pyramidal neurons, both between animals of different ages, but also within mid-layer 3 of any given animal (see Figure 29A, B and C; 4 days, 1.5 and 4.6 years). There were a large variety of apical dendritic and basal dendritic tree formations found in animals at all ages: some apical dendrites ended with terminal tufts only in layer 1, whilst others branched within layer 2 and furnished several major branches up into layer 1. Basal dendritic fields were also noted for their different architectures, with some pyramidal neurons possessing primarily downwardly-oriented dendrites, many had predominantly laterally-spreading dendrites, whilst others had a more uniformly distributed basal dendritic field.

9.1(B): Quantitative observations of mid-layer 3 pyramidal neurons.

9.1(B):(i). Changes in length and degree of branching for basal dendrites across postnatal development.

Using the Sholl ring analysis (see Methods 8.2), the mean number of dendritic ring intersections was measured for the basal dendritic fields of mid-layer 3 pyramidal neurons for cells in animals from 7 of the 10 ages (not the 22 day, 67 day or 2.5 year-old animals) employed in the study (see Table 1). There was found to be no significant difference in the mean number of dendritic intersections for the basal dendritic trees of mid-layer 3 pyramidal neurons at the various stages of postnatal development (see Figure 31B and Table 2, for raw data). Using a least sum-of-squares form of linear regression analysis, there was also found to be no significant difference between the mean total numbers of Sholl intersections for pyramidal neurons in each animal across the course of postnatal development.
Table 2. Sholl plots, i.e. number of intersections made by basal dendrites crossing each part of a circular grid aligned on the centre of the cell soma as shown for each animal age.

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Figure 30 A chart showing the stable relationship with age (for 7 out of the 10 animals used in the study), for the mean extent of dendritic branching at various distances from the cell soma for the basal dendritic fields of mid-layer 3 pyramidal neurons during postnatal development in areas 9 and 46 (DLPFC).
Figure 31 Measurements of dendritic length and dendritic branching. (A) Mean total apical dendritic length (in µm) for the mid-layer 3 pyramidal neuron sample used for the spine density analysis in Golgi study 1 of Andersen et al (1995), arranged in 5 age groups. (B) Mean number of Sholl ring intersections made by the basal dendritic fields of the mid-layer 3 pyramidal neuron sample - for 7 of the 10 animals - used in Golgi study 2 of Andersen et al (1995) - i.e. study 1 in the thesis. Standard errors indicated. N.B. the absence of an effect of age on apical dendritic length or the number of basal dendritic branches for mid-layer 3 pyramidal neurons. (From Andersen et al, 1995).
Figure 32. Bright-field photomicrographs illustrating the varied morphology of dendritic spines (indicated by arrow-heads), located on the basal dendrites of pyramidal neurons within mid-layer 3 of areas 9 and 46, in animals of various ages: (A) 4 days, (B) 78 days and (C) 7.0 years.
The distribution of dendritic branches around cell soma was found to be relatively invariant across development, in terms of their pattern, order of branching and their individual lengths. There were no statistically significant differences between animals, in terms of the dendritic length (see Figure 30), the mean number of basal dendritic branches or mean diameter of basal dendritic fields (see Figure 31B) for mid-layer 3 pyramidal neurons during the entire period of postnatal development between birth and 7.0 years of age, i.e. adulthood.

9.1(B),(ii): Changes in relative spine density for basal dendrites during postnatal development.

Regarding the spine density analysis conducted for selected basal dendrites of mid-layer 3 pyramidal neurons (see Figures 33A, B and Table 3 for raw data), values were initially found to be at low levels in the early neonatal animal (4 days of age) and were only slightly higher in the 22 day-old animal. Between 22 days and 10 weeks of age (in the 67 and 72 day-old animals) a dramatic rise in spine density was observed with an approximately 75% increase in values over this period of postnatal development. This high level of spine density for basal dendrites remained at a plateau until a 41% decrease occurred sometime between the animals of 2.5 and 2.6 years of age, bringing spine density values back down to levels close to that seen in the 22 day-old animal. This low level of spine density was maintained with a slight degree of variation from adolescence into adulthood (as seen for the 2.7, 4.6, 5.7 and 7.0 year-old animals).

The spine density data from the 10 animals examined, was originally grouped into 5 age groups in Anderson et al (1995), which when applied in this thesis using the non-parametric Kruskal-Wallis test as opposed to the parametric ANOVA test used in the original paper, resulted in a finding of no significant difference between the 5 age groups during postnatal development. However when the animals were grouped into just 2 age groups (4 days-22 days/4.6 yrs-7.0 yrs: n = 4 and 67 days-2.8 yrs: n = 6) for this thesis, on the basis of the similarity in values for the youngest and oldest animals and again the Kruskal-Wallis test was applied, a significant level (asymptotic) of $P < 0.055$, was found between the 2 groups of animals. That is between the youngest and mid-range animals and also between the oldest and mid-range animals. This was further confirmed by the use of the Mann-Whitney U test, which in addition to providing a 2-tailed (asymptotic) significance level of $P < 0.055$, also gave an exact significance level of $P < 0.067$. By grouping the results for the 10 animals used in the study in various ways, in terms of their ages, it was possible to find even more significant changes occurring for spine density during postnatal development. Maximally significant
Table 3. Mean spine density for basal dendrites of layer 3 pyramidal neurons.

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Figure 33 Mean relative spine density (per μm) on basal dendrites of mid-layer 3 pyramidal neurons (Golgi study 2 in Andersen et al (1995). (A) histogram showing results for individual animals on a proportional logarithmic time scale and (B) bar plot showing the results plotted in 5 age groups used for Golgi studies 1 and 2 in Andersen et al (1995). Standard errors indicated. (From Andersen et al, 1995).
differences were found to occur between the age groups containing the 4 youngest (4 days-72 days), 4 mid-age (1.5 yrs-2.8 yrs) and the 2 oldest (4.6 yrs and 7.0 yrs) animals: (asymptotic) significance, $P < 0.036$, with the strongest difference between the youngest and mid-age animals ($P < 0.001$) and equally when grouped together as the 2 youngest (4 days and 22 days), 4 mid-age (67 days-2.5 yrs) and 4 oldest (2.7 yrs-7.0 yrs) animals: (asymptotic) significance, $P < 0.036$, with the strongest difference between the mid-age and oldest animals ($P < 0.001$).

There were also significant variations in absolute spine density between proximal, medial or distal portions of basal dendrites (graph not shown, but see raw data in Table 3) the highest density occurring on the medial segment. However the relative differences in spine density along the extent of single dendrites were very similar at different ages, so there was not found to be a statistically significant age-related change in the overall mean proximal-to-distal distribution of spines along basal dendrites.


The time course of postnatal development for basal dendritic spines on pyramidal neurons in mid-layer 3 of areas 9 and 46, although showing a broadly-defined pattern of rise, plateau (stable values) and decline, only the stages of rising and falling spine density could be substantiated by statistical means, due to the small numbers of animals used in this study.

10.0: S. Anderson contribution to Anderson et al, 1995, (i.e. Golgi study 1).

10.1: The postnatal maturation of apical dendritic spines of mid-layer 3 pyramidal neurons in the monkey DLPFC.

The above results (Golgi study 2) have been published as part of an investigation (Anderson et al, 1995), which also included an independent analysis (by S. Anderson) of spine density on the apical dendrites (Golgi study 1) of PFC layer 3 pyramidal cells. Many cells were analysed from the same sections from the same animals in both analyses (but systematic dual analysis of the same neurons was not possible for reasons stated previously; see Methods 8.2 for details). The developmental time course for apical dendritic spine maturation showed a strong correlation with that seen for the basal dendritic spines seen in this thesis study.

A quantitative analysis was carried out for spine density on the apical dendrites of a comparable population of mid-layer 3 pyramidal neurons in area 9 and 46 of animals of various ages, several being the same animals used in the basal dendritic spine analysis. The
rationale for 2 comparable studies using different methods is discussed in the Methods section 8.2. Like the findings of changes in spine density across postnatal development for basal dendrites, apical dendrites showed a rapid rise in values within animals ranging from birth until 10 weeks of age when they reached their maximum and remained at this high density (plateau period) until at least within the 1.5 year-old animals (see Figure 56A in the appendix). Beyond this age, spine density dropped below the maximal level for the animal of 2.6 years of age and appeared to decrease steadily, initially reaching lowest values in the 4.6 year-old animal, when stable mature adult values were first observed. This mature level of apical dendritic spine density remained relatively constant throughout the rest of adult life, until at least 15.9 years of age (the oldest animal for which spine density was assessed in the apical dendritic analysis).

Statistically significant differences were found (see Figure 56B in the appendix) for the apical dendritic spine density of mid-layer 3 pyramidal neurons (F = 65.5, P < 0.0001). Specifically, there was a rise in spine density between the < 3-(0.49±0.02) and 10-week-(0.75 ±0.02) old age groups, a decrease between the 1.5-(0.74 ±0.01) and 2.6-year-(0.63 ±0.02) old age groups. Also there were reductions between the 10-week (0.75 ±0.02) and > 4.5-year-(0.45 ±0.01) old and between the 1.5-(0.74 ±0.01) and > 4.5-year-(0.45 ±0.01) old age groups. All differences were observed at a significance level of P < 0.05.

Also, differences were observed in the distribution of spines on the proximal, medial and distal portions of apical dendrites, corresponding with the ascending passage of the apical dendrite through-mid-layer 3, superficial layer 3 to layer 2 and layer 1, respectively (see Figure 57A, B and C in the appendix). The proximal portion of the apical dendrite, corresponding to a 10% band of cortical depth, from the soma in mid-layer 3 to the base of superficial layer 3, was shown to consistently possess the greatest absolute values for spine density during development; whilst the distal portion situated in layer 1, always demonstrated the smallest contribution to the overall spine density of apical dendrites at all ages. The medial portions of apical dendrites in layers 2 and superficial 3, initially exhibited high absolute levels of spine density (10 weeks age group), comparable to those for the proximal portions, but in the adult animals (> 4.5 years age group) the levels of spine density were much lower than the equivalent for the proximal dendritic portions.

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10.2: Comparison of postnatal time courses of maturation for changes in basal dendritic spine density in Golgi study 1 (J. Classey's work) with those in Golgi study 2 (S. Anderson's work from Anderson et al, 1995).

Changes in spine density for basal dendrites in Golgi study 1 were comparable to those seen for the basal dendritic analysis of Golgi study 2 with a similar time course and significant differences (study 1: P < 0.0001; study 2: P < 0.055) between almost identical age groups, but with lower absolute values for spine density in Golgi study 1 (compare figures 33A and B with Figure 58B in the appendix), due to the different sampling techniques employed for analysing spine density.

10.3. Summary: The postnatal time courses of maturation for pyramidal neuron basal and apical dendritic spines.

Over the course of postnatal development, apical dendritic spine density had much lower values than those observed for the basal dendritic spine analysis (e.g. 0.8 compared to 1.5 spines per μm at peak levels in the 72-day-old animal). Several factors account for this discrepancy, firstly the differences in the neuron reconstruction employed in each study (and different levels of magnification used) and secondly the variation in the methods of quantitative analysis. The basal dendritic spine analysis omitted the most proximal (0-10 μm) and very distal (mean = 10 μm) portions of the dendrites where spine densities are lower than for the rest of the dendrite. Despite these differences in methods and hence the absolute spine density values for the basal and apical dendrites of mid-layer 3 pyramidal neurons, the age-related changes in relative spine density with which we are primarily concerned were remarkably similar for both apical and basal dendrites of the mid-layer 3 pyramidal cells across the entire period of postnatal development.

11.0: Discussion.

Study 1: A quantitative Golgi study of the postnatal maturation of mid-layer 3 pyramidal neuron basal dendritic spines.

11.1: Introduction.

It is concluded that all dendritic compartments of mid-layer 3 pyramidal neurons in areas 9 and 46 share a generally concurrent pattern of refinement for the acquisition and loss of their spines, the sites of potential excitatory postsynaptic inputs, during the postnatal period (birth to 7.0 years of age, see extrapolated curve in Figure 34A). The only exception to this general rule was the variation between proximal and distal portions of both apical
Figure 34. Schematic summary curves illustrating the percentage of maximum and minimum values for spine density on the dendrites of mid-layer 3 pyramidal neurons during postnatal development, with a comparison to the density of chandelier neuron axon cartridges in deep layer 3 which exhibit PV-IR (data from Anderson et al., 1995). (A) Spine density (per μm), (B) Spine density (per μm) and PV-IR chandelier neuron axon cartridges (per μm²).
and basal dendrites in terms of their spine density. In the case of the apical dendrites (in S. Anderson’s work), this was manifested in terms of a more protracted plateau phase and a later refinement of maximal spine numbers for the proximal dendritic portions (decline between 10 weeks and 1.5 years age groups) situated in mid-layer 3 (see Figure 58C in the appendix) relative to the remainder of the apical dendrite located more superficially in layers upper 3, 2 or 1 (see Figure 58A and B in the appendix). The latter portions followed the general postnatal pattern of basal dendritic spine maturation (decline between 1.5 and 2.6 years age groups).

These changes in the relative density of dendritic spines located on a particular population of pyramidal neurons in mid-layer 3 of the DLPFC during postnatal development, may be related to the maturational time course of the unique intrinsic lattice circuitry which exists in the superficial layers of this region, including mid-layer 3 - where the majority of pyramidal neurons furnishing this circuitry are found to be located (Kritzer and Goldman-Rakic, 1995; Pucak et al, 1996. The postnatal refinement during puberty of the width of intrinsic lattice stripes in the superficial layers of areas 9 and 46, formed by the distribution of clusters of anterogradely-labelled axons and terminals been reported in a preliminary study (Woo et al 1997). There appeared to be a significant difference (30% > in size) in the both the length and width of stripes in pre-pubertal compared to young adult monkeys, when tangential reconstruction’s were made of axons in layers 1 and 3. In addition, a 50% loss in the numbers of varicosities and branch points of the pyramidal neurons axons was observed between the 2 age groups, demonstrating that significant changes in the intrinsic lattice circuitry of the DLPFC occurs during puberty, when overall numbers of axospinous synapses primarily associated with pyramidal neurons, are also declining.

11.2: Comparison of changes in dendritic spine density with other developmental events in layer 3:

11.2(A): Comparison with the time course of changes in density of parvalbumin immunoreactive (PV-IR) axon cartridges.

The time course of postnatal developmental changes in the inhibitory axon cartridges associated with GABAergic chandelier neurons as demonstrated by the density of PV-IR axon cartridges in mid-layer 3 can be compared to the changes in pyramidal neuron spine density in the same layer and region. These axon cartridges are constructed of a series of PV-IR varicosities forming a elongated columnar array below the unlabelled outlines of pyramidal neuron cell bodies (DeFelipe et al, 1989a; Akil and Lewis, 1992b). Comparative
imunohistochemical and Golgi studies in area 46 of monkey prefrontal cortex (Lewis and Lund 1990; Lund and Lewis, 1993) have identified these PV-IR cartridges to be the terminal portions of the axon arbours belonging to the chandelier class of inhibitory local circuit interneuron, as earlier described (Somogyi, 1977, 1979; Somogyi et al, 1982; DeFelipe et al, 1985, 1989a) to terminate on the initial axon segments of pyramidal neurons in other regions of rat, cat and monkey cerebral cortex. When observed at EM level in area 46 (Williams et al, 1992), these PV-IR axon cartridges can be seen to form symmetric (inhibitory) synapses with the axon initial segments of pyramidal neurons.

A quantitative analysis was conducted of the developmental changes in the density of PV-IR axon cartridges in the deep half of layer 3 in area 46 for a set of animals encompassing a similar age range (2 days-5.7 years) to that used in the dendritic spine study. A pattern of changes in density of PV-IR axon cartridges occurred that showed a rapid rise in numbers between the animals of 22 days and 10 weeks of age, a plateau phase present in 10-week- an 1.5-year-old animals, then a period of dramatic decline in density for the animals aged 1.5 years and 2.6 years of age and an almost entire loss of cartridges within deep layer 3 by the stage of adulthood represented in the 5.7-year-old animal.

The time course of changes in the density of chandelier neuron PV-IR axon cartridges during postnatal development, appears to follow the same time course of rise and fall (see Figure 34B) as that for spine density changes on their postsynaptic pyramidal cells (Anderson et al, 1995).

These events may reflect an increased activity by inhibitory inputs onto pyramidal neurons during the period when they concurrently possess elevated levels of excitatory synapses contacting their dendritic spines. That is mid-layer 3 pyramidal neurons may require greater regulatory control, by way of an increased level of release of the inhibitory modulatory neurotransmitter GABA, perhaps in order to balance the increased number of excitatory synapses. As a result, this may trigger the increasing of levels of PV, in a compensatory response to the overall increase in neuronal activity, whereby this specific calcium-binding protein can fulfil its neuroprotective role, acting to prevent - by intracellular buffering and ion transport - levels of calcium ions from rising to neurotoxic levels (Celio, 1986, 1990; reviewed by Rogers, 1989). This hypothesis (and the second one below) has been cited as possible explanations for the increased levels of PV-IR observed in layer 3 chandelier axon cartridges - known to terminate on pyramidal neuron AIS's - during the early postnatal period in DLPFC; showing a time course concurrent with that for an increase in the numbers of layer 3 pyramidal neuron dendritic spines - the sites of excitatory
synapses (Anderson et al, 1995). Likewise, both these components of DLPFC circuitry show parallel time courses during puberty when they decrease, in the case of PV-IR to levels below detectability and in the case of dendritic spines down to a level as low as that seen in the neonatal animal (Anderson et al, 1995).

11.2(B): Comparison with the postnatal maturation of dopaminergic (DAergic) axon varicosities in deep layer 3.

The density of TH-immunoreactive (IR) axons and varicosities in deep layer 3 of area 46 in the monkey prefrontal cortex has been observed to change markedly during the course of postnatal development (Rosenberg and Lewis, 1995). TH-IR and DAergic axons are distributed in a bilaminar pattern of innervation in area 46 at all ages with the highest density in layers 1-superficial 3 and a moderate density in layers 5 and 6 (Lewis et al, 1988; Williams and Goldman-Rakic, 1993). TH-IR and DAergic axons have been shown at EM level to primarily form symmetric (inhibitory) synapses with the somata and distal dendritic spines and shafts of pyramidal neurons in superficial layers of area 46 in the adult monkey prefrontal cortex (Goldman-Rakic et al, 1989; Smiley and Goldman-Rakic, 1993).

These observations have lead to the conclusion that there is likely to be close a functional relationship between the DAergic innervation and pyramidal neurons in layer 3 of the adult monkey DLPFC (Goldman-Rakic et al, 1989; Smiley and Goldman-Rakic, 1993). Correspondingly, during postnatal development it would not be surprising if there was a close relation between the time courses of maturation for pyramidal neuron basal dendritic spines and DAergic axon varicosities in layer 3 of the DLPFC.

However, a significant minority of symmetric synapses made by TH-IR varicosities have been observed at EM level associated with the distal dendrites of GABAergic interneurons in the superficial layers of lateral area 9 in the adult monkey DLPFC (Sesack et al, 1995b) which have been confirmed not to belong to the calretinin subclass of interneurons (Sesack et al, 1995a). Also, DAergic symmetric synaptic contacts have been found on the smooth, synaptically populous dendrites of presumed GABAergic interneurons (Smiley and Goldman-Rakic, 1993) in area 46 of adult monkey prefrontal cortex. Another recent EM-immunohistochemical double-labelling study of area 9 in monkey DLPFC (Lewis et al, 1996) has confirmed that TH-IR varicosities form close appositions with the PV-IR dendrites of either or both presumed chandelier or wide arbour "basket" neurons. One third of these contacts were concluded to be symmetric synapses, whilst other synapses were observed onto unlabelled dendrites in close proximity to PV-IR dendrites.
Figure 35. Schematic summary curves illustrating the maximum-minimum range of values for spine density (per μm) on mid-layer 3 pyramidal neurons, compared to the varicosity density (per 5000 μm²) for DAergic axons in deep layer 3 (data from Rosenberg and Lewis, 1995).
The time course for the changes in the varicosity density (per 5000 μm²) associated with DAergic axons in deep layer 3 of area 46 (from Rosenberg and Lewis, 1995) during postnatal development showed a pattern of progression which began with a small rise and plateau between birth and around 10 weeks of age, before rising considerably between 10 weeks and 2.0 years of age to reach a peak which was maintained between 2.0-3.0 years of age, before rapidly declining until a low stable level was achieved in the adult animals. The highest levels of DAergic varicosities for those animals aged between 2.0-3.0 years was found to be statistically significant compared to the levels in younger and older animals.

The time course of postnatal maturation for DAergic axon varicosities appeared to show a complex relationship (see Figure 35) to that for pyramidal neuron dendritic spines in layer 3 of DLPFC (Anderson et al, 1995). Firstly, between birth and approximately 10 weeks (or 2 months) of age both cortical components exhibited a rapid parallel increase in numbers, which in the case of the DAergic varicosities slowed to a less dramatic rate of growth once the spines had reached their maximum levels. High levels of spines were maintained between 10 weeks and 1.5 years of age before declining; prior to this decrease the levels of DAergic varicosities began to climb markedly once more, reaching their peak at around or just after the initiation of the down-turn in spine numbers has occurred. The levels of DAergic varicosities remained at peak values between 2.0-3.0 years, the same period during which the numbers of spines was observed to fall significantly, only once the levels of spines appeared to be slowing in their rate of decline did the numbers of DAergic varicosities begin to fall as well, so that both cellular components achieved a stable mature circuitry at about the same time in the adult monkey.

It is pertinent to mention here, that the time period over which the refinement of pyramidal neuron dendritic spines occurs, i.e. during puberty (around 3.0 years of age in the monkey) is likely to mark the crucial developmental stage for the emergence of the mature adult intrinsic lattice circuitry furnished by pyramidal neurons in the superficial layers of the DLPFC. This stage of development may be regarded as the "critical period" for DLPFC anatomical circuitry and the maturation of cardinal functions (i.e. working memory), comparable to that found in the monkey primary visual cortex and would hence be equally vulnerable to both experimental perturbation or to complex mechanisms of disease, in this case schizophrenia.
11.2(C) Comparison with the time course of maturation in asymmetric synaptic density in layer 3 of the DLPFC.

As described in detail in the pyramidal neuron review (section 5.8), a study has been conducted of the changes in density of synapses occurring in the different layers of area 46 during postnatal development in the monkey DLPFC (Bourgeois et al, 1994). A similar pattern of changes was observed in both the present thesis study and the Bourgeois et al (1994) study (see Figures 23A-C in section 5.7), with an initially rapid rise in spines or asymmetric (excitatory) synapses observed during the first 2 months or so of postnatal development, followed by a plateau period of high synaptic or spine density between 2 months and 1.5-2.5 years (spines) or 3.0 years (synapses) and finally a phase exhibiting either a gradual decline in the numbers of synapses from puberty through into adulthood (Bourgeois et al, 1994) or in the case of spines (present thesis study) a steady decline between 1.5 years of age (adolescence) and 4.6 years of age (early adulthood). The major difference between the time course of decline for spines and synapses shown by this present thesis study is that the period of decline begins earlier for the dendritic spines known to be located specifically on layer 3 pyramidal neurons than it appears to do for synapses generally located in the neuropil of layer 3 (Bourgeois et al, 1994), with no knowledge of the location of their parent cells.

The observation that synapses in layer 6 of the DLPFC did not show the same pattern of overshoot and decline in density as synapses in the superficial layers during postnatal development (Bourgeois et al, 1994), means that those synapses associated with the spines of apical dendrites passing through layer 3 which originate from layer 6 cells will contribute to the overall synaptic milieu sampled in that layer and provide an inaccurate representation of the changes occurring for pyramidal neurons whose cell body is actually present in layer 3. The majority of layer 3 pyramidal neurons are involved in providing and receiving corticocortical and corticothalamic (deep layer 3)/thalamocortical projections or they participate in intrinsic lattice connections, whereas layer 5 pyramidal neurons primarily project to sub-cortical targets such as the thalamus, superior colliculus and pulvinar etc, although they contribute 20% or more of corticocortical pathways and receive afferent input from other cortical areas. The differences between the projection targets and presumably in the functional influence of these 2 populations of pyramidal neurons, whose cell bodies and principal axon origins are located in different cortical layers, invalidates any suggestion that cell populations in all cortical layers show a concurrent time course in the maturation of their asymmetric synapses or dendritic spines. These considerations are found to be especially true, if no account has been taken of the fact that the different dendritic portions of the same
cell located in different cortical layers are likely to exhibit the same common patterns of change in the levels of their postsynaptic sites and synaptic contacts during development, irrespective of their laminar location. The only exception to this observation, seen in the work related to the current thesis study (contribution of S. Anderson) was for the different portions of the apical dendrites of layer 3 pyramidal neurons at their various levels in layers 1-3. The proximal portions of these dendrites demonstrated a slightly later attainment of their peak levels of spine density than the middle and more distal portions, thus the exact timing of the initial rise was varied, but there was a similar overall pattern of change in the amplitude of changes and a concurrence in the timing of the period of decline in spine density for all 3 portions of these apical dendrites.

The above implications along with the evidence for changes in spine density specific to layer 3 pyramidal neurons in this thesis study, show that various sub-populations of pyramidal neurons in different layers may exhibit distinct time courses of maturation both in terms of their levels of dendritic spines and asymmetric synapses during postnatal development. These changes in circuitry might correlate closely with the particular stages of emergence, consolidation and refinement of functional behaviours unique to the DLPFC, during the course of normal development in the macaque monkey. In humans, the same processes have been hypothesised to take place - albeit over a longer time scale - at the equivalent stages of postnatal development and the disruption of any one of these maturational stages may be an important clue to the underlying causes of the acute, florid symptoms (hallucinations, delusions and working memory deficits) of schizophrenia typically emerging in the late teenage years.

11.2(D) Other aspects of developing circuitry which may be relevant to the maturation of pyramidal neuron dendritic spines and the intrinsic lattice circuitry.

Extrapolation of the observations concerning dynamic biochemical changes occurring in dendritic spines during development and in adulthood (see Section 5.9) to the present study 1 of the thesis enables us to predict that similar phenomena may occur in the monkey DLPFC for the dendritic spines of mid-layer 3 pyramidal neurons. The 2 major forms of synaptic plasticity, i.e. long-term potentiation (LTP) and long-term depression (LTD) are triggered respectively by the opposed effects of Ca\(^{2+}\) ions flowing through NMDA activated glutamate receptor channels. LTP is thought to result from high-frequency stimulation facilitating high levels of postsynaptic NMDA receptor activation, while LTD is modulated by
low-frequency stimulation, e.g. resulting in low levels of postsynaptic NMDA receptor activation (Kirkwood et al, 1996). It is possible that the ongoing process of LTP could account for the rapid growth of spine numbers during early postnatal development (birth to 2-4 months postnatal), while during adolescence (2.5-3.0 years) and puberty (3.0-3.5 years) the conversely-acting process of LTD could provide a mechanism for synaptic activity to be "toned down" as a signal for individual excess spines and their postsynaptic contacts to be selectively detached from one another and retracted into their parent dendritic or axonal processes or perhaps engulfed by nearby glial cells. Spines and presynaptic processes which are either genetically-programmed to remain synaptically-active or which through a process of competitive inhibition via multicellular interactions (i.e. GABAergic neurons modulating pyramidal neurons and DA and 5-HT modulating both cell types) are not removed, are as a result incorporated into the mature adult cortical circuitry.

Since NMDAR1 and AMPA, AMPA/kainate and kainate receptor subunits have all been localised to various portions of pyramidal neurons in the monkey DLPFC as well as other cortical regions (see Section 5.10). It is likely that the activity state of these receptors plays an important role in enabling synaptic plasticity via changes in the density of dendritic spines, to occur during early development as well as in the normal mediation of excitatory neurotransmission between individual pyramidal neurons and their feedback influence on GABAergic interneurons (which also differentially express these glutamate receptors; see section 5.10). Observations of the involvement of NMDA receptors and non-NMDA receptors in visual plasticity in the cat and rat visual cortex provides a useful indication of what may occur in the monkey visual cortex and perhaps by a similar electrobiochemical mechanism, but with a different form of behavioural correlation (i.e. cognitive rather than sensory processing) in the monkey DLPFC.

Thus in the monkey DLPFC, plasticity of function is observed in the period of rapid synaptogenetic growth in the first 2 postnatal months and has been found to be accompanied by the ability to learn the delayed response (DR) task (Kubota, 1994). After 2 months of daily training beginning at 2 months of age, normal 4 month-old monkeys could perform with a 3 second delay on the DR task. This period of learning ability has been demonstrated to continue until around 2.5-3.0 years of age, when lesions of the DLPFC are first shown to produce profound deficits on DR task performance (reviewed in Goldman, 1972), which cannot be overcome even despite intensive retraining attempts. Infant or juvenile monkeys receiving DLPFC lesions seem to exhibit functional plasticity (Goldman, 1971) showing an initial deficit on the task, which with learning can be almost entirely reversed, so that in
adulthood they perform as well as do control unlesioned animals on a 10 second delay version of the DR task. Even lesions inflicted prenatally in monkeys (Goldman and Galkin, 1978), do not seem to prevent the emergence of DLPFC functions at their normal time in early postnatal development and their subsequent maturation at puberty, albeit in a region of cortex adjacent to that ablated.

It would appear that this contiguous region of cortex is modified at the cellular and connectional level - see section 5.6(C), to enable the appropriate cellular processes of anatomical and functional development to occur, including re-routing of thalamic pathways, which would seem to be important to the success of this functional sparing. Reversible cryogenic lesioning used in infant, juvenile and peri-pubertal monkeys (Alexander and Goldman, 1978), produces significant deficits (25% more errors) on DR task performance in the animals of around 3.0 years of age (puberty), compared to only minimal effects (7-8% more errors) in the 1.5-2.0 year-old animals, while there was no observable disruption of normal levels of correct responses for the youngest animals (9 months-1.5 years of age). These cooling experiments support the hypothesis that the maturation of DLPFC function is a gradual process, which does not begin to reach its full potential until around puberty (or between 3.0-4.0 years of age), although clearly the functional ability to learn tasks is present from early in the first few months of life, but there appears to be a requirement of 2.5 years of behavioural experience and anatomical refinement before the mature functional level of performance is possible.

The observation in this thesis study that levels of dendritic spines associated with pyramidal neurons in layer 3, begin to decline in their numbers earlier in development (from 1.5 years) than the asymmetric synapses (3.0 years), for which they are the postsynaptic contacts, may correlate with the findings of the behavioural studies that a certain level of functional maturation is detectable during early adolescence (1.5-2.0 years), prior to full functional maturation after 3.0 years of age, which would coincide with the start of the decrease in synaptic density in all layers at this time. Thus developmental events occurring in layer 3 may play an especially important role in this initial phase of the functional maturational sequence of the DLPFC and act to lay the anatomical framework of the intrinsic lattice circuitry (see section 8.1) which will eventually become the substrate for the mature diversity of fully-fledged DLPFC functions.

In summary, the functional and behavioural maturation of the primate DLPFC would appear to closely follow certain aspects of anatomical development including the formation, stability and loss of asymmetric synapses and also the time course of pyramidal neuron
dendritic spine growth, plateau and decline in layer 3, the site of intrinsic lattice connections, the maturation of which is thought to be instrumental in the fulfilment of normal adult functional ability in the monkey DLPFC. If a similar process of overproduction, consolidation and refinement of the excitatory pyramidal neuron circuitry occurs in the human DLPFC, then disruption of these process, either prenatally and/or postnatally, would be expected to have profound effects on the subsequent ability of the individual concerned to utilise the diverse aspects of DLPFC function such as involvement in behavioural patterns, requiring the "holding in mind" ability of working memory, multisensory integration with cognitive and motor schedules, goal-direction and planning of the future. All these functions are often found in one form or another to be disrupted in schizophrenia, in patients with lesions of the frontal lobe and to a lesser and more variable degree in subjects suffering Parkinson's disease, Alzheimer's disease, epilepsy and autism.

The synaptotrophic activity of steroid hormones as observed in rat hippocampal cell cultures (see section 5.9), may play a similar role in the initiation and/or maintenance of the rapid increase in spine numbers observed during neonatal development in the monkey DLPFC, when the mother's hormones (having crossed via the placenta prior to birth) are likely to be still active and still of major influence to the immature offspring. In later development when the maturation and refinement of dendritic spine complement is occurring on cortical pyramidal neurons, the reverse effect might come into play by means of the sexual maturation of the animal occurring concurrently with spine loss, when the levels of different intrinsic hormones are in a state of change, so that the most prevalent hormone would act with deleterious effects on spine density together with NMDA (LTD) mechanisms. There is also evidence to support hormonally controlled up-regulation of synapse numbers in adult animals (see section 5.9) as demonstrated by changes in sizes and types of presynaptic axon varicosity and axospinous synaptic interactions in the hippocampus of ovariectomised female rats.

As mentioned in Table 1 in the Methods section 6.0, several of the monkeys used in the study had previously undergone castrations for use in unrelated endocrinological studies. Examination of the data for the Spine study, showed that these animals did not appear to exhibit levels of spine density, dendritic length or degree of branching, outside the normal range of values either for the basal (thesis study) or apical (S.A. Andersen contribution) dendrites of layer 3 pyramidal neurons. For example, the castrated animals in the 2.6 years and 4.5 years age groups (see Figures 33A and B), possessed relative mean spine densities which were well within the overall range of values for the other animals in those age
Therefore, we draw the conclusion from these observations, that there is little evidence to suggest that gonadal hormones have a significant influence over the changes in spine density which we have observed for layer 3 pyramidal neurons in the monkey DLPFC in this study. However, in view of the findings of sex differences for several aspects of development in the literature for both rodents and monkeys, larger samples of animals and a comparison of values for both sexes across postnatal development (which was not possible in this thesis study, due to lack of age-matched males and females) need to be conducted before this issue can be resolved satisfactorily.

11.3: Final conclusions.

Thesis study 1, has examined the time course of development for dendritic spines on a population of pyramidal neurons in mid-layer 3 of the monkey DLPFC, as part of a wish to define the parameters of anatomical and presumably functional maturation in this important cortical region in primates. The study's findings indicate that the sequence of spine growth, stability and decline is intimately related to the presence of high densities and rapid acquisition of spines in the infant animal, consolidation of these high levels in the juvenile stage followed by a steady loss of spines from adolescence and through puberty, so by early adulthood low numbers of spines characterise the anatomical structure of the mature primate DLPFC.

Also, it was shown that the time course of changes in spines closely follows that for one form of inhibitory input that is known to contact pyramidal neurons in this layer, namely PV-IR chandelier neuron axon cartridges; whose levels of immunoreactivity for PV follow almost exactly the same amplitude and time frame of development as that for the density of dendritic spines.

The maturational development of pyramidal neuron dendritic spines in mid-layer 3 of the DLPFC was compared with the postnatal development of DAergic axons and varicosities in the same layer and a striking correlation was found to be shown by both markers. There appeared to be an intimate relation between the period of greatest rate of spine growth and raised DAergic activity (indicated by moderately increased levels of varicosities and axons between birth and 10 weeks of age). The rise in the density of the DAergic innervation in layer 3, continued during the period of highest spine density and then just prior to the decline in spine number, there was another rapid climb in the levels of DAergic axons and varicosities, whose peak levels coincided with the loss of spines and DAergic axons only decreased in density once spine number had matured to low adult levels.

Finally, we compared the time courses for dendritic spines and asymmetric
synapses in layer 3 and found that there was a coincidence for both markers of pyramidal
neuron maturation in the first half of development, i.e. from birth to about 1.5 years, whereby
both exhibited a rapid increase in their numbers in the first 2 postnatal months or so, which
was maintained for spines until 1.5 years of age and for the synapses until 3.0 years of age.
Since, the measures for excitatory synapses were not strictly limited to pyramidal neurons,
nor to those with their cell bodies in layer 3 (layer 5 apical dendrites included), while spine
density measures were restricted on both these counts, this could help explain the discrepancy
between the 2 closely-linked quantities during the second half of their developmental time
courses, although there might also be a genuine time-lag in the shedding of excitatory
synapses compared to that for their postsynaptic sites on pyramidal neuron dendrites.

Therefore, Study 1 of this thesis has demonstrated by comparison with other data in
the literature, for the same layer of the same region, that pyramidal neuron dendritic spine
development in layer 3 during the postnatal period in the monkey, is closely related to the
development of PV-IR inhibitory contacts on the same pyramidal neurons and also to
DAergic axons which are known to contact both pyramidal neuron dendritic spines and shafts
as well as PV-IR (GABAergic) interneurons in adult monkeys, whose axons terminate in the
PV-IR axon cartridges which contact the axon initial segments of pyramidal neurons - again
in adult monkeys. These closely related time courses are not surprising in view of the
apparent synaptic relationships between these 3 cortical components of layer 3 circuitry in the
adult monkey DLPFC, but the anatomy of these synapses has not yet been examined in
developing animals. It thus remains to be seen whether there is in fact a direct functional
synaptic mechanism which exists to co-ordinate events between these components or whether
it is simply due to the common action of trophic factors or large scale "volume transmission"
release of DA, GABA and glutamate, acting at DA (expressed on pyramidal cells and PV-IR
neurons), GABA (on pyramidal cells and interneurons) and NMDA/non-NMDA receptors
(on pyramidal cells and interneurons), respectively.

All of the issues discussed here are of major importance to the understanding of the
normal time course of development in the DLPFC of the macaque monkey, which at the
beginning of this thesis study we proposed as an animal model for the DLPFC in the human
brain (many of the cortical anatomical similarities between the two species have been covered
in the review sections). In this regard, it is likely that the disruption at any time-point of such
impeccably synchronised time frames for the development of the 4 (spines, PV-IR cartridges,
DAergic axons and asymmetric synapses) markers of layer 3 cortical circuitry in the DLPFC
which we have examined and compared in this thesis study, would lead to an
exceptional disturbance of the normal structure of anatomical circuitry and patterns of functional behaviour in adult life, as would seem to be exhibited in schizophrenic subjects.

We shall see in the next part of the thesis (Study 2; sections 12.0, 13.0 and 14.0) how the time course of development for another monoaminergic (like dopamine) neurotransmitter, serotonin, in layer 3 of the monkey DLPFC, may be related to the changes in spine density and the other components of the cortical circuitry examined in study 1 of the thesis. There will also be further discussion concerning the possible disruption of these maturational events in humans and their implication for the aetiology of schizophrenia (see General Discussion section 15.0).

12.0: Study 2. A quantitative immunohistochemical study of the postnatal development of 5-HTergic axons in mid-layer 3 of the ventral bank of area 46 in the monkey.

12.1: Introduction.

This study examines the maturation of the serotonergic innervation of layer 3 in the ventral bank of area 46 in the dorsolateral prefrontal cortex of the macaque monkey during postnatal life. We chose to examine the development of the serotonergic innervation in mid-layer 3 for this study, because it is the location of important intrinsic excitatory connectivity between pyramidal neurons and many of its pyramidal cells are the major source of corticocortical connections to other regions of the brain. Previous studies by our laboratory (study 1 in this thesis) had examined the postnatal development of other components of the cortical circuitry in layer 3 and we wished to compare how the time course of the mid-layer 3 serotonergic innervation might relate to these other developmental events.

In this study we used a quantitative analysis to examine whether there were significant changes in the density of the mid-layer 3 serotonergic innervation in the ventral bank of area 46 (visualised using a 5-HT-specific antibody) during the course of postnatal development. The study makes use of measures of total axon length (per 5000 μm²), varicosity density (per 5000 μm²) and varicosities per mm of axon to evaluate the degree of anatomical maturity at various ages, from birth into middle adulthood, for the overall population and for thick and thin varieties of 5-HTergic axons. These quantitative analyses are then used to reconstruct the time course of development for the 5-HTergic axon innervation in mid-layer 3 of the DLPFC. This time course is then related to the known functional roles of the serotonergic system during development and its interactions with the other anatomical circuitry and receptors in the prefrontal cortex of both adult and developing monkeys as well as for other species and other brain regions.
We also wished to establish whether the maturational time course of 5-HTergic axon development in mid-layer 3 was the same as or distinct from the time course of maturation for dendritic spines on Golgi-impregnated pyramidal neurons in mid-layer 3 of areas 9 and 46 (study 1 in this thesis; Anderson et al, 1995). In addition we wished to determine whether the time course of any changes in the 5-HT innervation during postnatal development, occurred in alignment with changes previously observed for other neurotransmitter-containing axons or chemically-defined neuronal populations, using quantitative immunohistochemistry, e.g. dopaminergic axons (Rosenberg and Lewis, 1995), and PV-IR axon cartridges (Anderson et al, 1995).

It seemed especially important, in view of the variation in the density of 5-HT innervation across the monkey cerebral cortex and in different layers within a given area (Takeuchi and Sano, 1983; Berger et al, 1986, 1988; Morrison and Foote, 1986; Hornung et al, 1990; Wilson and Molliver, 1991a; reviewed by Foote and Morrison, 1987a) to investigate how the serotonergic innervation of a particular PFC area develops during the postnatal period and within that area, to examine closely the time course of maturation for a specific layer - in this case layer 3 - since each cortical laminae contains neuron populations with distinctive morphological and immunohistochemical characteristics as well as unique functional attributes and intrinsic and extrinsic anatomical connectivity.

Serotonin acting via its many receptor subtypes, has been implicated in many diverse functional aspects of cortical and subcortical processing (reviewed in Spoont et al, 1992; Sirvio et al, 1994; Cassel and Jeltsch, 1995; Buhot, 1997), including associative learning (marmoset: Harder et al, 1996; rat: Meneses and Hong, 1997), spatial working memory (human: Grasby et al, 1992; rat: Buhot et al, 1995; Herremans et al, 1995; macaque monkey: Williams et al, 1996), visual recognition memory (squirrel monkey: DeNoble et al, 1991) and object discrimination (marmoset: Carey et al, 1992). Of particular relevance to Study 2 of the thesis are the observations of Williams et al (1996) in the DLPFC of the macaque monkey. In this study, the authors examined the effects of the 5-HT$_{2A/C}$ receptor antagonist ritanserin by iontophoresis on the firing properties of neurons in the DLPFC during the performance of the ODR task (a test of spatial working memory function; see Functional review section 4.0 for details). The overall activity of pyramidal neurons was shown to be reduced for 57% of recorded task-related cells, 75% of which exhibited memory fields and 14% of recorded task-related cells increased their firing activity in response to the antagonist, all of which demonstrated inhibitory activity during the delay period. This kind of modulatory action by serotonin probably via a restricted number of synapses and also by way of 5-HT receptors
located on GABAergic interneurons, supports the extensive evidence in clinical studies with various antipsychotic drugs in schizophrenic humans, that 5-HT via its receptors can act to mediate cognitive behaviour in regions such as the DLPFC in primates. Abnormal alterations in the 5-HT receptor expression or in the density of the 5-HT innervation in the DLPFC triggered during the course of their maturation during postnatal development, may be an instrumental part of the complex pathophysiology which generates the diversity and extent of the cognitively-disabling, delusional and hallucinatory symptoms commonly found in schizophrenic patients.

To our knowledge, this is the first quantitative immunohistochemical study to examine the postnatal development of 5-HTergic axons in the macaque monkey cerebral cortex. Qualitative studies have been conducted previously of the postnatal development of the 5-HTergic innervation in the cerebral cortex of the marmoset monkey (Hornung, 1992a) and in the primary visual cortex (area 17 or V1) in the macaque monkey (Foote and Morrison, 1984; reviewed in Morrison et al, 1984; Foote and Morrison, 1987b). Biochemical studies have been carried out for the tissue levels of serotonin and its breakdown-product 5-HIAA in blocks encompassing the entire PFC in developing and ageing macaque monkeys (Goldman-Rakic and Brown, 1981, 1982), but not for area 46 or the DLPFC specifically. This is despite the fact that moderate differences were seen between the tissue concentrations and in vivo synthesis rates of 5-HT in the dorsolateral and the orbital prefrontal cortices of adult monkeys in a previous study by the same group (Brown et al, 1979) i.e. their use of total PFC block tissue samples may have obscured a variability in the time course of changes occurring within different regions of the PFC during postnatal development.

12.2: Materials and Methods.

12.2(A): Animals.

This study includes analysis of tissue from 18 macaque monkeys, including many of the same animals used previously in the pyramidal neuron dendritic spine density study, (see Table 1 for details), thus giving the opportunity for within-animal comparisons between the two studies.

Eighteen rhesus monkeys (Macaca mulatta) of both sexes, covering an age range from 4 days postnatal to 16.7 years, were used in this study. Sixteen of the animals had undergone no prior experimental procedures. The 2.3-year-old (Rh127) and 4.5-year-old (Rh119) males were castrated at between 6-10 days after birth. The 3.0-year-old (Rh145) male had received 2-4 injections of gonadotrophin releasing hormone (GnRH)
over a period of time, the final injection being given not less than 8 months prior to perfusion as part of another study. In previous studies of prepubertal monkeys that have been through the same procedure, it is clear that even following protracted hypothalamic GnRH neuronal stimulation, discontinuation of that stimulation usually leads to a resumption of the animals original stage of sexual development (Gay and Plant, 1987, 1988). This was confirmed from 4 weekly measures of morning and evening plasma testosterone sampled just before perfusion. It was thus found that the 3.0-year-old had a level of testosterone secretion, characteristic of late puberty (which was appropriate for its calendar age, i.e. it was a normal monkey - see Rosenberg and Lewis, 1994, 1995 for further details). We could find no evidence to suggest that the procedures had altered any aspect of the development of the 5-HTergic system in these animals.

Animals were deeply anaesthetised with ketamine hydrochloride (25 mg/kg, intramuscularly) and pentobarbital sodium (30 mg/kg, intraperitoneally) and then perfused transcardially with cold 4% paraformaldehyde in phosphate buffer (buffer pH = 7.4) as described previously (Noack and Lewis, 1989, Anderson et al, 1995, Rosenberg and Lewis, 1995). The brain was removed immediately and the left frontal lobe cut into coronal blocks 3-5 mm thick. Tissue blocks were immersed in the same fixative for 6 hours; washed in a series of cold, graded sucrose solutions; and then sectioned (40 μm) on a cryostat. Tissue for the present study was obtained from stored sections of these animals which were stored in ethylene glycol as a cryoprotectant at -20 degrees C immediately subsequent to sectioning.

Appropriate sections which included (Walker's area 46, principal sulcus region) were chosen with the aid of a 1-in-4 series of interleaved Nissl-stained sections prepared immediately subsequent to sectioning. The rostral-caudal position of the sections was chosen to coincide with a region between the appearance of the caudate and putamen caudally and the start of area 10 rostrally. Stored sections from the 2.3-year-old (Rhl27) animal were taken from the right frontal lobe due to unavailability of sections from the left frontal lobe.

12.2(B): Immunocytochemical procedures.

Before any immunocytochemical procedures the sections were removed from the ethylene glycol storage solution, allowed to warm to room temperature before further processing and then given six 3-minute washes in phosphate-buffered saline (PBS). Sections were then pre-treated for 30 mins at room temperature on a shaker with PBS containing 0.3% Triton X-100 and 450 ml normal serum per 10 ml of solution. Since the secondary antibody was raised in goat, goat normal serum was used. For the primary antibody
stage, the pre-treatment solution was poured off and the sections were given a further six 3-
min washes, before being incubated for 24-48 hours at 4 degrees centigrade in PBS
containing 0.3% Triton X-100, 0.5 mg/ml bovine serum albumin (BSA), 300 ml normal
serum per 10 ml of solution and a rabbit anti-5-HT antibody (diluted 1:8000).

The anti-5-HT antibody (obtained from Incstar Corp, Stillwater, MN) was generated
in rabbit serotonin conjugated to BSA with paraformaldehyde. The serotonin molecule
identified by the antibody was 5-HT creatinin sulphate provided by Sigma. This antibody has
been previously shown to specifically recognise 5-HT in studies of rat (Barr et al, 1987;
McClean and Shipley, 1987a,b) and monkey (Smiley and Goldman-Rakic, 1996) brain. For
the secondary antibody stage, the primary antibody solution was poured off and the tissue
washed 3 times, 8 mins each time. The secondary antibody solution was used to incubate the
sections at room temperature for 1 hour, consisting of PBS, 0.3% Triton X-100, 300 ml
normal serum per 10 ml solution and 1 drop vectastain biotinylated antibody per 10 ml
solution. Sections were then processed with the avidin-biotin method of Hsu et al (1981),
using a standard Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and
diaminobenzidine (DAB). After the DAB reaction, sections were mounted on subbed (gel-
coated) slides and cover-slipped with TTX.

To control for potential variability of immunocytochemical staining between separate
experiments, sections from all the animals were processed together, three sections from
different animals were placed in each reaction compartment, in order to ensure equivalent
exposure to the antibody and hence eliminate age-specific processing bias. In a pilot study,
sections from animals of 3 widely spaced ages (2 days, 1.5 years and 16.7 year) were reacted
together. Different sets of sections from each animal were tested at 3 different dilution's of
the anti-5-HT antibody in order to determine the optimum dilution at which to use for the
main experiment. The ideal dilution of antibody was determined to be between 1:6000 and
1:12000, thus a compromise of 1:8000 was settled on for optimum staining intensity of both
5-HT axons and uniformity of background neuropil staining, indicating an equivalent uptake
of antibody throughout the tissue section. Two complete sets of sections from all the animals
were then reacted in separate experiments, in order to control for variability in the absorption
of the anti-5-HT antibody. There was found to be an overall difference in intensity of staining
between the 2 reacted series' of sections. Thus for the quantitative analysis, sections from
different animals were only used from the more intensely stained reaction series.
Figure 36. A bar plot illustrating the cortical depth location (as percentage of cortical pia-to-white-matter distance), for each animal age, of the topmost edge of the sampling box (100μm wide by 50μm depth) within which tracings were constructed of 5-HTergic axons (3 boxes per animal) in mid-layer 3 of the ventral bank of the principle sulcus (area 46). The plot shows that there was minimal variation between the locations of the 5-HTergic axons for different ages, with the topmost edge of tracing at all ages located within a cortical depth of between 32-36%, so the 5-HT axons would be at the equivalent percentage depth as the basal dendritic fields of the mid-layer 3 pyramidal neurons in the spine study.
12.2(C): Quantitative analyses.

The laminar locations of 5-HT immunoreactive (IR) axons in Walker's area 46 were ascertained by referring to the Nissl-stained sections mentioned previously, which occupied comparable (to within a few sections) rostral-caudal positions in the brain. Variations in the density of 5-HT-IR axon processes and varicosities across development, were determined by computer-tracing the labelled structures using the Eutectic Neuron Tracing System (Eutectic Electronics, Inc, Raleigh, SC). Three reconstruction's of 5-HT labelled axon processes were made in mid-layer 3 of area 46 in the ventral bank of the principal sulcus in each animal. Tracings were located at a position which represented a consistent percentage of the cortical depth, determined in all Nissl-stained sections to be reliably within mid-layer 3. This percentage depth, 32-36% (see Figure 36) was comparable to the 31-33% of cortical depth which represented the range of locations of mid-layer 3 pyramidal neuron cell bodies examined in Golgi study 1 (Anderson et al, 1995, see Figure 28) and similarly the locations of pyramidal neurons in Study 1 of this thesis.

Each tracing reconstructed the extent of all the axons and marked all the varicosities along those axons, located within the confines of a rectangular line-image, 50 μm high by 100 μm wide, which was superimposed over a bright-field image of the 5-HT-IR stained section at a total magnification of 630x. Three outlined reconstruction's were traced for each layer in each animal and the three outlines were positioned 100 μm apart in mid-layer 3 so as to avoid overlap in the reconstruction's. Since it was only practical to trace a very limited area of tissue in this study, three separated sampling areas were judged to give a more accurate representation of axon length and varicosity density in each layer than tracing within 3 contiguous outlines placed end-to-end. This was important since the axon processes were observed to be unevenly distributed across the lateral extent of layer 3, a clustering effect was often seen in some parts of the layer, producing alternately dense and sparse patches of 5-HT innervation. By spacing the sampling areas some distance apart, it was hoped to overcome tracing just low or just high density patches and to achieve an overall representative density measurement.

The criterion used for an axon varicosity was: an enlargement of the diameter of a fibre which was bordered by a smaller calibre intervaricosome segment on at least one side, but preferably on both sides (Oeth and Lewis, 1993). The total 5-HTergic axon length was calculated for each box (per 5000 μm²), by summing the lengths of individual axon processes. Likewise, the total density of 5-HTergic varicosities (per 5000 μm²) was found for each box.
and from these two measures a third could be quantified, i.e. varicosity density/total axon length (in \( \mu m \)) = varicosities per \( \mu m \) of 5-HTergic axon length. The results were then presented in terms of total axon length (per 5000 \( \mu m^3 \)), density of varicosities (per 5000 \( \mu m^3 \)) and varicosities per \( \mu m \) of axon length, respectively, for each reconstructed box area. A mean value for all three parameters was calculated from the 3 box reconstruction’s traced per animal. These measures provided a means of quantifying the postnatal maturation of the 5-HT innervation within mid-layer 3 in area 46 of the monkey dorsolateral prefrontal cortex. All measures were conducted in sections from animals of different ages, which were processed together in the same immunohistochemical reaction. The 5-HT axon innervation reconstruction’s were made on coded slides, such that the age of the animal was unknown to the investigator at the time of tracing.

12.2(D): Morphological criteria for 2 distinct classes of 5-HTergic axons.

To establish whether there may be changes during postnatal development in the proportions of 2 different classes of 5-HTergic axons, every effort was made to assign each 5-HTergic axon in the analysis to one or other of two main groups, i.e. the thick or thin axon classes. This was achieved by evaluating the size and shape of the varicosities, the calibre of the intervaricose segments and the overall impression of the thickness of each individual 5-HTergic axon in relation to those others surrounding it in the neuropil. This judgement was made for the convenience of the analysis and also on the basis of the previous observations in the literature of 2 populations of varicose 5-HTergic axons in adult animals (see Serotonin review section 7.0, for background and references). The other possibilities are that there may be more than two populations of varicose 5-HTergic axons or alternatively these morphological differences may be the manifestation of temporally differential maturational changes occurring within a single 5-HTergic axon population.

The choice to quantitatively evaluate the total 5-HTergic axon sample as the two separate thick and thin 5-HTergic axon sub-populations, made on the basis of their different morphological characteristics, was made despite the fact that the postnatal time courses of the varicosity density (per 5000 \( \mu m^3 \)) for each appeared very similar. The 2 sub-populations of 5-HTergic axons were also present in the tissue examined in the present thesis study. As shown in the Results, sections 13.1(C)(i), (iv) and (vi), we quantitatively evaluated the contribution of each fibre type on the basis of its morphological characteristics.

However because of the apparent heterogeneity of many 5-HTergic axons it was often difficult to assign them categorically to one type or another, so in order to justify
presenting the results for each type separately we decided to conduct a further statistical analysis comparing the concurrence or distinctions of the time courses for each 5-HTergic axon type.

12.2(E): Statistical analyses.

The statistical significance of changes across postnatal development in terms of total axon length for 5-HTergic axons, the density of 5-HTergic varicosities and the number of varicosities per μm of axon length, were all determined for the total 5-HTergic population in mid-layer 3 of prefrontal cortical area 46, as well as specifically for the morphologically distinguishable thick and thin types of 5-HTergic axons that make up this population. The non-parametric Kruskal-Wallis test was used to examine the statistical significance of changes in the 5-HTergic innervation over postnatal development. Between-group comparisons for age were carried out using the Bonferroni test.

13.0: Results. Study 2. A quantitative immunohistochemical study of the postnatal development of 5-HTergic axons in mid-layer 3 of the ventral bank of area 46 in the monkey DLPFC.


Three morphologies of 5-HTergic axons were observed throughout area 46 of the macaque monkey prefrontal cortex for each of the different ages examined, in agreement with previous observations in various regions of the adult rat, cat, ferret and primate cerebral cortex (Lidov et al, 1980; Mulligan and Törk, 1987, 1988; Blue et al, 1988; De Lima et al, 1988; Wilson et al, 1989; Hornung et al, 1990; Mamounas et al, 1991; Voigt and De Lima, 1991a; Wilson and Molliver, 1991a; Mulligan and Törk, 1993; Smiley and Goldman-Rakic, 1996; reviewed by Kosofsky and Molliver, 1987). The first variety of axon was termed the "thick" or "beaded" class of 5-HTergic axon and was characterised by large bead-like (round) varicosities interlinked by fine intervaricose segments. Secondly, there were the "thin" or "fine" class of 5-HTergic axon which was distinguished by its generally smaller, pleomorphic (many-shaped) varicosities and fine intervaricose segments. The third kind of axon (not quantified in this study) was termed the "thick non-varicose" type of 5-HTergic axon, on account of its lack of varicosities and very thick smooth appearance; they are presumed to represent the parent or pre-terminal trunks of the varicose terminal 5-HTergic axons i.e. the thick and thin types of 5-HTergic axons.
Figure 37. Bright-field photomicrographs, showing examples of the thick (large arrowheads) and thin (small arrowheads) types of 5-HTergic axon in area 46 of the monkey DLPFC at various ages during postnatal development: (A) 8 day-old animal, (B) 67 day-old animal. Scale bar represents 50 μm.
Figure 37. Bright-field photomicrographs, showing examples of the thick (large arrowheads) and thin (small arrowheads) types of 5-HTergic axon in area 46 of the monkey DLPFC at various ages during postnatal development: (C) 5 month-old animal (D) 1.5 year-old animal. Scale bar represents 50 μm.
Figure 37. Bright-field photomicrographs, showing examples of the thick (large arrowheads) and thin (small arrowheads) types of 5-HTergic axons in area 46 of the monkey DLPFC at various ages during postnatal development: (E) 2.5 year-old animal (F) 3.0 year-old animal. Scale bar represents 50 µm.
Figure 37. Illustrations of thick and thin varieties of 5-HTergic axons in area 46 of the monkey prefrontal cortex at various stages of postnatal development. (i) thick and (ii) thin 5-HTergic axon in an 8-day-old animal; (iii) thick and (iv) thin 5-HTergic axons in a 1.5-year-old animal and (v) thick and (vi) thin 5-HTergic axons in a 2.5-year-old animal. N.B. these tracings represent reconstructions of axons seen in photomicrographs: (A), (D) and (E), respectively.
As commented in a previous study of adult macaque monkey prefrontal cortex (area 46) at the light and electron microscope level (Smiley and Goldman-Rakic, 1996), the distinction between the first and second varieties of 5-HTergic axon were not always clear-cut. Their morphology seemed to form a continuum, ranging from the characteristics of the thick axons through to those of the thin variety, with many 5-HTergic axons being of intermediate morphology combining aspects of both sets of morphological criteria. For example, a single 5-HTergic axon process with large beaded varicosities may sometimes be observed to become finer and exhibit smaller pleomorphic varicosities further along its extent. This continuum of 5-HTergic axon morphologies was particularly evident in the tissue of the many immature animals examined in the present study, suggesting that perhaps the characteristics of the thick and thin 5-HTergic axon varieties develop over time, but even in the adult animal their morphological distinction is not always absolute.

13.1(B): Qualitative observations of 5-HTergic axons in mid-layer 3.

All sections of monkey PFC which were reacted for 5-HT immunohistochemistry were initially examined without knowledge of the age of the animal; then later, after quantitative data had been obtained, they were again observed now arranged in order of ascending age, in order to form a qualitative impression of the density and arrangement of 5-HT axons over postnatal development. The main objective was to compare the overall pattern of the maturing 5-HT axon organisation throughout layer 3 of the entire ventral bank of the principal sulcus, since it was only practical to sample a restricted area of this portion of the PS in the quantitative part of the study (see Methods section 8.2). This was evaluated in terms of the general density, morphology and orientation of 5-HT axons in layer 3 of the ventral PS of each animal and any evidence of patchiness, unevenness or clustering in the 5-HT axon distribution was also noted.

The 5-HTergic innervation of layer 3 was observed to show some common characteristics which were clearly evident at all stages of postnatal development:

(i) A pronounced radially and/or obliquely oriented criss-cross network of 5-HTergic axons was observed to extend through the dorsal-ventral extent of layer 3, with many axons often spanning across adjacent layers as well.

(ii) Long tangentially-aligned 5-HTergic axons could sometimes be observed to traverse hundreds of micrometers in some animals, most notably seen in layers 2-3 of the 2.8-year and 16.7-year-old animals and in layer 6 of the 1.0-, 2.8-, 3.0-, 5.7-year-old animals.
Figure 38 Low-power bright-field photomicrographs, showing a pia-to-white matter view of the developing serotonergic (5-HTergic) innervation in the ventral bank of the principal sulcus (area 46), as visualised by an anti-5-HT antibody in the neonatal, juvenile, adolescent and adult macaque monkey DLPFC, respectively at ages: A. 4 days, B. 5 months, C. 2.5 years and D. 4.5 years. Also shown are representations of the 3 sampling frames (100μm by 50 μm) used to conduct a quantitative analysis of 5-HTergic axons (see Materials and Methods section 11.2(C) for more details). Scale bar represents 100 μm.
Figure 39 Low-power dark-field photomicrographs showing a pia-to-white matter view of the developing serotonergic (5-HTergic) innervation in the ventral bank of the principal sulcus (area 46), as visualised by an anti-5-HT antibody in the neonatal, infant, juvenile and peripubertal macaque monkey DLPFC, respectively at ages: A. 8 days, B. 67 days, C. 7 months and D. 3.0 years. Scale bar represents 100 μm.
A patchiness or unevenness was evident in the distribution of the 5-HTergic axons, so that a given region of layer 3 in a single animal could be quite densely innervated, while less than a few hundred micrometers away there might be a very sparsely innervated expanse of neuropil.

There was a tendency for complex networks, comprising radial, oblique and tangentially-oriented axons, found at certain ages, to be more densely arranged near the lip of the sulcus in the ventral bank, than within the central portion of the sulcus or near the fundus, particularly seen in the animals of 8 days, 72 days, 78 days, 7 months, 1 year, 1.5 years, 2.3 years and 2.5 years of age.

Clusters of thick varicose axons were first observed in the 2.8-year-old animal and formed noticeably "basket-like" or "pericellular-like" arrangements in layer 3 and elsewhere particularly layers 1, 2 and 4; they were observed with varying levels of prominence in all other animals aged over 2.8 years. These structures have been described previously in the cerebral cortex and hippocampus of the developing (Vu and Törk, 1992) and adult cat (Mulligan and Törk, 1987, 1988, 1993), adult macaque monkey (Wilson and Molliver, 1991a) and the developing (Hornung, 1992a) and adult marmoset (Hornung et al, 1990; Hornung and Celio, 1992) - predominantly in the PFC and hippocampus; but have not been found in the developing or adult rat (reviewed in Kosofsky and Molliver, 1987). The age at which these "basket-like" 5-HT axon arrangements first appear and the maturational time course for their presence, has not to our knowledge been described in the cerebral cortex of the developing macaque monkey.

In a qualitative low power (50x and 100x total magnification) light microscope examination of the material from each animal, (see Figures 38 and 39) the time points between which the most dramatic changes in the density of the 5-HTergic axon innervation in layer 3 could be seen were for the animals of ages: 4 and 8 days (decrease), 1.0 and 1.5 years (decrease), 1.5 and 2.3 years (increase), 2.3 and 2.5 years (increase), 2.5 and 2.8 years (decrease), 4.5 and 5.7 years (increase) and 5.7 and 16.7 years (increase).

However, variations in 5-HTergic axon density observed at low magnification in layer 3 between animals of different ages were occasionally found to be unrepresentative of the variability in density within the layer when the same sections were observed in greater detail at a higher magnification. Many of the axons faintly stained with the 5-HT antibody could not be clearly seen at low power whereas axons which were intensely stained and thick in calibre would stand out and tend to give an inaccurate impression of the actual density of the 5-HTergic axons overall. For this reason and others outlined at the beginning of this section, we
undertook a quantitative analysis of the changes in the 5-HTergic axon innervation in layer 3 of area 46 during postnatal development.

N.B. All significance values in this section were derived from the absolute values rounded up conservatively, e.g. P = 0.003 would fall between the P < 0.001 and P < 0.005 bands and so was placed within the P < 0.005 band.

The quantitative analyses of the maturational time course of 5-HTergic axons in 3 sampling frames located within mid-layer 3 of the ventral bank of area 46 in the macaque monkey DLPFC (see Figure 38) are presented in 3 sections:

(i) Total axon length for all 5-HTergic axons (per 5000 µm²)
(ii) Total axon length for thick and thin 5-HTergic axons (per 5000 µm²).
(iii) Varicosity density for all 5-HTergic axons (per 5000 µm²).
(iv) Varicosity density for thick and thin 5-HTergic axons (per 5000 µm²).
(v) Varicosities per µm of axon length for all 5-HTergic axons.
(vi) Varicosities per µm of axon length for thick and thin 5-HTergic axons.

The statistical significance of these developmental changes in 5-HTergic axons in mid-layer 3 of area 46 was evaluated for age groups constructed from the results of the individual animals whilst maintaining their correct chronological age sequence. Two groupings were chosen: A and B. Grouping A will be used in the results section, whilst grouping B will be compared to grouping A in the Discussion section 13.0.

The groups tested for significance were as follows:

Grouping A.

Age sets in grouping A were chosen on the basis of comparing the most pronounced differences between animals in terms of the total axon length for 5-HTergic axons and both the density per 5000 µm² and per µm of axon length of their varicosities with increasing postnatal age. There are 5 age groups in grouping A: 4-22 days (<1 month), 37 days-5 months, 7 months-2.5 years, 2.8-4.5 years and 5.7-16.7 years (>4.5 years).

Grouping B.

Age sets in grouping B were derived from those used to present the data for the postnatal development of the DAergic axon innervation in layer 3 of area 46 (Rosenberg and Lewis, 1995). This was done to enable a comparison to be made of the time courses of maturation for the present 5-HTergic and the previously reported (Rosenberg and
Table 4A. Total axon length (in μm) for 3 boxes per animal.

<table>
<thead>
<tr>
<th>Age</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>4d</td>
<td>711.7</td>
<td>610.3</td>
<td>586.4</td>
<td>± 38.4</td>
</tr>
<tr>
<td>8d</td>
<td>491.5</td>
<td>819.2</td>
<td>795.8</td>
<td>±105.6</td>
</tr>
<tr>
<td>22d</td>
<td>697.8</td>
<td>520.0</td>
<td>703.9</td>
<td>± 60.3</td>
</tr>
<tr>
<td>37d</td>
<td>620.5</td>
<td>198.3</td>
<td>530.4</td>
<td>±128.4</td>
</tr>
<tr>
<td>67d</td>
<td>791.5</td>
<td>646.0</td>
<td>509.3</td>
<td>± 81.5</td>
</tr>
<tr>
<td>72d</td>
<td>573.9</td>
<td>855.4</td>
<td>738.3</td>
<td>± 81.6</td>
</tr>
<tr>
<td>78d</td>
<td>714.1</td>
<td>582.5</td>
<td>607.1</td>
<td>± 40.4</td>
</tr>
<tr>
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<td>849.0</td>
<td>832.6</td>
<td>618.0</td>
<td>± 74.4</td>
</tr>
<tr>
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<td>921.8</td>
<td>382.4</td>
<td>990.3</td>
<td>±192.2</td>
</tr>
<tr>
<td>1.0yr</td>
<td>821.7</td>
<td>714.8</td>
<td>943.6</td>
<td>± 66.1</td>
</tr>
<tr>
<td>1.5yr</td>
<td>692.3</td>
<td>588.4</td>
<td>911.5</td>
<td>± 95.2</td>
</tr>
<tr>
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<td>392.5</td>
<td>927.0</td>
<td>±178.1</td>
</tr>
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<td>1109.0</td>
<td>1067.5</td>
<td>± 72.0</td>
</tr>
<tr>
<td>2.8yr</td>
<td>860.5</td>
<td>314.2</td>
<td>184.1</td>
<td>±207.2</td>
</tr>
<tr>
<td>3.0yr</td>
<td>544.2</td>
<td>431.4</td>
<td>227.9</td>
<td>± 92.6</td>
</tr>
<tr>
<td>4.5yr</td>
<td>683.5</td>
<td>188.0</td>
<td>336.6</td>
<td>±146.8</td>
</tr>
<tr>
<td>5.7yr</td>
<td>893.7</td>
<td>1013.9</td>
<td>787.9</td>
<td>± 65.3</td>
</tr>
<tr>
<td>16.7yr</td>
<td>1001.2</td>
<td>711.2</td>
<td>1483.9</td>
<td>±225.4</td>
</tr>
</tbody>
</table>
Lewis, 1995) DAergic axon innervations in area 46. There were 5 age groups in grouping B: 4-22 days (< 1 month), 37-78 days (1-3 months), 5-7 months, 1.0-1.5 years, 2.3-3.0 years (2-3 years) and 4.5-16.7 years (> 4.5 years). See Discussion, 13.3(C):(ii).

13.1(C).(i): Total axon length for all 5-HTergic axons.

The total axon length of all 5-HTergic axons within 3 sampling areas of 5000 µm² each in the ventral bank of the PS was measured for each animal; the mean of these 3 samples was then calculated as a measure of mean total axon length (per 5000 µm²) at each age across postnatal development (see Figures 40A and 41A, also see Table 4A for raw data).

The total axon length for all 5-HTergic axons in early neonatal animals (4 to 22 days of age) remained constant at a moderate level until about the beginning of the first postnatal month (37 days), where it exhibited a much lower value. From 67 days to 1.0 year of age, the total axon length of all 5-HTergic axons showed a gradual but consistent rise. For those animals aged from 1.0 year through to 3.0 years of age there was a marked overall decline in total axon length with age.

One exception to this general trend was a high value for total length of 5-HTergic axons observed in the 2.5-year-old animal during the course of an otherwise uninterrupted decrease in total axon length for the animals aged between 1.0 and 3.0 years of age. The level of 5-HTergic axon innervation in the 2.5-year-old is almost double the value found in the 2.3-year-old animal and over twice the value for the 2.8-year-old animal - its immediate neighbours by age.

The low value of total axon length in the 3.0-year-old animal was maintained in the 4.5-year-old animal. The low levels of total axon length in young adults did not however, appear to remain stable in older adult animals, since those of 5.7 years and 16.7 years of age demonstrated much higher values than those seen for the 3.0- and 4.5-year-olds. A dramatic upward trend in total axon length was observed for the 5.7-year-old and a small further rise was evident for the 16.7-year-old.

Using the Kruskal-Wallis test, for grouping A (see Figure 42A), statistically significant changes were observed for total axon length (per 5000 µm²) with age (P < 0.05). Specifically, there was a marked difference between the 7 m-2.5 yrs and 2.8 yrs-4.5 yrs age groups (P < 0.10) and between the 2.8 yrs-4.5 yrs and > 4.5 yrs age groups (P < 0.10).

A modification of the groupings, i.e. the merging of age groups 1 and 2 (overall: 4 days-5 months) with the Kruskal-Wallis test, produced an increase in the overall significance of changes occurring between age groups (asymptotic, P < 0.01). The use of the Bonferroni
Figure 40. Results for individual animals on a linear scale: Total axon length (per 5000μm²) is shown for (A) all 5-HTergic axons; (B) thick 5-HTergic axons and (C) thin 5-HTergic axons.
Figure 41. Results for individual animals on a logarithmic scale, illustrating proportional age intervals between animals: Total axon length (per 5000 µm²) is shown for (A) all 5-HTergic axons; (B) thick 5-HTergic axons and (C) thin 5-HTergic axons.

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Table 4B. Axon length (in μm) for thick and thin axons for 3 boxes per animal.

<table>
<thead>
<tr>
<th>Age</th>
<th>Thick</th>
<th>Thin</th>
<th>Thick</th>
<th>Thin</th>
<th>Thick</th>
<th>Thin</th>
<th>SEM Thick</th>
<th>SEM Thin</th>
</tr>
</thead>
<tbody>
<tr>
<td>4d</td>
<td>515.1</td>
<td>196.6</td>
<td>353.2</td>
<td>257.1</td>
<td>424.5</td>
<td>161.9</td>
<td>± 46.9</td>
<td>± 27.8</td>
</tr>
<tr>
<td>8d</td>
<td>341.5</td>
<td>150.0</td>
<td>395.3</td>
<td>423.9</td>
<td>302.5</td>
<td>493.3</td>
<td>± 26.9</td>
<td>± 104.8</td>
</tr>
<tr>
<td>22d</td>
<td>381.4</td>
<td>316.4</td>
<td>244.4</td>
<td>275.6</td>
<td>339.0</td>
<td>364.9</td>
<td>± 40.5</td>
<td>± 25.8</td>
</tr>
<tr>
<td>37d</td>
<td>489.6</td>
<td>130.9</td>
<td>154.2</td>
<td>44.1</td>
<td>271.6</td>
<td>258.8</td>
<td>± 98.3</td>
<td>± 62.4</td>
</tr>
<tr>
<td>67d</td>
<td>489.8</td>
<td>301.7</td>
<td>530.4</td>
<td>115.6</td>
<td>374.9</td>
<td>134.4</td>
<td>± 46.6</td>
<td>± 59.1</td>
</tr>
<tr>
<td>72d</td>
<td>368.6</td>
<td>205.3</td>
<td>511.8</td>
<td>343.6</td>
<td>205.5</td>
<td>532.8</td>
<td>± 88.5</td>
<td>± 94.9</td>
</tr>
<tr>
<td>78d</td>
<td>629.2</td>
<td>84.9</td>
<td>335.1</td>
<td>247.4</td>
<td>167.6</td>
<td>439.5</td>
<td>±134.9</td>
<td>±102.5</td>
</tr>
<tr>
<td>5m</td>
<td>673.7</td>
<td>175.3</td>
<td>512.7</td>
<td>319.9</td>
<td>314.6</td>
<td>303.4</td>
<td>±103.9</td>
<td>± 45.7</td>
</tr>
<tr>
<td>7m</td>
<td>397.2</td>
<td>524.6</td>
<td>168.6</td>
<td>213.8</td>
<td>751.9</td>
<td>238.4</td>
<td>±169.7</td>
<td>± 99.8</td>
</tr>
<tr>
<td>1.0yr</td>
<td>341.3</td>
<td>480.4</td>
<td>419.0</td>
<td>295.8</td>
<td>641.7</td>
<td>301.9</td>
<td>± 90.0</td>
<td>± 60.5</td>
</tr>
<tr>
<td>1.5yr</td>
<td>439.3</td>
<td>253.0</td>
<td>323.2</td>
<td>265.2</td>
<td>111.6</td>
<td>799.9</td>
<td>± 95.9</td>
<td>±180.3</td>
</tr>
<tr>
<td>2.3yr</td>
<td>261.4</td>
<td>131.8</td>
<td>264.9</td>
<td>127.6</td>
<td>441.2</td>
<td>485.8</td>
<td>± 59.4</td>
<td>±118.7</td>
</tr>
<tr>
<td>2.5yr</td>
<td>683.3</td>
<td>192.0</td>
<td>607.9</td>
<td>501.1</td>
<td>667.7</td>
<td>399.8</td>
<td>± 23.0</td>
<td>± 91.0</td>
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<td>2.8yr</td>
<td>422.4</td>
<td>438.1</td>
<td>262.1</td>
<td>52.1</td>
<td>123.6</td>
<td>60.5</td>
<td>± 86.3</td>
<td>±127.3</td>
</tr>
<tr>
<td>3.0yr</td>
<td>328.5</td>
<td>215.7</td>
<td>205.9</td>
<td>225.5</td>
<td>110.2</td>
<td>117.7</td>
<td>± 63.2</td>
<td>± 34.4</td>
</tr>
<tr>
<td>4.5yr</td>
<td>321.3</td>
<td>362.2</td>
<td>33.4</td>
<td>154.6</td>
<td>48.1</td>
<td>288.5</td>
<td>± 93.6</td>
<td>± 60.8</td>
</tr>
<tr>
<td>5.7yr</td>
<td>583.7</td>
<td>310.0</td>
<td>442.0</td>
<td>571.9</td>
<td>194.4</td>
<td>593.5</td>
<td>±113.8</td>
<td>± 91.1</td>
</tr>
<tr>
<td>16.7yr</td>
<td>638.9</td>
<td>362.3</td>
<td>382.1</td>
<td>329.1</td>
<td>639.4</td>
<td>844.5</td>
<td>± 85.7</td>
<td>±166.5</td>
</tr>
</tbody>
</table>
correction following an ANOVA, further increased the levels of significant difference (P < 0.0005). Specifically, these changes occurred between the new age group and the > 4.5 yrs group (P < 0.02), as well as increased levels of significance for the differences in values between the 7 m-2.5 yrs and 2.8 yrs-4.5 yrs groups (P < 0.005) and between 2.8 yrs-4.5 yrs and the > 4.5 yrs groups (P < 0.001).

13.1(C).(ii): Total axon length for thick and thin 5-HTergic axons.

The total axon lengths for thick (see Figures 40B and 41B) and thin (see Figures 40C and 41C and Table 4B for raw data) types of 5-HTergic axons, which together comprise the total axon length (per 5000 µm²) of all 5-HTergic axons, were measured separately in order to examine their relative contribution to changes in overall 5-HTergic axon density that might be taking place during the course of postnatal development.

Total axon length for thick 5-HTergic axons.

The thick variety of 5-HTergic axons (see Figures 40B and 41B and also Table 4B for raw data) appeared to show a moderate-to-low level of innervation in the early neonatal period (4- to 37-day-old animals), with a moderate value for the 4-day-old and lower levels in successive ages, reaching a low point in the 37-day-old animal. There appeared to be a general trend of increasing total axon length between the animals ages 37 days and 5 months; only the 67-day-old animal showed a deviation from the general trend, with a much higher level of axon length than animals of a similar age (i.e. 72 and 78 day animals). There was very little variation in the levels of total axon length for the animals aged between 5 months and 1.0 year of age, but there was observed to be a large drop for the animals aged between 1.0 and 1.5 years of age; this lower level was sustained in the 2.3-year-old animal.

As seen for the measures of all 5-HTergic axons there was very high total axon length of thick 5-HTergic axons in the 2.5-year-old animal. Otherwise for the animals aged between 1.0 year and 4.5 years, there total axon length measures seemed to progressively decline for. There was observed to be a triple-fold higher level of axon length in the 5.7-year-old animal compared to the 4.5-year-old animal. In older adult animals, represented by the 16.7-year-old animal the total axon length was only slightly greater than that in the 5.7-year-old.

Non-significant (at the α = 0.05 level) but sizeable changes (asymptotic, P < 0.10) were observed in total axon length for the thick type of 5-HTergic axons (per 5000 µm²) during postnatal development, using grouping A (see Figure 42B). Specifically, there was a
Figure 42. Results for age groups arranged in grouping A (the number of animals in each group is indicated): Total axon length (per 5000μm²) is shown for (A) all 5-HTergic axons; (B) thick 5-HTergic axons and (C) thin 5-HTergic axons. The levels of significance for each difference between age groups is shown above the appropriate bars.
large difference between the values for the 7 m-2.5 yrs and the 2.8 yrs-4.5 yrs age groups (P < 0.10) and between those of the 2.8 yrs-4.5 yrs and > 4.5 yrs age groups (both P < 0.10).

A modification of the groupings, (i.e. groups 1 and 2 combined), did not produce any significant differences between the new age group (4 d-5 m) and the other age groups, although the overall significance for difference across all groups increased (asymptotic, P < 0.05). The ANOVA with the Bonferroni correction also appeared to raise the significance of the differences (P < 0.02), specifically between the 7 m-2.5 yrs and the 2.8 yrs-4.5 yrs age groups (P < 0.05) and of the latter and the > 4.5 yrs groups (P < 0.06).

**Total axon length for thin 5-HTergic axons.**

The thin class of 5-HTergic axons (see Figures 40C and 41C and also Table 4B for raw data) in the 4 day animal exhibited a low value for total axon length, whilst the level for the animals of 8 and 22 days of age was nearly twice this value. The lowest overall total axon length was observed for the 37-day old, which represented the beginning of a consistent rise in values from the animal aged 37 days through to the 1.5 year-old. The only exception to this smooth pattern was the high level for axon length found in the 72 day-old animal.

The levels of total axon length in the 2.3-year-old were about half those for the peak in the 1.5-year-old and apart from the consistently higher levels in the 2.5-year-old animal, a downward trend was followed which reached a low point in the 2.8- and 3.0-year-olds. A slightly higher total axon length was evident for the 4.5-year-old and a much higher level was present in the 5.7- and 16.7-year-olds. Thus very high levels of innervation appeared to be the norm for the older adult animals within the age limit of the series (i.e. 16.7 years of age).

Statistically significant changes (asymptotic, P < 0.05) were found in the total axon length for the thin 5-HTergic axons (per 5000 μm²) during postnatal development, as classified by grouping A. Specifically, (see Figure 42C) these differences were found between the < 1 m and > 4.5 yrs age groups (P < 0.10), between the 1 m-5 m and > 4.5 yrs age groups (P < 0.01) and between the 2.8 yrs-4.5 yrs and > 4.5 yrs age groups (P < 0.01).

A modification of the groupings (i.e. merging of age groups 1 and 2 ), with the Kruskal-Wallis test resulted in an increased overall level of significance for differences between age groups (asymptotic, P < 0.03). The use of an ANOVA with the Bonferroni correction, produced an increase in the overall level of significance for the changes evident between all age groups (P < 0.002). The strongest significant differences were observed to be between the new age group (4 d-5 m) and the 2.8 yrs-4.5 yrs age group (P < 0.005), as well between the 7 m-2.5 yrs and 2.8 yrs-4.5 yrs age groups (P < 0.005).
Table 5A. Total varicosity density for 3 boxes per animal.

<table>
<thead>
<tr>
<th>Age</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>4d</td>
<td>492</td>
<td>424</td>
<td>393</td>
<td>± 29.2</td>
</tr>
<tr>
<td>8d</td>
<td>394</td>
<td>638</td>
<td>624</td>
<td>± 79.1</td>
</tr>
<tr>
<td>22d</td>
<td>445</td>
<td>380</td>
<td>512</td>
<td>± 38.1</td>
</tr>
<tr>
<td>37d</td>
<td>476</td>
<td>153</td>
<td>476</td>
<td>±107.7</td>
</tr>
<tr>
<td>67d</td>
<td>290</td>
<td>244</td>
<td>216</td>
<td>± 21.6</td>
</tr>
<tr>
<td>72d</td>
<td>313</td>
<td>414</td>
<td>402</td>
<td>± 31.9</td>
</tr>
<tr>
<td>78d</td>
<td>271</td>
<td>323</td>
<td>358</td>
<td>± 25.3</td>
</tr>
<tr>
<td>5m</td>
<td>269</td>
<td>436</td>
<td>321</td>
<td>± 49.3</td>
</tr>
<tr>
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<td>572</td>
<td>261</td>
<td>658</td>
<td>±120.6</td>
</tr>
<tr>
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<td>486</td>
<td>378</td>
<td>593</td>
<td>± 62.1</td>
</tr>
<tr>
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<td>410</td>
<td>339</td>
<td>597</td>
<td>± 77.0</td>
</tr>
<tr>
<td>2.3yr</td>
<td>111</td>
<td>190</td>
<td>461</td>
<td>±106.0</td>
</tr>
<tr>
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<td>572</td>
<td>703</td>
<td>704</td>
<td>± 43.8</td>
</tr>
<tr>
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<td>182</td>
<td>217</td>
<td>123</td>
<td>± 27.4</td>
</tr>
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<td>152</td>
<td>286</td>
<td>166</td>
<td>± 42.5</td>
</tr>
<tr>
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<td>416</td>
<td>114</td>
<td>174</td>
<td>± 92.3</td>
</tr>
<tr>
<td>5.7yr</td>
<td>424</td>
<td>521</td>
<td>445</td>
<td>± 29.5</td>
</tr>
<tr>
<td>16.7yr</td>
<td>731</td>
<td>539</td>
<td>1085</td>
<td>±159.9</td>
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</table>
13.1(C).(iii): Varicosity density per 5000 μm² for all 5-HTergic axons.

The postnatal development of varicosity density for all 5-HTergic axons (see Figures 43A and 44A and also Table 5A for raw data) in mid-layer 3 of area 46 showed a broadly similar time course to that of the total axon length for all 5-HTergic axons and indeed is a product of total axon length and varicosities per μm of axon. The basically cyclical pattern of the density of innervation for developing 5-HTergic axons observed in mid-layer 3 of area 46 was even more pronounced in the case of the 5-HTergic varicosities.

The density of varicosities for all 5-HTergic axons during the early neonatal period was at moderate levels in the youngest animal of 4 days of age, whilst they were slightly higher in the 8 day animal. Then in the animals between 8 and 67 days of age varicosity density values appeared to decline at an even rate. These observations showed a generally parallel relationship with those for total axon length as described in section (i), although the lowest levels were found in the 37 day animal for the latter parameter, rather than in the 67-day-old.

The downward trend in varicosity density appeared to be reversed from 72 days onwards, to rise in value to the 7-month-old animal. The animals aged between 7 months and 1.5 years illustrated a plateau in their values for varicosity density, which began to fall as shown by the much lower levels seen for the 2.8-year-old. The 2.5-year-old demonstrated a level of varicosity density around three times that of the 2.8-year-old, appearing to be out of the range of all the other animals - apart from the oldest 2 adults - as observed before in the measurement of total axon length in section (i). The 2.8-year-old animal contained the lowest varicosity density of any age examined, but this low density was not maintained into the adult stage of development. A gradual increase was evident at first between the animals of 2.8 and 4.5 years of age, then an acceleration in the density of varicosities seemed to have occurred with a doubling of values between the animals of 4.5 and 5.7 years of age; the highest value of varicosity density at any age examined was seen in the 16.7-year-old, representing a doubling of values from that of the 5.7-year-old animal.

Statistically significant changes (asymptotic, P < 0.02) were found in varicosity density (per 5000 μm²) for the total 5-HTergic axon population across the postnatal period, using grouping A. Specifically, (see Figure 45A) there was a sizeable difference between the < 1 m and 2.8 yrs-4.5 yrs age groups (P < 0.10) and significant differences between the 1 m-5 m and > 4.5 yrs age groups (P < 0.10), between the 7 m-2.5 ys and 2.8 yrs-4.5 yrs age groups (P < 0.10) and between the 2.8 yrs-4.5 yrs and > 4.5 yrs age groups (P < 0.01).

The modification of age groups (i.e. combining 1 and 2), with the Kruskal-Wallis
Figure 43. Results for individual animals on a linear scale: Varicosity density (per 5000μm²) is shown for (A) all 5-HTergic axons; (B) thick 5-HTergic axons and (C) thin 5-HTergic axons.
Figure 44. Results for individual animals on a logarithmic scale, illustrating proportional age intervals between animals: Varicosity density (per 5000μm²) is shown for (A) all 5-HTergic axons; (B) thick 5-HTergic axons and (C) thin 5-HTergic axons.
Table 5B. Varicosity density for thick and thin axons for 3 boxes per animal.

<table>
<thead>
<tr>
<th>Age</th>
<th>Thick</th>
<th>Thin</th>
<th>Thick</th>
<th>Thin</th>
<th>Thick</th>
<th>Thin</th>
<th>SEM Thick</th>
<th>Thin</th>
</tr>
</thead>
<tbody>
<tr>
<td>4d</td>
<td>356</td>
<td>136</td>
<td>242</td>
<td>182</td>
<td>291</td>
<td>102</td>
<td>± 33.0</td>
<td>± 23.2</td>
</tr>
<tr>
<td>8d</td>
<td>282</td>
<td>112</td>
<td>298</td>
<td>340</td>
<td>244</td>
<td>380</td>
<td>± 16.0</td>
<td>± 83.5</td>
</tr>
<tr>
<td>22d</td>
<td>238</td>
<td>207</td>
<td>174</td>
<td>206</td>
<td>259</td>
<td>253</td>
<td>± 25.6</td>
<td>± 15.5</td>
</tr>
<tr>
<td>37d</td>
<td>382</td>
<td>94</td>
<td>119</td>
<td>34</td>
<td>286</td>
<td>190</td>
<td>± 76.8</td>
<td>± 45.4</td>
</tr>
<tr>
<td>67d</td>
<td>188</td>
<td>102</td>
<td>196</td>
<td>48</td>
<td>164</td>
<td>52</td>
<td>± 9.6</td>
<td>± 17.4</td>
</tr>
<tr>
<td>72d</td>
<td>207</td>
<td>106</td>
<td>258</td>
<td>156</td>
<td>108</td>
<td>294</td>
<td>± 44.0</td>
<td>± 56.2</td>
</tr>
<tr>
<td>78d</td>
<td>238</td>
<td>33</td>
<td>186</td>
<td>137</td>
<td>106</td>
<td>252</td>
<td>± 38.4</td>
<td>± 63.2</td>
</tr>
<tr>
<td>5m</td>
<td>230</td>
<td>39</td>
<td>257</td>
<td>179</td>
<td>175</td>
<td>146</td>
<td>± 24.1</td>
<td>± 42.3</td>
</tr>
<tr>
<td>7m</td>
<td>264</td>
<td>308</td>
<td>118</td>
<td>143</td>
<td>494</td>
<td>164</td>
<td>± 109.4</td>
<td>± 51.9</td>
</tr>
<tr>
<td>1.0yr</td>
<td>212</td>
<td>274</td>
<td>218</td>
<td>160</td>
<td>402</td>
<td>191</td>
<td>± 62.4</td>
<td>± 34.0</td>
</tr>
<tr>
<td>1.5yr</td>
<td>261</td>
<td>149</td>
<td>196</td>
<td>143</td>
<td>77</td>
<td>520</td>
<td>± 53.9</td>
<td>± 124.7</td>
</tr>
<tr>
<td>2.3yr</td>
<td>79</td>
<td>32</td>
<td>130</td>
<td>60</td>
<td>230</td>
<td>231</td>
<td>± 44.3</td>
<td>± 62.2</td>
</tr>
<tr>
<td>2.5yr</td>
<td>453</td>
<td>119</td>
<td>369</td>
<td>334</td>
<td>444</td>
<td>260</td>
<td>± 26.6</td>
<td>± 63.1</td>
</tr>
<tr>
<td>2.8yr</td>
<td>101</td>
<td>81</td>
<td>181</td>
<td>36</td>
<td>89</td>
<td>34</td>
<td>± 28.9</td>
<td>± 15.3</td>
</tr>
<tr>
<td>3.0yr</td>
<td>104</td>
<td>48</td>
<td>139</td>
<td>147</td>
<td>76</td>
<td>90</td>
<td>± 18.2</td>
<td>± 28.7</td>
</tr>
<tr>
<td>4.5yr</td>
<td>184</td>
<td>232</td>
<td>23</td>
<td>91</td>
<td>29</td>
<td>145</td>
<td>± 52.7</td>
<td>± 41.1</td>
</tr>
<tr>
<td>5.7yr</td>
<td>283</td>
<td>141</td>
<td>218</td>
<td>303</td>
<td>117</td>
<td>328</td>
<td>± 48.3</td>
<td>± 58.6</td>
</tr>
<tr>
<td>16.7yr</td>
<td>455</td>
<td>276</td>
<td>301</td>
<td>238</td>
<td>741</td>
<td>614</td>
<td>± 128.9</td>
<td>± 119.5</td>
</tr>
</tbody>
</table>
test revealed no alteration in the overall level of significance for changes in varicosity density during postnatal development (P < 0.02). The use of an ANOVA with the Bonferroni correction applied, did raise the overall level of significance for the difference between age groups (P < 0.01), but no rise in the case of the new age group (4 d-5 m) and the others, although there was a rise in the significant difference between the 7 m-2.5 yrs and 2.8 yrs-4.5 yrs age groups (P < 0.05).

13.1(C).(iv): Varicosity density per 5000 \( \mu m^2 \) for thick and thin 5-HTergic axons.

**Varicosity density for thick 5-HTergic axons.**

The density of varicosities counted for the thick variety of 5-HTergic axons (see Figures 43B and 44B and also Table 5B for raw data) was at moderate levels in the 4 day-old animal, but then exhibited a general decrease in values for most animals until 67 days of age. The animals of ages 67-78 days remained stable in their values, whilst those aged between 78 days and 5 months appeared to demonstrate a steady increase in varicosity density which continued until a peak was reached in the levels for the 7-month-old animal.

Following stable values for the 7-month and 1.0-year-olds, a major decline in varicosities was apparent for animals aged between 1.0 and 4.5 years of age; as before the 2.5-year-old animal was the exception in this trend. The levels for the 4.5-year-old were the lowest of any animal in the present study. A clear up-turn in varicosity density seemed to have taken place between the values for the animals of 4.5 and 5.7 years of age; whilst another large increase was observed to have occurred in the 16.7-year-old animal.

Statistically significant changes (asymptotic, P < 0.05) were found in terms of varicosity density (per 5000 \( \mu m^2 \)) for the thick 5-HTergic axons in grouping A (see Figure 45B), but no significant pair-wise differences between individual groups of animals could be differentiated.

Modification of the groupings (i.e. merging groups 1 and 2), with the Kruskal-Wallis test, did not produce any increase in the significance level of changes occurring for thick 5-HTergic axons, but in fact lowered the significance level (asymptotic, P < 0.10). The same result was found, even more prominently, when an ANOVA was employed with the Bonferroni correction (P = NS).

**Varicosity density for thin 5-HTergic axons.**

The thin type of 5-HTergic axons (see Figures 43C and 44C and also Table 5B for raw data) showed clear similarities with the overall population of 5-HTergic axons in the
Figure 45. Results for age groups arranged in grouping A (the number of animals in each group is indicated): Varicosity density (per 5000 μm²) is shown for (A) all 5-HTergic axons; (B) thick 5-HTergic axons and (C) thin 5-HTergic axons. The levels of significance for each difference between age groups is shown above the appropriate bars.
Table 6A. Total varicosities per μm for 3 boxes per animal.

<table>
<thead>
<tr>
<th>Age</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>4d</td>
<td>0.692</td>
<td>0.695</td>
<td>0.670</td>
<td>±0.0079</td>
</tr>
<tr>
<td>8d</td>
<td>0.802</td>
<td>0.779</td>
<td>0.784</td>
<td>±0.0070</td>
</tr>
<tr>
<td>22d</td>
<td>0.638</td>
<td>0.731</td>
<td>0.727</td>
<td>±0.0300</td>
</tr>
<tr>
<td>37d</td>
<td>0.767</td>
<td>0.772</td>
<td>0.939</td>
<td>±0.0570</td>
</tr>
<tr>
<td>67d</td>
<td>0.366</td>
<td>0.378</td>
<td>0.424</td>
<td>±0.0180</td>
</tr>
<tr>
<td>72d</td>
<td>0.545</td>
<td>0.484</td>
<td>0.544</td>
<td>±0.0200</td>
</tr>
<tr>
<td>78d</td>
<td>0.380</td>
<td>0.555</td>
<td>0.590</td>
<td>±0.0650</td>
</tr>
<tr>
<td>5m</td>
<td>0.317</td>
<td>0.524</td>
<td>0.519</td>
<td>±0.0680</td>
</tr>
<tr>
<td>7m</td>
<td>0.621</td>
<td>0.683</td>
<td>0.664</td>
<td>±0.0180</td>
</tr>
<tr>
<td>1.0yr</td>
<td>0.591</td>
<td>0.529</td>
<td>0.628</td>
<td>±0.0290</td>
</tr>
<tr>
<td>1.5yr</td>
<td>0.592</td>
<td>0.576</td>
<td>0.655</td>
<td>±0.0240</td>
</tr>
<tr>
<td>2.3yr</td>
<td>0.282</td>
<td>0.484</td>
<td>0.497</td>
<td>±0.0700</td>
</tr>
<tr>
<td>2.5yr</td>
<td>0.653</td>
<td>0.634</td>
<td>0.659</td>
<td>±0.0075</td>
</tr>
<tr>
<td>2.8yr</td>
<td>0.212</td>
<td>0.691</td>
<td>0.668</td>
<td>±0.1560</td>
</tr>
<tr>
<td>3.0yr</td>
<td>0.279</td>
<td>0.663</td>
<td>0.728</td>
<td>±0.1401</td>
</tr>
<tr>
<td>4.5yr</td>
<td>0.609</td>
<td>0.606</td>
<td>0.517</td>
<td>±0.0030</td>
</tr>
<tr>
<td>5.7yr</td>
<td>0.474</td>
<td>0.514</td>
<td>0.565</td>
<td>±0.0260</td>
</tr>
<tr>
<td>16.7yr</td>
<td>0.730</td>
<td>0.758</td>
<td>0.731</td>
<td>±0.0092</td>
</tr>
</tbody>
</table>
time course and relative amplitude of postnatal development for their varicosity density. The density of varicosities in the early neonatal animals was quite low in the 4 day animal, whilst the 8-day-old had double this level. From the 8 day animal onwards there appeared to be a trend of decreasing varicosity density until the 67 day animal. Then for the animals aged between 67 days and 1.5 years of age there was much variability in the levels of varicosity density, but overall there was a general upward trend in values, reaching a peak in the 1.5-year-old. A sharp drop in varicosity density was evident in the 2.3-year-old animal, a pattern of declining numbers which continued for the 2.8-year-old, with the 2.5-year-old animal at a much higher value. The 2.8-year-old animal formed a low-point for a steady and consistent rise in the varicosity density for the thin class of 5-HTergic axons over the next 14 year period of adult life, as represented by the age range 2.8-16.7 years of age.

Statistically significant changes (asymptotic, P < 0.05) were seen in varicosity density (per 5000 μm²) for the thin 5-HTergic axon subclass, using grouping A. Specifically, (see Figure 45C) significant differences were seen between the 1 m-5 m and > 4.5 yrs age groups (P < 0.02) and between the 2.8 yrs-4.5 yrs and > 4.5 yrs age groups (P < 0.02).

Modification of the groupings (i.e. combining 1 and 2), with the Kruskal-Wallis test, did not appear to raise the significance level of changes occurring across development for thin 5-HTergic axons, but in fact lowered it (asymptotic, P < 0.1). Although, the use of ANOVA with a Bonferroni correction did appear to increase the overall significance level of changes occurring over postnatal development for the varicosity density of thin 5-HTergic axons (P < 0.02). The new age group (4 days-5 months) and the 2.8 yrs-4.5 yrs age group demonstrated a moderately significant level of difference (P < 0.05) and the 2.8 yrs-4.5 yrs and the > 4.5 yrs age groups illustrating no change in their level of significant difference (P < 0.02).

13.1(C).(v): Varicosities per μm of axon length for all 5-HTergic axons.

The postnatal maturation of all 5-HTergic axons (see Figure 46A and 47A and see Table 6A for raw data) in terms of the varicosities per μm of axon length did not vary greatly over development, but there did seem to be a broad difference between the neonatal animals and the rest in that the youngest animals (4-37 days of age) exhibited higher values than most of the other ages for varicosities per μm of axon length in mid-layer 3 of area 46.

No statistically significant differences were observed between any of the age groups in either grouping A (see Figure 48A; P = NS ) for varicosities per μm of axon for the total 5-HTergic axon population. However when varicosities per μm of axon length for all 5-HTergic axons for the neonatal animals (4 d-37 d of age; 0.741 ± 0.050) was compared with
Figure 46. Results for individual animals on a linear scale: Varicosites per μm of axon length is shown for (A) all 5-HTergic axons; (B) thick 5-HTergic axons and (C) thin 5-HTergic axons.
Figure 47. Results for individual animals on a logarithmic scale, illustrating proportional age intervals between animals: Varicosities per μm of axon length is shown for (A) all 5-HTergic axons; (B) thick 5-HTergic axons and (C) thin 5-HTergic axons.
Table 6B. Varicosities per μm for 3 boxes per animal.

<table>
<thead>
<tr>
<th>Age</th>
<th>Thick</th>
<th>Thin</th>
<th>Thick</th>
<th>Thin</th>
<th>Thick</th>
<th>Thin</th>
<th>SEM Thick</th>
<th>Thin</th>
</tr>
</thead>
<tbody>
<tr>
<td>4d</td>
<td>0.691</td>
<td>0.692</td>
<td>0.685</td>
<td>0.708</td>
<td>0.686</td>
<td>0.630</td>
<td>±0.0019</td>
<td>±0.024</td>
</tr>
<tr>
<td>8d</td>
<td>0.826</td>
<td>0.747</td>
<td>0.754</td>
<td>0.802</td>
<td>0.807</td>
<td>0.770</td>
<td>±0.022</td>
<td>±0.016</td>
</tr>
<tr>
<td>22d</td>
<td>0.624</td>
<td>0.654</td>
<td>0.712</td>
<td>0.748</td>
<td>0.764</td>
<td>0.693</td>
<td>±0.041</td>
<td>±0.027</td>
</tr>
<tr>
<td>37d</td>
<td>0.780</td>
<td>0.718</td>
<td>0.772</td>
<td>0.771</td>
<td>0.807</td>
<td>1.250</td>
<td>±0.011</td>
<td>±0.016</td>
</tr>
<tr>
<td>67d</td>
<td>0.384</td>
<td>0.338</td>
<td>0.370</td>
<td>0.415</td>
<td>0.437</td>
<td>0.387</td>
<td>±0.020</td>
<td>±0.023</td>
</tr>
<tr>
<td>72d</td>
<td>0.562</td>
<td>0.516</td>
<td>0.504</td>
<td>0.454</td>
<td>0.526</td>
<td>0.552</td>
<td>±0.017</td>
<td>±0.011</td>
</tr>
<tr>
<td>78d</td>
<td>0.378</td>
<td>0.389</td>
<td>0.555</td>
<td>0.554</td>
<td>0.632</td>
<td>0.573</td>
<td>±0.075</td>
<td>±0.058</td>
</tr>
<tr>
<td>5m</td>
<td>0.341</td>
<td>0.222</td>
<td>0.501</td>
<td>0.560</td>
<td>0.556</td>
<td>0.481</td>
<td>±0.064</td>
<td>±0.102</td>
</tr>
<tr>
<td>7m</td>
<td>0.665</td>
<td>0.587</td>
<td>0.700</td>
<td>0.669</td>
<td>0.657</td>
<td>0.688</td>
<td>±0.013</td>
<td>±0.031</td>
</tr>
<tr>
<td>1.0yr</td>
<td>0.621</td>
<td>0.570</td>
<td>0.520</td>
<td>0.541</td>
<td>0.627</td>
<td>0.633</td>
<td>±0.035</td>
<td>±0.027</td>
</tr>
<tr>
<td>1.5yr</td>
<td>0.594</td>
<td>0.589</td>
<td>0.606</td>
<td>0.539</td>
<td>0.690</td>
<td>0.650</td>
<td>±0.030</td>
<td>±0.032</td>
</tr>
<tr>
<td>2.3yr</td>
<td>0.302</td>
<td>0.243</td>
<td>0.491</td>
<td>0.470</td>
<td>0.521</td>
<td>0.476</td>
<td>±0.069</td>
<td>±0.077</td>
</tr>
<tr>
<td>2.5yr</td>
<td>0.663</td>
<td>0.620</td>
<td>0.607</td>
<td>0.667</td>
<td>0.665</td>
<td>0.650</td>
<td>±0.019</td>
<td>±0.014</td>
</tr>
<tr>
<td>2.8yr</td>
<td>0.239</td>
<td>0.185</td>
<td>0.691</td>
<td>0.691</td>
<td>0.720</td>
<td>0.562</td>
<td>±0.156</td>
<td>±0.152</td>
</tr>
<tr>
<td>3.0yr</td>
<td>0.317</td>
<td>0.223</td>
<td>0.675</td>
<td>0.652</td>
<td>0.690</td>
<td>0.765</td>
<td>±0.122</td>
<td>±0.165</td>
</tr>
<tr>
<td>4.5yr</td>
<td>0.573</td>
<td>0.641</td>
<td>0.689</td>
<td>0.603</td>
<td>0.503</td>
<td>0.503</td>
<td>±0.054</td>
<td>±0.041</td>
</tr>
<tr>
<td>5.7yr</td>
<td>0.485</td>
<td>0.455</td>
<td>0.493</td>
<td>0.530</td>
<td>0.602</td>
<td>0.553</td>
<td>±0.038</td>
<td>±0.030</td>
</tr>
<tr>
<td>16.7yr</td>
<td>0.712</td>
<td>0.762</td>
<td>0.788</td>
<td>0.723</td>
<td>0.737</td>
<td>0.727</td>
<td>±0.022</td>
<td>±0.012</td>
</tr>
</tbody>
</table>
that for all other animals (67 d-16.7 yrs of age; 0.536 ± 0.010), (see Figure 49A) a statistically significant difference was found (P < 0.005). This difference represented a significant drop of 28% in varicosities per μm of axon between the youngest animals and the rest of the animals which occurred between 37 and 67 days of age.

13.1(C).(vi): Varicosities per μm of axon length for thick and thin 5-HTergic axons.

The maturational time course and levels of varicosities per μm of axon length were very similar for both thick (see Figures 46B and 47B and see Table 6B for raw data) and thin (see Figures 46C and 47C and see Table 6B for raw data) 5-HTergic axons and so exhibited very similar temporal changes to those seen for the overall 5-HTergic axon population in mid-layer 3 of area 46.

Again no statistically significant differences between age groups were seen for grouping A (see Figures 48B and 48C; thick: P = NS and thin: P = 0.093). However when varicosities per μm of axon length for the two 5-HTergic axon sub-populations in the neonatal animals (4 d-37 d of age; thick: 0.759 ± 0.048; thin: 0.722 ± 0.055) were compared separately (see Figures 49B and 49C) to that for all other animals (67 d-16.7 yrs of age; thick: 0.542 ± 0.026; thin: 0.530 ± 0.029), statistically significant differences were found for both thick (P < 0.01) and thin (P < 0.01) 5-HTergic axons. These differences represented significant drops of 29% and 27%, in varicosities per μm of axon, respectively for the thick and thin 5-HTergic axon populations.
Figure 48. Results for age groups arranged in grouping A (the number of animals in each group is indicated): Varicosities per μm of axon length is shown for (A) all 5-HTergic axons; (B) thick 5-HTergic axons and (C) thin 5-HTergic axons. There were no significant differences between age groups for varicosities per μm of axon length during postnatal development.
Figure 49. Results for age groups comparing animals aged between 4-37 days with the rest of the animals (>37days) in the set (the number of animals in each group is indicated): Varicosities per μm of axon length is shown for (A) all 5-HTergic axons; (B) thick 5-HTergic axons and (C) thin 5-HTergic axons. The levels of significance for each difference between age groups is shown above the appropriate bars.
14.0: Discussion

Study 2: A quantitative immunohistochemical study of the postnatal development of 5-HTergic axons in mid-layer 3 of the ventral bank of area 46 in the monkey DLPFC.

14.1: Reliability of the immunohistochemical technique in animals of different ages as a sensitive indicator of the changing levels of serotonergic axons during postnatal development.

As evident from the results above, we saw statistically significant differences between age groups across postnatal development for the 5-HTergic innervation in mid-layer 3 of area 46. There is however a concern regarding the validity of using results obtained from animals of different ages to attempt to reconstruct or recreate the time-course that might be expected to occur within the postnatal life of any one individual animal.

There are several pieces of technical evidence which we can offer in support of the contention that the majority of the changes which we see over age in this study represent a realistic temporal reconstruction of the changes in the density of the 5-HTergic innervation that are usually encountered within the lifetime of an individual animal and not the result of artifactual interference produced during the immunohistochemical tissue processing.

These points are:

1. The anti-rabbit 5-HT-specific antibody employed in this study has been used in previous studies by other investigators in animals of both different species and ages, including monkeys (Fischer et al, 1995; Smiley and Goldman-Rakic, 1996), cats (Gu et al, 1990; Mower, 1991) and rats (MacClean and Shiple, 1987a,b; Rhoades et al, 1990; Bennett-Clarke et al, 1991; Blue et al, 1991; Bennett-Clarke et al, 1994b; Osterheld-Haas et al, 1994). Control experiments show no detectable immunoreaction product when either the primary or secondary antibodies are omitted from the processing (replaced with normal rabbit serum) or the primary is reacted with an excess of 5-HT-bovine serum albumin conjugate (the molecule to which the antibody is directed) for the immunohistochemical reaction (e.g. Bennett-Clarke et al, 1991; Blue et al, 1991; Mower, 1991; Smiley and Goldman-Rakic, 1996). This demonstrates conclusively that the anti-5-HT antibody is specific for the 5-HT molecule only.

2. Two separate experimental 5-HT immunohistochemical reactions were carried out on different occasions, in each case sections from all 21 animals were included. Both sets of sections were qualitatively examined on separate occasions, by 2 investigators (D. Lewis and C. Kye for series 1 and D. Lewis and J. Classey for series 2: the one used in the quantitative analysis) with no knowledge of the tissue’s origin or animal age, so that each set was ranked
in order of the increasing density of 5-HT immunoreactive axons in area 46. In both cases the rank-order of sections for 5-HTergic axons tallied almost exactly - a few exceptions occurred when the densities of the innervation were very similar at some ages - with the order of ages derived from the quantitative analysis (also performed without knowledge of the age or number of the animal) as described in this thesis.

3. In both separate immunohistochemical experiments all sections were run together at the same time although not all in the same reaction receptacle, but sections from animals of diverse ages, chosen randomly, e.g. 4 days, 1.0 year and 16.7 years were reacted in the same wells and hence were exposed to the same experimental reaction conditions. Thus there was no opportunity for artificially-induced age differences to occur, which might have resulted from differences in the quality of the exposure to the immunoreaction if animals of all young ages or all old ages had been reacted together in separate groups.

4. Immunohistochemical experiments for different antibodies have previously been conducted in closely adjacent sections from the same age series of animals in areas 9 or 46 of the DLPFC, for dopaminergic axons and varicosities (Rosenberg and Lewis, 1995), CCK neurons (Oeth and Lewis, 1993) and for axons and varicosities immunoreactive for the 5-HT transporter or for the 5-HT-synthetic enzyme tryptophan hydroxylase (Kye and Lewis unpublished observations). In all of these studies, quite distinct sequences of changing levels of immunoreactivity could be visualised by employing antibodies specific to the particular molecules, e.g. CCK versus 5-HT. These previous observations suggest that there is no reason to suppose that any particular experimental parameters or idiosyncrasies (e.g. variable fixation time, use of different fixatives, i.e. paraformaldehyde or glutaraldehyde; thickness of sections; differences in antibody penetration due to increasing myelination with age) could be expected to have adversely affected the levels of immunostaining found for any of the animal tissue.

We have made the assumption on the basis of these 4 different lines of evidence, that the observed changes in levels of 5-HTergic axons and varicosities during the course of postnatal development are real and represent the closest possible indication of the actual levels that were present in each individual animal. We suggest that these levels would be found at these ages over the course of the lifetime for single animals. However, behavioural studies in adult male monkeys (Fontenot et al, 1995) show that 5-HT and 5-HIAA tissue levels measured in the frontal pole (area 10 and rostral area 46) of the PFC are significantly lower in animals who experienced past-stress (15-28 months ago) compared to those who have lived under no-stress social conditions prior to sacrifice, whilst animals experiencing
recent-stress (1-14 months ago) were intermediate in their 5-HT and 5-HIAA levels.

This kind of observation may be relevant to the 2.5-year-old animal, which consistently demonstrated seemingly abnormally high values for total axon length, varicosity density or varicosities per µm of axon, compared to its peers on either side of the time scale. However this animal was not alone in illustrating high values as some of the older animals (15.9-18.0 years of age) also exhibited levels of a very similar magnitude; the question thus arises as to whether all 4 of these animals may have been subject to social conditions which distinguished them from the rest of the age series or if just the 2.5 year old is genuinely exceptional in its 5-HT system compared to the other animals of a similar age.

14.2: Summary of Results.

The time course of development for the 5-HTergic innervation of layer 3 in the monkey DLPFC was found to represent a complex set of changes over time - expressed as a schematic curve; see Figure 50A, derived from the values plotted in Figure 41A - e.g. it did not show a simple linear or exponential relationship with increasing age. The key finding in terms of a steady set of progressive changes in the 5-HTergic innervation across development was in the levels of mean total varicosity density (per 5000 µm^2), i.e. derived from the multiple of mean varicosities per µm of axon length and the mean total axon length (in µm) for 3 sampling frames for each animal. The changes in the number of varicosities (potential release sites of 5-HT) present on the sampled population of 5-HTergic axons, were of a cyclic nature, with peaks and troughs in the population distribution across time, which were representative of the values for groups of animals at particular stages of postnatal development (see Figure 50A).

The most significant differences observed during development for total varicosity density (per 5000 µm^2) of 5-HTergic axons were exhibited by the large increases in values from the low values of the peri-/post-pubertal animals (2.8 yrs-4.5 yrs) and the infants (1 m-5 m) to the high values seen in the adults (5.7 yrs-16.7 yrs). Also significant was the change from the low values of the peri-/post-pubertal (2.8 yrs-4.5 yrs) animals to the high values of the juvenile/adolescent (7 m-2.5 yrs) animals.

We concluded - using the Spearman rank correlation coefficient - that the two 5-HTergic axon types - previously observed in other studies in monkeys and other species (see Serotonin review section 7.4) - were justified in being described as separate sub-populations. We show that each population possibly contributes somewhat differently to
Figure 50. Schematic summary curves illustrating the percentage of maximum and minimum values for varicosity density of all 5-HTergic axons in mid-layer 3 during postnatal development, with a comparison to the spine density on mid-layer 3 pyramidal neurons. (A) Varicosity density for all 5-HTergic axons (per 5000 μm²), (B) Varicosity density (per 5000 μm²) and spine density (per μm).
Figure 51. Schematic summary curves illustrating the percentage of maximum and minimum values for varicosity density of thick and thin classes of 5-HTergic axons in mid-layer 3 during postnatal development. Varicosity density (per 5000 μm²) for (A) thick and (B) thin 5-HTergic axons.
the overall changes observed for the maturation of the serotonergic axon innervation during postnatal development, as compared to their summed total for the 5-HTergic axon system over the same period. The possibly differential contribution of the individual axon sub-populations to the time course and pattern of maturation for the overall 5-HTergic axon innervation, could be indicative of diverse functional effects on cells postsynaptic to 5-HTergic axons. This would presumably be supported by virtue of the existence of multiple subtypes of 5-HT receptors, but also the presence of 5-HTergic axons exhibiting variably-sized 5-HT-filled varicosities would conceivably provide a differential mechanism by which to regulate the rate and magnitude of 5-HT release at various times both tonically, i.e. over a matter of seconds or minutes, as well as phasically, i.e. over a longer time period of hours or even days.

Of the significant differences in varicosity density exhibited by the total 5-HTergic axon population during postnatal development, two in particular coincided with significant changes in the varicosity density of the thin varicose 5-HTergic axons. These were observed for the low values of the infant animals and high values of the adults and between the low values of the peri-/post-pubertal (2.8-4.5yrs) and the high values of the adult animals (5.7-16.7yrs) expressed as an extrapolated curve (see Figure 51B). No statistically significant changes were observed for the varicosities of thick 5-HTergic axons during postnatal development (see Figure 51A), however, there were observed to be marked differences between the levels of varicosity density in the high values of the juvenile/adolescent animals (7m-2.5yrs) and the low values of the peri-/post-pubertal animals (2.8-4.5yrs) and also between the latter and the high values of the adults (5.7-16.7yrs).

Slight lags in the rate of change for varicosity density (per 5000 µm²) discerned between the two 5-HTergic axon sub-populations during postnatal development, hint that perhaps the form and magnitude of 5-HT release by the two 5-HTergic axon populations, defined in this thesis study, may vary at different times, with shifts between one or other population having the dominant modulatory influence over circuitry during particular stages of development. This hypothesis would also have to take into account the existence of the many 5-HT receptor subtypes some of which are known to be localised to different sub-cellular sites on the same class of neurons and/or in the same sub-cellular location for a variety of neuron populations and that the properties of these receptors may vary at different stages of postnatal development in both their levels of density and also in their degree of functionally active state.
Serotonergic $2_A$ receptors have been shown to be located both on 5-HTergic axon terminals (as autoreceptors) and glutamatergic afferent terminals (as heteroreceptors), on the cell bodies of GABAergic interneurons and also on the distal dendrites and cell bodies of pyramidal neurons in adult rats, monkeys and humans - see Serotonin review section 7.9(A).(ii) - while 5-HT$2_C$ receptors have been localised to pyramidal neurons and unidentified axon terminals in adult rats and humans - see Serotonin review section 7.9(G).(ii).

The functionally active state of 5-HT receptors has been observed to vary in the developing rat hippocampus (Ike et al, 1995), as investigated with pharmacological techniques. Specifically, between the first and third week of postnatal development the identity of the functionally active 5-HT receptor ($r$) subtype linked to the 5-HT-induced phosphoinositide (PI) hydrolysis effector pathway was observed to switch from 5-HT$2_A$ to 5-HT$2_C$.

It is possible that 5-HT$2_A$ and 5-HT$2_C$ receptors in the primate DLPFC may vary in their functionally active states during the course of various stages of development, such that the variations in the levels of 5-HT release or storage occurring at different ages, as indicated by the changes in varicosity density and axon length observed in this study may have relatively distinct effects at different stages of the postnatal period directly at 5-HT$2_A$ on pyramidal neurons or indirectly on pyramidal neurons via action at 5-HT$2_C$ localised to GABAergic interneurons (see Concluding Discussion section 15.0, for implications in schizophrenia).

The 5-HT system in the DLPFC has the potential for achieving a variety of modulatory effects on pyramidal neuron and interneuron activity, via different 5-HT receptor subtypes expressed specifically on the surfaces of different classes of neurons and on incoming thalamocortical and corticocortical afferents and those of other neurotransmitter systems (DAergic, NAergic and AChergic). The existence of indirect or direct actions of 5-HT on mid-layer 3 pyramidal neurons in the DLPFC, by way of diverse and perhaps temporally expressed receptor subtypes, may be integral to the prolonged maturational time course for pyramidal neuron spine acquisition and attrition seen in this cortical region during postnatal development in both monkeys and humans.

14.3: Comparison of the time courses of maturation for 5-HTergic axons and those for other neurotransmitter and neural components in layer 3.

Another rationale for testing the validity of the data for the 5-HTergic axon study was to directly compare the results with those of the other studies mentioned previously, which were carried out using material from all or part of the same age series of animals in area
46 of the monkey DLPFC. The premise being that if there are temporal correlation’s between the time courses of some but not every cellular or axonal chemical marker in layer 3 during postnatal development, it is very probable that the differences observed between animals of different ages actually do exist in vivo and are in such a temporal alignment due to their intimate relationship with or participation in common events occurring at certain developmental stages. Comparing the timing of the changes in the levels of 5-HT varicosity and pyramidal neuron spine density across postnatal development, we concluded that there is a clear relationship between the 2 time courses, such that a close functional interaction is likely to exist between them, with 5-HT released from 5-HTergic varicosities acting via the specific 5-HT receptor subclass located on dendritic spines.

14.3(A).(i): Comparison of the postnatal time courses for 5-HTergic varicosity density (per 5000 μm²) and pyramidal neuron spine density.

We find that there is some correlation between the timing of changes in total 5-HTergic varicosity density (per 5000 μm²) for all 5-HTergic axons and those for mean spine density in layer 3, with a fall in total 5-HTergic axon varicosity density occurring during the climbing phase of spine density, recovery of the levels of 5-HTergic varicosity density as spine density values plateau and both measures decline concurrently at around puberty.

As well as similarities during the time courses (see Figure 52A) there was a disparity in the development of thick and thin 5-HTergic axons compared to that of spine density, as the varicosity density of thin 5-HTergic axons climbed immediately after birth to match that of the already higher thick 5-HTergic varicosity density.

14.3(A).(ii): Significance of the postnatal time courses for 5-HTergic varicosities per μm of axon and pyramidal neuron mean dendritic spine density.

This inverse temporal correlation in the first 10 weeks of postnatal development is hard to account for on the basis of direct interactions, since the majority of 5-HTergic varicosities in layer 3 of area in the adult monkey do not seem to form conventional synapses and those that do - on average about 23% of 5-HTergic varicosities at EM level - contact the relatively spine-free dendrites of GABAergic interneurons (Smiley and Goldman-Rakic, 1996). Since EM studies of 5-HTergic axons have not so far been conducted in younger animals, it is not known how the microanatomy of the 5-HTergic innervation may be organised in the developing monkey between birth and adulthood. If the majority of the small numbers of synapses formed by 5-HTergic axon varicosities are in fact primarily
associated with GABAergic interneurons even during early postnatal development, they may be in a position to indirectly regulate the acquisition of pyramidal neuron dendritic spines via disinhibitory effects on those interneurons (chandelier and basket neurons) which have inhibitory action on pyramidal neuron activity levels.

Different 5-HT receptor subtypes have been localised to various sites on cortical pyramidal neurons in a variety of adult mammalian species, which would certainly provide 5-HT with the means to play an intimate role in the maturational development of these ubiquitously found cells. Despite the evidence for very little direct synaptic interaction between 5-HTergic axon varicosities and pyramidal neurons in the monkey DLPFC the expression of these 3 distinct 5-HTr subtypes on different and in some cases the same portions of pyramidal neurons in the adult - and presumably during postnatal development of the - DLPFC provides an indirect means for 5-HT to significantly influence particular aspects of pyramidal neuron activity for large number of cells over a large area of cortex in quick succession. It is presumed that each receptor subtype has its own unique functional role (via effector or signal transduction pathways) in modulating the activity of pyramidal neuron output (excitatory postsynaptic action potentials) and input (afferent "drive" or inhibitory or excitatory presynaptic potentials).

Several 5-HTr subtypes have been localised to inhibitory interneurons in the same regions and species as those 5-HTr's expressed by pyramidal neurons. Specifically, 5-HT_{1A}r's have been found on the cell bodies of various kinds of non-pyramidal cells in rat cerebral cortex, 5-HT_{2A}r's are located on the cell bodies of GABAergic interneurons in the rat neocortex and in the monkey DLPFC, 5-HT_{3}r's are present on the cell bodies of GABAergic basket interneurons in the rat neocortex (mainly PV-IR), 5-HT_{4}r's can be observed on the cell bodies and dendrites of non-pyramidal cells in the hippocampus and have been detected in the monkey (substantia nigra, striatum) and human (frontal cortex) brain and thus may at least be present in human cortical interneurons. This distribution of 5-HTr subtypes on GABAergic interneurons which are known to form extensive synaptic contacts with the cell bodies, AIS's and dendritic spines and shafts of pyramidal neurons in the adult monkey DLPFC and to have a strong inhibitory modulatory influence on their activity, provides another means by which the 5-HTergic axons in the DLPFC can potentially indirectly modulate pyramidal neuron activity in the DLPFC, i.e. by disinhibitive effects on GABAergic interneuron output to pyramidal neurons.
Figure 52. Schematic summary curves illustrating the percentage of maximum and minimum values for varicosity density of thick and thin 5-HTergic axons with a comparison to the spine density on mid-layer 3 pyramidal neurons and with the varicosity density for DAergic axons in deep layer 3. (data from Rosenberg and Lewis, 1995). (A) Varicosity density (per 5000 μm²) for thick and thin 5-HTergic axons compared to spine density (per μm), (B) Varicosity density (per 5000 μm²) compared for all 5-HTergic and DAergic axons.
14.3(B): Comparison of the postnatal maturation of the 5-HTergic axon innervation in mid-layer 3 and PV-IR neurons and PV-IR axon cartridges.

Two classes of local circuit neuron, i.e. chandelier and basket neurons, have been shown to express the calcium-binding protein parvalbumin (PV) in comparative Golgi and immunocytochemical studies in the monkey prefrontal cortex (Lewis and Lund, 1990; Lund and Lewis, 1993; Condé et al, 1994). The axons of chandelier neurons in Golgi preparations are seen to form characteristic termination's, i.e. "cartridges" composed of many varicosities targeting the axon initial segments (AIS) of un-impregnated pyramidal neurons in DLPFC (Lewis and Lund, 1990; Lund and Lewis, 1993, see Neurotransmitters review section 6.5(B), for details on earlier studies in other cortical areas). Golgi-impregnated and PV-IR wide arbour or "basket" neurons have been described in the monkey DLPFC, but were only visualised in infant animals (Lund and Lewis, 1993).

Another immunocytochemical study at both light microscope and EM level in area 46 of adult monkey PFC (Williams et al, 1992) has demonstrated the presence of PV-IR basket neurons and also the characteristic pericellular cluster arrangements of their axon terminals which surround and form symmetric synaptic contacts with the cell body and the proximal dendrites of pyramidal neurons in layers 2 and 3, as previously only seen at light microscope level in visual (layers 5 and 6) and motor (layers 3 and 5) regions of the cerebral cortex (Hendry et al, 1989; Akil and Lewis, 1992a,b).

A third subtype of PV-IR interneuron has also been hypothesised to play a role in the local inhibitory circuitry of the DLPFC, namely the short axon multipolar cell, which is likely to be the source of many of the symmetric synapses formed between PV-IR varicosities and the dendritic shafts and spines of presumed pyramidal cells seen at EM level in layers 2 and 3 of area 46 (Williams et al, 1992).

Recently, it has been shown that varicosities of 5-HTergic axons form synaptic contacts with the somata of PV-IR neurons at EM level in area 46 of the adult monkey DLPFC (Jakab and Goldman-Rakic, 1996). From the evidence of 5-HTergic axon interactions with PV-IR interneurons, it might be expected that the time course of maturation for a sub-population (perhaps thick or thin classes) of 5-HTergic axons in mid-layer 3 would show a partial similarity to the postnatal time course for the overall numbers of PV-IR neurons in layers 2 and 3 of area 46, the limitation being that it is not known yet which of the 3 subclasses of PV-IR neuron might be contacted by 5-HTergic axons.
14.3(C): Comparison of the postnatal time courses for the maturation of 5-HTergic and DAergic axons in mid-layer 3.

The time course of maturation for DAergic axons during postnatal development (from the data of Rosenberg and Lewis, 1995) has been described already in the Spine study discussion section 11.2(C). Here we will confine ourselves to a comparison of the changes in the levels of DAergic axons and varicosities during development relative to that for 5-HTergic axons and varicosities.

14.3(C).(i): Comparison of the varicosity density (per 5000 μm²) for 5-HTergic and DAergic axons.

The time course of postnatal maturation of 5-HTergic axon varicosity density (per 5000 μm²) appears to bear a reciprocal relationship to that of DAergic axon varicosities in layer 3 of area 46 (from Rosenberg and Lewis, 1995; see Figure 52B).

In order to test the degree of significance of the reciprocal pattern shown by the time courses for the varicosity density (per 5000 μm²) of 5-HTergic and DAergic axons (see Figure 52B), it was decided to compare the data from the 5-HT study in both the age groups used in the DA study (Rosenberg and Lewis, 1995; i.e. grouping B mentioned in the Results section 13.1(C), as well as in age grouping A used for the 5-HT results in this thesis. The results for 5-HTergic axons in the above modified DA age grouping B can be summarised as follows (see Figure 53A): No statistically significant changes (F-ratio = 0.702, P = NS) were observed in the varicosity density (per 5000 μm²) of the total 5-HTergic axon population during postnatal development. Neither were there any significant differences between age groups for the thick (F-ratio = 0.199, P = NS) or thin (F-ratio = 2.058, P = NS) 5-HTergic axon subclasses during the postnatal period (not shown).

The data for the complete set of animals used in the Rosenberg and Lewis (1995) study were modified by removing the measures for the 4 animals not used in the 5-HT study (mentioned in the Results section, 12.1(C). The consequences of this reduced number of subjects on the DAergic axon varicosity density (per 5000 μm²) measures during development can be summarised as follows (see Figure 53B): There was a significantly higher (P < 0.05) varicosity density (per 5000 μm²) for the 2-3yrs age group than the < 1m age group. Also there was a markedly (P < 0.10) higher level of varicosity density (per 5000 μm²) between 2-3yrs than for the 1-3m age group, which was just outside the α = 0.05 significance level used in this thesis. The use of only those 18 animals used in both the DA and 5-HT studies has lowered the significance levels of those differences between the high
Figure 53. (A). The results for varicosity density (per 5000 \( \mu \text{m}^2 \)) of all 5-HTergic axons in mid-layer 3 from the present study, re-plotted in the age groups used in the original DA study (Rosenberg and Lewis, 1995). No statistically significant changes over age were apparent for the 5-HTergic axons using age grouping B. (B). The results for DAergic axon varicosity density (per 5000 \( \mu \text{m}^2 \)) from the original DA study (Rosenberg and Lewis, 1995), re-plotted for this present study using the age groups from that study, using only the 18 animals used in both DA and the 5-HT studies.
Figure 54. (A). A comparison of the percentage maximum-minimum range of values for varicosity density of all 5-HTergic axons (present study) and the re-plotted data from the original DA study (Rosenberg and Lewis, 1995). (B). The results for DAergic axon varicosity density (per 5000 μm²) in their re-plotted format (from Rosenberg and Lewis, 1995) arranged in the age groups used in the present 5-HT study, for only the 18 animals used in both studies.
varicosity density (per 5000 μm²) of the 2-3yrs age group and the < 1m age group. Also the highly significant levels in the differences for the other age groups and the 2-3yrs age group have been rendered non-significant. This transformation illustrates the limitations of attempting to compare the data from the 5-HT and DA studies in the age grouping B used in the DA study (Rosenberg and Lewis, 1995).

The transfer of the data for DAergic axon measures of varicosity density during development (retaining all animal ages) to the age groupings used in the present study of 5-HTergic axon varicosity density, has in fact retains the high levels of significance for the differences in DAergic varicosity density between age groups observed at various stages of development in the original study (Rosenberg and Lewis, 1995). This evidence suggests that as well as being an ideal grouping for changes in 5-HTergic axons across development, age grouping A may permit a more representative assessment of the changes occurring for DAergic axons during the same time period than for age grouping B from the original DA study (Rosenberg and Lewis, 1995).

For instance, the differences between the high levels of varicosity density in the 2.8-4.5 years age group compared to the lower values of the < 1m, 1-5m and 7m-2.5yrs age groups are of greater significance than the nearest equivalent groupings in the original study - although in the latter the 2-3yrs age group was significantly higher (P < 0.05) than all the other age groups across development, these were of lower significance levels than for the reorganised groupings here. In addition, this regrouping has brought 2 other differences between age groups to significance levels, that were evident but not significant in the original study, namely the higher levels at 7m-2.5yrs than < 1m and also the higher value for the 2.8-4.5yrs age group compared to that between 5.7-18.0 yrs.

Removal from this analysis of the 4 animals not utilised in the 5-HT study (see Figure 54A), in order to directly compare the 2 sets of data for the 5-HT and DA studies results in a slight drop in the significance levels (F-ratio = 13.018, P < 0.0001) of the above age group differences for the varicosity density of DAergic axons during development. There are slight reductions in the significance levels for the higher value of varicosity density (per 5000 μm²) in the 7m-2.5yrs (P < 0.05) age group than in the < 1m age group and for the higher values at 2.8-4.5yrs compared to the 1-5m (P < 0.05) and 5.7-16.7yrs (P < 0.005) age groups. There is no change in the level of significantly higher varicosity density (per 5000 μm²) between the 2.8-4.5 and < 1m age groups (P < 0.0001). Overall the differences observed above for all ages used in the DA study between the values of varicosity density for DAergic axons are reasonably robustly conserved, even when the 4
Figure 55. Schematic summary curves illustrating the percentage of maximum and minimum values for tissue concentrations of 5-HT and DA in the PFC (data plotted for thesis from Goldman-Rakic and Brown, 1981, 1982) compared and the varicosity density of all 5-HTergic axons in mid-layer 3 compared to the tissue concentration of 5-HT in the PFC. (data from Goldman-Rakic and Brown, 1981, 1982) (A) 5-HT and DA tissue concentrations (nmol/g), (B) Varicosity density (per 5000 μm²) and 5-HT tissue concentration (nmol/g).
animals not used in the 5-HT study are omitted.

These observations provided a strong basis on which to directly compare the data for the 5-HT and DA studies in identical age groups with varicosity density values derived from analysis of tissue obtained from exactly the same animals. Comparing Figures 52B and 54B, it is possible in both to see a basically inverse relationship between the percentage of maximum and minimum values for the levels of varicosity density (per 5000 \( \mu m^2 \)) for 5-HTergic and DAergic axons during development. Thus at < 1m, 5-HT value is high and DA is low, at 1-5m the DA value is high, whilst the 5-HT one is low, for 7m-2.5yrs the 5-HT level is high again and DA is about the same.

The above rearrangements of the DA study data (from Rosenberg and Lewis, 1995) into age groupings appropriate for a comparison with the 5-HT results have shown that a strong degree of reciprocity exists between the relationship of the time courses of development for the DAergic and 5-HTergic axon populations in the monkey DLPFC. In this new format this relationship is more obvious than could be observed for the original DA data and the 5-HT study data separately. In addition the replotting of the DA study for both all its original animal ages and without those omitted in the 5-HT study, reveals a greater level of significance for the differences in DAergic axon varicosity density at various stages across development in the monkey DLPFC.

We also examined the degree of reciprocity between the time courses of development for the 5-HTergic and DAergic axon populations in Figure 52B. As we used in the comparison of thick and thin 5-HTergic axons in section 14.2 (see for rationale behind test), the Spearman rank correlation coefficient was employed to examine the relative strength of the temporal relationship between the 2 axon populations. In terms of total varicosity density (per 5000 \( \mu m^2 \)), for the 5-HTergic and DAergic axon populations, \( r_s = -0.362 \) (-36%; \( P = NS \)), i.e. there was greater evidence for a reciprocal relationship than one of concurrence, between 5-HTergic and DAergic axons during development. There was a much greater degree of reciprocity between the 2 neurotransmitter axon populations for total varicosity density compared to total axon length. The difference in rankings for the two axon populations, in terms of total axon length (in \( \mu m \); per 5000 \( \mu m^2 \)), was \( r_s = -0.117 \) (\( P = NS \)). Since \( r_s = -1.0 \), represents absolute reciprocity between 2 related populations, it can be only be tentatively concluded, that there is a greater degree of reciprocity (-12%), than of concurrence between the time courses of 5-HTergic and DAergic axons during postnatal development.
A comparison of the tissue concentration of 5-HT (Goldman-Rakic and Brown, 1981, 1982) and the varicosity density for 5-HTergic axons in the DLPFC (from this thesis; see Figure 55B) would suggest that the changes in density of 5-HT varicosities is the converse of the changes in the tissue levels of the neurotransmitter 5-HT itself. This suggests that high levels of 5-HTergic immunoreactivity in varicosities might reflect a local sequestering of 5-HT; as the levels of 5-HTergic varicosities decline, the tissue levels of 5-HT increase and vice versa, throughout the course of development and during normal adulthood.

A comparison of the developmental time courses for thick 5-HTergic axons and DAergic axons in terms of varicosity density (per 5000 µm²) using the Spearman rank correlation coefficient test, resulted in \( r_s = -0.441 \) (-44%; \( P < 0.05 \), 1-tailed; \( P = NS \), 2-tailed). The same comparison made between thin 5-HTergic and DAergic axons yielded a value of \( r_s = -0.406 \) (-41%; \( P < 0.05 \), 1-tailed; \( P = NS \), 2-tailed). In terms of axon length (in µm, per 5000 µm²), the value for thick 5-HTergic and DAergic axons was \( r_s = -0.313 \) (-31.0%; \( P < NS \), 1-tailed; \( P < NS \), 2-tailed), whilst the value for thin 5-HTergic and DAergic axons was \( r_s = 0.081 \) (+8%; \( P = NS \), 1-tailed; \( P = NS \), 2-tailed).

A similar finding of a reciprocal relationship between the time courses of 5-HT and DA system development seen in this thesis, was observed by Goldman-Rakic and Brown (1982) in their study of the postnatal maturation of monoamine tissue concentration levels in the monkey cerebral cortex (including the PFC). However, Goldman-Rakic and Brown (1982) found that the concentrations of 5-HT and DA tissue concentration were only inversely related in the youngest and oldest age groups (i.e. new-borns and 2.0-3.0 years of age) while there was a synchrony in the levels for the intervening age groups (see Figure 55A).

Goldman-Rakic and Brown (1982) found there to be a striking reciprocity in the rates of production of serotonin (as measured by 5-HTTP production) and dopamine (as measured by DOPA production). They stated that "a consistent finding at all ages is that the distribution of catecholaminergic (i.e. DA and noradrenaline) synthesis varies inversely with that of serotoninergic synthesis, indicating substantial interaction in the regulation of the two cortical systems."

In a study of the tissue concentrations of monoamines in adult and ageing monkeys Goldman-Rakic and Brown (1981) found that as for the developing animals, there was some evidence of reciprocity in the levels of 5-HT and DA over senescence, mostly due to relatively stable high levels of 5-HT and a significant decrease in the levels of DA in the 2
oldest groups of animals (see Figure 55A). Goldman-Rakic and Brown (1981) stated that there appeared to be no evident change in 5-HT levels throughout adulthood and neither did the levels of 5-HTP change significantly between 2.0-3.0 years and > 18.0 years of age.

In the present thesis study, however the levels of background neuropil 5-HT immunoreactivity (presumably representing tissue or extracellular levels of 5-HT released prior to the animals' perfusion) were proportionally related to the levels of 5-HT present within the axon innervation of layer 3. Thus, high neuropil levels of 5-HT were more often than not accompanied by high levels of 5-HT stored in axons and varicosities. These observations are the converse of that expected from the Goldman-Rakic and Brown studies, above.

Much more needs to be known regarding the mechanisms in vivo, which regulate and control the rates and absolute levels of turnover for 5-HT, i.e. its release, synthesis and re-uptake and what relative rates are expressed in the mid-brain cell synthesis, compared to levels in their cortical axon varicosities and release into cortical tissue. Many questions still remain unanswered as to what immunohistochemical findings in adults, as well as in developing animals may mean in terms of functional activity and modulatory influence. For now, in the absence of convincing evidence to the contrary, we shall presume that the high levels of 5-HT present in axons and varicosities at different ages in the DLPFC is representative of potentially high levels of release and availability of the neurotransmitter at that stage of development. These are believed to change dynamically over time, with regard to many variables in the short-term such as stress and physiological demands, as well as more long-term influences such as genetic predisposition, rearing conditions (again stress has an influence here) and any other effects such as the animal's diet, sexual development, other endocrine influences and history of disease.

Despite the apparent observation of concurrent tissue levels of 5-HT and DA during most of the pre-adult postnatal period (concerning relations to the development of other cortical components; i.e. 10 weeks to 4 years of age) by Goldman-Rakic and Brown (1981, 1982), there is compelling evidence from other studies in the adult monkey to suggest that 5-HT and DA exhibit a reciprocal relationship in both the relative distribution of their tissue levels (Brown et al, 1979) and axons (Lewis et al, 1985; Berger et al, 1986, 1988; Lewis, 1990; Schwartz and Mrzljak, 1992) in cortical and subcortical areas. This suggests that 5-HT and DA may play complementary roles in various aspects of behaviour which are likely to involve multiple brain regions.

The innervation of cortical regions by DAergic and 5-HTergic axons may show
a complementary laminar organisation, most notably in the DLPFC - Berger et al (1988); Lewis (1990); compare Lewis (1988) or Williams and Goldman-Rakic (1993) with Wilson and Molliver (1991a). This implies that the most dense portions of these innervations are likely exhibit their most potent modulatory influences over classes of neurons located in different layers, each with specific patterns of afferent projection targets and reciprocal incoming afferents. Also both DA and 5-HT demonstrate mutually inhibitory and excitatory modulation of each other's release via presynaptic or non-synaptic release-site heteroreceptors (e.g. 5-HT receptors sited on DAergic axon terminals and vice versa) and also via neuronal, i.e. somato-dendritically-located receptors - see Neurotransmitter review sections 6.1(E) and 6.2(E).

The significance of the variations in the tissue levels of 5-HT and DA in the early part of postnatal development and throughout adulthood (Goldman-Rakic and Brown, 1981, 1982) can be examined in the light of pharmacological studies conducted with 5-HT and DA receptor antagonists and agonists in the medial PFC of adult rats (Pehek, 1996) and the neostriatum in baboons (Dewey et al, 1995) and humans (Tiihonen et al, 1996; Smith et al, 1997). These studies have concluded that complex interactions exist between the levels of 5-HT and DA in both the DLPFC and the neostriatum, which are presumed to be mediated via the binding of each neurotransmitter to its variously located receptor subtypes. There thus exists indirect pharmacological evidence (see above studies) that 5-HT and DA can reciprocally modulate each others release, by way of the heteroreceptors located on the others axon varicosities.

The evidence from 5-HTr antagonist studies in the rat medial PFC (Nomikos et al, 1994; Pehek et al, 1996) is that 5-HT may inhibit intrinsic cortical DA release by its action at 5-HT2A's located on DAergic axons. Other studies comparing the effects of direct administration of 5-HT and 5-HTr agonists on DA release, usually demonstrate increased extracellular levels of DA, via the activation of 5-HT1B's or 5-HT3's located on DAergic axon varicosities in the anterior neostriatum (Benloucif and Galloway, 1991; Benloucif et al, 1993).

The PET receptor-binding studies of 5-HT and DA interactions in humans have been restricted to an examination of the neostriatum only. Tiihonen et al (1996) have found that the administration of a 5-HT transporter (or re-uptake site) inhibitor, citalopram, incurred a slight decrease in the binding of a DA antagonist ([11C]-raclopride) at D2's, thought to indicate increased levels of DA release, modulated by 5-HT. Another recent study (Smith et al, 1997) has used the same D2 antagonist to investigate the effects of another potent 5-
HT re-uptake inhibitor, fenfluramine on levels of DA release, again a significant increase in specific D_2r binding was observed, reflecting raised levels of extracellular DA in the striatum, modulated by 5-HT.

The results of these studies demonstrate that due to the different weightings of 5-HT and DA receptor subtypes in the cortex and neostriatum, care should be taken in trying to extrapolate the findings of 5-HT and DA interactions in the rat and human neostriatum, to those seen in the rat medial PFC. Despite the observation of similar 5-HT and DA receptor interactions in the baboon neostriatum (Dewey et al., 1995), it is not possible to say for certain, what interactions may be taking place between 5-HT and DA, via their different receptor subtypes in the adult (less still the developing) monkey DLPFC.

14.4: Final Conclusions.

The changes in the levels of 5-HT and its receptors in the monkey DLPFC during development are likely to be inextricably linked to the formation and refinement of intrinsic lattice circuitry by layer 2/3 pyramidal neurons and therefore associated with, perhaps the nearest equivalent measure of a critical period for the DLPFC to that found for example for ocular dominance columns in the visual cortex. It may be that 5-HT receptor subtypes, e.g. 5-HT_1D's or 5-HT_2A's are localised on the axon terminals of the recurrent collateral's arising from intrinsic pyramidal neurons which form the lattice structure through layers 1-3 in the DLPFC. Additionally, other 5-HT receptor subtypes present on pyramidal neurons participating in the intrinsic lattice (either furnishing these projections and/or the target of them) could express 5-HT_1A's on their axon initial segments and 5-HT_2C's on their cell bodies and dendrites.

The inhibitory GABAergic "basket" neurons proposed to reciprocally interact with this pyramidal neuron lattice (Lund et al., 1993; Goldman-Rakic, 1995b, 1996) may express 5-HT_2A's or 5-HT_3's on their cell bodies or 5-HT_1D's on their axon terminals in contact with pyramidal neurons. These various 5-HT receptor subtypes are known to be expressed on different portions of pyramidal neurons, GABAergic interneurons and axon terminals in various species and regions. Immunohistochemical studies of the adult monkey DLPFC with specific antibodies are required to show whether these various 5-HT receptor subtypes are in fact localised to the same or similar portions of the cellular components of the excitatory intrinsic lattice circuitry and its modulatory local circuit interneurons as have been found in previous studies in other regions and species. Secondly, the developmental time courses of the expression of these 5-HT receptor subtypes is needed to establish which of
them may exist at transiently high levels at different stages of development. In combination with the modulatory effects of other neurotransmitters, such as DA, and the expression of their receptor subtypes on the various neuronal portions of this circuitry, 5-HT and certain 5-HT receptors might prove to be important ingredients in the maturational "chemical cocktail" which could ensure the correct formation of axonal arbours, dendritic trees, dendritic spines and synaptic connectivity as a gradual process beginning prior to birth and continuing through development until puberty. This stage of development in primates (monkeys and humans) would seem to be the culmination of many maturational processes (anatomical and functional), seemingly with different time courses in different layers of the DLPFC region (dendritic spines on pyramidal neurons in layers 4 and 6 - unpublished observations from our laboratory; PV-IR axon cartridges, PV-IR neurons, CCK-IR neurons and DAergic axons; discussed in Anderson et al, 1995; Rosenberg and Lewis, 1995 and Condé et al, 1996; see in Concluding Discussion section 15.0).

The observations of this thesis study 2, regarding the maturation of the 5-HT innervation in layer 3, in relation to the expression of various 5-HT receptors, could have important implications for the normal development of the pyramidal neuron intrinsic excitatory lattice circuitry in the DLPFC of monkeys (as examined in study 1 of the thesis) and humans. The findings in both studies 1 and 2 of this thesis, could also be important for the reformulation of the various hypotheses of a neurodevelopmental cause underlying the emergence of an array of devastating symptoms - thought at least to be partly due to significant loss of integrative, cognitive and mnemonic functions in the DLPFC - exhibited in the early-onset (puberty/late adolescence) of schizophrenia (reviewed in Lewis and Anderson, 1995; Weinberger, 1995, 1996).

15.0: Concluding Discussion.

15.1(A): The postnatal maturation of the primate DLPFC: hierarchical or concurrent pattern of developmental time course within DLPFC and between DLPFC and other cortical areas.

In considering the broader implications of our findings from the two studies in this thesis, concerning the maturation of the monkey DLPFC, it should be asked if a similar time course for 5-HTergic axon and pyramidal neuron maturation occurs in all laminar depths in the DLPFC and even across all cortical areas; or if the events we have described for layer 3 are unique to that particular environment in the superficial layers in the DLPFC. We have reviewed evidence - see sections 2.2(A) and (B) - for two broad theories of cortical
development. One theory, which is the most recently formulated of the two - see section 2.2(A) - proposes that all cerebral cortical areas in the primate (i.e. monkeys and humans) show a synchronous developmental time course, including concurrent maturation of all layers in these areas. The second theory which has been long-standing for nearly a century - see section 2.2(B) - proposes that the various cortical regions reach their anatomical and hence functional maturity, in a sequence based on a hierarchy of the relative complexity of their information processing and their level of participation in sensory-, cognitive- and motor-guided behaviours. In this case, the sensory areas, such as primary visual and auditory cortices would reach their adult state earlier than the more cognitively-related association regions such as the DLPFC, with variations in the time of maturation among individual layers, both between and within each region.

15.1(B): The relation of the time courses of postnatal development for pyramidal neurons, 5-HTergic axons and other components in layer 3, to the overall maturation of the monkey DLPFC.

Comparing the results in studies 1 - see Spine study section 9.1(A) and (B) - and 2 - see Serotonin study section 13.1(B) and (C) - with other studies of anatomical maturation in area 46 of both macaque monkeys and humans - see Spine and Serotonin study Discussion sections 11.2(A-E) and 14.3(A-D) - several similarities and differences are apparent.

The general time course for pyramidal neuron dendritic spine development in our study of layer 3 in areas 9 and 46, was very similar to that for the maturational development of asymmetric (excitatory) synaptic density in layer 3 of area 46 (Bourgeois et al, 1994), with a rapid phase of synaptic and spine growth, during the first 10 weeks of life reaching a peak at around 2 months of age, remaining at a plateau level and declining in later life. However, the exact timing of these events in the 2 studies also demonstrated important differences, with the declining phase in our study, characterised by a steady reduction in the numbers of dendritic spines, occurring earlier in development (from 1.5 years of age to 4.6 years) than the markedly protracted plateau phase and later more gradual decline (from 3.0 years of age) in the density of asymmetric synapses throughout adulthood (Bourgeois et al, 1994). The difference in the rate of decline between asymmetric synapses and the majority of their postsynaptic sites, i.e. spines, may be due to the fact that synaptic loss is more protracted than that for spines. This difference may be accounted for by the variation between the time courses of maturation for asymmetric synapses, many associated with the apical
dendrites passing through layer 3, of pyramidal neurons in deeper layers, by inclusion of asymmetric contacts onto non-spiny cells, i.e. smooth interneuron dendrites or changes in neuropil volume during development.

Comparison with other postnatal developmental studies of components of cortical circuitry in various layers of areas 9 and 46 in the monkey DLPFC (Mrzljak et al, 1990, 1992; Rosenberg and Lewis, 1995; Condé et al, 1996) reveals that different cortical layers show different schedules of development for pyramidal neurons, neurotransmitter-specific axons and interneurons, e.g. PV-IR axon cartridges and dopaminergic axons. There is also variation between these components within the confines of a particular laminar environment, e.g. differences between DAergic axons in middle and deep layer 3.

Different time courses of morphological development have been observed for layer 3 and 5 pyramidal neurons in the human DLPFC on the basis of changes in their basal dendritic tree (Mrzljak et al, 1990; Mrzljak et al, 1992; Koenderink et al, 1994; Koenderink and Uylings, 1995). Layer 3 pyramidal neurons appeared to mature earlier than layer 5 pyramidal neurons, since they attained a mature level of dendritic length and branching by the beginning of the first year of postnatal life, while layer 5 pyramidal neurons grew rapidly in the first year and continued to develop in their dendritic extent and branching up until 5.0 years of age.

Although we demonstrate that the developmental time courses for the elements of 2 distinct but synaptically-connected cell populations in layer 3 are virtually identical (pyramidal neuron dendritic spines and parvalbumin-IR changes in chandelier neuron axon cartridges), in the same layer the developmental progression of maturation for dendritic spines and dopaminergic axons shows marked differences in the form of their time course. Nonetheless, coincidence at several points during the developmental time course of dendritic spines and dopamine, suggests that dopamine may play a role in the developmental regulation of the processes of spine acquisition and loss on the dendrites of pyramidal neurons in layer 3 of the monkey DLPFC. The temporal concurrence of the periods of maximal change for both of these components of the cortical circuitry in this layer, implies that there is some functional interrelation and it is known that DAergic synapses occur on pyramidal neuron dendritic spines - see Neurotransmitters section 6.1(C). Additionally, both systems could also be under the influence of a common regulatory substance, e.g. hormones or growth factors.

In summary, our observations in the DLPFC give support to a hierarchical mode of cortical development in the macaque monkey brain, with a later completion of maturation for the DLPFC than for the primary sensory regions, e.g. area 17, as well as differential rates of development even within single layers of a region, for different cellular components.
15.2(A): The relevance of developmental anatomical studies in monkeys to observations made in post-mortem human material and to developmental hypotheses for the aetiology of schizophrenia.

There have been several theories proposed in the last decade or so which have highlighted the possibility of an aberrant process of maturation in cortical regions - including the DLPFC - during postnatal development as a cause for schizophrenia (reviewed in Jones, 1995; Lewis and Anderson, 1995; Weinberger and Berman, 1996). These hypotheses are based on the observation of many and varied anatomical abnormalities found to exist in the post-mortem schizophrenic human brain, which could contribute to the cognitive dysfunction's evident in the performance of tasks requiring high levels of focused attention, mnemonic ability, goal-directed behaviour or planning and also the implied alterations in levels of neuronal activity observed in functional imaging studies.

It is of importance for us to examine the anatomical and functional evidence which supports the above hypotheses and consider how the disruption of developmental events in the normal monkey DLPFC described in this thesis might contribute to behavioural disturbance in later life.

Various cortical components have been implicated in the aetiology of schizophrenia, including abnormal levels of synaptic proteins, e.g. synaptophysin and GAP-43, altered levels of dopamine, serotonin, GABA or glutamate and their receptors, aberrant sets of certain CBP-containing GABAergic interneurons as well as overall alterations in the densities of neurons and changes in the proportions of cells based on their somal size.

Observations indicating that normal neuronal migration may be disrupted for many classes of neurons as part of the aetiology of schizophrenia, are important in that this could result in the malformation of intrinsic lattice circuitry in the superficial layers of the DLPFC, as well as an abnormal balance of excitatory and inhibitory neural elements and their interrelations during development.

15.2(A).(i): Pyramidal neurons and dendritic spines.

As mentioned in the Pyramidal neuron review section 5.0, there have been several reports of abnormally low levels of dendritic spines on the pyramidal neurons in the DLPFC of the post-mortem schizophrenic brain (Garey et al, 1995; Glantz and Lewis, 1995, 1996), a finding which has been localised particularly to pyramidal neurons in layer 3 (Glantz and Lewis, 1996). On the basis of the normal process of synaptic "pruning" during puberty in the DLPFC, observed both in monkeys (Rakic et al, 1986) and in humans (reviewed in...
Huttenlocher, 1993), it has been hypothesised that the DLPFC, with a more prolonged time course of maturation than other cortical regions, is more vulnerable to long-lasting effects of abnormal developmental events (Feinberg, 1987, 1992; Saugstad, 1994). Aberrant developmental processes might occur prenatally or in the early postnatal period (reviewed in Jones, 1995) and the resultant anatomical and functional ramifications may not become apparent until puberty, the typical time of onset for the symptoms of schizophrenia. With regard specifically to dendritic spines, hypotheses (Feinberg, 1982; Keshavan et al, 1994) have predicted that excessive synaptic "pruning" or the opposite, i.e. ineffecual synaptic "pruning", leading to depleted or excessive levels of synapses, respectively might account for the timing and types of symptoms emerging around the time of puberty in schizophrenic patients.

Our developmental studies in the macaque monkey would suggest that if the same processes do occur in normal humans, then puberty would be a very vulnerable period for the disruption of various cortical components in the DLPFC, which would seem to mature around this time. For the monkey DLPFC, puberty is known to be a time of complex interactions between neurotransmitters, excitatory and inhibitory neurons and the refinement of excitatory synapses. Abnormal patterns of events occurring for any one of these components, e.g. 5-HT, DA or pyramidal neurons themselves, would almost certainly derail the normal sequence of events in the formation of the intricately-organised intrinsic lattice circuitry of the superficial layers. The result of this failure in normal maturational processes in the DLPFC would undoubtedly be manifested in the form of dysfunctional behaviours due to deficits in cognitive and mnemonic information processing, as are observed in most cases of schizophrenia.

15.2 (A).(ii): Glutamate receptors.

As reviewed in the Pyramidal neuron review section 5.10, many pyramidal neurons in monkey and human neocortex, express NMDA and non-NMDA receptor subunits, as do many GABAergic interneurons. The presence of these receptors in the DLPFC on various pyramidal neurons in layers 2 and 3, involved in intraareal intrinsic lattice circuitry, as well as sending extrinsic projections to other cortical and subcortical regions and receiving incoming glutamatergic afferents from pyramidal neurons in other cortical regions allows for activity-dependent modulation, which is likely to be most susceptible to aberrant disruption during the late maturational phase, i.e. around puberty.

Changes in the levels of mRNA signal coding for particular NMDAR's have
been reported for the DLPFC of the post-mortem schizophrenic brain. These changes have been hypothesised to be at least partially the result of an abnormal series of developmental errors in the maturation of the reciprocal corticocortical and thalamic-connecting pyramidal neuron circuitry which ultimately may lead to subtle specific alterations in many diverse neurotransmitters, synaptic proteins and receptor classes (Akbarian et al, 1996).

Such potential alterations in the normal complements of both NMDA and non-NMDA receptors in the human DLPFC, each known to play an important role in the normal modulation of information processing via excitatory cortical projections in the monkey neocortex, would almost certainly impinge on the maturation of the intrinsic lattice circuitry of the DLPFC, as well disrupt its numerous interactions with interareal connections during development and in their subsequent refinement. The presence of NMDA and non-NMDA receptor subunits on asymmetric synapses in adult monkeys, implies that they may have been present at these sites associated with pyramidal neuron dendritic spines from the early stages of postnatal development. Increased levels of these receptors might thus be expected to accompany the rising levels of excitatory synapses and dendritic spines seen during the first two months of postnatal life. It might also be expected that the density of glutamate receptors might decrease in line with either the decline in spine numbers (from 1.5 years of age) or synapses (from 3.0 years of age). No studies have yet been conducted of NMDA or non-NMDA receptor development in the monkey DLPFC, although a preliminary study in the visual cortex (area 17) has shown that several subtypes of these receptors, exhibit changes of density that seem to show some temporal coincidences in line with the synaptic development of this sensory cortical region.

15.2(B): γ-aminobutyric acid (GABA)ergic interneurons and GABA receptors.

An increase in the density of the CB-IR class, but not PV-IR or CR-IR classes of GABAergic interneurons has been found within layer 3 of the DLPFC in schizophrenics. Since the axons of CB-IR neurons are known to form synaptic contacts with the dendritic shafts and spines of pyramidal neurons in many cortical areas - see Neurotransmitters section 6.5(D) - a rise in the numbers of cells exhibiting CB-IR, might indicate an alteration in the degree of inhibitory regulation of at least some of the excitatory pyramidal neuron intrinsic and corticocortical circuitry as part of the aetiology of this disease. In terms of the symptoms which are manifested by patients, such as the disintegration of cognitive and mnemonic functions; these could clearly be interpreted as a lack of appropriate modulation of pyramidal neurons by GABAergic cells. Since CB-IR neurons may also form synaptic contacts
with other interneurons, abnormally high numbers of neurons demonstrating CB-IR in schizophrenia, may also reflect abnormal inhibitory modulation among subtypes of interneurons, which could also adversely affect their regulation influence over pyramidal neuron excitatory activity.

Lack of appropriate GABAergic modulation could permit the abnormal excitation of intrinsic connectivity in the DLPFC, since a constant flow of information from diverse afferent inputs converges on these pyramidal neurons as well as inputs from other layers. Hence, an unregulated or inappropriate modulation of excitatory neurotransmission might ensue within the pyramidal neuron lattice circuitry, which could cause extensive disruption throughout the PFC as a whole, since the layer 3 intrinsic lattice circuits in DLPFC appear not to be restricted to cytoarchitectonic borders but spread continuously across areas 9 and 46 and probably across other PFC areas.

There is also known to be extensive interaction of callosal and associative corticocortical pathways (see Anatomical connections review section 3.6) between different PFC regions (including the DLPFC, cingulate, orbital and ventrolateral areas) primarily mediated by layer 3 pyramidal neurons. In schizophrenia, a lack of inhibitory regulation in just one region e.g. area 46, producing overactivity or inappropriate patterns of activity might invoke disturbance of processing in more distant regions as well as the possible dissociation of limbic functions (in orbital PFC) from those of mnemonic (DLPFC, FEF and ventrolateral areas) and motor-related functions (cingulate areas). A general overactivity or imbalance in the information flow among these projection pathways correlates with some reports of "hyperactivity" of blood-flow observed in the DLPFC region in rCBF studies of schizophrenics, which may in turn be indicative of the phases of florid symptoms (hallucinations and delusions, etc) which have been described for most patients.

Changes have also been noted in the density of GABA receptors in the post-mortem DLPFC of schizophrenic patients, with a selective up-regulation of the GABAA receptor subtype reported in cingulate and DLPFC (area 10) regions, but no changes in the levels of GABAA receptor mRNA expression in the DLPFC (area 46). The increase in the presence of GABAA receptors in the DLPFC and cingulate cortex has been hypothesised to correlate with the findings of "hypoactivity" of blood-flow in the DLFPC region from rCBF studies in schizophrenic patients. These observations might perhaps be indicative of the phase of negative symptoms (loss of affect, blunting of emotion, social isolation) often observed in chronic, so-called untreatable schizophrenics, in between their acute bouts of positive symptoms or manic episodes. The lack of alteration in gene expression for GABAA
receptors, means that the increase in numbers of binding sites would most likely be due to post-translational or post-synthesis changes in the production of these receptors. The extrinsic connections between different areas of the PFC and the cingulate cortex would suggest these GABAergic receptor changes to have widespread disruptive effects even if limited to specific areas.

15.2(C): Serotonergic and dopaminergic neurotransmitter and receptor interactions.

The DAergic axon innervation of the DLPFC in monkeys and humans is present at a moderate density in layer 3 and is known to directly contact the dendritic spines and shafts of pyramidal neurons as well as their PV-IR interneuron modulators - see Neurotransmitters review section 6.1(B). In view of the remedial effects of neuroleptic drugs on DA release and binding at its variously located receptors in primates (Lidow and Goldman-Rakic, 1994; Lundberg et al, 1996) dopamine has been implicated in playing a major role in the biochemical mechanisms of dysfunction in schizophrenia, including its role in the DLPFC (Carlsson, 1988; Weinberger, 1988).

This thesis provides anatomical evidence in the macaque monkey that there are likely to be extensive interactions between 5-HT and DA during the course of postnatal development in layer 3 of the DLPFC and there is considerable pharmacological evidence to support the existence of extensive modulatory interactions between 5-HT and DA. The enormous range of potential locations for 5-HT-DA interaction in the primate brain, including within the DLPFC, at the level of both presynaptic and postsynaptic receptors located on axon terminals and neurons, may account for many of the complex effects of atypical antipsychotic drugs which appear to alleviate both the positive and to some extent the negative symptoms exhibited in schizophrenia (Lundberg et al, 1996; reviewed in Hagan et al, 1993; Kahn and Davidson, 1993; Schmidt et al, 1995; Kapur and Remington, 1996).

This interaction of 5-HT and DA is exhibited also in the demonstration of a reciprocal relationship for the tissue concentrations of 5-HT and DA (Goldman-Rakic and Brown, 1981, 1982) and for the densities of 5-HTergic and DAergic axons and varicosities - Rosenberg and Lewis (1995) and 5-HT study Discussion section 14.3(C).(i) - during the course of postnatal maturation and senescence. In addition the hourly rates of 5-HT and DA neurotransmitter turnover and synthesis (Brown et al, 1979) within individual adult monkeys have been found to show reciprocity in their values, whilst a complementary laminar distribution for 5-HTergic and DAergic axons has been described using a thin section autoradiographic visualisation technique in the DLPFC of the adult monkey (Berger et
There appears to be a process of structural or biochemical compensation by the 5-HTergic system for reduced levels of DA or DAergic axons implying that the 5-HT and DA systems are interactive or mutually inhibitory. The emergence of high levels of 5-HT and an excessive 5-HTergic axon innervation could be an attempt to correct the imbalance between the 5-HTergic and DAergic innervations by a form of plasticity involving 5-HT in a facilitatory role, e.g. by activating growth factors such as S100B (Azmitia et al, 1990).

15.3: Summary.

The major findings of the two studies which form the basis for this thesis are:

1. Using quantitative analysis, changes in the numbers of dendritic spines present on Golgi-stained pyramidal neurons in layer 3 of the monkey DLFPC (areas 9 and 46), were used as an indication of the degree of pyramidal neuron synaptic maturation across the course of postnatal development. The time course of spine development consisted of an initially rapid rise in spines in early postnatal life, with a plateau phase characterised by high levels of spines from 10 weeks to 1.5 years of age and a gradual but steady decline in spine numbers thereafter until around 4.5 years of age. This time course parallels that for asymmetric synapses reported previously for all layers in area 46 (Bourgeois et al, 1994). However, there appeared to be an earlier initiation (at 1.5 years of age) to the phase of refinement for dendritic spines, compared to that found for excitatory synapses (3.0 years of age onwards). Since mid-layer 3 pyramidal neurons provide unique intrinsic intraareal and extrinsic interareal connectivity patterns, their developmental time course should thus be indicative of the "critical period" for the anatomical and functional maturation of the DLPFC.

2. The time course of pyramidal neuron spine development shows a striking similarity to the time course of development of a particular class of inhibitory input, the PV-IR axon cartridges of chandelier neurons in area 46 (Anderson et al, 1995), known to terminate synaptically onto the axon initial segments of layer 3 pyramidal neurons in the DLPFC. This close match of developmental time courses of pre- and postsynaptic sites is thought to imply the balance of weight of inhibitory modulation to the weight of excitatory input to the layer 3 pyramidal neurons during the phases of rapid growth and refinement of high numbers of dendritic spine synapses.

3. The schedule of dendritic spine maturation in layer 3 was found to show a temporal relationship to stages of the maturational time course of DAergic axons in the same layer of area 46 (Rosenberg and Lewis, 1995). The varicosity density of DAergic axons rises in
2 stages during postnatal development, the first rise coinciding with the increase in pyramidal neuron spine density and the second rise is in temporal alignment with the reduction of spines. These close temporal relations are taken to indicate a modulatory involvement of DA in spine development. This hypothesis is supported by previous evidence of DAergic axon varicosities forming synaptic contacts with pyramidal neuron dendritic spines and shafts as well as with the smooth dendrites of PV-IR neurons (modulators of pyramidal neurons) in the monkey DLPFC.

The initial rise in DAergic varicosities and dendritic spines may reflect indirect DAergic disinhibitory action on spines, via the suppression of the PV-IR neuron inhibitory influence over pyramidal neurons, while the second rise in DAergic varicosities with concurrent decline in spines, may indicate the direct inhibitory modulation of pyramidal neuron spines.

4. In a quantitative analysis of immunohistochemically-labelled 5-HTergic axons in layer 3 of the monkey DLPFC (area 46), the postnatal time course of development for all 5-HTergic axons (in terms of their varicosity density per 5000 μm²) exhibits a complex cyclical time course, consisting of 2 distinct stages (infancy and puberty/early adulthood) when low varicosity densities predominate and 3 stages (neonatal period, juvenile phase and later adulthood) when there is a high density of varicosities.

5. Evidence from previous studies has suggested that 2 forms of 5-HTergic axons exist in the adult macaque monkey cerebral cortex. In this thesis, we have attempted to differentiate the individual developmental time courses for these 2 sub-populations of axons in layer 3 of the DLPFC. Despite careful analysis of 5-HTergic axons, clear differentiation of 5-HTergic axons into 2 morphological forms was not clearly evident. Using a criterion of larger versus finer diameter varicosities and intervaricose segments to attempt to analyse separate sub-populations, it was not possible to demonstrate a different pattern of maturational time course for two 5-HTergic axon sub-populations, although the timing of each stage was slightly different for the 2 groups we defined. This led to the conclusion that 5-HTergic axon sub-populations are either not clearly distinguishable in their forms during development or the 2 types of 5-HTergic axons almost certainly follow a common time course of maturation.

6. A comparison of the maturational sequences of 5-HTergic axons and dendritic spines in layer 3, revealed that there may be some correspondence between the timing of rises and falls in the 5-HTergic varicosity density and the rise and fall of dendritic spines during postnatal development. Coincidence between the 5-HT system and pyramidal neurons occurred between the fall in 5-HTergic varicosity density and the rise in dendritic
spines, recovery in 5-HTergic varicosity density with the plateau phase of spine numbers, the fall in the density of 5-HTergic varicosities and the decline in dendritic spines and finally the rise in 5-HTergic varicosity density and achievement of mature levels of spine density. Previous evidence suggests that 5-HTergic axon varicosities in the adult monkey DLPFC form synaptic contacts primarily with GABAergic interneurons (PV-IR and CB-IR types) and only a minority with pyramidal neurons, suggesting a lack of direct synaptic modulation of pyramidal neuron maturation by 5-HT. However, as for DA and its receptor distribution on pyramidal neurons, some 5-HT receptors are known to be localised to pyramidal cells (5-HT1A, 2A and others to GABAergic interneurons, providing 5-HT with an alternative extrasynaptic means to modulate these neurons.

7. Comparing the maturational time courses for 5-HTergic axons, as defined in this thesis and DAergic axons (as defined by Rosenberg and Lewis, 1995), in terms of their varicosity density (per 5000 μm²) in area 46, revealed a remarkable degree of reciprocity in density between these 2 neurotransmitter innervations in layer 3, across the entire age range of animals examined (the same animals were analysed in both studies). This inverse relationship in the numbers of potential 5-HT and DA release sites over postnatal development suggests a complementary modulation of each other's activity, via receptors known to be located on their axon varicosities.

The maturation of dendritic spines may be partially mediated by both these neurotransmitters, with 5-HT mediating GABAergic interneuron (e.g. CB-IR and PV-IR varieties) inhibitory influence over pyramidal neuron excitatory activity, while DA provides direct innervation to postsynaptic sites of excitatory input to pyramidal neurons. Alternatively, other components of the layer 3 circuitry not examined in this study may be modulated by the actions of 5-HT and DA release and these in turn might affect the pyramidal neuron excitatory lattice circuitry.

8. By re-plotting the DAergic axon data of Rosenberg and Lewis (1995) in the age groups that showed significant changes for 5-HTergic axons during development, we were able to show significant reciprocity between the changes in varicosity density for 5-HTergic and DAergic axons across postnatal development. This led to the conclusion that our selection of age groups may provide a more exact representation of the time-scale for interactions across postnatal development, between the different components of the cortical circuitry in layer 3 of the monkey DLPFC.

9. The time courses of development for 5-HTergic axon varicosity density and 5-HT tissue concentration (Goldman-Rakic and Brown, 1981, 1982) in the DLPFC, also
appeared to show a reciprocal relationship during postnatal development. It is not immediately apparent why the mean levels of released 5-HT free in the neuropil should be the inverse of the mean levels of 5-HT stored in varicosities at each age, but it is possible that this reflects variations in rate of synthesis and turnover of 5-HT at each age, although it is significant that previous studies in the DLPFC of developing and adult monkeys suggest that the rate of 5-HT synthesis and breakdown remains at relatively the same mean rates throughout postnatal life.

However, preliminary evidence supporting changes in 5-HT production, release and re-uptake during development is shown by the changes in the density of varicosities positive for an antibody specific to the 5-HT transporter in PFC area 9 (Kye and Lewis, unpublished observations). This transporter molecule mediates the re-uptake of released 5-HT and exhibits a time course synchronous with our analyses of 5-HT varicosity density during development. Also, the varicosity density of axons positive for the 5-HT synthetic enzyme, tryptophan hydroxylase in layer 3 of area 46 have been found to show a reciprocal developmental relationship to the time course for 5-HTergic axon varicosity density in this same location (Kye and Lewis, unpublished observations). Both these additional observations would seem to support our finding of a cyclical time course of progression for 5-HTergic axons in area 46, during postnatal development.

10. All of these temporal relationships for pyramidal neuron dendritic spines and 5-HTergic axons described above in points 1-9, are relevant to the postnatal development of the uniquely stripe-like terminal fields of pyramidal neuron intrinsic lattice circuitry in layers 1-3 of the DLPFC (area 9 and 46). The dendritic spines of pyramidal neurons in layer 3, studied in this thesis reflect both pre- and postsynaptic sites contributing to this intrinsic lattice structure, which is on of the chief anatomical substrates for DLPFC function in primates. From the above observations, it is apparent that DA and 5-HT are likely to play very important roles in the modulation of this structural development in the superficial layers of the DLPFC; this modulation of most probably includes both synaptic and non-synaptic components, involving the participation of a variety of 5-HT and DA receptor subtypes.

11. In conclusion, the implications of the results from the 2 studies in this thesis and the comparative developmental relationships demonstrated between them has provided an important contribution to the continuing process of establishing the temporal relations of events occurring in the normal postnatal development of the monkey DLPFC.

As described in the detailed introduction: section 2.3, the developmental time course for different cortical layers, as well as maybe differing between distinct, cortical regions,
may even vary within the same cortical area, or perhaps differs for particular neuronal populations and their various cellular compartments, according to their immediate laminar environment, i.e. the hierarchical hypothesis. In the case of basal dendritic spine density for layer 3 pyramidal neurons, the pattern of changes in levels of spines with time, indicates that anatomical - and by extrapolation - the functional maturation of area 46, is achieved much later (around 2.5-3.0 years) than for the corresponding layer 3 pyramidal neurons in primary visual cortex (1.0 year; area 17; Boothe et al, 1979). The alternative pattern of cortical development, i.e. the concurrent hypothesis, as described in section 2.2, can be partially discounted on the basis that the developmental study of excitatory synapses in layers 1-6 in area 46 (Bourgeois et al, 1994), did not take into account the fact that different compartments of a given pyramidal neuron are likely to exhibit different rates of developmental time course. The same is true of synaptic development studies conducted in other cortical areas (Rakic et al, 1986), of which careful re-examination may later bring to light that these different regions do indeed show a similar level of concurrent maturation at the gross laminar level, but at the neuronal level, development may be found to be more specifically related to their immediate laminar environment.

In the case of 5-HTergic axons, the evidence for differential rates of development between cortical areas, is less clear since we only have the current quantitative thesis study in area 46 and a qualitative study conducted in area 17 (Foote et al, 1984). It would appear from a comparative examination of the 5-HTergic axon development in the 2 regions, that area 46 achieves its mature level of 5-HTergic axon innervation density at a later age (around 3.0 years) than area 17 (5-7 months). In addition, in area 46, the mature levels of 5-HTergic axon innervation do not appear to remain stable for long, but from 6.0 years onwards they appear to vary throughout the rest of adulthood.

12. The accumulation of information forming the basis of this thesis will potentially provide a firm anatomical basis for modelling human DLFPC maturation and will facilitate further investigation of anatomical, neurophysiological and pharmacological aspects of the superficial layer intrinsic lattice circuitry of the monkey DLFPC. These studies lay a better foundation for the pathological studies of disrupted structure and function in the human DLPFC, disruption which would seem to be integral to the dysfunction of working memory and cognitive processing that is characteristic of schizophrenia. The developmental studies in this thesis may ultimately help to reveal the contribution that the abnormal development of the human DLPFC may make towards symptoms of working memory deficit, as observed in schizophrenic patients.
15.4: Appendix.


Figure 56. Mean relative spine density (per μm) on apical dendrites of mid-layer 3 pyramidal neurons (Golgi study 1 in Anderson et al., 1995). (A) histogram showing the results for individual animals on a proportional logarithmic time scale and (B) bar plot showing the results plotted in 5 age groups used for Golgi studies 1 and 2 in Anderson et al., (1995). Standard errors indicated. Figure from Anderson et al., (1995).
Fig. 57. Variability of mean relative spine density (per μm) for different laminar locations on apical dendrites of mid-layer 3 pyramidal neurons (Golgi study 1 in Anderson et al, 1995). (A) shows the spine density (per μm) for proximal portions close to the cell body in mid-layer 3, (B) shows spine density (per μm) for middle portions located in layer 2 and superficial layer 3 and (C) shows spine density (per μm) for distal portions situated in layer 1. Notice a slightly different time course of spine development in the proximal portions compared to the middle and distal portions of apical dendrites, i.e. in mid-layer 3 relative to layers 1 and 2, indicating that there might be a slightly later maturation of spine numbers in mid-layer 3. Standard errors are shown. Figure from Anderson et al., (1995).
Figure 58. Mean relative spine density (per μm) on apical oblique (A and B) and basal (C and D) dendrites of mid-layer 3 pyramidal neurons (Golgi study 1 in Anderson et al, 1995). (A and C) histogram showing results for individual animals on a proportional logarithmic time scale, (B and D) bar plot showing the results plotted in 5 age groups used for Golgi studies 1 and 2 in Anderson et al, (1995). Standard errors are shown. Figure from Anderson et al, (1995).
16.0: References.


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18.0: Abbreviations.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AIS</td>
<td>Axon initial segment</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid</td>
</tr>
<tr>
<td>AS</td>
<td>Arcuate sulcus</td>
</tr>
<tr>
<td>BDA</td>
<td>Biotinylated dextran amine</td>
</tr>
<tr>
<td>c</td>
<td>Caudal</td>
</tr>
<tr>
<td>CalB</td>
<td>Calbindin-positive</td>
</tr>
<tr>
<td>CalR</td>
<td>Calretinin-positive</td>
</tr>
<tr>
<td>CBP</td>
<td>Calcium-binding protein</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>ChAT</td>
<td>Cholineacetyltransferase</td>
</tr>
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<td>Central sulcus</td>
</tr>
<tr>
<td>CtB</td>
<td>Cholera toxin subunit-B</td>
</tr>
<tr>
<td>d</td>
<td>Dorsal</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>2DG</td>
<td>2-[14C]-deoxyglucose</td>
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<tr>
<td>DLPFC</td>
<td>Dorsal lateral prefrontal cortex</td>
</tr>
<tr>
<td>DR</td>
<td>Delayed response task</td>
</tr>
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<td>DRN</td>
<td>Dorsal raphe nucleus</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalography</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
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<td>5-HTr</td>
<td>5-HT receptor</td>
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<tr>
<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>la-p</td>
<td>Insula, agranular periallocortical</td>
</tr>
<tr>
<td>Idg</td>
<td>Insula, dysgranular</td>
</tr>
<tr>
<td>Ig</td>
<td>Insula, granular</td>
</tr>
<tr>
<td>IP</td>
<td>Intraparietal</td>
</tr>
<tr>
<td>IPa</td>
<td>Anterior inferior parietal area</td>
</tr>
<tr>
<td>IPL</td>
<td>Inferior parietal lobule</td>
</tr>
<tr>
<td>IPS</td>
<td>Intraparietal sulcus</td>
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<tr>
<td>IR</td>
<td>Immunoreactive</td>
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<td>KA</td>
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<tr>
<td>LCGU</td>
<td>Local cerebral glucose uptake</td>
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<td>LIP</td>
<td>Lateral intraparietal area</td>
</tr>
<tr>
<td>LOS</td>
<td>Lateral orbital sulcus</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>LTD</td>
<td>Long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>mc</td>
<td>Magnocellular</td>
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<tr>
<td>MD</td>
<td>Mediodorsal nucleus</td>
</tr>
<tr>
<td>MDmf</td>
<td>Mediodorsal nucleus multiform subdivision</td>
</tr>
<tr>
<td>MOS</td>
<td>Medial orbital sulcus</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging (structural)</td>
</tr>
<tr>
<td>MRN</td>
<td>Median raphe nucleus</td>
</tr>
<tr>
<td>MT</td>
<td>Middle temporal area</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR1</td>
<td>NMDA receptor 1</td>
</tr>
<tr>
<td>OA</td>
<td>Area 19 (lower bank of STS)</td>
</tr>
<tr>
<td>OAA</td>
<td>Caudal depth and ventral bank of STS</td>
</tr>
<tr>
<td>OBF</td>
<td>Orbitofrontal cortex</td>
</tr>
<tr>
<td>ODR</td>
<td>Oculomotor delayed response</td>
</tr>
<tr>
<td>Opt</td>
<td>Caudalmost IPL</td>
</tr>
<tr>
<td>P</td>
<td>Pyramidal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Pa-Alt</td>
<td>Lateral parakoniocortex zone (in caudal dorsal STG)</td>
</tr>
<tr>
<td>pc</td>
<td>Parvocellular</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<td>Caudal-dorsal parietal region</td>
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<td>Rostral-most IPL</td>
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<td>Prefrontal cortex</td>
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<tr>
<td>PFG</td>
<td>Rostral middle IPL</td>
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<tr>
<td>PG</td>
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<td>Inferior pulvinar nucleus</td>
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<td>PL</td>
<td>Lateral pulvinar nucleus</td>
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<tr>
<td>PM</td>
<td>Medial pulvinar nucleus</td>
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<td>POa</td>
<td>Ventral bank of IPS</td>
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<td>POa-e</td>
<td>External portion of ventral of IPS</td>
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<td>ProA</td>
<td>Prokoniocortex</td>
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<td>ProM</td>
<td>Rostral premotor cortex</td>
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<td>Principal sulcus</td>
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<td>PV</td>
<td>Parvalbumin-positive</td>
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<td>r</td>
<td>Rostral</td>
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<tr>
<td>S</td>
<td>Smooth, interneuron</td>
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<td>S-I</td>
<td>Primary somatosensory cortex</td>
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<td>SN</td>
<td>Substantia nigra</td>
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<td>ST</td>
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<td>STG</td>
<td>Superior temporal gyrus</td>
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<td>Superior temporal polysensory cortex</td>
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<tr>
<td>STS</td>
<td>Superior temporal sulcus</td>
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<tr>
<td>TAA</td>
<td>Rostro-caudally extensive strip of cortex in dorsal bank of STS</td>
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<td>TEa</td>
<td>Rostral portion of ventral bank of STS</td>
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<tr>
<td>TEM</td>
<td>Middle dorsal inferior temporal cortex on lip of ventral bank of STS</td>
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<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
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<tr>
<td>TPO-1/2/3/4</td>
<td>Rostral, rostral middle, caudal middle and caudal sectors of dorsal STS (STP)</td>
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<td>Temporoparietal zone (caudal STG)</td>
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<td>Ventral</td>
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<td>Ventral tegmental area</td>
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<tr>
<td>WBS</td>
<td>Whole blood serotonin</td>
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19.0: Published work resulting from this thesis.


