Experience-dependent regulation of functional maps & protein expression in visual cortex

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Summary

Despite great progress in understanding of how experience modifies cortical circuitry in primary visual cortex (V1), the underlying physiological and molecular mechanisms still remain to be understood fully. Although some of the molecules associated with the critical period for ocular dominance plasticity in cats have been examined, the role of downstream signalling molecules that form pathways with receptor subunits has received scant attention. The present study demonstrates using optical imaging of intrinsic signals that sensory experience is not required for initial establishment of ocular dominance column layout and iso-orientation domains but is required for maintenance of these properties; its absence leads to their eventual breakdown. Animals were sacrificed and V1 was removed and homogenised, followed by immunoblotting for quantification of protein expression. The immunoblotting findings point to a set of proteins (including NR2A, PSD-95, αCaMKII, NR2B and $GABA_A\alpha 1a$) that are regulated developmentally and the effects of dark-rearing indicate that sensory activity regulates mechanisms associated with both excitatory (NR2A and NR2B) and inhibitory ($GABA_A\alpha 1a$) transmission and synaptogenesis (synaptogenesis) so as to maintain a homeostatic balance.

Pattern or form information is necessary in both eyes to maintain normal maps in both eyes while differences in illumination between two eyes did not affect ocular dominance and orientation maps in normally reared or in dark-reared cats subsequently exposed to light. Monocular deprivation (MD) for 2 days and 7 days resulted in similar depression of deprived eye responses. In contrast, potentiation of non-deprived eye responses was almost double in magnitude after 7 days compared to 2 days of MD. The immunoblotting findings demonstrate that MD regulates signalling molecules (PSD-95, αCaMKII and synGAP) downstream of NMDA receptors and GluR1 subunit; it appears that different mechanisms are activated depending on the nature of sensory experience.

Contents

List of Figures

List of Tables

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
APV	2-amino-phsophonovaleri acid
AP-5	2-amino-5-phosphonopentanoic acid
BCM	Bienenstock, Cooper and Munro
BD	binocular deprivation
BDNF	brain derived neurotrophic factor
BOLD	blood oxygen level dependent
C	control
CaMKII	Ca2+/calmodulin-dependent protein kinase
CB1	cannabinoid receptor 1
cDNA	complementary deoxyribonucleotide acid
CNQX	cyano-7-nitroquinoxaline-2,3-dione
CO	cytochrome oxidase
CO_2	carbon dioxide
CSF	cerebrospinal fluid
CSPG	chondroitin-sulfate proteoglycans
D	dark-reared
2D-DIGE	2-dimensional difference gel electrophoresis
2-DG	2 deoxyglucose
DE	deprived eye
dLGN	dorsal lateral geniculate nucleus
DMEM	1g/LD-Glucose, L-Glutamine, 25mM HEPES, sodium pyruvate
DR-LR	Dark-rearing followed by a week of sensory experience
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
EPSP	excitatory post synaptic potential
ERK	extracellular signal-regulated kinase 1.2
fMR1	functional magnetic resonance imaging
GABA	γ- amino butyric acid
GAD	glutamic acid decarboxylase
IGF1	insulin-like growth factor
IPSC	inhibitory post synaptic potential
KCC2	K^+-Cl^- cotransporter 2
LTD	long term depression
LTP	long term potentiation
LR-DR	sensory experience followed by a week of darkness
MAPK	p42/p44 mitogen activated protein kinase
θm	modification threshold
O_2	oxygen

MD	monocular deprivation
mEPSC	miniature excitatory postsynaptic current
mGluR	metabotropic glutamate receptors
NDE	non-deprived eye
N_2O	nitrous oxide
NMDARs	N-methyl-D-aspartate receptors
ND	Neutral density
OIS	optical imaging using intrinsic signals
PET	Positron Emission Tomography
PKA	protein kinase A
PLC	phospholipase C
PSD	post synaptic density
RNA	ribosomal nucleotide acid
ROI	region of interest
SAP-102	synaptic associated protein
SDS	sodium dodecyl sulfate
SF	spatial frequency
synGAP	Synaptic GTPase Activating Protein
TTX	tetradotoxin
V1	primary visual cortex
VEP	visually evoked potentials
VGCC	voltage-gated calcium channels

CHAPTER I

INTRODUCTION

1.1 Introduction to visual processing system

The visual cortex is the most intensively studied of the sensory areas of the cerebral cortex since the pioneering work on its functional architecture by Hubel and Wiesel. Research in the visual cortex is not only targeted towards understanding how vision works but also in understanding mechanisms that are involved in cortical organization following normal and abnormal visual experience. In many mammalian species primates and humans in particular, a larger part of the cortex is devoted to vision than to any other sensory modality. This is because of the extreme complexity of the task required for vision from classifying and interpreting the wide range of visual stimuli that we confront in the physical world to the highest levels of processing such as object recognition that help us experience visual perception.

In addition to understanding the mechanisms of visual processing, there is increasing amount of evidence that visual deficits are associated with many neurological diseases such as schizophrenia (Dorph-Peterson et al, 2007), Alzheimer's disease (Asensio-Sanchez et al, 2006) and Charles Bonnet syndrome (Tan et al, 2006) to name a few. Therefore continuing research into visual processing and visual development is imperative for diagnostic as well as for therapeutic purposes. Abnormalities in any aspect of visual processing from the retina to primary visual cortex (V1) and from occipital to parietal and temporal cortex may affect visual perception.

1.1.1 Early visual system

Light enters through the pupil and is projected at the back of the retina where an upside-down image of the visual world is created. In the retina, the first stage of visual processing takes place and here visual information is transformed into neuronal signals by photoreceptors.

The retina contains a number of different neuronal cell types. The ganglion cells are the innermost cells of the retina whose axons form the optic nerve carrying retinal

information to downstream areas of the visual pathway. There are three distinct retinal ganglion cells namely X, Y and W (Boycott and Wassle, 1974). Y or α cells have a large cell body (~30 μm) and they tend to respond faster to visual stimulus and are sensitive to low spatial frequency stimulus (Derrington and Fuchs, 1979). On the other hand, X or β cells have a medium sized cell body (~20 μm) and resolve high spatial frequency details (Derrington and Fuchs, 1979). In addition, W or γ are characterized by small cell body with small dendritic tree (Boycott and Wassle, 1974) and tend to respond rather sluggishly to visual stimuli (Wilson et al, 1976).

In addition to morphological and physiological properties of retinal ganglion cells, they also differentially respond to stimulus depending on where on the cell light falls and this is known as cell's receptive field. The receptive field was first described by Kuffler (1953) and is defined as the region over which a cell can be excited or inhibited by visual stimulus (Hubel and Wiesel, 1961). The receptive field of retinal ganglion cells is divided into two categories: ON-centre with OFF-surround and OFF-centres with ON-surround, with two being mutually antagonistic. The retinal ganglion cells have concentric receptive fields, with large receptive fields in the periphery but much smaller ones in the centre of the retina known as fovea in primates or area centralis in cats, where there is maximum photoreceptor density.

The optic nerves leave the eye at the optic disc (also know as blind spot) and meet at the optic chiasm. In cats as in primates and humans there is a partial decussation of optic tract fibres: the fibres from the nasal retina cross the midline while those from the temporal retina do not. The thalamus is a relay station for all the sensory information from the periphery except for olfaction. The part of the thalamus known as the dorsal lateral geniculate nucleus (dLGN) imparts visual information from the retina to the primary visual cortex. The topographic arrangement of retinal afferents is accurately mapped onto dLGN. The dLGN of the cat consists of A laminae (A and

A1) and C laminae (C, C1-C3). Layers A, C and C2 receive afferents from the contralateral eye and A1, C1 and C3 receive afferents from the ipsilateral eye (Guillery 1970). The laminae A, A1 and C receive afferent inputs from Y and X retinal ganglion cells while laminae C1-C3 receive inputs from W cells in the retina (Rodieck, 1979). In contrast to cats, macaque LGN is organized in six layers with layers 1, 4 and 6 receiving inputs from the contralateral eye while layers 2, 3 and 5 receive inputs from the ipsilateral eye. The geniculate receptive fields resemble those of the retinal ganglion cells, having concentric antagonistic centre-surround organization (Hubel and Wiesel, 1961).

1.1.2 Primary visual cortex (V1)

The axons from the dLGN travel through the optic radiation and project to V1. Just like the dLGN, V1 is also retinotopically organized. The striate cortex is divided into six functional layers and layer IV in macaque is additionally subdivided into four layers namely IVA, IVB, IVα, and IVβ. The geniculate afferents first synapse onto layer IV where segregation is still maintained and subsequent projections to upper and lower layers can be excited by the left eye and the right eye stimulation. In feline visual cortex, there is a significant overlap of the inputs from the two eyes within layer IV (Shatz and Stryker, 1978), resulting in much less area that is strictly monocular than in the case of macaque visual cortex (Hubel and Wiesel, 1977).

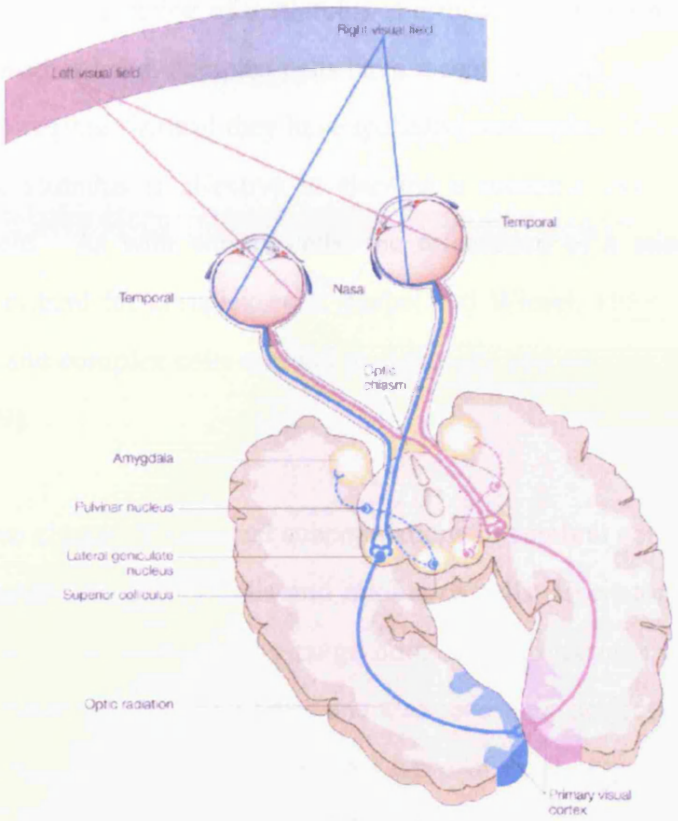


Figure 1.1: *Schematic visual pathway.* Axons from retinal ganglion cells converge at the optic disc and form the optic nerve. The fibres from the nasal hemiretina of each eye decussate at the optic chiasm while fibres from the temporal hemiretina do not cross. The fibres then project onto their target cells in lateral geniculate nucleus and finally project to primary visual cortex. The dashed line shows the subcortical pathway which is thought to pass information from the retina to superior colliculus, pulvinar and amygdala. (Adapted from Gazzaniga MS, 2002).

1.1.2.1 Types of cells in V1

The cells in the visual cortex are divided into simple and complex cells. Unlike the concentric shape of retinal and geniculate cell receptive fields, the simple cells have receptive fields with side-by-side arrangement of ON and OFF regions (Hubel and Wiesel, 1959). Also, the antagonism between excitatory (ON) and inhibitory (OFF) regions appears to be more pronounced in the cortical cells (Hubel and Wiesel, 1959)

compared to geniculate and retinal receptive fields. In contrast to retinal ganglion and dLGN cells, the orientation of a stimulus is critical for optimum response of simple cells. On the other hand, complex cells have receptive fields that lack segregated ON and OFF sub-regions; instead they have spatially overlapping ON and OFF responses. Therefore, a stimulus is effective in eliciting a response over a wide area in the receptive field. As with simple cells, the orientation of a stimulus for optimum response is critical for complex cells (Hubel and Wiesel, 1959; 1961). In addition, both simple and complex cells respond to stationary and moving stimulus (Hubel and Wiesel, 1959).

There are two classes of neuronal subpopulations in cerebral cortex and elsewhere in the brain namely excitatory cells and inhibitory cells. Excitatory cells make dense local projections, as well as long-range horizontal projections that usually contact cells with similar response properties and also send their projections to other cortical areas. Anatomically there are two distinct types of excitatory neurons namely spiny stellate and pyramidal cells. Spiny stellate cells give rise to intrinsic connections which usually make synapses with other spiny stellate cells (Saint Marie and Peters, 1985). These cells are the major recipient of thalamocortical axons and their dendrites tend to be restricted to single OD columns (Katz et al, 1989). On the other hand, inhibitory cells make only local projections, which are further spread in space than local excitatory connections (Kisvarday et al. 1986). Excitatory and inhibitory neurons receive different patterns of synaptic inputs and differ in their receptive field properties. For instance, excitatory neurons are orientation and direction selective whereas most inhibitory are orientation but poorly direction-tuned (Azouze et al, 1997; Gibber et al, 2001). A huge variety of anatomically distinct types of inhibitory interneurons has been described in the visual cortex which includes basket cells and chandelier cells (reviewed in Monyer and Markram, 2004). In addition, the inhibitory

interneurons are also classified according to peptides they express; the peptides include parvalbumin, calbindin and neuropeptide Y.

1.1.2.2 Ocular dominance

The modular organization of the cortex was first proposed by Mountcastle (1957) where cells extending through all cortical layers share similar response properties. Such columnar property is set by specific afferent inflow from the thalamus or by intracortical links such as horizontal connections, feedforward or feedback connections (Mountcastle, 1997).

The pioneering studies by Hubel and Wiesel (1962) using single cell recording, initially in cats and later in macaque monkeys (1968), elaborated the columnar organization of V1 by revealing that neurons are preferentially driven by stimuli delivered to one eye or the other (ocular dominance (OD) columns). The precise function of OD columns is not known though they are present in cats, ferrets, most monkeys, chimpanzees and man but absent in rodents and tree shrews. In animals that do have OD columns, there is variability in the layout of such columns. For example in cats, the layout and shape of OD columns is much more irregular than in the macaque. While OD columns in the macaque have stripy appearance with constant width of about 500μm (LeVay et al, 1975), OD columns in cats as revealed by optical imaging of intrinsic signals (OIS), have the shape of patches or curved bands running across the cortex over a distance of few millimetres (Hubener et al, 1997). The OD columns in humans are organized in a similar fashion to macaque except that human columns are much wider (~1000μm) as revealed by cytochrome oxidase (CO) activity (Horton et al, 1990). The OD columns in humans appear to be wider near the boundary of the primary visual cortex and within the representation of the peripheral visual field (Horton et al, 1990). Subsequent studies using fMRI (functional magnetic resonance imaging) (Menon et al, 1997; Cheng et al, 2001) confirmed the presence of

OD columns in humans and recently direct mapping of human ocular dominance columns was performed in response to monocular stimulation (Dechent et al, 2000).

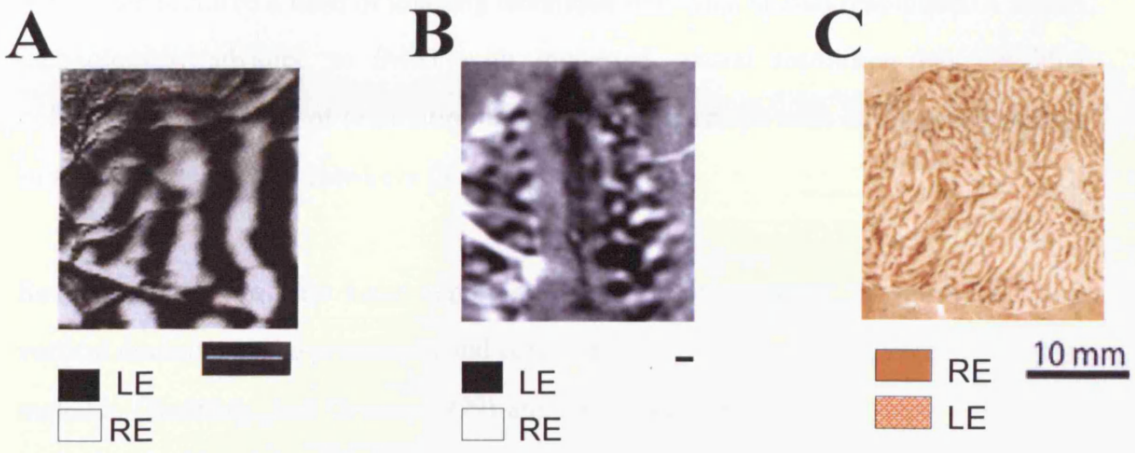


Figure 1.2: *Ocular dominance columns.* In visual cortex the proportion of neurons respond to stimulation of either eye resulting in OD columns. **(A)** Architecture of OD columns obtained from primate visual cortex using OIS (From Frostig et al, 1990). Neurons with similar eye preference are clustered together and respond more strongly to stimulation of one eye than the other eye forming stripy layout (scale bar 1mm). **(B)** Architecture of OD columns obtained from feline visual cortex using OIS. Neurons with similar eye preference are grouped together forming columnar organization (scale bar 1mm). The overall layout in feline visual cortex is more irregular compared to primate visual cortex. **(C)** Complete montages of OD columns obtained from human visual cortex by staining it with cytochrome oxidase (From Adams et al, 2007).

1.1.2.3 Orientation selectivity

Another major contribution of Hubel and Wiesel (1963) was that they showed that neurons in the same vertical penetration have the same preferred orientation while neurons in oblique penetrations change their preferred orientation in a systematic way. This leads to a mosaic of varying orientation columns across the cortex, whereby a complete set of orientation columns are arranged in pinwheels fashion (Bonhoeffer and Grinvald, 1991). These pinwheel centres tend to lie in the centre of

OD columns in both cats (Hubener et al, 1997) and in monkeys (Bartfeld and Grinvald, 1992). The property of orientation selectivity is functional and therefore it cannot be discerned via anatomical methods as with OD columns and its visualisation in humans required a need of imaging technique with high spatial resolution. A recent technological advance in fMRI with increased spatial resolution has revealed columnar organization of orientation in human visual cortex with striking similarities in spatial features with monkeys (Yacoub et al, 2008).

Several studies on cats have reported unequal representation of horizontal and vertical contours in the primary visual cortex. Investigators using electrophysiological methods (Kennedy and Orban, 1979) and OIS (Wang et al, 2003) reported that in both developing and adult cats, larger areas of visual cortex are activated by the horizontal and vertical contours than by oblique orientations. Such cardinal bias has also been reported in other species such as ferrets (Chapman et al, 1996; Chapman and Bonhoeffer, 1998) and this may not be a surprise considering most of the orientations encountered naturally and even more so in man-made environments are either vertically or horizontally based (Coppola et al 1998).

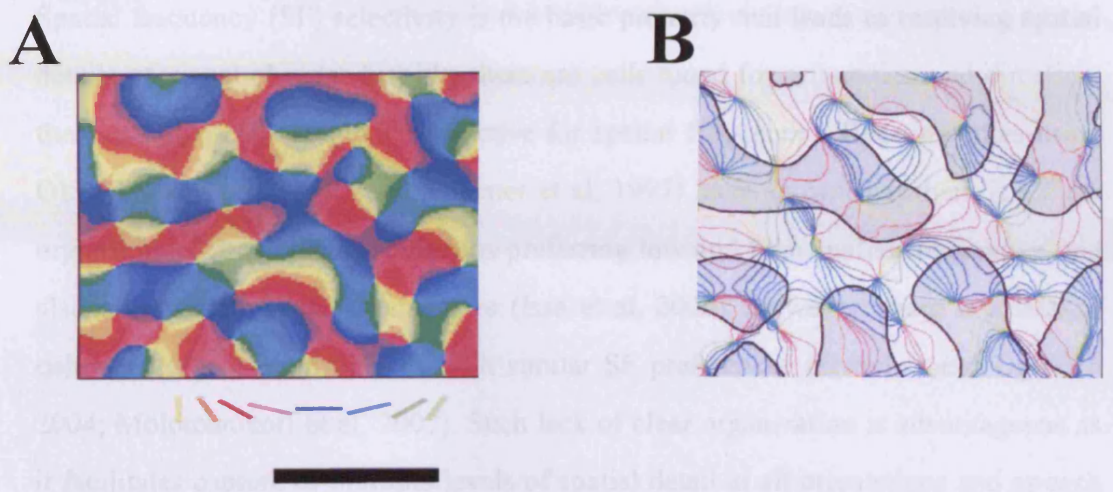


Figure 1.3: *Relationship between orientation selectivity and ocular dominance.* V1 neurons are selective to stimulus orientation and neurons with similar preferred orientation tend to cluster together forming columnar domain. **(A)** Orientation map obtained from feline V1; the angle of preferred orientation is colour coded according to the key shown below (scale bar 1mm). **(B)** The map shows iso-orientation domains and all points with a similar colour prefer same orientation. The black solid lines indicate the boundaries of ocular dominance columns and these intersect approximately at right angles with orientation preference iso-lines. (Figures from Hubener et al, 1997).

1.1.2.4 Directional selectivity

Similar to orientation selectivity and binocularity, direction selectivity is also organized in a columnar fashion, forming clusters, where units preferring similar direction of movements are grouped together in areas 17 and 18 of the cat visual cortex (Payne et al, 1981). The degree of clustering according to preference of direction is considerably weaker than that observed for orientation (Payne et al, 1981; Shmuel and Grinvald, 1996). Using OIS, it has been shown that iso-orientation patches in V1 exhibit preference for opposite directions (Weliky et al, 1996; Shmuel and Grinvald, 1996) suggesting that motion information is processed in early stages of visual processing.

1.1.2.5 Spatial frequency

Spatial frequency (SF) selectivity is the basic property that leads to resolving spatial details of visual objects. Just like there are cells tuned for orientation and direction, there are also cells within V1 selective for spatial frequency. Recent studies using OIS (Shoham et al, 1997 and Hubener et al, 1997) have shown that there is a clear organization of neuronal populations preferring low and high spatial frequencies and also intermediate spatial frequencies (Issa et al, 2000). However, there is a lack of columnar organization of cells with similar SF preferences (Sirovich and Uglesich 2004; Molotchnikoff et al, 2007). Such lack of clear organization is advantageous as it facilitates capture of multiple levels of spatial detail at all orientations and at each location in the visual field (Molotchnikoff et al, 2007). In fact computational models have shown that the geometrical relations between orientation, OD and spatial frequency maps are such as to optimise uniform combinations of map features across the cortex; this is commonly referred as coverage uniformity (Swindale et al, 2000). If one of the map features is perturbed, the coverage value decreases, suggesting that uniformity is important during development of cortical features (Swindale et al, 2000).

1.1.2.6 Cytochrome oxidase (CO) blobs

Cytochromes are responsible for electron transport and oxidative phosphorylation, yielding ATP which is vital for protein synthesis and maintenance of the resting membrane potential. Different neuronal groups with diverse functional demands may exhibit different levels of CO activity and that such levels may change when the degree of maintained neuronal activity changes (Wong-Riley, 1979). Histochemical staining for CO produces a regular pattern of darkly stained patches in the upper layers of the V1 in monkeys (Horton and Hubel, 1981), in cats (Murphy et al, 1995) and in humans (Duffy et al, 2007). The darker stained patches are known as blobs and the lighter intervening patches regions are known as interblobs. In both monkeys and

cats the blobs are located near the centre of OD columns. In monkeys, neurons within the blobs tend to be less binocular and less selective for orientation, but more colour selective with centre-surround receptive fields (Livingstone and Hubel, 1984). In contrast to monkeys, CO blobs in cats are correlated with the spatial frequency map; the blobs correspond to regions preferring low spatial frequency and lighter stained interblobs correspond to high spatial frequency domains (Shoham et al, 1997). CO blobs are therefore indeed a general feature in different species even though functionally they may serve a different purpose.

The histochemical technique for CO has been used effectively to demonstrate enzymatic changes in response to deprivation in cat visual cortex (Murphy et al, 1995) and in a patients suffering from long term monocular blindness (Duffy et al, 2007). In feline MD, CO dense bands within layer IV were replaced by alternating columns of dark and light reactivity corresponding to high and low neuronal activity respectively (Murphy et al, 1995). In human patients, CO staining loss was greatest in the superficial layer IVβ while a less obvious reduction was observed in layers V and VI (Duffy et al, 2007). Therefore CO staining in particular in humans is useful in determining the effect of MD on neuronal activity

1.2 Early development of the visual system

Connections in the visual pathway from the retina to the dLGN and then to cortex and subcortical areas are remarkably specific and researchers have been intrigued as to how and when such specific connectivity is established. Several studies carried out in the last 20 years have shed light on the time-line of development of the visual system in several species including cats.

The eye specific afferents in cats are initially (between E38-E43) intermixed with each other throughout dLGN as revealed by labelling individual ganglion cells with horseradish peroxidase (Sretavan and Shatz, 1986). By E55, the eye-specific layers emerge gradually by retraction and expansion of axonal arbors appropriate to their eye of origin. This retinogeniculate segregation takes place even before photoreceptors become capable of responding to light (Galli and Maffei, 1988). These retinogeniculate axons are thought to be guided by molecular cues such as ephrins (reviewed in McLaughlin and O'Leary, 2005) and retinal activity (Shatz and Stryker, 1988).

The process of segregation of retinogeniculate afferents is widely believed to involve competitive interactions between ganglion cell axons from the two eyes. Some of the first evidence in favour of the idea that competitive interactions underlie segregation has come from studies of enucleation. Chalupa and Williams (1984) enucleated one eye in kitten fetuses two weeks prior to birth and found that the intact eye in adult cats occupied the entire territory of dLGN including the area that would have been normally innervated by the enucleated eye (Chalupa and Williams, 1984). These morphological changes in dLGN remarkably did not alter the functional responses of dLGN neurons with retinotopy being intact (Chalupa and Williams, 1984). This suggests that neuronal activity from both eyes is necessary for segregation to take place and retinogeniculate axons are not intrinsically programmed for their ocular identity.

1.2 Early development of the visual system

Retinal ganglion cells are the first principle sites of visual processing, and they fire action potentials spontaneously during very early stage of development (Galli and Maffei, 1988). Shatz and Stryker (1988) have shown that silencing activity of retinal ganglion cells with tetradotoxin (TTX) in kitten fetuses aged between E45-65 (around the time when segregation takes place) prevents the retinogeniculate segregation. Interestingly, the segregation is not prevented by arrested growth but instead by growth of inappropriate branches that would normally have been selectively eliminated (Shatz and Stryker, 1988).

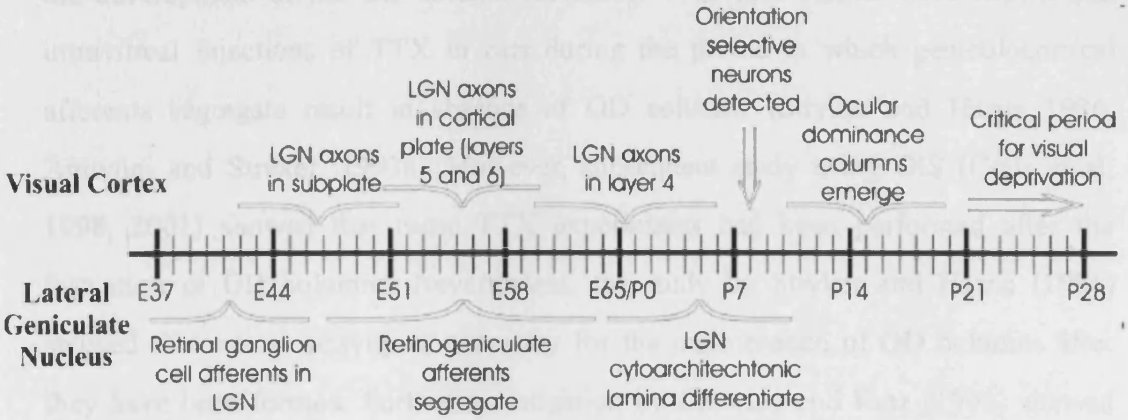


Figure 1.4: *Cat visual development time line.* Emergence of visual cortical properties is indicated in the top panel while the bottom panel indicate the time line development lateral geniculate properties. Segregation of retinogeniculate axons takes place in embryonic stage and the emergence of orientation selective neurons and ocular dominance columns appear in post natal stage before the onset of the critical period (Figure adapted from Crair et al, 2001).

1.2.1 Emergence of ocular dominance columns

As described above, the retinogeniculate afferents are established during a very early stage of development. Several studies have been carried out to determine when eye-specific segregation (formation of OD columns) occurs at the cortical level in cat visual cortex. Earlier anatomical studies using transneuronal tracer (proline) to label dLGN axons found that the patterns of proline was continuous in young animals and

an alternating pattern of label was observed in cats between three to six weeks of age (LeVay et al, 1978). The disadvantage of using transneuronal tracers is that in young animals there is a problem of spill-over in the dLGN into neighbouring layers (LeVay et al, 1978) thus resulting in continuous pattern even in the presence of segregated columns. Subsequent studies using OIS (Crair et al, 1998, 2001) and anatomical studies (using transneuronal and retrograde labelling) showed that geniculocortial segregation is present by the second postnatal week in cats.

Several studies have been carried out to determine the role of spontaneous activity in the development of the OD column formation. Previous studies have shown that intravitreal injections of TTX in cats during the period in which geniculocortical afferents segregate result in absence of OD columns (Stryker and Harris 1986; Antonini and Stryker, 1993a). However, subsequent study using OIS (Crair et al, 1998, 2001) showed that those TTX experiments had been performed after the formation of OD columns. Nevertheless, the study by Stryker and Harris (1986) showed that retinal activity is necessary for the maintenance of OD columns after they have been formed. Further investigation by Crowley and Katz (1999) showed that enucleation of both eyes in ferrets between P0 and P18 (before geniculocortical afferents even reach layer 4) remarkably revealed patches (using anterograde tracer) in adult ferret resembling OD columns. This study showed that retinal activity is not requisite for the formation of OD columns and the authors put forward the hypothesis that molecular cues such as ephrin may guide the segregation of geniculocortical afferents (Crowley and Katz, 1999). However, even in the absence of eyes, it is possible that spontaneous activity in the dLGN and V1 may provide the basis of activity-dependent formation of OD segregation (Hubener and Bonhoeffer, 1999). Nevertheless, the study by Crowley and Katz challenged the widely accepted model in which retinal activity controls the process of thalamocortical segregation.

Recently, a study by Huberman and Chapman 2006 challenged Crowley and Katz's (1999) view on the role of spontaneous activity in the formation of OD columns. Their study showed that blocking retinal activity in both eyes in ferrets with epibatidine (a nicotinic cholinergic agonist that can block retinal waves) between P1-P10 disrupted the anatomical patterning of the OD columns when labelled (using transneuronal tracer) at P100. Labelling in adult animals offers the advantage of avoiding spill-over problems usually encountered in young animals (Huberman et al, 2006). Blocking retinal activity specifically resulted in no discernible OD segregation in the cortical hemisphere contralateral to the proline-injected eye. In contrast, in the cortical hemisphere ipsilateral to the proline-injected eye, OD columns were observed, however, they appeared broader than normal (Huberman et al, 2006). In addition, retinal blockade increased receptive field size of binocular cells but not monocular cells, perhaps in the absence of retinal activity, the binocular cells fail to undergo refinement (Huberman et al, 2006). It is not quite clear as to why enucleation (Crowley and Katz, 1999) results in normal OD segregation whereas blockade of retinal activity does not. It can be argued that blockade of spontaneous activity is different from eye removal (Crowley and Katz, 1999) in terms of its impact on the levels and pattern of activity in remaining thalamocortical afferents (Huberman et al, 2006). In fact enucleation is likely to induce dramatic secondary effects due to deafferentation (Del Rio and Feller, 2006).

1.2.2 Development of orientation selectivity

The development of orientation selectivity was first studied in monkeys and in cats. In kittens, orientation-specific responses are detected as early as at P8 before natural eye opening (Hubel and Wiesel, 1963). However, the degree of orientation tuning during development remained controversial in the literature, from 0% of cells exhibiting orientation selectivity (Barlow and Pettigrew, 1971) to 25-30% (Blakemore and Van Sluyters, 1975; Buisseret and Imbert, 1976), to 100% (Hubel

and Wiesel, 1963). These discrepancies could be due to difficulty in performing electrophysiological recordings in young kittens where cells tend to respond sluggishly (Hubel and Wiesel, 1963). Also, maintaining optimum physiological conditions can be difficult in very young kittens and any slight changes in blood pressure or expired CO_2 levels can make orientation-selective cells non-responsive (Blakemore and Van Sluyters, 1975). For this reason, more recent studies use ferrets as animal model to study orientation selectivity development. The ferret is born around three weeks earlier in the development compared to cats (Linden et al, 1981) and therefore serves as an ideal model for studying early stages of development. Studies on ferret primary visual cortex have shown that some degree of orientation tuning exist before natural eye opening and have been recorded through closed eye lids (Krug et al, 2001) but adult-like tuning levels are not reached until a week after eye opening (Chapman and Stryker, 1993) suggesting that visual experience is not required for initial establishment of orientation selectivity but is required for its subsequent refinement and maintenance.

In addition, studies on the development of orientation maps using OIS in ferret V1 have shown that orientation maps are observed around the time of natural eye opening (P31-P35) and that the overall layout of these maps remains stable throughout development (Chapman et al, 1996). This suggests that patterns of iso-orientation domains are established early during development and are not affected by anatomical rearrangement of geniculocortical afferents (Chapman et al, 1996).

Although visual experience is not necessary for the initial development of orientation selectivity, spontaneous activity is necessary. This was well documented in a study by Chapman and Stryker (1993) which showed that silencing all spontaneous neuronal activity in the ferret visual cortex (TTX treatment was induced between 21-23 days, before eye opening, until 7 weeks of age) completely abolished maturation of orientation selectivity. Several experiments have addressed whether activity is playing an instructive role in orientation selectivity development. Computational models have suggested that spontaneous activity from ON and OFF centres of retinal

ganglion instruct the development of orientation tuning in cortical cells (Miller, 1994).Silencing pharmacologically ON-centre retinal ganglion cells in ferrets (age between 28-35 days) prevented the maturation of orientation maps as revealed by OIS (Chapman and Godecke, 2000). This suggests that patterns of neuronal activity carried in the separate ON and OFF centre visual pathways are necessary for the development of orientation selectivity and activity carried by both these channels is indeed playing an instructive role (Chapman and Godecke, 2000).

1.2.3 Development of direction selectivity

The property of direction selectivity is not innate rather this property is acquired later during development (Li et al (2006). Rearing ferrets in complete darkness from birth prevents the development of direction selectivity and only restoring vision within a week of eye opening established motion selectivity whereas later did not (Li et al, 2006). Therefore there appears to be a narrow time-window which allows the formation of direction selectivity to be shaped by visual experience (Sengpiel, 2006), this unique brief dependency distinguishes direction selectivity from other mapped properties of the visual cortex which are present in ferrets in the absence of light (White et al, 2001; Li et al, 2006). This finding also suggests that visual experience plays an instructive role and is not merely permissive for the emergence of cortical direction selectivity (Huberman et al, 2008).

1.3. Experience-dependent plasticity

The development of the visual system and the emergence of receptive field properties in V1 can be divided into three stages. The first stage occurs during early embryonic life, and is an activity-independent stage, during which for example, the retinal ganglion cell afferents form synapses in the dLGN. The second stage is an activity-dependent but experience-independent stage; it comprises processes such as retinogeniculate segregation and emergence of OD columns. The third stage occurs postnatally during a defined time window (critical period) where the visual system is remarkably sensitive to sensory manipulation (experience-dependent plasticity).

The critical period is defined as the time period when neural development is susceptible to environmental manipulation and appropriate stimulation allows maturation and refinement of neuronal networks. The critical period is different for different species and receptive field properties. For example, in cats, the critical period for ocular dominance plasticity commences at 3 weeks of age (Hubel and Wiesel, 1970) although Olson and Freeman (1978) reported that some susceptibility to deprivation effects is present even during the second postnatal week. The critical period then peaks at 4-6 weeks of age and declines at 3-4 months of age; however, cats deprived at 4-6 months of age still show plasticity (Cynader and Mitchell, 1980). In contrast to cats, in macaque, the critical period for visual function extends to about 2 years of age and in humans it ends at about the age of 8-10 years. The critical period for OD plasticity varies in different cortical layers in cats; it first declines first in layer IV (Mower et al, 1985) and more slowly for layers II, III, V and VI (Daw et al, 1992).

Early studies by Wiesel and Hubel (1963a) were the first to demonstrate that when kittens undergo monocular deprivation (MD) for 2-3 months during the peak of the critical period, the majority of cells found using single-cell recording were driven by the non-deprived eye (NDE) and they failed to find cells responding to the deprived eye (DE). Anatomical studies based on transneuronal transport of radioactive tracers

injected in one eye of kittens monocularly deprived from the period of eye-opening till the end of critical period, have demonstrated that cortical domains devoted to the DE undergo substantial shrinkage in layer IV while those innervated by the NDE expand (Shatz and Stryker, 1978).

In addition to anatomical changes observed in striate cortex, there are morphological changes observed in dLGN during MD, for instance, cells in DE layers tend to be 30-40% smaller and appear pale and more densely packed than cells in the NDE layers, however, their responses are normal physiologically (Wiesel and Hubel 1963b, Guillery and Stelzner, 1970). As mentioned previously, adjacent laminae of dLGN receive their afferents from opposite eyes and in addition, there is a monocular segment of the nucleus which only receives afferents from the temporal part of the visual field known as the monocular segment of nucleus. The morphological changes that occur following MD are seen most clearly in the binocular segment of the dLGN, in laminae A and A1 (Wiesel and Hubel, 1963b; Guillery and Stelzner, 1970) and not in the monocular segment (Guillery and Stelzner, 1970). This observation suggests that in the binocular segment of dLGN competition between two eyes take place whereas in monocular segment there is no competition and so cells are unaffected by imbalanced activity. It should be noted that even though the cell bodies and dendrites are in the dLGN, most of the axon terminals are in the cortex whose shrinkage and expansion is evident in MD. Therefore competition is thought to take place in V1 and consequently has a retrograde effect in the dLGN.

In another study, Hubel and Wiesel (1965) sutured the lids of both eyes (binocular deprivation, BD) and found that the cortex was affected with an overall reduction in responsiveness and orientation selectivity. However, the effects of BD on ocular dominance were less detrimental than the effects of MD. Thus this suggests that the damage caused by MD is not simply due to disuse or absence of patterned input but indeed depends largely on the balanced activity in the afferent pathways from the two eyes (Hubel and Wiesel, 1965). However, it should be noted that prolonged periods of BD lead to devastating effects resulting in a high percentage of visually

unresponsive cells with abnormal receptive fields and fewer cells with direction and orientation selective properties (Mower et al, 1981).

1.3.1 Plasticity in adults

The classical studies by Hubel and Wiesel led to the notion that there is a time window during the juvenile age where the visual cortex is most susceptible to the effects of sensory manipulation. However, recent studies (Guire et al, 1999; Sawtell et al, 2003; Pham et al, 2004; He et al, 2006; Hofer et al, 2006; Fischer et al, 2007) have proven beyond doubt that adult rodent visual cortex has far greater potential for experience-dependent plasticity than previously thought. The mechanism underlying OD plasticity appears to differ between juvenile and adult animals. For instance, in juvenile animals, OD shift occurs in mice (similar to cats, Friedlander et al. 1991) by depression of DE responses and/or enhancement of NDE responses (Frenkel and Bear, 2004) while in adults, enhancement of NDE eye precedes depression of DE responses (Sawtell et al, 2003; Hofer et al, 2006). It should be noted that plasticity during development is essential for refining the neuronal network and plasticity in adults has to be restricted in some way as drastic remodelling can be detrimental (Karmarkar and Dan, 2006). It should also be noted, however, that so far adult OD plasticity has been demonstrated primarily in mice and it will be interesting to see if such plasticity exists in higher animals such cats or monkeys, whose visual system bears more resemblance to the human than that of rodents.

1.4 Concepts of plasticity

1.4.1 Cellular mechanisms of plasticity

The term synaptic plasticity was first coined by Tim Bliss and Terje Lomo in 1973 and is defined as the ability of synapses to change in strength depending on activity. Hebb was one of the first people to suggest that learning takes place by strengthening transmission at a single synapse. His widely accepted postulate on synaptic modification has become a keystone in understanding activity dependent neural development and the cellular basis of learning and memory. He postulated in his book (Hebb, 1949):

"when an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth processes or metabolic changes takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased".

One of the key features of Hebb's postulate is that an increase in synaptic strength or "weight" of the synapse connecting A to B is dependent upon the correlated firing of A and B – commonly referred to as "neurons that fire together wire together". One of the central features to Hebb's postulate is temporal specificity which means that the synaptic connection is strengthened only if cell A takes part in firing cell B i.e.; cell A fires before cell B (Bi and Poo, 2001). In fact experimental evidence supports the notion that the activation of both pre- and postsynaptic elements induces synaptic modifications by strengthening or weakening synaptic weights depending upon the temporal order of these activations (Rauschecker and Singer 1981). Similarly synaptic efficiency diminishes or is reduced remarkably if either the pre- or postsynaptic neuron is silenced (Rauschecker and Singer 1981).

Hebbian mechanisms have provided a useful explanation of some of the experimental evidence of experience-dependent plasticity in the visual cortex. For instance in a

normal kitten, a given point in the visual field is mapped on corresponding points of the two retinas therefore the presynaptic activity from the two eyes converging on a postsynaptic target cell is synchronous. However, when one eye is deprived of vision, the pattern activity is abolished in that eye hence the pre-synaptic activity from the two eyes is not in synchrony with their post-synaptic target neuron (Stent, 1973). Therefore, in such condition, anti-Hebbian mechanisms prevail where neurons do not fire together (Stent, 1973). Hebbian and anti-Hebbian learning rules permit bidirectional modification of synaptic strengths depending on correlated pre- and postsynaptic activity, or the lack of it (Lisman, 1989). Such specificity is not only useful in stabilizing neuronal networks but may serve a useful purpose in physiological functions such as learning and memory.

During early postnatal development of cat visual cortex; there is asymmetry between responses in two eyes, the response to stimulation of contralateral eye is stronger and more selective than response to stimulation of ipsilateral eye in cat visual cortex (Crair et al, 1998). The ipsilateral eye responses become as strong as the contralateral eye at about 3 weeks of age (Crair et al, 1998). It appears that the rapidly maturing contralateral eye inputs serve as a template for ipsilateral eye maturation and indeed depriving contralateral eye results in poorly organized retinopy map in the ipsilateral eye as shown using OIS (Smith and Trachtenberg 2007). The transition from overall contralateral dominance to an almost equal partition of the cortex between the two eyes poses some problems for strictly Hebbian activity-dependent process and therefore it is possible that anti-Hebbian prevails during development in cat visual cortex (Crair et al, 1998).

Long-term potentiation (LTP; an increase in the efficacy of synaptic transmission) and long-term depression (LTD; a decrease in the efficacy of synaptic transmission) have been accepted as paradigmatic examples of Hebbian plasticity and these two processes are thought to be basis of learning and memory. The processes of LTP (Kirkwood et al 1994a) and LTD (Kirkwood et al, 1994b) have been proposed to contribute to experience-dependent synaptic modifications in the visual cortex during

MD and in dark-reared animals. Also, susceptibility to LTP coincides with the critical period and rarely occurs in adulthood; while LTD occurs in developing as well as in mature cortex (Yoshimura et al, 2003).

Perhaps one of the biggest shortcomings of Hebb's postulate is that it does not define a point where the strength of synaptic weight is prevented to reach maximum or zero. Therefore more and more correlation or decorrelation would potentiate or weaken synaptic weights reaching maximum or zero respectively (Bienenstock et al, 1982, Turrigiano and Nelson, 2000). Furthermore, in order to maintain stable neuronal network, strengthening of some inputs should be accompanied by weakening of other inputs, but the simplest Hebbian rules are not competitive, in that the strengthening of some inputs does not necessarily lead to the weakening of others (Turrigiano and Nelson, 2000). Competition is a ubiquitous feature of synaptic plasticity and is important in OD plasticity, where inputs from the normal eye are retained whereas inputs from the deprived eye are lost. However, a study by van Rossum et al (2000) has shown that stable correlation-based plasticity can be achieved without introducing competition, suggesting that competition may not be desirable at all developmental stages.

The limitations of Hebbian mechanisms required a need of mechanism that maintains an appropriate level of total excitation within a neuronal network. Bienenstock, Cooper and Munro (1982) suggested another cellular mechanism of plasticity, where a certain level of post-synaptic activity (known as modification threshold, θm) maintains overall synaptic weight by preventing synaptic activity to reach maximum or zero (this model is referred to as BCM model). According to this model, the overall magnitude of postsynaptic response determines whether synaptic strength will increase or decrease (Bienenstock et al, 1982). In addition, the change in efficacy of a synapse depends not only on instantaneous pre- and postsynaptic activities, but also on temporal order of their activation (Bienenstock et al, 1982). Therefore the timing of inputs arriving at the post synapse determines whether the strength of synapse is increased or decreased.

A fixed modification threshold would lead to certain problems, for example, during BD when the post synaptic response to all patterns of input activity slips below θm, then the synaptic strength would decrease to zero and the cell would cease responding to any stimulus (Bienenstock, 1982). On the other hand if the response is greater than θm, then all synapses would potentiate to their saturation and the cell would lose its selectivity (Bienenstock et al; 1982; Bear, 1996). Therefore, the BCM model predicts that θm is a non-linear function and slides depending on the average firing rate of the postsynaptic cell (Bienenstock et al, 1982). The threshold increases if the postsynaptic neuron is highly active, making LTP more difficult and LTD easier to induce. And when the average postsynaptic activity is low, it slides so as to make LTP more likely. This sliding of the modification threshold (also known as 'meta plasticity') therefore stabilizes the neuronal network.

One of the theoretical requirements for the BCM modification threshold is that its value reflects the history of the postsynaptic activity, i.e. the depression vs. potentiation crossover point, θm, varies depending on the history of cortical activity. An elegant study by Kirkwood et al (1996), showed experimentally the evidence for a sliding modification threshold in visual cortex. Their study revealed that in slices of visual cortex of light-deprived rats LTP is enhanced and LTD is diminished and exposing these animals to light for only two days, returned the magnitude of LTD to normal levels. These findings support the concept that θm is set according to the activation history of the cortex.

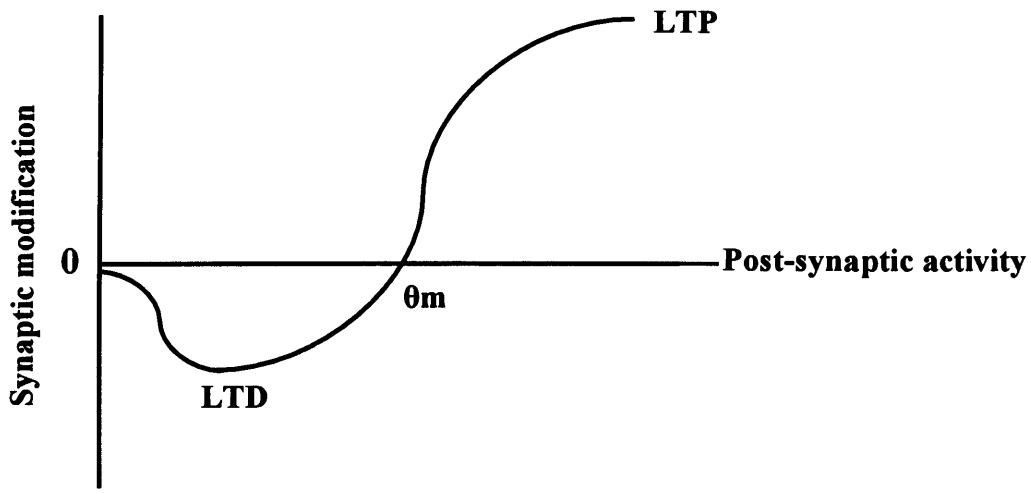


Figure 1.5: *BCM model.* The θm threshold slides along as a function of post synaptic activity. The θm threshold increases if the postsynaptic neuron is highly active, making LTP more difficult and LTD easier to induce. And when the average postsynaptic activity is low, it slides so as to make LTP more likely.

The BCM model and its modification threshold allows neuronal activity to remain relatively constant, however, in a physiological environment neuronal network are subject to frequent perturbations such as changes in synapse number or strength or release of neurotransmitters that alter excitability constantly. Therefore there is a need of homeostatic plasticity that serves to stabilize neuronal activity in the face of such perturbations. Synaptic scaling, a form of homeostatic plasticity, is thought to adjust firing rates of neurons by globally scaling excitatory synaptic strengths up or down thus maintaining the relative strengths of individual synapses (Turrigiano et al, 1998).

Synaptic scaling was first demonstrated by Turrigiano et al, (1998) in rat visual cortical pyramidal neurons in vitro. Their study showed that abolishing firing activity using TTX increased the amplitude of miniature excitatory postsynaptic currents (mEPSC) without changing their kinetics. These changes occur relatively slowly and cumulatively requiring several hours of altered activity to produce measurable

changes in synaptic strength (Tuirrigiano et al, 1998). This slowness is important because if homeostasis occurred too rapidly, it would dampen moment-to-moment fluctuations that are used to transmit information (Turrigiano and Nelson, 2004). Synaptic scaling has also been successfully demonstrated in vivo where monocular injection of TTX in one eye for 2 days increases the amplitude of mEPSCs in pyramidal neurons in the DE hemisphere while leaving the ones in the NDE hemisphere unaffected (Desai et al, 2002). In addition, synaptic scaling has also well been demonstrated in experience-dependent plasticity using two-photon calcium imaging, where long durations (4-8 days) of MD in mice not only strengthened overall open eye responses but also increased deprived eye responses of the few neurons devoid of non-deprived eye input (Mrsic-Flogel et al, 2007). This suggests that synaptic scaling is involved in activity-dependent refinement of neuronal circuitry and reflects recent cortical history.

1.4.2 Molecular correlates of plasticity

Molecular mechanisms underlying the development and plasticity of visual cortex are a key towards understanding the role of experience in shaping and refining neuronal networks. Several studies have been done in an attempt to find the molecular players involved in ocular dominance plasticity; however, the mechanism underlying the cortical response to sensory deprivation still remains an elusive phenomenon.

1.4.2.1 The role of excitatory transmission

N-methyl-D-aspartate receptors (NMDARs) are a class of ionotropic excitatory L-glutamate neurotransmitter receptors. They require the simultaneous binding of glutamate and glycine (co-agonist) and their activation is voltage-dependent. The voltage dependent activation is necessary to alleviate blockade by magnesium ions (Mayer et al, 1984). The NMDAR is a heteromeric ion channel composed of two NR1 subunits and two NR2 subunits. There are four types of NR2 subunits, namely NR2A, B, C and D. In the cerebral cortex, NR1, 2A and 2B are the dominant subunits (Monyer et al, 1994); NR2 subunits confer functional variability to the receptors and

NR1 is a mandatory subunit required for the function of the receptor and binding of glycine.

In cat visual cortex, NR1 and NR2B are highly expressed during the first post-natal week and NR2A is expressed at very low levels at first and then peaks at about 5 weeks of age (Chen et al, 2000). The switch from NR2B to NR2A shortens the NMDAR-EPSP (excitatory post synaptic potential) current duration (Flint et al, 1997), i.e., the duration of the open time for the NMDAR channels becomes shorter. The long duration of the NMDA receptor-mediated synaptic current is caused by slow dissociation of glutamate from the receptor, and this long duration current lasts for 100msec (Bourne and Nicoll, 1993). The evidence for the role of NR2A in shortening of NMDAR-EPSP currents is further provided in transgenic mice with targeted disruption of NR2A, where the long duration EPSP is maintained (Roberts et al 1998; Fagiolini et al, 2003). Also in mice with NR2B knock-out, the duration of EPSC was not different from wild-type, suggesting that NR2A determines the duration of the EPSP current (Philpot et al, 2001).

Originally, the shortening of NMDAR-EPSP currents was thought to correlate with the elimination of visual cortical plasticity in rats (Carmignoto and Vicini, 1992). However, subsequent studies have shown that the switch takes place near the start (not the end) of the critical period in ferrets (Roberts and Ramoa, 1999), in rats (Fagiolini et al, 2003) and in cats (Fox et al, 1989; Chen et al, 2000). The increased NR2A expression and consequent changes in NMDAR-EPSC kinetics at a relatively early stage in development implies that fast kinetics are necessary to transfer the sensory cortex from an immature state to a more mutable state (Roberts and Ramoa, 1999; Carmignoto and Vicini, 1992).

NMDA receptors mediate a slow component of synaptic transmission (100msec) and are involved in synaptic plasticity in the visual cortex (Artola and Singer, 1987). Antagonists of NMDA receptors such as APV (2-amino-phsophonovaleri acid) abolish OD plasticity that normally occurs after MD (Bear et al, 1990) and also

prevent the development of orientation selectivity (Ramoa et al, 2001). Several studies have been carried out to determine the role of NR2A and NR2B in visual cortical plasticity. The evidence for the role of NR2A and NR2B in critical period plasticity is well documented by raising animals in complete darkness (Quinlan et al, 1999; Chen et al, 2000; Philpot et al, 2001). Dark-rearing attenuates the developmental increase in NR2A, and levels of NR2A increase rapidly (in < 2 hours) when dark-reared rats are exposed to light, and decrease gradually over the course of 3-4 days when they are deprived of light (Philpot et al, 2001). These results suggest that experience-induced changes occur within hours while deprivation-induced changes take days (Philpot et al, 2001). However, brief periods (2-3 days) of dark-rearing in mice result only in an increase in NR2B levels while the levels of NR2A levels are unaltered (Chen et al, 2007). This indicates that the earliest response to visual deprivation is a decrease in the ratio of NR2A/2B which is achieved by increased expression of NR2B followed by a later reduction in the expression of NR2A (Chen et al, 2007). These results demonstrate that NMDAR subunit composition in the visual cortex is remarkably dynamic and can be modified bidirectionally as a result of visual experience (Quinlan, 1999; Philpot et al, 2001; Chen et al, 2007). Consistent with the dark-rearing studies described, brief period (3 days) of MD also results in elevation of NR2B levels followed by a delayed reduction in NR2A levels after 7 days of MD in the contralateral hemisphere (Chen et al, 2007). The MD-induced changes in NR2B and NR2A expression map closely map onto time points when physiological changes (DE depression occurs first followed by a delayed (7 days) potentiation of NDE responses.) are known to occur (Frenkel and Bear, 2004). In addition brief MD in NR2A-knockout mice failed to exhibit deprivation-induced depression instead demonstrated potentiation of the NDE inputs (Cho et al, 2009). Therefore this suggests that a reduction in the ratio of NR2A/2B during MD is permissive for the compensatory potentiation of the NDE inputs (Cho et al, 2009).

In contrast to NMDA receptor mediated transmission, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) are responsible for fast excitatory currents in conditions of basal neuronal activity. Activation of NMDA receptors

results in calcium influx into post-synaptic spine, which regulates kinases and phosphotases which then regulate AMPA receptors. A wealth of studies has been carried out to determine the role played by AMPA receptors in OD plasticity. The expression of AMPA receptors appears to be species-specific for instance, in mice GluR1 is predominantly expressed in cortical layers II/III, V and VI with highest density in layers II/III (Kim et al, 2005). In contrast to mice, GluR1 expression is dominant in layer VI in rats (Gutierrez-Ibarluzea et al, 1997). In cat visual cortex, GluR1 is relatively abundant in layers II-VI whereas GluR3 dominates layers II, III, V and VI (Gutierrez-Igarza et al, 1996). GluR2 is expressed in cortical layers II/III, the upper part of the layer V and layer VI, and is scarce in layer IV (Van Damme et al, 2003). GluR4 is sparse in cat visual cortex and is predominantly found in layers III and V (Gutierrez-Igarza et al, 1996). The reason for such species differences is not known.

The four subunits are expressed differentially during normal and altered visual experience. In rats, expression of GluR1 and GluR2 is absent during embryonic stage and emerges during early postnatal days (Akaneya, 2007). In contrast, expression of GluR4 is relatively abundant in embryonic stage and then declines gradually to become almost absent in adulthood. Dark-rearing does not influence the expression of GluR1 and GluR2 subunits while the expression of GluR4 remains elevated (Akaneya et al, 2007). This suggests that GluR4 expression is regulated in an activity-dependent manner and sensory experience is required for its decline during development. This is reminiscent of the NR2A subunit which is also regulated in an activity-dependent manner.

Several studies have been carried out to determine the role of excitatory transmission underlying cellular mechanisms of plasticity. For instance, the mechanism underlying synaptic scaling is thought to be mediated by AMPA transmission (Turrigiano et al, 1998) and blockade of AMPA receptors with CNQX (cyano-7-nitroquinoxaline-2, 3-dione) increases mEPSC (miniature excitatory post synaptic potential). A study by Watt et al, (2000) demonstrated that when recording mEPSCs from cultured cortical

neurons expressing both NMDA and AMPA receptors, the ratio of NMDA and AMPA mEPSCs was similar from neuron to neuron. Both the AMPA and NMDA mEPSCs increase proportionately by activity blockade (with TTX) and decrease proportionately by activity enhancement (using bicuculline). Therefore these receptors are tightly co-regulated by activity at synapses at which they are both expressed (Watt et al, 2000).

The molecular mechanism underlying LTP and LTD has generated a wide interest largely because of its link with learning and memory. Pharmacological experiments suggest that NR2A and NR2B-containing NMDARs contribute selectively to LTD and LTP induction respectively (Yoshimura et al, 2003). In addition, the developmental decline of NR2B subunit was paralleled by a decline in the incidence of LTP, and these were both prevented by rearing rats in darkness (Yoshimura et al, 2003). Therefore, the long duration of NMDAR-mediated current facilitates generation of LTP and inclusion of NR2A subunits (increasing the ratio of NR2A/NR2B) results in subsequent shortening of NMDAR- mediated currents which then facilitates LTD. In addition, studies by Bear et al (1987) have proposed a possible physiological mechanism underlying the sliding θm. Their study suggested that θm is the membrane potential at which NMDAR activation results in calcium entry; a modest but sustained elevation in postsynaptic calcium results in LTD and LTP is elicited by large calcium influx.

1.4.2.2 The role of inhibitory transmission

The primary inhibitory neurotransmitter in the brain is γ- amino butyric acid (GABA) and the large majority of $GABA_A$ receptors is composed of two α, two β, and one γ subunit (Baumann et al, 2001). In most juvenile brain regions post-synaptic $GABA_A$ receptors incorporate α2 and α3 subunits (Fritschy et al, 1994) which mediate long-lasting inhibitory post-synaptic currents (IPSCs) (Bosman et al, 2002). During development, the α1 subunit which is initially rare is strongly up-regulated and forms the dominant α subunit in most brain regions while the α2 and α3 subunits diminish

(Fritschy et al, 1994; Heinen et al, 2004). The incorporation of α1 subunit results in short-lasting IPSCs (Bosman et al, 2002; Heinen et al, 2004) and mice lacking α1 subunit retain juvenile IPSC kinetics until adulthood (Bosman et al, 2005). Additionally mice with "knockin" mutation to alpha subunits which rendered individual $GABA_A$ receptors insensitive to diazepam prevents the induction of OD plasticity (Fagiolini et al, 2004). This suggests that the inclusion of the α1 subunit and the subsequent shortening of IPSCs are essential for OD plasticity. The expression of α1 subunit and its dependence on sensory experience appears to be species specific. For example, raising rats in complete darkness did not have a major impact on the expression pattern of α1 subunits (Heinen et al, 2004) while rearing cats in complete darkness elevated its expression (Chen et al. 2001).

The functional maturation of GABAergic synapses occurs much later than maturation of excitatory transmission. GABA is synthesized by glutamic acid decarboxylase (GAD) which is encoded by two distinct genes, *GAD65* and *GAD67*; the former synthesizes GABA at synaptic terminals and the latter synthesizes GABA throughout the cell. Deletion of *GAD65* gene prevents ocular dominance plasticity in response to MD, and this plasticity is restored by infusion of GABA agonists such as diazepam (Fagiolini and Hensch, 2000). This form of rescue is possible at any age in *GAD65* knockout mice; suggesting that the onset of the critical period is dependent on a threshold level of inhibitory transmission (Fagiolini and Hensch, 2000). The basic receptive field properties, such as size, retinotopy, and orientation and direction selectivity are unaffected in *GAD65* deletion (Hensch et al, 1998; Fagiolini and Hensch, 2000). However, it is possible that GABA synthesized by *GAD67* is sufficient to maintain receptive field properties. A recent study by Kanold et al (2009) has demonstrated that mice with *GAD65* knock-out not only have diminished inhibition but also show reduced NR2A levels and slower NMDAR currents. And application of benzodiazepines also increase NR2A levels suggesting that changes in both excitation and inhibition engage in homeostatic mechanisms to maintain neuronal stability (Kanold et al, 2009). Mice with *GAD67* deletion are not viable and therefore it was essential to generate a conditional knock-down of *GAD67* to

determine its role in visual cortex. A recent study (Chattopadhyaya et al, 2007) has shown that conditional knock-down of *GAD67* resulted in deficits in axon branching and perisomatic synapse formation, and this was rescued by suppressing GABA reuptake and by infusion of $GABA_A$ agonist, diazepam. This suggests that GABA synthesized by *GAD67* is involved in the maturation of inhibitory innervation patterns (Chattopadhyaya et al, 2007).

The onset of the critical period can be accelerated prematurely by enhancing inhibition with benzodiazepines (Fagiolini and Hensch, 2000), as well as by over expressing BDNF (brain derived neurotrophic factor) to facilitate the maturation of GABAergic interneurons (Huang et al, 1999). The maturation of GABAergic inhibitory transmission is experience dependent, and it has been shown that raising animals in darkness from birth reduces BDNF levels (Castren et al, 1992) and GABA mediated transmission (Morales et al, 2002) due to impaired maturation of functional GABAergic synapses (Kohara et al, 2007). This then leads to delayed onset of the critical period and can be prevented by direct infusion of GABA in total absence of visual input (Iwai et al, 2003). Also over-expression of BDNF levels in complete darkness abolishes the expected delay of the critical period (Gianfransceshi et al, 2003), possibly by promoting the growth of GABAergic interneurones and facilitating maturation of inhibitory transmission by acting locally on GABAergic synapses containing BDNF producing neurons (Kohara et al, 2007). In addition, GABAergic inhibition is impaired in mice with heterozygous BDNF knock-out (Abidin et al, 2008) further demonstrating that the level of BDNF affects the maturation and function of GABAergic inhibition.

In contrast to the hyperpolarizing and thus inhibitory action of $GABA_A$ in mature neurons, $GABA_A$ receptor mediated currents are depolarizing and excitatory in immature neurons (Luhmann and Prince, 1991). $GABA_A$ receptor-mediated current depolarization activates voltage-gated calcium channels (VGCC), which leads to transcriptional induction of the K^+-Cl^- cotransporter 2 (KCC2). This early excitatory action of GABA is attributable to a high intracellular chloride concentration which

sets the reversal potential for chloride currents through GABA receptors at a more positive level than the resting potential (Ben-Ari et al, 1989). During development, there is upregulation of KCC2 which lowers chloride currents resulting in shift from depolarization to hyperpolarization (Cancedda et al, 2007), an event that seems to be activity dependent (Ben-Ari 2002). A recent study by Cancedda et al (2007) eliminated excitatory GABA actions by using a construct encoding KCC2, which was then transferred into neural progenitor cells using in utero electroporation. This resulted in severe impairment of the morphological maturation of cortical neurons in vivo suggesting that early depolarizing action of GABA is essential for the maturation of inhibitory cortical neurons (Cancedda et al, 2007).

The BCM model, Hebbian synaptic plasticity (such as LTP and LTD) and synaptic scaling all require correlated firing between pre and post-synaptic neurons. Interestingly the substrates for all this forms of plasticity are essentially the same, suggesting they are all intertwined at the molecular level. According to the BCM model, the sliding threshold for synaptic plasticity determines stability of overall neuronal activity hence θm may serve as a homeostatic point. In essence the BCM model is one of the forms of homeostatic mechanisms, which maintains a stable neuronal network while Hebbian mechanisms encode incoming information. Hebbian plasticity and synaptic scaling also work concurrently and cooperatively with the former encoding information and the latter controlling overall electrical activity and stabilizing neuronal network (Maffei and Turrigiano, 2008). Therefore, visual cortical plasticity cannot be explained solely by one mechanism, but rather arises through a complex interplay of many excitatory and inhibitory mechanisms occurring at many sites within a cortical circuit (Maffei and Turrigiano, 2008).

1.4.2.3 Downstream mechanisms of synaptic plasticity

Using immunoblotting and mass spectrometry, Husi and Grant (2001) found that the complex of NMDARs comprises of other glutamate receptors (mGluRs), adaptor proteins (e.g. PSD-95), second messenger enzymes (kinases and phosphotases), cytoskeletal proteins and cell adhesion proteins (Figure 1.6). Assembly of not only

NMDA receptors, but also AMPA receptors with signal transduction proteins is thought to be a general mechanism of cellular signalling (Pawson and Scott, 1997).

NMDA receptors, specifically NR2A subunit is known to bind to PSD-95 which then binds to complex ras activating protein, synGAP (Synaptic GTPase Activating Protein), which interacts with Ca2+/calmodulin-dependent protein kinase (CaMKII) (Oh et al, 2004). Post synaptic density-95 (PSD-95) and CaMKII have been documented to play a role in plasticity. For instance, in mice with PSD-95 knock-out, show marked inability to learn the position of the hidden platform in water-maze task, suggesting that PSD-95 is important in coupling with NMDA receptor pathway that control learning and memory (Migaud et al, 1998). Similarly, in mice with CaMKII-α (the most abundant isoform, Tighilet et al, 1998) knock-out, demonstrate clear impairments in spatial learning in the Morris water maze task showing a direct role of CaMKII-α in learning and memory (Gordon et al, 1996). Also, these mice failed to show measurable plasticity in response to MD (Gordon et al, 1996). Furthermore, mice possessing a mutant form of αCaMKII that is unable to autophosphorylate also show impairments in OD plasticity (Taha et al, 2002; Taha and Stryker, 2005). These results suggest that autophosphorylation of this enzyme is essential for synaptic plasticity.

In addition to αCaMKII, protein kinase A (PKA) also phophorylates other proteins and consists two of catalytic and regulatory subunits. The catalytic subunits include Cα and Cβ and the regulatory subunits include RIα, RIβ, RIIα and RIIβ (Cadd and Mcknight, 1989). Pharmacological blockade of PKA blocks OD shift early in development (Beaver et al, 2001b) and LTP and LTD in vitro (Liu et al, 2003). In PKARIβ deficient mice neuronal response properties including receptive field size, retinotopy, orientation selectivity, ocular dominance and response strength were indistinguishable from wild type mice (Hensch et al, 1998). However, these mice lacked OD plasticity and LTD but LTP was preserved (Fischer et al, 2004). Taken together these studies suggest a role of PKA function in OD plasticity with different subunits contributing differentially to plasticity.

1.4 Concepts of plasticity

It is unclear how NMDA receptors and its signalling molecules downstream integrate signal. Ras belonging to monomeric GTPases, which are involved in receptor-mediated signal transduction pathways, are thought to be involved integrating downstream signal (Gille and Downward, 1999). The major target of Ras is extracellular signal-regulated kinase 1.2 (ERK) (also known as p42/p44 mitogen activated protein kinase (MAPK)), which can be activated in response to a diverse range of extracellular stimuli including growth factors and in response to glutamatergic signalling by activation of NMDA receptors or voltage gated calcium channels (Rosen et al, 1994). ERK1/p44 and ERK2/p42 phosphorylation occurs through activation residues of Thr202/Tyr204 and Thr185/Tyr187, respectively. The first evidence to show that ERK signalling is involved in plasticity was provided by English and Sweatt (1997) who showed that selectively inhibiting MAPK cascade with PD098059 blocked ERK activity in response to NMDAR activation and LTP induction in the hippocampus. Furthermore, a study by Blum et al (1999) showed that training in water-maze task leads to activation of ERK in the hippocampus and ERK inhibitor blocked long term memory storage thus providing a direct role of ERK's involvement in plasticity.

Phospholipase C (PLC) couples downstream to metabotropic receptors (Husi and Grant, 2001) which are known to play a central role in signal transduction releasing the second messenger inositol triphosphate (IP3). PLC consists of subunits: PLCβ, PLCγ and PLCδ and they are distinguished from each other by their structure and activation mechanisms. Furthermore PLCβ has four isoforms: PLCβ1- β4 (Suh et al, 1988). Phospholipase C-β1, the predominant isoform in the brain, has shown to be regulated developmentally and its down-regulation is prevented in cats raised in complete darkness suggesting that its expression is dependent on sensory experience (Kind et al, 1994).

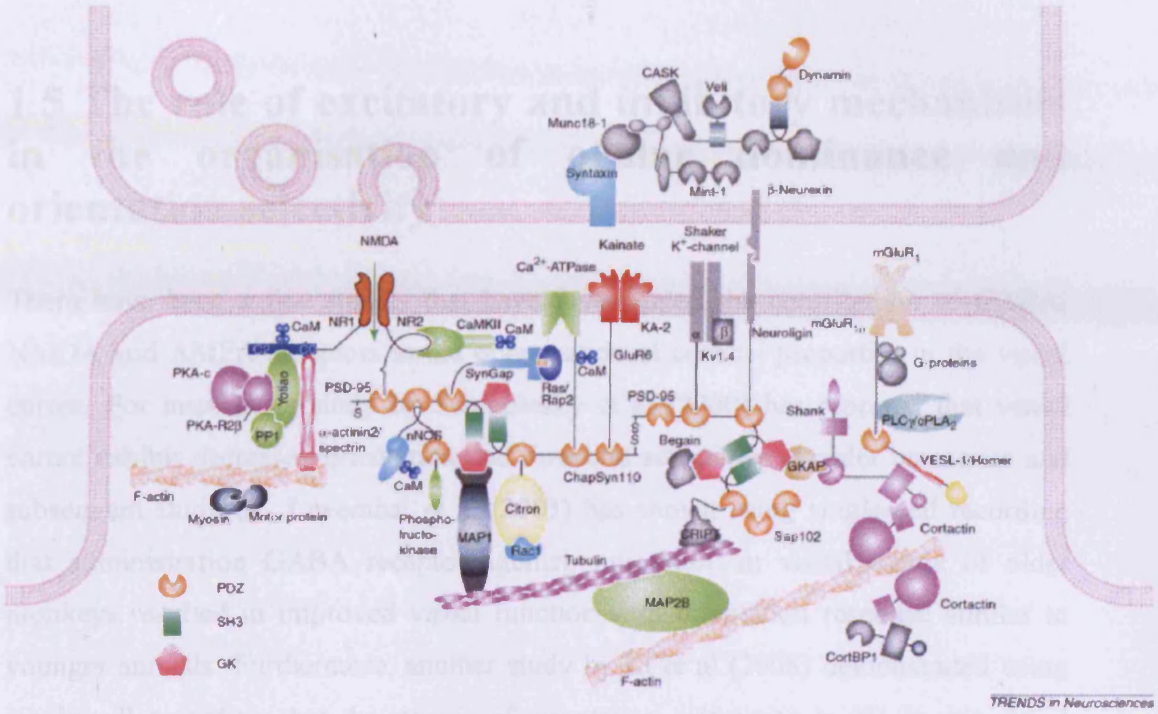


Figure 1.6: *Assembly of proteins found in the NMDA receptor complex.* The figure is showing assembly of proteins found in the NMDAR complex and their interaction with other neurotransmitter receptors such as metabotropic and glutamate receptors (Figure adapted from Husi and Grant, 2001).

1.5 The role of excitatory and inhibitory mechanisms in the organisation of ocular dominance and orientation selectivity

There have been a few studies that have investigated the contribution of GABA, NMDA and AMPA receptors in the organisation of cortical properties in the visual cortex. For instance, a study by Schmolesky et al (2000) has reported that visual cortex exhibit decreased orientation and direction selectivity in older macaques and subsequent study by Leventhal et al (2003) has shown using single-cell recording that administration GABA receptor agonist, muscimol, in visual cortex of older monkeys resulted in improved visual function with orientation response similar to younger animals. Furthermore, another study by Li et al (2008) demonstrated using single-cell recording that the degree of orientation selectivity in V1 in cat visual cortex correlated with GABAergic inhibition strength, cells with high orientation selectivity receive strong inhibition and further increase in inhibition does not improve the orientation selectivity. Therefore these studies show that inhibition is one of the mechanisms of improving orientation selectivity response in the visual cortex.

In addition to the role of inhibitory mechanism in the formation of orientation selective response, the role of excitatory mechanism has also been investigated. For instance, a study by Yu et al (2008) has shown that infusion of NMDA receptor blocker, AP-5, (2-amino-5-phosphonopentanoic acid) or AMPA receptor blocker, CNQX, (6-cyano-7-nitroquinoxalin -2, 3-dinoe) in cat visual cortex weakened the 0°-90° differential orientation maps as revealed using optical imaging of intrinsic signals. On the other hand a mixture of both AP-5 and CNQX erased the orientation-selective patches. These results demonstrate that both NMDA and AMPA receptors play a critical role in the formation of orientation maps. Similarly, mice with NMDA receptor, in particularly NR2A knock-out failed to mature orientation preference. On the other hand infusion of $GABA_A$ receptor antagonist (bicuculline) resulted in

different effects depending on drug concentration. When a relatively low dose (20μM) was applied to area 17, the cortical area became darker and the contrast of 0°-90° differential maps was enhanced suggesting that the cortex became more active resulting in increased consumption of more oxygen and blood flow. However, increasing further the concentration of bicuculline (40μM), the orientation-selective patches diminished in the differential maps. Therefore, it appears that a balance between excitatory and inhibitory inputs is necessary to maintain orientation-selective response.

In addition to orientation-selective response, the role of inhibitory mechanism in the formation of ocular dominance columns in cat visual cortex has also been investigated in a study by Hensch and Stryker (2004). They showed that chronic treatment with diazepam (GABA agonist) revealed robust orientation maps using optical imaging of intrinsic signals and single-unit recordings showed normal ocular dominance distribution but with few binocular cells. In addition, Hensch and Stryker examined the overall layout of proline-labelled ocular dominance columns (layer IV) in flattened visual cortex, and found that columns near the diazepam infusion site were wider than columns in regions further away from the infusion site. Infusion of inverse agonist DMCM (methyl-6, 7-dimethoxy-4-ethyl-β-carbo-line) resulted in less discreet or narrower columns in the infusion site than the ones further away. Thus it appears that intracortical inhibitory circuits shape the geometry of afferent thalamic neurons (Hensch and Stryker, 2004).

1.6 Aims and Objectives

Even though there are several single unit studies that have investigated the role of sensory experience on sculpting neuronal responses understanding the functional organisation of primary visual cortex is a key towards revealing information processing that takes place. One of my aims was to visualise the functional architecture of primary visual cortex of subjects raised in a normal visual environment from birth using optical imaging of intrinsic signals (OIS) and in order to understand how cortical organisation develops with age, the age groups examined were 5 weeks (peak of the critical period), 12 weeks (decline of the critical period) and 1 year (end of the critical period) of age. Previous studies have shown that sensory experience is not required for the initial establishment of cortical properties but is required for its maintenance; therefore I set out to obtain OIS maps from subjects raised in complete darkness from birth until 5 and 12 weeks of age. In line with previous single unit studies it was hypothesized that cortical organisation would develop normally until 5 weeks of age but would then degrade increasingly with age. To corroborate the findings from OIS, I also aimed at obtaining an electrophysiological estimate of visual acuity by recording visually evoked potentials (VEPs) in control and dark-reared subjects. I expected that visual acuity would increase with age in control subjects whereas in dark-reared subjects a delayed development and subsequent decline in visual acuity was expected.

A previous VEP study by Frenkel and Bear (2004) has shown that MD in mice occurs in two distinct processes, the first one being depression of deprived eye responses followed by a delayed potentiation of non-deprived eye responses. My second aim was to determine whether there are visible differences in the functional maps following 2 days and 7 days MD using OIS, that could be related to the two-stage processes involved in MD. The advantage of using OIS over VEPs is that one is directly able to visualise patches (ocular dominance columns) representing each eye and how they are affected by MD. Here I set out to test whether depression of

deprived eye responses and potentiation of non-deprived eye responses show similar temporally distinct processes in cat V1 as they are in mice.

In addition to MD with lid suture, several studies have employed other methods of MD to determine which aspect of visual stimulation has the greatest impact on the relative dominance of each eye. I therefore wanted to test whether using different MD regimens such as eye patch, frosted lens and neutral density filter had visibly different effects on functional maps of ocular dominance using OIS. Furthermore, I also aimed at obtaining an electrophysiological estimate of the visual acuity in MD subjects by means of VEP recordings. Both eye patch and frosted lens abolish pattern information and therefore I expected marked take-over of cortical territory by the non-deprived eye. In contrast, neutral density filters preserve pattern information but reduce illumination in the affected eye, which should have little effect on the relative dominance of each eye according to a previous study (Blakemore, 1976).

During the critical period, the responsiveness of neurons in the visual cortex reflects the recent history experienced as shown in previous studies (Buisseret et al, 1978; Buisseret et al, 1982; Mower et al, 1983; Philpot et al, 2001; Hofer et al, 2009). For instance, neurons in the visual cortex of animals raised in complete darkness lack stimulus selectivity (Blakemore and van Sluyters, 1975) and this effect of dark-rearing can rapidly be reversed within hours of visual experience (Buisseret et al, 1978) as revealed using single-cell recordings. The use of single-cell recordings faces the problem of sampling bias and in order to determine how the overall functional architecture of V1 is affected, I set out to obtain OIS maps from subjects that experienced a change in the nature of visual stimulation at the peak of the critical period. Also, I aimed to establish the effects of this change on visual acuity as determined from VEPs.

In order to relate the physiological responses observed in different experimental conditions, I aimed at determining how the expression levels of glutamatergic receptor subunits and their downstream signalling molecules as well as GABAergic

receptor subunits are regulated developmentally and by the nature of visual experience. Although several molecules have been associated with ocular dominance plasticity, there are no studies that have identified sets of proteins that form part of NMDA receptor complex and that are co-regulated in different paradigms of experience-dependent plasticity in cat visual cortex. Therefore, I aimed at identifying proteins (such as NMDA receptor subunits and their downstream signalling molecules) whose expression is regulated in an activity-dependent manner. This was achieved by harvesting visual cortices and carrying out immunoblotting. In addition, I also aimed at isolating synaptosomes and PSD components to determine pre- and postsynaptic expression of the glutaminergic proteins since it is known that changes that occur at the synaptic level are most relevant to the functional changes associated with altered sensory experience.

CHAPTER II

GENERAL METHODS

2.1 Introduction

Our understanding of the functional organization of the visual cortex and its developmental plasticity has been transformed over the last 20 years. Several neuroimaging techniques have become available to study functional architecture related to cortical plasticity. Ideally a brain imaging technique should have spatial resolution at single-cell level in order to observe individual neurons and have temporal resolution within milliseconds to follow dynamics of cortical processing. In addition, the technique should have the ability to sample simultaneously from large cortical regions and be non-invasive. Unfortunately such an ideal technique does not exist, but the current imaging techniques available offer one or more of the advantages.

Single cell recording is one of the most popular techniques used widely as a measure of understanding basic computational elements in anaesthetized animals. This technique, though invasive, offers the advantage of recording action potentials directly from the cortical neurons with high spatial and temporal resolution sufficient to follow real time changes. However, this technique requires several hours of recording and sometimes the recordings are limited due to cortical tissue damage caused by prolonged exposure. This technique is also associated with the problem of sampling bias. Recent development of microelectrode arrays provides simultaneous recording from many individual neurons and repetitive recordings are possible by implanting microelectrodes in the brain. However, in general electric recordings do not provide essential information about cortical architecture and therefore one is unable to relate neuronal responses with the cortical structure.

Another popular technique is 2-deoxyglucose (2-DG) autoradiography (Sokoloff et al, 1977), which is capable of recording simultaneously from a large cortical region with high spatial resolution. This technique essentially measures the level of neuronal metabolic activity as an indirect measure of neuronal activity using 2-deoxy-D-glucose. It permits postmortem visualization of active brain areas and therefore lacks

glucose. It permits postmortem visualization of active brain areas and therefore lacks the ability to track real time changes. Although strong metabolic activity is associated with high neuronal activity, it remains unclear whether the signal observed using 2-DG technique is also a reliable indicator of activation of brain areas that have low neuronal activity (Hess and Scheich, 1996). The other major disadvantage of 2-DG technique is that it is limited to one assessment only per animal.

Positron Emission Tomography (PET) is non-invasive imaging technique which allows repetitive measurements. PET utilises small amounts of a compound of interest labelled with radioactive tracers. The radiation emitted as the radioactive label decays is detected by external detectors resulting in image showing distribution of the radioactive tracer. The image generated offers high spatial and temporal resolution. The use of PET scan for laboratory animals (such as rats and mice) posed a caveat relating to low spatial resolution. However, recently high resolution PET scanners (1.5mm) have been specifically designed to image mice and rats (Tai et al, 2005). Its use is limited in research by the need for clearance by ethics committees to inject radioactive material into participants. A further limitation arises from the high cost of cyclotrons needed to produce the short lived radionuclide for PET scanning. Also the use of such technique requires extensive training of qualified personnel, therefore placing further limitation to its use.

Functional Magnetic Resonance Imaging (fMRI) is one of the most recently developed techniques (Belliveau et al, 1991) and is widely employed in research as well as in clinical settings. fMRI is non-invasive and therefore allows repetitive measurements with time. This technique has moderately good spatial resolution but with relatively poor temporal resolution. fMRI detects the BOLD (blood oxygenation level-dependent) signal, associated with altered blood flow and oxygen metabolism within the brain. In active cortex, the signal initially dips (1 sec) reflecting a transient decrease in oxyhaemoglobin followed by a much larger secondary (2-4 sec) increase in local oxyhaemoglobin which dominates the BOLD signal. The BOLD signal is however only an indirect measure of neuronal activity.

Recently Ohki et al, 2005, were the first to apply 2-photon imaging technique which permits functional imaging with cellular resolution. This technique employs use of a calcium-sensitive indicator which labels thousands of neuronal populations which is then imaged at single cell resolution with two photon microscopy. More recently (Mank et al, 2008), a genetically encoded calcium indicator has been developed which allows repeated imaging of response properties of neurons which will be crucial for gaining new insights into cellular mechanisms of plasticity.

2.1.1 Optical Imaging using intrinsic signals (OIS)

Roy and Sherrington (1890) first discovered that changes in the blood flow and blood oxygenation in the brain are closely linked to the neuronal activity under normal physiological conditions. More active neurons have a higher metabolic demand which is reflected in increase in blood flow in surrounding vasculature. The high demand of neuronal activity leads to local changes in the relative concentration of deoxyhaemoglobin and oxyhaemoglobin. Intrinsic signals are changes of light reflectance that occur in active neuronal tissue and this has been known since 1962 (Chance et al, 1962), however it was not until 1986 (Grinvald et al, 1986) that its use was first exploited in mapping cortical activity.

The advantage of OIS is that it offers high spatial resolution (100μm) with moderate temporal resolution (in the order of seconds after stimulus onset). The limited temporal resolution of OIS, however, does not pose a problem for the mapping of relatively stable functional architecture such as orientation and ocular dominance columns. Another advantage of OIS is that it does not rely on the use of extrinsic substances like dyes or radioactively labelled substances which could potentially damage the brain and therefore permits repeated recordings over time. However, the use of voltage sensitive dyes offers a better temporal resolution and therefore obtains real time images of brain activity. OIS offers also another advantage of being less costly compared to other imaging techniques such as fMRI. The use of OIS is

invasive in higher animals such as feline or primates and requires one to perform craniotomy while in rodents; one can acquire images through a thinned skull.

2.1.1.1 Intrinsic signals and their sources

The precise physiological mechanism underlying the intrinsic signal seen during cortical activation remains unclear. There are three identifiable components to the intrinsic signal. **1)** The fastest component of OIS arises from light scattering changes that accompany cortical activation caused by ion and water movement, expansion and contraction of extracellular spaces, capillary expansion and release of neurotransmitters (Grinvald et al, 1999). **2)** Another component of the intrinsic component arises from activity-dependent changes in the oxygen saturation of haemoglobin. The first part of this component is an increase in the deoxyhaemoglobin concentration, resulting from elevated oxygen consumption of the neurones due to their metabolic activity. The second part includes a decrease in deoxyhaemoglobin as the blood rushing in contains high levels of oxyhaemoglobin. This blood related components dominate at signal 400-600nm (Bonhoeffer and Grinvald, 1996). **3)** The slowest component of the intrinsic signal originates from the changes in blood volume due to vasodilation or capillary recruitment in an area containing electrically active neurons in a process known as neurovascular coupling and this is achieved through orchestrated communication between neurons and microvessels. Although the various components of the intrinsic signal originate from different sources, the functional maps obtained from various sources are very similar (Frostig et al, 1990).

The contribution of the above mentioned sources to the intrinsic signal are dependent on the wavelength of light used to illuminate the cortex. For example, when using visible wavelengths, blood-related sources dominate the intrinsic signal, and light scattering changes dominate in the infra-red part (Frostig et al, 1990). However, in terms of signal-to-noise ratio and contrast between active and non active regions, the best images of cortical activity are obtained using red (630nm) illumination (Frostig et al, 1990) with its greater ability to penetrate the tissue. Also the artefacts from the large blood vessels are larger in shorter wavelengths than in longer wavelengths

(Frostig et al, 1990). This is because in shorter wavelengths (570nm-illumination) capillary recruitment and vasodilation is not highly localized to the site of spiking neurons whereas in longer wavelengths (600nm-illumination) haemoglobin absorption is highly localized to the site of spiking neurons (Frostig et al, 1990).

Since the intrinsic signals are sensitive to changes in blood flow and oxygenation state of haemoglobin, it is important to maintain a stable basal physiological state of the animal. Fluctuations in heart rate, depth of anaesthesia, core temperature and oxygen content of the blood can markedly affect the intrinsic signal. It is well known that anesthesia has a strong effect on the coupling between cerebral blood flow and neuronal activity, however, the effect of anesthesia on various components of the intrinsic signal require more investigation (Shytoyerman et al, 2000). Changes in anaesthesia level can affect the quality of imaging data; if the animal is lightly anesthetized, there is an increased contribution to biological noise (especially surface vasculature) while if the animal is very deeply anaesthetized it can lead to a decrease in the ratio of stimulus-evoked signal and spontaneous background activity (caused by oxygenation of blood) (Frosting, 2002). Therefore choice of appropriate level of anesthetic and careful maintenance of systemic physiology is of paramount importance.

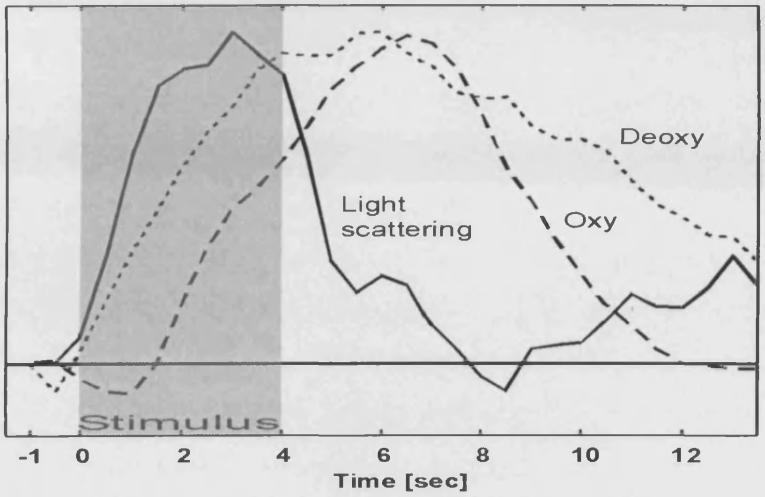


Figure 2.1: *Time course of the optical imaging intrinsic signal.* In response to a stimulus of 4 seconds duration, the light scattering signal component peaks before oxyhaemoglobin and deoxyhaemoglobin component. The light scattering is relatively fast and locked to cortical electrical activity while components originating from microcirculation peak after cessation of stimulus display and are present in the cortex for much longer than the electrical activity. (Figure from Bonhoeffer & Grinvald, 1996).

2.1.1.2 Application of OIS in humans

The use of OIS is not only restricted to animal research but has also been applied successfully in humans largely because of its high spatial resolution. Optical imaging using intrinsic signals in humans has proven to be useful in surgical treatment of neocortical epilepsy (Haglund et al 1992; Haglund and Hochman, 2004). Haglund and colleagues were the first to demonstrate optical signals in both Broca's and Wernicke's areas during naming of a task (Haglund et al 1992). Moreover, Pouratian and colleagues investigated language cortices in response to performing tasks in two different languages (Pouratian et al, 2000). Also, OIS has been a useful tool in investigating the organization of the human primary somatosensory cortex (Sato et al, 2002). The application of OIS in humans has indeed shed further light in the

organization of the sensory and motor cortices, language, and other cognitive processes.

2.2 Methodology

2.2.1 Preparation of an animal for optical imaging

All the procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and Cardiff University regulations. Cats were bred in a closed laboratory colony. Initially the cat was anaesthetised with ketamine (20-40mg/kg) in combination with xylazine (4mg/kg) injected intramuscularly. Ketamine-xylazine is a standard veterinary combination anaesthesia used for its rapid onset and its relative safety. Ketamine is a dissociative anaesthetic and therefore it was combined with a sedative, xylazine, to provide adequate anaesthetic effect. Xylazine also has analgesic property and when combined with ketamine causes muscle relaxation and visceral analgesia inducing periods of surgical anaesthesia. Anaesthesia developed within a few minutes after intramuscular injection and during this time the animal was placed on a heating blanket to minimize drastic reduction in body temperature induced by anaesthesia. The animal was judged to be adequately anaesthetised when withdrawal reflexes were abolished. In some cases when the initial dose was not adequate, an additional low dose of ketamine was administered and in some cases isoflurane was administered via a face mask at a rate of 2.5- 3.5%. Atropine (0.2mg/kg) was injected intramuscularly after ketamine injection to counteract bradycardia (induced by xylazine) and to prevent excessive mucous secretion in particular in the lungs and trachea. Dexamethasone, an anti-inflammatory steroid, was injected subcutaneously in order to prevent cortical oedema.

The onset of surgical anaesthesia results in loss of all protective airway reflexes and so tracheotomy is crucial to provide ventilation in acute experiments. Tracheotomy is a surgical procedure in which a cut or an opening is made in trachea and a tube is inserted into the opening to allow artificial ventilation. This procedure involved incision from below the Adam's apple to the top of the breast bone. The tissue surrounding the trachea was pulled apart carefully (avoiding large vessels running through the tissues) and once the trachea was located, a cut of sufficient size was

made into the tough walls of trachea to allow insertion of the tube. Once the tube was inserted, it was securely tied with a thread. The animal was then mounted on a stereotaxic frame with its head and neck extended to ensure the airway is clear and unobstructed. Hypoxia often results after the onset of surgical anaesthesia and so it is essential to provide continuous oxygen. The animal was connected to pressure controlled respirator (Fohr Medical Instrument, GmBH) which delivered a mixture of oxygen (O_2) and nitrous oxide (N_2O) at a ratio of 2:3 and was supplemented with isoflurane at levels of 2.0-2.5% to maintain general anaesthesia throughout the course of the experiment. The level of isoflurane was decreased to 1-1.5 % during imaging. Nitrous oxide is not a very potent anaesthetic in animals but as an analgesic it is useful in reducing the concentration of isoflurane required.

All the animal's physiological parameters were monitored by means of a patient monitoring software written in LABVIEW (National Instruments, Austin, Texas). The end tidal carbon dioxide (CO_2) was monitored continuously and was maintained within normal physiological bounds (usually 3-4.5%). In small animals (<500g), the expired CO_2 was measured intermittently because the sample volume taken by the patient monitor (Ohmeda 4700 OxiCap) would have been too large compared with the ventilated volume to allow continuous sampling. End-expiratory pressure was monitored and maintained at 60-120 mmHg by adjusting O_2 and N_2O flow rates and respiration rate as well as anaesthesia levels. If pressure changed suddenly, the animal's neck and body position were checked to ensure there was no obstruction to the airways.

Anaesthetics depress thermoregulation and so monitoring body temperature is vital. The rectal temperature was maintained in the range of 37-38°C during the course of the experiment with a feedback-controlled using homeothermic blanket unit (Harvard Apparatus). In addition, the animal was also covered to minimise heat loss. Electrocardiogram electrodes (syringe cannules) were inserted to monitor heartbeat throughout the course of the experiment. The heartbeat was monitored and maintained within normal physiological limits at a rate of 150-200 beats/min by

adjusting anaesthesia levels. In addition, an intravenous catheter was inserted in the hind limb to allow continuous infusion of saline, glucose, atropine, and dexamethasone at a rate of 3ml/kg/hr. Gallamine triethiodide (a neuromuscular blocking agent) was added intravenously after the surgery to stabilize ventilation and prevent ocular drifts.

Once stable physiological conditions for the animal were established, craniotomy was performed (Horsley-Clarke coordinates between P3.5 and A2.0). A cut was made from the frontal part of the head to the back of the head to expose the skull. This was then followed by scraping carefully to remove any overlying tissue on the skull. The lambda point and midline clearly became visible once the scraping was effectively done. Trepanations were made anterior of the lambda point and it is imperative not to drill very close to lambda as the bone is very thick and there is a risk of rupturing a major sinus running along the midline. In young animals, a heart-shaped opening was made with the dip of the heart just anterior of lambda. In older animals, the midline was not drilled due to risk of bleeding from the major sinus and therefore two openings were made. The drilling was done carefully to avoid damage to the underlying cortex and it is always advisable not to drill on one side continuously as excessive heat generated could damage the cortex. Bleeding from the bone was inevitable and bone wax was used to seal any holes. The use of saline for rinsing is advantageous as it cools down the heat generated from the drilling and also reduces friction. The bone debris were removed by short pulses of pressured air or by flushing with saline.

Once the drilling was performed, the bone was removed very gently. This was done by carefully placing a thin spatula as horizontally as possible under the bone and moving it back and forth while at the same time holding the bone from the opposite end with a pair of tweezers. The bone was then carefully lifted and any blood was quickly flushed away with saline to prevent it from drying out. The process of detaching the bone has to be performed with extreme caution as there is always a risk of going through the dura and damaging underlying blood vessels or cortical tissue.

The bone surrounding the exposed area was then dried out with swabs and scraped clean. In order to reduce interference of brain movement caused by heart beat pulsations and respiration during image acquisition, a titanium chamber (with an inlet and outlet tubes) was glued on the skull and fixed with dental cement. In addition, to achieve a perfect seal the inside of the chamber was treated with dental wax. This process had to be done thoroughly to ensure the inside of the chamber is gap-free.

The cortical surface was carefully dried and kept free from any traces of blood, cerebrospinal fluid (CSF) and saline using Sugi sterile swabs made from cellulose fibres (Kettenbach, Eschenburg, Germany). In areas of the exposed cortex where bleeding was persistent, haemostatic sponge (1X Lyostypt) was used to contain it. The dura was carefully removed from the cortex in cases where its opacity would have compromised image quality. The chamber was then filled with de-gassed silicone oil and any floating particles such as bone debris or wax were removed before sealing the chamber as these would introduce artefacts during image acquisition. Thereafter, the chamber was closed with a cover slip which was pressed down on to a silicone gasket with a threaded ring and both the inlet and the outlet tubes were then blocked. At this point the level of isoflurane was decreased gradually to a level of 1-1.5% prior to image acquisition. In some cases electroencephalogram (EEG) electrodes (silver wire electrodes) were placed anterior to the chamber to monitor adequate anaesthesia.

Gas permeable contact lenses were placed in the eyes to prevent the cornea from drying out (blink reflexes are lost once the cat is anaesthetised) and to correct refractive error. The lenses used were circular with pupil size of 3.5 mm at the centre to decrease light scattering and increase depth of focus. The animal's eyes were focused onto a 21 inch monitor at a distance of 33cm. A few drops of atropine were administered to facilitate pupil dilatation and relaxation of the ciliary eye muscle (i.e. to prevent accommodation) and phenylephrine was administered to retract eyelids.

2.2.2 Camera set-up

The camera was then mounted above the animal's head and initially the brain was illuminated using a green filter (band-pass, 546nm) using two light guides to obtain a vascular surface reference image. A region of interest (ROI) was then defined on the reference image restricted to the putative primary visual cortex. A super pixel area was also defined which is used to establish the overall time course of the signal during image acquisition and it is a good indication of the signal strength and reliability. A red filter (700nm) was used for imaging; at this wavelength light penetrates sufficiently into cortical layers II and III and intrinsic signal is dominated by the light scattering component which is known to yield better spatial resolution than other components of the intrinsic signal. Images were captured using an enhanced differential imaging system (Imager 2001, Optical Imaging Inc, Mountainside, NJ) with the charged couple device (CCD) camera focused 500μm below the cortical surface (after switching to the red filter). The lens employed in the camera is a tandem-lens which has exceedingly shallow depth of field and this ensures that the surface vasculature is sufficiently blurred and artefacts are minimised (Ratzlaff and Grinvald, 1991).Computer-controlled shutters were then placed in front of the eyes to ensure that only one eye was stimulated at a time.

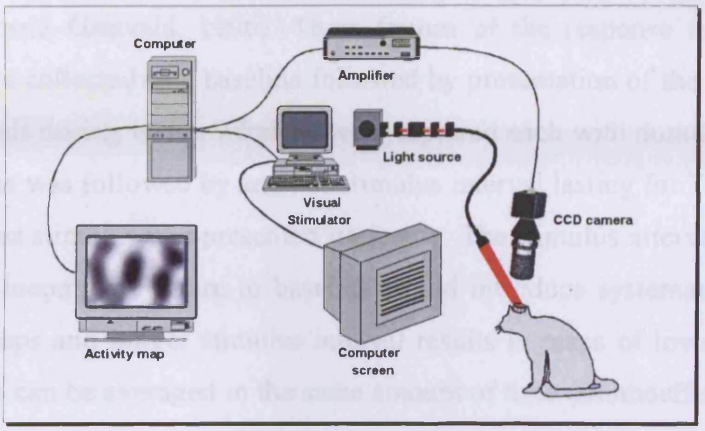


Figure 2.2: *Optical Imaging set-up.* The cortex is illuminated with red light and images are acquired with a CCD camera whilst the animal is viewing grating stimuli on a computer screen. The activity maps are displayed on a second screen after being digitised (From Bonhoeffer & Grinvald, 1996).

2.2.3 Stimulation protocol

The stimulus that is most commonly used in studying the primary visual cortex is a sine wave grating, the main attributes being spatial frequency, contrast, orientation and direction and speed of drift. Combination of such right attributes can be used to construct visual stimulus for a particular study.

For OIS stimuli were produced by a visual stimulus generator (VSG Series 3; Cambridge Research Systems) and consisted of high contrast sinusoidal gratings with low spatial frequency (0.1-0.2cyc/deg) and high spatial frequency (0.4-0.6cyc/deg). The exact values were chosen depending on the age and the rearing history of the animal. The gratings moved back and forth at 2cyc/sec and were presented at four different orientations (0, 45, 90 or 135 degrees). In addition four blank inter-stimuli of the same mean luminance as the grating stimuli (40cd/m^2) were presented.

Reconstruction of the time course of the intrinsic signal is important for its proper evaluation and this is achieved dividing the data acquisition time into frames where data of each frame are stored separately which can then later be averaged together (Bonhoeffer and Grinvald, 1996). Three frames of the response to the stationary stimulus were collected as a baseline followed by presentation of the moving grating for 5.4 seconds during which 9 frames were captured each with duration of 600msec. Each stimulus was followed by an inter-stimulus interval lasting for 7 seconds during which the next stimulus was presented stationary. The stimulus interval should not be too short as incomplete return to baseline would introduce systematic errors in the functional maps and longer stimulus interval results in maps of lower quality since fewer images can be averaged in the same amount of time (Bonhoeffer and Grinvald, 1996). Each trial consisted of 20 stimuli (4 orientations x 2 spatial frequencies x 2 eyes + 4 blank screen stimuli) and the data collected was then saved in blocks which comprised of 4 trials each.

The camera is an eight-bit frame grabber which cannot digitise intensity changes of <1/256 and therefore it is insufficient to resolve minute reflectance changes (~0.1%) associated with intrinsic signals. In order to resolve this, 8-bit data is converted back to analogue data and thereafter is subtracted from a reference frame taken with blank stimulus. The resulting reference image is converted back to a digital data in a file format of 16–bit floating point. For visualisation purpose, an 8-bit scale is used.

2.2.4 Image analysis

Optical imaging of intrinsic signals in biological preparations has small signal-to-noise ratio and usually no better than 1 in 1000 parts (Frostig, 2002) in single trial. In order to extract meaningful signal for appropriate off-line analysis, signal acquired from repeated presentations of stimuli are accumulated in consequent trials and subsequently resulting in enhanced signal. The noise from repeated presentations is minimised, and so one is able to extract meaningful signal.

2.2 Methodology

Data acquired from OIS is large and therefore massive compression of data is essential. The first reduction of data occurs by averaging of video frames into data frame. This usually reduces amount of data by a factor of 20. Also 2x2 binning of pixels further compresses the data by a factor of 4. The accumulation of 4 trials per block achieves another 4-fold compression. It should be noted that the compression of data does not result in loss of signal and if anything, it minimises time needed for analysis.

In order to obtain activity maps from the cortex, the images are acquired while the cortex is being stimulated. From these images, a baseline image has to be subtracted (a cortical stimulus independent image) and they have to be corrected for uneven illumination. This is accomplished in two ways notably by using a blank image or cocktail-blank image. The blank image is obtained from unstimulated cortex and the difficulty with using this image is that any visual stimulus will cause an overall elevation in absorption and thus it is difficult to ascertain selective neuronal response associated with a specific grating orientation or eye of presentation. The blank image is commonly associated with artefacts and so obscures underlying neuronal activity. In contrast to the blank image, a "cocktail-blank" image is obtained by summing the responses to a set of stimuli which activates the cortex in a uniform manner. Therefore the cocktail-blank corrected images from each eye are complementary to each other but the blank corrected images are not. We used both blank and cocktail blank analysis before making any judgements on activity maps as the assumptions made for analysis can strongly influence the appearance of the maps.

An offline analysis was carried IDL software. The biological noise associated with OIS is large and in order to combat this, first frame analysis was used, where the first three baseline frames were subtracted from subsequent frames and this removed slow noise with a frequency lower than 0.3 Hz and also removed artefacts that are more or less time-invariant within the duration of the stimulus (Zepeda et al, 2004).

2.2 Methodology

To map the cortical regions activated by a grating of a particular orientation, one captured the image while the animal was viewing that orientation and divided this image by the cocktail blank, i.e. the sum of images obtained when the animal was stimulated with gratings of all four orientations. Ocular dominance (OD) maps were obtained by taking images acquired from one eye and dividing them by images obtained during visual stimulation of the other eye. A more comprehensive analysis of orientation maps was achieved by colour coding preferred orientation to form angle maps. This was achieved by summing response of four different gratings vectorially on a pixel by pixel basis. In addition, the magnitude of the resulting vector was also displayed as the brightness of each pixel resulting in a polar map. The resulting polar map shows the preferred orientation (hue of the colour) and the magnitude of the vector (intensity of the colour). However, this should be treated with caution, as dark areas in the polar map may represent either a genuinely low response or an equal response to all orientations (as seen around pinwheel centres) which would result in null vector.

Filtering of orientation and OD images were carried out in two stages, a low pass filtering and a high pass filtering. A low pass filtering generates smooth surface by averaging across the set number of pixels using a Gaussian weighting factor. This removes outliers on the greyscale and results in a less grainy image. High pass filtering is essential to eliminate effects of uneven illumination and baseline level (DC) changes across the images. The resulting maps increase overall responsiveness within the maps to reveal specific responses. The high pass filtering should be done with caution, because firstly DC level differences between different stimulus conditions may have biological significance, and secondly one might inadvertently remove very low frequency image components.

Since the reflectance values of pixels were approximately normally distributed, "clipping" of the 1 or 2% of pixels of the highest and lowest values removed all outliers. The resulting pixel range was rescaled to an 8-bit (256-step) greyscale, where a value of 0 appears as black (maximal light absorption) and a value of 255

appears white (maximal light reflectance). In the case of cocktail-bank divided images, the clipping values were then adjusted in a way that the low-clip value and the high clip value are approximately equidistant from 1.This is important for the later quantification process (see below).

The actual signal used for subsequent quantitative analysis was reflectance change for each pixel given. A ROI (region of interest) was defined manually using IDL software comprising the visually responsive part of one hemisphere within the image ensuring to exclude blood vessel artefacts. For quantification, IDL software shifted the ROI by ±10 pixels in x and y coordinates and calculated mean results across all shift conditions to minimise subjectivity in the defining of the ROI. The pixels within the area of interest were assigned to the left and right eye depending on whether their value was greater or less than 1 and from this the resulting percentage of cortical surface within the ROI responding to each eye was obtained. For analysis of the relative strength of responses through the two eyes, blank images were only low-pass filtered and pixel strength was calculated. This was done by calculating how much a pixel deviates from 1 (which is equivalent to blank response) and this results in responsive pixel. The actual response strengths of all pixels within the ROI were added and response strength of the left and the right eye was calculated. In addition to ocular dominance, orientation selectivity was also analysed. A ROI was defined as described previously. The pixels within the areas of interest were assigned to a specific orientation by calculating their absolute mean deviation from 127 (the centre value) on the grey scale. The absolute mean deviation was used as signal strength for a specific orientation.

The optical imaging data analysis employed here works well in juvenile cats, however in adult cats; more sophisticated analysis is usually required to extract any signal. The OIS tends to deteriorate with age possibly due to increase in myelination in older animals which reduces tissue transparency and therefore the intrinsic signal. However, several studies have shown that it is possible to extract statistically significant spatial and temporal response from optical imaging data from adult cats

using more sophisticated analysis such as principal component analysis (PCA) (Stetter et al. 2000) and independent component analysis (ICA) (Bell and Sejnowski, 1995; Chen et al, 2007).

2.3 Visually evoked potentials

Visually evoked potential (VEP) is a measure of pooled activity from enormous number of neurons in response to broad range of spatial frequency and this results in reliable electrophysiological measure of visual acuity (Snyder and Shapley 1979; Freeman et al, 1983). After imaging; the inlet and the outlet tubes of the imaging chamber were reopened to release pressure, the cover slip was removed and silicone oil was replaced with saline. A silver ball electrode was placed on the surface of V1 near the representation of area centralis (Horsley–Clarke coordinates P3.0 and A2.0). The recording signal was amplified by a factor of 20000 (sometimes 10000 or 30000) and band pass filtered at 0-300Hz. During the recording session, four recordings were made (one per hemisphere and eye). The recording electrode was approximately matched stereotaxically by a mirror symmetric relocation on the opposite side. Whenever the waveforms were barely distinguishable from the noise, the electrode position was changed to obtain better signal.

2.3.1 Stimulation protocol

Stimuli were displayed on a computer screen at a distance of 100cm generated by a visual stimulus generator (VSG Series 3; Cambridge Research Systems). The stimuli consisted of high contrast phase-reversing square-wave horizontal gratings that varied in spatial frequency in the range of 0.14-3.2 cyc/deg. Gratings drifted upwards at a velocity of 0.1cps. A blank stimulus of the same mean luminance as the gratings was used to measure the baseline response. A stimulus presentation consisted of a block of six phase reversals each 500ms apart and every stimulus was repeated 10 times before the next was presented with inter-stimulus interval of 3 seconds; the stimulus order was pseudo random.

2.3.2 VEP analysis

As a physiological measurement of visual acuity, the VEP cut-off point was determined for each eye from the VEP amplitude versus spatial frequency curve. A

straight line was fitted through the final 3-4 descending points, and spatial frequency was calculated at its intersection with a line corresponding blank response plus standard error. The total amplitude of the VEP signal amplitude was defined as the difference in voltage between the signal peak and subsequent trough within a 500 msec window. And a ratio of VEP amplitudes through the two eyes was calculated by dividing the sum of amplitudes in response to the four lowest SF for the right eye by the same sum obtained for the left eye.

2.4 Removal of the primary visual cortex

Once the VEPs had been recorded, the saline was removed from the chamber and an overdose of barbiturate was injected via the intravenous line inserted at the start of the experiment. The chamber was then quickly detached and the surrounding bone was removed carefully to have a clear view of the tissue to be removed. The primary visual cortex from each hemisphere (Horsley-Clarke coordinates between P3.0 and A2.0) was removed and stored at -80°C until further use.

2.5 Synaptosome and post-synaptic density preparations

Synaptosome is defined as a membrane-bound body containing three or more vesicles of 40-60nm in diameter (Dunkley et al, 1986). Synaptosome is sometimes used to denote the presynaptic component although morphologically it is characterised by a cleft region and a post-synaptic component (Whittaker, 1971). Synaptosomes should be distinguished from synaptoneurosome, which is defined as a subcellular preparation enriched in presynaptic structures with attached post-synaptic entities (Quinlan et al, 1999). Synapses feature prominent thickening at the cytoplasmic surface of the post synaptic membrane termed post-synaptic density (PSD). The PSD contains glutamate receptors with associating signalling and scaffolding proteins that organise signal transduction pathways for the post synaptic membrane.

Synaptosome preparations were prepared according to the methods of Dunkley et al, (1986) using Percoll gradients. The major advantage of Percoll over other media such as sucrose and Ficoll is its low viscosity, which allows for rapid sedimentation and the use of low centrifugal forces (Dunkley et al, 1986). A second advantage is that its isotonicity can be maintained and therefore leads to homogenous material that is depleted of damaged synaptosomes myelin and extrasynaptosomal mitochondria (Dunkley et al, 1986). This method of fractionation is rapid hence reduces the possibility of any protein degradation (Dunkley et al, 1986).

The Percoll solutions used were 3% (containing 3ml Percoll, 25ml 4x Sucrose/EDTA/Tris (pH7.4, containing 1mM EDTA (ethylenediaminetetraacetic acid) and 5mM Tris) and 72ml dH_20), 10% (containing 10ml Percoll, 25ml 4x Sucrose/EDTA/Tris, and 65ml dH_20), and 23% (containing 23ml Percoll, 25ml 4x Sucrose/EDTA/Tris, and 52ml dH_20) and pH was adjusted to 7.4 before use. The Percoll gradients were prepared by first adding 3ml of 23% Percoll solution followed by 3ml of 10% and 3ml of 3% using peristaltic pump.

2.5 Synaptosome and post-synaptic density preparation

The visual cortex sample was thawed and transferred into a tube containing ice cold 0.32M sucrose, 1mM EDTA and 5mM Tris solution. Sucrose is the most commonly used homogenising medium and at 0.32M concentration; it is isotonic for the osmotically sensitive synaptosomes (Whittaker, 1959). Protease and phosphatase inhibitors were also added in Sucrose/EDTA solution. The sample was then homogenised with approximately 6 strokes up and down in a Teflon/glass homogeniser at about 700rpm (rotations per minute). The consequence of homogenisation is the release of subcellular particles into a suspending medium. The homogenate (0.75ml -1ml per gradient) was then gently poured on the top layer of Percoll gradients. This was then centrifuged at 4°C with acceleration speed of 9 and the brake of 1 for 12 min. at 15000 rpm. After centrifugation, a synaptosome layer was found between 23% and 10% layer while the myelin layer was found between 10% and 3% layer. These fractions which are the last to come down in a centrifugation procedure are the most highly enriched. Carefully the myelin layer was sucked off and the synaptosomes were removed and spun for 20-30 minutes in ice cold solution containing 0.32M, 1mM EDTA and 5mM Tris at 13000rpm with same temperature and speed as before. After spinning, the supernatant was discarded and the resulting pellet was then resuspended in cold Kreb's buffer (containing NaCl, KCl, $MgSO_4$, glucose, $Na_2HPO_4.12.H_2O$ and HEPES, but lacking Ca^{2+}) and spun at 13000rpm for 10 minutes, twice in succession. The resulting pellet is the synaptosomes and this was then resuspended in lysis buffer (containing 50mM HEPES pH 7.5, 1% Triton X-100, 50mM NaCl, protease inhibitors, phosphatase inhibitor mixtures I and II (P2850 and P5276 respectively). For PSD preparation, after treating the synaptosomes with lysis buffer, ultracentrifugation was carried out at 35000 X g for 45 minutes to generate a crude sample The supernatant was removed and stored and the resulting pellet was re-suspended in lysis buffer (containing 50mM HEPES pH 7.5, 1% Triton X-100, 50mM NaCl, protease inhibitors, phosphatase inhibitor mixtures I and II (P2850 and P5276 respectively) and stored at -80 until required.

2.5 Synaptosome and post-synaptic density preparation

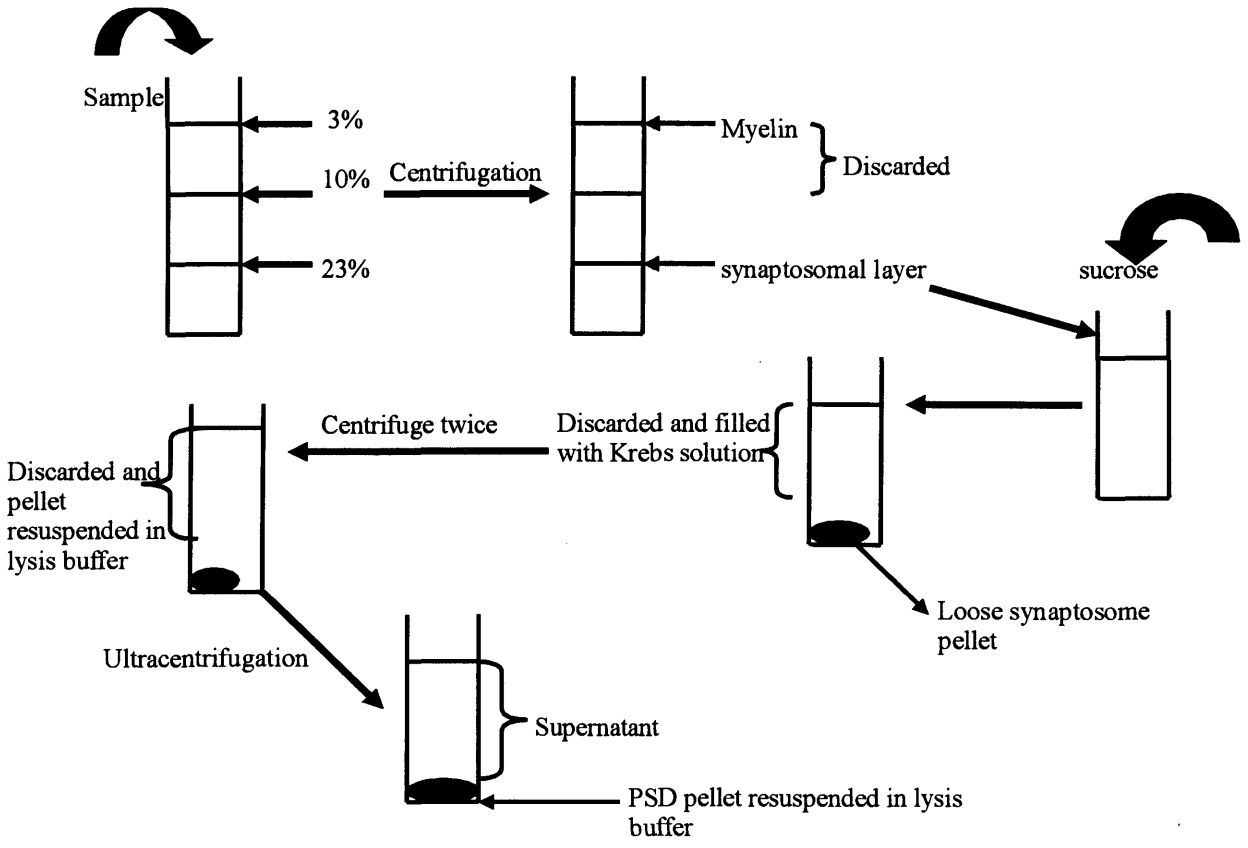


Figure 2.3: *An outline of the method employed in preparation of synaptosomes and PSD.* Percoll solution was used to create gradients as first introduced by Dunkley et al, 1986.

2.6 Western blotting

Western blotting is also known as immunoblotting because an antibody is used to detect its specific antigen. The term blotting refers to the transfer of biological samples from a gel and their subsequent detection on the surface of the membrane. It was first introduced by Towbin et al, 1979, and is now a routine technique for protein analysis. The specificity of antibody-antigen interaction enables a single protein to be identified in the midst of a complex protein structure.

All the equipments for Western blotting were thoroughly washed and the plates were additionally treated with 70% ethanol before proceeding with the experiment. The first step in a Western blotting procedure is to separate the macromolecules using gel electrophoresis. Usually 6-7% gel was prepared for all the immunoblotting experiments. 10% ammonium persulfate and TEMED were added just before loading of the gel to circumvent polymerisation. The gel was then carefully poured between the plates and a layer of butanol was added on top to remove any bubbles. The gel was left to set for 15 minutes for polymerisation to ensue. Thereafter, butanol was poured off and 4% stack solution was layered and allowed to polymerise for 45 -60 minutes. Additionally, cones were placed to facilitate formation of wells.

Lowry assay was carried out to determine protein concentration and usually 10µg of sample was loaded unless otherwise indicated. Lysis buffer (containing protease inhibitors, phosphatase inhibitor mixtures I and II (P2850 and P5276) and Laemmli buffer were also added with samples. The Laemmli buffer consists of mercaptoethanol which reduces the intra and inter-molecular disulphide bonds. It also contains SDS detergent which denatures the proteins and gives each an overall negative charge so that they separate based on size. The bromophenol blue in the buffer serves as a dye front that runs ahead of the proteins. The mixture was then heated for 5 minutes prior to loading. Kaleidoscope prestained standards were added for estimating the molecular weights of sample proteins. These standards were also useful in assessing the quality of transfer. The tank was then filled with running

buffer (containing Tris, glycine, SDS) to provide a conducting medium for electrophoresis to take place. The process of electrophoresis was carried out at 15mA per gel where the proteins separated according to size: charge ratio. Following electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane. First, the gel was carefully detached from the glass plate and was then carefully adhered to the blotting paper. This was sandwiched in between a pad and a nitrocellulose membrane in a transfer medium (containing Tris, glycine, 20% methanol).

The most common method employed for transfer is electrophoretic transfer because of its speed and transfer efficiency. A fast transfer was carried out within 1-2 hours at 200mA per tank while a slow transfer was kept overnight at 50mA per tank. Electrophoretic transfer uses electrophoretic mobility of proteins to transfer them from the gel to the matrix. When the electric field is applied, the proteins move out of the polyacrylamide gel and onto the surface of the membrane where the proteins become tightly attached. The resulting membrane is a copy protein pattern that was found in the polyacrylamide gel. Transfer efficiency can vary dramatically among proteins based upon the ability of protein to migrate out of the gel. The efficiency of transfer depends on factors such as the composition of the gel, whether there is complete contact of the gel with the membrane, the position of the electrodes, the transfer time, the size and composition of proteins, field strength and the composition of transfer buffer.

After successful transfer process, the membrane was stained with amido black staining solution (containing 0.1% Amido black, 25% Isopropanol and 10% acetic acid) to confirm equal loading and any membrane that was not equally loaded was discarded. The membrane was then destained with Tris base solution (containing 200mM Trisbase, 600mM sodium chloride and 0.2% triton) for 30 minutes. Thereafter incubation was followed in Immomedia (containing 5% foetal calf serum (as a blocking agent), 0.2% sodium azide DMEM and 0.5% triton). For homogenate samples PKARIIβ (BD transduction laboratories, 1:10000) was used as an internal

control and for synaptosomes and post-synaptic density fraction, β actin (ABCAM, 1:5000) was used. The primary antibody incubation was kept overnight and following incubation; the membrane was rinsed with Tris base solution (containing 200mM Trisbase, 600mM sodium chloride and 0.2% triton) twice in succession to remove any unbound primary antibody. The fluorescently tagged secondary antibody at a concentration of 1:10000 [Alexa Flour goat anti-mouse and donkey anti-goat (Molecular probes) and goat anti-rabbit (LI-COR Odyssey)] was then incubated in immunomedia for an hour only. Primary antibodies used were: GluR2/3 (Upstate 1:1000), GluR1 (Frontiers Science 1:300), NR1 (BD transduction laboratories 1:500), NR2B (BD transduction laboratories 1:500), NR2A (Millipore 1:500), mGluR5 (Chemicon, 1:4000), $GABA_A\alpha 1a$ (Imgenex, 1:4000), PKC (BD transduction laboratories 1:10000), PSD-95 (Upstate, 1:10000), SAP-102 (NE-dlg, Santa Cruz. 1:5000), αCaMKII (SIGMA, 1:5000), SynGAP (Bioaffinity Reagents, 1:4000), pMAPK (Cell signalling, 1:4000), synaptophysin (Synaptic systems 1:1000), PLCβ1 (Santa Cruz, 1:4000) and PLCβ4 (Santa Cruz, 1:4000).

The membrane was then visualised using LI-COR system. Fluorescent detection offers many advantages compared to standard chemiluminescent detection. The latter relies on enzymatic reaction that produces light which can then be viewed on a film and is highly dependent on time and exposure. The enzymatic reaction used to produce light is dynamic and constantly changing over time. The advantage of LI-COR system is that it offers the advantage of producing linear results because when the fluorescent light is excited, the amount of light produced is constant. This therefore makes fluorescent detection a more precise and accurate measure of the differences in signal produced by labelled antibodies bound to protein of interest (Ambroz, 2006). In addition, the membrane can be imaged again even after a few months. The Odyssey system uses two infrared wavelengths for detection, advantage not available with chemiluminescent detection. Two colour Western analysis makes normalisation easy and eliminates error introduced by stripping or reprobing.

2.6.1 Quantification of Western blots

The quantification was carried out using NIH Image J software. The scanning in Odyssey scanner generated tiff files which saves the spatial and density calibration. Using Image J, the tiff files were altered in brightness and in contrast and it displayed images by linearly mapping pixel values in the range of 0-255 scale. Pixels with a value of less than minimum were displayed as black and those with a value greater than maximum were displayed as white. A rectangle was made on the biggest band visible and a constant band size was used to measure band intensity. The band intensity of protein of interest was normalized with the band intensity of internal control.

CHAPTER III

EXPERIMENTAL CHAPTER

Optical Imaging and intrinsic signal and VEP results

3.1 Introduction

Since the first pioneering studies by Hubel and Wiesel, several studies have been carried out to explore different forms of visual deprivation and the impact it has on cortical properties at the peak of the critical period. Such investigation has undoubtedly shed light on the postnatal development of the visual system and in particular, how experience modifies visual cortical responses at the peak of the critical period and in adult animals.

3.1.1 Dark-rearing

The nature versus nurture controversy prompted several researchers to investigate how dark-rearing (DR) affects cortical maturation and the extent to which visual experience is necessary to promote development and maintain the integrity of visual function throughout development.

Previously, it was thought that the effects of dark-rearing and binocular lid suture (BD) are similar (Blakemore and van Sluyters et al, 1975), however, it is now widely accepted that the two forms of deprivation are different hence have different impact on cortical cells (Mower et al, 1981; Blais et al, 2008). Similar to MD, BD is characterized by deprivation of form that permits light perception while DR means complete abolition of visual experience. BD during the critical period in cats results in overall reduction in responsiveness with abnormal receptive fields and with few binocular cells are encountered (Wiesel and Hubel, 1965; Mower et al, 1981; Blais et al, 2008). In contrast, after a period of dark-rearing many neurons retain their binocular property: however similar to BD, cortical physiology is abnormal with many cells encountered that are unresponsive or are weakly selective in their responses, although receptive fields remain mappable (Mower et al, 1981; Blais et al, 2008).

3.1 Introduction

The two forms of deprivation differ in their effect on subsequent MD. MD experienced after BD results in only slight physiological changes and selective expansion of connections from the open eye does not occur, but MD after dark-rearing leads to re-establishment of connections from the open eye (Mower et al, 1981; Mower et al, 1982; Fagiolini et al, 1994). These results indicate that BD appears to produce permanent developmental effects on cortical physiology whereas dark-rearing leaves cortex in a state which can be modified by subsequent visual experience. In addition, a recent study has shown that after 3 days of MD followed by 4 days of BD, the VEP magnitudes are indistinguishable from the ones obtained from normal animals (Blais et al, 2008). In contrast, when MD is followed by dark-rearing, the OD shift towards the non-deprived eye (NDE) is retained (as determined by measuring VEP amplitude) and is similar to the shift obtained after MD alone (Blais et al, 2008). In addition, light entering through sutured eyelids is sufficient to evoke cortical responses albeit weaker than non-deprived eye responses (Blais et al, 2008). Therefore it is possible that light entering through sutured eye lids during BD generates correlated activity between the two eyes which subsequently facilitates recovery (Blais et al, 2008). Another possibility is that during BD, the feedback pathways are recruited from the cortex which could influence correlated activity in LGN, which then leads to correlated input back to the cortex (Blais et al, 2008).

One of the key properties of dark-rearing is that it slows the time course of the critical period resulting in developmental delay in the maturation of cortical properties (Cynader and Mitchell, 1980; Mower, 1991; Fagiolini et al, 1994). The progression of the critical period is also delayed even after it has been initiated by normal vision (Mower, 1991). Therefore, during the critical period, the responsiveness of neurons in the visual cortex reflects the recent history experienced. For example, neurons in the visual cortex of animals raised in complete darkness respond sluggishly and lack stimulus selectivity (Blakemore and van Sluyters 1975; Fagiolini et al, 1994). This effect of dark-rearing can be rapidly reversed; within hours of light exposure in rodents (Philpot et al, 2001) and in cats (Buisseret et al, 1978; Buisseret et al, 1982; Mower et al, 1983). Conversely, the robust visual responsiveness of cortical neurons

in light-reared animals can be degraded by 3-6 days of dark-rearing (Freeman et al, 1981). A brief period of dark-rearing results in twice as many unresponsive cells with responses generally weaker compared to normally reared cats as determined using single cell recording (Freeman et al, 1981). It should be noted that such bidirectional processes have different temporal profiles: experience can induce detectable changes in synaptic transmission within hours while deprivation induced changes take days (Philpot et al, 2001). According to the BCM model (Bienenstock et al. (1982), absence of sensory experience leads to enhanced LTP and LTD is diminished and conversely re-exposure to visual environment favours LTD (Kirkwood et al, 1996). Active synapses undergo LTD when the post synaptic activity is less than the modification threshold θm and responses greater than θm lead to LTP of active synapses (Kirkwood et al, 1996, Philpot et al, 2001). The fact that cortical physiology can be altered so dramatically by manipulations of sensory experience has made the visual cortex a favourite model system for studying how experience modifies neuronal properties.

3.1.2 Monocular deprivation (MD)

The physiological shift towards the NDE observed during MD is also accompanied by anatomical shrinkage of the deprived-eye (DE) and expansion of the NDE ocular dominance columns (Shatz and Stryker, 1978, Antonini and Stryker, 1993b) and vision through the closed eye is severely degraded (Wiesel and Hubel, 1963a). In addition, the geniculocortical afferents from the DE branch less frequently and form fewer and smaller synaptic terminals, which contain fewer mitochondria (Tieman, 1985). Recently two-photon laser scanning microscopy has revealed the effect MD has on dendritic spines (Hofer et al, 2009). Following MD, the rate of spine formation is doubled and restoring binocular vision returned spine dynamics to baseline levels but absolute spine density remained elevated in the binocular zone of the visual cortex (Hofer et al, 2009). This suggests that altering dendritic spine dynamics is one of the mechanisms contributing to experience-dependent plasticity (Hofer et al, 2009).

3.1 Introduction

The anatomical changes that accompany MD have not only been observed after prolonged period of deprivation but also in short-term MD lasting 6-7 days (Antonini and Stryker, 1993b). The latter study evaluated and compared geniculocortical axonal arbours in cats that underwent MD for 4 weeks and 6-7 days at the peak of the critical period by labelling with anterograde tracer (phaseolusLectin). Antonini and Stryker (1993b) found that following both long-term and short-term MD, the labelled afferents serving the DE showed a reduction in the complexity of terminal arborisation while the afferents serving the NDE expanded. Further quantification revealed that axonal length was smaller for DE afferents following both long-term and short-term MD. Similarly, the total length of the arbours (as a measure of growth) was smaller for the DE after short-term MD but arbour length for the NDE was not different from normal cats suggesting that 7 days of MD is not sufficient to induce significant outgrowth of geniculocortical NDE afferents (Antonini and Stryker,1993b).This is in accordance with physiological studies which have shown that MD occurs in two distinct temporal processes: depression of DE responses followed by a delayed potentiation of NDE responses (Frenkel and Bear, 2004).

In addition to MD with lid suture, several studies have employed other methods of MD to determine the effect they may have cortical binocularity. Binocularity is defined as the ability of neurons to respond to stimulation of either eye. A study by Blakemore (1976) showed that merely attenuating retinal illumination (using a neutral density (ND) filter) in one eye does not diminish the influence in the cortex of that eye; however, the number of binocular cells was reduced with an increase in domination of monocular cells by 28%. This effect is similar to what is observed in artificial strabismus, where neither eye seems to suffer relative to the other eye in terms of overall dominance but there is a marked reduction in the number of binocular driven neurons (Hubel and Wiesel, 1965; Blakemore et al, 1975). In another kitten, Blakemore (1976) used a translucent opal diffuser (eliminating pattern information) over the left eye and a transparent ND filter (reducing retinal illumination) over the right eye and found that all cells were strongly dominated by the right eye. Furthermore, Blakemore (1976) inserted opal diffusers in both eyes and

placed an additional ND filter in front of the left eye only and found that the number of binocular cells was not reduced. In addition, Blakemore (1976) also used goggles that presented vertical contours to the left eye and horizontal to the right eye, and found that binocularity is also reduced in this animal with 53% of the units were monocularly driven. There was a strong tendency for those units dominated by the left eye to have near vertical orientations while those dominated by the right eye usually had preferences to horizontal orientations (Blakemore, 1976). Similarly, binocularity is also reduced (59% of visually responsive cells were monocular) when the animals were exposed with alternate monocular occlusion in a striped environment with both eyes viewed same kind of pattern but not simultaneously (Blakemore, 1976). Therefore there are several parameters that are important for preserving binocularity: cortical neurons should be stimulated by similar retinal illumination and by congruent patterned images being present simultaneously in receptive fields of both the eyes (Blakemore, 1976). In contrast to binocularity, the relative dominance between the two eyes does not require equal retinal illumination (as seen in ND filter) or congruent stimulation (as seen in strabismus).

In contrast to MD induced in animals raised in normal environment, monocular experience after dark-rearing is more useful in evaluating recovery in the NDE rather than loss of connections in DE (Mower and Christen 1985; Mower, 1991). The effects of MD in dark-reared cats are partly due to monocular recovery of the NDE and partly a binocular competition process (Mower, 1991). MD in dark-reared animals has received scant attention and therefore the temporal profile of depression of DE and potentiation of NDE responses is not known. Since it is known that deprivation induced changes take place within days while experience-induced changes can take place within hours (Philpot et al, 2001), it is possible that the delayed potentiation of NDE responses observed in normal animals (Frenkel and Bear, 2004) perhaps takes place sooner in dark-reared animals. In addition, in normal development, susceptibility to MD is low at 3 weeks of age, rises sharply at 6 weeks of age and then gradually declines from 12 weeks onwards (Mower, 1991). Dark-rearing alters this developmental profile, and dark-reared animals tend to be more

plastic over the first 12 weeks of age (Mower et al, 1991), and in fact MD remains effective in altering cortical OD even in cats which have been dark-reared for up to 2 years (Cynader, 1983).

I assessed functional architecture of V1 using OIS in subjects raised in complete darkness from birth and compared them with subjects raised in 12 hour light/dark cycle. The advantage of OIS is that one records from population of neurons and thus overcomes the problem of sampling bias inherent in single cell recording (Bonhoeffer and Grinvald, 1996). In order to determine how bidirectional plasticity alters the functional architecture of V1, I assessed how a brief (7 days) period of either sensory experience or sensory deprivation modifies the functional architecture of V1 following 4 weeks of dark or normal rearing, respectively. Additionally, I determined the magnitude of deprivation effects in animals that had undergone MD for 2 days compared with 7 days after 4 weeks of normal rearing. In order to compare the effects of MD by different methods, I also set out to obtain optical imaging maps of subjects that were exposed to monocular vision using either a frosted lens, a neutral density filter or eye patch. This was done in subjects raised in normal 12 hour light/dark cycle compared with subjects raised in complete darkness. Lastly, I also obtained V1 surface VEPs from subjects under all the conditions examined. As a measure of pooled activity from a large number of neurons, VEPs recorded in response to a broad range of spatial frequencies can be used to obtain a physiological estimate of visual acuity (Snyder and Shapley 1979; Freeman et al, 1983).

3.2 Methods

3.2.1 Surgery

All procedures were approved by local ethical review and covered by UK Home Office licenses. At four weeks of age (postnatal days 28-30), kittens were anaesthetised with ketamine and xylazine (see General Methods chapter) and placed in a stereotaxic frame. Lid suture was then performed by first detaching the conjunctiva from the inside of the upper and lower lids and suturing it carefully using absorbable vicryl 5/0 sutures. The size of the knots was minimized in order not to cause any abrasions to the cornea. Thereafter, the outer lids were carefully sutured with 4/0 silk thread using mattress stitches, leaving a small nasal drainage hole. The animals were monitored and antibiotic (chloramphenicol) ointment was applied daily until the day of the experiment. On the day of the experiment, the sutures were carefully reopened and the eye was treated with saline and examined for any sign of corneal damage. Preparation of subjects for surgery, camera set-up, VEP recordings and data analysis were carried out as explained in detail in Chapter II (General Methods).

3.2.2 Rearing

Light and dark-rearing

The control subjects were raised in normal 12 hour light/dark environment until 10 days (before eye opening, n=2), 3 weeks (onset of the critical period, n=2), 5 weeks (peak of the critical period, n=4), 12 weeks (late phase of the critical period, n=4) and 1 year of age (adult, end of the critical period, n=2). Dark-reared subjects were placed in the dark room as soon as they were born along with their mother until 3 weeks (n=2), 5 weeks (n=3) and 12 weeks (n=2) of age. Animals that are raised in complete darkness may be at greater risk of infection and tend to gain weight less rapidly than the ones raised in normal environment (Movshon and van Sluyters, 1981). The cats used in my study were checked on daily basis using a night vision camera and none of them showed any signs of ill health.

Another group of subjects was divided into two subgroups: one subgroup (n=2) was raised in a normal 12 hour light/dark cycle until 4 weeks of age and then was kept in the dark room for a week (LR-DR). The other subgroup (n=2) was raised in

complete darkness for four weeks from birth along with their mother and was then exposed to a 12 hour light/dark cycle for a week (DR-LR).

Monocular deprivation

Fourteen subjects were raised in a normal 12 hour light/dark cycle and selective rearing was carried out at 4 weeks of age (between postnatal days of 28-30). Subjects were divided into five groups: one group underwent MD with lid suture for 2 days only (n=2), the second group underwent lid suture for 7 days (n=3), the third group (n=3) underwent MD with a frosted lens (with optical density of 0.3), the fourth group (n=2) wore a ND filter (with optical density 2.0) and the fifth group (n=4) wore an eye patch. All these groups were exposed to a lighted environment for 4 hours daily and the rest of the time they were kept in the dark room together with their mother. In order to achieve a maximum effect of MD, the first two experienced monocular vision for 2 weeks (total monocular experience, 56 h) while the third group wore the eye patch for 1 week only (total monocular experience, 28 h). Five additional subjects were raised in complete darkness from birth until four weeks of age when selective rearing was started. One group underwent MD by lid suture (n=2), the second group wore an eye patch (n=3) and the third group wore a ND filter (n=2). The latter two groups were exposed to lighted environment for four hours daily and the rest of the time they were kept in the dark room along with their mother. Subjects that had undergone MD by lid suture or eye patch experienced monocular vision for a week only whereas the subject that wore ND filter experienced monocular vision for two weeks.

I first used plastic goggles for the placement of lenses and tied them with Velcro bands for fixation; however, the disadvantage of using goggles was that minimal indirect illumination from the edges could not be prevented, which may be sufficient to override the effects of MD. I therefore made individual masks using cardboard material to hold the lenses which where then fastened with Velcro bands. The eye patches were made from opaque surgical face masks that were fastened with Velcro bands. During the period of visual exposure the animals were monitored regularly to readjust the masks or the eye patch, if necessary, and were encouraged to stay awake. After the first few days, the subjects usually adapted

well to wearing the masks or patches, and any unintended binocular exposure caused by removal of a mask rarely exceeded a few minutes.

For statistical purpose, t-test was used to determine significant difference between two experimental conditions. However, in experimental conditions where n=2, it was not possible to use any parametric tests such as t-test or ANOVA which relies on the normal distribution of the data. Therefore, a non-parametric test (Kruskal-Wallis test) was used as a method of testing equality of population median among groups. It is identical to ANOVA but does not rely on the assumption that the data is normally distributed.

3.3 Results

3.3.1 Control and dark-reared

Due to difficulty in maintaining a stable physiological state of 3 weeks old subjects, these were not imaged and only tissue samples of primary visual cortex were extracted (see Chapter II). OD maps obtained from control subjects raised in a normal environment at 5 weeks (5C) and 12 weeks (12C) of age, showed equal proportions of cortex dominated by each eye. For instance, at 5 weeks the average proportion of cortex dominated by the left eye was 50.15% (±4.9% SEM) and that dominated by the right eye was 49.83% (± 4.94% SEM). At 12 weeks, the average proportion of cortex representing the left eye was 50.95% (± 8.29% SEM) and that representing the right eye was 49.05% (±8.29 SEM). However, at 12 weeks of age (Figure 3.1A and B), OD maps showed much less distinct patches than in younger animals. Finally, OD maps obtained from one year old cats did not reveal any observable patches (Figure 3.2A). This could be due to increase in myelination in older animals which subsequently compromises the intrinsic signal. However, some studies have shown discrete OD and orientation patches in cats aged 5-12 months (Chen et al, 2007; Wong et al, 2007) using alfaxolone as opposed to isoflurane for maintenance of general anaesthesia. In fact it is known that isoflurane suppresses cortical responses more than halothane and the magnitude of responses using OIS in both cats (Fukuda et al, 2005) and monkeys (Shtoyerman et al, 2000) is reduced with isoflurane. Therefore in the present study the choice of anaesthetic may be one of the contributing factors for the absence of signal in adult cats and possibly for weaker signal observed at 12 weeks of age compared to 5 weeks of age.

Orientation maps obtained from the 5C and 12C groups showed equally strong response to all orientations examined (Figure 3.1C and E respectively). However, the orientation patches, as with OD patches, at 12 weeks (Figure 3.1A and B) appeared less distinct compared to orientation patches obtained at 5 weeks of age (Figure 3.1C). Furthermore, the angle maps from both age groups (5C and 12C) had distinct

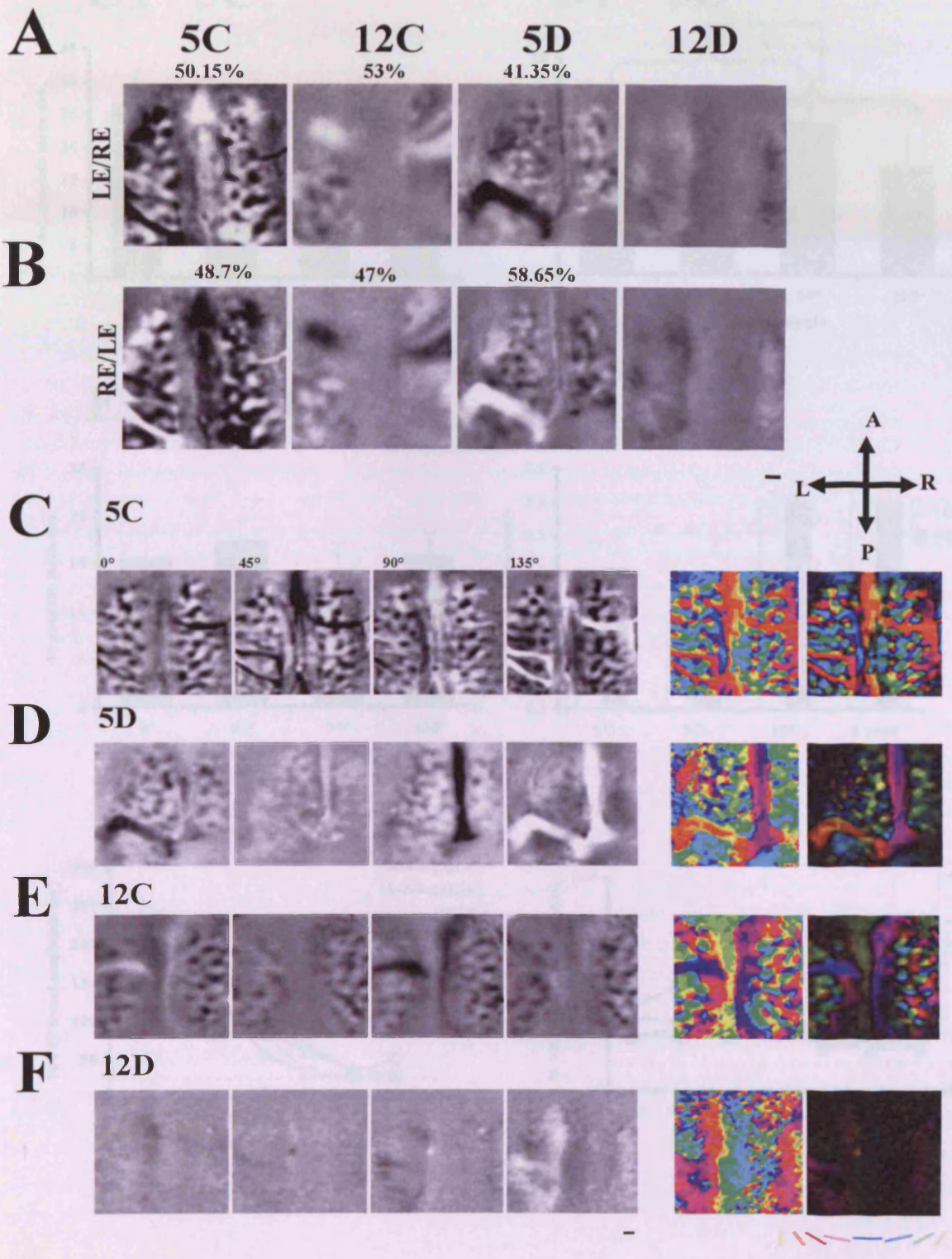
iso-orientation domains which were not biased towards any particular orientation and pinwheel centres were evident in both age groups (Figure 3.1C and E respectively). The polar maps in both age groups showed intense response selectivity as judged by the brightness of the map (Figure 3.1C and E). Quantification of orientation maps revealed that the response strength, measured as average absolute mean deviation, seems to be more or less equal across all orientations examined in groups 5C and 12C (Figure 3.1C1and Figure 3.1E1 respectively). Similar to OD maps, orientation selective patches were not visible in one year old subjects (Figure 3.2B).

Similar to control animals of the same age, OD maps obtained from dark-reared subjects at 5 weeks of age (5D) showed distinct patches (Figure 3.1A and B), with equal proportion of cortical domains dominated by the left eye (48.68 ±11.42% SEM) and the right eye (51.32% ±11.42% SEM). In contrast, OD maps acquired from dark-reared subjects at 12 weeks (12D) of age did not show any observable patches suggesting that the columnar organization had degraded after a prolonged period of darkness (Figure 3.1A and B). The quantification analysis of ocular dominance performed on high-pass filtered maps is not well suited to deal with more or less uniform maps (as in this case) where the overall signal is greatly affected by the filtering and therefore the quantification may not accurately reflect the presence or absence of activity. I therefore calculated the OD response strength of unfiltered images in which the overall responsiveness (DC level) was preserved. The response strength dominated by the right eye was 90.44 while the response strength dominated by the right eye was 62.36 suggesting that even in the absence of visible patches; the cortex is not entirely unresponsive to stimulation of either eye.

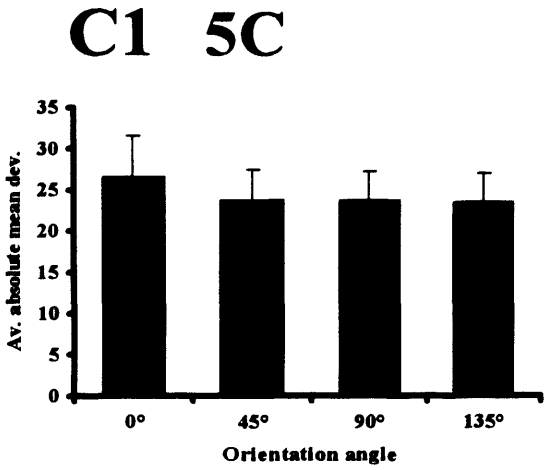
In contrast to control animals, the orientation maps obtained from 5D cats showed stronger responses (with more prominent patches) to cardinal orientations (0° and 90°) compared to the responses to oblique angles (paler patches) (Figure 3.1D). This is further supported by quantifying the orientation selective response where the response strength, measured as average absolute mean deviation, for cardinal orientations is significantly (t-test, $p=0.03$ (significant at $p<0.05$)) higher than the

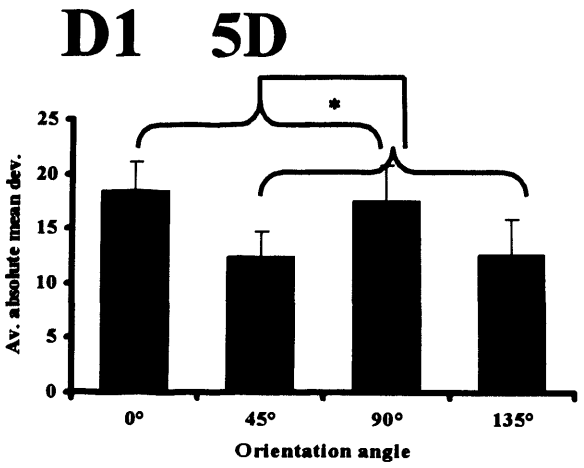
response strength calculated for oblique angles (Figure 3.1D1). Furthermore, the angle maps showed irregular layout of iso-orientation domains with few pinwheel centres visible (Figure 3.1D). Overall, the responses in 5D animals (Figure 3.1D) appeared less orientation selective than control animals (Figure 3.1C), as judged by the reduced contrast in the polar maps in the former compared to the latter. However, this should be treated with caution, as a dark area in the polar map may represent either a genuinely low response or an equal response to all orientations (which would also result in a null vector). Assessing the orientation maps qualitatively, there were no discernible patches observed in 12D animals (Figure 3.1F). Moreover, the angle map did not show any organised layout of orientation domains, further emphasizing the breakdown of orientation selectivity (Figure 3.1F). This point is underlined by the almost complete absence of any signal in the orientation polar map in a 12D animal (Figure 3.1F) compared to the polar map obtained from a control animal (Figure 3.1E) of the same age.

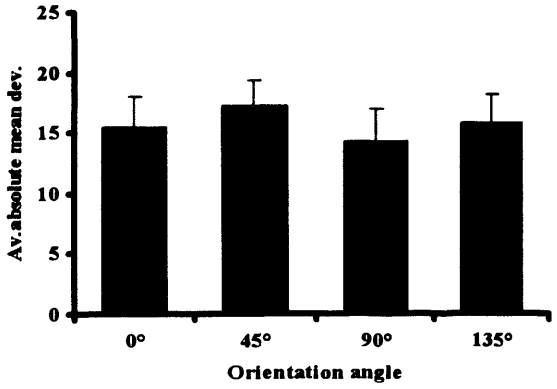
Visual acuity as measured from VEPs revealed that in control animals, visual acuity increased from 2.26 cyc/deg in 5 weeks to 3.2 cyc/deg (± 0.28 SEM) in 12 weeks and was maintained in 1 year old subjects (Figure 3.1G). Subjects that were dark-reared for 5 weeks showed reduced acuity (1.6 cyc/deg ± 0.29 SEM) compared to control of the same age (2.26 cyc/deg; Figure 3.1G). In agreement with the OIS maps no responses could be elicited at any spatial frequency in animals dark-reared for 12 weeks. I also plotted average VEP signal amplitude against spatial frequency for each eye for control and dark-reared subjects at 5 weeks of age and no inter-ocular differences were observed suggesting that the relative dominance of each eye is not different in control and dark-reared subjects (5C, Figure 3.1H and 5D, Figure 3.1I). However, overall the amplitude of VEP signal was higher in control compared to dark-reared subjects.

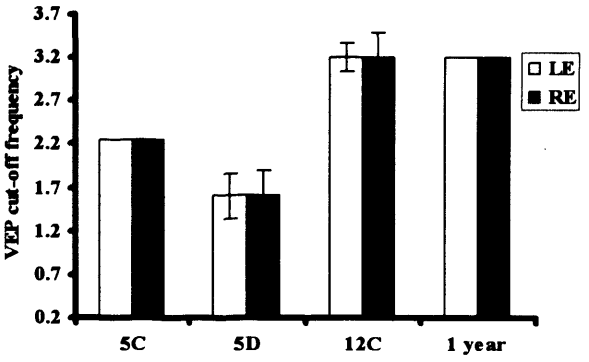

A
5C
12C
5D
12D
50.15%
53%
41.35%
LE/RE
B
48.7%
47%
58.65%
RE/LE
A
L
R
P
C
5C
0°
45°
90°
135°
D
5D
E
12C
F
12D

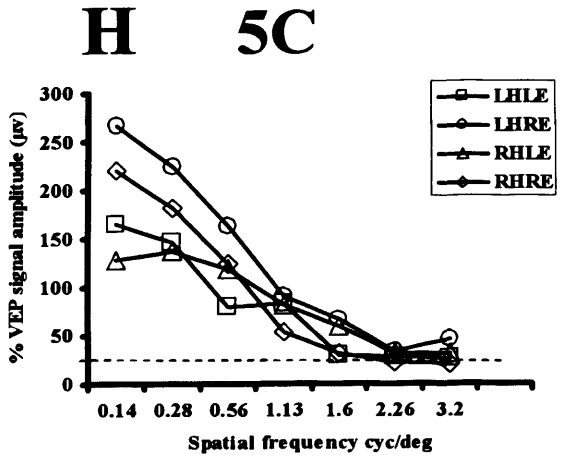
3.3 Results


C1 5C
Av. absolute mean dev.
35
30
25
20
15
10
5
0
0°
45°
90°
135°
Orientation angle


D1 5D
*
Av. absolute mean dev.
25
20
15
10
5
0
0°
45°
90°
135°
Orientation angle


E1 12C
Av.absolute mean dev.
25
20
15
10
5
0
0°
45°
90°
135°
Orientation angle


G
VEP cut-off frequency
3.7
3.2
2.7
2.2
1.7
1.2
0.7
0.2
LE
RE
5C
5D
12C
1 year


H 5C
% VEP signal amplitude (μv)
300
250
200
150
100
50
0
LHLE
LHRE
RHLE
RHRE
0.14 0.28 0.56 1.13 1.6 2.26 3.2
Spatial frequency cyc/deg

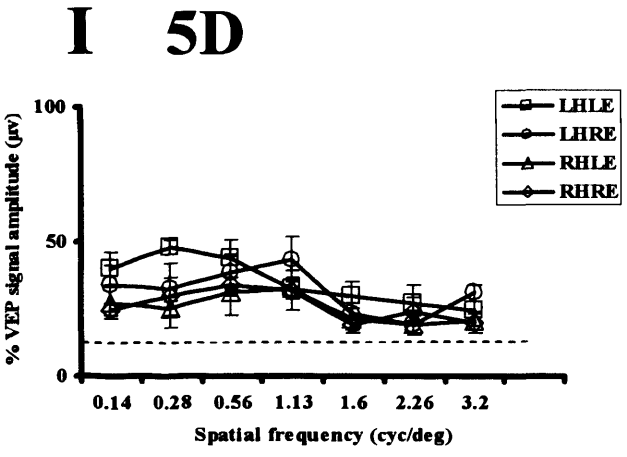

I 5D
% VEP signal amplitude (μv)
100
50
0
LHLE
LHRE
RHLE
RHRE
0.14 0.28 0.56 1.13 1.6 2.26 3.2
Spatial frequency (cyc/deg)

Figure 3.1: *Representative ocular dominance maps, orientation maps and VEPs obtained from control and dark-reared subjects:* Panel **A)** and **B)** show differential ocular dominance maps of control **(C)** and dark-reared (D) subjects at 5 and 12 weeks of age. In the row labelled LE/RE **(A)**, dark areas correspond to cortical domains activated by the left eye (LE), and the row labelled RE/LE **(B)** dark areas correspond to cortical domains activated by the right eye (RE). The numbers above each map indicate percentage of cortical territory dominated by each eye for the representative maps shown. Panel **C, D, E** and **F** show single-condition, cocktail-blank divided iso-orientation maps of 5C (n=4), 5D (n=3), 12C (n=4) and 12D (n=2) subjects respectively (scale bar 1mm). The number above the maps in **C** indicates the angle of orientation. The coloured maps on the far right are angle and polar maps. The angle map shows orientation-selective domains, in which the colour indicates the angle of orientation and the right most column shows polar maps in which response strength is indicated by intensity (see colour key). Bar charts labelled **C1, D1** and **E1** show average quantification of orientation maps obtained by calculating average mean deviation from 127 on a grey scale (see Chapter II) of 5C, 5D and 12C subjects. The average absolute mean deviation for cardinal orientations in 5D is significantly higher than the response calculated for oblique angles. **G)** The average VEP cut-off difference is plotted for control (5C, 12C and 1 year; no error bars indicate n=1) and dark-reared subjects (5D), and no responses at any spatial frequency could be elicited in 12 weeks dark-reared subjects. Panels **(H-I)** show VEP signal amplitude for the same three subjects plotted against spatial frequency of the stimulus (no error bars in 5C as n=1) obtained from left hemisphere (LH) and right hemisphere (RH) for both the eyes (RE and LE). The dotted line indicates average baseline levels of VEP amplitude. All data represent mean ± SEM (* $p=<0.05$).

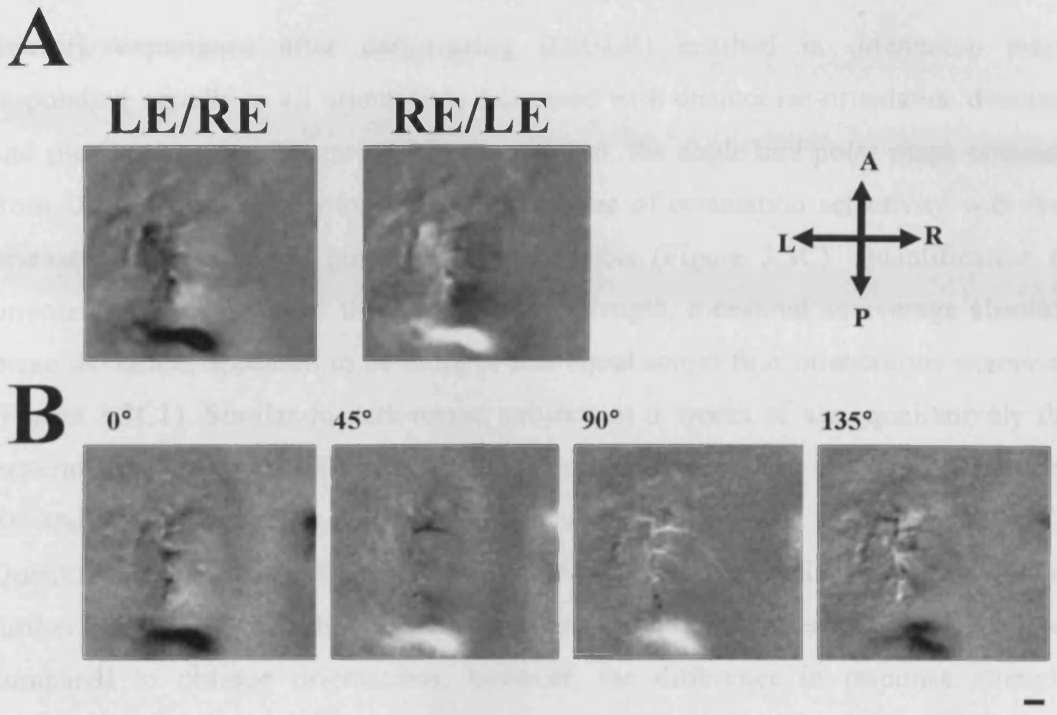


Figure 3.2: *Representative ocular dominance and orientation maps obtained from 1 year old subject.* **A)** OD maps obtained from a year old subject raised in normal 12 hour light/dark cycle. **B)** The first four maps show single-condition, cocktail-blank divided iso-orientation maps and the number above indicates the angle of orientation (n=2) (Scale bar 1mm).

3.3.2 Sensory experience and deprivation

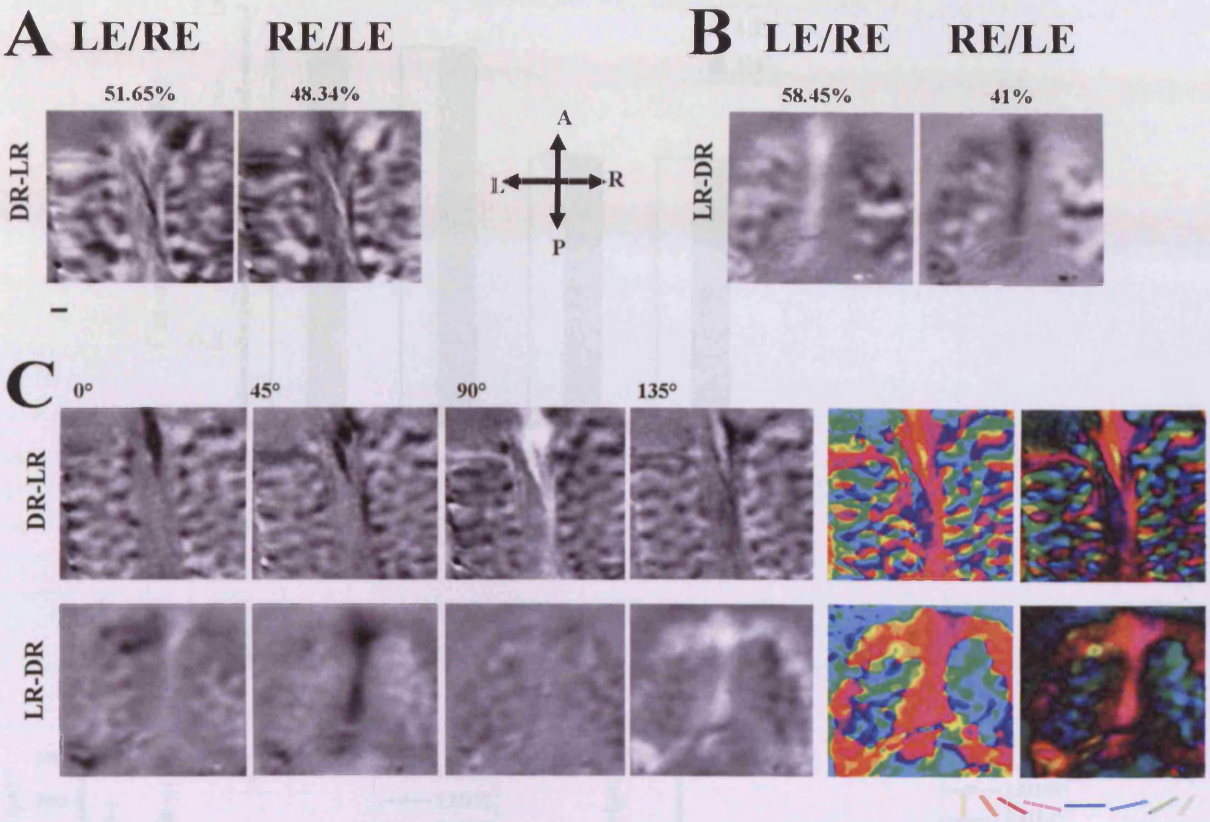
Qualitatively, the OD maps obtained from subjects raised in complete darkness until four weeks of age followed by a week of visual experience (DR-LR) showed distinct patches (Figure 3.3A) as obtained in control animals of the same age (Figure 3.1A and B). On the other hand, the OD maps obtained from subjects raised in a normal 12 hour light/dark cycle until four weeks of age followed by a week of dark-rearing (LR-DR) appeared weak (Figure 3.3B) and were comparable to the ones obtained from dark-reared subjects of the same age (Figure 3.1A and B). Quantitatively, experimental group DR-LR showed equal proportions of cortex dominated by the left eye (51.6% ± 7.6% SEM) and the right eye (48.4% ± 9.59% SEM, Similarly, group LR-DR also showed equal proportions of cortex dominated by the left eye (53.65% ± 7.6% SEM) and the right eye (46.35% ± 8.6% SEM).

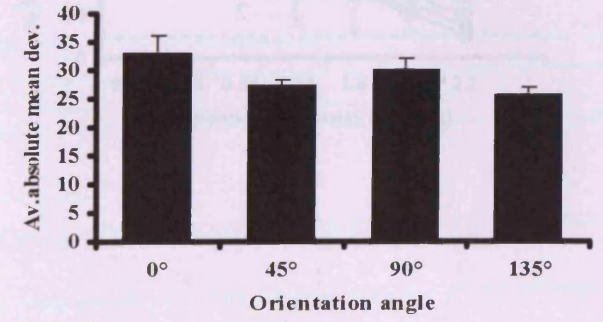
3.3 Results

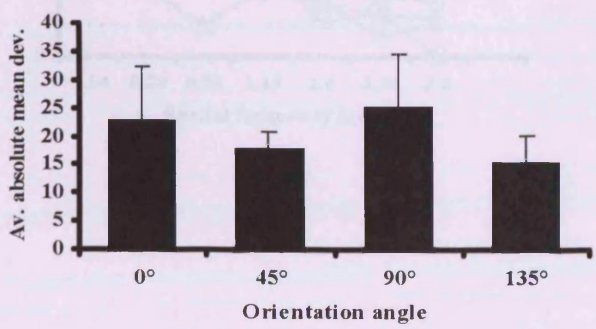
Sensory experience after dark-rearing (DR-LR) resulted in orientation maps responding equally to all orientations examined with distinct iso-orientation domains and pinwheel centres (Figure 3.3C). In addition, the angle and polar maps obtained from DR-LR subjects demonstrated high degree of orientation selectivity with iso-orientation domains and pinwheel centres visible (Figure 3.3C). Quantification of orientation maps revealed that the response strength, measured as average absolute mean deviation, appeared to be more or less equal across four orientations examined (Figure 3.3C1). Similar to dark-reared subjects at 5 weeks of age, qualitatively the experimental group LR-DR also revealed stronger responses to cardinal orientations (0° and 90°) compared to responses to oblique angles (45° and 135°) (Figure 3.3C). Quantification of orientation maps, measured as average absolute mean deviation, further confirmed that the response strength to cardinal orientations was higher compared to oblique orientations, however, the difference in response strength between cardinal and oblique angles was not statistically significant (Kruskal-Wallis test, $p=0.462$) (Figure 3.3C2). Furthermore, in contrast to DR-LR, angle maps from the experimental group LR-DR had poorly organised angle maps with few pinwheel centres and the polar map showed a lesser degree of orientation selectivity as judged by the brightness of the map (Figure 3.3C).

In order to assess visual acuity, VEP was recorded from these two experimental groups. Abolition of sensory experience for a week (LR-DR) decreased visual acuity to 1.6 cyc/deg (± 0.02 SEM) compared to control subjects of the same age (2.26cyc/deg) (Figure 3.3D). In fact the reduced acuity in LR-DR subjects is indistinguishable from dark-reared subjects (5D, see Figure 3.1G). On the other hand, subjects exposed to only one week of visual experience (DR-LR) showed comparable acuity to control animals at 2.26 cyc/deg (Figure 3.3D). This is in accordance with imaging data suggesting that during the critical period, cortical physiology reflects the recent history of visual experience. I also plotted VEP response amplitude against spatial frequency and both the experimental conditions did not show inter-ocular differences. Overall the VEP response amplitude was much higher in subjects that

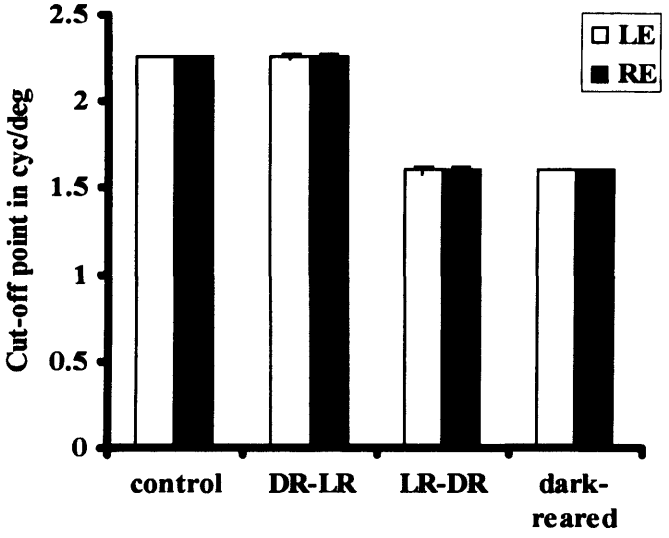
experienced a week of visual experience compared to subjects that were dark-reared for a week.


A LE/RE RE/LE
51.65%
48.34%
DR-LR
A
L
R
P
B LE/RE RE/LE
58.45%
41%
LR-DR
C
0°
45°
90°
135°
DR-LR
LR-DR

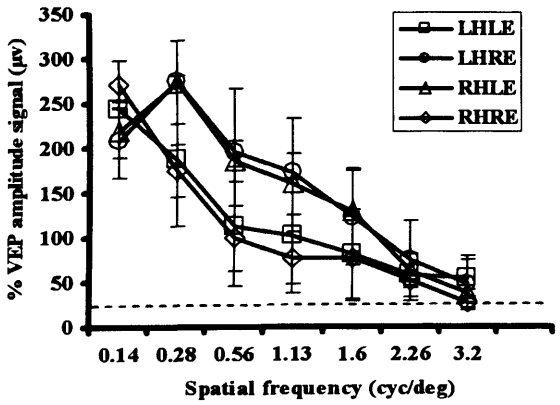

C1 DR-LR
Av. absolute mean dev.
40
35
30
25
20
15
10
5
0
0°
45°
90°
135°
Orientation angle


C2 LR-DR
Av. absolute mean dev.
40
35
30
25
20
15
10
5
0
0°
45°
90°
135°
Orientation angle

D



E1 DR-LR



E2 LR-DR

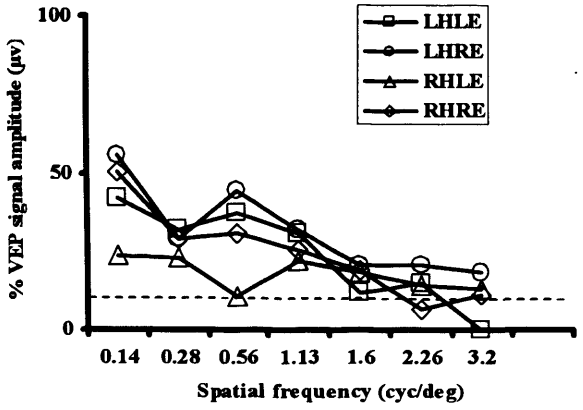


Figure 3.3: *Ocular dominance, orientation maps and VEPs obtained from subjects exposed to brief sensory alteration.* **A)** Differential ocular dominance maps of subjects raised complete darkness until four weeks of age followed by a week of visual experience (DR-LR) (n=2) **B)** and of subjects raised in 12 hour light/dark cycle until four weeks of age followed by a week of complete darkness (LR-DR) (n=2). In the column labelled LE/RE, dark areas correspond to cortical domains activated by the left (LE) eye, and the column labelled RE/LE dark areas correspond to cortical domains activated by the right (RE) eye. The numbers above each map indicate the percentage of cortical territory dominated by each eye for the representative maps shown. **C)** Orientation maps obtained from experimental groups DR-LR and LR-DR show single-condition, cocktail-blank divided iso-orientation maps and the number on top indicates the angle of orientation (scale bar 1mm). The angle map shows orientation-selective domains, in which the colour indicates the angle of orientation and the right most column shows polar maps in which response strength is indicated by intensity (see colour key). The histograms below show strength of orientation selectivity response determined by calculating the average absolute mean deviation from 127 on a gray scale of experimental groups DR-LR (**C1**) and LR-DR (**C2**). Bar chart (**D**) shows average VEP cut-off points for each of the experimental condition. Panels (**E1**) and (**E2**) show VEP signal amplitude for the same two subjects plotted against spatial frequency of the stimulus obtained from left hemisphere (LH) and right hemisphere (RH) for both the eyes (RE and LE). The dotted line indicates average baseline levels of VEP amplitude. All data represent mean ± SEM.

3.3.3 Monocular deprivation by lid suture (2 and 7 days)

Judging the maps qualitatively, it is apparent that OD maps obtained from subjects that exhibited 2 days and 7 days of MD responded to almost equal area dominated by the DE (Figure 3.4A). I only analysed absolute strength of responses through the DE and the NDE across the regions of interest in both cortical hemispheres in images that were not high-pass filtered. With this analysis, the DC component was preserved and any variability caused by differences in DC offsets is minimised. This form of analysis revealed that subjects that exhibited 2 days of MD had significantly (Kruskal-Wallis test, $p=0.04$) reduced DE response strength (43.9 ± 3.9 SEM) compared to the NDE response strength (77.8 ± 11.9 SEM). Similarly, subjects that had experienced 7 days of MD had reduced DE response strength (32.23 ± 15.98 SEM) while the NDE response strength (141.03 ± 9.3) was significantly higher (Kruskal-Wallis test, $p=0.03$) than DE strength (Figure 3.4B). In addition, the absolute strength of responses through the NDE following 7 days of MD was significantly higher than 2 days of MD (Kruskal-Wallis test, $p=0.04$; Figure 3.4B) and almost double in magnitude. I also plotted average response strength of the right and the left eye of control subjects and it was clear that the deprived eye strength after 2 and 7 days of MD was reduced compared to control animals. In addition, the response strength of the non-deprived eye was higher after 7 days of MD but after 2 days of MD, the response strength was not different from control animals. This is in accordance with previous studies (Friedlander et al, 1991; Frenkel and Bear, 2004) which have shown during MD there are two distinct processes: the depression of the deprived eye responses takes places after 3 days of MD followed by potentiation of the non-deprived eye responses which takes place after 5-7 days.

Qualitative analysis of orientation maps obtained from subjects that had undergone 7 days of MD showed that there were no visible patches observed in the DE and strong orientation selective patches were observed in the maps obtained for the NDE (Figure 3.5A). Therefore one week of deprivation during the peak of the critical period leads to the apparent elimination of responses through the DE. Polar maps obtained from the DE map showed minimal response strength after 7 days of MD as judged by the

brightness of the map. On the other hand the polar map for the NDE showed high response strength (Figure 3.5A). In contrast, orientation maps obtained after 2 days of MD revealed weaker orientation-selective patches in the DE compared to NDE patches (Figure 3.5A) but the normal layout of the map was maintained. Quantification of orientation maps, measured as average absolute mean deviation, revealed that the response strength to DE was weaker than NDE responses, however, the difference was not significant (Kruskal-Wallis test, $p=0.083$) (Figure 3.5B).

In order to assess if MD had any effect on visual acuity, I also recorded VEPs to obtain an electrophysiological estimate of visual acuity. MD after 2 days resulted in reduced visual acuity in the DE (1.13 cyc/deg) compared to the NDE (2.26 cyc/deg). On the other hand MD after 7 days did not elicit any response through the DE while the visual acuity through the NDE was 2.26cyc/deg. I also plotted response amplitudes against spatial frequency in both experimental conditions. MD for 2 days caused a significantly reduced amplitude in the DE (Kruskal-Wallis test, $p=0.000$) compared to the response amplitude of the NDE (Figure 3.6A). In contrast, MD for 7 days resulted in virtually no response through the DE compared to response amplitude of the NDE (Figure 3.6B).

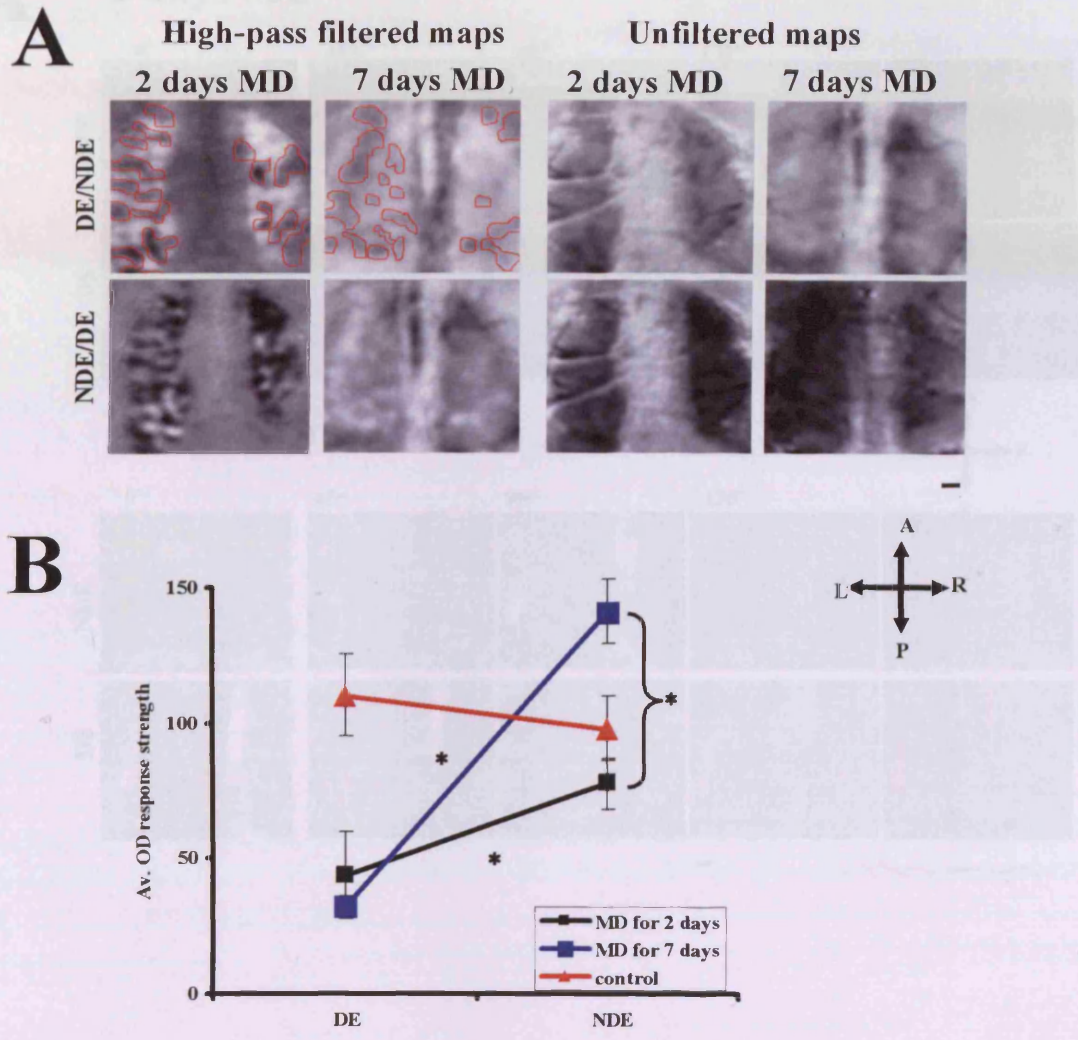
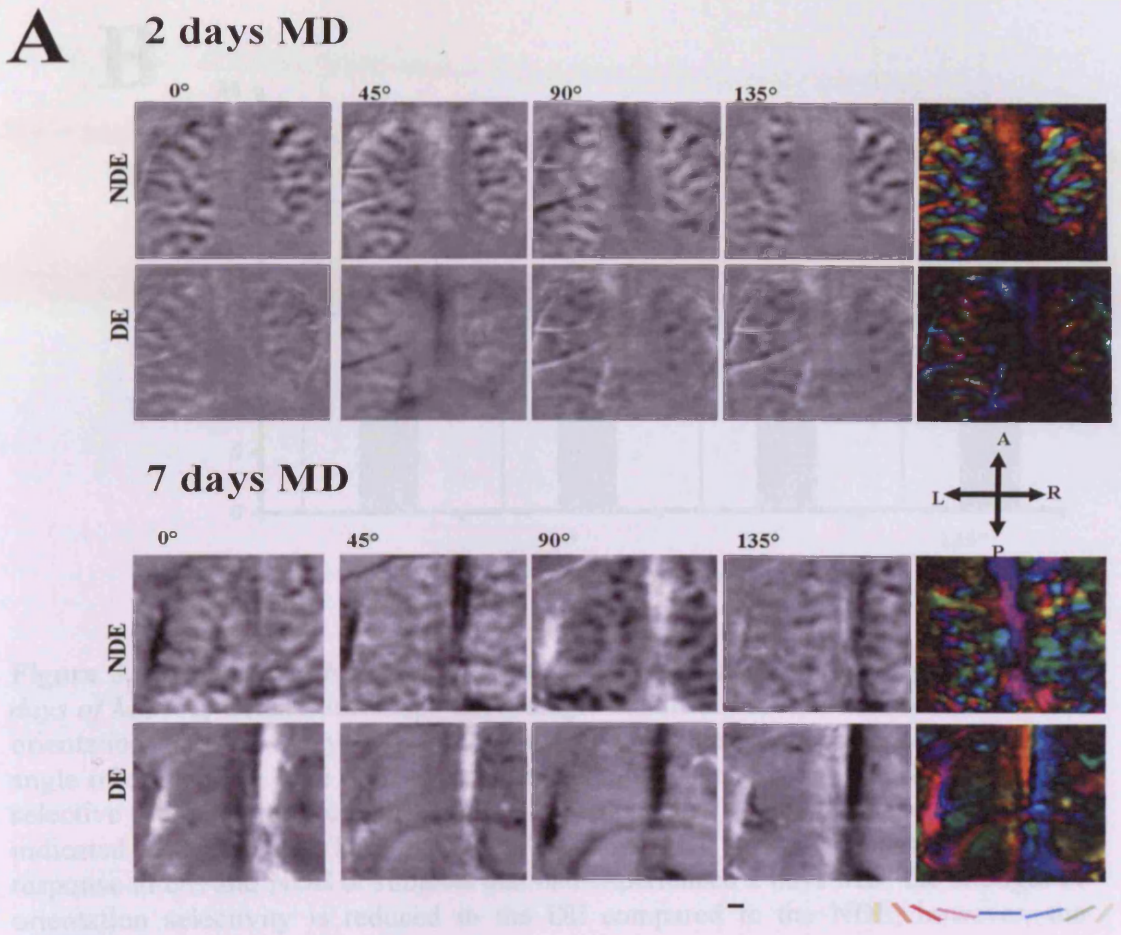


Figure 3.4: *Representative ocular dominance maps obtained from subjects after 2-days and 7-days of MD.* **A)** Differential ocular dominance maps of subjects that underwent 2 days (n=2) or 7 days (n=3) of MD. In the row labelled DE/NDE, dark areas correspond to cortical domains activated by the deprived right eye (DE), and the row labelled NDE/DE dark areas correspond to cortical domains activated by the (NDE) eye. The DE dominated patches are highlighted in red. On the far right, the maps show unfiltered activity maps through the DE and the NDE divided by the blank response (scale bar 1mm). **B)** The graph shows average OD response strength calculated for the two MD conditions compared with control subjects. The OD strength is significantly reduced through stimulation of the DE after 2 and 7-days of MD while NDE strength is significantly higher after 7-days of MD. All data represent mean ± SEM (* denotes $p<0.05$).


A
2 days MD
0°
45°
90°
135°
NDE
DE
7 days MD
A
L
R
P
0°
45°
90°
135°
NDE
DE

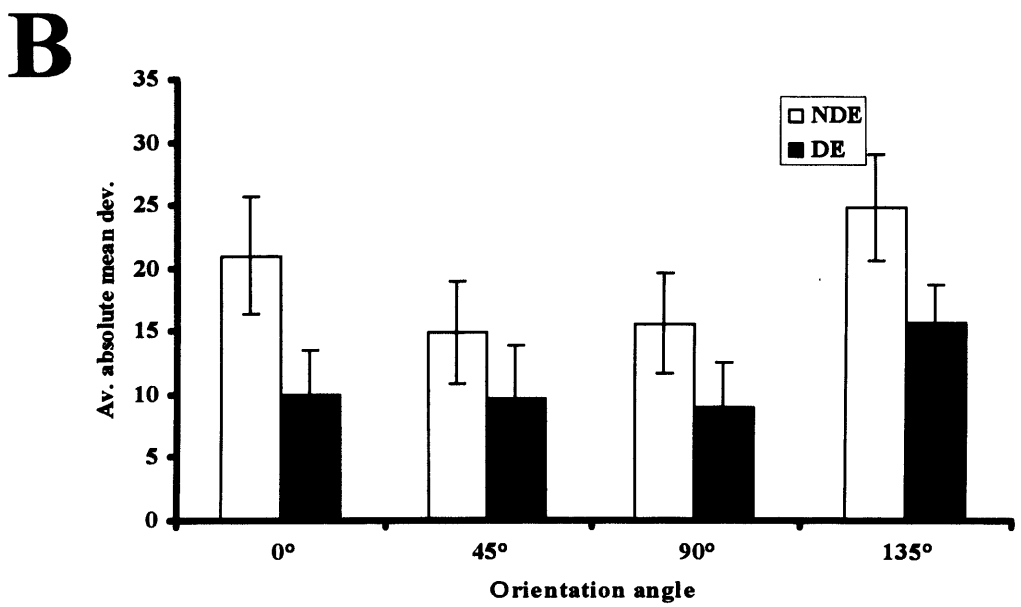


Figure 3.5: *Representative orientation maps obtained from subjects after 2 and 7-days of MD.* **A)** Orientation maps show single-condition, cocktail-blank divided iso-orientation maps of 2 days MD and 7 days MD and the number above indicates the angle of orientation. The right most column shows polar maps in which orientation-selective domains are colour-coded (see colour key) and response strength is indicated by intensity. The histogram in panel **(B)** shows orientation selective response in DE and NDE of subjects that had experienced 2 days MD; the strength of orientation selectivity is reduced in the DE compared to the NDE, however, the difference was not significant.

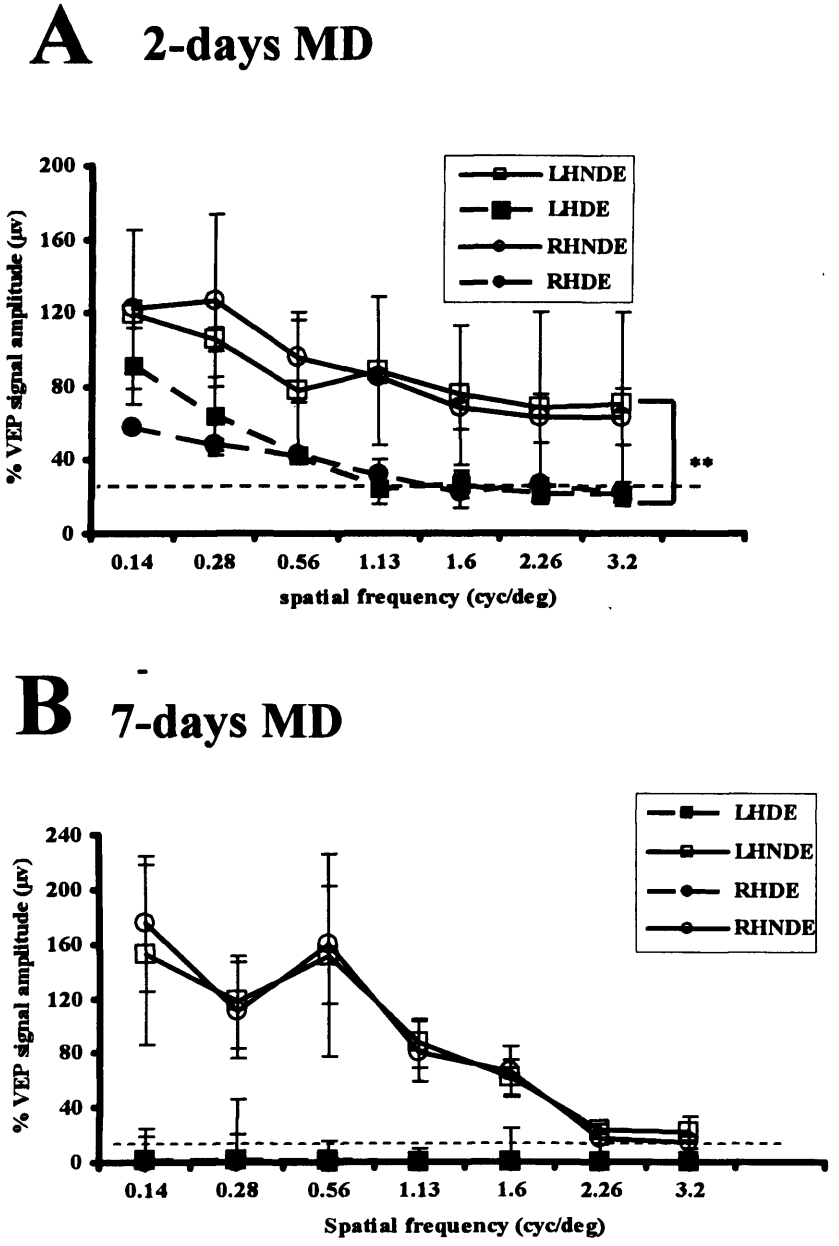


Figure 3.6: *VEP response amplitude obtained from subjects after 2 and 7- days of MD.* Panels **(A-B)** show VEP signal amplitude plotted against spatial frequency of the stimulus of subjects that had undergone different durations of deprivation. 2-days of MD show significantly reduced amplitude in the DE (deprived eye) in both the hemispheres (right hemisphere: RH and left hemisphere: LH) compared to the NDE (non-deprived eye); however MD after 7 days resulted in virtually no VEP signal through the DE. The dotted line indicates average baseline levels of VEP amplitude. All data represent mean ± SEM (** denotes $p<0.05$).

3.3.4 Other MD conditions

In order to assess if other forms of deprivation paradigms induce deprivation effects similar to those observed with lid suture, I reared subjects wearing either an eye patch, ND filter or a frosted lens in one eye (see section 4.1.2). Initially, I reared subjects monocularly with frosted lens (using goggles) for a week as done with MD with lid suture except that the subjects experienced monocular vision daily for 4 hours only. Qualitatively, the OD maps obtained from subjects that had experienced MD with frosted lens (n=4) (Figure 3.7A) and ND filter (n=1) (Figure 3.8A) showed equal area responding to DE and NDE stimulation. In order to make sure that the deprivation effect, if any, was not obscured by high-pass filtering, only unfiltered images were used for analysis to calculate OD response strength. Monocular deprivation with frosted lens revealed that the strength of responses through the DE and the NDE was not much different from each other (DE: 51.9 ± 13.8 and NDE: 50.8 ± 11.8). In addition, qualitative analysis of the orientation maps showed distinct orientation selective patches through stimulation of both the DE and the NDE (Figure 3.7B). Similarly polar maps obtained through stimulation of the DE and the NDE showed strong responses as judged by the brightness of the maps (Figure 3.7B).These results were surprising as a previous study by Blakemore (1976) showed that 30-40 hours of monocular exposure with an opal diffuser lens (in which the pattern information is abolished similar to frosted lens) was sufficient to cause an OD shift towards the non-deprived eye. Similarly, MD with ND filter resulted in almost equal responses through the DE (95.40 ± 7.6) and the NDE (112 ± 13.8) and orientation maps showed distinct patches for both eyes (Figure 3.8A and 3.8B respectively). Moreover, polar maps obtained through stimulation of the DE and the NDE showed equally strong responses as judged by the brightness of the map with clear iso-orientation domains (Figure 3.8B). In order to test the possibility that perhaps one week of deprivation was not sufficient to cause any OD shift, I deprived one subject with a frosted lens for 2 weeks. Qualitatively, it is apparent that there is a reduced response through DE compared to NDE stimulation (Figure 3.9A). Further quantification revealed that the DE response strength was 25.62 while the NDE

strength was 43.53. Qualitative analysis of orientation maps from the latter subject showed strong orientation patches dominated by the NDE but weaker represented by the DE (Figure 3.9B). Furthermore, polar map obtained from the DE revealed weaker orientation selectivity as judged by the brightness of the map compared to the one obtained from the NDE (Figure 3.9B). In order to eliminate the possibility that the goggles permitted indirect illumination, I used individual masks made from cardboard to hold the lenses (frosted lens or ND filter).

Qualitatively it is apparent that MD with frosted lens (using cardboard to hold lenses) for two weeks resulted in reduced responses to DE stimulation while MD with ND filter resulted in a significantly larger area of cortex responding to the DE than the other MD regimens (Figure 3.10A). In order to minimize variability caused by high-pass filtering only unfiltered images were used for analysis to calculate OD response strength. Quantitative analysis of cortical area exposed to monocular vision by frosted lens showed that the DE strength was significantly lower (56.64 ± 21.61 SEM, t-test $p=0.03$) than the NDE response strength (157.2 ± 36.8 SEM) (Figure 3.10B). Therefore it appears that if MD is limited to 4 hours per day an additional week of exposure is required to achieve the same MD effect as that observed in MD with lid suture. Since previous studies have shown that MD with ND filter does not affect the relative balance of ocular dominance (Blakemore, 1976), I additionally also deprived subjects with ND filter for two weeks, such that the exposure time was identical to subjects wearing a frosted lens. As after one week, MD with ND filter for two weeks did not cause any OD shift towards the NDE (DE response 95.58 ± 13.82 SEM and NDE response 80.43 ± 10.19 SEM; Kruskal-Wallis test, $p=0.20$) (Figure 3.10B). On the other hand, MD with eye patch for one week only showed a reduced area responding to the DE compared to the NDE (Figure 3.10A) and this was further confirmed by quantifying the response strength of the DE (45.84 ± 20.96) and the NDE (79.90 ± 8.04 SEM), however the difference between the two eyes was not significant (t-test, $p=0.23$) (Figure 3.10B).

3.3 Results

In addition to OD maps, orientation maps were also analysed from subjects that underwent different MD regimens. Qualitative analysis of orientation maps obtained from subjects that had experienced MD with frosted lens did not reveal any visible orientation selective patches following stimulation of the DE eye while strong orientation-selective patches were obtained through the NDE (Figure 3.11A). Moreover, polar maps obtained from the DE appeared flat with no orientation selective response visible while polar maps from the NDE showed clear iso-orientation domains (Figure 3.11A). Consistent with the results obtained for OD maps, subjects that experienced MD with ND filter exhibited distinct orientation-selective patches following stimulation of both the DE and the NDE (Figure 3.11B) and further quantification did not show a significant difference (Kruskal-Wallis test, $p=0.39$) between the two eyes (Figure 3.11B1). In addition, polar maps obtained from DE and NDE (Figure 3.11B) showed equally strong responses as indicated by the brightness of the maps with clear iso-orientation domains visible. In contrast, MD with eye patch revealed weaker orientation patches in the DE compared to the NDE (Figure 3.11C). Further quantification (Figure 3.11C1), however, revealed that the difference in response strength between DE and NDE was not quite significant (t-test, $p=0.058$). Moreover, polar maps obtained from the DE revealed weaker selectivity as judged by the brightness of the map compared to the one obtained from the NDE (Figure 3.11C).

In order to assess if different regimens of MD had different effects on visually evoked responses and visual acuity, I recorded VEPs from the surface of V1. I plotted average VEP response amplitude against spatial frequency for different MD regimens. MD with frosted lens (Figure 3.12A) resulted in virtually no response through the DE compared with NDE response. In contrast, MD with ND filter and MD with eye patch did not show differences in VEP amplitude responses through DE and NDE stimulation (Figure 3.12B and C respectively). Similarly, the acuity from these two experimental conditions was indistinguishable from control animals (2.26cyc/deg).

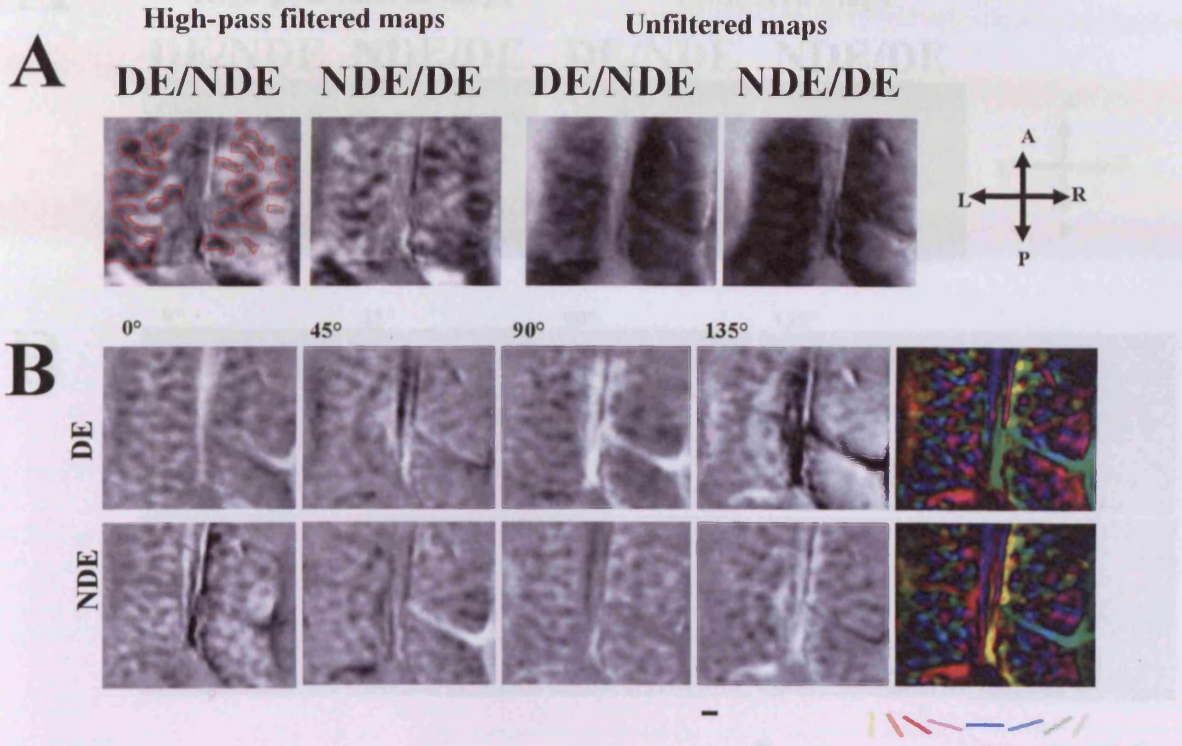


Figure 3.7: *Representative ocular dominance maps and orientation maps obtained from subject that had undergone MD with frosted lens for a week* (n=3). **A)** Differential ocular dominance maps of subjects and in the row labelled DE/NDE, dark areas correspond to cortical domains activated by the deprived (DE) eye, and the row labelled NDE/DE dark areas correspond to cortical domains activated by the non- deprived eye (NDE). The patches in the DE for all the experimental conditions are highlighted in red. On the far right, the maps show unfiltered activity maps of DE and NDE divided by the blank response. **B)** Orientation maps show single-condition, cocktail-blank divided iso-orientation maps of DE and NDE and the number on top of the maps indicates the angle of orientation. The right most column shows polar maps of DE and NDE in which orientation-selective domains are colour-coded (see colour key) and response strength is indicated by intensity. (Scale bar 1mm).

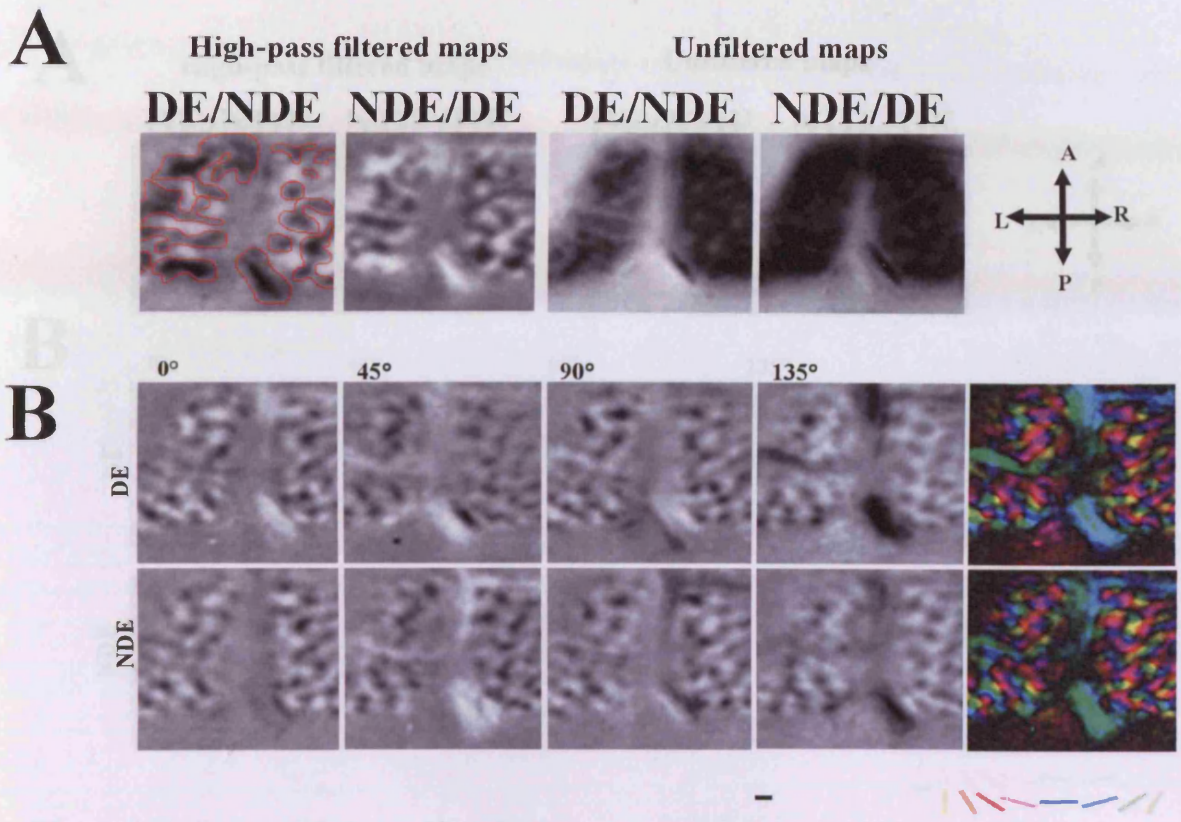


Figure 3.8: *Representative ocular dominance maps and orientation maps obtained from subject that had undergone MD with ND filter for a week* (n=1). **A)** Differential ocular dominance maps of subjects and in the row labelled DE/NDE, dark areas correspond to cortical domains activated by the deprived (DE) eye, and the row labelled NDE/DE dark areas correspond to cortical domains activated by the deprived (right) eye (NDE). The patches in the DE for all the experimental conditions are highlighted in red. On the far right, the maps show unfiltered activity maps of DE and NDE divided by the blank response. **B)** Orientation maps show single-condition, cocktail-blank divided iso-orientation maps of DE and NDE and the number on top of the maps indicates the angle of orientation. The right most column shows polar maps of DE and NDE in which orientation-selective domains are colour-coded (see colour key) and response strength is indicated by intensity. (Scale bar 1mm).

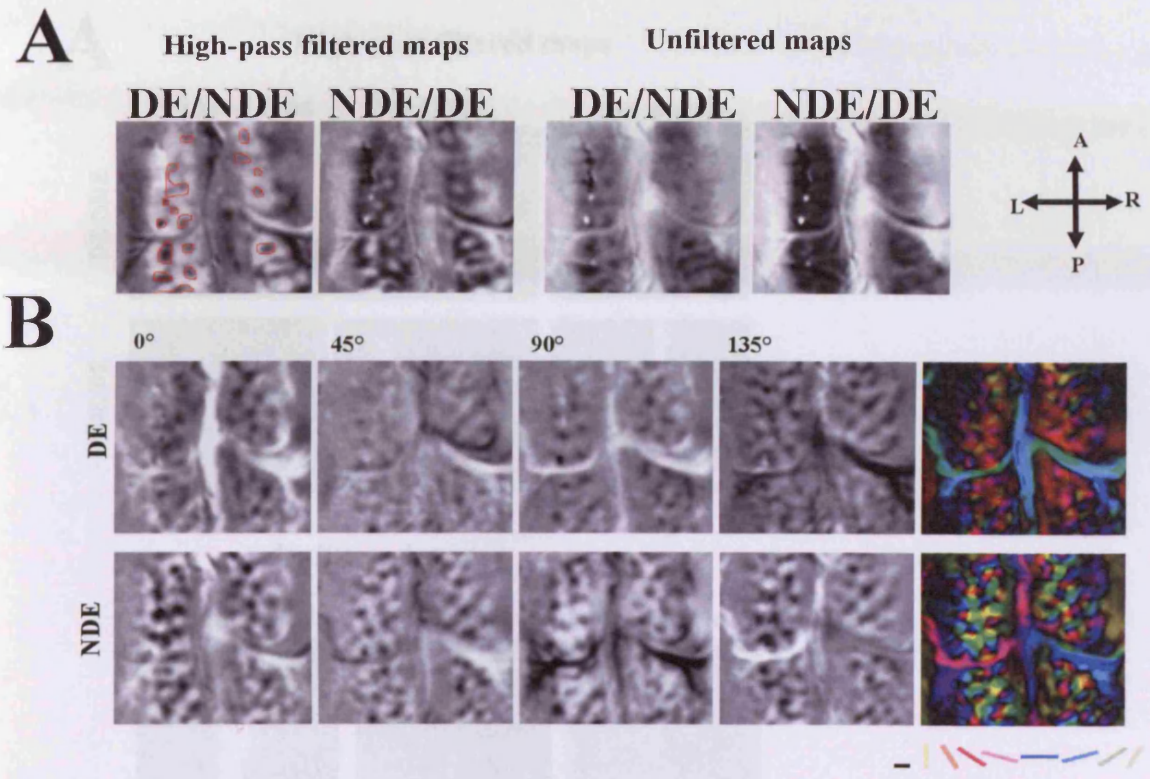
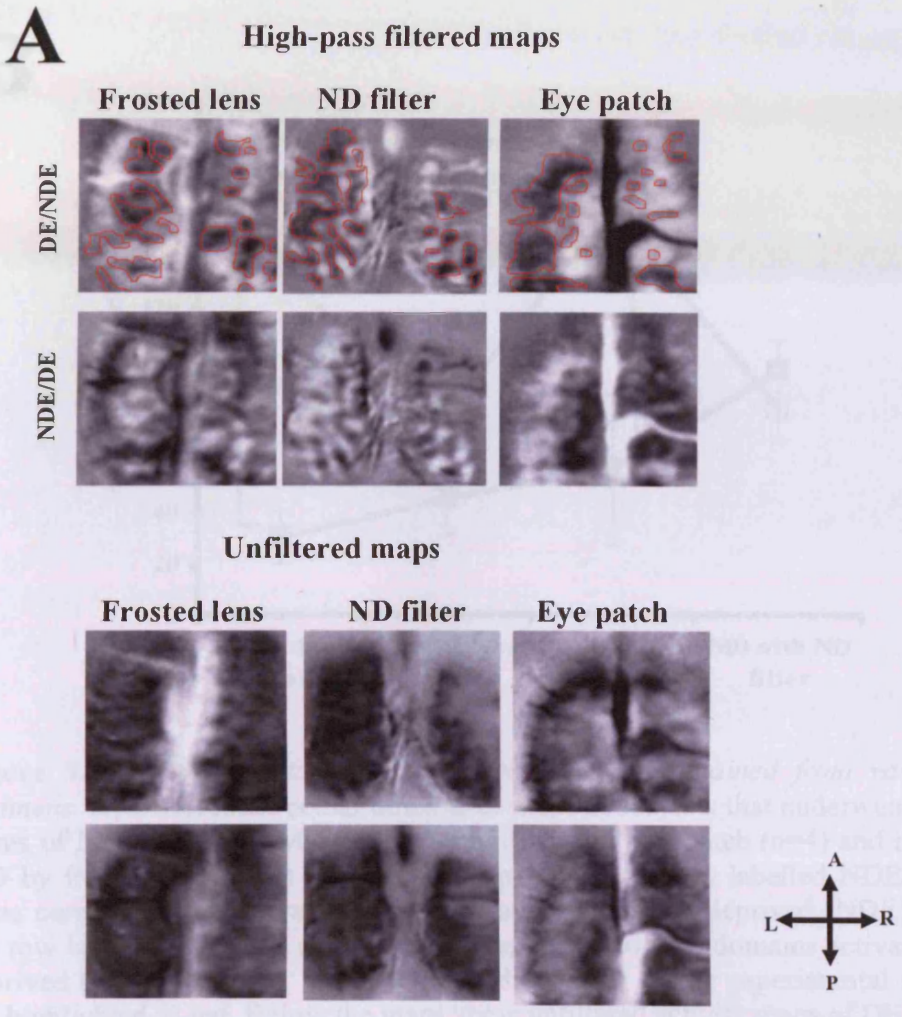


Figure 3.9: *Representative ocular dominance maps and orientation maps obtained from subject that had undergone MD with frosted lens for two weeks* (n=3). **A)** Differential ocular dominance maps of subjects and in the row labelled DE/NDE, dark areas correspond to cortical domains activated by the deprived (DE) eye, and the row labelled NDE/DE dark areas correspond to cortical domains activated by the non-deprived eye (NDE). The patches in the DE for all the experimental conditions are highlighted in red. On the far right, the maps show unfiltered activity maps of DE and NDE divided by the blank response. **B)** Orientation maps show single-condition, cocktail-blank divided iso-orientation maps of DE and NDE and the number on top of the maps indicates the angle of orientation. The right most column shows polar maps of DE and NDE in which orientation-selective domains are colour-coded (see colour key) and response strength is indicated by intensity. (Scale bar 1mm).


A
High-pass filtered maps
Frosted lens
ND filter
Eye patch
DE/NDE
NDE/DE
Unfiltered maps
Frosted lens
ND filter
Eye patch
A
L
R
P

B

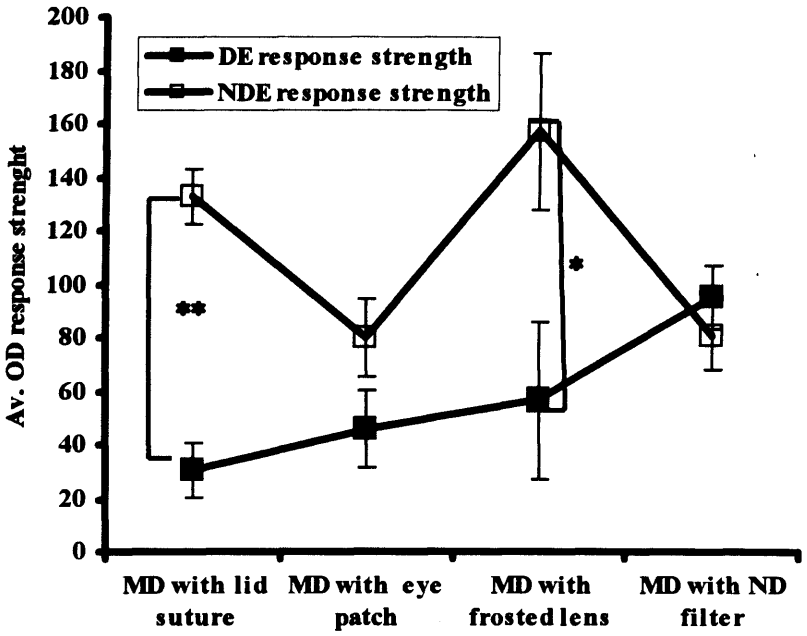
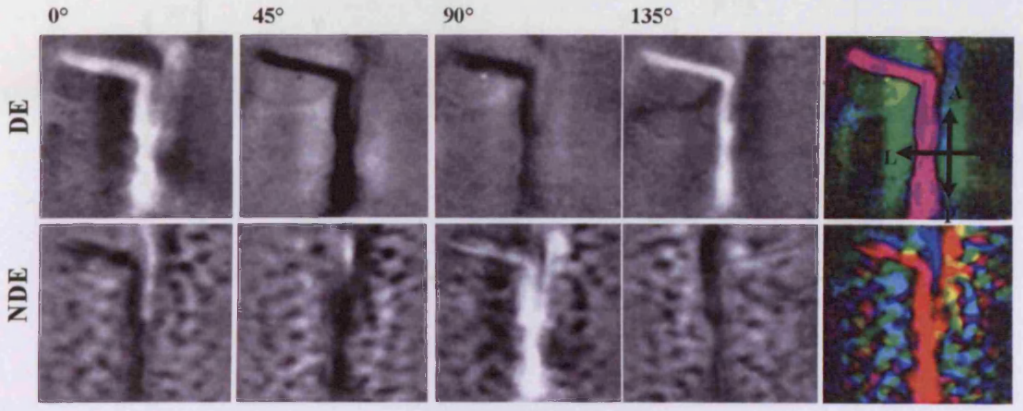
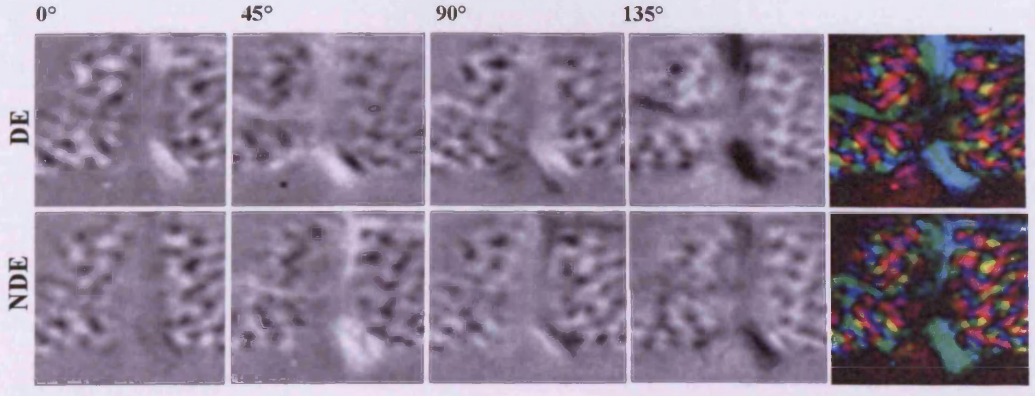


Figure 3.10: *Representative ocular dominance maps obtained from various MD regimens.* **A)** Differential ocular dominance maps of subjects that underwent different forms of MD (one week MD by lid suture (n=3) and eye patch (n=4) and two weeks MD by frosted lens (n=3) and ND filter (n=2)). In the row labelled NDE/DE, dark areas correspond to cortical domains activated by the non-deprived (NDE) eye, and the row labelled DE/NDE dark areas correspond to cortical domains activated by the deprived (right) eye (DE). The patches in the DE for all the experimental conditions are highlighted in red. Below the maps show unfiltered activity maps of DE and NDE divided by the blank response. (Scale bar 1mm). **B)** Average strength of responses was calculated for DE and NDE of different MD conditions. Only MD with lid suture and MD with frosted lens showed significant difference between DE and the NDE. (** denotes $p<0.005$ and * denotes $p<0.05$).

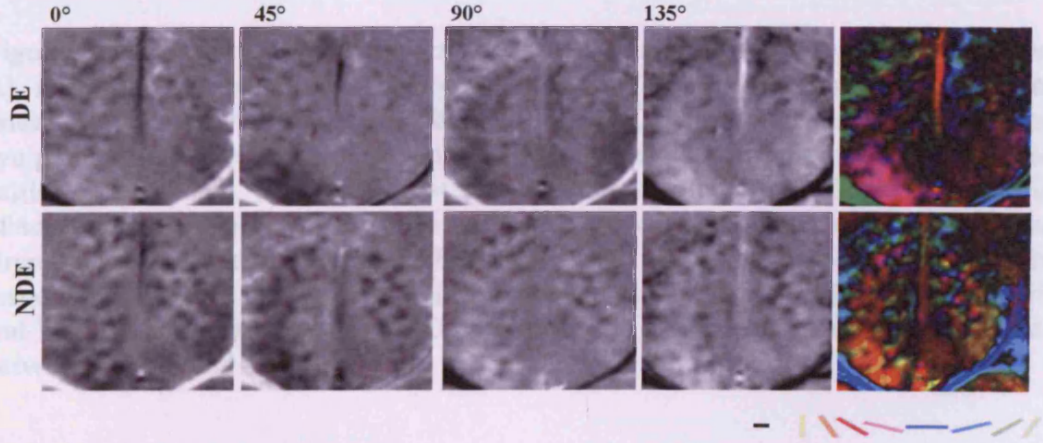
A **MD with frosted lens**



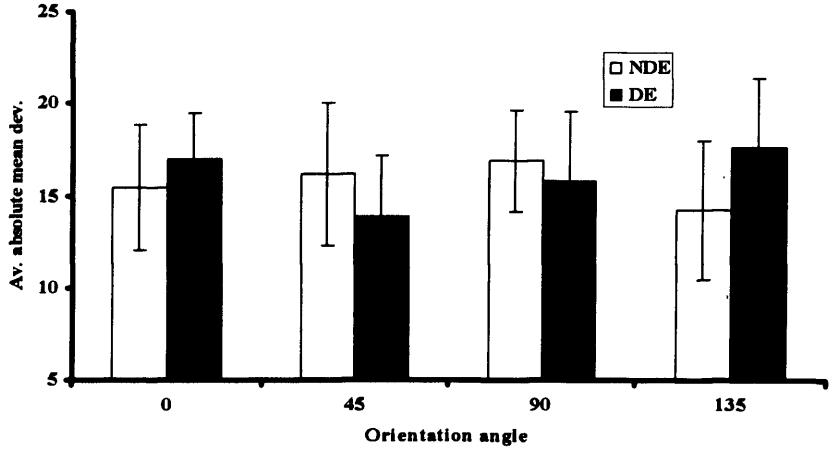
B **MD with ND filter**



C **MD with right eye patch**



B1 **MD with ND filter**



C1 **MD with right eye patch**

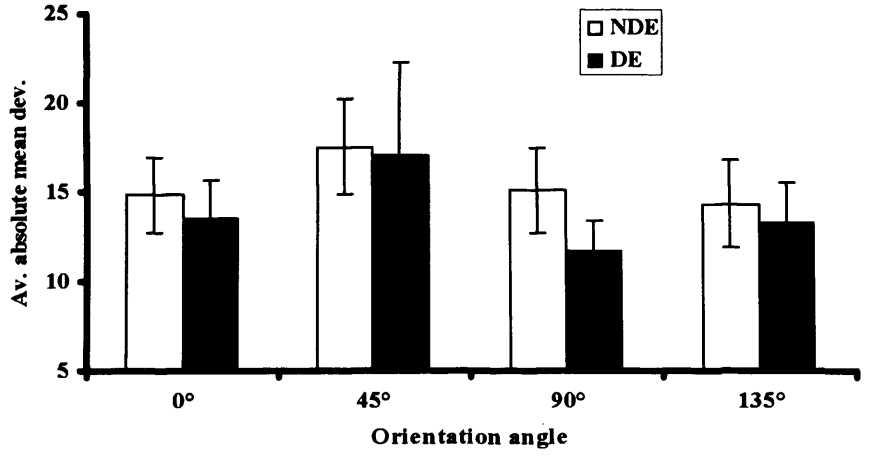


Figure 3.11: *Representative orientation maps obtained from various MD regimens.* **A), B) and C)** Orientation maps show single-condition, cocktail-blank divided iso-orientation maps of DE and NDE obtained from MD with frosted lens, ND filter and eye patch respectively (scale bar 1mm). The number on top of the maps indicates the angle of orientation. The right most column shows polar maps of DE and NDE in which orientation-selective domains are colour-coded (see colour key) and response strength is indicated by intensity. Panel **(B1)** and **(C1)** show orientation selective response of DE and NDE obtained from subjects that underwent MD with eye patch and ND filter respectively; both MD conditions did not reveal significant differences between the DE and the NDE.

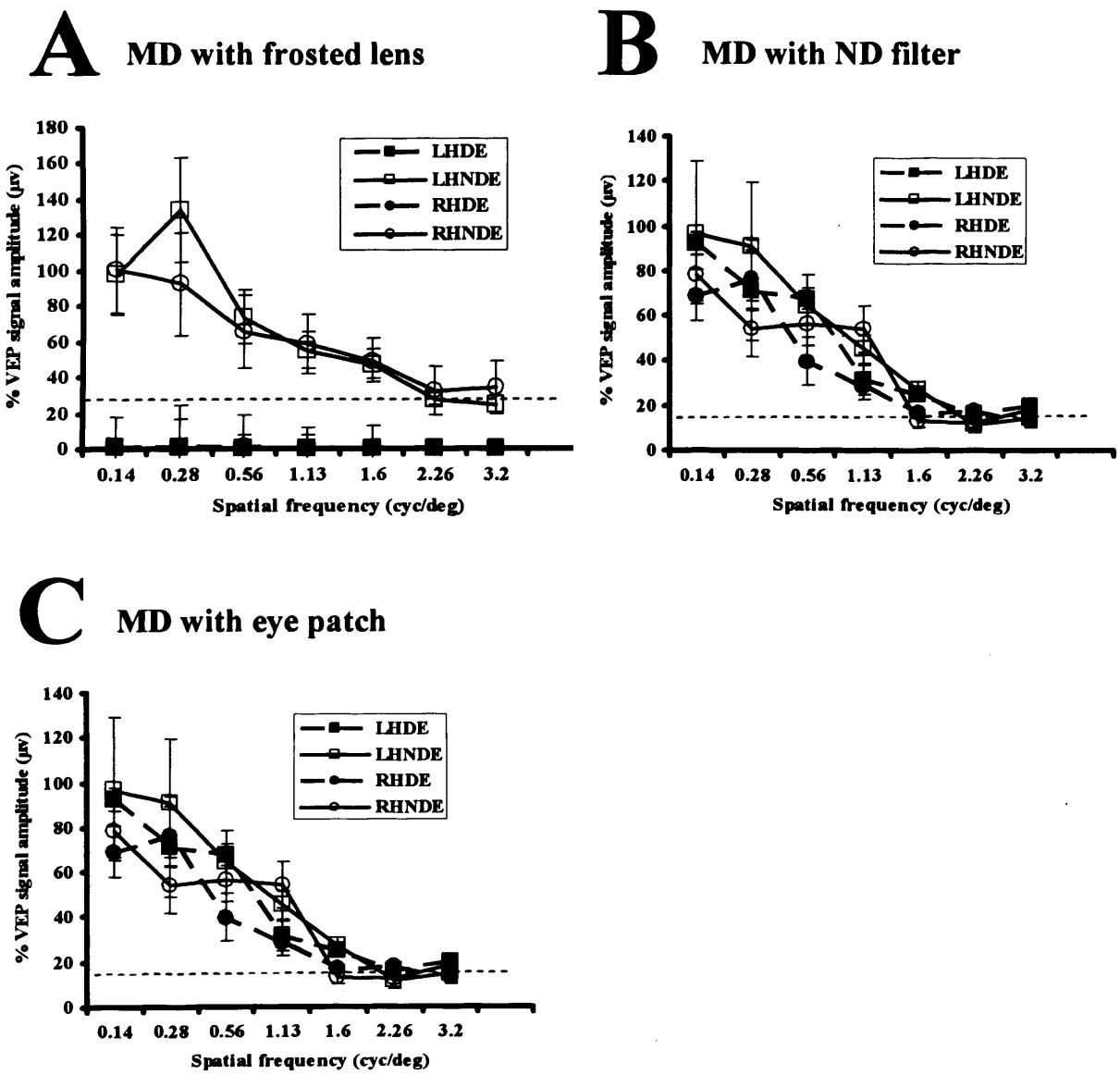


Figure 3.12: *VEP response amplitude obtained from MD of various regimens.* Panels (A-C) show VEP signal amplitude plotted against spatial frequency of the stimulus of subjects that underwent various MD conditions; MD with frosted lens elcited vitually no responses through stimulation of the deprived eye (DE) while MD with ND filter and eye patch did not show interocular differences through stimulation of the DE and the non-deprived (NDE) eye in both the hemispheres (right hemisphere: RH and left hemisphere: LH).The dotted line indicates baseline levels.All data represent mean ± SEM.

3.3.5 Dark-rearing followed by MD

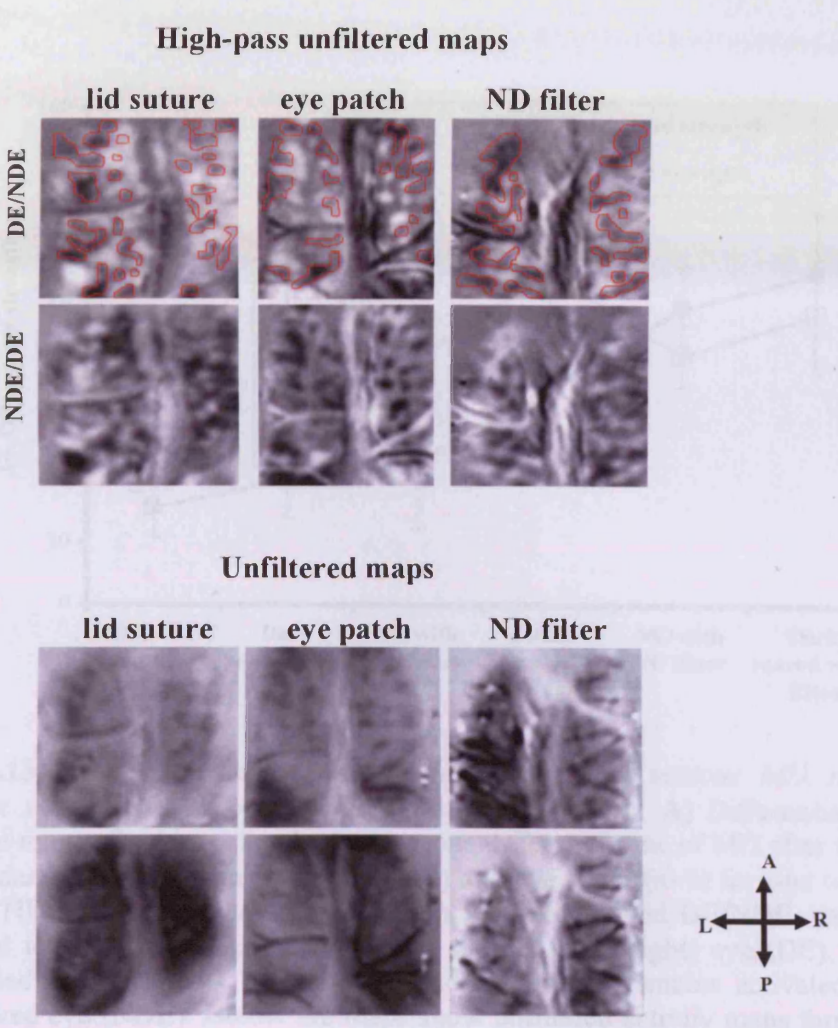
In order to assess if deprivation induced in dark-reared subjects has similar effects compared to subjects raised in a normal 12 hour light/dark cycle, subjects were raised in complete darkness for four weeks followed by a week of MD with lid suture, eye patch or ND filter. Qualitatively, it is apparent that animals who experienced MD with lid suture and MD with eye patch exhibited similarly reduced responses to deprived eye stimulation while MD with ND filter resulted in much larger areas of cortex responding to the DE than in the former two MD regimens (Figure 3.13A). In order to make sure that the deprivation effect, if any, is not obscured by high-pass filtering, only unfiltered images were used for quantitative analysis to calculate OD response strength. Subjects that were raised in complete darkness followed by a week of MD with lid suture showed significantly reduced response through the DE (40.31 ± 12.78 SEM) compared to NDE responses (110.44 ± 18.52 SEM) (Kruskal-Wallis test between DE and NDE, $p=0.04$) (Figure 3.13B). This is consistent with the data obtained for MD induced in normal subjects raised in 12 hour light/cycle.

Similar to normal subjects, animals that were raised in completed darkness followed by a week of MD with eye patch, the absolute strength of responses through the DE was 70.11 (± 5.1 SEM) while that of the NDE was 106.85 (± 14.4 SEM), however, the difference was not significant (t-test $p=0.23$). On the other hand, after MD with ND filter, the absolute response strength through the DE and the NDE was almost the same (DE =106.96 and the NDE 93.43; Figure 3.13B) and this is consistent with the results obtained from subjects raised in 12 hour light/dark cycle followed by MD with ND filter. Therefore, it appears that a change in luminance levels in one eye even after raising subjects in complete darkness did not affect the functional architecture of the cortex.

In addition to OD maps, qualitative analysis of orientation maps obtained from subjects raised in complete darkness followed by MD with lid suture revealed distinct orientation selective patches for the NDE, responding equally to all orientations, while maps obtained from the DE appeared flat with no visible patches (Figure

3.14A). Similarly, the polar map for DE stimulation appeared flat with no orientation selective response whereas the one responding through the NDE demonstrated high responsiveness with iso-orientation domains and pinwheel centres visible (Figure 3.14A). On the other hand, orientation maps obtained from subjects raised in complete darkness followed by MD with eye patch revealed orientation selective patches through stimulation of both DE and the NDE, however the former maps appeared weaker than the latter (Figure 3.14B). Quantitative analysis measured as absolute mean deviation, however, did not show significant difference in orientation selective response between the DE and the NDE (t-test, $p=0.097$) (Figure 3.14B1). Similarly, the polar maps obtained from the DE and the NDE revealed clear iso-orientation domains and pinwheel centres, however, the former appear weaker than the latter as judged by the brightness of the maps (Figure 3.14B). The orientation maps from these two MD conditions are consistent with the ones obtained from subjects raised in a 12 hour light/dark cycle followed by MD (see Figure 3.5A and 3.12A). In contrast, orientation maps obtained from subjects that had undergone MD with ND filter showed equally strong orientation selective patches through stimulation of both DE and NDE. Similarly, polar maps dominated by the DE and the NDE revealed clear iso-orientation domains and pinwheel centres (Figure 3.14C).

In order to assess if MD after dark-rearing had any effect on visual acuity, I recorded VEPs from the surface of V1. I plotted VEP response amplitude against spatial frequency for DE and NDE stimulation. Dark-reared subjects with MD by lid suture exhibited virtually no response when stimulated through the DE while a larger response was observed when stimulated through the NDE (Figure 3.15A). On the other hand, dark-reared subjects that had experienced MD by eye patch did not show significant change in the VEP amplitude represented by the DE and the NDE (Figure 3.15B). In accordance with OI data, MD with ND filter did not result in a difference in the VEP amplitude for DE and NDE stimulation (Figure 3.15C). In addition, visual acuity through the DE and the NDE (in both MD with eye patch and ND filter) was indistinguishable from control subjects (2.26cyc/deg).


A
High-pass unfiltered maps
lid suture
eye patch
ND filter
DE/NDE
NDE/DE
Unfiltered maps
lid suture
eye patch
ND filter
A
L
R
P

B

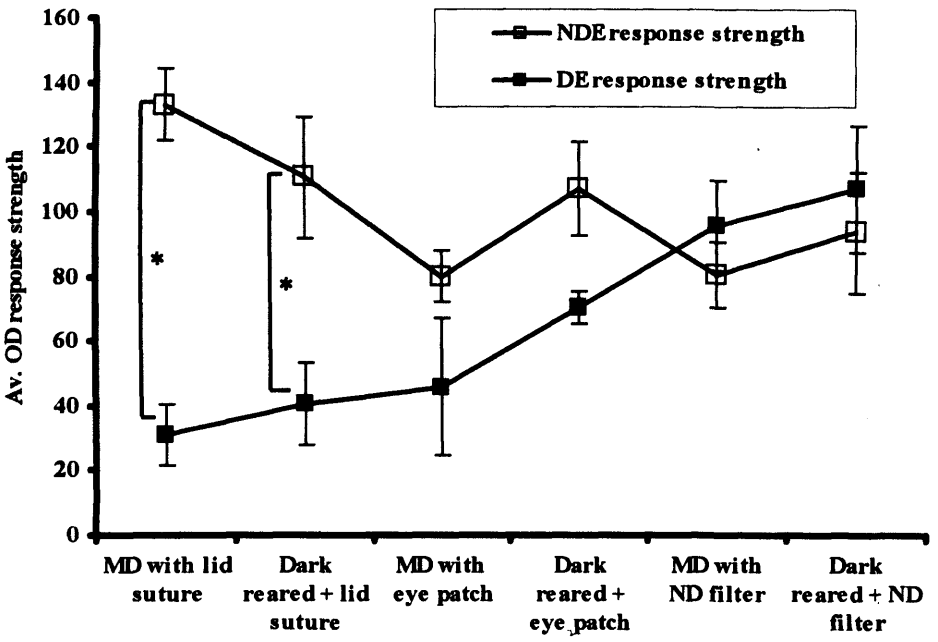
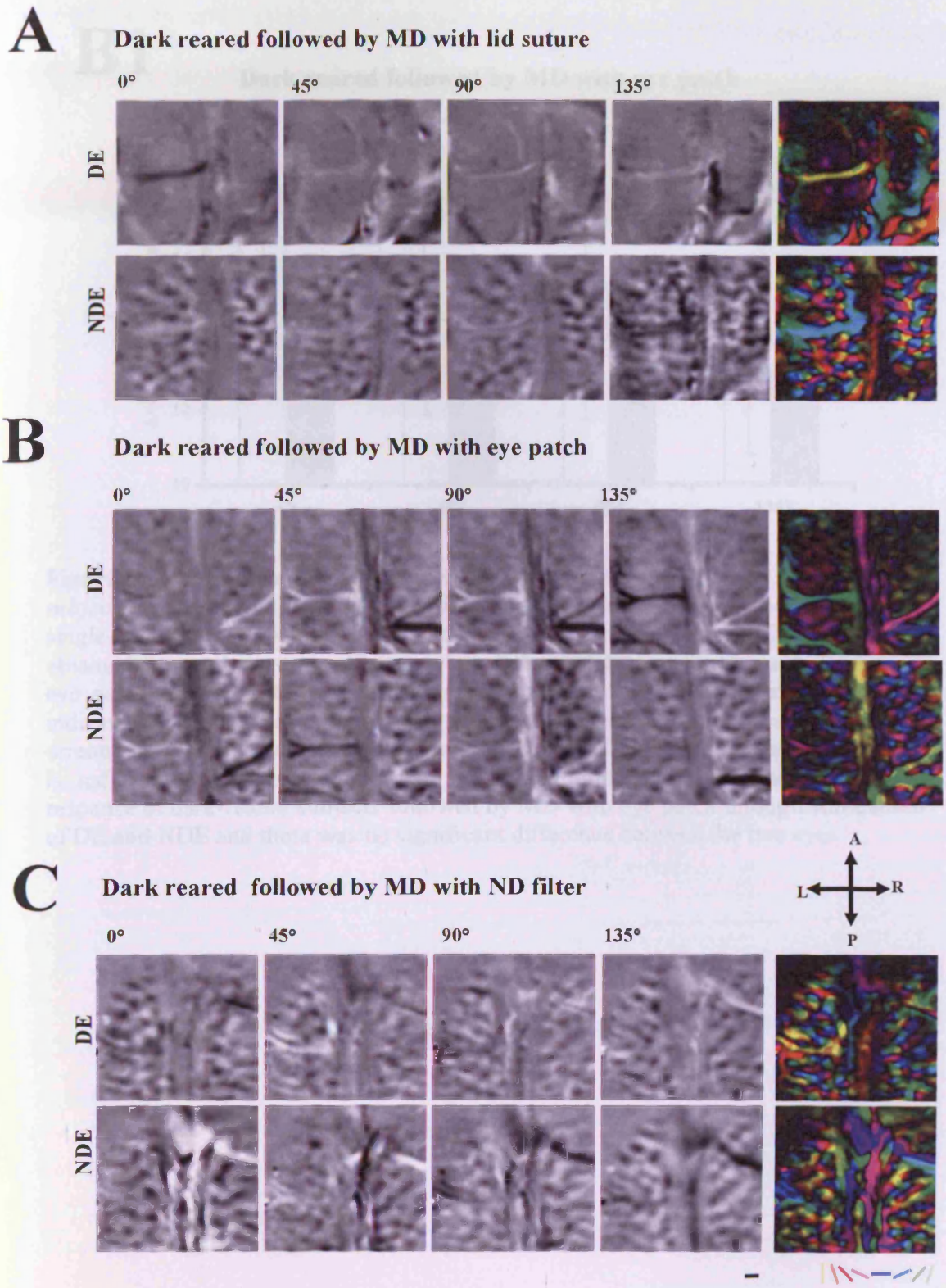


Figure 3.13: *Representative ocular dominance maps of various MD regimens induced in subjects raised in complete darkness from birth.* **A)** Differential ocular dominance maps of subjects that had undergone different forms of MD after raised in complete darkness (MD with lid suture (n=2) and eye patch (n=3) for one week and MD with ND filter (n=2) for two weeks). In the row labelled DE/NDE, dark areas correspond to cortical domains activated by the deprived (right) eye (DE), and the row labelled NDE/DE dark areas correspond to cortical domains activated by the non-deprived eye (NDE). Below the maps show unfiltered activity maps through the DE and NDE divided by the blank response (Scale bar 1mm). **B)** Average strength of responses was calculated for DE and NDE of different MD conditions induced in subjects raised in normal 12 hour light/dark cycle and compared with MD induced in subjects raised in complete darkness. Only MD with lid suture showed significant difference between response strength of the DE and the NDE (* denotes $p<0.05$).


A
Dark reared followed by MD with lid suture
0°
45°
90°
135°
DE
NDE
B
Dark reared followed by MD with eye patch
0°
45°
90°
135°
DE
NDE
C
Dark reared followed by MD with ND filter
A
L
R
P
0°
45°
90°
135°
DE
NDE

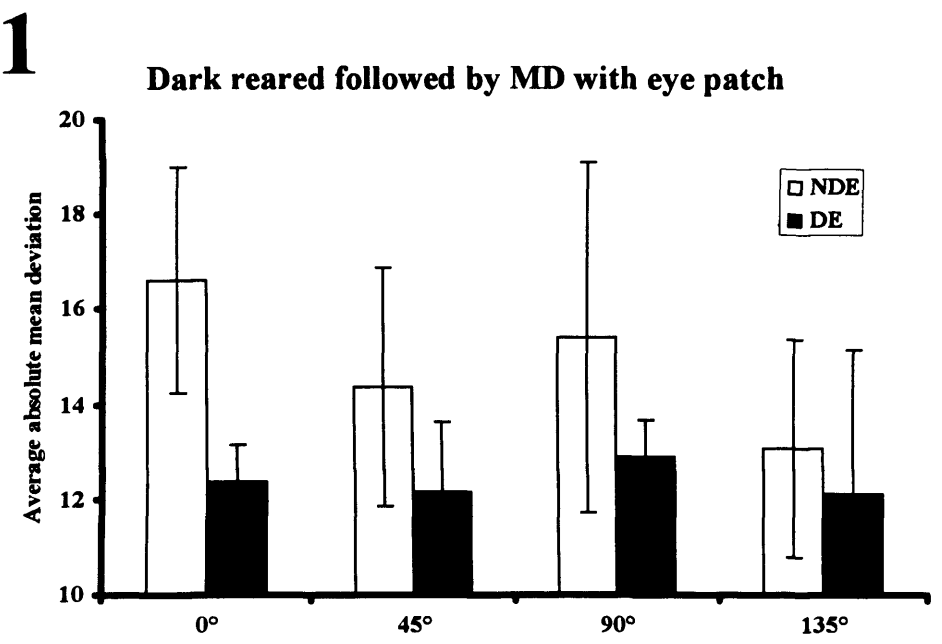
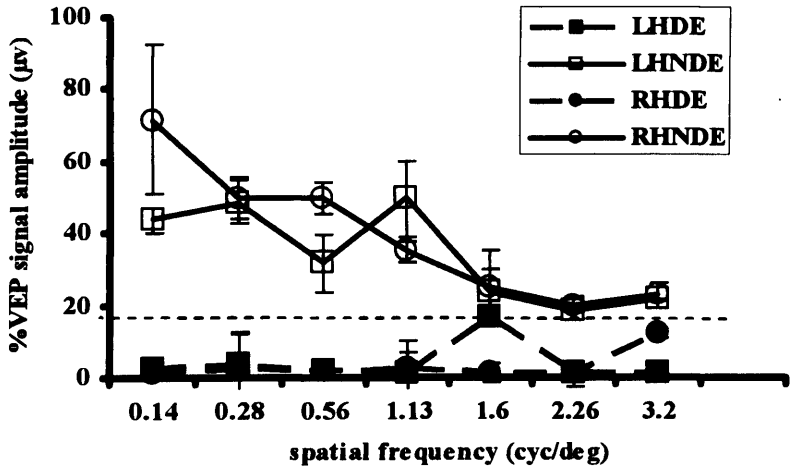
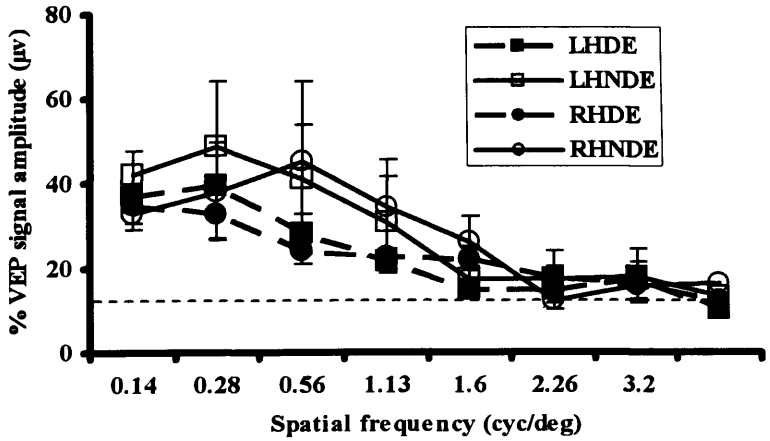


Figure 3.14: *Representative orientation maps of various MD regimens obtained from subjects raised in complete darkness from birth.* **A,B and C)** Orientation maps show single-condition, cocktail-blank divided iso-orientation maps of DE and NDE obtained from subjects raised in complete darkness followed by MD with lid suture, eye patch and ND filter respectively (scale bar 1mm). The number above the map indicates the angle of orientation. The right most column shows polar maps in which orientation-selective domains are colour-coded (see colour key) and response strength is indicated by intensity. Panel **B1** shows quantification of orientation selective response of dark-reared subjects followed by MD with eye patch through stimulation of DE and NDE and there was no significant difference between the two eyes.

A **Dark rearing followed by MD with lid suture**



B **Dark rearing followed by MD with eye patch**



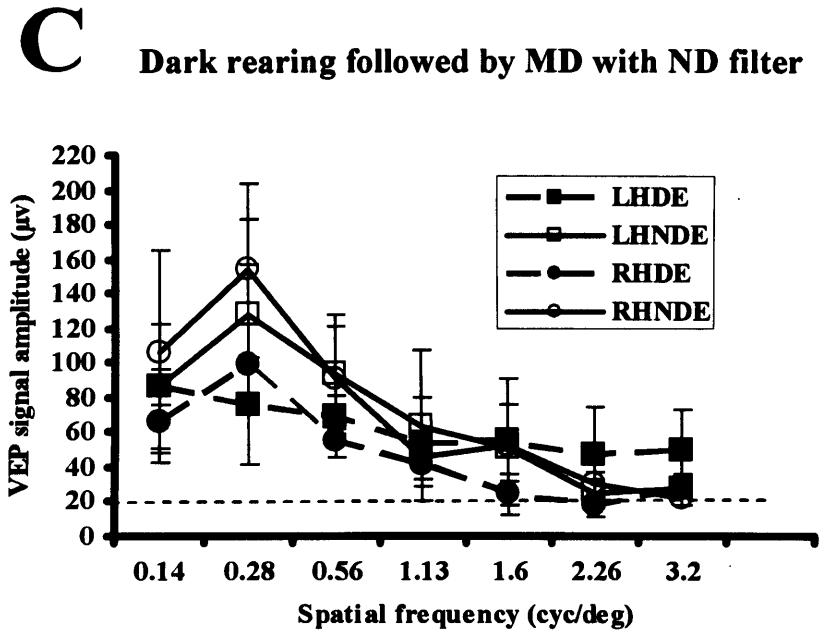


Figure 3.15: *VEP response amplitude of various MD regimens preceded by dark-rearing.* Panels **(A-C)** show VEP signal amplitude plotted against spatial frequency of the stimulus of subjects that underwent various MD conditions after being raised in complete darkness from birth. MD with lid suture **(A)** elicited virtually no response through stimulation of the deprived eye (DE) while MD with eye patch **(B)** and ND filter **(C)** did not show interocular differences through stimulation of the deprived and the non-deprived eye (NDE) in both the hemispheres (right hemisphere: RH and left hemisphere: LH). The dotted line indicates the baseline levels. All data represent ± SEM.

3.4 Discussion

3.4.1 Summary of OIS and VEP results

One of the key findings of my study is that my imaging results support the notion suggested by previous studies using single-cell recording (Imbert and Buisseret, 1975; Buisseret and Imbert, 1976) that sensory experience is not required for the initial establishment of ocular dominance columns and orientation selectivity but is required for maintenance of these cortical properties. Even though the OD and orientation maps appeared normal in dark-reared cats at 5 weeks, there were slight variations compared to control subjects of the same age. For instance, qualitative and quantitative analysis of the orientation maps revealed that there was a bias towards to cardinal orientations in dark-reared subjects whereas in control subjects, this bias was not evident. On the other hand, dark-reared subjects at 12 weeks of age failed to show any orientation-selective patches. In addition, visual acuity from control subjects showed a developmental increase while dark-reared cats had reduced acuity and no responses could be elicited at any spatial frequency in dark-reared cats at 12 weeks of age.

Secondly, imaging results obtained from animals with brief sensory experience (DR-LR) and brief sensory deprivation (LR-DR) support the notion that during the peak of the critical period the responsiveness of neurons in the visual cortex reflects the recent history of visual experience and that the cortical responses are bidirectionally modifiable (Buisseret et al, 1978; Buisseret et al, 1982; Mower et al, 1983; Philpot et al, 2001). Sensory experience after dark-rearing resulted in OD maps and orientation maps indistinguishable from control subjects whereas a week of darkness, similar to 5 weeks of dark-rearing from birth, resulted in normal OD layout (weaker though as judged by the reduced contrast in those maps) but an orientation bias towards cardinal orientations. In addition, visual acuity was indistinguishable in DR-LR subjects to control subjects while in LR-DR subjects the acuity was similar to dark-reared subjects.

Thirdly, 2 days of monocular deprivation by lid suture resulted in depression of deprived eye strength, the magnitude of which was similar after 7 days of MD. In contrast, potentiation of NDE strength after 7 days of MD was almost double in magnitude compared to 2 days of MD. These imaging results are consistent with previous studies in cats (by observing individual labeled boutons) (Friedlander, 1991) and in mice (by recording visually evoked potentials) (Frenkel and Bear, 2004). In addition, VEP recordings revealed that there was virtually no response through stimulation of the DE after 7 days of MD while 2 days of MD resulted in reduced visual acuity through the deprived eye compared to non-deprived eye.

Monocular deprivation by eye patch resulted in reduced area of cortex dominated by the DE; however, the extent of deprivation was not as strong as MD by lid suture. Similarly, VEPs recorded through the DE had reduced signal amplitude compared to stimulation of the NDE. On the other hand, MD by frosted lens resulted in virtually no response to stimulation of the DE and OD maps revealed that the extent of deprivation was as strong as for MD by lid suture. In contrast, MD with ND filter had no discernible effect as judged by cortical maps and VEP recordings.

3.4.2 Technical considerations and caveats

Because of the sensitive issue of using cats as experimental models, I was limited in the number of animals I could use in each of the experimental conditions. Therefore in some experimental conditions, I could only use 2 animals and this posed a problem for statistics, however, the use of non-parametric test (Kruskal-Wallis test) circumvented this problem.

Optical Imaging using intrinsic signal (OIS) offers several advantages in understanding experience-dependent plasticity in the visual cortex (see CHAPTER II). However, this technique obtains intrinsic signal from layers II/III only and therefore in order to understand plasticity in different layers, one would benefit from improved ability to resolve signals from different depths in the visual cortex. Therefore, the results from the present study cannot be generalized for other cortical

layers; they nonetheless provide insight into how response of population of neurons is affected in response to altered sensory experience.

OIS lacks the ability to record from individual neurons and therefore one cannot ascertain how neuronal spiking activity is shaped by experience. The 2 photon-imaging technique has recently been applied successfully in several studies to study experience-dependent plasticity in the visual cortex (Mrsic-Flogel et al, 2007; Hofer et al, 2009) and it permits functional imaging with cellular resolution.

One of the drawbacks of using OIS is that its signal is dependent on the choice of anaesthetics used. For instance, in the present study, the use of isoflurane in juvenile animals did not appear to affect the intrinsic signal, however, in the older animals (3 months and 1 year), the signal was compromised despite all the physiological parameters of animals being within normal range. Future studies will require the use of halothane or other anaesthetics to obtain a more reliable signal.

The use of optical imaging using intrinsic signals in biological preparation suffers from a small-to-noise ratio and therefore several precautions were taken to extract meaningful signal during image analysis. First of all, when defining the region of interest (ROI), the IDL software shifted the ROI by ±10 pixels in x and y coordinates and calculated mean results across all shift conditions to minimise subjectivity in defining of the ROI. Secondly, in order to ensure that responses were not obscured by high pass filtering, both filtered and unfiltered maps were used for analysis. Thirdly, a common approach to image analysis is differential imaging, where the two experimental conditions are contrasted. For instance, for ocular dominance analysis, images obtained from one eye were divided by images obtained from the other eye. Similarly, to map the cortical regions activated by a grating of a particular orientation, one captured the image while the animal was viewing that orientation and divided this image by the sum of images obtained when the animal was stimulated with gratings of all four orientations. In these cases it is straightforward to choose two appropriate conditions for image analysis, however, in certain cases, for example,

when studying the representation of colour in the cortex, any choice of appropriate condition strongly depends on the particular theory of colour perception one is investigating at that moment (Frostig, 2002). In order to circumvent this problem, one would like to analyse the response to the experimental condition against a 'blank' control and such images are referred to as single condition images. For instance, in the present study, blank images were used for calculating ocular dominance strength so that overall response of each eye could be ascertained.

Visually evoked potentials (VEPs) provide a reliable electrophysiological estimate of visual acuity. During VEP recording session, the electrode was placed near area centralis (Horsley-Clarke P3.0 and A2.0). However, in cases where the cortex was exposed for a long time, it was very difficult to obtain a reliable VEP signal and this is possibly due to decline in the state of the cortex with time. Therefore, in such cases the electrode was moved away from the representation of the area centralis until a good signal could be detected. This subjectiveness of electrode placement could not be avoided, however, in stimulation protocol, measures were taken to minimize subjectiveness, for instance, each trial consisted of randomized presentation and a blank response was used as baseline for calculating the response cut-off point.

3.4.3 The role of sensory experience in maturation of ocular dominance and orientation maps

Several studies have been carried out to investigate how dark-rearing affects cortical maturation using single-cell recording and the extent to which visual experience is necessary to promote development and maintain the integrity of visual function. The use of optical imaging using intrinsic signals (OIS) has the advantage of eliminating sampling bias associated with single-cell recording and therefore makes this technique well suited to examine plasticity and functional architecture of normal visual cortex and in response to altered sensory experience. Earlier reports using OIS (Crair et al, 1998; White et al, 2001; Coppola and White, 2004) have shown that the basic structure of cortical maps is innate, but experience is essential for specific

features of these maps, as well as for maintaining the responses and selectivity of cortical neurons. The results obtained from the present study are in agreement with previous findings as 5 weeks of dark-rearing resulted in normal ocular dominance (OD) and orientation maps layout while 12 weeks of dark-rearing abolished those features of the maps.

The maintenance of orientation maps after 5 weeks of dark-rearing is in contrast to what is observed after binocular deprivation (Crair et al, 1998; White et al, 2001). During development the naïve maps are dominated by the contralateral eye and experience is necessary for responses through the other eye to become strong; the responses become strong and nearly equal between two eyes by the fourth postnatal week in cat visual cortex (Crair et al, 1998). Binocular deprivation of patterned visual experience by bilateral lid suture from before the time of eye opening in cats has little effect during the first three weeks of life (Crair et al, 1998). With continued binocular deprivation, ipsilateral eye responses never become strong or selective and orientation maps from the two eyes begin to deteriorate and the two maps become less similar (Crair et al, 1998). A study by White et al, (2001) showed that ferrets that had undergone binocular lid suture, two weeks prior to eye opening, did not reveal any orientation selectivity in orientation maps using OIS. The electrophysiological assessments of orientation-selectivity were consistent with the results obtained by OIS; dark-reared ferrets showed an intermediate level of tuning that was sharper than the lid-sutured groups but not as selective as seen in normal ferrets (White et al, 2001). Therefore deprivation of pattern information has more devastating effect on orientation maps than complete absence of sensory experience. Anatomically both binocular deprivation and dark-rearing resulted in less extensive clustering of horizontal connections than normal (Callaway and Katz, 1991; White et al, 2001). Such restricted horizontal connections underlie the effects of dark-rearing while the more devastating effects of lid suture can be explained by alteration in the functional organization of other cortical circuits (White et al, 2001).

Optical Imaging of the visual cortex in both cats (Bonhoeffer and Grinvald, 1993;

Sengpiel et al, 1999; Muller et al, 2000; Wang et al, 2003) and in immature and in young adult ferrets (Chapman and Bonhoeffer, 1998; Coppola 1998; Coppola and White, 2004) have revealed bias towards the representation of the cardinal axes. The bias is weak and not quite significant in cats whereas in ferrets a small but significant bias exists towards the cardinal orientations. Such cardinal bias has also been reported recently in human primary visual cortex using fMRI (Furmanski and Engel, 2000), suggesting orientation anisotropy could be a general feature. This cardinal bias is usually a transient feature in the developing ferret visual cortex observed near the time of eye opening (Coppola and White, 2004) and this finding from optical imaging are in accordance with an electrophysiological study in cats, which showed a significant cardinal bias in young kittens but after 4 weeks of age, equally selective responses to all orientations were observed (Fregnac and Imbert, 1978). In the present study, the optical imaging maps were acquired at 5 weeks of age, and therefore by that age it was not surprising that responses were similar to cardinal and oblique orientations in normally reared animals. Dark-reared ferrets did not show orientation anisotropy until postnatal weeks 8 and 9, but anisotropy then persisted throughout the period in which robust orientation-selective responses were observed (Coppola and White, 2004). Similarly, electrophysiological studies of dark-reared kittens also showed higher percentage of responsive units tuned to cardinal than oblique orientations (Fregnac and Imbert, 1978). Consistent with these studies, the present study using OIS also showed a cardinal bias at 5 weeks of age in dark-reared cats. This along with other studies suggests that the cardinal bias is an endogenous property of the visual cortical network and that normal visual experience during cortical maturation serves to equalize the representation of orientation preference (Coppola and White, 2004). Orientation selectivity gradually declines with continued deprivation (White et al, 2001 and Coppola and White, 2004) and in accordance with previous studies, the orientation maps obtained from dark-reared cats at 12 weeks of age did not show any selectivity. This suggests that sensory experience is not required to establish the map of orientation preference but is needed for its maintenance and fully mature levels of selectivity are achieved only with the benefit of normal experience.

3.4 Discussion

In line with results obtained from subjects dark-reared from birth, subjects that were raised in a normal environment followed by a week of complete darkness (LR-DR), showed normal OD map layout (equal proportion of cortex dominated by each eye), albeit weaker than control subjects as judged by the brightness of the map. In contrast, subjects raised in complete darkness followed by a week of visual exposure (DR-LR) showed OD maps as strong as the ones obtained from control subjects (as judged by the brightness of the map) suggesting that the cortex must have fully recovered after a visual exposure. Similar to OD maps, orientation maps obtained from DR-LR (one week of visual experience) demonstrated strong responses to all orientations with normal layout of iso-orientation domains and pinwheel centres as seen in control subjects of the same age. This is consistent with previous electrophysiological recordings which have shown that 6 hours (Mower et al, 1983) or 1 to 2 days of visual experience (Mower and Christen, 1985) is sufficient to trigger the refinement of orientation-selective response. On the other hand, orientation maps obtained from LR-DR subjects (one week of darkness), as with dark-reared cats at 5 weeks of age, showed a bias to cardinal orientations. This once again supports the notion that cardinal bias is an innate property of the visual cortical network and that continued visual experience is necessary to equalise the representation of orientations (Fregnac and Imbert, 1978; White et al, 2001; Coppola and White, 2004).

The role of visual experience in triggering maturation and refinement of visual cortical properties was first revealed by electrophysiological studies. Imbert and Buisseret (1975) demonstrated that a cat reared in complete darkness until 6 weeks of age has few orientation selective cells but a 6 hour period of vision leads to a rapid increase in the proportion of orientation selective cells approaching normal levels. Similarly Mower and colleagues (1983) demonstrated that cats that were dark-reared for 4-5 months (i.e. beyond the end of the critical period) followed by prolonged MD showed a measurable shift towards the non-deprived eye. However, cats that were exposed to 6-12 hours of visual experience during the period of dark-rearing failed to show a measurable ocular dominance shift after MD. This suggests that a few hours

of sensory experience appears to be sufficient to eliminate plasticity in dark-reared cats and allows completion of the critical period in a normal time course with or without further visual experience (Mower et al, 1983). The main question however, is how does light trigger maturation and refinement of visual cortical properties? Recent studies have attempted to determine the molecular substrates required for maturation of visual cortical responses after dark-rearing. For instance, Quinlan et al, (1999) have shown using immunoblotting that levels of NR2A increase within 2 hours when dark-reared rats are exposed to light and gradually decrease over the course of 3-4 days when the animals are deprived of light. Similarly, a study by Philpot et al (2001) has shown that visual experience decreases the proportion of NR2B receptors and shortens the duration of NMDA receptor-mediated synaptic currents while visual deprivation has an opposite effect. Therefore visual experience and deprivation bidirectionally modify NMDA receptor subunit composition. The rapidity with which the experience-induced changes take place appears to be surprising; one would have thought that protein synthesis or insertion of NMDA receptor subunits may take more than 2 hours. However, it is very unlikely that cortical properties such as ocular dominance and orientation selective response become adult-like after 2 hours of visual experience. It is possible that one of the first effects of light exposure is alteration of NMDA receptor kinetics which then possibly drives the refinement of cortical properties.

A study by Krahe et al, (2005) measured whether cortical protein synthesis plays a role in loss and recovery of deprived eye after short term MD (6 days) by using protein inhibitors such as cycloheximide and rapamycin and assessed cortical properties such as ocular dominance and orientation selectivity in ferrets using optical imaging of intrinsic signals. Their study revealed that recovery of deprived eye responses after monocular deprivation was not prevented in ferrets that were cortically infused with protein synthesis inhibitors, however, TTX infusion prevented cortical recovery suggesting a critical role of activity (Krahe et al, 2005). Therefore it appears that after short term MD, the recovery process does not involve any major anatomical rearrangements which would have required protein synthesis but instead it

is mostly due to changes in the function of a latent set of geniculocortical and cortical intrinsic connections (Krahe et al, 2005). Morphologically, dark-rearing results in a significantly lower spine density with large head diameter in rats raised in complete darkness compared to normal rats (Wallace and Bear, 2004). The average spine head diameter is reduced to normal levels after 10 days of light exposure but spine density is not (Wallace and Bear, 2004). It appears that as with short term MD, a brief period of dark-rearing does not involve major remodelling of anatomical organisation and therefore it is possible that recovery after dark-rearing does not require protein synthesis. However, this will need to be validated using protein synthesis inhibitors as done previously (Krahe et al, 2005).

Visual acuity measured from visually evoked potentials (VEPs) in the present study showed a progressive increase in acuity from 2.26cyc/deg (5 weeks) to 3.2cyc/deg (12 weeks). The electrophysiological estimate of visual acuity obtained from control subjects at 5 weeks of age is in contrast to a study by Freeman and Marg (1975) which showed through electrophysiological recordings that the acuity was 1 cyc/deg. This discrepancy in visual acuity could be due to differences in measuring visual acuity. For instance, in the latter study, visual acuity was taken as the highest spatial frequency for which electrical response was visible (Freeman and Marg, 1975) whereas in the results obtained herein, the blank response was the baseline level from which the cut-off point was extrapolated by visual inspection of the stimulus response curve. In addition, the acuity calculated in control subjects of 5 weeks of age was based on only n=1 and therefore more animals are required to draw any firm conclusion.

In dark-reared subjects at 5 weeks of age, there is a delay in the developmental increase in visual acuity and the acuity was lower (1.16cyc/deg) than in normal subjects of the same age further supporting the concept that sensory experience is necessary for the maturation of cortical properties. This is consistent with previous studies (Timney et al, 1978; Fagiolini et al, 1994) which also demonstrated that visual acuity is immature in dark-reared animals. In addition, one week of darkness (LR-

DR) also resulted in reduced acuity which was indistinguishable from subjects raised in complete darkness while one week of visual experience (DR-LR) resulted in acuity comparable to control subjects. Furthermore, visual acuity obtained from electrophysiological estimates was somewhat lower than in behavioral studies using the jumping stand (Mitchell et al 2003) and this is possibly due to the fact that the VEP responses were not sampled from the most sensitive neurons that mediate behaviourally determined acuities (Schwarzkopf et al, 2007). It is also possible that differences in electrophysiological and behaviourally estimates may be attributed to the state of the animal, since Sebel and colleagues (1986) have shown that with increasing concentration of isoflurane, there was a reduction in VEP amplitude and increase in VEP latency.

3.4.4 Monocular deprivation

Monocular deprivation by lid suture prevents form vision and studies have reported that it reduces the levels of retinal illumination by 4-5 log units (Wiesel and Hubel, 1963a), others have reported 2 log-units (Spear et al, 1978) and others 3-4 log units (Loop and Sherman, 1977). The differences between studies could be due to variability among cats in terms of lid transmittance and/or subtle differences in techniques employed (Loop and Sherman, 1977). Monocular deprivation, however, does permit entry of diffuse light and a study by Spear et al, (1978) demonstrated that 30% of the cells respond to visual stimuli presented through the sutured lids. The abnormalities that result from deprivation therefore occur in two possible ways. First of all, since the animals cannot fixate through sutured eye lids, the visual stimulus may arrive asynchronously to the two eyes (Hubel and Wiesel, 1965; Spear et al, 1978). The second possibility is that light transmission through the open and closed eye lids is not the same and a reduction in retinal illumination produces an increase in the latency and a decrease in the rate of retinal ganglion cell discharge (Enroth-Cugell and Lennie, 1975). Therefore, interocular intensity differences result in dissimilar discharge in the timing and rate of activity evoked by visual stimulus (Spear et al, 1978).

3.4 Discussion

In the present study, deprived eye responses were weaker within 2 days of monocular deprivation and robust potentiation of open-eye responses emerged after 7 days of deprivation. Therefore, the present study has extended previous findings that MD results in two temporally distinct processes: depression of deprived eye responses followed by a delayed potentiation of non-deprived eye responses (Friedlander et al, 1991; Frenkel and Bear, 2004; Mrsic-Flogel et al, 2007). However, this is the first study to employ optical imaging of intrinsic signals to show two different processes involved during MD in cat visual cortex. In addition to OD maps, the present study has also demonstrated that orientation selective response was virtually absent after 7 days of MD while weak orientation-selective response was present after 2 days of MD through stimulation of the deprived eye, suggesting that the full deprivation effect is only achieved after 7 days of MD. It is indeed after 6-7 days of MD, that the anatomical changes that accompany physiological changes become evident (Antonini and Styker, 1993) and therefore it is not surprising to observe such devastating effects at the same time. Consistent with the results obtained from OIS, no responses could be elicited through stimulation of the deprived eye at any spatial frequency after 7 days of MD while MD by lid suture for 2 days resulted in reduced VEP signal amplitude through stimulation of the deprived eye with reduced acuity compared to non-deprived eye.

Longer period of MD (5-7 days) do not only strengthen open-eye responses but also increase deprived eye responses of neurons devoid of open-eye input as shown using two-photon imaging (Mrsic-Flogel, 2007). In the present study, the use of optical imaging which records from populations of neurons makes it impossible to differentiate between monocular and binocular cells and since a large part of the visual cortex is binocular, it will be difficult if not impossible to image the relatively small and inaccessible area of the monocular zone. It is possible only with techniques like two-photon imaging which offers the advantage of imaging at the single-cell resolution. In addition to results obtained by Mrsic-Flogel (2007), a study by Shatz and Stryker (1978) showed using electrophysiological recordings in cats that following deprivation (for 8-11 months); the deprived eye dominated a substantial

proportion of all cortical cells (22%) in layer IV while in other layers only 7% of the cells were dominated by the deprived eye. Since their study did not establish responses of those cells before deprivation, it is difficult to explain in light of the recent study by Mrsic-Flogel (2007). Nevertheless, Shatz and Stryker suggested those cells are most likely monocular and may be resistant to the effects of deprivation, while those cells driven equally by the two eyes are taken over through competitive processes (Guillery and Stelzner, 1970; Hubel et al, 1977; Shatz and Stryker, 1978). It should however, be noted that in cat visual cortex, there are more binocular cells than monocular cells (Hubel and Wiesel, 1962) whereas in mouse visual cortex, there is a strong contralateral bias with few binocular cells responding equally to stimuli presented in either eye. Another difference between Mrsic-Flogel (2007) and Shatz and Stryker (1978) is that the latter sampled cells from layer IV (which receives direct geniculate input) and other cortical layers while the former sampled cells from layers II/III only (which receives indirect geniculate input via layer IV). Nonetheless, both findings cannot be explained by Hebbian mechanisms but are better explained using homeostatic mechanisms which act to maintain overall visual drive by increasing neuronal responsiveness and maintain firing rates within a certain range during MD (Desai et al, 2002; Turrigiano and Nelson, 2004; Mrsic-Flogel, 2007).

Other MD conditions

As the present study and other studies have shown that deprivation of form or pattern information in one eye results in marked take-over by the other eye, therefore, one would have expected that MD with eye patch would have caused similar extent of OD shift towards the non-deprived eye as in MD with lid suture. However, MD with eye patch for a week resulted in slight but not significant take-over by the other eye in both normal subjects and in subjects raised in complete darkness. This is further corroborated by VEPs which showed no differences in amplitude through stimulation of the deprived eye and the non-deprived eye. These discrepancies could be due to several reasons; first of all, it is possible that any abnormal physiological conditions during image acquisition or factors related to the animal during rearing such as the amount of time they spent being awake would have an effect on the overall signal and

possibly could have confounded the results. Secondly, a previous study from the same laboratory (Schwarzkopf et al, 2007) employed the same method of eye patching and found marked take-over by the non-deprived eye; however, the duration of deprivation was for 3 weeks. Therefore, it is possible that in order to have observed significant take-over by the non-deprived eye, one should have had deprived animals for more than one week, considering that monocular experience only amounted to 4 h per day. But in order to minimise the number of animals used in the study, we did not use more animals to test if longer exposure times causes a greater effect. The other possibility is that a period of complete darkness (20 hours) after daily monocular exposure (4 hours) for 7 days induced in normal subjects and in dark-reared subjects is sufficient to diminish some consequences of deprivation. In fact a study by Freeman and Olson (1979), demonstrated that a period of darkness (48 hours) after a day of MD, did not produce consolidation effect but instead diminished any consequences of MD by increasing the number of cells driven by both the eyes (Freeman and Olson, 1979).

Monocular deprivation by frosted lens resulted in marked take-over of V1 by the non-deprived eye but only after 2 weeks and not 1 week of deprivation. The extent of deprivation after 2 weeks was similar in magnitude to MD by lid suture for a week. Furthermore, no responses could be elicited through stimulation of the deprived eye at any spatial frequency as revealed by VEP recordings following 2 weeks of MD by frosted lens. Previous studies have shown that 24 hours of MD using opaque lens resulted in fewer cells controlled by the deprived eye compared to the non-deprived eye (Freeman and Olson, 1979). Therefore, it appears that continued monocular exposure is necessary to cause substantial OD shift. Similarly, monocular exposure using an opaque lens for 8 hours a day for 6-7 weeks resulted in marked take-over by the non-deprived eye (Christen and Mower, 1987). It appears therefore if animals experience few hours of monocular exposure per day, a significant take-over by the non-deprived eye is achieved only after 2 or more weeks of deprivation. It is possible that in the first week of monocular exposure the effect of deprivation is overridden by the daily periods of darkness (Freeman and Olson, 1979). It has been shown that there

is enhancement of cortical plasticity in response to MD after a 6 hour sleep period in darkness as measured by microelectrode recording and optical imaging (Frank et al, 2001); however, in the present study the sleep patterns of the cats were not ascertained, therefore I cannot comment on whether sleep during the periods of darkness contributed to the overall outcome.

Monocular deprivation by ND filter (optical density 2) for 2 weeks which leaves the pattern information intact but reduces illumination in that eye did not result in take-over of V1 by the non-deprived eye and this extends previous findings by Blakemore (1976) and Rittenhouse et al (2006). The latter study compared the effects of 2 days of MD by lid suture and monocular blur using an overcorrecting contact lens in cat visual cortex. They found comparable OD shifts towards the non-deprived eye suggesting that depression of the deprived eye was not a result of reduced illumination but rather absence of pattern information in both forms of MD (Rittenhouse et al, 2006). Even though the relative dominance of each eye did not change following MD by ND filter, the study by Blakemore (1976) demonstrated that the number of binocular cells had decreased. The analysis of optical imaging maps in that respect however, is limited and therefore I cannot ascertain the degree of binocularity following MD. Any loss of binocularity may be partly due to an increase in response latencies (Barlow and Levick, 1969; Enroth-Cugell and Lennie, 1975) which result in difference in the timing of signals arriving from the two eyes (Barlow and Levick 1969) and partly caused by differences in the strength of the discharges in the two eyes (Blakemore, 1976). Consistent with imaging results, VEP recordings showed visual acuity comparable to normal subjects.

Raising animals in complete darkness followed by monocular deprivation did not affect the consequences of deprivation and the effects were comparable to MD induced in subjects raised in a normal environment as revealed by OIS and VEP recordings. This is consistent with a study by Beaver et al, (2001a) which compared the effects of monocular vision in light reared and dark-reared animals at 6 weeks of age using single-cell recording and found that both groups of animals exhibited

similar OD shift towards the non-deprived eye. In another study, using single-cell recording, a study by Mower and Christen (1985) demonstrated that monocular deprivation by lid suture imposed in cats after 4 months of dark-rearing resulted in selective development of connections from the open-eye with many orientation selective cells encountered compared to cats that were dark-reared for several months (Mower and Christen, 1985). These results suggest that during dark-rearing the visual cortex retains many immature response properties and on exposure to visual experience the responses become selective and refined. This is evident in the present study where orientation maps obtained from the NDE exhibited orientation-selective responses with clear iso-orientation domains and pinwheel centres. Even though there is a clear evidence of take-over by the non-deprived eye after MD in cats raised in complete darkness, there are however reported laminar differences in OD shift in normal and dark-reared cats (Mower and Christen 1985). For instance, in MD in dark-reared cats, the NDE take-over was evident only outside layer IV, while MD in normal cats, the cells driven by the NDE dominated in all layers (Mower and Christen, 1985; Beaver et al, 2001).

3.4.5 Conclusion

Despite great progress in our understanding of how experience modifies cortical circuitry, the underlying physiological mechanism still remains to be understood fully. The present study demonstrates that sensory experience is not required for initial establishment of ocular dominance layout and iso-orientation domains but is required for maintenance of these properties; its absence leads to their eventual breakdown. Pattern or form information is necessary in both eyes to maintain balanced dominance of the left and the right eye while interocular differences in illumination do not affect the ocular dominance and iso-orientation organisation in normally reared and dark-reared cats. The present study raises several important questions that still need to be answered. For instance, how does sensory experience maintain cortical properties once they are established? The present study did not examine intermediate time period between 5 and 12 weeks of age, therefore it is not known over what time period sensory experience becomes necessary in refining

initial established cortical properties. This study has also demonstrated monocular deprivation must be continuous to be most effective as monocular exposure by lid suture for 7 days is sufficient to cause a marked take-over by the non-deprived eye while 4 hours a day of monocular deprivation using a frosted lens only achieves such noticeable effect after 14 days of deprivation.

CHAPTER IV
EXPERIMENTAL CHAPTER

Biochemistry results

4.1 Introduction

Several receptors and downstream signalling pathways are involved in OD plasticity. For example, NMDA, AMPA and metabotropic receptors are activated upon the arrival of afferent activity in the visual cortex. This in turn activates kinases such as such as CaMKII, PKA and MAPK and transcription factors such as CREB and OTX2 which then facilitates gene expression and protein synthesis. In order to balance the levels of excitation, growth factors such as BDNF are activated to facilitate maturation of inhibitory interneurons. Some feedback pathways from intracellular signalling molecules may be also acting directly on AMPA and NMDA or metabotropic receptors at the post-synaptic density to change the efficacy of the synapses (Daw, 2006).

A considerable number of studies have been carried out to determine the sequence of signals that are regulated by age and whose expression is dependent on activity. For instance, expression of NR2B and NR2A subunits of NMDA receptors (Quinlan et al, 1999; Chen et al, 2000; Philpot et al, 2001), GluR4 subunit of AMPA receptors (Akaneya et al, 2007), mGluR5 (Reid and Romano 2001) and $GABA_A$ receptors (Morales et al, 2002; Iwai et al, 2003) is dependent on sensory experience as revealed by raising animals in complete darkness. Such dark-rearing studies are useful in helping us understand how experience not only modifies functional architecture and response properties but also how different glutamate receptor-dependent signalling pathway may modify neuronal properties. Subsequently, signalling molecules downstream of the above mentioned receptor subunits such as αCaMKII (Taha et al, 2002), PKA (Hensch et al, 1998b; Fischer et al, 2004), CREB (Pham et al, 2004; Lalonde and Chaudhuri, 2007), PSD-95 (Cotrufo et al, 2003) ERK (Di Cristo et al, 2001; Oliveira et al, 2008) and PLCβ1 (Kind et al, 1994; Choi et al, 2005) have also been associated with ocular dominance plasticity in the visual cortex.

The cellular mechanism underlying experience-dependent plasticity has been a topic of interest for several researchers. It has been proposed that during monocular deprivation (MD) depression of deprived eye responses occurs via LTD mechanisms. Daw and colleagues were the first to point out that the mechanisms of

LTD vary in different cortical layers (Daw et al, 2004). A subsequent study by Crozier et al, (2007) applied low frequency synaptic stimulation in the mouse visual cortex and revealed that LTD is induced in layers IV and III via activation of NMDA receptors while in layer IV only LTD is mediated via AMPA receptor insertion and MD reduced LTD in both layers III and IV suggesting that MD induces LTD in different layers but by different molecular mechanisms (Crozier et al, 2007). The delayed potentiation of non-deprived eye responses is thought to occur via the mechanisms of LTP and according to the BCM model, the neurons respond to decreased post synaptic activity by lowering the modification threshold (θm) thus facilitating LTP. The molecular mechanism underlying open eye potentiation has received scant attention relative to that mediating deprived eye depression. A study by Heynen and Bear (2001) has shown that theta burst stimulation of the dLGN reliably induces LTP in layers III and IV in rat visual cortex and this LTP is sufficient to increase the magnitude of visually evoked potentials. This enhancement of responses is dependent on NMDA receptor activation and open eye potentiation is absent in targeted deletion of NR1 in layers II-IV (Sawtell et al, 2003). In addition to NMDA receptors, AMPA receptors have also been demonstrated to play a role in the expression of LTP. It has been shown that the expression of a region of the GluR1-C terminal is sufficient to prevent delivery GluR1 to synapses and block LTP in hippocampal neurons (Shi et al, 2001). A recent study has shown that GluR1 subunit is not required for LTP in pyramidal neurons of somatosensory cortex and therefore it appears that the role of GluR1 in LTP appears to be region specific (Frey et al, 2009). In addition, another study by Wright et al (2008) showed that removal of all whiskers but one results in depression of principal whisker responses in layers II/III. However, in GluR1 knock-out mice, principal whisker responses were indistinguishable from non-deprived and control values (Wright et al, 2008). Although experience-dependent depression was prevented in GluR1 knock-out mice potentiation was not, suggesting that LTP expression is not dependent on GluR1 (Wright et al, 2008). The cellular mechanism underlying ocular-dominance plasticity has well been documented in rodents; however, such mechanism in cats has not received much attention.

Most of the molecular studies have been carried out in genetically modified mice or

using pharmacological approaches and the wealth of knowledge acquired has undoubtedly contributed towards underpinning the molecular mechanisms underlying experience-dependent plasticity. Mice display rapid and robust ocular dominance plasticity in response to monocular deprivation and the kinetics and behavioural consequences of this plasticity is similar to those observed in cats (Gordon and Stryker, 1996; Mrsic-Flogel et al, 2007). More importantly, the rate of synaptogenesis, that is, the mean number of axosynaptic synapses increase steadily between P3-P30 in rats (Miller et al, 1986), between P7-P14 in mice (Li et al, 2009) and between P8-P37 in cat visual cortex (Cragg, 1976). However, the mouse visual system is different from the cat or the primate visual system. First of all, mouse or rat visual cortex lacks any columnar organization for ocular dominance or orientation preference. Secondly the mouse visual cortex has a small binocular visual field and therefore only a small portion of cortex receives inputs from both eyes. Thirdly, the critical period in cats commences at 3 weeks of age, peaks at 4-6 weeks of age and declines at 3-4 months of age while in mice the critical period was thought to be between P19 and P32 (Gordon and Stryker, 1996); however since then several studies have been carried out that showed that adult mouse visual cortex retains the capacity to undergo ocular dominance shift after monocular deprivation (Sawtell et al, 2003; Pham et al, 2004; He et al, 2006; Hofer et al, 2006; Fischer et al, 2007). There are several questions that need readdressing: does a mouse have a well defined critical period for experience-dependent plasticity and does it serve a good experimental model for studying ocular-dominance plasticity? Many experiments that have shed light in uncovering the underlying cellular and molecular mechanisms of ocular-dominance plasticity in mice will require additional experiments to take into account of age. One of the other disadvantages of studying ocular dominance plasticity in mouse visual cortex is that mouse strains derived from mixed backgrounds tend to show highly varied effects in response to MD (Heimel et al, 2008). For instance, C57BL/6J and DBA/2J mice showed the most significant shift in OD while some strains such as BXD02 and BXD28 do not show any shift towards the non-deprived eye in response to monocular deprivation (Heimel et al, 2008). Therefore, it appears that insufficient back-crossing could lead to variable results; however, the use of littermate controls may partially solve this problem (Heimel, 2008).

4.1 Introduction

The critical period in cats has been well defined and therefore serves a good model for studying possible cellular and molecular mechanism underlying ocular dominance plasticity. In order to determine if there are any interspecies differences in these molecular mechanisms, I analysed the expression of various proteins using Western blotting/Immunoblotting (see General Methods Chapter II). Signalling molecules downstream of NMDA receptors have not received much attention in OD plasticity in cat visual cortex. I was particularly interested in how molecules within a pathway were co-regulated during development and in altered sensory experience. I therefore focused on NMDA receptors and their downstream signalling molecules such as PSD-95, SAP-102, synGAP and ERK and kinases such as αCaMKII, PKARIIβ and PKC. Furthermore, I also assessed the expression of mGluR5 and its associated enzymes such as PLCβ1 and PLCβ4. There is a wealth of studies providing evidence of AMPA receptors and the role they play in plasticity and therefore I examined the expression of GluR1 and GluR2/3 subunits during development and in altered sensory experience in cat visual cortex. In addition to excitatory transmission, a study by Fagiolini and colleagues (Fagiolini et al, 2004) have shown that mice with $GABA_A\alpha1a$ subunit knock-out failed to cause an ocular dominance shift towards the non-deprived eye after monocular deprivation (Fagiolini et al, 2004). I was interested to determine how the expression of $GABA_A\alpha1a$ (the dominant α1 subunit) changes during development and in altered sensory experience. Synaptophysin, an integral protein of synaptic vesicle membrane, has been shown to be regulated developmentally largely by an increase in the number of vesicles per synapse and synapse elimination (Kierstein et al, 1996) and therefore is a good indicator of synaptogenesis taking place. Therefore, I was interested in replicating the results obtained previously and determine if its expression is regulated by sensory experience.

I also wanted to determine the expression of various proteins at the synaptic level and this entailed preparing synaptosomes and post synaptic densities (PSD) from homogenate samples of primary visual cortex. The biochemical changes occurring at the synaptic level are may be most relevant to the functional changes (Chen and Bear, 2007) because at the PSD site, there are glutamate receptors with their associated signalling and scaffolding proteins that ultimately organize signal

transmission pathways at the post-synaptic membrane. These Western blotting experiments may shed a light into possible cellular mechanism underlying experience-dependent plasticity in the cat visual cortex.

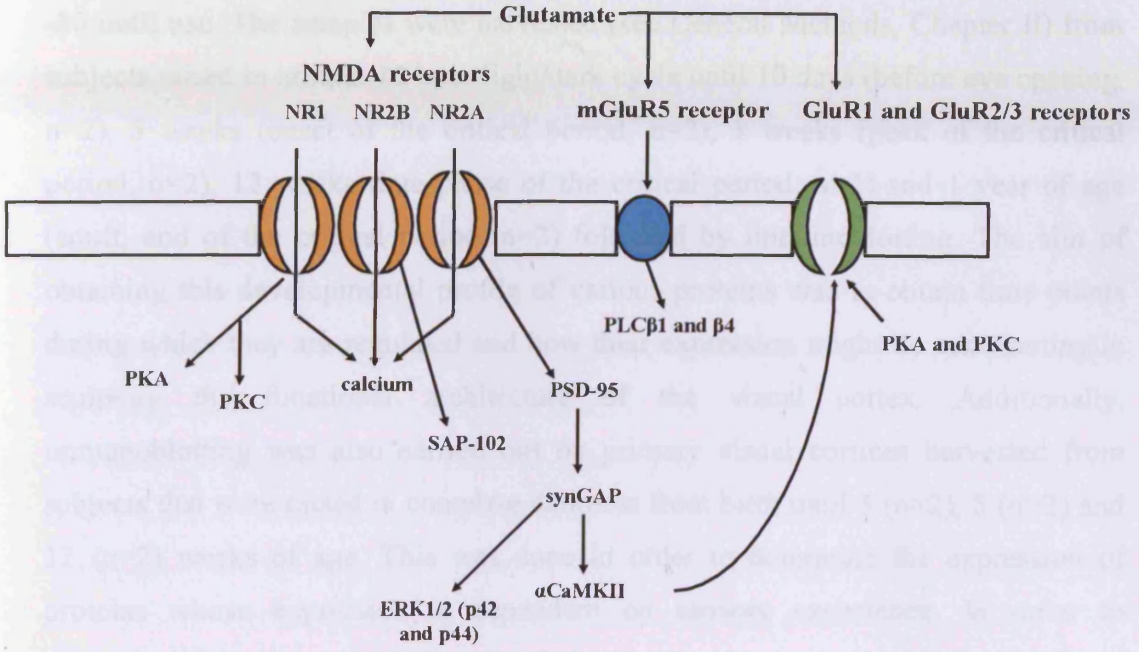


Figure 4: *Signalling pathways in NMDA receptor complex*. Only those components in the pathways are illustrated that were investigated in this study. It should be noted that some downstream signalling molecules may be linked to NMDA receptor at multiple location. For instance αCaMKII also interact with AMPA receptors and these receptors also interact with kinases such as PKA and PKC.

4.2 Methods

Following optical imaging and VEP session, the subjects were terminated with an overdose of pentobarbitone and primary visual cortices were harvested and stored at -80 until use. The samples were harvested (see General Methods, Chapter II) from subjects raised in normal 12 hour light/dark cycle until 10 days (before eye opening, n=2), 3 weeks (onset of the critical period, n=2), 5 weeks (peak of the critical period, n=2), 12 weeks (late phase of the critical period, n=2) and 1 year of age (adult, end of the critical period, n=2) followed by immunoblotting. The aim of obtaining this developmental profile of various proteins was to obtain time points during which they are regulated and how their expression might be contributing in sculpting the functional architecture of the visual cortex. Additionally, immunoblotting was also carried out on primary visual cortices harvested from subjects that were raised in complete darkness from birth until 3 (n=2), 5 (n=2) and 12 (n=2) weeks of age. This was done in order to determine the expression of proteins whose expression is dependent on sensory experience. In order to determine if there are any bidirectional changes in the expression of proteins in cat visual cortex after brief sensory experience and deprivation, primary visual cortices were harvested from subjects that were raised in complete darkness until four weeks of age followed by only a week of visual experience (DR-LR, n=2) and from subjects that were exposed to normal 12hour light/dark cycle followed by a week of darkness (LR-DR, n=2).

In rodents when a contralateral eye is deprived, most of the connections dominated by that eye shrink since a very small portion of cortex receives inputs from both the eyes. On the other hand, a very large part of the visual cortex in cats and other higher animals is binocular. This difference in the organisation of the visual cortex may be different at the molecular level for functional effects seen after MD in cat visual cortex. I was interested to determine if there are any molecular changes associated with physiological shift observed after 2-days MD (see CHAPTER III) and samples from those subjects were extracted (n=2). I also wanted to determine if a longer period of MD (7-days, n=2), which results in anatomical retraction of

afferents (Antonini and Stryker, 1993b), is also accompanied by molecular changes and if those changes differ from brief (2-days) period of MD.

Synaptosome and PSD fractions were prepared using the technique employed by Dunkley et al, (1989) (see General Methods Chapter II) as done previously (Watson et al, 2006). In order to minimise variability in preparations, each of the samples was prepared in duplicate and yields usually were sufficiently large with total protein concentration of 5-6μg (determined using Lowry assay).The protein samples were stored at -80°C until use (details of Western blotting can be found in Chapter II).

Due to fewer animals used it this chapter, it was not possible to use any parametric tests such as one-way ANOVA which relies on the normal distribution of the data. Therefore, a non-parametric test (Kruskal-Wallis test) was used as a method of testing equality of population median among groups. It is identical to ANOVA but does not rely on the assumption that the data is normally distributed. However, due to fewer numbers of animals, a significant difference was not always achieved.

4.3 Results

4.3.1 Protein expression during development

The results herein are presented in the order of the way they are linked with NMDA receptor subunits; however, this is an oversimplification, as some downstream molecules may be linked to NMDA receptor complex by multiple routes. For instance phospholipases also indirectly interact with PKA and PKC while the latter kinases also complex with αCaMKII. AMPA receptors are also indirectly interact NMDA receptor complex regulated by kinases and phosphotases. The results obtained from AMPA receptors (even though they interact indirectly with NMDA receptor complex) and GABAAα1a subunit and synaptophysin are dealt as separate pathways from NMDA receptor complex.

The expression of NR2A complex during development

In accordance with previous studies (Chen et al, 2000; Philpot et al, 2001), NR2A expression was absent in 10 days and 3 weeks old cats and a band corresponding to NR2A subunit was detected from 5 weeks onwards (Figure 4.1A and Figure 4.1.2A). However, in the PSD fraction a band corresponding to NR2A was not observed in all the age groups examined (Figure 4.1.1A) and since this subunit is tightly anchored in synaptic compartments (Groc et al, 2006), it should have been detected in reasonable amounts in the PSD fraction. It is possible that during purification process of PSD, the epitope to which the antibody binds might have been altered or lost. The NR2A subunit forms a complex with PSD-95 and the expression of the latter increased substantially by 47% at 3 weeks of age and thereafter its expression was maintained through out the course of the development (Figure 4.1B and Figure 4.1.2B). The results obtained herein are consistent with the results obtained by Sans et al, (2000), who also showed that the expression of PSD-95 was low at early postnatal ages in the hippocampus of rats and its expression increased substantially with age. Consistent with the results obtained from the homogenate, the expression of PSD-95 in the synaptosomes and in the PSD fraction also increased substantially with age (Figure 4.1.1B).

The NR2A and PSD-95 then forms a complex with synGAP (Kim et al, 1998) and similar to the expression of PSD-95, the expression of synGAP also increased substantially from 3 weeks onwards and thereafter its expression was maintained through out development (Figure 4.1C and Figure 4.1.2C). In the PSD fraction, however, the expression of synGAP showed a distinct band at 10-days till 12 weeks and at 1 year old, its expression declined (Figure 4.1.1C). This is consistent with the results obtained by Porter et al, (2005) who demonstrated that gene expression levels of synGAP increased substantially in early developmental but then declined in adulthood. The NR2A/PSD-95 and synGAP complex then interact with αCaMKII (Oh et al, 2004) and the expression of the latter was low at 10 days old and increased at 3 weeks of age and thereafter was maintained throughout development (Figure 4.1D and Figure 4.1.2D).The developmental expression of α-CAMKII is consistent with gene expression study by Neve and Bear (1989), which also showed that αCaMKII, was initially present at very low levels and gradually increased during development. In contrast to the homogenate, the expression of αCaMKII in the PSD fraction showed a distinct band at 12 weeks and 1 year old (Figure 4.1.1D) and this is consistent with the results obtained by Petralia et al, (2005) which also demonstrated that the expression of αCaMKII increases with age in rat hippocampus.

SynGAP mediates its effects by differential activation of several downstream effectors pathways including MAPK. In contrast to the expression of synGAP, the expression of pMAPK (p42 and p44) increased dramatically from 5 weeks onwards and thereafter did not change while its expression at 10-days and 3 weeks appeared low (Figure 4.1E and Figure 4.1.2E). The time period during which the expression increased coincides with the critical period. This is consistent with the results obtained by Oliveira et al, (2008) which have shown that during postnatal development between p15-p45 in rat visual cortex, the expression of ERK1/2 phosphorylation increased substantially using Western blotting.

The NR2A and its complex should have been highly enriched in the synaptosomal fraction since they contain molecular machinery necessary for the uptake, storage and release of neurotransmitters. Much to my surprise, I could only detect PSD-95 in the synaptosomal fraction (see Discussion for possible reasons).

The expression of NR2B complex during development

The expression of NR2B was highly expressed at 10 days and 3 weeks of age and thereafter its expression declined with age (Figure 4.1F and Figure 4.1.2F), and this consistent with previous studies (Chen et al, 2000; Philpot et al, 2001). Likewise, in the PSD fraction, the expression of NR2B was highly expressed in 10 days old and 3 weeks old and thereafter its expression declined with age (Figure 4.1.1E). SAP-102 (synaptic associated protein) is known to be associated with NR2B (Sans et al, 2000) and during development the NR2B-SAP102 complex in immature synapses tends to be replaced by NR2A-PSD-95/PSD-93 in mature synapses (Townsend et al, 2003), resulting in shortening of EPSPs. Consistent with previous studies (Shi et al, 1997), the present study also revealed that the expression of SAP-102 also declined with age (Figure 4.1G and Figure 4.1.2G). Similarly, in the PSD fraction, the expression of SAP-102 also declined with age (Figure 4.1.1F). It appears that there is a close correlation between changes in protein levels seen in homogenate and in PSD fractions suggesting that regulation of levels of NR2B and SAP-102 at synapses is directly related to synthesis of these proteins in the visual cortex (Petralia et al, 2005).

The expression of NR1 complex during development

Immunoblotting results demonstrated that NR1 expression (Figure 4.1H and Figure 4.1.2H) did not change throughout development. Consistent with the results obtained from the homogenate, the expression of NR1 also did not change throughout development in the PSD fraction (Figure 4.1.1G). Kinases such as PKA is known to be linked to NR1 (Husi and Grant, 2001) and the expression of the former also appeared to be stable through out development (Figure 4.1I and Figure 4.1.2I). Similarly, the expression of PKC (Figure 4.1J and Figure 4.1.2J) did not change through out development. This is in contrast to a study by Jia et al, (1990) who showed using immunoreactivity that PKC expression was high at early postnatal ages (6 weeks) and declined afterward till adulthood in cat visual cortex. The difference in results could be due to different methodological approaches employed and a disadvantage of Western blotting is that any layer differences cannot be detected.

The expression of mGluR5 and downstream signalling molecules during development

The expression of mGluR5 subunit was low at 10 days old and then peaked at 3 and 5 weeks of age and thereafter declined again (Figure 4.1K and Figure 4.1.2K). This is consistent with a previous study by Reid et al (1996) which demonstrated that production of cAMP induced by activation of metabotropic receptors, peaked at the critical period and thereafter declined. In contrast to mGluR5, the expression of downstream signalling molecules such as such as PLCβ1 (Figure 4.1L and Figure 4.1.2L) and PLCβ4 (Figure 4.1M and Figure 4.1.2M) did not change through out development. Previous study has shown that the expression of PLCβ1 is down-regulated developmentally using immunohistochemistry (Kind et al, 1994) and the discrepancy with the results obtained herein could be due to differences in splice variants in the commercially available antibody and cat-301 used in the latter study.

The expression of AMPA receptors, GABAAα1a and synaptophysin during development

AMPA receptor subunits such as GluR1 and GluR2/3 did not change their expression throughout the course of the development (Figure 4.1N, Figure 4.1.2N and Figure 4.1O and Figure 4.1.2O respectively).This is in contrast to previous study by Gordon et al (1995) in cats which showed that AMPA binding sites increased from P7-P42 and thereafter declined, however, no specific receptor subunit was determined developmentally. Previous studies have shown that the expression of GluR1 appears to be species specific in the visual cortex, for instance in rats, GluR1 expression increases with age (Akaneya, 2007) while in ferrets, immunohistochemical studies have shown that GluR1 immunoreactivity is present only in few neurons during early postnatal age and this staining intensity increases dramatically during the first two postnatal weeks and then declines to adult levels (Herrmann, 1996). The GluR1 subunit was not observed in the PSD fraction (Figure 4.1.1H) and due to lack of availability of GluR2/3 antibody (discontinued from the supplier), its expression in the PSD fraction could not be determined.

The $GABA_A\alpha1a$ subunit was not detected at 10 days old; its expression increased at 3 weeks of age and was maintained throughout development Figure 4.1P and Figure 4.1.2P) and this is consistent with the results obtained by Chen et al (2001).

Synaptophysin level was examined as an indicator of synaptogenesis and its expression increased substantially at 3 weeks of age and was then maintained throughout the course of development (Figure 4.1Q and Figure 4.1.2Q). This is consistent with a previous study showing time course of synaptogenesis in primary visual cortex (Cragg, 1976).

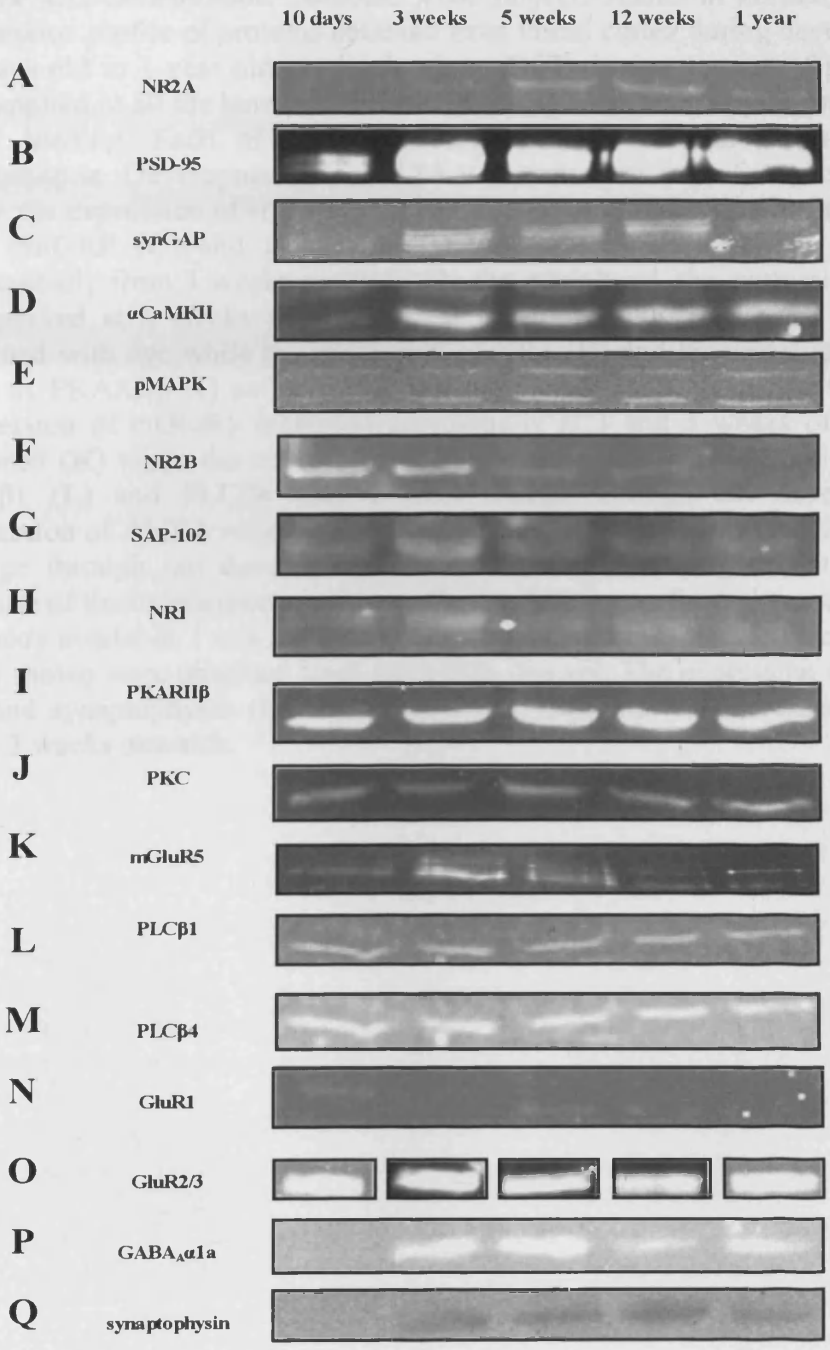

10 days
3 weeks
5 weeks
12 weeks
1 year
A
NR2A
B
PSD-95
C
synGAP
D
αCaMKII
E
pMAPK
F
NR2B
G
SAP-102
H
NR1
I
PKARIIβ
J
PKC
K
mGluR5
L
PLCβ1
M
PLCβ4
N
GluR1
O
GluR2/3
P
GABAAα1a
Q
synaptophysin

Figure 4.1: *Immunoblots obtained from subjects raised in normal environment.* Expression profile of proteins obtained from visual cortex during development from 10 days old to 1 year old cat (each age n=2). The same amount of protein (10μg) was applied in all the lanes and PKARIIβ was used as an internal control to confirm equal loading. Each of these blots was carried out four times or more for confirmation. Developmentally, NR2A was expressed from 5 weeks onwards (**A**) while the expression of signaling molecules NR2A complexes with such as PSD-95 (**B**), synGAP (**C**) and αCaMKII (**D**) was low in 10 days old and increased substantially from 3 weeks onwards. On the other hand, the expression of pMAPK (**E**) peaked at 5 weeks of age. The expression of NR2B (**F**) and SAP-102 (**G**) declined with age while the expression of NR1 (**H**) and kinases it is linked with it such as PKARIIβ (**I**) and PKC (**J**) did not change through out development. The expression of mGluR5 increased substantially at 3 and 5 weeks of age and then declined (**K**) while the expression of its downstream signalling molecules such as PLCβ1 (**L**) and PLCβ4 (**M**) did not change through out development. The expression of AMPA receptor subunits such as GluR1 (**N**) and GluR2/3 (**O**) did not change through out development. The band corresponding to GluR1 was faint because of the inconsistency of the antibody. And due to limited amount of GluR2/3 antibody available, I was not able to run all the control samples in one gel, hence the blots shown were obtained from more than one gel. The expression of $GABA_A\alpha 1a$ (**P**) and synaptophysin (**Q**) was absent in 10 days old and increased substantially from 3 weeks onwards.

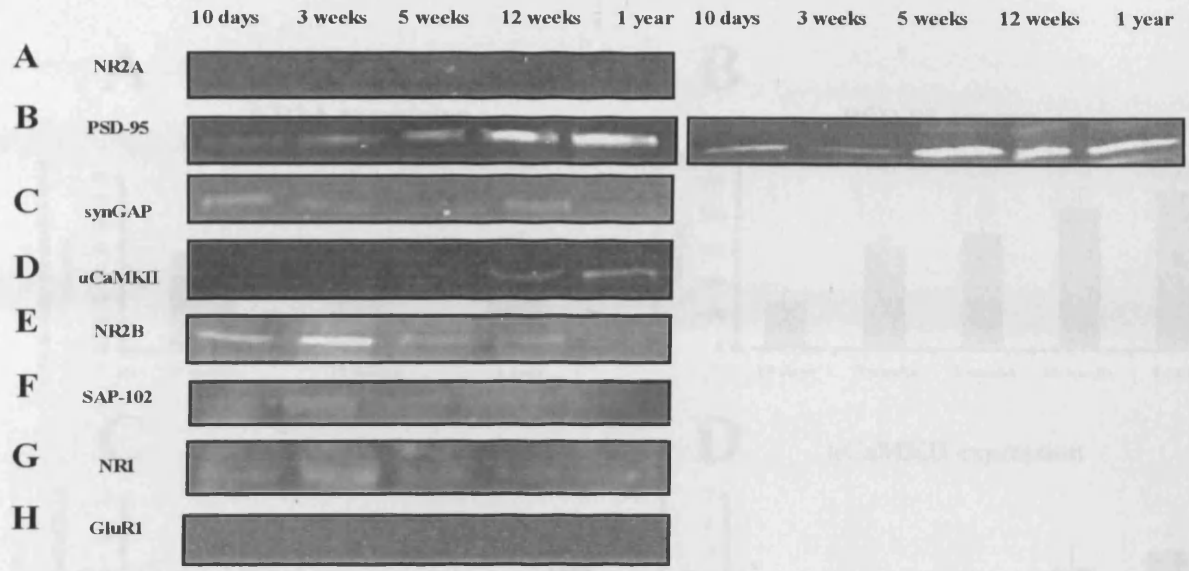
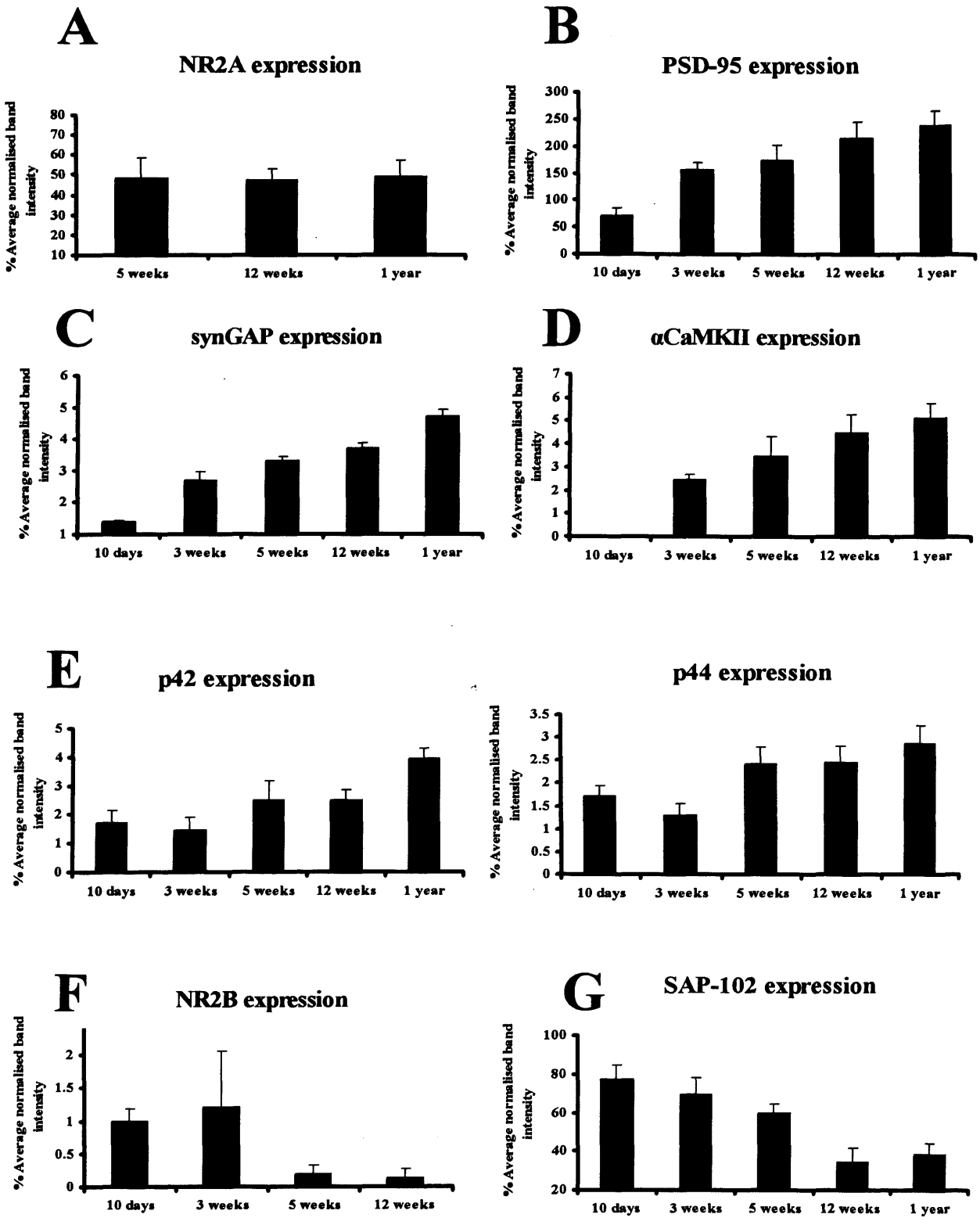
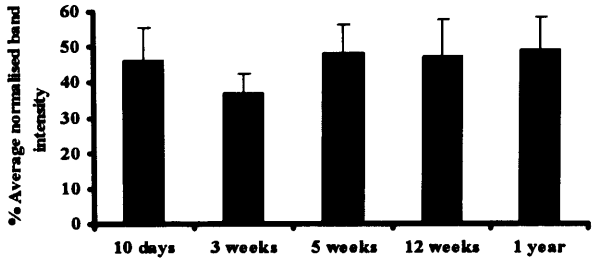


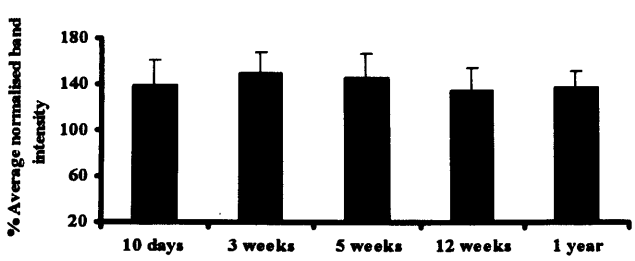
Figure 4.1.1: *Immunoblots obtained from PSD fraction of subjects raised in normal environment.* Expression profile of proteins obtained from visual cortex during development from 10 days old to 1 year old cat obtained from isolated post synaptic density (PSD) fraction. An equal amount of protein (30μg) was added in all the lanes and β-actin was used as in internal control to confirm equal loading. A band corresponding to NR2A was not detected in the PSD fraction in all the age groups (**A**). Consistent with the results obtained from the homogenate samples, the expression of PSD-95 increased from 3 weeks onwards (**B**). A blot on the right is obtained from a synaptosomal preparation and shows that the expression of PSD-95 increased with age. On the other hand, in contrast to homogenate (see Figure 4.1), the expression of synGAP declined in 1 year old (**C**) and a band corresponding to αCaMKII was detected in samples obtained from 12 weeks and 1 year old cats only (**D**).Consistent with the results obtained from the homogenate samples, the expression of NR2B (**E**) and SAP-102 (**F**) down-regulated with age while the expression of NR1 (**G**) did not change through out development in the PSD fraction. A band corresponding to GluR1 was not observed in all the age groups (**H**).


A
NR2A expression
% Average normalised band intensity
5 weeks
12 weeks
1 year
B
PSD-95 expression
% Average normalised band intensity
10 days
3 weeks
5 weeks
12 weeks
1 year
C
synGAP expression
% Average normalised band intensity
10 days
3 weeks
5 weeks
12 weeks
1 year
D
αCaMKII expression
% Average normalised band intensity
10 days
3 weeks
5 weeks
12 weeks
1 year
E
p42 expression
% Average normalised band intensity
10 days
3 weeks
5 weeks
12 weeks
1 year
p44 expression
% Average normalised band intensity
10 days
3 weeks
5 weeks
12 weeks
1 year
F
NR2B expression
% Average normalised band intensity
10 days
3 weeks
5 weeks
12 weeks
G
SAP-102 expression
% Average normalised band intensity
10 days
3 weeks
5 weeks
12 weeks
1 year

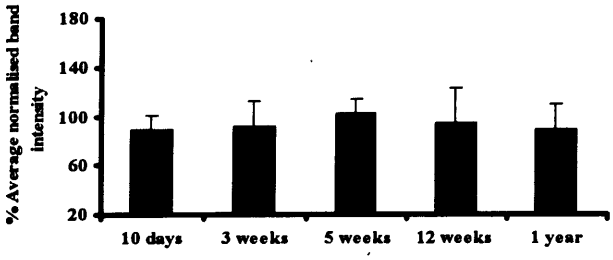
H


NR1 expression
% Average normalised band intensity
60
50
40
30
20
10
0
10 days
3 weeks
5 weeks
12 weeks
1 year

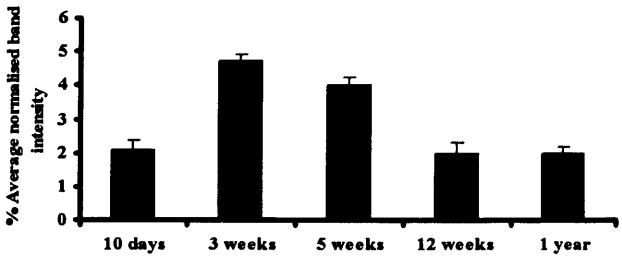
I


PKARIIbeta expression
% Average normalised band intensity
180
140
100
60
20
10 days
3 weeks
5 weeks
12 weeks
1 year

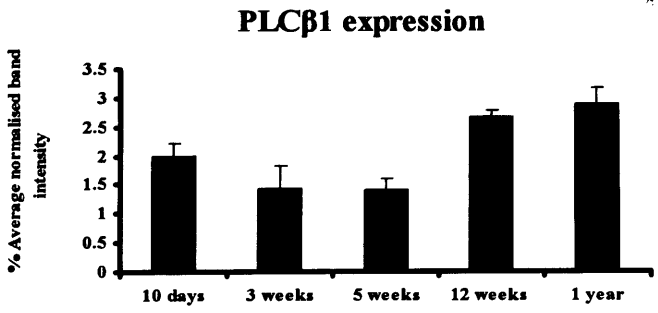
J


PKC expression
% Average normalised band intensity
180
140
100
60
20
10 days
3 weeks
5 weeks
12 weeks
1 year

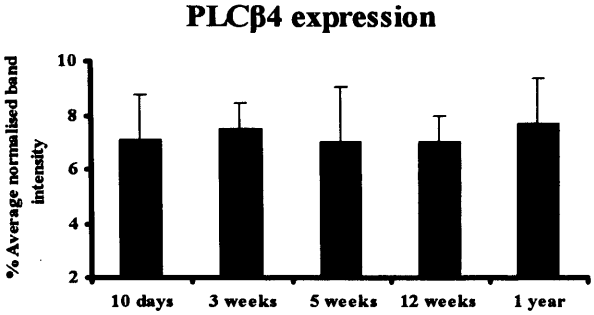
K


mGluR5 expression
% Average normalised band intensity
6
5
4
3
2
1
0
10 days
3 weeks
5 weeks
12 weeks
1 year

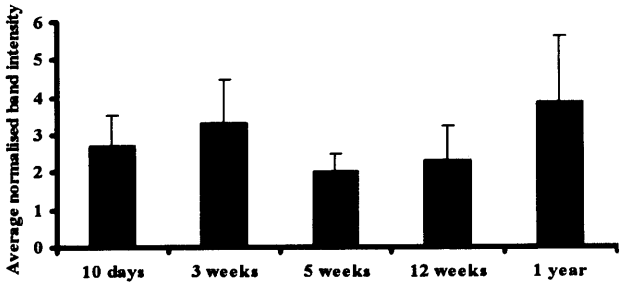
L


PLCβ1 expression
% Average normalised band intensity
3.5
3
2.5
2
1.5
1
0.5
0
10 days
3 weeks
5 weeks
12 weeks
1 year

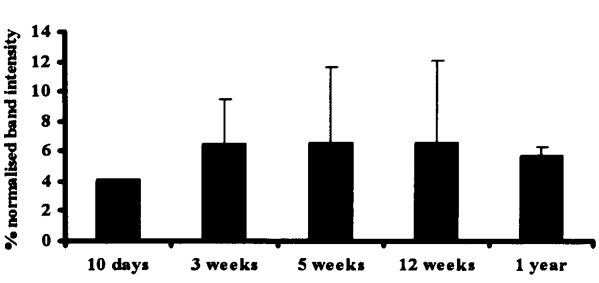
M


PLCβ4 expression
% Average normalised band intensity
10
8
6
4
2
10 days
3 weeks
5 weeks
12 weeks
1 year

N


GluR1 expression
Average normalised band intensity
6
5
4
3
2
1
0
10 days
3 weeks
5 weeks
12 weeks
1 year

O


GluR2/3 expression
% normalised band intensity
14
12
10
8
6
4
2
0
10 days
3 weeks
5 weeks
12 weeks
1 year

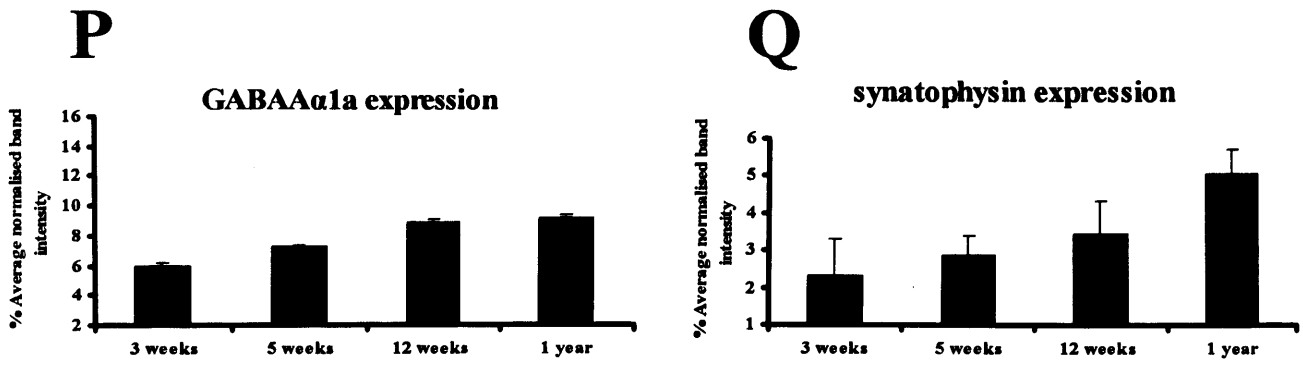


Figure 4.1.2: *Quantitative analysis of immunoblots obtained from homogenate samples prepared from visual cortices of subjects raised in normal environment.* The band intensity of protein of interest was normalized to the internal control and the error bars indicate ± SEM. Developmentally, NR2A was expressed from 5 weeks onwards (**A**) and thereafter its expression did not change throughout development while the expression of signaling molecules NR2A complexes with such as PSD-95 (**B**), synGAP (**C**) and αCaMKII (**D**) was low in 10 days old and increased substantially form 3 weeks onwards. On the other hand, the expression of pMAPK (**E**) peaked at 5 weeks of age and thereafter its expression was maintained. The expression of NR2B (**F**) declined with age and a band corresponding to NR2B could not be detected in 1 year old cat. Similarly, the expression of SAP-102 (**G**) declined with age while the expression of NR1 (**H**) and kinases it is linked with it such as PKARIIβ (**I**) and PKC (**J**) did not change through out development. The expression of mGluR5 increased substantially at 3 and 5 weeks of age and then declined (**K**) while the expression of downstream signalling molecules such as PLCβ1 (**L**) and PLCβ4 (**M**) did not change through out development. Similarly, the expression of AMPA receptor subunits such as GluR1 (**N**) and GluR2/3 (**O**) did not change through out development. The expression of $GABA_A\alpha1a$ (**P**) and synaptophysin (**Q**) was absent in 10 days old and increased substantially from 3 weeks onwards.

4.3.2 Changes in protein expression after dark-rearing

The expression of NR2A complex in dark-reared cats

The developmental increase of NR2A expression was prevented in subjects raised in darkness at 5 or 12 weeks (5D, 12D) but not at 3 weeks of age (3D) compared to control (C) animals of the same age (Figure 4.2A) consistent with previous studies (Quinlan et al, 1999; Chen et al, 2000; Philpot et al, 2001). In the PSD fraction, despite numerous attempts a band corresponding to NR2A was not detected (Figure 4.2.1A) and this is possible that during isolation of PSD, the epitope to which NR2A antibody binds was somehow altered or degraded. The expression of PSD-95 did not appear to be regulated by activity as its expression levels in dark-reared animals was indistinguishable from control subjects in all age groups (Figure 4.2B and Figure 4.2.2A (Kruskal-Wallis test, $p=0.342$)). Similarly, in the synaptosomal preparation and in the PSD fraction, the expression of PSD-95 (Figure 4.2.1B) did not appear to be regulated by sensory activity (Kruskal-Wallis test, $p=0.65$) as its expression was not different from control subjects. The results obtained here are consistent with the data obtained from mice raised in complete darkness (Fagiolini et al, 2003), however, studies by Cotrufo et al, 2003 and Yoshii et al, 2003 illustrated that rearing rats in darkness reduced the expression of PSD-95. It is possible that there are species differences in the experience-dependent regulation of PSD-95. It should be noted that only PSD-95 was detected in the synaptosomes while other excitatory subunits and signalling molecules were not detected (see Discussion for possible reasons).

SynGAP is known to interact with PDZ domain of PSD-95, localized at the post synaptic membrane (Walikonis et al, 2000) has been shown to play key role hippocampal plasticity (Komiyama et al, 2002). Similar to the expression of PSD-95, the expression of synGAP was not regulated by sensory activity as its expression in dark-reared subjects was not different from control subjects in all age groups examined (Kruskal-Wallis test, $p=0.631$; Figure 4.2C and Figure 4.2.2B). Similarly, in the PSD fraction, the expression of synGAP did not appear to be regulated by sensory experience (Kruskal-Wallis test, $p=0.31$ (Figure 4.2.1C).

CaMKII is also part of the NR2A/PSD-95/synGAP complex (Oh et al, 2004). The expression of αCaMKII did not change in dark-reared compared to control subjects (Kruskal-Wallis test, $p=0.330$; Figure 4.2D and Figure 4.2.2C). This is in contrast to a study by Neve and Bear, 1989 which showed that αCaMKII gene expression is elevated in dark-reared relative to normal animals in kitten visual cortex in the peak of the critical period. It should be noted that the latter study focused on gene expression and not relative protein expression as done in the present study, and the elevated gene expression does not necessarily mean that all those genes will be translated into functional proteins. Interestingly, in the PSD fraction, the expression of αCaMKII showed variable band intensities across control (C) and dark-reared (D) age groups. For instance, the expression of αCaMKII did not differ in control and dark-reared at 3 weeks of age. On the other hand, at 5 weeks of age, there was an intense band in the dark-reared group compared to control (this is consistent with gene expression study by Neve and Bear, 1989) while at 12 weeks of age, its expression was higher in control than in dark-reared (Figure 4.2.1D). Previous study has shown that blocking activity with TTX in cortical neurones obtained from rat embryos reduced the expression of αCaMKII in PSD fraction (Ehlers, 2003) and this is consistent with the results obtained herein in dark-reared cats at 12 weeks of age. However, blocking neuronal activity with TTX and the impact it may have on cortical activity is different (Linden et al, 2009) than raising subjects in complete darkness. It is possible that even in the absence of sensory activity, calcium levels become elevated as a homeostasis mechanism comes into play and therefore may explain high levels of α-CaMKII. However, even if homeostasis were to play a role, it is still not quite known as to why only dark-reared subjects at 5 weeks of age showed elevated levels and not at 3 or 12 weeks of age.

Expression of pMAPK (p42 and p44) was also investigated in dark-reared subjects relative to control subjects. Using immunoblotting the expression of p42 and p44 did not change significantly in dark-reared subjects compared to control in all age groups examined (p42: Kruskal-Wallis test, $p=0.873$ and p44: Kruskal-Wallis, $p=0.631$) (Figure 4.2E and Figure 4.2.2D). In contrast, a previous study has shown that there was a rapid increase in the expression of pMAPK (ERK1 and 2) upon visual stimulation in rats raised in complete darkness in cortical layers I-II/III using immunohistochemistry (Boggio et al, 2007). The disadvantage of Western blotting

is that it escapes any layer-specific or cell type regulation but is quantitative whereas immunohistochemistry is not and any changes in layer are perhaps diluted by no change in other layers. It is possible that in the present study the expression of pMAPK might have increased in response to visual stimulation in dark-reared subjects due to time involved in acquiring OIS maps as well as VEP recordings. Therefore in order to observe changes reported in Boggio et al, (2007) in cats, one would have to record before and after visual stimulation.

The expression of NR2B complex in dark-reared cats

Consistent with previous studies (Quinlan et al, 1999; Chen et al, 2000; Philpot et al, 2001), NR2B was highly expressed from birth and its expression declined with age and this down-regulation was prevented in subjects raised in complete darkness only at 5D and 12D (Figure 4.2F and Figure 4.2.2E). In accordance with the results obtained in the homogenate, the down-regulation of NR2B was also prevented in dark-reared subjects in the PSD fraction (Figure 4.2.1E). In contrast to NR2B, the expression of SAP-102 did not change significantly in subjects raised in complete darkness compared to control (Kruskal-Wallis test, $p=0.873$; Figure 4.2G and Figure 4.2.2F), however, its expression in the PSD fraction was not detected. It was surprising to see that the expression levels of SAP-102 are not affected in dark-reared subjects, since its counterpart, NR2B, was regulated by sensory experience. A study by Elias et al (2008) had demonstrated that SAP-102 is necessary for the trafficking of AMPA and NMDA receptors during synaptogenesis and maturation of synapses is not prevented in SAP-102 knockout mice. Since synaptogenesis proceeds normally in dark-reared animals but overall the spine density is reduced (Wallace and Bear, 2004), it is probable that maintained expression levels of SAP-102 permits maturation of synapses in dark-reared subjects.

The expression of NR1 complex in dark-reared cats

NR1, exhibited no significant change in expression levels in control versus dark-reared subjects in all age groups examined (Kruskal-Wallis test, $p=0.395$) and this is consistent with the results obtained previously (Chen et al, 2000) (Figure 4.2H and Figure 4.2.2G). Similarly, NR1 expression in the PSD fraction did not change in control and dark-reared subjects (Figure 4.2.1F). The expression of kinases such as PKARIIβ (Figure 4.2I and Figure 4.2.2H) and PKC (Figure 4.2J and Figure

4.2.2I) did not appear to be regulated by sensory experience as their expression was indistinguishable from control in age groups examined (PKARIIβ, Kruskal-Wallis test, *p=0.171*; and PKC, Kruskal-Wallis test, *p=0.817*). This is in contrast to a study by Elkabes et al (1993) which showed that the expression of PKC was lower in the visual cortex of dark-reared than in light reared rats. However, the latter study used a phorbol ester binding technique to assess the neuronal pool of PKC and it is possible that the actions of these esters have limited access to the pool of PKC, since they are likely to be sequestered by the plasma membrane (Murphy et al, 1999). PKA and PKC have also been demonstrated to play a role in long term potentiation in layers II/III in the visual cortex (Schrader et al, 2004) and applying PKA inhibitors such as KT5720 for 20 minutes and inducing theta burst stimulation in visual cortical slices prevented the induction of both LTP and LTD (Liu et al, 2003). It is possible that the presence of PKARIIβ and PKC may be required for the functional effects of dark-rearing rather than their regulation by sensory activity.

The expression of mGluR5 and downstream signalling molecules in dark-reared cats

The group 1 metabotropic receptor such as mGluR5 is the most abundant metabotropic receptor (Romano et al, 1996). Previous studies using immunostaining and Western blotting have revealed that dark-rearing from birth prevents developmental down-regulation of mGluR5 and its expression remains elevated in cat visual cortex (Reid et al, 1997). Using immunoblotting, the present study failed to reveal any difference in mGluR5 expression in dark-reared subjects relative to control subjects in all age groups examined (Kruskal-Wallis, *p=0.909*; Figure 4.2K and Figure 4.2.2J) It is possible that any modest differences in the expression levels could not be detected nonetheless, the results obtained herein need to be confirmed by using more animals. The expression of signaling downstream signalling molecules such as PLCβ1 (Figure 4.2L and Figure 4.2.2K) and PLCβ4 (Figure 4.2M and Figure 4.2.2L) did not change significantly in dark-reared subjects compared to control (PLCβ1; Kruskal-Wallis test, *p=0.612* and PLCβ4; Kruskal-Wallis test, *p=0.376*). Previous study by Kind et al (1994) has shown using immunohistochemistry that the developmental down-regulation of PLCβ1 (cat 307) is prevented in cats raised in complete darkness. The inconsistency with results obtained herein could be due to differences in techniques employed, for instance,

with immunoblotting any layer differences cannot be recognized but it is quantitative whereas immunohistochemistry is not. Also, the latter study used cat-307 antibody which has the same molecular weight as PLC β1 but it is possible that it detects a different splice variant than the commercially available antibody used herein.

The expression of AMPA receptors, $GABA_A\alpha 1a$ and synaptophysin in dark-reared cats

Dark-rearing from birth did not significantly change expressions levels of GluR1 (Figure 4.2N and Figure 4.2.2M) and GluR2/3 (Figure 4.2O and Figure 4.2.2N) compared to control (GluR1; Kruskal-Wallis test, $p=0.06$ and GluR2/3; Kruskal-Wallis test, $p=0.724$). In the PSD fraction, the expression of GluR2/3 was up-regulated in dark-reared animals at 5 and 12 weeks of age (Figure 4.2.1G). This is consistent with a previous study (Tropea et al, (2006) which also showed up-regulation of AMPA receptor subunits in mice that were dark-reared from birth. However, in the PSD fraction a band corresponding to GluR1 was not detected (Figure 4.2.1H). On the other hand, the expression of $GABA_A\alpha 1$ subunit was elevated in dark-reared subjects compared with control; however a significant difference was obtained in only dark-reared subjects at 5 and 12 weeks of age (Kruskal-Wallis test, $p=0.025$; Figure 4.2P and Figure 4.2.2O). This is consistent with the results obtained previously (Chen et al (2001), which also showed that only expression levels of α1, is up-regulated in the absence of sensory experience while α2 subunit is not.

The expression of synaptophysin was also determined in dark-reared subjects to determine if it is regulated by sensory experience. It was found that the expression of synaptophysin was significantly reduced (Kruskal-Wallis test, $p=0.025$) in dark-reared relative to control subjects (Figure 4.2Q and Figure 4.2.2P). Previous study has shown that dark-reared animals have relatively low spine density compared to normal animals (Wallace and Bear, 2004) and since synaptophysin is a good indicator of synapses being formed and eliminated (Kierstein et al, 1996), the results obtained herein are consistent with results obtained in the former study.

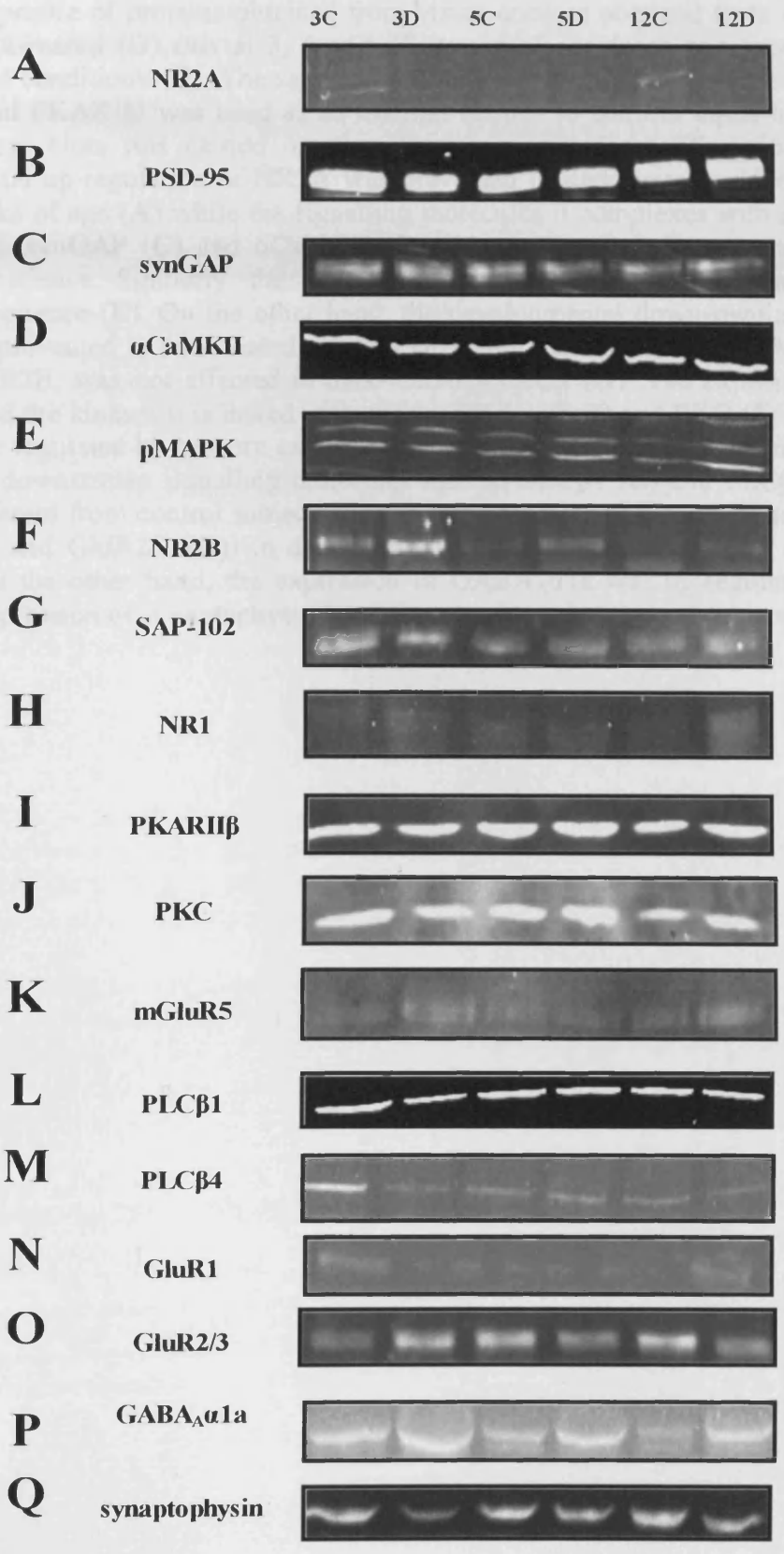

3C
3D
5C
5D
12C
12D
A
NR2A
B
PSD-95
C
synGAP
D
αCaMKII
E
pMAPK
F
NR2B
G
SAP-102
H
NR1
I
PKARIIβ
J
PKC
K
mGluR5
L
PLCβ1
M
PLCβ4
N
GluR1
O
GluR2/3
P
GABA$_A$α1a
Q
synaptophysin

Figure 4.2: *Immunoblots obtained from visual cortex from subjects raised in normal environment and those that were raised in complete darkness from birth.* Expression profile of proteins obtained from visual cortices obtained from control (C) and dark-reared (D) cats at 3, 5 and 12 weeks of age (each age group and experimental condition n=2). The same amount of protein (10μg) was loaded in all the lanes and PKARIIβ was used as an internal control to confirm equal loading. Each of these blots was carried out four times or more for confirmation. The developmental up-regulation of NR2A was prevented in dark-reared subjects at 5 and 12 weeks of age (**A**) while the signalling molecules it complexes with such as PSD-95 (**B**), synGAP (**C**) and αCaMKII (**D**) did not appear to be regulated by sensory experience. Similarly, the expression of pMAPK was not regulated by sensory experience (**E**). On the other hand, the developmental down-regulation of NR2B was prevented in dark-reared subjects (**F**) while the expression of SAP-102, bound to NR2B, was not affected in dark-reared subjects (**G**). The expression of NR1 (**H**) and the kinases it is linked with such as PKARIIβ (**I**) and PKC (**J**) did not appear to be regulated by sensory experience. Similarly, the expression of mGluR5 (**K**) and its downstream signalling molecules such as PLCβ1 (**L**) and PLCβ4 (**M**) was not different from control subjects. The expression of AMPA receptor subunits (GluR1 (**N**) and GluR2/3 (**O**)) in dark-reared was indistinguishable from control subjects. On the other hand, the expression of $GABA_A\alpha 1a$ was up-regulated (**P**) while the expression of synaptophysin (**Q**) was reduced in dark-reared subjects.

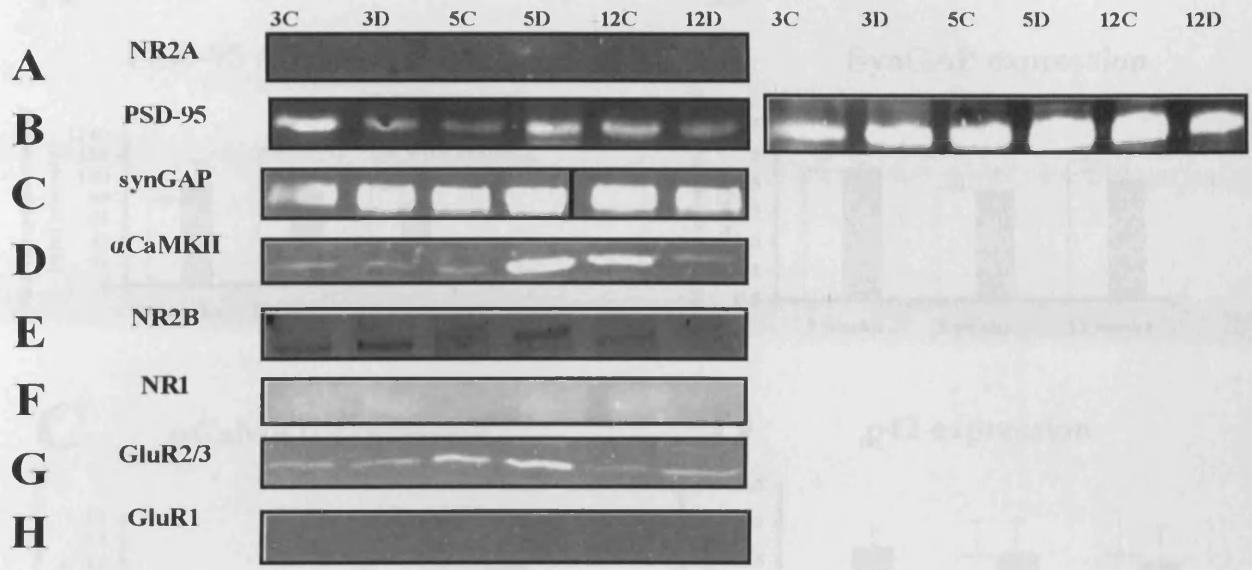
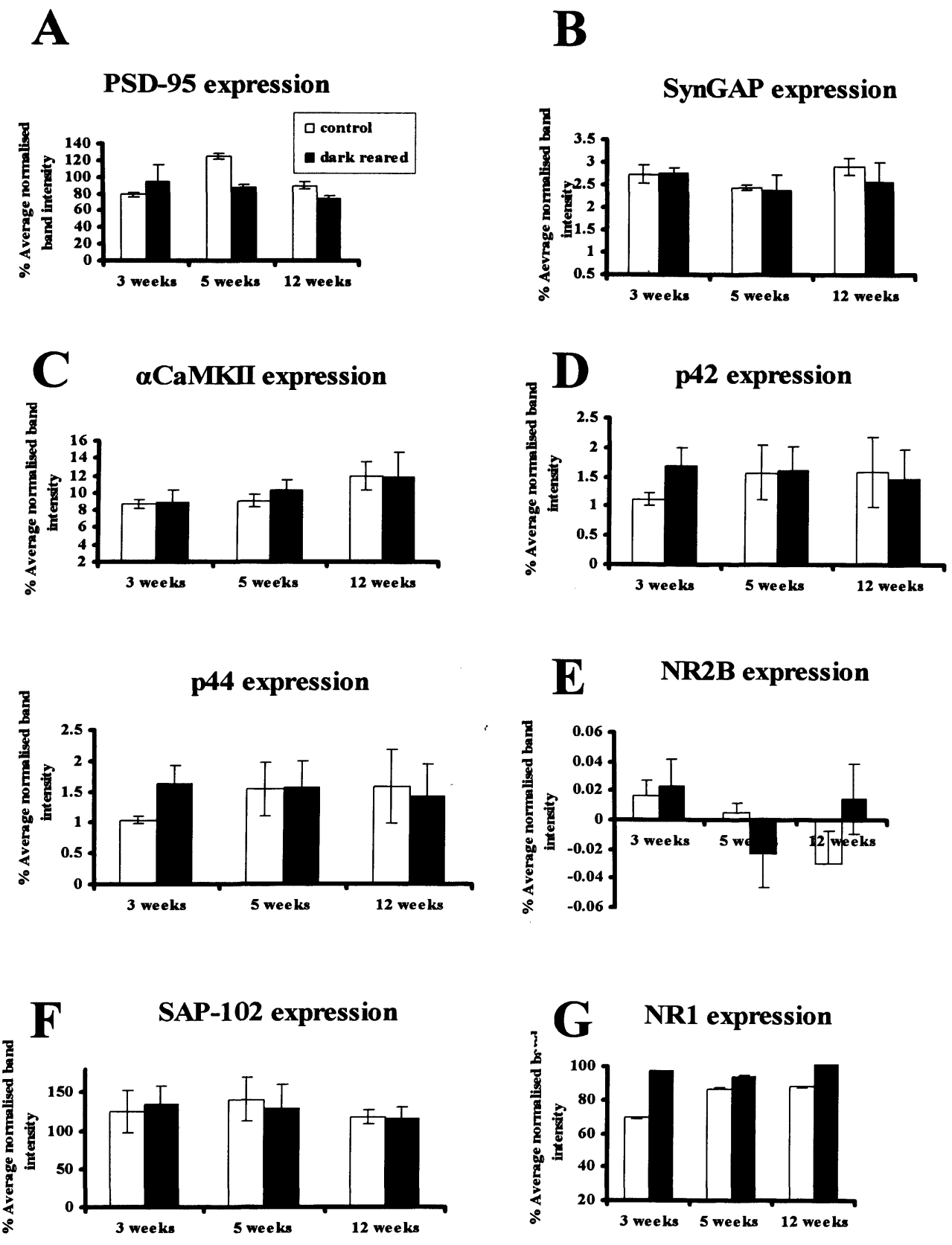
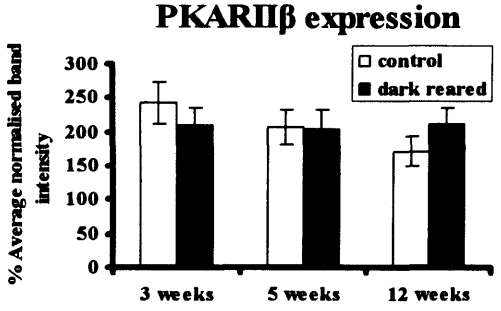


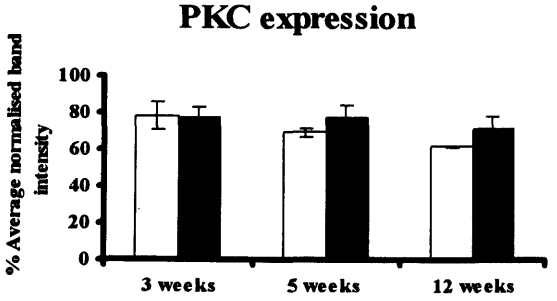
Figure 4.2.1: *Immunoblots obtained from PSD fraction obtained from subjects raised in normal environment and those that were raised in complete darkness from birth.* Expression profile of proteins obtained from visual cortices obtained from control (C) and dark-reared (D) cats at 3, 5 and 12 weeks of age. An equal amount of protein (30μg) was added in all the lanes and β-actin was used as in internal control to confirm equal loading. A band corresponding to NR2A was not detected, however, signalling molecules it complexes with such as PSD-95 (**B**) (far right shows immunoblot obtained from synaptosomes), synGAP (**C**) did not appear to be regulated by sensory experience in the PSD fraction, and this is consistent with the results obtained from the homogenate (see Figure 4.2). On the other hand, signalling molecule such as αCaMKII (**D**) showed variable band intensity with intense band at 5D and 12C of age. In accordance with the results obtained from the homogenate, the developmental down-regulation of NR2B was prevented in dark-reared cats (**E**) while the expression of NR1 was indistinguishable from control (**F**) (see Figure 4.2). The expression of GluR2/3 showed variable band intensity with up-regulation at 5D and 12D (**G**) while a band corresponding to GluR1 was not detected in the PSD fraction (**H**).


A
PSD-95 expression
control
dark reared
% Average normalised band intensity
140
120
100
80
60
40
20
0
3 weeks
5 weeks
12 weeks
B
SynGAP expression
% Aevrage normalised band intensity
3.5
3
2.5
2
1.5
1
0.5
3 weeks
5 weeks
12 weeks
C
αCaMKII expression
% Average normalised band intensity
16
14
12
10
8
6
4
2
3 weeks
5 weeks
12 weeks
D
p42 expression
% Average normalised band intensity
2.5
2
1.5
1
0.5
0
3 weeks
5 weeks
12 weeks
p44 expression
% Average normalised band intensity
2.5
2
1.5
1
0.5
0
3 weeks
5 weeks
12 weeks
E
NR2B expression
% Average normalised band intensity
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
3 weeks
5 weeks
12 weeks
F
SAP-102 expression
% Average normalised band intensity
150
100
50
0
3 weeks
5 weeks
12 weeks
G
NR1 expression
% Average normalised band intensity
100
80
60
40
20
3 weeks
5 weeks
12 weeks

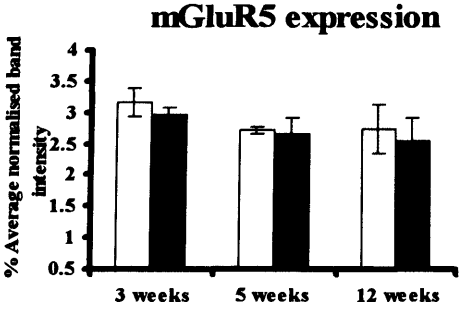
H


PKARIIβ expression
% Average normalised band intensity
300
250
200
150
100
50
0
control
dark reared
3 weeks
5 weeks
12 weeks

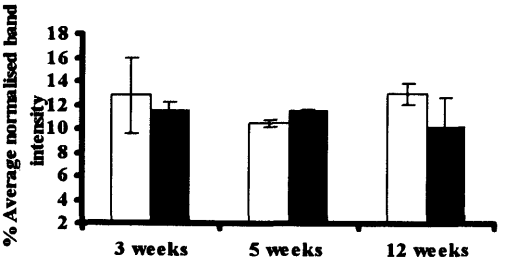
I


PKC expression
% Average normalised band intensity
100
80
60
40
20
0
3 weeks
5 weeks
12 weeks

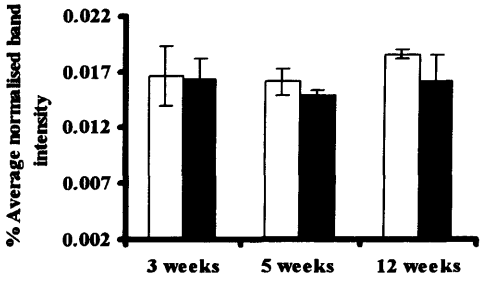
J


mGluR5 expression
% Average normalised band intensity
4
3.5
3
2.5
2
1.5
1
0.5
3 weeks
5 weeks
12 weeks

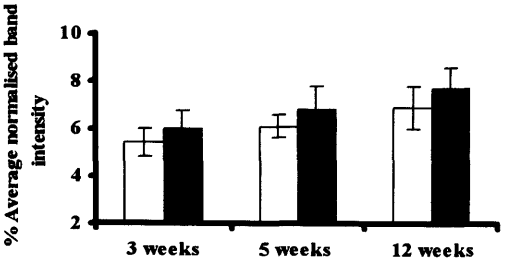
K


PLCβ1 expression
% Average normalised band intensity
18
16
14
12
10
8
6
4
2
3 weeks
5 weeks
12 weeks

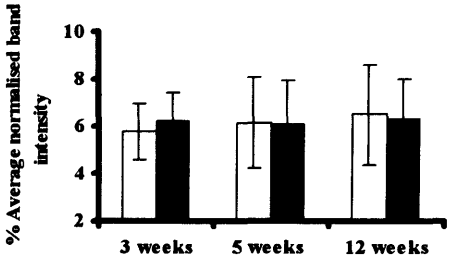
L


PLCβ4 expression
% Average normalised band intensity
0.022
0.017
0.012
0.007
0.002
3 weeks
5 weeks
12 weeks

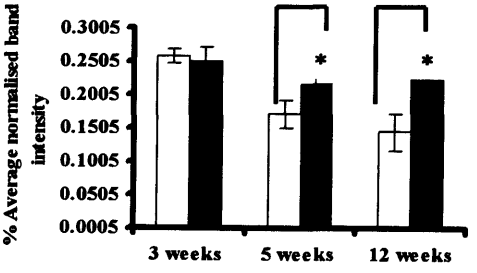
M


GluR1 expression
% Average normalised band intensity
10
8
6
4
2
3 weeks
5 weeks
12 weeks

N


GluR2/3 expression
% Average normalised band intensity
10
8
6
4
2
3 weeks
5 weeks
12 weeks

O


GABAAα1a expression
% Average normalised band intensity
0.3005
0.2505
0.2005
0.1505
0.1005
0.0505
0.0005
*
*
3 weeks
5 weeks
12 weeks

P

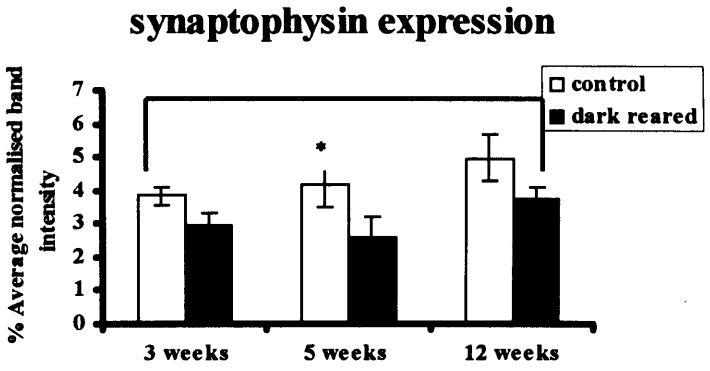


Figure 4.2.2: *Quantitative analysis of immunoblots obtained from homogenate samples prepared from visual cortices of subjects raised in normal environment and those that were raised in complete darkness from birth.* Expression profile of proteins obtained from visual cortices obtained from control (C) and dark-reared (D) cats at 3, 5 and 12 weeks of age. The band intensity was normalised to internal control and the error bars indicate ± SEM. The signalling molecules which complex with NR2A such as PSD-95 (**A**), synGAP (**B**), αCaMKII (**C**) and pMAPK (**D**) did not appear to be regulated by sensory experience as their expression was not different in control and dark-reared subjects. On the other hand, the developmental down-regulation of NR2B was prevented in dark-reared subjects at 5 and 12 weeks of age (**E**) while the expression of SAP-102, bound to NR2B, was not affected in dark-reared subjects (**F**). The expression of NR1 (**G**) and the kinases it is linked with such as PKARIIβ (**H**) and PKC (**I**) had their expression in dark-reared subjects indistinguishable from control subjects. Similarly, the expression of mGluR5 (**J**) and its downstream signalling molecules such as PLCβ1 (**K**) and PLCβ4 (**L**) was not different from control subjects. Furthermore, the expression of AMPA receptor subunits (GluR1 (**M**) and GluR2/3 (**N**)) in dark-reared was indistinguishable from control subjects. On the other hand, the expression of $GABA_A\alpha 1a$ was significantly up-regulated in dark-reared cats at 5D and 12D of age (**O**) while the expression of synaptophysin (**P**) was significantly down-regulated in dark-reared subjects (* denotes $p<0.05$ (Kruskal-Wallis test).

Protein	Up-regulated		Down-regulated		No change	
	H	PSD	H	PSD	H	PSD
NR2A			√			
PSD-95					√	√
synGAP					√	√
αCaMKII		√			√	
pMAPK					√	√
NR2B	√	√				
SAP-102					√	
NR1					√	√
PKARIIβ					√	
PKC					√	
mGluR5					√	
PLCβ1					√	
PLCβ4					√	
GluR1					√	
GluR2/3		√			√	
$GABA_A\alpha 1a$	√					
synaptophysin			√			

Table 1: ***A summary of results showing changes in expression of proteins after dark-rearing relative to control subjects in the homogenate (H) and in the post synaptic density (PSD).*** The molecular substrates are tabulated in the order they complex with receptor subunits, and substrates belonging to one complex are indicated by the same colour. However, GluR1 and GluR2/3 subunits are colour coded similarly because they belong to the same group of receptors while $GABA_A\alpha 1a$ and synaptophysin do not form a complex with the rest of the substrates and therefore they fall in a different group indicated by a different colour. It should be noted that some downstream signalling molecules may be linked to NMDA

receptor complex in multiple locations. The up-regulation of NR2A subunits was prevented in dark-reared subjects while the expression of signalling molecules it complexes with such as PSD-95, synGAP, αCaMKII and pMAPK did not appear to be regulated by sensory experience. But in the PSD fraction, the expression of αCaMKII was up-regulated in dark-reared subjects at 5 weeks of age only. On the other hand the developmental decline of NR2B was prevented in dark-reared subjects while the expression of SAP-102 was not affected in dark-reared subjects. The expression of NR1 in dark-reared was indistinguishable from control subjects and similarly, the expression of kinases it is linked with such as PKARIIβ and PKC did not appear to be regulated by sensory experience. Likewise, the expression of mGluR5 and its downstream signalling molecules did not show changes in their expression after dark-rearing. Also, the expression of AMPA receptor subunits such as GluR1 and GluR2/3 did not appear to be regulated by sensory experience, however, in the PSD fraction, the expression of GluR2/3 was up-regulated in dark-reared subjects at only 5 and 12 week of age. The expression of $GABA_A\alpha 1a$ was significantly up-regulated (denoted by√) while the expression of synaptophysin was significantly down-regulated (denoted by√) in dark-reared subjects.

4.3.3 Changes in protein expression after sensory experience (DR-LR) and sensory deprivation (LR-DR)

The physiological experiments on DR-LR and LR-DR cats (see Experimental chapter III) suggested that a change in visual exposure for a week is sufficient to alter the functional architecture of the visual cortex. A few studies have shown that the molecular changes such as NR2A and NR2B associated visual experience after raising animals in completed darkness take place within hours (Quinlan et al, 1999; Philpot et al, 2001). However, the role of downstream molecules associated with brief period of dark-rearing or visual experience has not been investigated. Also, the bidirectional changes of NMDA receptor subunits observed have been well documented in rodent visual cortex but not in higher animals such as feline or primates. Therefore, I set out to determine if the same bidirectional changes exists in cat visual cortex.

For direct comparison, homogenate samples obtained from DR-LR and LR-DR (5 weeks of age) were run on the same gel along with control and dark-reared subjects of the same age. Due to limited amount of PSD fraction obtained from dark-reared subjects, only PSD fraction obtained from control subjects were run along with samples obtained from LR-DR and DR-LR subjects. For analysis purposes, data obtained from the homogenate from DR-LR was normalised to dark-reared subjects and data obtained from LR-DR was normalised to control, generating two data sets respectively. And the data points obtained from control and dark-reared will therefore be 100% respectively. For statistical purposes, a non-parametric test (Kruskal-Wallis test; see Methods section 4.1.1) was carried out between DR-LR and dark-reared subjects and between LR-DR and control subjects.

The expression of NR2A receptor complex

The expression of NR2A expression was significantly higher in DR-LR subjects compared to dark-reared subjects (Kruskal-Wallis test, $p=0.021$) while its expression in LR-DR subjects was indistinguishable from control (Kruskal-Wallis test, $p=0.123$); Figure 4.3A and Figure 4.3.2A). In the PSD fraction, NR2A was not detected in DR-LR and LR-DR subjects (Figure 4.3.1A). The expression of

downstream signalling molecule, PSD-95, was elevated in DR-LR subjects compared to dark-reared subjects but not significantly so (Kruskal-Wallis test, *p=0.121*) while its expression in LR-DR subjects was indistinguishable from control subjects (Kruskal-Wallis test, *p=0.22*; Figure 4.3B and Figure 4.3.2B). Similarly, in the synaptosomal preparation and in the PSD fraction, the expression of PSD-95 expression was not different in the two experimental conditions compared to control Figure 4.3.1B). The expression of synGAP (Kruskal-Wallis test between DR-LR and dark-reared, *p=0.18*; LR-DR and control, *p=0.15*; Figure 4.3C and Figure 4.3.2C) and αCaMKII (Kruskal-Wallis test between, DR-LR and dark-reared, *p=0.18* and LR-DR and control, *p=0.09*; Figure 4.3D and Figure 4.3.2D) did not appear to be regulated after a change in sensory experience. However, in the PSD fraction, the expression of synGAP showed a significant increase in LR-DR compared to control (Kruskal-Wallis test, *p=0.04*) while its expression did not change significantly in DR-LR subjects compared to control (Kruskal-Wallis test, *p=0.24*; Figure 4.3.1C and Figure 4.3.2Q). In the PSD fraction, αCaMKII expression was surprisingly absent in DR-LR subjects and was only visible in LR-DR subjects (Figure 4.3.1D). Ras GTPase-activating protein such as synGAP is known to mediate its effects by differential activation of downstream effectors such MAPK and in the present study, the expression of pMAPK (Figure 4.3E and Figure 4.3.2E) (Kruskal-Wallis test,, p42; DR-LR and DR, *p=0.19* and p44; *p=0.16*. t-test, p42; LR-DR and control, *p=0.42* and p44; *p= 0.36*) did not appear to be regulated after sensory experience and deprivation.

The expression of NR2B and SAP-102

At the homogenate level, NR2B expression was higher in DR-LR subjects compared to dark-reared subjects, however, the difference was not significant (Kruskal-Wallis, *p=0.08;* Figure 4.3F and Figure 4.3.2F). However, its expression in LR-DR subjects was not different relative to control subjects (Kruskal-Wallis test, *p=0.121;* Figure 4.3F and Figure 4.3.2F). In the PSD fraction, NR2B subunit was not detected in both the experimental groups (LR-DR and DR-LR) (Figure 4.3.1E). The binding partner of NR2B, SAP-102, its expression was not different in DR-LR compared to dark-reared subjects (Kruskal-Wallis test, *p=0.121*; Figure 4.3G and Figure 4.3.2G). On the other hand, the expression of SAP-102 in LR-DR subjects was higher compared to control subjects, however, the difference in

expression was not significant (Kruskal-Wallis test, $p=0.07$; Figure 4.3G and Figure 4.3.2G). This is in contrast to results obtained after prolonged period of darkness and this could be due to the fact that during brief period of dark-rearing, the expression of SAP-102 adjusts homeostatically to permit continuation of maturation of synapses (Elias et al, 2008). In the PSD fraction, however, the expression of SAP-102 in both LR-DR and DR-LR groups was indistinguishable from control subjects (Figure 4.3.1F).

The expression of NR1 and kinases

The expression of NR1 did not appear to be regulated by activity following brief sensory experience (DR-LR) and sensory deprivation (LR-DR) (Kruskal-Wallis test between DR-LR and dark-reared, $p=0.48$ and Kruskal-Wallis test between LR-DR and control, $p=0.25$) (Figure 4.3H and Figure 4.3.2H). Similarly, in the PSD fraction, the expression of NR1 expression did not change in DR-LR and LR-DR compared to control subjects Figure 4.3.1G). Kinases such as PKARIIβ (Kruskal-Wallis test between DR-LR and dark-reared, $p=0.42$; LR-DR and control, $p=0.55$; Figure 4.3I and Figure 4.3.2I) and PKC (Kruskal-Wallis test between DR-LR and dark-reared, $p=0.22$; LR-DR and control, $p=0.35$; Figure 4.3J and Figure 4.3.2J) did not appear to be regulated after sensory experience and deprivation.

The expression of mGluR5 and downstream signalling molecules

Consistent with the results obtained in control and dark-reared subjects, the expression of mGluR5 did not change significantly between DR-LR and dark-reared subjects (Kruskal-Wallis test, $p=0.121$) and between LR-DR and control subjects (Kruskal-Wallis test, $p=0.08$) (Figure 4.3K and Figure 4.3.2K). Similarly, the expression levels of PLCβ1 (Figure 4.3L and Figure 4.3.2L) and PLCβ4 (Figure 4.3M and Figure 4.3.2M) downstream to metabotropic receptors did not change after a period of dark-rearing (LR-DR) compared to control (PLCβ1, Kruskal-Wallis test, $p=0.38$ and PLCβ4, Kruskal-Wallis test, $p=0.24$) and after sensory experience (DR-LR) compared to dark-reared subjects (PLCβ1, Kruskal-Wallis test,, $p=0.16$ and PLCβ4, Kruskal-Wallis test, $p=0.38$).

The expression of AMPA receptors, $GABA_A\alpha 1a$ and synaptophysin

The expression of GluR1 expression did not change significantly in DR-LR compared to dark-reared subjects (Kruskal-Wallis test, *p=0.26*) and in LR-DR compared to control subjects (Kruskal-Wallis test, *p=0.06*) (Figure 4.3N and Figure 4.3.2N). In contrast to the results obtained from homogenate samples, one week of dark-rearing (LR-DR) significantly increased the expression of GluR1 in the PSD fraction compared control subjects (Kruskal-Wallis test, *p=0.019*), while its expression in DR-LR was not significantly different from control subjects (Kruskal-Wallis test, *p=0.121*) (Figure 4.3.1H and Figure 4.3.2R). This is consistent with a previous study by Goel et al (2006) who demonstrated that a brief period of dark-rearing increased surface expression of GluR1 in rat visual cortex. This was further corroborated by findings that one week of dark-rearing produced an increase in mEPSC amplitude that was reversed by re-exposing rats to lighted environment for 2 days (Goel and Lee 2007). Therefore, AMPA receptors bidirectionally and homeostatically modify their expression depending on the recent history of visual experience.

As mentioned previously, dark-rearing from birth increased the expression of $GABA_A\alpha 1a$ compared to control. Similarly LR-DR also elevated $GABA_A\alpha 1$ expression compared control subjects, however, a non-parametric test did not show a significant difference (Kruskal-Wallis test, *p=0.124;* Figure 4.3O and Figure 4.3.2O). Interestingly enough, the expression of $GABA_A\alpha 1a$ remained as elevated in DR-LR subjects as in dark-reared subjects (Figure 4.3O and Figure 4.3.2O). It should be noted that the expression of $GABA_A\alpha 1$ appeared higher in DR-LR than in LR-DR subjects (Figure 4.3O and Figure 4.3.2O) but the difference was not significant (Kruskal-Wallis test, *p=0.121*). In contrast to rapid bidirectional changes of NR2A/NR2B in response to altered sensory experience (Quinlan et al, 1999; Philpot et al, 2001), the expression of GABA and *GAD65* to normal levels takes place over a much longer period of time (2 weeks) after returning dark-reared rats to normal environment (Lee et al, 2006). Therefore, in the present investigation, for $GABA_A1a$ expression to attain levels similar to control in DR-LR subjects, would perhaps require more than a week of sensory experience.

Synaptophysin (Kruskal-Wallis test, DR-LR and DR, *p=0.22* and LR-DR and

control, $p=0.31$) did not appear to be regulated by experience (Figure 4.3P and Figure 4.3.2P) as its expression was indistinguishable in DR-LR and LR-DR compared with control and dark-reared subjects. Therefore it is possible that a week of sensory deprivation is not sufficient to cause a reduction in spine density, however, the results obtained herein need to be validated by using more animals.

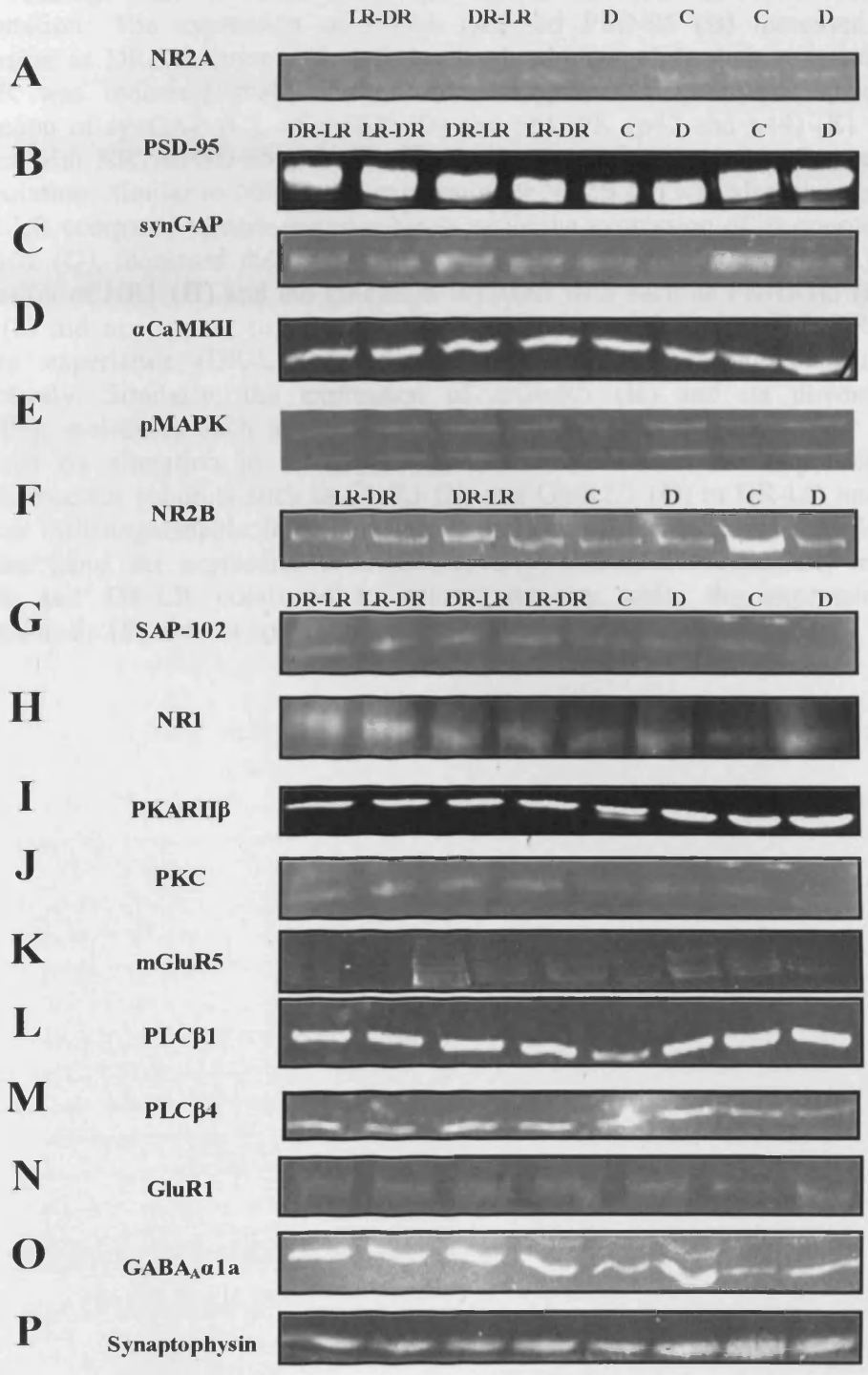


Figure 4.3: *Immunoblots obtained from subjects that were raised in complete darkness from birth followed by a week of visual exposure (**DR-LR**) (n=2) and subjects that were exposed to only a week of darkness after raised in normal environment (**LR-DR**) (n=2).* The gels were run with control (**C**) and dark-reared

(**D**) for direct comparison of expression. The same amount of protein (10μg) was loaded in all the lanes and PKARIIβ was used as an internal control to confirm equal loading. Each of these blots was carried out four times or more for confirmation. The expression of NR2A (**A**) and PSD-95 (**B**) increased their expression in DR-LR compared to dark-reared subjects while their expression in LR-DR was indistinguishable from control subjects. On the other hand, the expression of synGAP (**C**), αCaMKII (**D**) and pMAPK (p42 and p44) (**E**) which interact with NR2A/PSD-95 complex did not appear to be regulated after sensory manipulation. Similar to NR2A, the expression of NR2B (**F**) was also up-regulated in DR-LR compared to dark-reared subjects while the expression of its counterpart, SAP-102 (**G**), increased their expression after sensory deprivation (LR-DR). The expression of NR1 (**H**) and the kinases it is linked with such as PKARIIβ (**I**) and PKC (**J**) did not appear to be regulated after sensory deprivation (LR-DR) and sensory experience (DR-LR) compared to control and dark-reared subjects respectively. Similarly, the expression of mGluR5 (**K**) and its downstream signalling molecules such as PLCβ1 (**L**) and PLCβ4 (**M**) did not appear to be regulated by alteration in sensory experience. Furthermore, the expression of AMPA receptor subunits such as GluR1 (**N**) and GluR2/3 (**O**) in DR-LR and LR-DR was indistinguishable from dark-reared and control subjects respectively. On the other hand, the expression of GABAAα1a (**P**) increased substantially in both LR-DR and DR-LR compared to control subjects while the expression of synaptophysin (**P**) did not appear to be regulated after sensory manipulation.

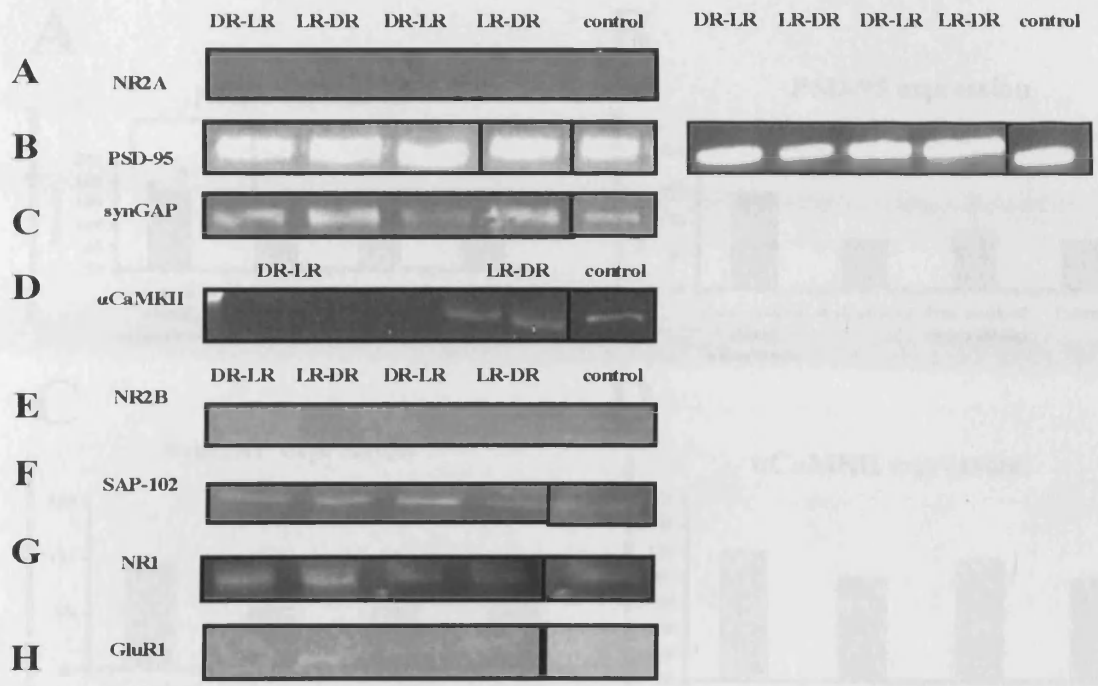
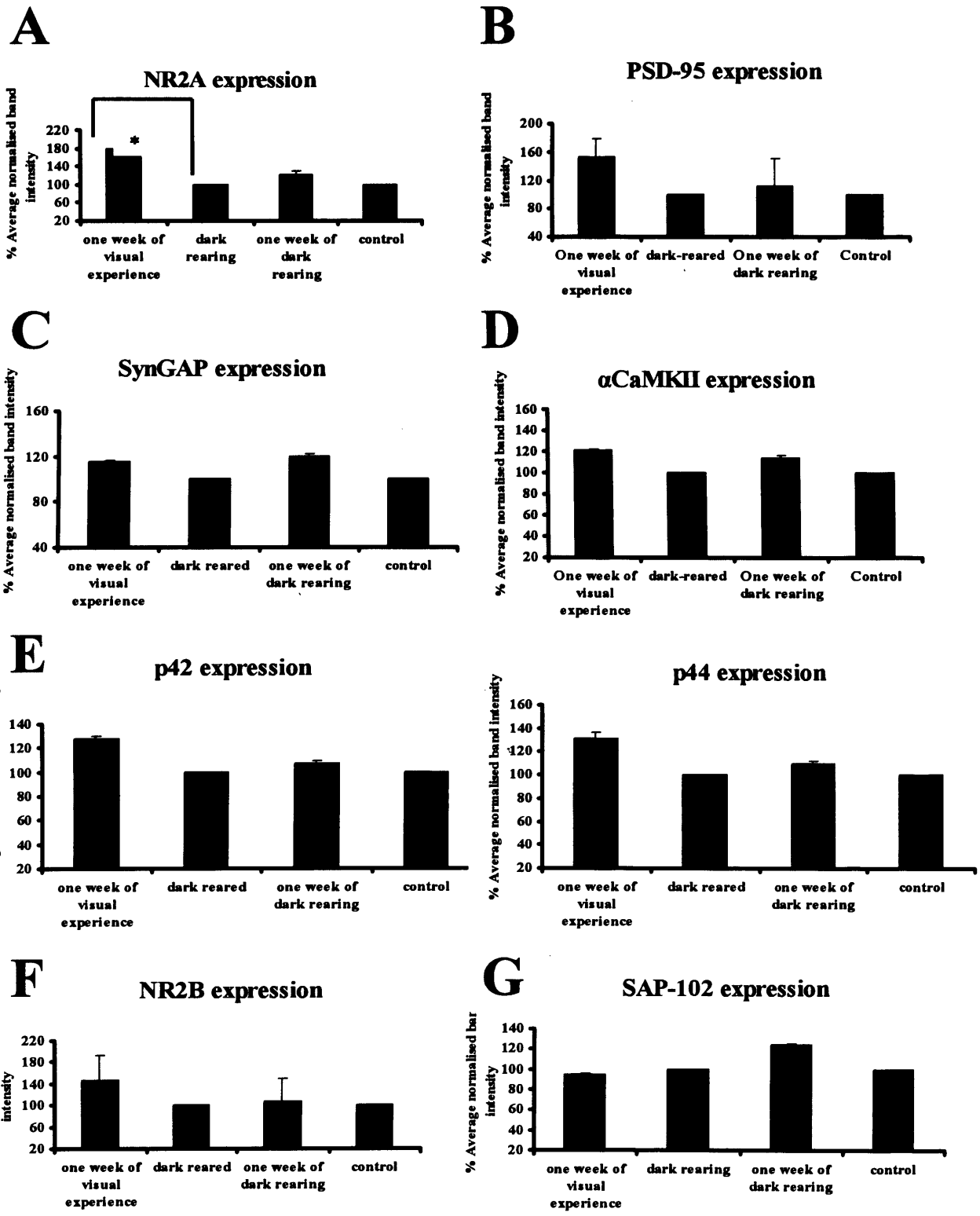
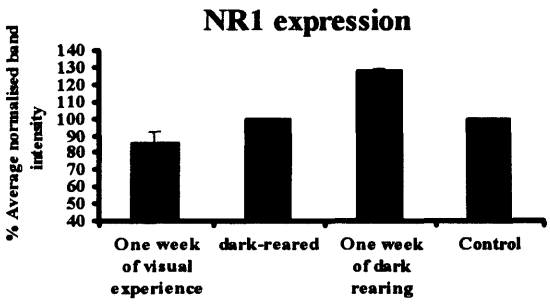


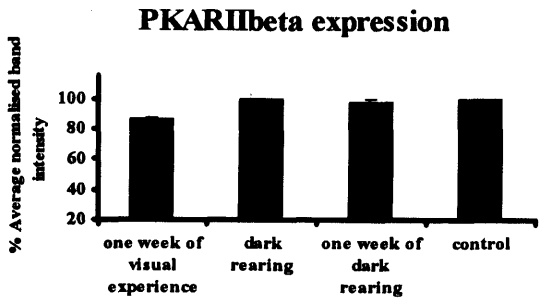
Figure 4.3.1: *Immunublots obtained from PSD fraction obtained from subjects that were raised in complete darkness from birth followed by a week of visual exposure (**DR-LR**) and subjects that were exposed to only a week of darkness (**LR-DR**).* Unfortunately, due to limited amount PSD fraction obtained from dark-reared subjects, only PSD fraction obtained from control were run on the same gel along with samples obtained from DR-LR and LR-DR subjects. For representative purposes, a band corresponding to control animals was attached obtained from other gels. The same amount of protein (30μg) was loaded in all the lanes and β-actin was used as an internal control to confirm equal loading. Each of these blots was carried out four times or more for confirmation. A band corresponding to NR2A (**A**) was not detected in both DR-LR and LR-DR subjects while the expression of PSD-95 was indistinguishable from control subjects (**B**). A blot on the far right is obtained from synaptosomes and also shows that the expression of PSD-95 did not change after sensory deprivation and experience. On the other hand, the expression of synGAP (**C**) was up-regulated in LR-DR while in DR-LR, the expression was not different from control. Interestingly, a band corresponding to αCaMKII was not found in DR-LR subjects while it was expressed in LR-DR (**D**). Similar to NR2A, a band corresponding to NR2B was not detected in DR-LR and LR-DR subjects (**E**) while the expression of SAP-102 did not differ in its expression in DR-LR and LR-DR subjects (**F**). Furthermore, the expression of NR1 (**G**) did not change in DR-LR and LR-DR subjects while the expression of GluR1 (**H**) was elevated in LR-DR subjects.


A
NR2A expression
% Average normalised band intensity
220
180
140
100
60
20
*
one week of visual experience
dark rearing
one week of dark rearing
control
B
PSD-95 expression
% Average normalised band intensity
200
160
120
80
40
One week of visual experience
dark-reared
One week of dark rearing
Control
C
SynGAP expression
% Average normalised band intensity
160
120
80
40
one week of visual experience
dark reared
one week of dark rearing
control
D
αCaMKII expression
% Average normalised band intensity
160
140
120
100
80
60
40
20
One week of visual experience
dark-reared
One week of dark rearing
Control
E
p42 expression
% Average normalised band intensity
140
120
100
80
60
40
20
one week of visual experience
dark reared
one week of dark rearing
control
p44 expression
% Average normalised band intensity
160
140
120
100
80
60
40
20
one week of visual experience
dark reared
one week of dark rearing
control
F
NR2B expression
% Average normalised band intensity
220
180
140
100
60
20
one week of visual experience
dark reared
one week of dark rearing
control
G
SAP-102 expression
% Average normalised bar intensity
140
120
100
80
60
40
20
one week of visual experience
dark rearing
one week of dark rearing
control

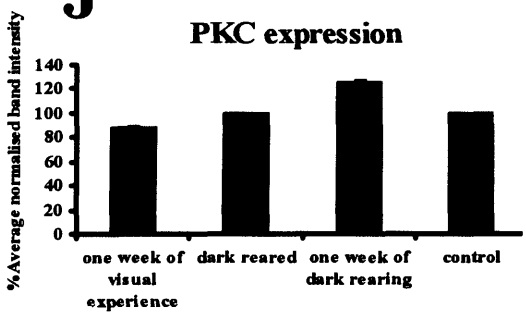
H


NR1 expression
% Average normalised band intensity
140
130
120
110
100
90
80
70
60
50
40
One week of visual experience
dark-reared
One week of dark rearing
Control

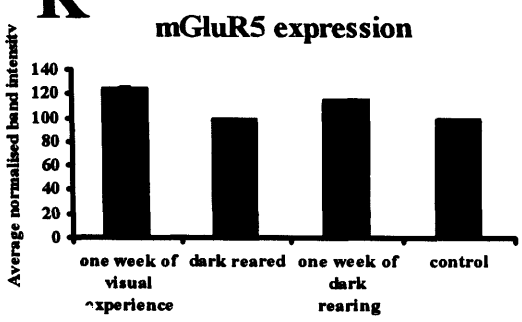
I


PKARIIbeta expression
% Average normalised band intensity
100
80
60
40
20
one week of visual experience
dark rearing
one week of dark rearing
control

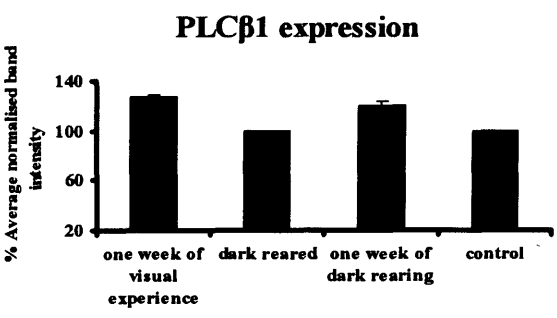
J


PKC expression
%Average normalised band intensity
140
120
100
80
60
40
20
0
one week of visual experience
dark reared
one week of dark rearing
control

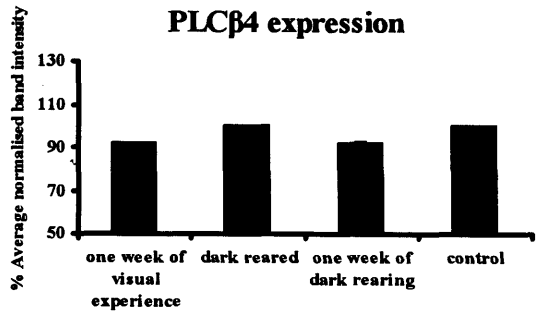
K


mGluR5 expression
Average normalised band intensity
140
120
100
80
60
40
20
0
one week of visual experience
dark reared
one week of dark rearing
control

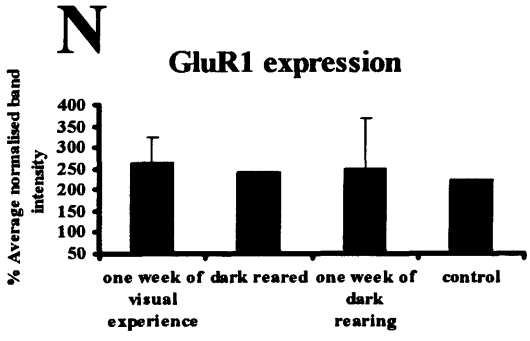
L


PLCβ1 expression
% Average normalised band intensity
140
100
60
20
one week of visual experience
dark reared
one week of dark rearing
control

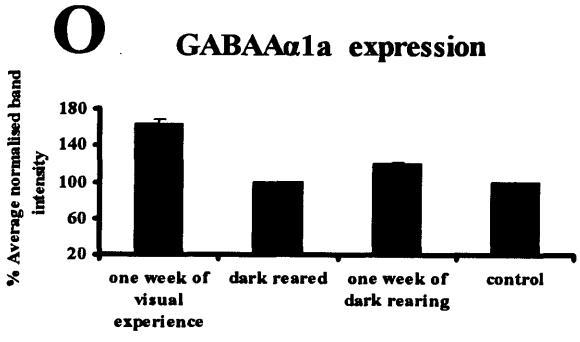
M


PLCβ4 expression
% Average normalised band intensity
130
110
90
70
50
one week of visual experience
dark reared
one week of dark rearing
control

N


GluR1 expression
% Average normalised band intensity
400
350
300
250
200
150
100
50
one week of visual experience
dark reared
one week of dark rearing
control

O


GABAAα1a expression
% Average normalised band intensity
180
140
100
60
20
one week of visual experience
dark reared
one week of dark rearing
control

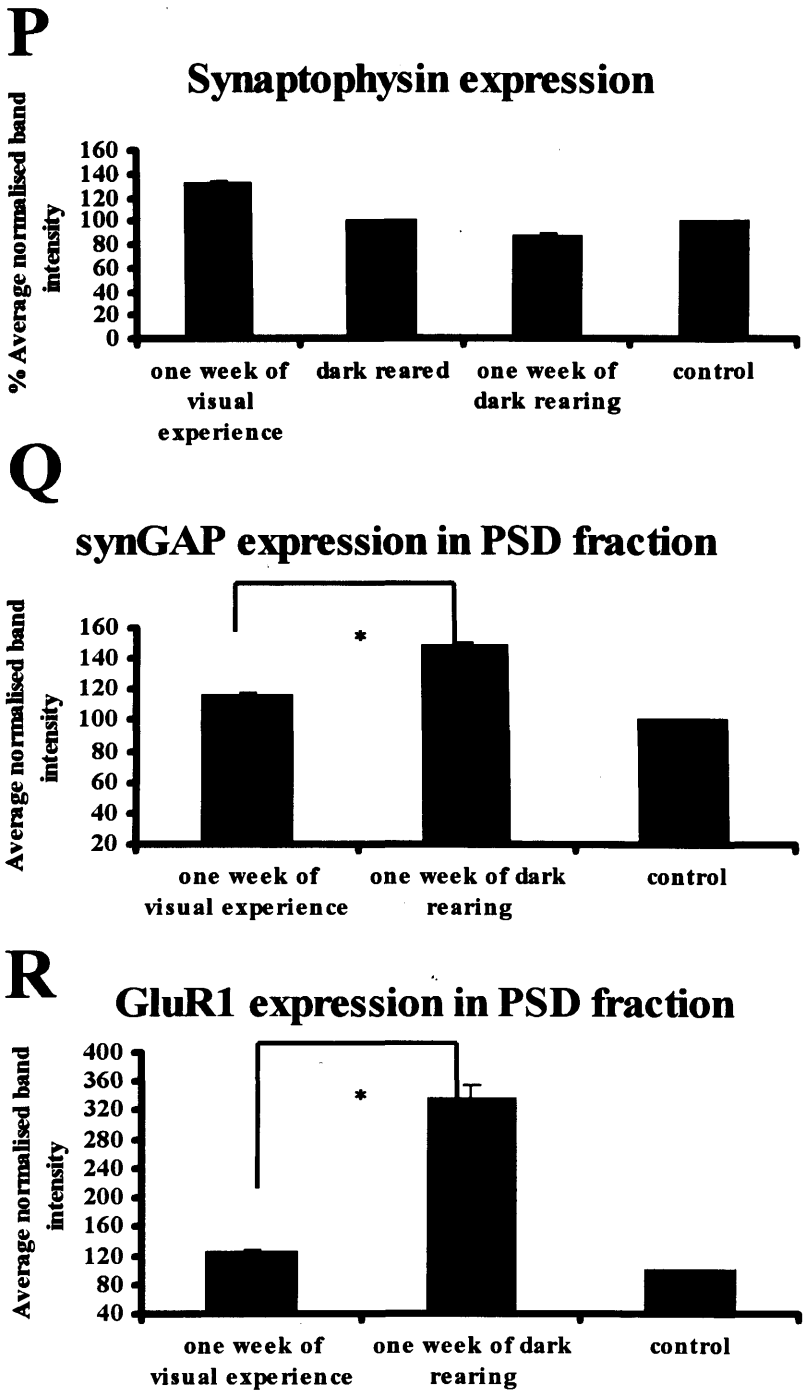


Figure 4.3.2: *Quantitative analysis of immunoblots obtained from samples prepared from visual cortices of subjects that experienced sensory experience and deprivation for a week.* The band intensity was first normalised to the internal control and then the data obtained from DR-LR (one week of sensory experience) was normalised to dark-reared subjects while data obtained from LR-DR (one week of sensory deprivation) was normalised to control, generating two data sets. Since the data is normalised to 100% of control and dark-reared subjects, every data point will be 100% and therefore there is no variation in this sample hence no error bars. The error bars indicate ± SEM (normalised to control or dark-reared). The

expression of NR2A (**A**) and its counterpart, PSD-95 (**B**) was higher in DR-LR subjects compared to dark-reared but only NR2A showed a significant difference. On the other hand, the expression of synGAP (**C**), αCaMKII (**D**) and pMAPK (**E**) did not appear to be regulated after a week of sensory experience and deprivation. However, in the PSD fraction, the expression of synGAP was significantly elevated in LR-DR compared to control (**Q**). Similar to the expression of NR2A, the expression of NR2B was also up-regulated after sensory experience (DR-LR) compared to dark-reared subjects (although the difference was not significant) (**F**) while the expression of SAP-102 increased in LR-DR compared to control subjects (**G**) but the difference was not significant. The expression of NR1 (**H**) and its kinases PKARIIβ (**I**) and PKC (**J**) did not change their expression after sensory experience and deprivation. Similarly, the expression of mGluR5 (**K**) and its signalling molecules such as PLCβ1 (**L**) and PLCβ4 (**M**) did not change after sensory experience and deprivation. Furthermore, the expression of GluR1 subunit (**N**) did not appear to be regulated after sensory alteration, however, its expression in the PSD fraction was elevated significantly in LR-DR compared to control subjects (**R**). The expression of GABAAα1a increased substantially in both LR-DR and DR-LR compared to control (**P**) while the expression of synaptophysin in LR-DR and DR-LR was indistinguishable from control and dark-reared subjects respectively (* denotes $p<0.05$ (Kruskal-Wallis test).

Protein	Up-regulated		Down-regulated		No change	
	H	PSD	H	PSD	H	PSD
NR2A	√					
PSD-95	√					√
synGAP					√	√
αCaMKII				absent	√	
pMAPK					√	
NR2B	√					
SAP-102					√	√
NR1					√	√
PKARIIβ					√	
PKC					√	
mGluR5					√	
PLCβ1					√	
PLCβ4					√	
GluR1					√	√
$GABA_A\alpha 1a$					√	
synaptophysin					√	

Table 2: ***A summary of results showing changes in expression of proteins in subjects raised in complete darkness followed by a week of sensory experience (DR-LR) relative to dark-reared in homogenate (H) and in post synaptic density (PSD).*** The molecular substrates are tabulated in the order they complex with receptor subunits, and substrates belonging to one complex are indicated by the same colour. However, GluR1 subunit even though it binds to NMDA receptor indirectly, it has been colour coded differently to show a different receptor subunit, while $GABA_A\alpha 1a$ and synaptophysin do not form a complex NMDA receptor complex and therefore they fall in a different group indicated by a different colour. It should be noted that some downstream signalling molecules may be linked to NMDA receptor complex in multiple locations. The expression of NR2A and PSD-95 is up-regulated after sensory experience but only NR2A showed a significant

difference (denoted by√). In the PSD fraction, however, the expression of PSD-95 was not different from control. On the other hand, the expression of synGAP, αCaMKII and pMAPK did not appear to be regulated by sensory experience, however, in the PSD fraction, there was no band corresponding to αCaMKII. The expression of NR2B was up-regulated while the expression of its counterpart, SAP-102, was not different from dark-reared subjects. The expression of NR1 and its kinases, PKARIIβ and PKC did not appear to be regulated by sensory experience. Similarly, the expression of mGluR5 and its downstream signalling molecules such as PLCβ1 and PLCβ4 was not regulated by sensory experience. The expression of GluR1 and synaptophysin was not regulated by sensory experience while the expression of $GABA_A\alpha 1a$ was up-regulated after sensory experience.

Protein	Up-regulated		Down-regulated		No change	
	H	PSD	H	PSD	H	PSD
NR2A					√	
PSD-95					√	√
synGAP		√			√	
αCaMKII					√	√
pMAPK					√	
NR2B					√	
SAP-102	√					√
NR1					√	√
PKARIIβ					√	
PKC					√	
mGluR5					√	
PLCβ1					√	
PLCβ4					√	
GluR1		√			√	
$GABA_A\alpha 1a$	√					
synaptophysin					√	

Table 3: ***A summary of results showing changes in expression of proteins in subjects raised in normal environment followed by a week of dark-rearing (LR-DR) relative control in homogenate (H) and in post synaptic density (PSD).*** The molecular substrates are tabulated in the order they complex with receptor subunits, and substrates belonging to one complex are indicated by the same colour. However, GluR1 subunit even though it binds to NMDA receptor indirectly, it has been colour coded differently to show a different receptor subunit, while $GABA_A\alpha 1a$ and synaptophysin do not form a complex with NMDA receptor complex and therefore they fall in a different group indicated by a different colour. It should be noted that some downstream signalling molecules may be linked to NMDA receptor complex in multiple locations. The expression of NR2A and

signalling molecules it complexes with such as PSD-95, synGAP, αCaMKII and pMAPK did not change after a week of dark -rearing. However, in the PSD fraction, the expression of synGAP was significantly (denoted by √) up-regulated after a week of dark-rearing. The expression of NR2B did not change while the expression of SAP-102 was up-regulated after a week of dark-rearing but only in the homogenate. The expression of NR1 and kinases such as PKARIIβ and PKC did not appear to be regulated after a week of dark-rearing. Similarly, the expression of mGluR5 and downstream signalling molecules such as PLCβ1 and PLCβ4 did not change after a week of dark-rearing. On the other hand, the expression of GluR1 was not different from control in the homogenate but in the PSD fraction, its expression was significantly elevated (denoted by √). The expression of $GABA_A\alpha 1a$ was up-regulated relative to control while the expression of synaptophysin was not different from control.

4.3.4 Monocular deprivation

Since the pioneering studies by Hubel and Wiesel, several studies have been carried out to underpin the physiological and the molecular mechanisms underlying experience-dependent plasticity. There are several advantages in understanding the molecular as well as physiological basis of OD plasticity. Firstly, the processes involved in OD plasticity help us to understand how cortical circuitry is refined in response to qualities of sensory experience during development. Secondly, the rapid OD plasticity induced by MD is one of the most widely studied cellular examples of learning and memory in the brain. Thirdly, the detailed physiological and molecular understanding of how synaptic connections are weakened and strengthened by sensory deprivation will offer therapy to overcome amblyopia (Smith et al, 2009).

Because of the high degree of decussation of the retinofugal projection in rodents; even the binocular regions of visual cortex are dominated by input from the contralateral eye. This is in contrast to cat visual cortex, where there is only partial decussation of optic fibers and therefore the contralateral bias is not as great as in rodents. I therefore carried out immunoblotting experiments in both contralateral and ipsilateral hemispheres to the deprived eye. In order to make a direct comparison, the samples (contralateral and ipsilateral) from 2 days and 7 days of MD were run on the same gel along with control subjects of the same age. When quantifying the band intensity, I normalised MD (contralateral and ipsilateral) to control and, therefore every data point for this group will be 100%. For statistical purposes, Kruskal-Wallis test (non-parametric test) was carried out to determine expression levels of proteins relative to control.

The expression of NR2A and its complex after monocular deprivation

Despite my numerous attempts, a band corresponding to NR2A could not be obtained from contralateral and ipsilateral hemispheres after 2 and 7 days of MD in the homogenate (Figure 4.4A) and in the PSD fraction (Figure 4.4.1A). The same NR2A antibody was used as in other experimental conditions and therefore it was surprising

that no band could be detected. NR2A is known to bind to PSD-95, and the expression of the latter did not change its expression in the contralateral (Kruskal-Wallis test: $p=0.121$) and in the ipsilateral hemispheres (Kruskal-Wallis test: $p=0.98$) after 7 days of MD relative to control. Similarly, its expression was indistinguishable in contralateral (Kruskal-Wallis test: $p=0.12$) and ipsilateral hemispheres (Kruskal-Wallis test: $p=0.11$) after 2 days of MD (Figure 4.4B and Figure 4.4.3A).Further statistical analysis revealed that there was no interhemispheric difference after 2 (Kruskal-Wallis test: $p= 0.28$) and 7 days of MD (Kruskal-Wallis test, $p=0.19$). However, in synaptosomes and in the PSD fraction, the expression of PSD-95 was significantly higher in the contralateral compared to ipsilateral hemisphere after 7-days MD (Kruskal-Wallis test, $p=0.03$). On the other hand, 2-days of MD resulted in significant reduction in both the contralateral and ipsilateral hemispheres relative to control (Kruskal-Wallis test: $p=0.02$, Figure 4.4.1B and Figure 4.4.3P).

Another downstream signalling molecule, synGAP (which binds to NR2A and PSD-95 complex), was significantly higher after 7-days of MD relative to control (contralateral hemisphere: Kruskal-Wallis test, $p=0.02$ and ipsilateral hemisphere: Kruskal-Wallis test, $p=0.019$; Figure 4.4C and Figure 4.4.3B) and there was no interhemispheric difference in its expression (Kruskal-Wallis test, $p=0.386$). However, in the PSD fraction, the expression of synGAP was higher in the contralateral compared to the ipsilateral hemisphere after 7-days of MD (Figure 4.4.1C). In contrast to 7 days of MD, MD for 2 days resulted in significantly reduced expression of synGAP in the contralateral (Kruskal-Wallis test: $p=0.02$) and in the ipsilateral hemisphere (Kruskal-Wallis test: $p=0.01$) relative to control and there was no difference in its expression between the two hemispheres (Kruskal-Wallis test: $p=0.262$) (Figure 4.4C and Figure 4.4.3B)

The complex NR2A/PSD-95/synGAP is known to bind to αCaMKII (Oh et al, 2004). The expression of αCaMKII was higher in both the hemispheres after 7-days of MD relative to control, although a non-parametric test did not show a significant difference (contralateral hemisphere: Kruskal-Wallis test, $p=0.15$ and ipsilateral

hemisphere: Kruskal-Wallis test, $p=0.20$). In addition, there was no interhemispheric difference in the expression of αCaMKII after 7 days of MD (Kruskal-Wallis test, $p=0.127$). In contrast, after 2-days of MD, the expression of αCaMKII was indistinguishable from control (contralateral hemisphere: Kruskal-Wallis test, $p=0.513$ and ipsilateral hemisphere: Kruskal-Wallis test, $p=0.51$ (Figure 4.4D and Figure 4.4.3C)). A previous study has shown that autophosphorylation of αCaMKII is necessary for OD plasticity (Taha et al, 2002) and mice with αCaMKII knock-out failed to show measurable plasticity in response to MD after 4 days of MD but prolonged period of MD induced OD plasticity indistinguishable from wild types (Taha and Stryker, 2005). This suggests that αCaMKII plays a role in OD plasticity; however, its expression may not necessarily be regulated by experience. In the PSD fraction, the expression of αCaMKII was not detected in either hemisphere after 7-days MD while after 2-days of MD, there appeared to be elevated expression of αCaMKII in the contralateral compared to the ipsilateral hemisphere (Figure 4.4.1D).

The expression of phosphorylated form of MAPK, specifically p42 and p44, did not appear to be regulated by activity after 2 days and 7 days of MD relative to control (2 days of MD: p42; contralateral hemisphere: Kruskal-Wallis test $p=0.33$ and ipsilateral hemisphere: Kruskal-Wallis test, $p=0.43$ and p44 contralateral hemisphere: Kruskal-Wallis test $p=0.07$ and ipsilateral hemisphere: Kruskal-Wallis test $p=0.09$) (7 days of MD: p42; contralateral hemisphere: Kruskal-Wallis test, $p=0.35$ and ipsilateral hemisphere: Kruskal-Wallis test $p=0.43$ and p44 contralateral hemisphere: Kruskal-Wallis test $p=0.17$ and ipsilateral hemisphere: Kruskal-Wallis test $p=0.27$ (Figure 4.4E and Figure 4.4.3D)). This is in contrast to a study by Majdan and Shatz (2006) which demonstrated that MAPK was not itself regulated trancriptionally by monocular enucleation, but protein levels of phosphorylated MAPK were decreased in the deprived cortex (Majdan and Shatz, 2006). It should however be noted that monocular enucleation is likely to induce dramatic secondary effects due to deafferentation (Rio and Feller, 2006) and therefore may employ different molecular substrates for functional effects.

The expression of NR2B and SAP-102 after monocular deprivation

The expression levels of NR2B was not different from control in both contralateral (Kruskal-Wallis test, $p=0.513$) and ipsilateral hemispheres (Kruskal-Wallis test, $p=0.827$) after 7 days of MD. In addition, there was no inter-hemispheric difference in the expression of NR2B after 7 days of MD (Kruskal-Wallis test, $p=0.513$). In contrast, after 2-days MD, a band corresponding to NR2B was not detected in either contralateral or ipsilateral hemispheres to the deprived eye (Figure 4.4F and Figure 4.4.3E). This is in contrast to a study by Chen and Bear, (2007), which showed that following 3 days of MD, NR2B levels were significantly elevated in the contralateral hemisphere to the DE in mouse visual cortex. In the PSD fraction, a band corresponding to NR2B was not detected in either contralateral or ipsilateral hemispheres after 7 and 2 days MD (Figure 4.4.1E). On the other hand, the expression level of SAP-102 was not different from control in the contralateral (Kruskal-Wallis test, $p=0.513$) and the ipsilateral hemispheres (Kruskal-Wallis test, $p=0.49$) after 7 days of MD and there was no interhemispheric difference in its expression (Kruskal-Wallis test, $p=0.96$). Similarly, after 2 days of MD, the expression of SAP-102 was indistinguishable from control (contralateral hemisphere: Kruskal-Wallis test, $p=0.127$ and ipsilateral hemisphere: Kruskal-Wallis test, $p=0.186$) and its expression was not different between two hemispheres (Kruskal-Wallis test: $p=0.80$ (Figure 4.4G and Figure 4.4.3F)). Unfortunately due to depletion of SAP-102 antibody, its expression in the PSD fraction could not be determined.

The expression of NR1 and kinases after monocular deprivation

The expression level of NR1 in the contralateral (Kruskal-Wallis test: $p=1.00$) and the ipsilateral hemisphere (Kruskal-Wallis test: $p=0.439$) to the deprived eye was indistinguishable from control and there was no interhemispheric difference in its expression (Kruskal-Wallis test: $p=0.44$). Similarly, the expression of NR1 was indistinguishable from control after 2 days of MD in the contralateral (Kruskal-Wallis test: $p=0.413$) and in the ipsilateral hemisphere (Kruskal-Wallis test: $p=0.55$) and there was no difference in its expression between the two hemispheres (Kruskal-Wallis: $p=0.642$) (Figure 4.4H and Figure 4.4.3G). Furthermore, in PSD fraction, the

expression of NR1 was indistinguishable from control and after 2 and 7 days of MD (Figure 4.4.1F).

Similarly, the expression of PKARIIβ was not different from control after 2 and 7 days of MD (2 days of MD: contralateral hemisphere; Kruskal-Wallis test, $p=0.36$ and ipsilateral hemisphere; Kruskal-Wallis test, $p=0.16$) (7 days of MD: contralateral hemisphere; Kruskal-Wallis test, $p=0.38$ and ipsilateral hemisphere; Kruskal-Wallis test, $p=0.26$ (Figure 4.4I and Figure 4.4.3H)). Likewise, the expression of PKC was indistinguishable from control after 2 days (contralateral hemisphere; Kruskal-Wallis test, $p=0.22$ and ipsilateral hemisphere; Kruskal-Wallis test, $p=0.16$) and 7 days of MD (contralateral hemisphere; Kruskal-Wallis test, $p= 0.25$ and ipsilateral hemisphere; Kruskal-Wallis test, $p=0.66$ (Figure 4.4J and Figure 4.4.3I)).

The expression of mGluR5 and downstream signalling molecules after monocular deprivation

The expression of mGluR5 was indistinguishable from control after 7 days of MD in the contralateral (Kruskal-Wallis test: $p=0.542$) and in the ipsilateral (Kruskal-Wallis test: $p=0.84$) hemispheres relative to control and there was no interhemispheric difference in its expression (Kruskal-Wallis test: $p=0.92$). Similarly, the expression of mGluR5 was not different from control after 2 days of MD in either contralateral (Kruskal-Wallis test: $p=0.66$) or ipsilateral hemispheres (Kruskal-Wallis test: $p=0.78$) and its expression was not different between the two hemispheres (Kruskal-Wallis test: $p=0.54$) (Figure 4.4K and Figure 4.4.3J).

Likewise, the expression of PLCβ1 (Figure 4.4L and Figure 4.4.3K) was indistinguishable from control after 2 days of MD in both the contralateral (Kruskal-Wallis test, $p=0.92$) and the ipsilateral hemisphere (Kruskal-Wallis test, $p=0.62$). Furthermore, the expression of PLCβ1 was not different from control after 7 days of MD in both the contralateral (Kruskal-Wallis test, $p=0.340$) and the ipsilateral (Kruskal-Wallis test, $p=0.461$) hemispheres. Similarly the expression of PLCβ4 (Figure 4.4M and Figure 4.4.3L) was not regulated by activity after 2 and 7 days of

MD in both the contralateral and the ipsilateral hemispheres relative to control (2 days of MD: contralateral hemisphere; Kruskal-Wallis test, $p=0.22$ and ipsilateral hemisphere; Kruskal-Wallis test,, $p=0.60$) (7 days of MD: contralateral hemisphere; Kruskal-Wallis test, $p=0.34$ and ipsilateral hemisphere; Kruskal-Wallis, $p=0.52$).

The expression of AMPA receptors, $GABA_A\alpha 1a$ and synaptophysin after monocular deprivation

The expression of GluR1 subunit was not significantly different from control in the contralateral (Kruskal-Wallis test, $p=0.483$) and in the ipsilateral hemispheres (Kruskal-Wallis test, $p=0.121$) after 7-days MD. In addition, the expression of GluR1 did not reveal any interhemispheric differences (Kruskal-Wallis test, $p=0.439$). In contrast, 2 days of MD resulted in reduced expression of GluR1 subunits in both the contralateral and the ipsilateral hemispheres relative to control, however a non-parametric did not show a significant difference (Kruskal-Wallis, $p=0.12$) (Figure 4.4N and Figure 4.4.3M). In addition, there was no interhemispheric difference in the expression of GluR1 between contralateral and ipsilateral hemispheres after 2 days of MD (Kruskal-Wallis test, $p=0.121$). This is consistent with the results obtained by Heynen et al, 2003 which demonstrated that brief MD (24 hours) in rats is accompanied by a loss of surface-expressed GluR1 and GluR2 subunits in the hemisphere contralateral to the deprived eye. In the PSD fraction, a band corresponding to GluR1 was not detected in either MD experimental conditions (Figure 4.4.1G).

Several studies have been carried out to determine the role of GABAergic inhibition in OD plasticity but with contrasting results. For instance, MD in cats did not result in any detectable effects on the distribution of *GAD* activity in the binocular and monocular zone and therefore the effect of MD does not appear to require emergence of GABAergic interneurons (Bear et al, 1985). In contrast, another study by Shaw and Cynader (1988) demonstrated that $GABA_A$ receptor densities were 100% higher after MD in all layers of cat visual cortex compared to normal counterparts. Another study by Maffei et al (2006) demonstrated that MD increases the amplitude of IPSC

by threefold in the monocular zone of the rat visual cortex. This suggests that potentiation of inhibition is one of the cellular mechanisms underlying the deprivation-induced degradation of visual function (Maffei et al, 2006). The present study showed that the expression of $GABA_A\alpha1a$ receptor subunit was not different from control after 2 and 7 days of MD (2 days of MD: contralateral hemisphere; Kruskal-Wallis test, $p=0.230$ and ipsilateral hemisphere; Kruskal-Wallis test, $p=0.18$) (7 days of MD: contralateral hemisphere; Kruskal-Wallis test, $p=0.102$ and ipsilateral hemisphere; Kruskal-Wallis test, $p=0.121$). In addition, there was no interhemispheric difference after 2 days (Kruskal-Wallis test, $p=0.994$) and 7 days (Kruskal-Wallis test, $p=0.586$) of MD (Figure 4.4O and Figure 4.4.3N).

The expression of synaptophysin was indistinguishable from control after 2 days (contralateral hemisphere; Kruskal-Wallis test, $p=0.66$ and ipsilateral hemisphere; Kruskal-Wallis test, $p=0.78$) and 7 days (contralateral hemisphere; Kruskal-Wallis test, $p=0.80$ and ipsilateral hemisphere; Kruskal-Wallis test, $p=0.37$ (Figure 4.4P and Figure 4.4.3O). This is consistent with a previous study by Silver and Stryker (2000) which have shown using immunohistochemistry that the expression of synaptic vesicle proteins (such as synaptophysin and synaptotagmin) did not show difference in expression in columns dominated by the deprived eye and non-deprived eye after 2 and 7 days of MD in kitten visual cortex (Silver and Stryker, 2000).

Changes in protein expression after MD with frosted lens in the PSD fraction

In addition, I also prepared PSD fractions from subjects that underwent MD with frosted lens. Since most of the biochemical changes associated with MD with lid suture (after 7 days) were observed at the PSD site, I wanted to determine if the changes observed were similar using MD with frosted lens. PSD fractions obtained from MD with lid suture and MD with frosted lens were run on the same gel along with the PSD fraction obtained from control subjects. However, in certain experiments PSD fraction obtained from control subjects was exhausted and therefore only comparison with previously run gels were made. Similar to MD with lid suture, MD with frosted lens did not a show a band corresponding to NR2A (Figure 4.4.2A).

4.3 Results

Similar to MD with lid suture, MD with frost lens also resulted in significant elevation of PSD-95 in the contralateral hemisphere compared to the ipsilateral hemisphere (Kruskal-Wallis test, $p=0.02$) (Figure 4.4.2B) while a band corresponding to αCaMKII was not observed (Figure 4.4.2C). In addition, similar to MD with lid suture, the PSD fraction obtained from subjects with MD with frosted lens did not show any detectable band corresponding to NR2B (Figure 4.4.2D). NR1 expression did not change significantly in either of the hemispheres after MD with frosted lens compared with control (contralateral hemisphere, (Kruskal-Wallis test, $p=0.068$ and ipsilateral hemisphere, Kruskal-Wallis test, $p=0.54$) (Figure 4.4.2E). And in accordance with the data obtained from MD with lid suture, the PSD fraction obtained from subjects with MD with frosted lens did not show a band corresponding to GluR1 (Figure 4.4.2F). It appears that the molecular changes observed at the PSD site are similar regardless of the method of form deprivation, suggesting that the effects observed here share a common molecular mechanism.

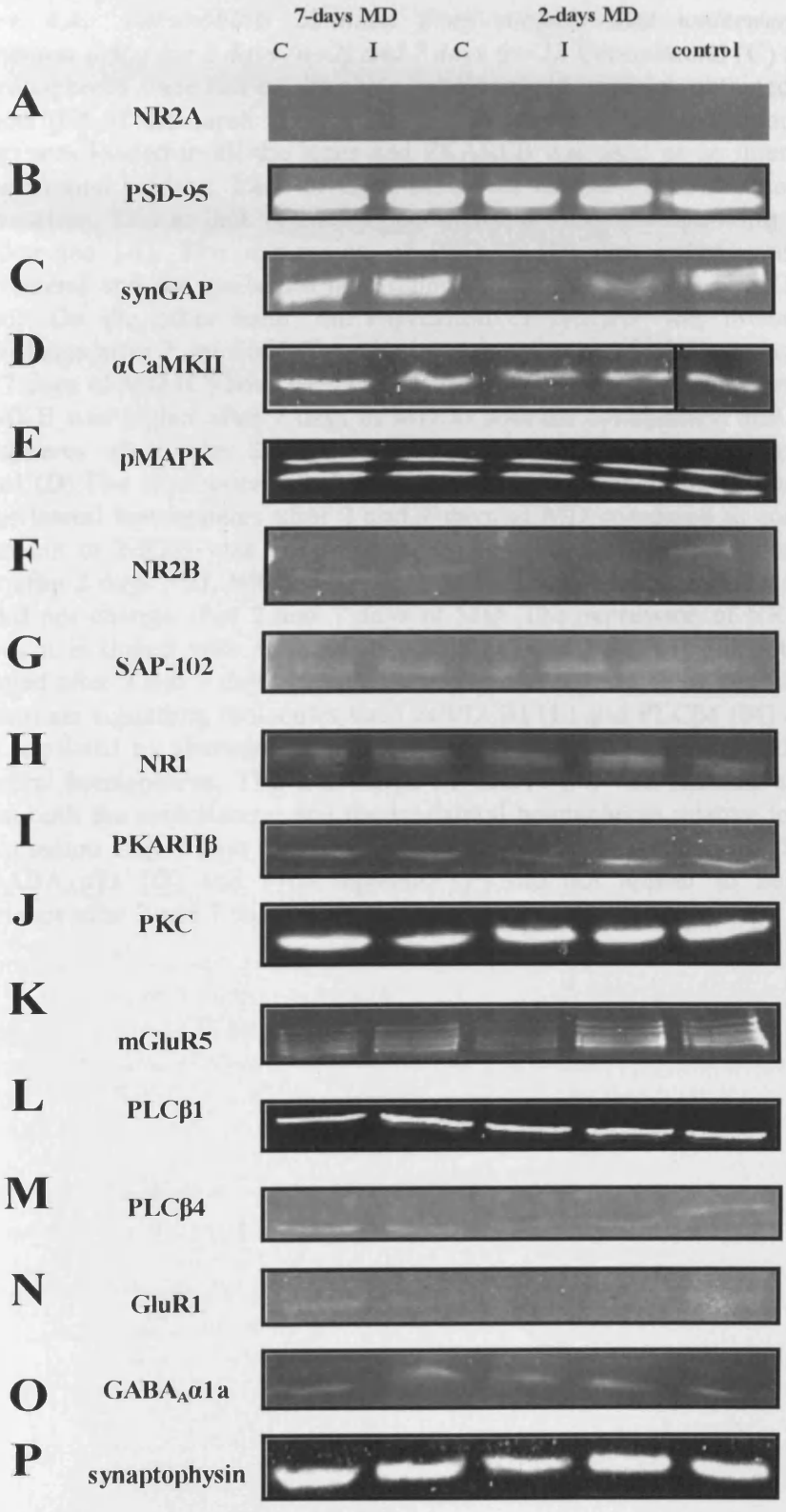

7-days MD
2-days MD
C
I
C
I
control
A
NR2A
B
PSD-95
C
synGAP
D
αCaMKII
E
pMAPK
F
NR2B
G
SAP-102
H
NR1
I
PKARIIβ
J
PKC
K
mGluR5
L
PLCβ1
M
PLCβ4
N
GluR1
O
GABA$_A$α1a
P
synaptophysin

Figure 4.4: *Immunoblots obtained from subjects that underwent monocular deprivation (MD) for 2 days (n=2) and 7 days (n=2).* Contralateral (**C**) and Ipsilateral (**I**) hemispheres were run on the same gel along with samples obtained from control subjects (**C**) of the same age for direct comparison. The same amount of protein (10μg) was loaded in all the lanes and PKARIIβ was used as an internal control to confirm equal loading. Each of these blots was carried out four times or more for confirmation. Due to lack of NR2A specificity, a band corresponding to NR2A was not detected (**A**). The expression of PSD-95 (**B**) was indistinguishable in the contralateral and the ipsilateral hemispheres after 2 and 7 days of MD compared to control. On the other hand, the expression of synGAP was lower in both the hemispheres after 2 days of MD while its expression was higher compared to control after 7 days of MD (**C**) compared to control. In contrast to synGAP, the expression of αCaMKII was higher after 7 days of MD in both the contralateral and the ipsilateral hemispheres while after 2 days of MD, its expression was indistinguishable from control (**D**).The expression of pMAPK was not different in the contralateral and in the ipsilateral hemispheres after 2 and 7 days of MD compared to control (**E**). The expression of NR2B was not different in both the hemispheres after 7-days MD, while after 2 days MD, NR2B was not detected (**F**) while the expression of SAP-102 (**G**) did not change after 2 and 7 days of MD. The expression of NR1 (**H**) and the kinases it is linked with such as PKARIIβ (**I**) and PKC (**J**) did not appear to be regulated after 2 and 7 days of MD. Similarly, the expression of mGluR5 (**K**) and its downstream signalling molecules such as PLCβ1 (**L**) and PLCβ4 (**M**) did not appear to be regulated by alteration in sensory experience in both the contralateral and the ipsilateral hemispheres. The expression of GluR1 (**N**) was reduced after 2 days of MD in both the contralateral and the ipsilateral hemispheres relative to control while its expression after 7 days of MD was indistinguishable from control. The expression of $GABA_A\alpha 1a$ (**O**) and synaptophysin (**P**) did not appear to be regulated by experience after 2 and 7 days of MD.

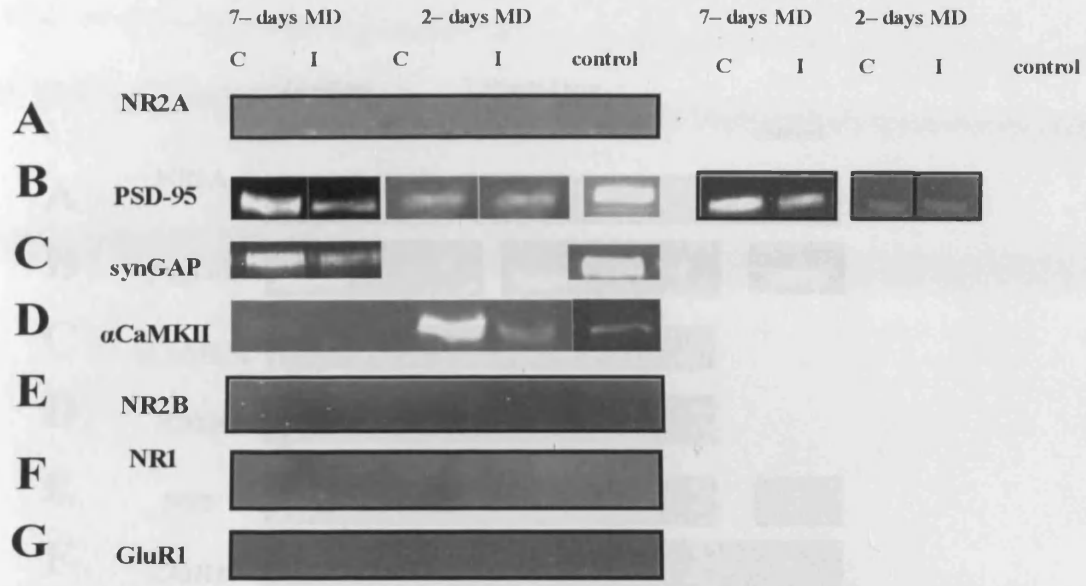


Figure 4.4.1: *Immunoblots obtained from PSD fraction of 7-days MD and 2-days MD.* Contralateral (**C**) and ipsilateral (**I**) hemispheres were run on the same gel, but due to limited amount of PSD fraction obtained from control subjects, they could not be run on the same gel. The same amount of protein (30μg) was loaded in all the lanes and β-actin was used as an internal control to confirm equal loading. Each of these blots was carried out four times or more for confirmation. A band corresponding to NR2A (**A**) was not detected in both hemispheres after 7 days and 2 days MD. On the other hand, the expression of PSD-95 was elevated in the contralateral hemisphere compared to the ipsilateral hemisphere after 7 days of MD, while 2 days of MD resulted in reduction in both the hemispheres compared to control (**B**) and in the synaptosomes (on the far right). The expression of synGAP was higher in the contralateral hemisphere compared to ipsilateral hemisphere (**C**) after 7 days of MD; however, due to lack of availability of synGAP antibody, its expression in the PSD fraction after 2 days of MD could not be determined. The expression of αCaMKII was not detected in either the contralateral or the ipsilateral hemispheres after 7 days MD while 2 days of MD resulted in significant elevation in the contralateral hemisphere compared to ipsilateral hemisphere (**D**). A band corresponding to NR2B was not detected after 2 and 7 days of MD in the PSD fraction (**E**). Consistent with data obtained from homogenate, in the PSD fraction, NR1 expression was not different in the contralateral and the ipsilateral hemispheres after 2 and 7 days MD (**F**). On the other hand, a band corresponding to GluR1 was not detected after 2 and 7 days of MD (**G**).

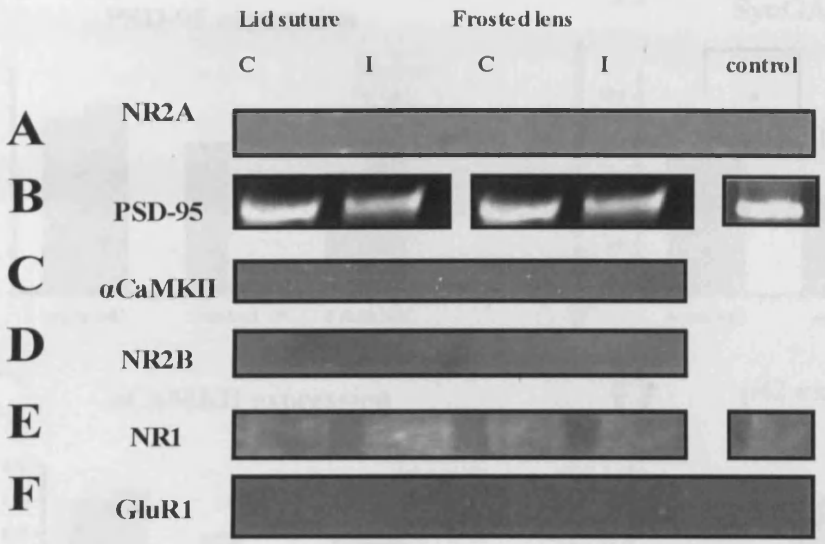
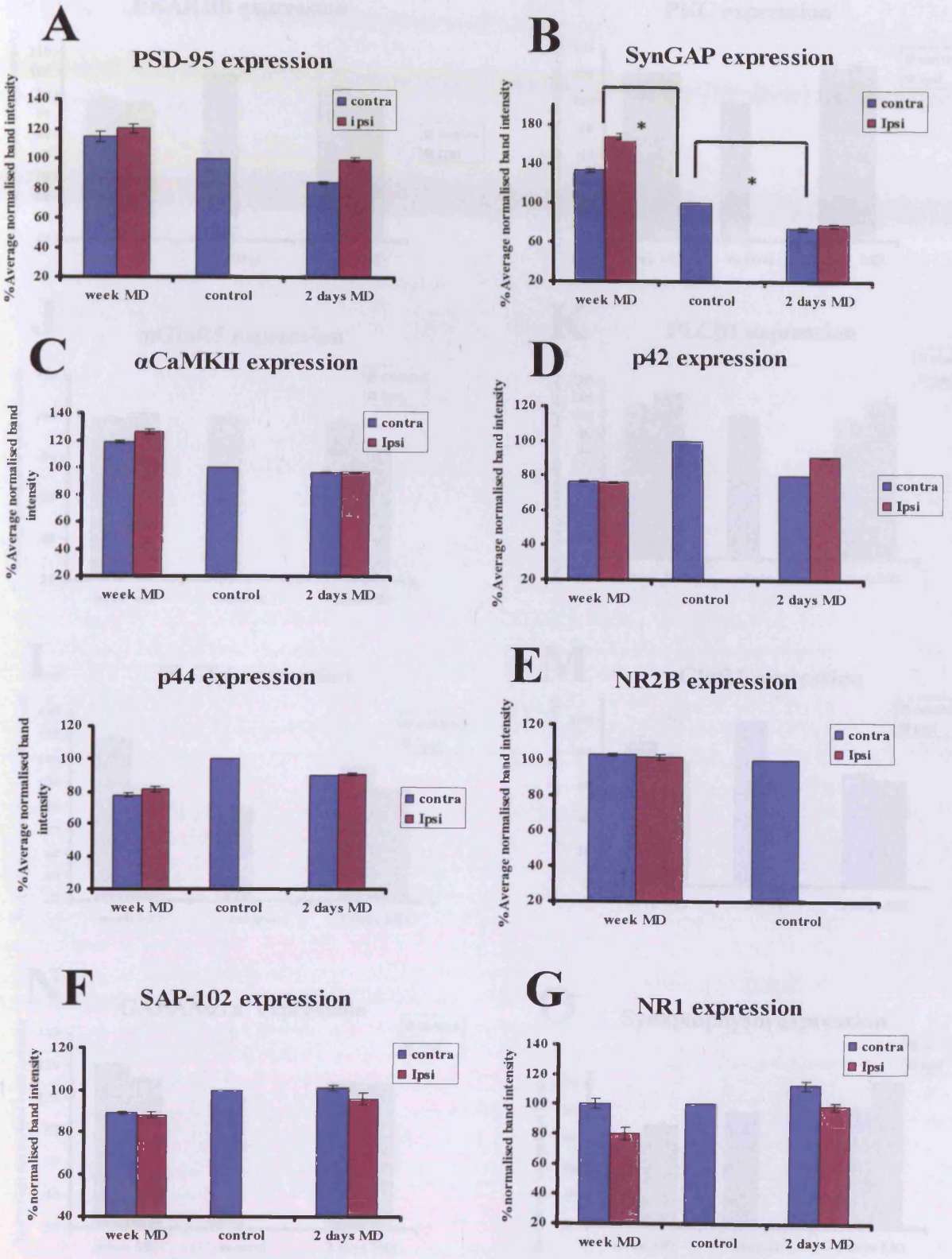
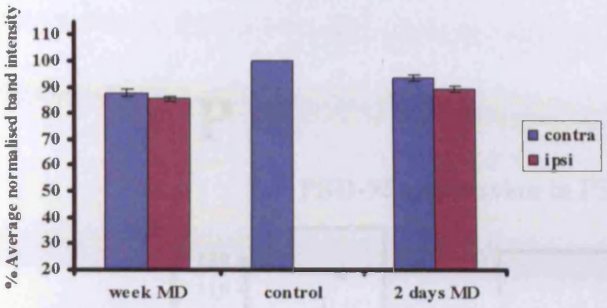


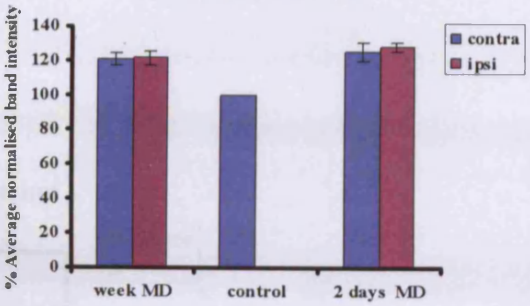
Figure 4.4.2*: Immunoblots obtained from PSD fraction of MD with lid suture and MD with frosted lens.* Contralateral (**C**) and ipsilateral (**I**) hemispheres were run on the same gel, but due to limited amount of PSD fraction obtained from control subjects, they could not always be run on the same gel. The same amount of protein (30μg) was loaded in all the lanes and β-actin was used as an internal control to confirm equal loading. Each of these blots was carried out four times or more for confirmation. Similar to PSD fraction obtained from MD with lid suture, a band corresponding to NR2A was not detected (**A**). On the other hand, the expression of PSD-95 was elevated in the contralateral hemisphere than in the ipsilateral hemisphere (**B**). Similar to MD with lid suture, a band corresponding to αCaMKII (**C**) and NR2B (**D**) was not detected after MD with frosted lens. The expression of NR1 was indistinguishable from control after MD with frosted lens (**E**) while a band corresponding to GluR1 was not detected (**F**).


A
PSD-95 expression
%Average normalised band intensity
contra
ipsi
week MD
control
2 days MD
B
SynGAP expression
%Average normalised band intensity
contra
Ipsi
*
*
week MD
control
2 days MD
C
αCaMKII expression
%Average normalised band intensity
contra
Ipsi
week MD
control
2 days MD
D
p42 expression
%Average normalised band intensity
contra
Ipsi
week MD
control
2 days MD
p44 expression
%Average normalised band intensity
contra
Ipsi
week MD
control
2 days MD
E
NR2B expression
%Average normalised band intensity
contra
Ipsi
week MD
control
F
SAP-102 expression
%normalised band intensity
contra
Ipsi
week MD
control
2 days MD
G
NR1 expression
%normalised band intensity
contra
Ipsi
week MD
control
2 days MD

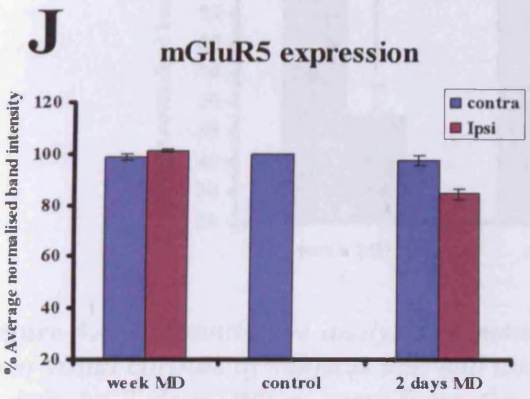
H


PKARIIβ expression
% Average normalised band intensity
110
100
90
80
70
60
50
40
30
20
contra
ipsi
week MD
control
2 days MD

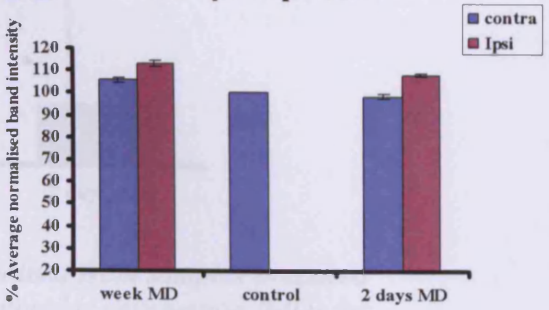
I


PKC expression
% Average normalised band intensity
140
120
100
80
60
40
20
0
contra
ipsi
week MD
control
2 days MD

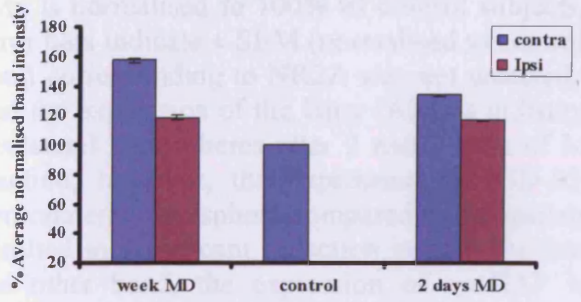
J


mGluR5 expression
% Average normalised band intensity
120
100
80
60
40
20
contra
Ipsi
week MD
control
2 days MD

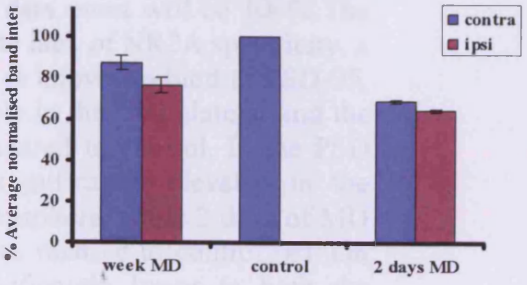
K


PLCβ1 expression
% Average normalised band intensity
120
110
100
90
80
70
60
50
40
30
20
contra
Ipsi
week MD
control
2 days MD

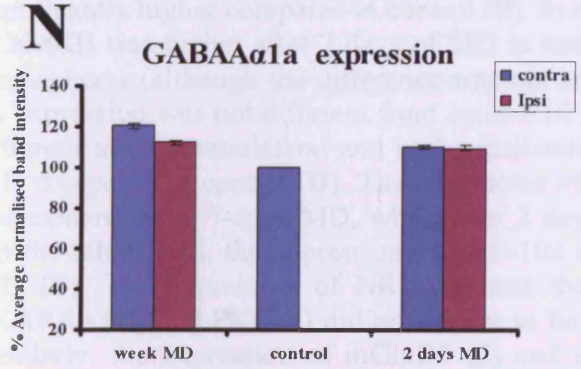
L


PLCβ4 expression
% Average normalised band intensity
180
160
140
120
100
80
60
40
20
contra
Ipsi
week MD
control
2 days MD

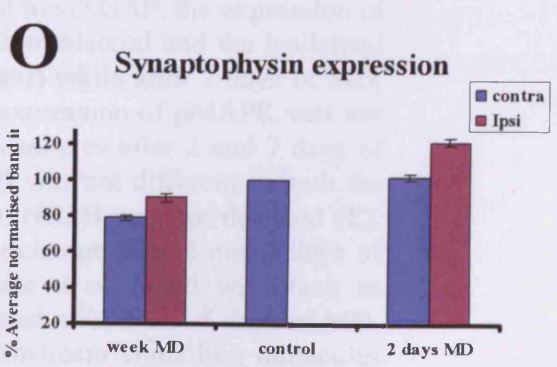
M


GluR1 expression
% Average normalised band inter
100
80
60
40
20
0
contra
ipsi
week MD
control
2 days MD

N


GABAAα1a expression
% Average normalised band intensity
140
120
100
80
60
40
20
contra
Ipsi
week MD
control
2 days MD

O


Synaptophysin expression
% Average normalised band i
120
100
80
60
40
20
contra
Ipsi
week MD
control
2 days MD

P

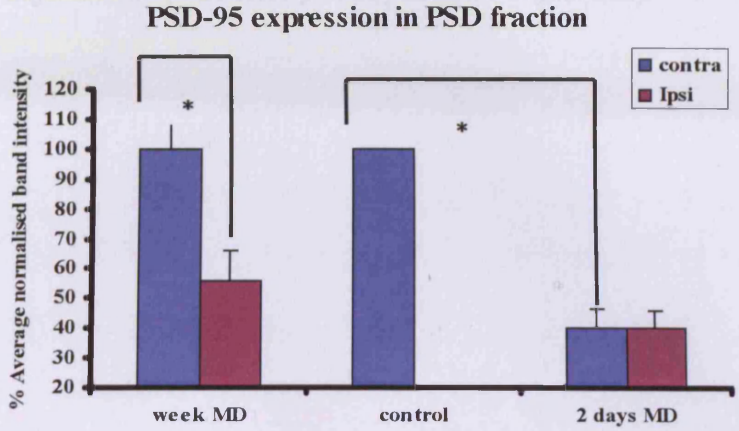


Figure 4.4.3: *Quantitative analysis of immunoblots obtained from samples prepared from visual cortices of subjects that had undergone monocular deprivation (MD) for 2 days or 7 days.* When quantifying the band intensity, band intensity was first normalised with internal control, and the data (contralateral and ipsilateral hemispheres) was then normalised to data obtained from control subjects. Since the data is normalised to 100% of control subjects, every data point will be 100%.The error bars indicate ± SEM (normalised to control). Due to lack of NR2A specificity, a band corresponding to NR2A was not detected. NR2A is known to bind to PSD-95, and the expression of the latter (**A**) was indistinguishable in the contralateral and the ipsilateral hemispheres after 2 and 7 days of MD compared to control. In the PSD fraction, however, the expression of PSD-95 was significantly elevated in the contralateral hemisphere compared to the ipsilateral hemisphere, while 2 days of MD resulted in significant reduction in both the hemispheres relative to control (**P**). On the other hand, the expression of synGAP was significantly lower in both the hemispheres after 2 days of MD while its expression after 7 days of MD was significantly higher compared to control (**B**). In contrast to synGAP, the expression of αCaMKII was higher after 7 days of MD in both the contralateral and the ipsilateral hemispheres (although the difference was not significant) while after 2 days of MD, its expression was not different from control (**C**).The expression of pMAPK was not different in the contralateral and in the ipsilateral hemispheres after 2 and 7 days of MD compared to control (**D**). The expression of NR2B was not different in both the hemispheres after 7-days MD, while after 2 days MD, NR2B was not detected (**E**). On the other hand, the expression of SAP-102 did not change after 2 and 7 days of MD (**F**). The expression of NR1 (**G**) and the kinases it is linked with such as PKARIIβ (**H**) and PKC (**I**) did not appear to be regulated after 2 and 7 days of MD. Similarly, the expression of mGluR5 (**J**) and its downstream signalling molecules

such as PLCβ1 (**K**) and PLCβ4 (**L**) did not appear to be regulated in both the contralateral and the ipsilateral hemispheres after 2 and 7 days of MD. The expression of GluR1 (**M**) was reduced after 2 days of MD in both the contralateral and the ipsilateral hemispheres (although the difference was not significant) while its expression after 7 days of MD was indistinguishable from control. The expression of $GABA_A\alpha 1a$ (**N**) and synaptophysin (**O**) did not appear to be regulated by experience after 2 and 7 days of MD. (* denotes $p<0.05$, Kruskal-Wallis test).

Protein	Up-regulated				Down-regulated				No change			
	C		I		C		I		C		I	
	H	PSD	H	PSD	H	PSD	H	PSD	H	PSD	H	PSD
PSD-95						√		√	√		√	
synGAP					√		√					
αCaMKII		√							√		√	
pMAPK									√		√	
NR2B					A		A					
SAP-102									√		√	
NR1									√	√	√	√
PKARIIβ									√		√	
PKC									√		√	
mGluR5									√		√	
PLCβ1									√		√	
PLCβ4									√		√	
GluR1					√		√					
$GABA_A\alpha1a$									√		√	
synaptophysin									√		√	

Table 3: ***A summary of results showing changes in expression of proteins in subjects that had undergone monocular deprivation for 2 days only relative to control in the homogenate (H) and in the post synaptic density (PSD).*** The molecular substrates are tabulated in the order they complex with receptor subunits, and substrates belonging to one complex are indicated by the same colour. However, GluR1 subunit even though it binds to NMDA receptor indirectly, it has been colour coded differently to show a different receptor subunit, while $GABA_A\alpha1a$ and synaptophysin do not form a complex with the rest of the substrates and therefore they fall in a different group indicated by a different colour. It should be noted that some downstream signalling molecules may be linked to NMDA receptor complex in

multiple locations. Due to lack of specificity of NR2A antibody, a band corresponding to NR2A was not detected. The expression of PSD-95 did not change at the homogenate while in the PSD fraction; the expression of PSD-95 was significantly (√) down-regulated in both the contralateral and the ipsilateral hemispheres. On the other hand, the expression of synGAP was significantly (√) reduced in the contralateral and in the ipsilateral hemispheres while the expression of αCaMKII was not regulated in the homogenate but in the PSD fraction, its expression was elevated in the contralateral compared to the ipsilateral hemisphere. The expression of pMAPK was not regulated after 2 days of MD. Surprisingly, a band corresponding to NR2B was not detected after 2 days of MD, while the expression of SAP-102 was indistinguishable from control. The expression of NR1 and kinases such as PKARIIβ and PKC did not appear to be regulated after 2 days of MD. Similarly, the expression of mGluR5 and downstream signalling molecules such as PLCβ1 and PLCβ4 did not change their expression relative to control after MD. On the other hand, the expression of GluR1 was reduced in both the contralateral and in the ipsilateral hemispheres (although the difference was not significant) while the expression of $GABA_A\alpha 1a$ and synaptophysin was unaltered after MD for 2 days.

Protein	Up-regulated				Down-regulated				No change			
	C		I		C		I		C		I	
	H	PSD	H	PSD	H	PSD	H	PSD	H	PSD	H	PSD
PSD-95		√							√		√	
synGAP	√	√	√									
αCaMKII	√		√			A		A				
pMAPK									√		√	
NR2B									√		√	
SAP-102									√		√	
NR1									√		√	
PKARIIβ									√		√	
PKC									√		√	
mGluR5									√		√	
PLCβ1									√		√	
PLCβ4									√		√	
GluR1									√		√	
$GABA_A$α1a									√		√	
synaptophysin									√		√	

Table 4: ***A summary of results showing changes in expression of proteins in subjects that had undergone monocular deprivation (MD) for 7 days only relative to control in the homogenate (H) and in the post synaptic density (PSD).*** The molecular substrates are tabulated in the order they complex with receptor subunits, and substrates belonging to one complex are indicated by the same colour. However, GluR1 subunit even though it binds to NMDA receptor indirectly, it has been colour coded differently to show a different receptor subunit, while $GABA_A$α1a and

synaptophysin are not part of NMDA receptor complex and therefore they fall in a different group indicated by a different colour. It should be noted that some downstream signalling molecules may be linked to NMDA receptor complex in multiple locations. Due to lack of specificity of NR2A antibody, a band corresponding to NR2A was not detected. The expression of PSD-95 did not change after 7 days of MD relative to control while its expression in the contralateral hemisphere was elevated compared to the ipsilateral hemisphere in the PSD fraction. On the other hand, the expression of synGAP was increased significantly (√) in both the hemispheres in the homogenate while in the PSD fraction, the expression of synGAP was elevated only in the contralateral hemisphere. Similarly, the expression of αCaMKII was up-regulated in both the contralateral and the ipsilateral hemisphere (although the difference was not significant) while its expression in the PSD fraction was not detected. The expression of pMAPK did not appear to be regulated after 7-days of MD. Similarly, the expression of NR2B and SAP-102 was indistinguishable from control in both the contralateral and the ipsilateral hemispheres after 7 days of MD. The expression of NR1 and kinases such as PKARIIβ and PKC did not appear to be regulated after 7 days of MD. Likewise, the expression of mGluR5 and downstream signalling molecules such as PLCβ1 and PLCβ4 did not change their expression relative to control after 7-days of MD. On the other hand, the expression of GluR1, $GABA_A\alpha 1a$ and synaptophysin was indistinguishable from control after 7-days of MD.

4.4 Discussion

4.4.1: Summary of Immunoblotting results

During development, the expression of $GABA_A\alpha1a$, PSD-95, αCaMKII, synGAP and synaptophysin increased substantially from 3 weeks of age and onwards while the expression of NR2A, pMAPK and mGluR5 peaked at 5 weeks of age. On the other hand the expression of NR2B and SAP-102 declined during development while the expression of NR1, GluR1, GluR2/3, PKC, PKARIIβ, PLCβ1, and PLCβ4 did not change throughout development. In the PSD fraction, consistent with homogenate samples NR2B and SAP-102 also showed developmental down-regulation in their expression while the expression of NR1 did not change throughout development. On the other hand, the expression of PSD-95 increased significantly with age while in contrast to homogenate a band corresponding to αCaMKII was only detected at 12 weeks and 1 year old in the PSD fraction. In addition, in contrast to homogenate, a band corresponding to synGAP was detected at 10 days until 12 weeks of age and thereafter its expression declined.

Dark-rearing from birth prevented developmental down-regulation of NR2B and up-regulation of NR2A subunits. In addition, the expression of $GABA_A\alpha1a$ increased substantially while the expression of synaptophysin was reduced after dark-rearing. On the other hand, the expression of NR1, mGluR5, GluR1, GluR2/3, PKC, PKARIIβ, PLCβ1, PLCβ4, SAP-102, PSD-95, αCaMKII and pMAPK did not appear to be regulated by sensory experience. In PSD fraction, however, the expression of αCaMKII and GluR2/3 was up-regulated in dark-reared subjects.

Monocular deprivation for 7 days did not result in change in expression of NR2B in contralateral and ipsilateral hemispheres relative to control while NR2B was not detected after 2 days of MD. AMPA receptor subunit, GluR1, showed down-regulation in both the contralateral and the ipsilateral hemispheres after 2 days of MD while no change in its expression was observed after 7 days of MD relative to control. In contrast, the expression of αCaMKII was up-regulated after 7 days of MD but not

after 2 days of MD while synGAP was down-regulated after 2 days of MD but up-regulated after 7 days of MD relative to control. In the PSD fraction, αCaMKII was not detected after 7 days of MD, but 2 days of MD resulted in elevated expression of αCaMKII in the contralateral compared to the ipsilateral hemisphere. On the other hand, the expression of synGAP and PSD-95 was higher after 7 days of MD in the contralateral hemisphere compared to the ipsilateral hemisphere while 2 days of MD resulted in down-regulation of PSD-95 in the PSD fraction.

4.4.2 Technical considerations and caveats

Western blotting is a critical tool in protein analysis and is largely dependent on the quality and specificity of the antibody used to probe the protein of interest. Although reproducibility of Western blotting can be difficult, the use of house-keeping proteins such as β-actin or PKARIIβ as internal control offers the advantage of determining how accurately the samples were loaded. Obviously, Western blotting data cannot provide information about cell-type or layer-specific protein expression. Future experiments using techniques with higher spatial and/or cell-type resolution are required to address this question. One of the disadvantages of Western blotting, however, is its lack of sensitivity and therefore one cannot detect any modest changes (less than 30%) in protein expression. Due to difficulty in reproducing gel patterns and because of variability between animals, I carried out experiments four times or more for confirmation.

The unique expression of proteins in cat visual cortex was aimed at gaining preliminary insight into possible plasticity mechanisms and was not meant to be an exhaustive study which would require use of more animals in each of the experimental conditions. Due to great amount of time involved in carrying out immunoblotting experiments, I could only use 2 animals for each of the experimental conditions. For samples acquired from control and dark-reared, each of the hemispheres was analysed for protein expression separately and therefore the hemispheres of the animals were treated independently. But this was possible only in control and dark-reared cats and not in monocularly deprived cats where both the

hemispheres have differential effects. For statistical purpose, the number of animals used in this study posed a problem for a parametric test; therefore I used a non-parametric test to show any significant difference.

In order to minimise variability in the few samples I had and maximise their use, I prepared synaptosomes and post synaptic density fractions in duplicate for each hemisphere in each experimental condition. The synaptosomes and PSD fraction were then assayed for protein concentration and then stored at -80°C until further use. It was surprising that in all synaptosomal preparations; only a band corresponding to PSD-95 was detected whereas excitatory subunits and other signalling molecules were not found using Western blotting. Previous studies by Johnson et al (1997) and Watson et al, (2006) have demonstrated that synaptosomes contain a variety of synaptic proteins that are found enriched in synaptosome fraction prepared by sucrose-gradient and Percoll-gradient methods respectively. It is very unlikely that insufficient transfer during Western blotting might have affected the outcome of the results because its efficiency was confirmed by staining the nitrocellulose membrane with amido black to confirm equal loading of protein samples and any membrane that appeared unequally loaded was discarded.

It is known that the metabolic state or viability of synaptosomes is largely dependent on the methodology employed to isolate them (Rayne et al, 1997). Calcium appears to be necessary to maintain stability of synaptosomes and storage in low calcium increases the chance of membrane leakage leading to eventual breakdown of neurotransmitters (Rayne R, 1997). The method employed in the present study did not use buffers containing calcium but it is very unlikely that lack of calcium might have compromised synaptosomes as sufficient amount of PSD proteins were detected. Also, the amount of lysis buffer used to suspend synaptosomes was reduced considerably to avoid any possibility of over-diluting samples which could have resulted in not detecting synaptosomal proteins. The synaptosomes and PSD fraction were instantaneously diluted in Laemmli buffer and boiled for 5-7 minutes before storing them at -80°C and therefore it is very unlikely that these samples were

degraded with time. Also, with long-term storage, aggregation of protein samples is inevitable and mild sonication was applied to both synaptosomes and PSD to solubilise contents in Laemmli buffer before loading them in gels. In all the synaptosomes prepared, only a band corresponding to PSD-95 was detected and this is consistent with a study by Gylys et al, (2004) which showed using electron microscopy that a majority of large particles in synaptosomes are PSD-95.

In contrast to synaptosomes, I was able to detect glutamate receptors with associated signalling and scaffolding proteins in the post synaptic density (PSD) fraction. As with synaptosomes, the PSD fraction was also diluted with Laemmli buffer and boiled for 5-7 minutes before storing them at -80°C. In all the experimental conditions examined and despite my several attempts, a band corresponding to NR2A subunit was not detected in the PSD fraction. Similarly, in certain experimental conditions such as monocular deprivation with lid suture and a week of sensory experience (DR-LR) and deprivation (LR-DR), a band corresponding to NR2B was not detected. On the other hand, GluR1 subunit was only observed in the PSD fraction obtained from subjects that had undergone sensory experience (DR-LR) and deprivation (LR-DR) but not in other experimental conditions. These discrepancies could be due to the fact that the epitope to which the antibodies (NR2A, NR2B and GluR1) bind to in the PSD fraction are perhaps in some way altered or washed away during isolation of PSD. This may not always be the case, as GluR1 subunit was detected in DR-LR and LR-DR PSD fraction and NR2B was detected in PSD fraction obtained from control and dark-reared animals.

4.4.3 Protein expression during development

Developmentally, immunoblotting results have shown that there appear to be two time points where the expressions of proteins appear to be regulated. The two time points include 10 days (before eye opening) and 5 weeks of age (peak of the critical period). For instance, the expression of $GABA_A\alpha1a$, PSD-95, αCaMKII, synGAP and synaptophysin increased from 3 weeks onwards with little or no expression at 10 days old. It is tempting to speculate that this lack of expression is due to lack of visual stimulation through the as yet unopened eye lids and increase in expression could be positively correlated to an increase in visual cortex activity upon eye opening. The expression of synaptophysin is a good indicator of synaptogenesis and its heightened expression at 3 weeks of age coincides around the time when synaptogenesis is at its peak and this is consistent with a previous study (Cragg, 1976). Similarly, the time period during which the expression of $GABA_A\alpha1a$, PSD-95, αCaMKII and synGAP peaks is around the time when the synaptogenesis is at its peak, i.e. at 3 weeks of age. Another time point where there appears to be developmental regulation of expression of proteins is at 5 weeks of age (peak of the critical period). For instance, the expression of mGluR5 and pMAPK peaked at 5 weeks of age. It is tempting to conclude that proteins whose expression is highest at the peak of the critical period such as mGluR5 and pMAPK may play a role in ocular dominance plasticity. However, a study by Hensch and Stryker (1996) demonstrated that blocking metabotropic glutamate receptors with α-methyl-4-carboxyphenylglycine (MCPG) in cat visual cortex did not block ocular dominance (OD) plasticity following monocular deprivation, suggesting that the expression of metabotropic glutamate receptors at the peak of the critical period does not underlie experience-dependent plasticity in the primary visual cortex. However, it is widely accepted nowadays that MCPG blocks mGluRII and not mGluR5 and subsequent study by Dolen et al, (2007) found that mice with FMRP (Fragile X mental retardation protein) knock-out showed substantial open-eye potentiation after short period of MD. This defect in plasticity can be rescued by reducing the expression of mGluR5 by 50% (Dolen et al, 2007) suggesting that mGluR5 does indeed play a role in OD plasticity. Similarly, the peak

expression of pMAPK during the critical period plays a direct role in OD plasticity as intracortical administration of inhibitors of ERK pathway such as UO126 and PD98059 in rat visual cortex prevented the OD shift towards the non-deprived eye following monocular deprivation (Di Cristo et al, 2001).

The expression of NMDA receptor subunits NR2A and NR2B also changed at 5 weeks of age. The expression of NR2A was observed from 5 weeks onwards while the expression of NR2B declined at 5 weeks of age. The switch appears to take place during the critical period and this is consistent with previous studies (Fox et al, 1989; Chen et al, 2000). A previous study by Fagiolini and colleagues (2003) has shown that genetic deletion of NR2A did not prevent OD shift towards the non-deprived eye after monocular deprivation; however, the magnitude of OD shift was weaker than in normal mice. The OD plasticity was restored fully after infusion of GABA agonist such as diazepam suggesting that a balance between excitation and inhibition is critical for OD plasticity (Fagiolini et al, 2003). The role of NR2B in OD plasticity has also been documented in mice by injecting a recombinant adenovirus containing NR2B oligonucleotide between P26-P29 in the right cortex (Cao et al, 2007). Monocular deprivation of the right eye after the infusion of the virus prevented the OD shift towards the non-deprived eye but when the left eye was deprived, the OD shift was observed (Cao et al, 2007). Therefore, it appears that NR2B is required in the ipsilateral hemisphere but not in the contralateral hemisphere to the deprived eye (Cao et al, 2007). However, their study did not determine if OD plasticity is affected after bilateral injection of adenovirus containing NR2B oligonucleotide. Nonetheless, the results obtained herein suggested that expression of proteins regulated at the peak of the critical period correlates with increase in susceptibility of visual cortex to environmental manipulation.

Another finding from the developmental profile is that the expression of proteins obtained from the homogenate did not necessarily correlate with the expression at the post synaptic density. For instance, at the homogenate level, the expression of αCaMKII and synGAP is very low before eye opening (P10) and plateaued from 3

weeks onwards while in the PSD fraction, a distinct band corresponding to αCaMKII was visible only at 12 weeks and 1 year of age and a band corresponding to synGAP declined at 1 year of age. On the other hand, the expression of NR1, NR2B, PSD-95 and SAP-102 in the PSD fraction correlated well with their expression at the homogenate level. It is not known whether changes in protein composition of the PSD, during postnatal development, involve mainly changes in the amounts of protein synthesis, or whether there are radical changes in protein types needed during different stages of development, for example, replacement of one receptor or protein type for another (Petralia et al, 2005). The differences in expression in the homogenate and in the PSD fraction could also be regulated by other postnatal developmental mechanisms, such as the formation of new synaptic connections or changes in protein trafficking during development. Nevertheless, the analysis of protein expression during development in cat visual cortex has shown that there are profound changes in protein composition and abundance during development.

So far I have looked at the expression of proteins individually but grouping them according to the way the receptor subunits complex with downstream signalling molecules, one is able to determine how a pathway is regulated developmentally and if the protein in the pathway act together or individually. For instance, the expression of NR2B and SAP-102 declined with age and therefore it appears that these two act together during development. Similarly, the expression of NR1 and its kinases maintained their expression throughout development and therefore one is tempted to say that these three may be acting together. The NR2A is expressed from 5 weeks onwards while the expression of signalling molecules it complexes with such as PSD-95, synGAP and αCaMKII peaked at 3 weeks of age. Similarly, the expression of mGluR5 peaked at 3 and 5 weeks of age while the expression of its downstream signalling molecules such as PLCβ1 and PLCβ4 did not change throughout development. There are several questions that arise from the developmental profile obtained herein. For instance, why are some proteins expressed in a similar fashion in a pathway whereas others are expressed differentially? And how expression of these different proteins contributes to the maturation of cortical properties? A previous

study in mice has shown that targeted disruption of PSD-95 prevented the maturation of orientation selectivity but OD plasticity was unaffected (Fagiolini et al, 2003). On the other hand, mice with CaMKII-α knock-out failed to show measurable plasticity in response to MD (Gordon et al, 1996). It appears that NR2A receptors and their downstream signalling molecules play different roles in the maturation and refinement of cortical properties and in experienced-dependent plasticity. Therefore proteins within a complex need not necessarily all be expressed in a similar fashion. It is worth emphasising that the pathways considered in this study are an oversimplification and some downstream molecules may be linked to receptor subunits at multiple locations.

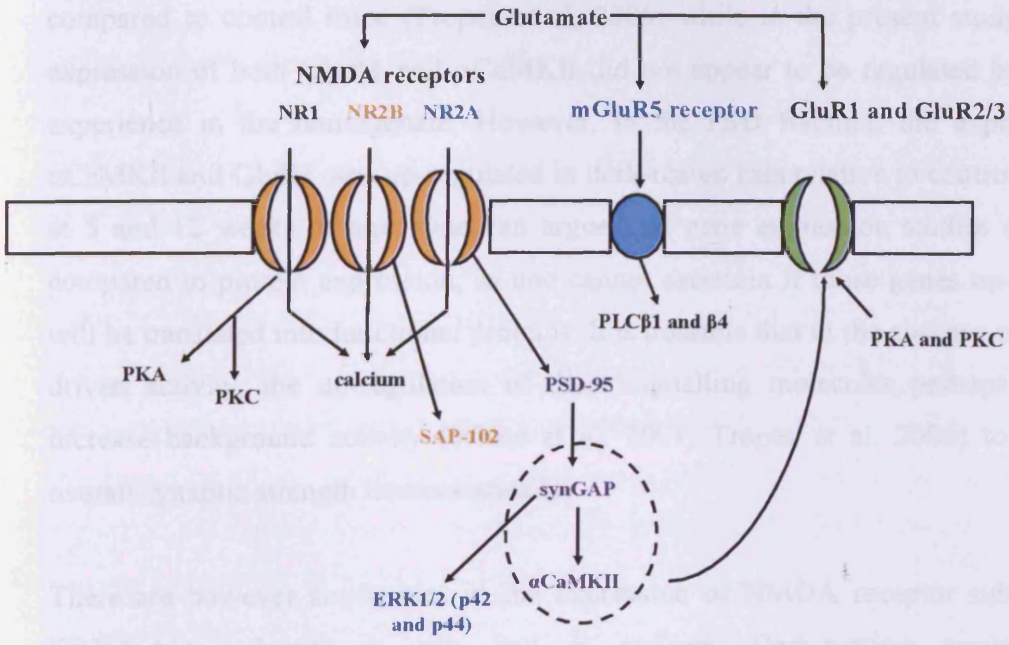


Figure 4.5: *Signalling pathways in NMDA receptor complex during development.* Only those components of the pathways are illustrated that were investigated in this study. The expression of proteins regulated developmentally is illustrated in a different font colour while the expression of proteins whose expression was maintained throughout development is indicated by a black font. The expression of NR2B and SAP-102 declined from 5 weeks of age while NR2A, mGluR5, pMAPK (ERK1/2) peaked at the peak of the critical period (5 weeks of age). In contrast to NR2A, the expression of PSD-95, synGAP and αCaMKII was low at P10 and peaked at 3 weeks of age. However, in the PSD fraction, the expression of synGAP declined

by 1 year of age while αCaMKII was expressed at 12 weeks and 1 year of age. (Figure modified from Husi and Grant, 2001).

4.4.4 The role of sensory experience in protein expression

The present analysis of protein expression in cat visual cortex after dark-rearing has highlighted several important findings. It is known that cats raised in darkness almost totally lack spontaneous activity in V1 (Fregnac and Imbert, 1984) while the rate of spontaneous activity is high in rats raised in darkness (Benevento et al, 1992). These differences may imply that there are differences in the organization of the circuitry in the visual cortex following sensory deprivation. At the molecular level, for example, after dark-rearing the gene expression of *GluR1* and *αCaMKII* was up-regulated compared to control mice (Tropea et al, 2006) while in the present study, protein expression of both GluR1 and αCaMKII did not appear to be regulated by sensory experience in the homogenate. However, in the PSD fraction, the expression of αCaMKII and GluR1 was up-regulated in dark-reared cats relative to control but only at 5 and 12 weeks of age. One can argue that gene expression studies cannot be compared to protein expression, as one cannot ascertain if those genes up-regulated will be translated into functional proteins. It is possible that in the absence of visually driven activity, the up-regulation of these signalling molecules perhaps serve to increase background activity (White et al, 2001, Tropea et al, 2006) to maintain overall synaptic strength homeostatically.

There are however similarities in the expression of NMDA receptor subunits and $GABA_A\alpha1a$ subunits in cats and in rodents. Dark-rearing prevented the developmental increase of NR2A subunit expression and the developmental down-regulation of NR2B at 5 and 12 weeks of age consistent with previous results obtained from rodent visual cortex (Quinlan, 1999: Philpot et al, 2001); the changes in NMDA receptor subunit expression were observed at the time when the cortex is most susceptible to environmental manipulation. The expression of $GABA_A\alpha1a$ was elevated in dark-reared subjects compared to control consistent with previous studies (Chen et al, 2001; Tropea et al, 2006). It is known that the effect of raising animals in

complete darkness delays the onset and decline of the critical period (Mower 1991; Fagiolini and Hensch, 2000). And since critical level of inhibition is necessary to restore plasticity and resume normal time course of the critical period (Fagiolini et al, 2000), one would have expected that dark-reared cats would have had reduced levels of $GABA_A\alpha1a$. It is possible that enhanced expression of $GABA_A\alpha1a$ combined with depressed expression of NR2A subunits adjust homeostatically to maintain the balance between excitation and inhibition (Chen et al, 2001; Turrigiano and Nelson, 2004). However, NR2A subunit is not the sole determinant of excitatory transmission as other glutamate receptor subunits examined herein such as NR1 and GluR1 may also be contributing to maintaining the balance between excitation and inhibition. Similarly, $GABA_A\alpha1a$ is not the only $GABA_A$ subunit affected by dark-rearing as Chen and colleagues (2001) demonstrated that the α3 subunit was also elevated in dark-reared animals while α2 was not.

It appears that proteins that were regulated after dark-rearing were the ones associated with neurotransmitter receptors (GluR2/3, NR2A, NR2B and $GABA_A\alpha1a$), NMDA receptor signalling (αCaMKII, which associates with NR2A subunit) and synapse formation (synaptophysin). For proteins such as synGAP and SAP-102, no such link to experience-dependent plasticity has been established and therefore their expression could potentially be regulated by other postnatal developmental mechanisms such as the formation of new synaptic connections or reduction in spine density as one would expect in dark-reared animals (Wallace and Bear, 2004). It was surprising that dark-rearing from birth identified few proteins that appear to be regulated by sensory experience. It is possible that for the functional effects of dark-rearing the presence of proteins investigated herein is sufficient rather than their regulation by sensory experience. There are several questions that arise: for instance, what makes some proteins more susceptible to environmental manipulation than others and how do those that are regulated by sensory experience, contribute to the altered cortical properties seen in dark-reared subjects? This can be answered by using pharmacological blockers and targeting those proteins whose expression is dependent on sensory experience (as the ones identified herein) and to determine how cortical

properties are affected. However, not all the proteins that are regulated developmentally may be amenable to pharmacological blockade.

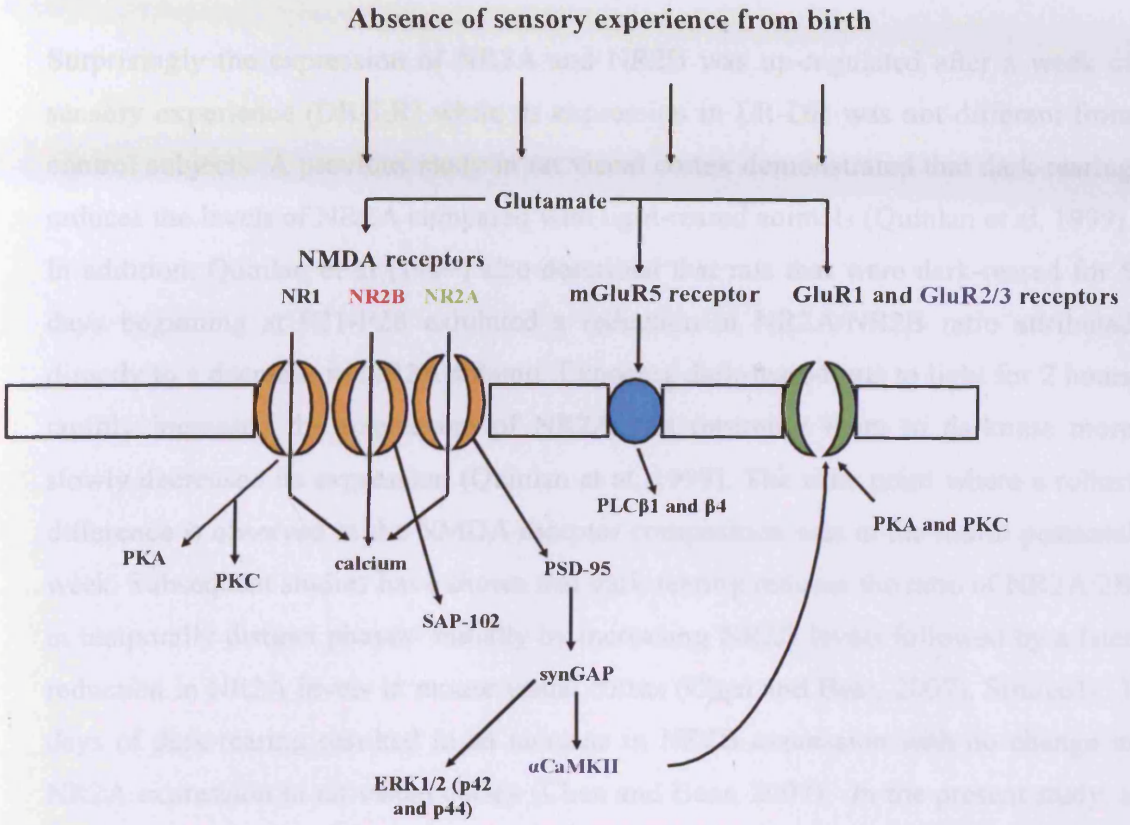


Figure 4.6: *Signalling pathways in NMDA receptor complex in the absence of sensory experience.* Only those components of the pathways are shown that were investigated in this study. The expression of proteins regulated by sensory experience is indicated in a different font colour while those whose expression is not affected by sensory experience is indicated by a black font. The developmental down-regulation of NR2B is prevented in dark-reared subjects while NR2A is not expressed in dark-reared subjects. The expression of αCaMKII and GluR2/3 was up-regulated in dark-reared subjects in the PSD fraction only but not in the homogenate. (Figure modified from Husi and Grant, 2001).

4.4.5 Differential effects of experience on protein regulation

In order to determine if shorter periods of dark-rearing might have more disruptive effects on protein expression than prolonged period of darkness, some subjects were

exposed to a period of darkness for a week (LR-DR) whereas other subjects were exposed to a week of visual experience (DR-LR) after having been raised in complete darkness from birth.

Surprisingly the expression of NR2A and NR2B was up-regulated after a week of sensory experience (DR-LR) while its expression in LR-DR was not different from control subjects. A previous study in rat visual cortex demonstrated that dark-rearing reduces the levels of NR2A compared with light-reared animals (Quinlan et al, 1999). In addition, Quinlan et al (1999) also described that rats that were dark-reared for 5 days beginning at P21-P28 exhibited a reduction in NR2A/NR2B ratio attributed directly to a decrease in NR2A subunit. Exposing dark-reared rats to light for 2 hours rapidly increased the expression of NR2A and returning them to darkness more slowly decreased its expression (Quinlan et al, 1999). The time point where a robust difference is observed in the NMDA receptor composition was in the fourth postnatal week. Subsequent studies have shown that dark-rearing reduces the ratio of NR2A/2B in temporally distinct phases- initially by increasing NR2B levels followed by a later reduction in NR2A levels in mouse visual cortex (Chen and Bear, 2007). Similarly, 3 days of dark-rearing resulted in an increase in NR2B expression with no change in NR2A expression in rat visual cortex (Chen and Bear, 2007). In the present study, a week of dark-rearing did not change the expression levels of either NR2A or NR2B and only sensory experience after dark-rearing (DR-LR) increased the expression levels of both NR2A and NR2B. It is possible that bidirectional changes which have so far been observed in rodents require shorter time scale of deprivation in order to be observed in higher animals such as felines. The shorter period of deprivation will determine transient changes in the expression of NMDA receptor subunits while a week of deprivation used herein perhaps homeostatically adjusts the expression of these subunits to normal levels. More importantly, since the difference in NMDA receptor subunit composition is manifested in the fourth postnatal week in rat visual cortex after brief sensory deprivation coincident with the onset of functional vision (Quinlan et al, 1999), it is possible that one needs to determine any difference in

4.4 Discussion

NMDA receptor composition much earlier in feline life; at about 2-3 weeks, i.e. the onset of functional vision.

Interestingly, a brief period of sensory alteration (DR-LR) resulted in change in expression of downstream signalling molecules such as PSD-95, synGAP and αCaMKII which are known to associate with NR2A subunits. For instance, the expression of PSD-95 increased in DR-LR relative to dark-reared subjects while brief sensory deprivation did not change its expression. However, in the PSD fraction neither experimental condition resulted in a change in the expression of PSD-95, suggesting that one of the responses to visual stimulation after a week of darkness is an increase in the cytoplasmic pool of PSD-95. A study by Yoshii et al (2003) using immunoblotting showed that 6 hours of visual experience increases the expression of PSD-95 while four days of dark-rearing resulted in reduced expression of PSD-95 in rat visual cortex. However, in the present study a brief period of dark-rearing did not affect the expression levels of PSD-95 and this is possibly due to differential regulation of PSD-95 in these two species.

The association of synGAP with NMDA receptor complex specifically PSD-95, suggests that synGAP may specifically respond to a change in calcium levels mediated by activation of NMDA receptors (Kim et al, 1998). The expression of synGAP did not change between the two experimental conditions at the homogenate level but in the PSD fraction, synGAP expression increased after a week of sensory deprivation (LR-DR). This is consistent with findings that increased expression of synGAP retards synapse formation and reduces spine heads (Vazquez et al, 2004) and this reflects the morphological changes seen after a period of dark-rearing (Wallace and Bear, 2004). SynGAP has been known to play a significant role in excitatory transmission as demonstrated in a study by Rumbaugh et al (2006) which showed that mice lacking synGAP exhibited a significant increase in AMPA receptor mediated mEPSC when compared to wild type littermates. And conversely when synGAP protein was present at high levels, AMPA receptor mediated transmission was depressed (Rumbaugh et al 2006). Therefore synGAP appears to be interacting

positively with NMDA receptors but negatively with AMPA receptors. In the present study, subjects that were exposed to a week of dark-rearing (LR-DR) did not only up-regulate the expression of synGAP but also GluR1 in the PSD fraction. Therefore it appears that in the absence of sensory experience, synGAP and GluR1 may not be acting negatively with each other. SynGAP is known to regulate ERK/MAPK (Komiyama et al, 2002) and in contrast to the expression of synGAP, the expression of pMAPK was not affected after sensory experience (DR-LR) and deprivation (LR-DR). This is consistent with the results obtained after prolonged period of darkness; however, the expression of pMAPK has previously been shown to increase upon visual stimulation in rats raised in complete darkness (Boggio et al, 2007). It is possible that as with prolonged period of darkness, the expression of pMAPK might have increased in response to visual stimulation in both the experimental groups (DR-LR and LR-DR) due to time involved in acquiring OIS maps as well as VEP recordings.

Similar to the expression of synGAP, the expression of αCaMKII did not change in the homogenate after brief sensory deprivation and experience. This is in contrast to a study by Neve and Bear (1989) which showed that the transcripts of αCaMKII is up-regulated in visual cortex of kittens raised in darkness and remains elevated after 12 hours of visual experience. On the other hand, a study by Wu et al (1998) demonstrated synaptoneurosome fractions prepared from dark-reared rats exposed to light for 30 minutes to 6 hours contained more αCaMKII than those from dark-reared rats with no visual experience. However, it should be noted that the former study was carried out at the gene expression level while the latter looked at the expression of αCaMKII at the synaptic level, therefore apart from possible species differences, it is probable that there are differences in the amount of transcripts and the amount of protein synthesised. The discrepancy in the expression of αCaMKII could be due to lack of sensitivity of Western blotting and may have failed to detect any subtle changes in expression. In the PSD fraction, however, the expression of αCaMKII in LR-DR was indistinguishable from control (and this is consistent with the results obtained from prolonged period of dark-rearing) while αCaMKII was not detected

after sensory experience (DR-LR). This is in contrast to studies by Wu et al, (1998) and (Thiagarajan et al, 2002) which have shown that the expression of αCaMKII adjusts itself depending on the levels of neuronal activity. Since homogenisation was not carried out until few months after harvesting the tissues, it is possible that αCaMKII may have been modified chemically and in fact a previous study has shown that homogenisation of the brain immediately after harvesting is essential to obtain highly active αCaMKII in good yield (Goldenring et al, 1984; Suzuki et al, 1994). A study by Suzuki et al (1994) has also shown that cytosolic αCaMKII is susceptible to deterioration after harvesting the tissue and somehow the inactive form is then immobilised to PSD cytoskeleton during PSD preparation (Suzuki et al, 1994). Therefore, the regulation of αCaMKII after a brief period of sensory experience needs to be validated by minimising the time lapsed between harvesting the tissue and its homogenisation.

The expression of NR1 and the kinases it is linked with such as PKARIIβ and PKC did not appear to be regulated after sensory experience and deprivation. Similarly, the expression of mGluR5 and its associated signalling molecules such as PLCβ1 and PLCβ4 in DR-LR and LR-DR was not different from control and dark-reared subjects. This is in line with the results obtained from subjects that were dark-reared for a prolonged period of time from birth (5-12 weeks). It is possible that since these proteins are synaptic modulators (such as kinases and phospholipases) they perhaps maintain a certain level of cellular signalling in the absence of sensory experience. The observations from a week of darkness and experience suggest that sensory alteration has a profound effect on the expression of not only neurotransmitter subunits but also downstream signalling molecules especially the ones that complex with NR2B and NR2A subunits.

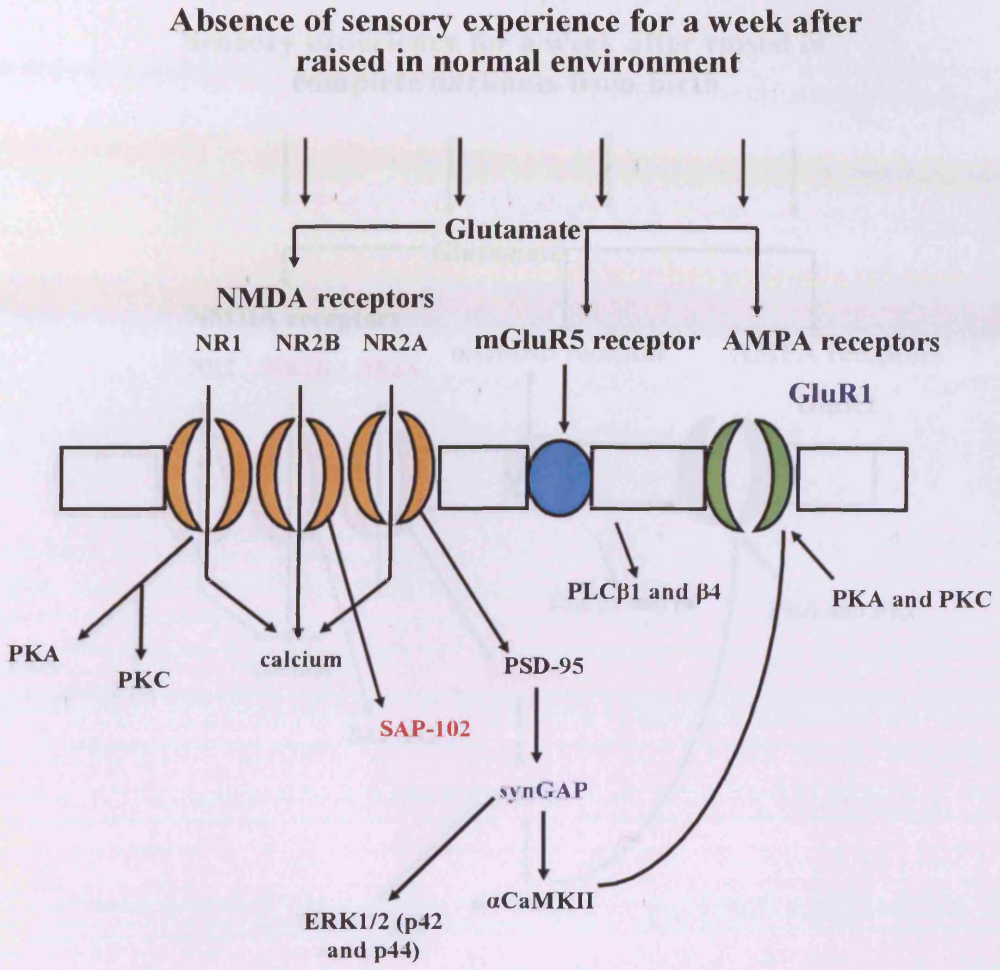


Figure 4.7.1: *Signalling pathways in NMDA receptor complex in the absence of sensory experience for a week only after raising subjects in normal environment.* Only those components of the pathways are shown that were investigated in this study. The expression of proteins regulated by sensory experience is indicated in different font colour while those whose expression is not affected by the nature of sensory experience is indicated by a black font. The expression of GluR1 and synGAP is up-regulated in the PSD fraction but not in the homogenate while the expression of SAP-102 is enhanced in the homogenate after a week of darkness. (Figure modified from Husi and Grant, 2001).

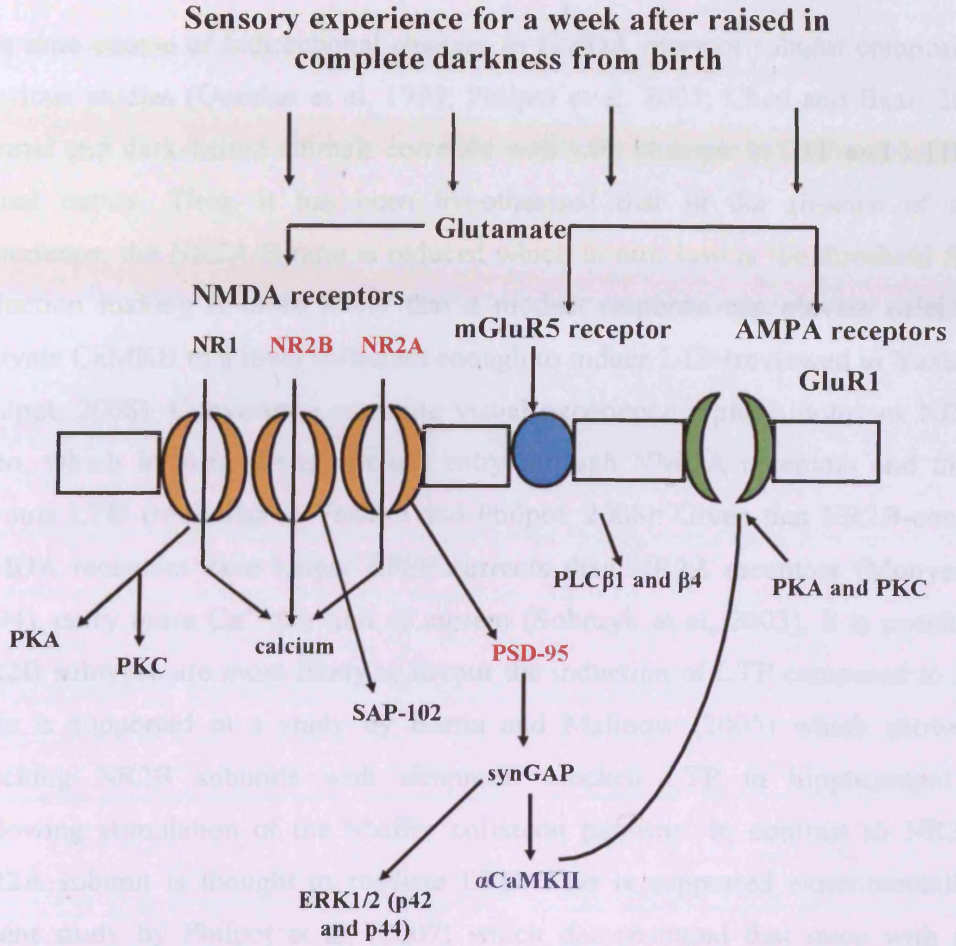


Figure 4.7.2: *Signalling pathways in NMDA receptor complex after sensory experience for a week only after raising subjects in darkness from birth.* Only those components of the pathways are illustrated that were investigated in this study. It should be noted that some downstream signalling molecules may be linked to NMDA receptor complex at multiple locations. Proteins whose expression is regulated by sensory experience are shown in a different font colour while those whose expression is not affected by sensory experience is indicated by a black font. The expression of NR2B, NR2A and PSD-95 in the homogenate was up-regulated after a week of sensory experience while in the PSD fraction, αCaMKII was not observed. (Figure modified from Husi and Grant, 2001).

Cellular mechanisms after sensory deprivation and experience

The time course of bidirectional changes in NMDA receptor subunit composition in previous studies (Quinlan et al, 1999; Philpot et al, 2001; Chen and Bear, 2007) in normal and dark-reared animals correlate well with changes in LTP and LTD in the visual cortex. Thus, it has been hypothesised that in the absence of sensory experience, the NR2A/B ratio is reduced which in turn lowers the threshold for LTP induction making it more likely that a modest response can elevate calcium and activate CaMKII to a level sufficient enough to induce LTP (reviewed in Yashiro and Philpot, 2008). Conversely, restoring visual experience rapidly increases NR2A/2B ratio, which in turn, limits calcium entry through NMDA receptors and therefore favours LTD (reviewed in Yashiro and Philpot, 2008). Given that NR2B-containing NMDA receptors have longer EPSP currents than NR2A receptors (Monyer et al, 1994), carry more Ca^{2+} per unit of current (Sobczyk et al, 2005), it is possible that NR2B subtypes are more likely to favour the induction of LTP compared to NR2A. This is supported in a study by Barria and Malinow (2005) which showed that blocking NR2B subunits with ifenprodil blocked LTP in hippocampal slices following stimulation of the Shaffer collateral pathway. In contrast to NR2B, the NR2A subunit is thought to mediate LTD. This is supported experimentally in a recent study by Philpot et al, (2007) which demonstrated that mice with genetic ablation of NR2A exhibited LTP after raising them in darkness but failed to produce LTD after restoring visual experience. Therefore, in the context of the BCM model, the changes in NR2A/2B subunits caused by sensory experience provided a mechanism of adjusting the synaptic modification threshold homeostatically (Bienenstock et al, 1982). However, in the present study and in contrast to previous studies (Quinlan et al, 1999; Philpot et al, 2001; Chen and Bear, 2007) the expression of both NR2A and NR2B was up-regulated after sensory experience but brief period of dark-rearing failed to show any differences in their expression. One should keep in mind that the models of LTP and LTD demonstrated so far have been on rodent visual cortex and may not necessarily bear resemblance to molecular mechanisms in feline visual cortex. Therefore, it appears that in contrast to rodents, increasing levels of both NR2A and NR2B will most likely favour the induction of LTD; however, this

will need to be validated by first increasing the number of samples and then blocking NR2B or NR2A with their specific antagonists and inducing LTD in cortical slices. Also, determining the expression of NR2B and NR2A at the synaptic level will give more of an indication of how each of these subunits contributes to LTP and/or LTD.

In addition to the role of NMDA receptor subunits in LTP and LTD, there is now abundant evidence that PSD-95, SAP-102 and αCaMKII also participate in NMDAR-dependent forms of synaptic plasticity. For instance, mice lacking PSD-95 show enhanced LTP in wide range of frequencies of synaptic stimulation (Migaud et al, 1998) while SAP-102 knock-out mice showed impairments in the induction of LTP (Cuthbert et al, 2007). However, these studies have been carried out in mouse hippocampus while their roles in visual cortical plasticity have not received much attention. It is possible that there might be regional and species differences in the regulation of PSD-95 and SAP-102 in the mechanism of plasticity. In the present study, similar to the expression of NR2A, the expression of PSD-95 was also up-regulated in DR-LR subjects and it is tempting to say that along with NR2A, PSD-95 could also be contributing to LTD. In contrast to PSD-95, the expression of SAP-102 was up-regulated in LR-DR subjects since it directly interacts with NR2B subunits; it is possible that it also plays a role in LTP in the visual cortex. However, this will need to be validated by increasing the number of samples, and using pharmacological blockers will give us an insight into the role SAP-102 plays in visual cortical plasticity. Kirkwood and colleagues (1997) have demonstrated that adult mice with αCaMKII knock-out exhibited little LTP or LTD using standard conditioning protocols in visual cortical slices while substantial LTP and LTD could be induced in 4-5 weeks old animals. Therefore, the role of αCaMKII in experience-dependent plasticity is restricted depending on the age of the animal. Future studies will require the use of αCaMKII blockers to investigate the role it plays in experience-dependent plasticity in cat visual cortex.

4.4.6 Monocular deprivation

Although the physiological effects of MD are more pronounced after 7 days of MD, there are also significant changes after 2 days of MD. For instance orientation maps dominated by the deprived eye after 7 days of MD appeared flat with no orientation-selective patches visible while the ones obtained after 2 days of MD showed orientation-selective patches albeit weaker than the ones represented by the non-deprived eye (see CHAPTER III for details). To examine similarities and differences between a short (2 days) and a longer period (7 days) of MD, I carried out immunoblotting experiments to determine protein expression. Monocular deprivation after 2 and 7 days did not result in change in expression of proteins associated with synaptic transmission such as NR1, mGluR5 and $GABA_A\alpha 1a$. In addition, the expression of kinases such as PKARIIβ and PKC which form a complex with NR1 did not appear to be regulated after monocular deprivation for 2 and 7 days. Previous studies have shown that the absence of PKARIIβ in mice (Fischer et al, 2004) and pharmacological blockade of PKA (Beaver et al, 2001b) in cats prevented the ocular dominance shift towards the non-deprived eye in response to MD. This is in contrast to the results obtained herein; it is possible that the presence of PKA may be sufficient to initiate cascade of events for the functional effects of MD rather than their regulation by sensory experience.

Phospholipases such as PLCβ1 and PLCβ4 couple downstream to metabotropic receptors (Husi and Grant, 2001) and their role in monocular deprivation has not been investigated. In the present study, the expression of PLCβ1 and PLCβ4 was not regulated in response to MD. Kind et al (1994) using immunoprecipitation demonstrated that rearing cats in complete darkness for four months prevented the developmental down-regulation of PLCβ1. However, dark-rearing and monocular deprivations are two different deprivation paradigms and therefore may employ different molecular mechanisms for their functional effects.

The expression of NR2B expression was indistinguishable from control after 7 days of MD while NR2B was not detected at all after 2 days of MD. On the other hand, the

expression of SAP-102 which is known to associate with NR2B was indistinguishable from control. Lack of NR2B after 2 days of MD was surprising as a recent biochemical study indicated that NR2B levels are significantly elevated at 3 days of MD while NR2A levels are reduced after 7 days of MD in the hemisphere contralateral to the deprived eye in mouse visual cortex (Chen and Bear, 2007). This suggests that the delay in potentiation of the open-eye response may be a result of a deprivation-induced reduction in NR2A/NR2B ratio (Chen and Bear, 2007). The absence of NR2B in the homogenate after 2 days of MD is astonishing and needs confirmation by increasing the number of samples. It is also probable that the expression of NR2B was reduced below the level of detection using Western blotting. Unfortunately, due to difficulty in obtaining a reliable band corresponding to NR2A, I could not determine how expression levels of NR2A are affected in response to 2 and 7 days of MD. Therefore, any conclusions regarding differential regulation of NMDA receptor subunits cannot be drawn. The role of the NR2A subunit in monocular deprivation is shown in a recent study by Cho et al (2009) which demonstrated that mice with genetic deletion of the NR2A subunit fail to exhibit potentiation of NDE inputs suggesting that a reduction in NR2A/B ratio during MD is permissive for the compensatory potentiation of NDE inputs at least in mouse visual cortex (Cho et al, 2009).

The NMDA receptor subunit, NR2A, interacts with PSD-95 and the expression of the latter was not regulated by imbalance of visual experience between the two eyes at the homogenate level. However, in the PSD fraction, the expression of PSD-95 was higher in the hemisphere contralateral to the DE compared to the ipsilateral hemisphere after 7 days of MD. On the other hand, after only 2 days of MD, the expression of PSD-95 was reduced in both the hemispheres. A recent study (Ehrlich and Malinow, 2004) has demonstrated that over-expressing PSD-95 in the barrel cortex increases AMPA receptor mediated currents and occludes LTP and enhances LTD perhaps as a result of internalisation of AMPA receptors. Complementary to this finding, mice that do not express PSD-95 show reduced AMPA receptor mediated currents and enhanced LTP (Elias et al, 2006). In light of these studies it is possible

that increased expression of PSD-95 observed in the contralateral hemisphere after 7 days of MD and its reduced expression combined with down-regulation of GluR1 after 2 days of MD could be contributing to LTD (Heynen et al, 2003).

The expression of synGAP increased after 7 days of MD but decreased after 2 days of MD in both contralateral and ipsilateral hemispheres compared to control. On the other hand, in the PSD fraction, the expression of synGAP was higher in the contralateral compared to the ipsilateral hemisphere after 7 days of MD. It has been shown using 2-photon imaging that MD accelerates spine formation thereby increasing the spine density (Hofer et al, 2009) and studies in hippocampal cultures have shown that the developmental increase in synGAP retards spine formation (Vazquez et al, 2004). Therefore it is possible that elevated expression of synGAP seen after 7 days of MD in the contralateral hemisphere homeostatically adjusts to changes in calcium levels and further prevents reduction in spine density. On the other hand, it is probable that reduced expression of synGAP seen after 2 days of MD perhaps permits spine formation and increase in spine density (Vazquez et al, 2004).

SynGAP is known to form a complex with αCaMKII and calcium influx results in dissociation of αCaMKII accompanied by dephosphorylation of synGAP and potentiation of synaptic AMPA responses (Krapivinsky et al, 2004). The expression of αCaMKII was not regulated by activity after 2 days of MD but 7 days of MD resulted in increased expression of αCaMKII in both the hemispheres. Previous studies (Hendry and Kennedy, 1986 and Tighilet et al, 1998) have shown that monocular deprivation by intraocular injection of TTX (7, 14 and 16 days) or lid suture (9-11 weeks) lead to up-regulation of αCaMKII in macaque primary visual cortex. Therefore, the expression of αCaMKII is uniquely sensitive to levels of activity. In the present study, the expression of αCaMKII was up-regulated after 7 days of deprivation but not after 2 days suggesting that prolonged period of deprivation is necessary for up-regulation of αCaMKII. After 7 days of MD, there is retraction of axons dominated by the deprived eye and expansion of axons dominated by non-deprived eye (Antonini and Stryker, 1993b) and the up-regulation of

4.4 Discussion

αCaMKII can be seen as an attempt to compensate for reduced activity by engaging cellular mechanisms that should enhance excitatory transmission (Tighilet et al, 1998). In contrast to results from homogenate, αCaMKII was not detected in the PSD fraction after 7 days of MD, suggesting that that up-regulation of αCaMKII is restricted to cytoplasm but is not inserted on the cell surface. On the other hand, after 2 days of MD, there was a significant elevation of αCaMKII in the contralateral hemisphere compared to the ipsilateral hemisphere to the deprived eye, and this is consistent with previous studies (Tighilet et al, 1998; Hendry and Jones, 1986), which showed using immunocytochemical methods that there is up-regulation of this enzyme only in domains dominated by the deprived eye in macaque visual cortex.

SynGAP mediates its effects by differential activation of several downstream pathways including ERK (pMAPK). In the present study, in contrast to synGAP, the expression of pMAPK was not altered in response to MD and this is in contrast to a study by Majdan and Shatz (2006) which showed that protein levels of pMAPK were decreased after monocular enucleation. As mentioned previously, monocular enucleation and monocular deprivation are two different forms of deprivation paradigms and the former is most likely to induce secondary effects due to deafferentation (Rio and Feller, 2006) and therefore may employ or activate different molecular mechanisms than the latter.

My results regarding the expression of AMPA receptor subunits in response to MD are in accordance with previous studies in the mouse visual cortex (Heynen et al, 2003; Yoon et al, 2009). In the present study, after 2 days of MD, GluR1 expression was reduced in the contralateral and in the ipsilateral hemispheres but after 7 days of MD, the expression of GluR1 was indistinguishable from control. This is consistent with a study by Heynen et al (2003) which demonstrated that 24 hours of MD in mice is accompanied by loss of surface-expressed GluR1 and GluR2 subunits using immunoblotting and these changes were apparent within 6 hours of MD but disappeared after 2 days of MD. In addition, their study also revealed that blocking NMDA receptors with CPP (3-[(R)-2-carboxypiperazin-4-yl]-prop-2-enyl-1-

phosphonic acid) did not cause surface loss of expression of AMPA receptor subunits therefore dephosphorylation of GluR1 and their subsequent degradation is dependent on activation of NMDA receptors (Heynen et al, 2003). A study by Yoon et al (2009) used herpes simplex virus (HSV) vector to express G2CT peptide designed to block internalization of AMPA receptors; they demonstrated that OD shift and depression of deprived-eye responses were blocked after MD. This further confirms that internalisation of AMPA receptors is essential for the loss of synaptic strength caused by MD (Yoon et al, 2009). The transient loss of AMPA receptor subunits sets in motion the same molecular changes as LTD and thus the cellular mechanism underlying loss of visual responsiveness after MD is likely LTD (Heynen et al, 2003). It is also possible that loss of AMPA receptors is permissive for subsequent potentiation of non-deprived eye responses and that both NMDA and AMPA receptors work in harmony to set in motion the effects of MD.

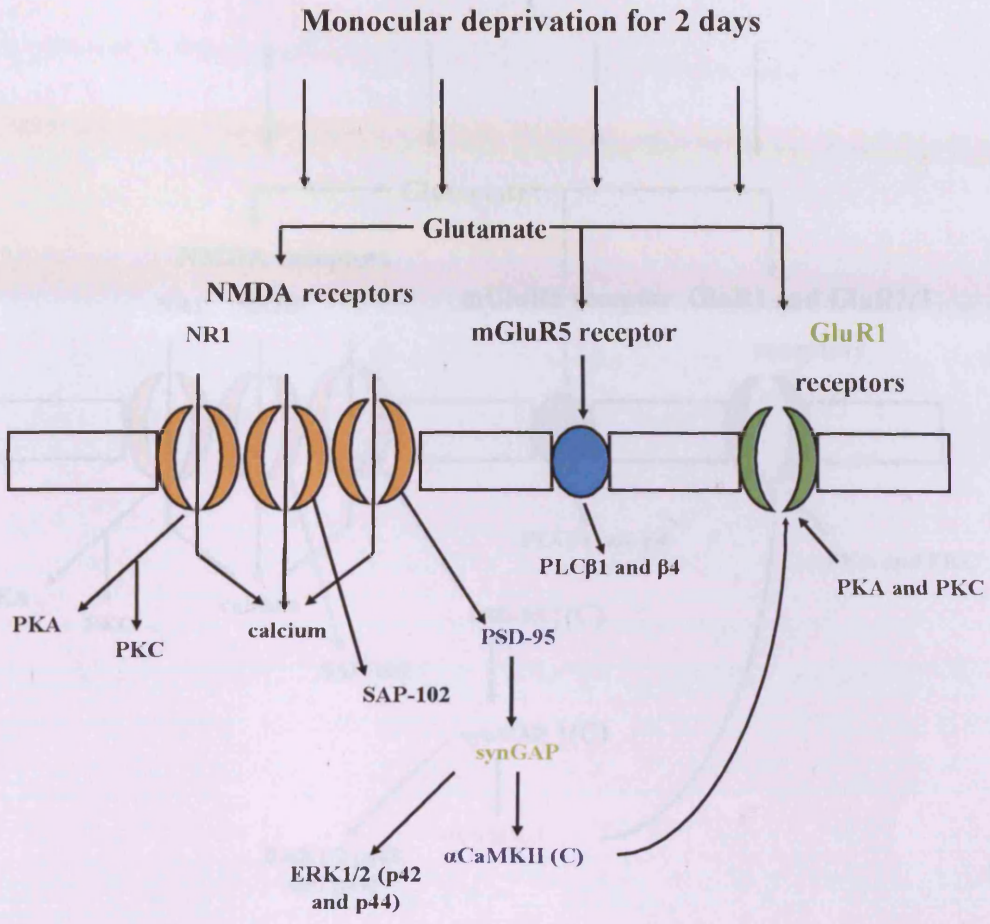


Figure 4.8.1: *Signalling pathways in NMDA receptor complex after monocular deprivation (MD) for 2 days only.* Only those components of the pathways are illustrated that were investigated in this study. It should be noted that some downstream signalling molecules may be linked to the NMDA receptor complex at multiple locations. The expression of proteins regulated by sensory experience is indicated in a different font colour while those whose expression is not affected by change in sensory experience is indicated by a black font. The expression of NR2B and NR2A was not observed and therefore both these subunits have been omitted from the figure above while the expression of GluR1 and synGAP was down-regulated after 2 days of MD. On the other hand, in the PSD fraction, the expression of PSD-95 was down-regulated in both the hemispheres while the expression of αCaMKII was up-regulated only in the hemisphere contralateral to the deprived eye after 2 days of MD. (Figure modified from Husi and Grant, 2001).

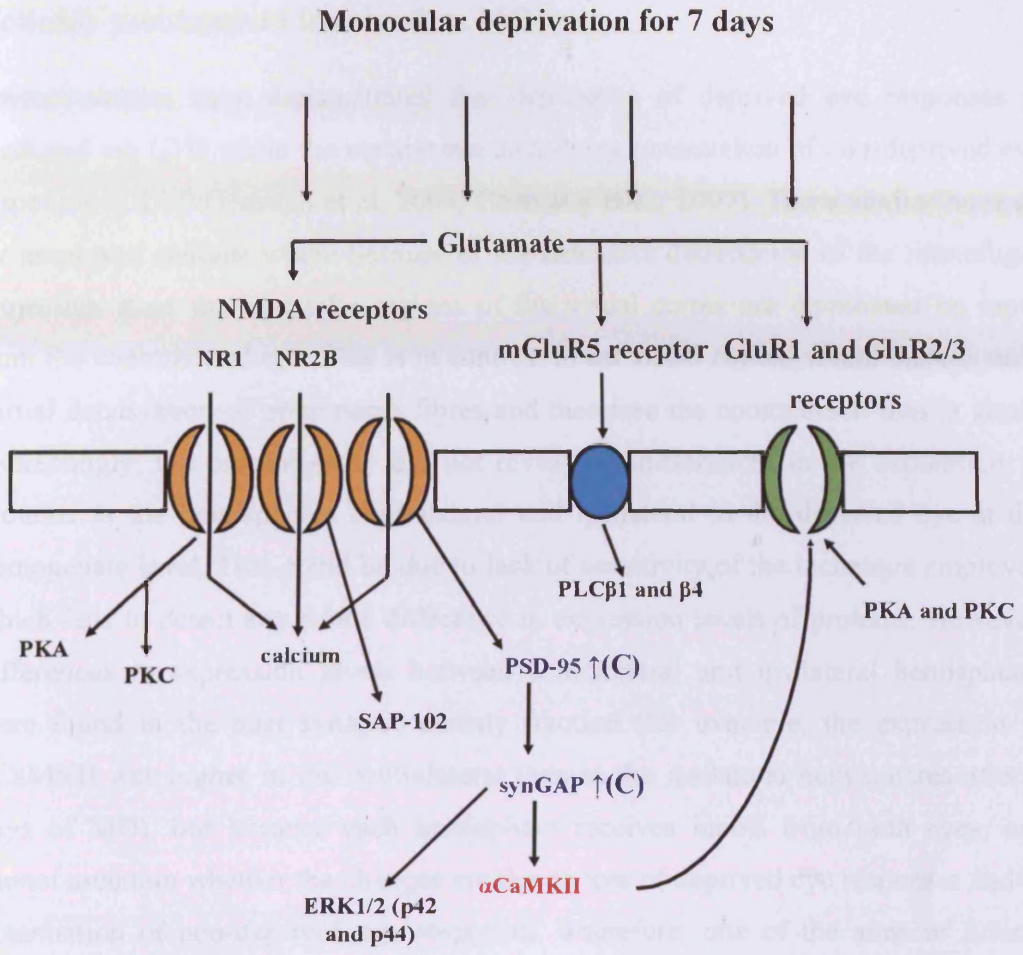


Figure 4.8.2: *Signalling pathways in NMDA receptor complex after monocular deprivation (MD) for 7 days.* Only those components of the pathways are illustrated that were investigated in this study. It should be noted that some downstream signalling molecules may be linked to the NMDA receptor complex at multiple locations. The expression of proteins regulated by sensory experience is indicated in a different font colour while those whose expression is not affected by change in sensory experience is indicated by a black font. The expression of NR2A was not observed and therefore has been omitted from the figure above while the expression of αCaMKII was up-regulated after 7 days of MD. On the other hand, in the PSD fraction, the expression of PSD-95 and synGAP was up-regulated only in the contralateral hemisphere after 7 days of MD. (Figure modified from Husi and Grant, 2001).

Cellular mechanism involved in MD

Several studies have demonstrated that depression of deprived eye responses is mediated via LTD while the mechanism underlying potentiation of non-deprived eye responses is LTP (Heynen et al, 2003; Chen and Bear, 2007). These studies have so far employed rodents where because of the extensive decussation of the retinofugal projection even the binocular regions of the visual cortex are dominated by input from the contralateral eye. This is in contrast to cat visual cortex, where there is only partial decussation of optic nerve fibres and therefore the contralateral bias is weak. Interestingly, the present study did not reveal any differences in the expression of proteins in the hemispheres contralateral and ipsilateral to the deprived eye at the homogenate level. This could be due to lack of sensitivity of the technique employed which fails to detect any subtle difference in expression levels of proteins. However, differences in expression levels between contralateral and ipsilateral hemispheres were found in the post synaptic density fraction (for example, the expression of αCaMKII was higher in the contralateral than in the ipsilateral hemispheres after 2 days of MD), but because each hemisphere receives inputs from both eyes, one cannot ascertain whether the changes are due to loss of deprived eye responses and/or potentiation of non-deprived eye responses. Therefore, one of the aims of futures studies should be to sample visual cortices from left and right eye ocular dominance columns separately.

It is tempting to speculate that reduced expression of GluR1 and possibly loss of NR2B after 2 days of MD contributes to depression of deprived eye responses (as seen in ocular dominance maps), probably by LTD as demonstrated in a previous study (Heynen et al, 2003). However, the latter study only showed loss of AMPA receptor subunits in the hemisphere contralateral to the deprived eye but not in the ipsilateral hemisphere in mouse visual cortex. After 7 days of MD, the expression of GluR1 was indistinguishable from control animals while the expression of αCaMKII was substantially higher compared to 2 days MD in both the contralateral and ipsilateral hemispheres. It is possible that reduced expression of GluR1 after 2 days of

MD is transient and permissive for subsequent potentiation of open-eye responses as seen after only 7 days of MD. This however needs to be validated by increasing the number of samples and isolating ocular dominance columns representing the left and the right eye so that molecular candidates responsible for depression of deprived eye and potentiation of non-deprived eye can be established. Also, such studies should take into account that the mechanisms of LTD (Daw et al, 2004; Crozier et al, 2007) and OD plasticity (Trachtenberg et al, 2000) may vary across cortical layers.

4.4.7 Conclusion

My findings point to a set of proteins that transduce input activity during development into maturation and refinement of cortical circuitry. Different forms of visual experience, such as dark-rearing and monocular deprivation, activate some similar but also different mechanisms that underlie experience-dependent plasticity in the visual cortex. The effects of dark-rearing indicate that sensory activity regulates mechanisms associated with both excitatory and inhibitory transmission and synaptogenesis. On the other hand, the effects of monocular deprivation demonstrate that sensory experience additionally regulates signalling molecules downstream of NMDA receptor subunits. It appears that these multiple signalling molecules provide a means to initiate cascades of events that are unique to different paradigms of sensory experience.

CHAPTER V

OUTLOOK

5.1 Relating functional maps with proteomics

In the present study, dark rearing from birth resulted in substantial increase in the expression of $GABA_A\alpha1$ at 3, 5 and 12 weeks of age while the developmental decline of NR2B and inclusion of NR2A was prevented in dark-reared cats at 5 and 12 weeks of age. Relating these expression profiles to the physiological response observed and in light of previous studies (Hensch and Stryker, 2004; Li et al, 2008) it is possible that elevated expression of $GABA_A\alpha1$ is one of the mechanisms that maintained the orientation selective response and ocular dominance organisation observed in dark-reared cats at 5 weeks of age. The enhanced expression of $GABA_A\alpha1a$ combined with depressed expression of NR2A subunits maintained the overall excitation and inhibition balance (Chen et al, 2001; Turrigiano and Nelson, 2004; Yu et al, 2008) and this balance is possibly one of the mechanisms underlying the maintenance of orientation selective response and ocular dominance organisation in dark-reared cats at 5 weeks of age. However, it appears that these two mechanisms are not solely responsible for maintaining cortical properties in dark-reared cats as the columnar organisation disappeared in dark-reared cats at 12 weeks of age despite elevated $GABA_A\alpha1a$ expression levels. It is plausible that combined sensory experience and the balance between excitation and inhibition at later stages become necessary for the maintenance of initially established cortical properties.

A week of dark rearing (LR-DR) only up-regulated the expression of $GABA_A\alpha1a$ while the expression of NR2A and NR2B remained unchanged, but in contrast to prolonged period of dark rearing, the expression of GluR1 and synGAP also increased in LR-DR subjects. However, the cortical properties such as the layout of ocular dominance and orientation maps after a week of darkness did not differ from maps obtained from subjects that were raised in complete darkness from birth until 5 weeks of age. In light with previous studies (Hensch and Stryker, 2004; Yu et al, 2008), it is possible that enhanced expression of $GABA_A\alpha1a$ maintained orientation selectivity and ocular dominance organisation while elevated expression of GluR1 maintained overall excitation and inhibition balance (Yu et al, 2008). This suggests that even though the cortical properties tend to be affected in a similar way (in subjects that were raised in complete darkness and subjects that were exposed to a

week of dark rearing at 5 weeks of age), at the molecular level, different molecular mechanisms tends to be employed depending on the nature of the sensory experience.

Subjects that were raised in complete darkness from birth followed by a week of sensory experience (DR-LR) attained ocular dominance and orientation preference maps similar to normal animals. At the molecular level, the expression of $GABA_A\alpha1a$ remained elevated as in LR-DR subjects, but the expression of NR2A, NR2B and PSD-95 was also up-regulated. It is possible that combined elevated expression of $GABA_A\alpha1a$ and excitatory subunits maintained the overall balance between excitation and inhibition which subsequently facilitated the orientation selective response (Yu et al, 2008) and ocular dominance organisation as in normal animals. It is tempting to say that combined elevation of $GABA_A\alpha1a$, NR2A and NR2B subunits resulted in more neuronal spiking activity resulting in strong intrinsic signal and therefore clear and distinct ocular dominance and orientation selective patches.

Downstream signalling molecules have not received much attention in terms of the role they play in sculpting ocular dominance and orientation map layout. Many of the studies have focused on the role the downstream signalling molecules play in ocular-dominance plasticity (Taha et al, 2002; Hensch et al, 1998; Fischer et al, 2004; Di Cristo et al, 2001; Oliveira et al, 2008; Kind et al, 1994; Choi et al, 2005). A study by Fagiolini and colleagues (2003) is one of the few studies that explored the role of downstream signalling molecule such as PSD-95 in the organisation of cortical properties and found that orientation selective response fails to mature in mice with targeted disruption of PSD-95. It is possible that elevated expression of PSD-95 observed in DR-LR subjects might also be contributing to strong orientation selective patches.

Monocular deprivation after 2 days resulted in reduced expression of GluR1 and loss of NR2B while the expression of $GABA_A\alpha1a$ was indistinguishable from control. It is tempting to speculate that maintained levels of $GABA_A\alpha a1$ combined with reduced expression of excitatory subunits maintained the balance between excitation and inhibition and therefore permitted normal orientation map layout

through stimulation of the deprived eye, albeit weaker than the ones dominated by non-deprived eye after 2 days of monocular deprivation. However, after a longer period of MD (7 days), the orientation maps through stimulation of the deprived eye disappeared even though the expression of $GABA_A\alpha a1$ and GluR1 was indistinguishable from control subjects. But the expression of downstream signalling molecules such as αCaMKII and synGAP was up-regulated after 7 days of MD; however, their contribution to the organisation of orientation or ocular dominance maps requires further investigation. One can argue that up-regulation of αCaMKII and synGAP is an attempt to compensate for reduced activity by engaging a mechanism that should enhance excitatory transmission.

Several studies have shown that small changes in the balance between excitation and inhibition can profoundly alter experience-dependent plasticity (Hensch et al, 1998; Huang et al, 1999). The maintenance of this balance occurs via homeostatic mechanisms and when activity is reduced, the strengths of all excitatory synapses increase (Turrigiano et al, 1998) while increasing the levels of activity (by blocking inhibitory synapses) decrease the strength of all excitatory synapses (Kilman et al, 2002). Consistent with previous studies, the present study also demonstrated that in the absence of sensory experience, the expression of $GABA_A\alpha 1a$ and excitatory subunits such as NR2A, NR2B and GluR1 adjust homeostatically to compensate for reduced activity. This indicates that excitation and inhibition must be delicately balanced to keep cortical networks functional (Turrigiano and Nelson, 2004).The expression of $GABA_A\alpha 1a$, NR2A and NR2B was up-regulated in response to sensory experience (DR-LR) and this is in contradiction to the homeostasis rule. However, it is possible that elevated activity initially increases the expression of excitatory and inhibitory subunits which then increase the overall firing rate, but with continued sensory experience, the expression of these subunits may return to normal levels (Turrigiano et al, 1998).

5.2 Strengths and weaknesses of the study

The present study is unique as it is the first that employed the use of combined physiological recordings and proteomics to study experience-dependent plasticity in cat visual cortex. The combined use of these two techniques offered the advantage of relating physiological responses to the expression levels of various proteins and how those changes in expression levels might be contributing to the functional organisation of the visual cortex in different rearing conditions. Since cortical protein expression is crucial for rapid ocular dominance plasticity (Krahe et al, 2005), it is of utmost importance to identify at the protein level the different molecules that contribute to experience-dependent plasticity. Although some of the molecules (such as PLCβ1 (Kind et al, 1994); NR2A (Chen et al, 2000); mGluR5 (Reid et al, 2001); $GABA_A\alpha 1a$ (Mower and Chen, 2003) associated with the critical period for ocular dominance plasticity in cats have been examined, the role of downstream signalling molecules that form pathways with receptor subunits has received scant attention. Additionally, there are no data on sets of proteins that form part of NMDA receptor complex that are up- or down-regulated in different paradigms of activity-dependent plasticity in cat visual cortex. Therefore the present study offered the advantage of identifying changes in protein expression in the visual cortex in different rearing paradigms.

Even though there is no evidence, one way or the other it is possible that the time involved in acquiring optical imaging maps and recording visually evoked potentials might be sufficient to involve slight if not major changes in protein expression because of the prolonged visual stimulation. Future studies will require infusion of protein synthesis inhibitors such as cycloheximide or rapamycin (Krahe et al, 2005) before physiological recordings so that it is certain that no further changes in protein expression take place after completion of the rearing process.

Due to the lack of commercially available feline specific antibodies, the success of such immunoblotting experiments relied on sequence homology to allow cross-reactivity between species. The antibodies were expensive and results were not always reproducible, for instance, NR2A did not always show a band. After long-term storage (more than 6 months at 4°C or -20°C) of antibodies, some of them lost

their efficacy and therefore failed to yield any specific bands. Furthermore, the immunoblotting experiments were laborious and very time consuming and therefore it was not possible to achieve the sort of sample sizes that would have been desirable for statistical analysis.

In contrast to Western blotting, two-dimensional gel electrophoresis (2-D DIGE) offers the advantage of characterisation of many proteins simultaneously as done previously (Van den Bergh et al, 2006). The two-dimensional gel electrophoresis relies on separating proteins based on iso-electric point or protein mass and offers the advantage of comparing multiple samples by internal standard or by comparing each sample with a sample that is kept constant throughout the experiment (Van den Berg et al, 2006). Proteins that are differentially expressed from the internal standard are the ones that are regulated and one can validate only those proteins using Western blotting. However, due to the lack of necessary equipment for two-dimensional gel electrophoresis, I chose to use Western blotting for characterisation of few selected proteins.

5.3 Future outlook

In cats because each hemisphere receives inputs from both eyes, one cannot determine whether the molecular changes observed after monocular deprivations are due to loss of deprived eye responses and/or potentiation of non-deprived eye responses. Therefore in order to dissect further the mechanisms underlying experience-dependent plasticity, ocular dominance columns representing the left or the right eye need to be isolated in subjects that have undergone monocular deprivation. The present study and other studies (Friedlander et al, 1991; Frenkel and Bear, 2004) have shown that the effects of MD occur in two distinct temporal profiles: depression of deprived eye response followed by a delayed potentiation of non-deprived eye response. In order to determine molecular changes related to the two-stage processes, one could isolate ocular dominance columns from subjects that had undergone 2 and 7 days of monocular deprivation. The columns representing the left or the right eye can be isolated by stereotactically targeting patches obtained from ocular dominance maps during the optical imaging session, using the blood-vessel pattern (reference image) for alignment with the optical imaging map. Since the size of the ocular dominance column is only few millimetres in width (Hubener et al, 1997), the small volume of tissue that could be harvested per column would require extraction of at least 5 or more patches for each eye for microarrays and Western blotting (see below).

Microarrays could then be performed to measure changes in expression levels of RNA (ribosomal nucleotide acid) most commonly referred as cDNA (complimentary deoxyribonucleotide acid after reverse transcription) in isolated columns. Such high density cDNA array-based analysis will determine gene expression patterns of thousands of genes simultaneously. This will reveal large number of genes that are orchestrated in OD plasticity, however, it should be noted that mismatches do occur between the levels of RNA and proteins expressed (Anderson and Seilhamer, 1997).

Microarrays have been successfully applied in several species such as mice (Tropea et al, 2006; Majdan and Shatz, 2006), cats (Prasad et al, 2002) and monkeys

(Lachance and Chaudhuri, 2004). Prasad and colleagues (2002) found that there is at least 85% identity in cats and human gene coding sequence and therefore used membrane-based assays containing human cDNAs in cat visual cortex for microarray analysis. This analysis examined gene expression over different postnatal time points covering the visual critical period (P0, P10, P30 and adult animals) and comparing to dark-reared animals (4 months). The candidate plasticity genes identified by Prasad et al. (2002) comprised 21 known genes plus another 31 uncharacterized expressed sequence tags. Those plasticity candidate genes included participants in second messenger systems, in cell adhesion, in transmitter recycling and cytokines. However, their study did not examine the effects of monocular visual input on gene expression profile. Therefore, this method could be employed to analyse expression profile of columns represented by one eye or the other. This would then help to identify gene expression levels that are tightly coupled to sensory input and delineate more precisely the molecular events underlying experience-dependent plasticity. In order to identify expression at the protein level, once the regulated gene set is established using microarrays; Western blotting could then be carried out to determine the expression of those proteins.

The mechanism of OD plasticity varies across cortical layers (Trachtenberg et al, 2000) and therefore it is important to determine any layer differences in the molecular mechanism underlying this form of plasticity. It would be interesting to use another set of animals to mark deprived-eye and non-deprived-eye columns with fluorescent tracers such as green fluorescent protein (GFP). A recombinant adenovirus bearing the GFP gene could be used as done previously to label intrinsic connections in macaque primary visual cortex (Stettler et al, 2002). The animals could then be perfused with 4% paraformaldehyde so that coronal sections of visual cortex can be obtained. One could then label proteins identified in the regulated gene set (using microarrays) to determine laminar differences in plasticity mechanism.

It is expected that deprived eye columns should reflect LTD-linked processes such as down-regulation of NR2A and GluR1 subunits while non-deprived eye columns

should show LTP-linked processes such as up-regulation of NR2B. The possible synaptic mechanism underlying monocular deprivation has well been documented in mice (Heynen et al, 2003; Cho and Bear, 2007; Yoon et al, 2009) and it is proposed that in juvenile animals the deprived-eye responses rapidly weaken through LTD while open-eye responses are strengthened more slowly via LTP. However, limited research has directly addressed the question of possible cellular mechanisms in higher mammals such as felines or primates. A study Komatsu et al (1988) was one of the first studies to provide evidence that repetitive stimulation of the white matter in slice preparations and stimulation of LGN and optic chiasm in vivo in kittens produced potentiation of synaptic transmission lasting for several hours. Their study also revealed that both in vivo and in vitro, LTP occurred more frequently in juvenile animals (P25-P35) than in older animals and occurred more strongly in layers II/III than in any other layers. Another study from the same laboratory (Komatsu et al, 1991) made intracellular recordings from layers II/III obtained from slice preparation of kitten (P30-P40) visual cortex and showed that low frequency stimulation evoked two responses: EPSP followed by IPSP. The falling phase of EPSP was blocked by APV (an NMDA receptor blocker) while the rising phase of EPSP was insensitive to APV (Komatsu et al, 1991), therefore it appears that the induction of LTP is not mediated by NMDA receptors. However, their study did not examine the potential involvement of AMPA receptors in LTP. Several studies from mice have revealed that AMPA receptors are rapidly delivered to synapses during LTP and mice lacking the GluR1 subunit cannot generate LTP (Zamanillo et al, 1999). The involvement of AMPA receptors in LTP and LTD processes has not received much attention in cat visual cortex. The present study has highlighted some important candidate molecules such as PSD-95, αCaMKII, synGAP GluR1 and NR2B which are likely to contribute to LTP and LTD mechanisms, and therefore examining their expression levels in deprived eye and non-deprived eye columns may yield additional insight into cellular mechanisms of experience-dependent plasticity in cats.

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