



**BIOINFORMATICS AND GENETICS ANALYSIS
OF EXPERIENCE DEPENDENT PLASTICITY IN
THE MOUSE BARREL CORTEX**

SUBMITTED FOR THE DEGREE OF Ph.D.

BY

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ABSTRACT

Formation of neuronal circuits represent memories, making synaptic plasticity the root of learning and memory (Buonomano and Merzenich 1998). Neuronal plasticity has been studied using facial vibrissae deprivation paradigm in rodents (Fox 1992). Whisker deprivation alters the balance of activity in cortical neurons and their responses to sensory input, providing good grounds to study experience dependent plasticity (Simons and Land 1987; Fox 1992). Alterations in gene expression underpinning changes in cortical activity have been investigated in this thesis. The molecular signature underlying the temporal effect of repeated anaesthesia was identified and provided a fertile area for future work, revealing the necessity to separate anaesthesia from deprivation induced changes. Changes in gene expression were gender specific, with the females exhibiting quicker neuronal organisation. Taking under consideration the two confounding factors; anaesthesia and gender, a new normalisation protocol was developed underpinning investigations of plasticity dependent transcriptional alterations. The present study confirmed the two molecular mechanisms underlining synaptic plasticity (Shi *et al.* 1999); with early time points (Day 1) revealing alterations of existing synaptic proteins and later time points (Day 8 and 16) indicating neurotransmitter release regulating gene expression. Day 8 was identified as the critical time point for plasticity, exhibiting the peak of transcriptional changes. Gender specificity was evident, indicating a role for hormonal-dependent gene expression, which future studies should consider. Ontological analysis has confirmed the role of Ca^{2+} trafficking (via AMPARs and NMDARs) and calcium dependent binding (involving molecules like Calmodulin) in a variety of pathways, such as transporter activity, channel activity and neurogenesis, associated with gene transcription and regulation of plasticity. A significant up-regulation of the expression profiles of transcripts associated with plasticity, NOS1, NOS3 and Bassoon was observed at Day 8 in wild type mice. GluR1^{-/-} mice revealed the direct relationship of these genes with the GluR1 subunit of AMPA receptors. A delayed up-regulation was detected after 16 days, suggesting a plausible delayed compensatory mechanism in the absence of the GluR1 subunit of the AMPA receptor. Gene ontology provided a functional footprint for plasticity even in the GluR1^{-/-} mice, known to exhibit impaired post-synaptic plasticity (Schmitt *et al.* 2005).

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Understanding our surroundings is critical to our survival. Our awareness of the world is dependent on the sensory information we gather and the way our brains interpret this information. Our sensory inputs come via vision, smell, taste, hearing and touch whilst due to the nocturnal nature of rodents, such as mice and rats; they rely on mystacial vibrissae (whiskers) to sense their environment. Understanding of the physical mechanism by which a stimulus is registered and transmitted from the sensory organ (such as an eye or a whisker) to the appropriate area of the brain is one of the major challenges of neuroscience. Conversely, the response of the brain to this stimulus comes mainly through the processing of the signal being transmitted between and within the neuronal cells leading to the formation of memories and storage in the appropriate brain areas. Processing of the signal transferring the information from the sensory organs to the brain takes place in the thalamus which is thought to be the station for all sensory signals before they are distributed to the appropriate cortical areas; whether that is the visual cortex or the barrel cortex. Studying the architectural remodelling of the neural pathways that occurs upon a change in sensory input has been used to understand the interplay of neuronal circuits and determine their relationship to memory. This project aims to investigate the molecular modifications brought about by sensory alterations, via whisker removal and the resulting neural plasticity in a well defined model system, the mouse barrel field.

1.2. Historical overview: Mapping the anatomy of sight

The basic understanding of sensory mechanisms was investigated by the Greeks circa 600 – 400 B.C. who studied the visual system. However, little further progress was made until the 1900s, when chemical and histological advancements identified and facilitated studies on a cellular level of the brain region responsible for sensory processing, the cortex. Electron microscopy provided visualisation of synaptic

connections, which revealed the true complexity of the cerebral cortical architecture (Wagor *et al.* 1980).

A seminal series of experiments by Hubel and Wiesel investigated the visual cortex of cats (1959, 1962, and 1963) and monkeys (Hubel & Wiesel 1968, 1974; Hubel *et al.* 1977; Wiesel & Hubel 1974). Their preliminary experiments showed that alterations in the sensory input led to long lasting anatomical and physiological changes in the cortex (Hubel and Wiesel 1962), thus providing evidence that the brain changes synaptic connections in response to the external stimuli.

1.3. The visual cortex

Hubel and Wiesel in the 1960's were the first ones to introduce the scientific world to the concept of experience dependent visual cortex plasticity (Hubel and Wiesel, 1962), by showing that alteration at the balance of sensory input from the eyes results in long-lasting anatomical and physiological changes in the cortex. Long term changes to the synaptic connections in the visual cortex can be introduced by depriving animals of visual experience during a *critical period* in their life.

Afferents from the thalamus connect with layer IV neurons of the primary visual cortex, where the formation of ocular dominance columns is achieved (Purves *et al.*, 2001). Ocular dominance columns can be visualised by the injection of radioactively labelled tracers into the eye (Stryker and Harris, 1986). The ocular dominance columns can be seen as stripes of neurons corresponding to cells that respond to either the left or right eye when viewed in horizontal sections in layer IV (Le Vay *et al.*, 1980). Cortical layers above and below layer IV form the binocular zone that contains neurons responsive to either eye, whose activity can be recorded. Hubel and Wiesel have developed a scoring system dependent upon the degree of response of the seven ocular dominance categories:

- category 1 neurons respond to stimulation of the right eye only
- category 7 neurons respond only to the left eye
- category 4 neurons are responsive to both eyes equally
- categories 2, 3, 5 and 6 contain cells that respond to both eyes but with extra vigour to either the right (5 and 6) or left (2 and 3).

In control animals the majority of neurons are driven by both eyes and a much smaller proportion is driven by either the left or right eye (Hubel and Wiesel, 1962). In order to understand the developmental processes of this well defined and organised visual cortex, cats were subjected to eye suturing at the day of birth for 2.5 months followed by re-opening. A significant difference in the distribution of neurons amongst the ocular dominance categories is detected when recordings were performed in the visual cortex three years later with all neurons being driven by the eye contralateral to the deprived eye (Hubel and Wiesel, 1963). The same experiment has also been performed in adult cats around the age of 12 months and the eye has been kept closed for 2 years with most neurons being driven by either eye (Hubel and Wiesel, 1970). Hence, the term *critical period* was introduced in order to determine the time during which experience has the potential to change the synaptic connections and shape the brain's connections. The physiological changes mentioned above are followed by anatomical changes with a severe decrease in size of the ocular dominance columns of the deprived eye and an enlargement of the columns corresponding to the undeprived eye (Le Vay et al., 1978, 1980).

Similar responses to monocular deprivation have been found not only in cats (as mentioned above) but also in sheep (Kennedy et al., 1980), rabbits (Van Sluyters and Stewart, 1974), hamsters (Emerson et al., 1982) and mice (Gordon and Stryker, 1996). Monocular deprivation for 4 days in mice, during the critical period, is sufficient to shift the responsiveness of neurons in layer IV to the undeprived eye. The critical period occurs from birth and is maximal at postnatal day 28 until 32 where effects of monocular deprivation diminish rapidly (Gordon and Stryker, 1996). Interestingly enough, when neuronal responses are recorded using visual-evoked cortical potentials (VEP) the effects of monocular deprivation extend past puberty into adulthood (Guire et al., 1999, Lickey and Gordon, 2002). However, these changes lack the stability and solidity of those observed in younger animals (Pham et al., 2004).

1.4. Anatomy of touch: Mapping the neocortex

The cerebral hemispheres are surrounded at the extreme distal surface by the neocortex, which are comprised of four lobes: frontal, parietal, temporal and occipital. The primary somatosensory cortex (S1) is located within the parietal lobe, and is responsible for the processing of tactile senses (Woolsey 1978). The neocortex is divided into six layers – layers I-VI (Figure 1.2) (Hubel and Wiesel 1962).

Cortical neurons and barrel boundaries between layers I to V have been studied in more detail (Simons and Woolsey 1984). Three classes of neurons were recognised: pyramidal cells, class I non-pyramidal cells and class II non-pyramidal cells. Pyramidal cells are characterized by conical somata, a stout apical dendrite and spines. Class I neurons have small somata with proximal dendritic branching and shorter dendrites, whereas class II neurons have smoother dendrites. Furthermore, there are two subdivisions of class I neurons: the star pyramids and the spiny stellate cells. Likewise, class II neurons are comprised of two subcategories: the multiform cells and the bipolar cells (Simons and Woolsey 1984). Layer I is the outermost layer of the neocortex, containing glial cells. The axons and dendrites of this layer's neurons extend laterally (Caviness and Frost 1980; Frost and Caviness 1980; Jensen and Killackey 1987).

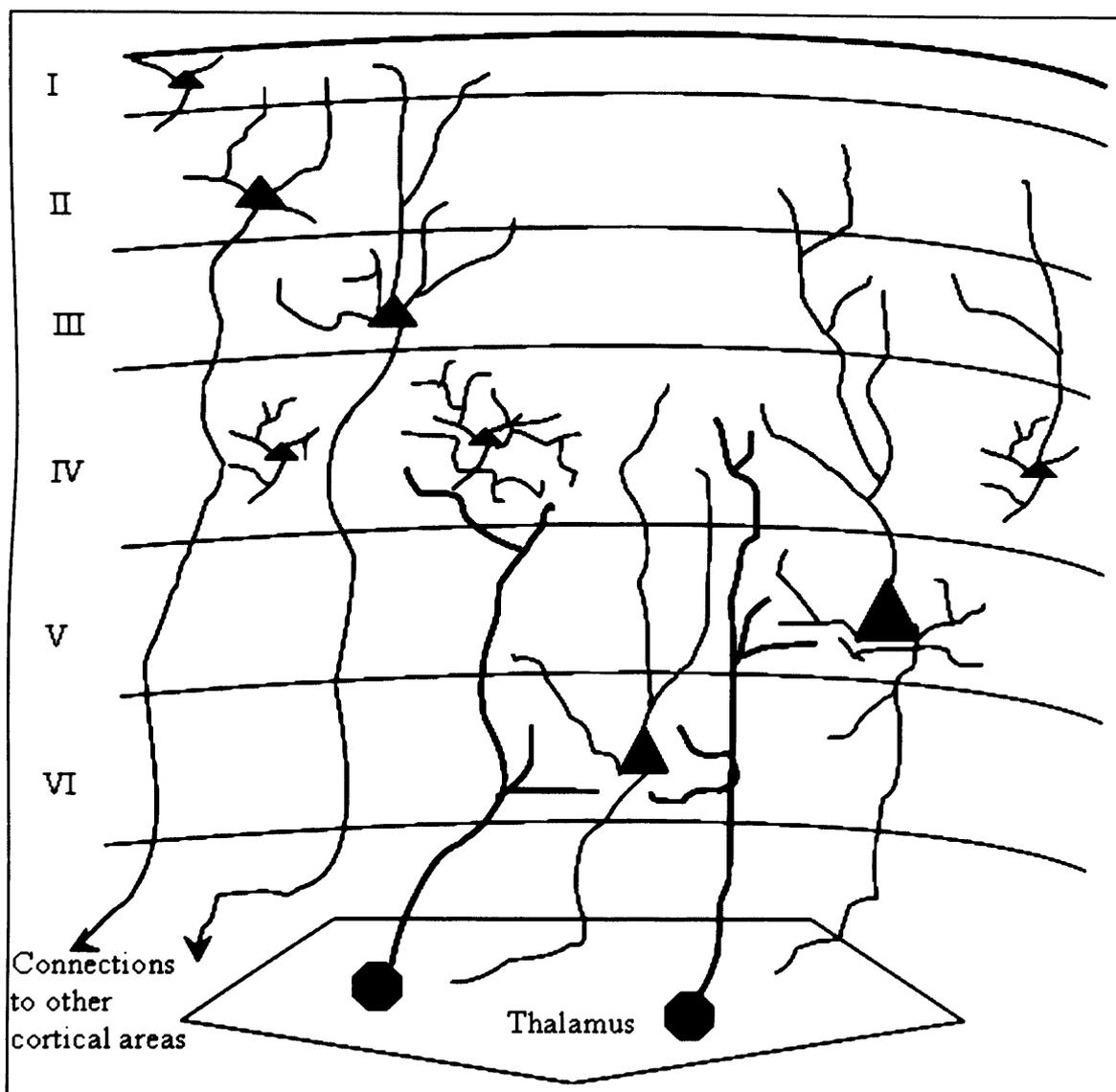


Figure 1.2: Simple diagrammatic representation of cortical layers I-VI. Cortical neurons are shown as triangles, with their endings protruding to other cortical layers (above or below) and into the thalamus.

Small pyramidal neurons are found in layers II and III. The dendrites of neurons in these layers run laterally, as well as towards layer I. The majority of output is provided by layers II and III to other cortical regions (Frost and Caviness 1980; Jensen and Killackey 1987).

Layer IV, the densest of the layers, also contains two types of neurons: pyramidal and non pyramidal; with the latter in abundance (~80%). Layer IV neurons primarily

receive thalamic input and process locally-produced information (Jensen and Killackey 1987). It is also believed that layer IV cells amplify the thalamic signal and redirect it to other layers. When layers II/III receive this amplified signal, they spread it laterally and vertically (Armstrong-James and Fox 1987).

The apical dendrites, contained in layer V, are large pyramidal cells which project predominantly into upper cortical layers (Armstrong-James and Fox 1987; Markram 1997; Reyes and Sakmann 1999).

Large pyramidal cells are the major components of Layer VI. Their axons run towards the thalamus, forming the major output of the cortex to the thalamus (Wise and Jones 1977a, 1977b; White and Keller 1987). The white matter, lying immediately below layer VI, carries axons to and from the cortex.

1.5. The thalamus and its relation to the somatosensory cortex

The thalamus lies deep in the cerebral hemispheres (Figure 1.3) in each side of the forebrain. It has the key role of transmitting information to the neocortex (Sherman and Guillery 1996). The thalamic nuclei serve as “stations” for all sensory messages before they are transmitted to the cortex (Figure 1.4). The thalamus is divided into three regions: **dorsal**, **ventral** and **epithalamic**. The thalamus is connected to the cerebral cortex through its dorsal and ventral regions (Sherman and Guillery 2002; Lopez-Bendito and Molnar 2003).

The dorsal thalamus is further sub-divided into two regions: the ventrobasal complex (VB) and the posterior complex (POM) (Rose and Mountcastle 1952). First order nuclei mainly constitute the VB, transferring information into the cortex. The neurons from the ventrobasal complex project in layers III and IV (Jensen and Killackey 1987; Sherman and Guillery 1996). In contrast, the POM consists mainly of higher order nuclei. Neurons from this complex project to layers III and IV, whereas they receive input from layers V and VI (Sherman and Guillery 1996). The ventral thalamus contains the thalamic reticular nucleus, lying between the thalamus and the cortex. Information is received from the thalamus and cortical layer VI.

There are two thalamocortical pathways for signalling sensory information to the barrel cortex. Neurons in the ventral posterior medial (VPM) nucleus are glutamatergic and are primarily responsible for signaling information relative to deflections of a single whisker. The axons of these neurons terminate at barrels present in layer IV with minor innervations in the upper areas of layer VI. The POM innervates the septal regions of barrel cortex via the paralemniscal pathway, with cortical neurons typically exhibiting broad receptive fields (Brecht, 2007). Corticothalamic inputs from the POM predominantly innovate layer Va, although other inputs to layer IV, III and II are evident (Brecht, 2007).

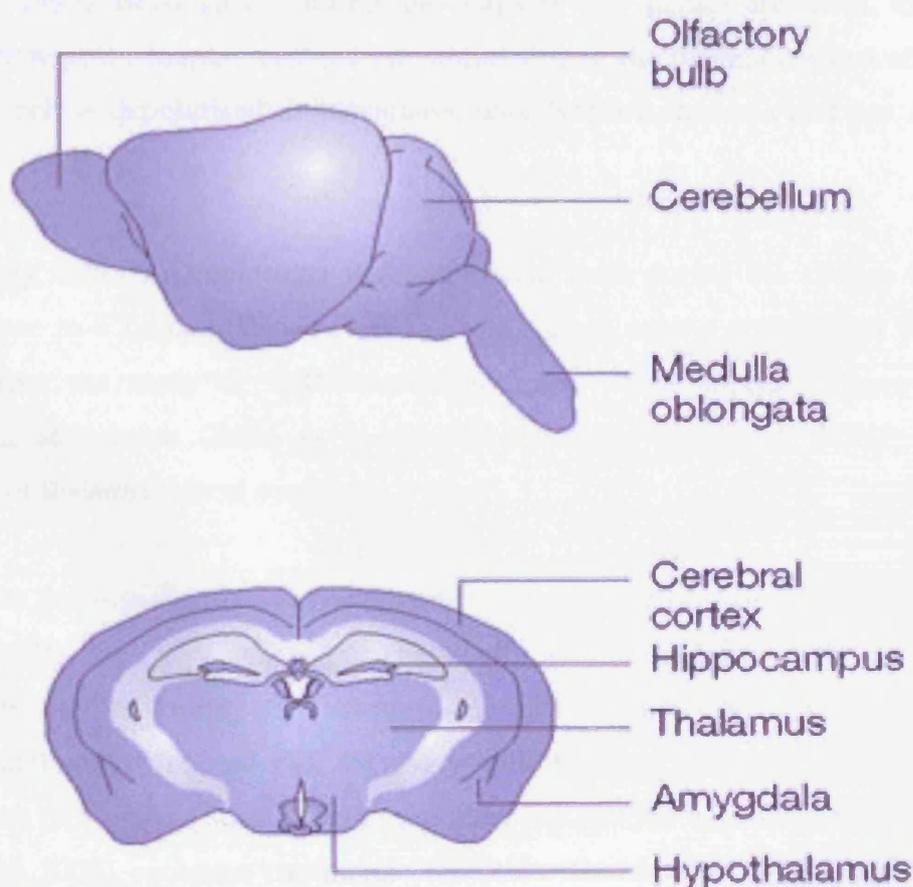


Figure 1.3: Representation of the mouse brain and its main parts. Brain areas such as the cerebral cortex, the hippocampus and the thalamus are visible (Cryan and Holmes 2005).

Thalamic afferents are organised as early as P0 in the rodent. At the time that the thalamic axons arrive at the position that will give rise to layer IV; the cortical plate has a non-differentiated and homogenous form. Between stages P0 and P2, thalamocortical arbors overlap several barrels, before being restricted to the one barrel during the first postnatal week of the rodent's life (Agmon *et al.*, 1995). Neuronal activity is required for the refinement of TCA targeting both before and after birth. TCA arbors will no longer exhibit the expected topography and the excitatory connections will be inhibited (Fox *et al.*, 1996; Inan and Crair, 2007).

Synaptic transmission can be measured on cortical plate neurones as early as P0, from immature synapses. The number of these synapses is low during barrel formation (Kim *et al.* 1995). Between P2 and P5, the majority of synapses are silent, meaning that they show post-synaptic responses to stimulation of the thalamocortical afferents only if the cell is depolarised as it contains only NMDA channels and not AMPA (Isaac *et al.* 1997).

One of many important molecules with significant roles during the change from a silent synapse to a functional one is BDNF. Its role in such a process has become apparent from the study of BDNF knockout mice which appear to have silent synapses in abundance (Itami *et al.* 2003), indicating a role of BDNF in the maturation of thalamocortical synapses.

Not only do thalamocortical synapses mature via the insertion of AMPA channels postsynaptically, they also mature presynaptically with an increase of the density of thalamocortical arbors during the first postnatal week (White *et al.* 1997). During the early postnatal stages (around P4), the presynaptic terminal consists of a number of receptors and transporters such as serotonin receptors, serotonin transporters (Young-Davies *et al.* 2000), nicotinic cholinergic receptors (Broide *et al.* 1996) and kainate receptors (Kidd *et al.* 2002). Although, the above receptors and transporters show an initial increase, they demonstrate a decrease between P10 and P21 during which period NMDA-dependent AMPA insertion takes place as well.

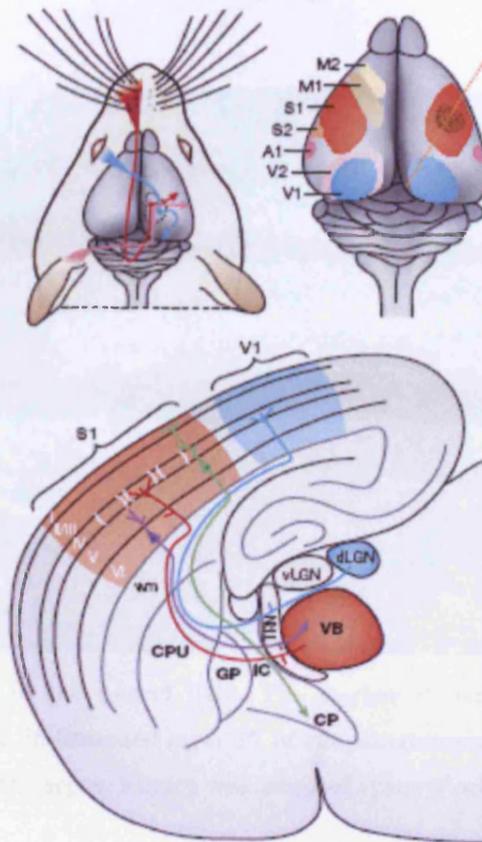


Figure 1.4: Representation of thalamocortical connections important for processing input from sensory organs to their way towards the appropriate brain areas (Lopez-Bendito and Molnar 2003).

1.6. The rodent somatosensory cortex: Barrels and barrel field

In rodents, neurons within layer IV are arranged in cylinder-like structures called barrels (Figure 1.5) and they form in the cortex during the first 4 post-natal days of a rodent's life (Woolsey 1967; Woolsey and Van der Loos 1970; Fox 1995). The thalamic input from VB (Figure 1.4) projects into cortical neurons within the barrels whereas POM inputs project to the space between the barrels (Koralek *et al.* 1988). This structure of aligned barrels surrounded by POM inputs is termed the barrel field and is the topographical representation of the mystacial vibrissae, with a 1:1 relationship between barrels and whiskers (Hubel and Wiesel 1962). The inter and intra barrel connections and their relation to the thalamus are important features of the

barrel field and could be altered in an attempt to study and understand the architecture of neuronal circuits and their relationship to external stimuli changes.

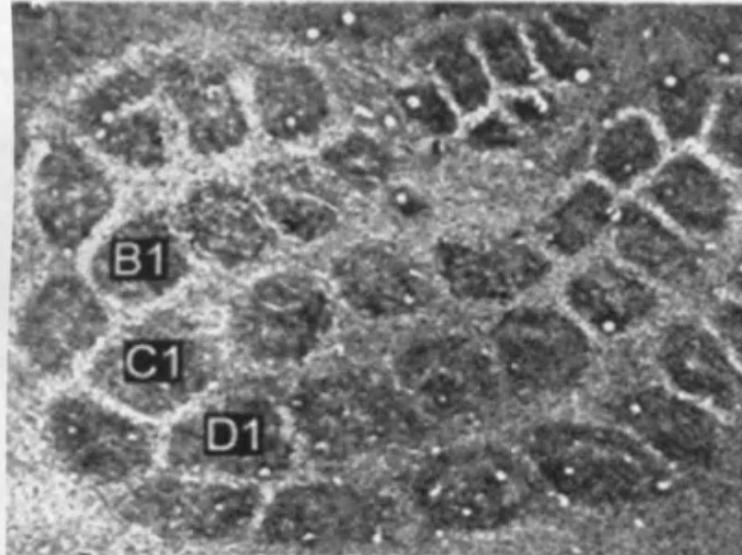


Figure 1.5: Representation of the barrel field. The darker structures are the barrels under Cytochrome oxidase staining, in flattened layer IV of rat somatosensory cortex. The intermediate regions between barrels are the septa. Figure was adapted from (Foeller and Feldman 2004).

1.7. Structure and function of the rodent's mystacial vibrissae

A rodent has flexible, moving whiskers on each side of its nose, which are thicker and have deeper roots than ordinary hairs. Due to the plethora of nerve endings, they receive vital sensory information about the environment. Whiskers are characterised by their sensitivity to external stimuli and are directly connected to the nervous system, identifying environmental messages by vibrations in the air. Arranged into rows and columns; each whisker is represented by a barrel, i.e. a group of cortical cells in the brain. Indeed, the barrel cortex in the brain is organized with remarkable similarity to the mystacial vibrissae (with a 1:1 relationship).

Early anatomical and physiological observations revealed that each vibrissal follicle is surrounded by two categories of muscles: **extrinsic** and **intrinsic**. The extrinsic muscles, so-called because they originate outside the mystacial region, are facial muscles coordinating the movement of the upper lip and the nose (Figure 1.5; Dorfl 1982). In contrast, the intrinsic, or follicular, muscles (Figure 1.6) connect two

adjacent follicles within a row of whiskers. The size of the muscles is associated with the size of the follicle, with the biggest being the one corresponding to follicles α , β , γ and δ (Dorfl 1982).

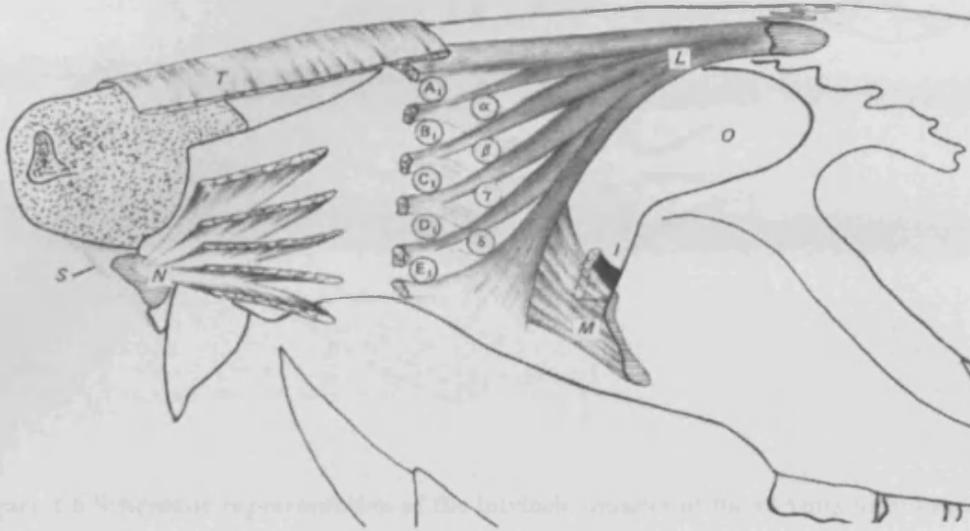


Figure 1.5: Schematic representation of the extrinsic muscles of the rodent's face; in particular the left whisker pad. The five follicle rows are marked on the Figure as A-E. "T" is the infraorbital nerve; L is the levator labii superioris; M is the maxillolabialis; O is the orbit; T is the transverses nasi and S is the septum intermusculare. Figure was obtained from Dorfl (1982).

The nerve supply of the rodent's mystacial vibrissae is highly ordered. Two nerves enter each whisker; the main vibrissal nerve enters the hair from the lower end and the small (conus) nerve enters the hair at the top. The main nerve, containing around 150 axons divides as it enters the hair, forming a network surrounding the follicle (Figure 1.7; (Renehan and Munger 1986)

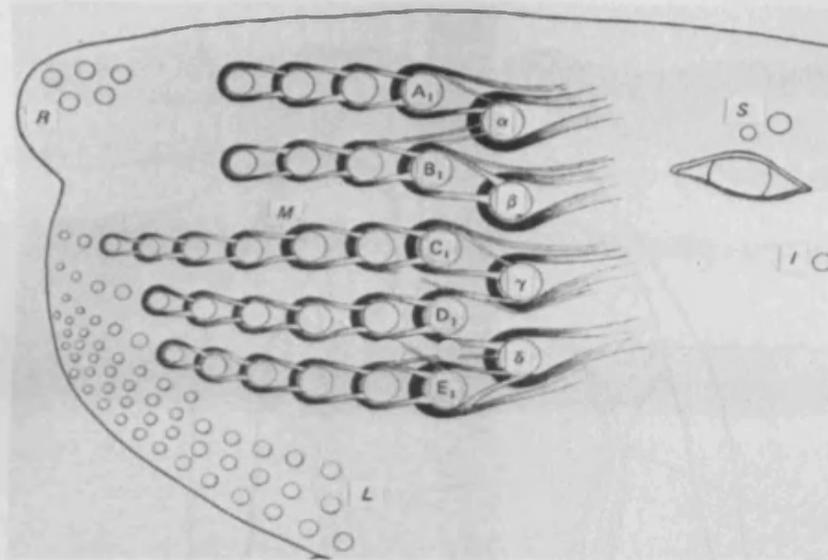


Figure 1.6 Schematic representation of the intrinsic muscles of the rodent's face; in particular the left region. In more detail, "I" is the infraorbital; L is the labial; M is the mystacial; R is the rhinal and S is the supraorbital. The black lines in the rodent's face represent the muscles embracing each facial follicle. Muscles were seen around all the rows, A-E. However, the more rostral follicles (just at the front of rows C, D and E) were not surrounded by intrinsic muscles. Figure was obtained from Dorfl (1982).

A very well constructed system of nerves and muscles are responsible for control of whisker movement (Figure 1.8). Moreover, whiskers fall into two size categories, each with distinct sensory tasks. It is believed that the larger whiskers withdraw information from the surrounding environment, whereas the smaller, more rostrally positioned, whiskers discriminate between different textured objects. Whisking – the process via which the whiskers move – is a two-phase movement: approach and withdrawal. Whilst sniffing, the nose is moved towards the object, but also the whole mystacial region is directed appropriately. In sleeping or anaesthetised rodents, whiskers are in their resting position. The extreme positions and the constant changes of direction of the hair are due to the elasticity of the connective tissue around the follicles (Dorfl 1982). It has been suggested that the sensitivity of the whiskers is comparable to the human fingertip (Carvell and Simons 1990).

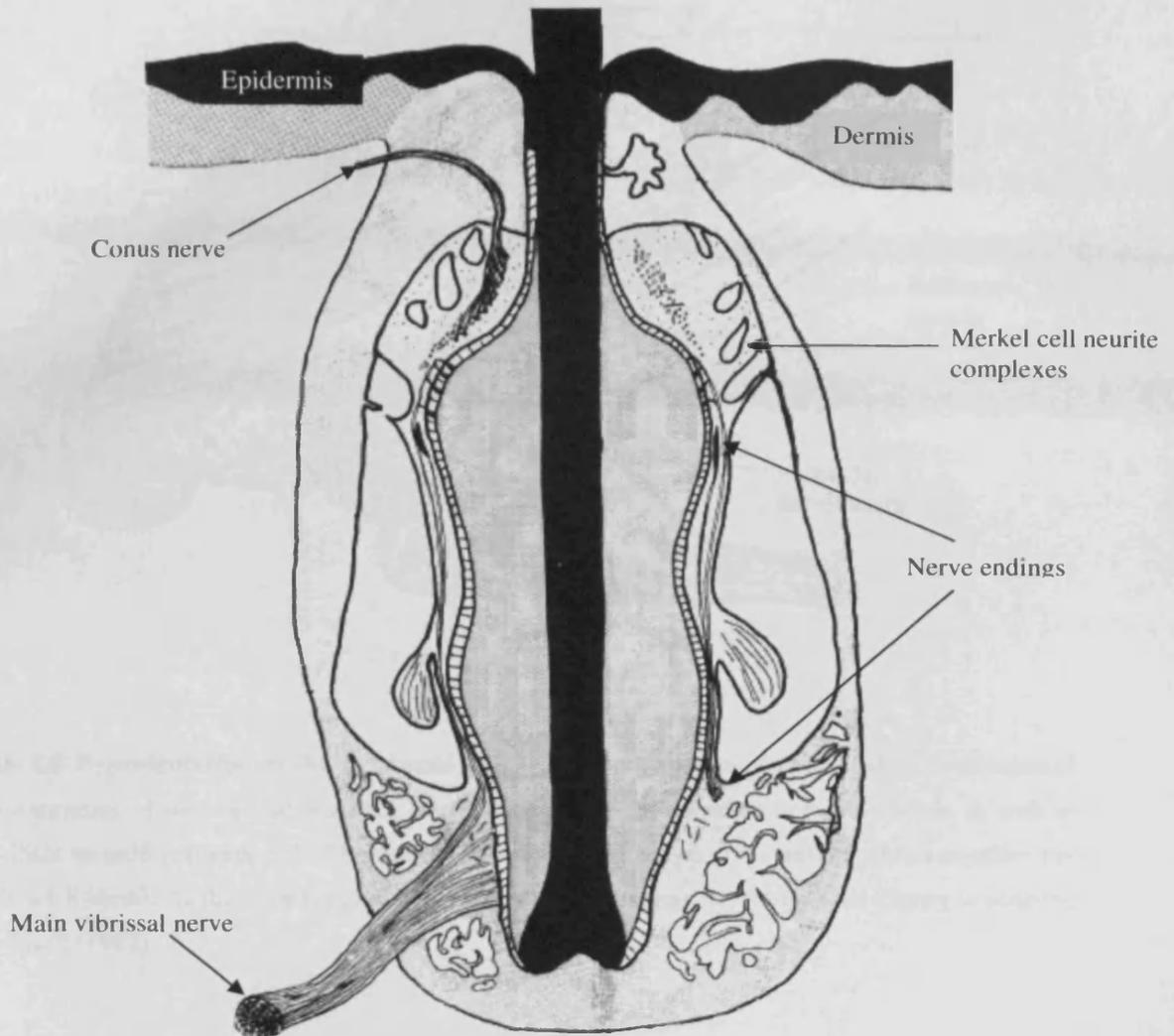


Figure 1.7 Representation of the enervation of the mystacial vibrissae. The main nerve enters the follicle from below and supplies free nerve endings at the rest of the follicle. The smaller nerve, the conus, enters from the top enervating other parts of the follicle. Figure is modified from Ranehan and Munger (1986)

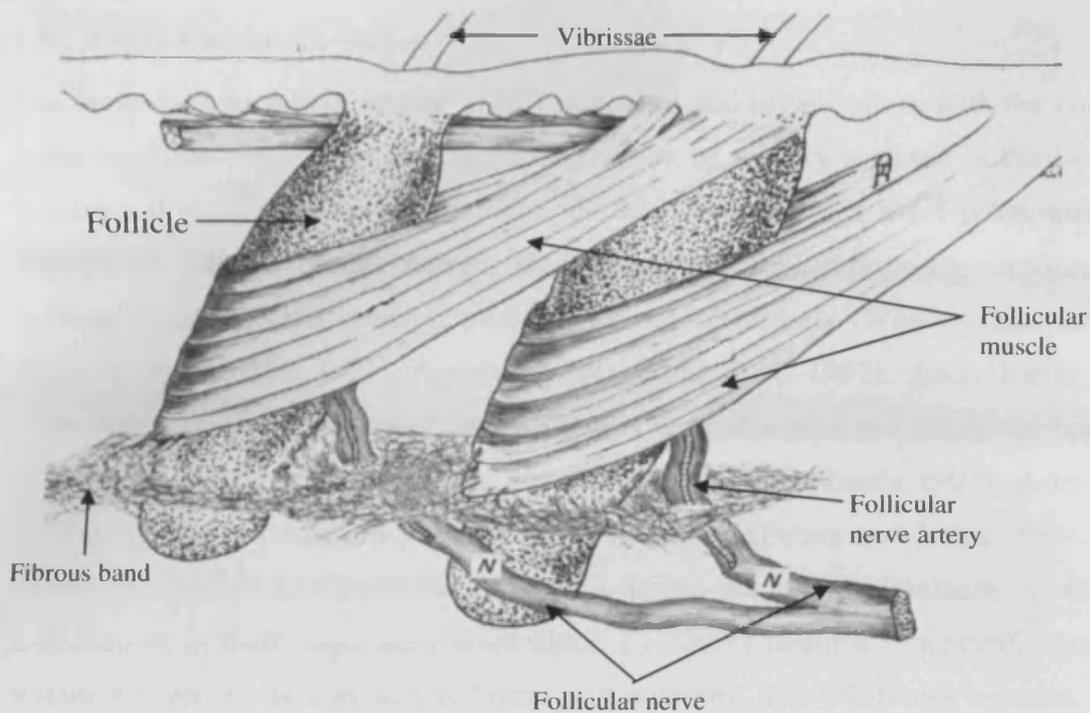


Figure 1.8 Representation of the mystacial follicle. The movement of the whisker is dependent upon a number of nerves and muscles. In this Figure the main follicle nerve is visible, as well as the follicle muscle embracing it. The nerve is also attached to the nerve artery which supplies the follicle with blood. At the very top, the beginning of the vibrissae is visible. This Figure is adapted from Dorfl (1982)

As mentioned earlier, the facial vibrissae are represented by barrels in the somatosensory cortex which, rather like the whiskers themselves, are organized in rows and columns. Cytochrome oxidase staining of the barrel field of the rodent's brain has been used to visualize and construct the somatosensory map (Woolsey and Van der Loos 1970). Woolsey and Van der Loos used tangential and coronal sections to answer questions about the morphology of the barrels, their connections and their precise position (Woolsey and Van der Loos 1970). The barrel field occupies a relatively large area of the cortex, revealing its importance and the degree of dependency the rodents have on their whiskers for a better understanding of their environment. Each barrel receives input from one specific whisker (Van der Loos and Woolsey 1973) and only a limited number of cortical neurons respond to movements of multiple vibrissae (Welker 1976).

1.8. From whisker to cortex

The cortex shows a high degree of differentiation and organisation, with the cortical areas organised into layers and the representation of sensory surfaces in the form of topological maps with the whiskers and the barrels maintaining a 1:1 relationship as mentioned above. Each barrel is responsible for processing information predominantly from its principle whisker but not exclusively (Woolsey and van der Loos 1970, Welker 1971, Armstrong-James and Fox 1987). Each barrel also represents a functional group of neurons that is vertically arranged across the borders of layers; a structure known as the cortical column (Mountcastle 1997). A cortical column consists of excitatory and inhibitory neurons (Peters and Jones 1984). The excitatory neurons, also known as principal neurons due to their dominant nature, use L-glutamate as their major neurotransmitter. Excitatory neurons synaptically interact within a layer as well as across layers and columns. The inhibitory neurons, also known as local-circuit cells, use GABA as their neurotransmitter and their axon usually stay within the column. It is believed that the full understanding of the size, texture and form of any given object is based upon intracolumn communications and the exchange of information between the neurons (Schubert *et al.* 2007).

The dynamic function of the barrel cortex can only be determined if the complete pathway between the whisker and the specific cortical area of response is determined. The deflection of a whisker evokes action potentials in sensory neurons of the trigeminal nerve releasing glutamate at the first synapse in the brain stem. In turn, the brain stem neurons are sending sensory information to the thalamus which leads to yet another glutamatergic synapse that excites thalamocortical neurons with projections onto the barrel cortex (Petersen 2007).

Neurons in the principal trigeminal nucleus are organized into distinct structures called barrelettes (Veinante and Deschenes, 1999). The principal trigeminal neurons project to the ventral posterior medial (VPM) nucleus of the thalamus, which also consists of specific anatomical units termed barreloids (Brecht and Sakmann, 2002). The axons of VPM neurons within individual barreloids project to the somatosensory

neocortex forming the barrels. The distinctive formation of the barrel map is arranged identically to the layout of the whiskers (Woolsey and Van der Loos, 1970).

1.9. Barrel Cortex Development

Understanding how the cortex develops and how it changes throughout the rodent's life is crucial for our comprehension of general brain function and possible treatments of neurological diseases. During cortical development, the formation of distinct areas that will become the message retrievers is crucial. These areas, or layers, are characterised by specific sets of input, output and information processing. Throughout time, studies have tried to characterise the formation of these layers and have tried to create a time line on the events taking place during neurogenesis (Butt *et al.* 2005). Two early hypotheses have been the main focus of research on the formation of cortical layers; the "protomap hypothesis" (Rakic 1988) and the "protocortex hypothesis" (O'Leary 1989). The first hypothesis required specific genes being expressed early in the ventricular zone in such a way that facilitated the formation of the cortex and the latter hypothesis requires thalamic afferents being the main cause of cortical differentiation. Nowadays, the development of the cortex is believed to be due to a combination of thalamic afferents and genetic information. The majority of the barrel cortex; which will come to be consisted of 6 layers, is developed before birth with progenitor cells, which in the cortex are radial glial cells, giving rise to neurons (Noctor *et al.* 2004). Progenitor cells have been shown to give rise to cells in a single column (Luskin *et al.* 1988).

Layer patterning involves a number of transcripts encoding transcription factors, cell adhesion molecules as well as molecules that will in time regulate projections to other parts of the brain (Zhong *et al.* 2004; Rubenstein *et al.* 1999). Several transcripts take place in the formation of patterns in the cortex, with the family of cadherins demonstrating their role in the differentiation between primary motor and somatosensory cortex (Miyashita-Lin *et al.* 1999). What is more, the establishment of the A-P axis of the neocortex is facilitated by the expression of Fgf8 as illustrated by *in utero* electroporations (Fukuchi-Shimogori & Grove 2001, Garel *et al.* 2003).

The generation of neurons is a rather complex process, involving a number of steps and a vast combination of transcripts, some of which have been mentioned above. Neurogenesis is regulated by a number of proneural genes, encoding typical basic helix-loop-helix (bHLH) transcriptional activators that form dimmers with E proteins. These dimmers, in turn, bind to E boxes present in the promoter regions of target genes leading to transcription activation. Ectopic expression of a proneural gene is sufficient to initiate neuronal differentiation. In specific, three proneural genes are expressed in the mouse cortex; *Neurogenin 1* (*Ngn1*), *Neurogenin 2* (*Ngn2*; this being the most important for corticogenesis) and *Mash1*. Distinct corticogenesis defects have been found only in the *Ngn2* mutants; where the regulatory effect of *Ngn2* upon *Ngn1* and *Mash1* has been revealed (Fode *et al.* 2000). Double mutant mice for the presence of *Ngn2* and *Mash1* have shown reduced cortical neurogenesis and reduced cortical plate (Fode *et al.* 2000, Nieto *et al.* 2001). The role of *Ngn1* and *Ngn2* in wild type mice is to activate a cycle of events that will provide neurons with glutamatergic phenotypes. Interestingly enough, double mutants for *Ngn1* and *Ngn2* have defects on neurons being born between E11.5 and E14.5; however, neurons born between E14.5 and E17.5 appear normal and show glutamatergic markers. Also, *Ngn2* single mutants present defects in early born neurons (layers V and VI) whereas late-born neurons in layers IV and II/III appear to be normal (Schuurmans *et al.* 2004). One of the most important molecules for the regulation of late-born neurons (migrating into the upper cortical layers) is *Pax6* which has been characterised as a pattern-regulatory gene (Tarabykin *et al.* 2001). Patterning of the cortex in the mouse and specification of the different layers is a long process which involves a number of transcripts that have been studied throughout the years from a variety of groups. A table presented below gives a summary of some of those molecules and the layers they have been found to act predominantly. Some transcripts, do not act only on one layer but they act in synergy in order to obtain the well known cortical pattern.

Neurons are generated in the late embryonic stages (between E15 and E17), with cells that leaving the cell-cycle earlier will form the deeper cortical layers and cells that are being born later will form the more superficial layers (Sur & Leamey 2001). Thalamic axons are seen leaving the thalamus at around E16, whereas there is a clear view of the thalamic axons entering the lower layers of the cortex by E18. At E17 layer VIb is being differentiated and by E19 the discrimination of layer VIa is obvious. The

differentiation of layer V is beginning at E21 with thalamocortical axons branching into it to establish connections (Catalano *et al.* 1996). Layer V neurons are characterised by their long projections in the brainstem and the spinal cord; whereas layer II/III neurons project mainly to other cortical areas. The long-distance projection of layer V neurons are severely affected in *fezl/zfp312* deficient mice; making these transcripts crucial for the regulated formation of layer V neurons (Chen *et al.* 2005, Chen *et al.* 2005, Molyneaux *et al.* 2005). The expression of *fezl/zfp312* has been observed much earlier than E21 which the time of differentiation for layer V which may suggest an involvement in the birth of these neurons but not in their specification (Chen *et al.* 2005, Rash & Grove 2006).

At P0, some of the thalamic afferents have reached the point where layer IV will be formed and by P3 layers V and VI can be clearly seen (Erzurumlu and Jhaveri 1990). At P0 most of the axons are radially oriented and are branching mostly into layers VIa and V. By P1, axons can be seen travelling through layer V and forming branches in the region of the emerging layer IV. Studies have shown that the thalamic afferents show a somatotopic pattern by P1, which would suggest that they carry the pattern formation rather than the cortex containing it beforehand (Catalano *et al.* 1996, Erzurumlu and Jhaveri 1990). The last group of cells migrates into place by P7 (post-natal stage); completing the six-layered barrel field. The whole process occurs with the migration of cells to the cortical plate, which is called the pre-plate during the initial pre-migration stages (Ghosh and Shatz 1992).

The formation and completion of the cortical pattern has been studied in relation to cortical activity in an attempt to determine whether activity affects the morphology of the barrel cortex. Experiments where cortical activity (Chiaia *et al.* 1992) and/or the infraorbital nerve (Henderson *et al.* 1992) have been blocked by tetrodotoxin during development have shown that the cortical pattern still developed normally. However, neuronal activity does appear to be of significance when refining of the barrel field is concerned. Studies have indicated that an activity-dependent mechanism is responsible for eliminating errors in thalamocortical projections accumulated during development (Rebsam *et al.* 2002). Thalamocortical axons beyond the borders of the barrels have been identified in animals lacking NMDA receptor function when compared to control animals (Lee *et al.* 2005).

1.10. The Critical Period for Neural Plasticity

Hubel and Wiesel (1963) have performed the first steps towards identifying the critical age for neural plasticity. They started investigating whether cortical cells have normal receptive fields responding to sensory input by the surrounding environment in 1-3 week old kittens (1963). Their first conclusion was that neuronal connections, underlying functional architecture of the cortex of young kittens, were present at the time of birth, implying that visual experience is not necessary for the development of the optic nerve. In control animals, where both eyes were left intact throughout their life, a normal distribution was observed and the majority of cells responded to stimuli from both eyes (Hubel and Wiesel 1962). In an attempt to disturb the normal processes of the neurons in the visual cortex, they performed monocular deprivation on kittens on the time of birth for 2.5 months. Recordings showed that all neurons were exclusively responding to sensory input from the undeprived ipsilateral eye; even 3 years after normal binocular vision had been restored (Hubel and Wiesel 1963).

In their later years of research, Hubel and Wiesel repeated these experiments using adult cats, which have been subjected to 2 years of monocular deprivation beginning at 12 months of age after birth. Recordings showed that, in contrast to the younger kittens, neurons were still responding to information by either eye. Subsequent experiments during different stages of development showed that visual deprivation exerted its effect between the fourth week and the third month of age, after birth. Thus, the critical period during which synaptic connections could be altered in an experience-dependent manner leading to permanent brain patterns was identified (Hubel and Wiesel 1970).

Visual deprivation was also employed by Le Vay *et al.* (1980), to study the development of ocular dominance columns, a structure similar to the columns of which the barrel field consists in rodents, in monkeys. Similarly to cats, these studies involved differing ages (from birth) and various deprivation periods. At the 1st week, from birth, ocular column segregation has started. By the 3rd week, from birth, segregation was complete with just slight overlaps at the borders within the ocular columns, making this the critical period for anatomical changes. At the 5th week, since

birth, the columns presented a mature organization. Binocular deprivation of 3-day-old monkeys showed normal patterns of segregation, implying that ocular dominance columns develop independently to visual experience (LeVay *et al.* 1980).

Similar to visual cortex organization, barrel cortex development is affected by deprivation; in this case vibrissal removal. When whisker deprivation is applied to rats on the 4th week, after birth, cortical layers undergo structural changes, indicating the importance of the cortex for plasticity (Fox 1992). It has also been indicated that different cortical layers have different critical periods. Particularly, neurons in layer II/III are characterised by greater plasticity at all developmental stages and ages and have longer critical periods for experience-dependent plasticity than cells in layer IV (Fox 1992). The critical period during which the refinement of the neuronal projections occurs in layers II/III ranges from the 14th day to the 21st day of age, after birth, however the critical period for layer IV is much earlier - in the first week since birth (Fox 1992).

Receptors and trophic factors present at synaptic connections are regulated during development and associated with the critical period for plasticity in the rodent. NMDA receptors (Section 1.10.2) are present at the synapses from birth and are the main component of the synaptic current in the first postnatal week; which coincides with the critical period of layer IV connections (Fox and Zahs 1994). Expression of neurotrophins, such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Section 1.11), coincides with synaptogenesis (Jin 2005); the formation of synapses, which takes place throughout an organism's life but it is particularly important during its critical period in order for neuronal connections to be formed. In cat's cortex NGF was detected from early post-natal ages into adulthood and BDNF was found in adult animals. Activity dependent expression of these neurotrophins was studied in cats. Experiments have shown decreased mRNA levels of NGF and BDNF in cats which were kept in the dark for several days; whereas BDNF mRNA levels were back to normal when the animals were returned into a normal light cycle (for review see Fox and Zahs 1994).

1.11. Effects of vibrissae removal

Somatic sensation arises from all the non-vibrissal parts of the body as well as the facial whiskers; however all somatic sensation from the vibrissae is transmitted to the barrel field underlining its vital importance (Section 1.6). Furthermore, it was noticed that the number (25) of whiskers, and barrels, remains constant between individuals, simplifying studies of the effect of injury or removal of a particular vibrissae on the corresponding barrel (Van der Loos and Woolsey 1973). For example, permanent damage to whisker follicles at birth creates dramatic architectural changes in the cortex, corresponding to loss of the barrels in the barrel field matching the removed vibrissae (Van der Loos and Woolsey 1973).

Changes in cortical organisation also occur simply as a result of trimming the vibrissae (Simons and Land 1987; Fox 1995). In this case sensory input is merely reduced rather than completely blocked, and it originates from spontaneous activity (Fox 1992). The absence of follicle damage during these experiments allows the study of experience dependent plasticity and possible recovery – if regrowth of the whiskers is allowed (Waite and Cragg 1982; Rhoades *et al.* 1987; Fox 1992).

Experiments where the D1 whisker of rats was spared (Figure 1.9) on one side of the face, while the other side was left intact, have shown a shifting of the sensory input to the spared whisker. The experiment involved raising rats from P0, P2, P4 and P7 (numbers indicate the age of the rat in post-natal days) with only the D1 whisker spared on one side and the other side left intact. The deprived whiskers were left to regrow before the recordings took place at P30 and P90 in layers II, III and IV (Fox 1992). When the brain was sliced and stained with cytochrome oxidase, it was revealed that the barrel corresponding to the spared D1 whisker was enlarged but the rest of the barrel field retained its normal anatomical characteristics (Fox 1992).

A considerable decrease in plasticity in layer IV at P0 and P4 was observed by measuring the cells outside the barrel that responded to the stimulation of the spared D1 whisker. Indeed, the percentage of neighbouring cells showing greater responses to the stimulation of D1, rather than the stimulation of their own regrown whiskers, decreased as deprivation was applied at later developmental stages. Similar recordings

in layer II/III have shown that cells from neighbouring columns respond to the spared whisker stimulation more vigorously than cells from the D1 barrel when deprivation occurs from P4 and P7 (Fox 1992).

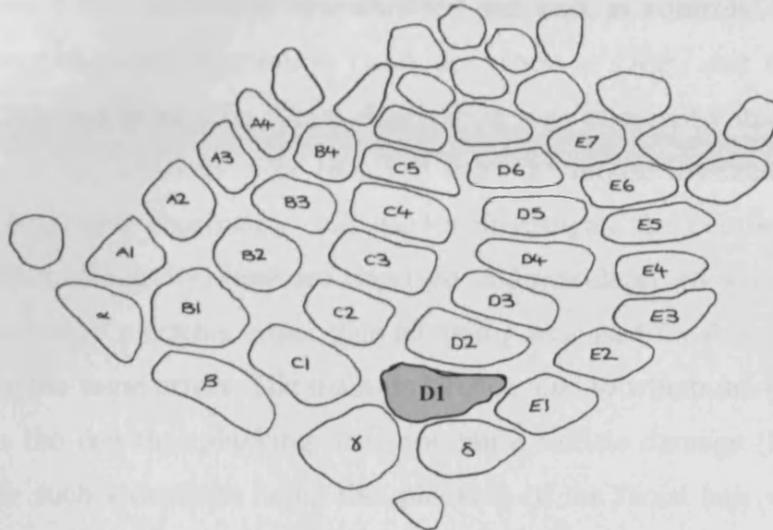


Figure 1.9: Representation of the cortical map showing the deprivation experiment during which the D1 whisker (shown in grey) is spared while the rest of the field is fully deprived (diagram kindly provided by James Dachtler).

Another deprivation method, via which plasticity could be induced and subsequently could be studied, is the “chessboard” pattern where every other whisker is deprived ending up with a pattern where every deprived whisker is surrounded by four spared whiskers and every spared is surrounded by four deprived (Fox 2002). The above pattern of removed facial hair corresponds to a similar pattern of active and less active barrels in the barrel field due to the 1:1 relationship between the two maps (Section 1.6). Visual cortex studies have identified that the distance between the active and inactive barrel is important for plasticity induction (for review see Fox 2002). In the case of chessboard deprivation, electrophysiological studies have detected potentiation in the deprived barrel when stimulating a neighbouring spared whisker and depression when stimulating its corresponding whisker. Furthermore, these responses are greater if a neurone corresponding to a deprived column is closer to an

active column that accepts sensory inputs from a spared whisker (Glazewski and Fox 1996).

In the present study, as it will be described later in detail, three conditions were used to investigate the effect of whisker removal in gene expression in the mouse neocortex. One of the conditions consisted of control subjects that have not been deprived; instead they have been anaesthetised and kept as controls. The other two conditions were the total deprivation (later described as DEP) and the chessboard deprivation (referred to as CB). Total deprivation was chosen to study the lack of activity since all the whiskers were removed from the mouse mystacial pad. On the other hand, chessboard deprivation was used to investigate the competition of input (hence the effect of activity) between deprived and non-deprived whiskers. Overall, the chosen method of plucking rather than trimming was used for this study; although they both have the same effect. The main difference, due to which the preference was established, is the fact that plucking does not cause follicle damage (Li et al 1995). The reason for such a decision being that plucking of the facial hair would produce less chance of activity by activation of the whisker stubs that one would get by whisker trimming.

1.12. Mouse Plasticity

1.12.1. The mouse as a model organism

As described in Molnar *et al.* (2006), comparative developmental analysis has helped researchers identify significant variations in the basic pattern of forebrain organization in different vertebrates. As described in previous sections monkeys and cats were the organisms of choice for visual deprivation experiments due to their similar visual capacities to humans (Guire *et al.* 1999). Understanding the evolutionary alterations in cortical development provides a better insight into the similarity between the human brain and that of other mammals.

The mouse is the favoured animal model system for studying developmental abnormalities and neurological disorders associated with plasticity, as it provides cortical similarity with humans (Figure 1.10) at a cellular level (Guire *et al.* 1999).

Furthermore, of particular relevance to genetic studies, mouse developmental periods are much shorter than other animal models such as cats or monkeys, and murine husbandry is relatively cheap and facile. The utility of the mouse as a model is considerably enhanced due to the ability to perform targeted genetic manipulation (Guire *et al.* 1999)

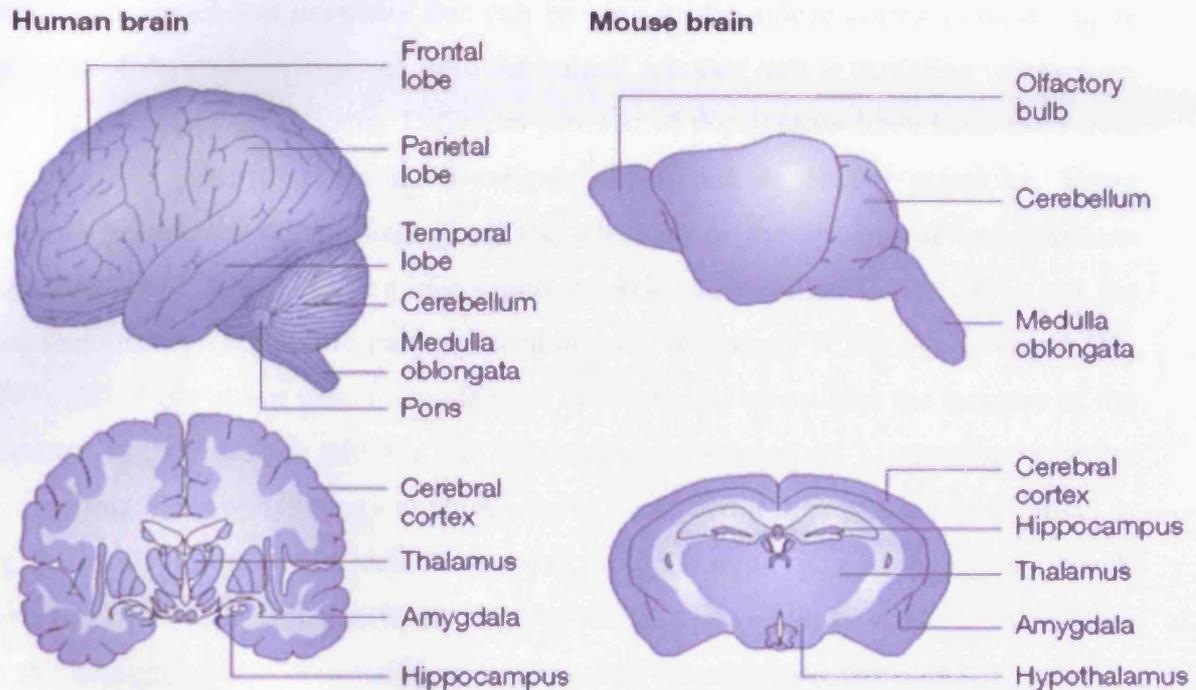


Figure 1.10: Gross comparison of the human and mouse brain representing main areas, including the cerebral cortex, the hippocampus and the thalamus (Cryan and Holmes 2005).

1.12.2. Developmental Mechanisms & Neuronal Plasticity

Neuronal connections are achieved via two main mechanisms (Goodman and Shatz 1993) those which are neuronal “**activity dependent**” and “**activity independent**”. Activity dependent synaptic plasticity is an ongoing process which takes place throughout the life of an organism, and is directly related to environmental inputs, which modify the strength and structure of dendritic connections (Purves *et al.* 1986a; Purves *et al.* 1986b). It can involve modifications of existing synaptic proteins leading to alterations in their function (Hawkins *et al.* 2006). The activity independent system

involves the molecular pathways for target recognition and memory formation (Goodman and Shatz, 1993). The second mechanism is associated with neurotransmitter release regulating gene transcription as well as protein changes at synapses. Similar to the first mechanism it also involves protein phosphorylation but in this case alterations take longer to occur and their effects last longer indicating an association of this mechanism with long-term memory (Hawkins *et al.* 2006).

Experience dependent plasticity that can be seen in the mouse cortex is reliant upon the kind of deprivation imposed onto the animal whether that is trimming or plucking or even damaging the follicle. Different patterns of deprivation have been used over the past few years in order to investigate experience dependent plasticity. These patterns include the deprivation of all the whiskers or the sparing of two whiskers (Diamond *et al.* 1993) or even of a complete row (Simons and Land, 1994) and the chessboard deprivation (the pattern of choice for this study (CB); Wallace and Fox 1999). All of the above used methods have attempted to investigate the increase of the response of the spared whisker or whiskers. In the case of a complete whisker deprivation, electrophysiological studies have identified a depression of responses to the re-grown whiskers (which have been initially deprived). It has been noted, however, that the distance between a spared and a deprived whisker has a crucial role on the strength of the depression observed when the deprived whisker is allowed to re-grow (Glazewski *et al.* 1998). The above is confirmed in the case when chessboard deprivation is used to alter plasticity in the rodent's barrel cortex, where every spared whisker is surrounded by four deprived and every deprived whisker is surrounded by four spared. Hence, greater depression is seen in the deprived whisker of a CB animal as it interacts with its four surround more active spared whiskers.

Sensory experience has been known to refine sensory cortical maps (Hubel & Wiesel 1965, 1970). Early postnatal stages during the rodent's life show signs of extensive plasticity within cortical areas of the brain; however the ability to remap expands into adulthood (Diamond *et al.* 1993, Glazewski & Fox 1996, Buonomano & Merzenich 1998). The barrel cortex, the system used in this thesis, is an excellent model for studying experience-dependent plasticity. The excellence of this paradigm lays in the property of layer 4 neurons to receive input from a primary single whisker. These cortical barrels develop between P0 and P5 (Agmon *et al.* 1993). Crair & Malenka

(1995) have demonstrated that thalamocortical connections show a critical period. Between P8 and P12, major mobility changes are observed in filopodia and spines within layer 2/3 (Lendvai *et al.* 2000). By the end of the second postnatal week and the beginning of the third, the neurons of layer 2/3 are mature and layer 4 to layer 2/3 synapses exhibit NMDA-dependent plasticity (Feldman 2000, Maravall *et al.* 2004). In the barrel cortex, during the second postnatal week a great amount of experience-dependent plasticity occurs in layer 2/3 (Stern *et al.* 2001, Fox 2002, Foeller & Feldman 2004). If sensory deprivation is subjected during that time, short term synaptic changes occur along side longer lasting synaptic plasticity (Allen *et al.* 2003). Mierau *et al.* (2004) have observed that the properties of synaptic NMDA receptors depend upon sensory experience during the second postnatal week; however the ratio of AMPA/NMDA receptors progressed independently of sensory experience.

1.12.3. Experience-dependent & -independent changes

The developmental refinement of sensory cortical maps depends upon sensory experience; as stated by Hubel in the late 60s. Plasticity of sensory cortical maps could be detected at early postnatal stages; however the ability to remap extends even into adulthood (Glazewski & Fox 1996). The way that the barrel cortex is organised into barrels (hence the name) representing the facial vibrissae makes it a very useful tool for manipulation of sensory experience. Layer 4 – where the barrels form between P0 and P5 in mice (Agmon *et al.* 1993) – neurons in a given barrel receive primary input from their principal whisker. A critical developmental time period for plasticity has been shown with LTP induction being achievable only during the first postnatal week (Crair & Malenka 1995). Layer 2/3 basal dendrites receive the majority of their input from layer 4 spiny neurons. A significant amount of changes occur during P8 and P12 in the morphology of layer 2/3 cells. It is believed that soon after the end of the second postnatal week, layer 2/3 pyramidal neurons are mature enough and that synapses between layers 4 and 2/3 show evidence of NMDA-receptor-dependent spike timing-dependent plasticity (Maravall *et al.* 2004).

In the visual cortex, NMDA-receptor mediated responses are subject to change during postnatal development; with EPSCs becoming faster with age (Carmignoto & Vicini 1992). This change is associated with an increased expression of NMDA-receptor

subunit 2A during postnatal development which is experience dependent (Philpot *et al.* 2001). Experience dependent plasticity also occurs in the barrel cortex during the second postnatal week (Fox 2002). During that period, sensory deprivation can cause significant alterations in short-term synaptic dynamics and can induce longer-lasting synaptic plasticity (Finnerty *et al.* 1999, Allen *et al.* 2003).

When studying cortical development, it is crucial to consider the degree upon which synaptic circuitry depends on sensory experience. Mierau *et al.* (2004) have studied experience dependent and independent changes in glutamatergic transmission in the barrel cortex of deprived and non-deprived mice during the second postnatal week. They observed an increase in the ratio of AMPA to NMDA receptor mediated responses in relation to developmental stages. They observed no profound effect due to deprivation at the development of the AMPA to NMDA ratio during the second postnatal week. However, they report that the properties of synaptic NMDA receptors were dependent upon experience, during the same developmental stage (second postnatal week; Mierau *et al.* 2004).

Holtmaat *et al.* (2006) have provided evidence that stabilization of new spines is driven by experience; indicating an experience dependent remodelling of neocortical circuits. In their paper, Holtmaat *et al.* (2006) have used the trimming of the whiskers as the preferred paradigm for inducing adaptive functional changes in the neocortex. They have observed the stabilization of new spines; which almost always formed synapses in layer 5, and the destabilisation of previously persistent spines. Their data indicates that stabilisation of new spines in cortical neurons is dependent upon novel sensory experience; underlying experience-dependent remodelling of the mouse neocortex.

1.12.4. Synaptic plasticity in different developmental stages

1.12.4.1. Overview

In the early stages of development, functional neuronal circuits are created through synaptogenesis and activity-dependent refinement of synaptic connections. Later in development, the already established neuronal connections are prone to experience-

dependent changes and modifications. Different cortical layers have variable critical periods and this facilitates the above changes (Brainard & Knudsen 1998a, b).

When the rodent is sexually mature, the ability to form new synapses and adapt to changes in external stimuli is reduced (Fox and Zahs 1994), though manifestation of activity-dependent plasticity is still present in some cortical layers (apart from layer IV) (Diamond *et al.* 1993; Glazewski and Fox 1996). It is believed that this ability is still present in the rodent's brain in order to allow cortical reorganisation after injury (Buonomano and Merzenich 1998). Especially, after 28 days, since birth, animals are mature enough to have finished with main parts of cortical development and most of the synaptogenesis but they are still prone to synaptic changes (Fox 2002) and they show plasticity in some cortical layers (Fox and Zahs 1994; Glazewski and Fox 1996).

1.12.4.2. Postnatal Stages and Plasticity

At the time of birth, the rodent's cortex is relatively immature and incomplete. Such is the extent of immaturity that a high percentage of cortical cells have not yet migrated to their final position (see previous sections), which is still taking place during the first week of the rodent's life; a period while which the brain is affected by the external environment. Early experiments, by Fox (1992), have shown that experience affects cortical development during the first postnatal week; a period crucial for the development of the thalamic inputs into layer IV of the cortex. Postnatal stage P0 is characterised by the greatest degree of receptive field plasticity with thalamocortical afferents showing LTP at the same time as the conversion of silent synapses to active ones (see previous sections) via insertion of AMPA channels in an NMDA-dependent manner. A significant decrease in plasticity is observed in a progressive manner in layer IV, reaching its low levels at around P4 (Fox 1992).

1.12.4.3. Plasticity during adolescence and adulthood

Rodents reach adolescent around the first month of age (Spires *et al* 2005) when major organisation events in the cortex have occurred and the animal is almost sexually mature. However, complete neuronal development has not been achieved at

this stage giving space to some aspects of plasticity to still make an appearance that will not be present any longer in much older animals (Glazewski & Fox 1996). When the cortex of adolescent animals is compared to the one of one-week old animals, it is apparent that plasticity in all layers is somehow decreased. However, this decrease varies between layers; with layer IV being the most affected with no signs of LTP and experience-dependent plasticity as shown by the spared-whisker experiment. On the other hand, layers II/III show a much smaller decrease in plasticity between the time of birth, the first postnatal week and adolescence (one month old). This gives the opportunity to study plasticity in those layers and gives the animal the freedom to undergo upregulation of the spared input and downregulation of the deprived input (Fox 1992, Fox 1996, Isaac *et al.* 1997). As far as adulthood is concerned, a rodent is thought to enter adulthood around the sixth month of its age with signs of plasticity still present in the cortex. These signs of plasticity can be detected until the fifteenth month of age (Chapman *et al.* 1999). Adult mice do not show depression of deprived input anymore; however, potentiation is still present in layers II/III in the cortex making the study of synaptic plasticity in adult mice as important as in earlier postnatal ages.

1.12.5. Potentiation and depression of synapses

Synapses serve cellular communications and modifications of neuronal transmission (Debanne *et al.* 2003). These modifications can occur pre-synaptically and post-synaptically (Daoudal and Debanne 2003; Debanne *et al.* 2003). Pre-synaptic changes of neurotransmitter release are involved in short-term depression (STD), whilst post-synaptic modifications are involved in long-term potentiation (LTP) of the synaptic strength (Zucker and Regehr 2002; Daoudal and Debanne 2003; Debanne *et al.* 2003). Understanding the cellular and molecular mechanisms that alter the efficiency of synaptic connections has been achieved through the study of long term potentiation, which is a sustained increase in the efficiency of synaptic transmission that occurs in response to heightened neuronal activity.

Depression is the weakening of the synapses, which can last from hours to days. It could be short-term (STD) and long-term (LTD) (Gaiarsa *et al.* 2002). As stated by their names, short-term depression is a quick transitory effect. It is believed that

decrease of the synaptic efficacy might as important as strengthening (potentiation) for synaptic circuits development (Feldman *et al.* 1999). Depression is usually observed in early developmental stages, mostly at P4-P5, and declines gradually until later in life. Some animals do not show depression by P9, whereas others can take up to P12 until any sign of depression is abolished (Figure 1.11; (Feldman *et al.* 1999).

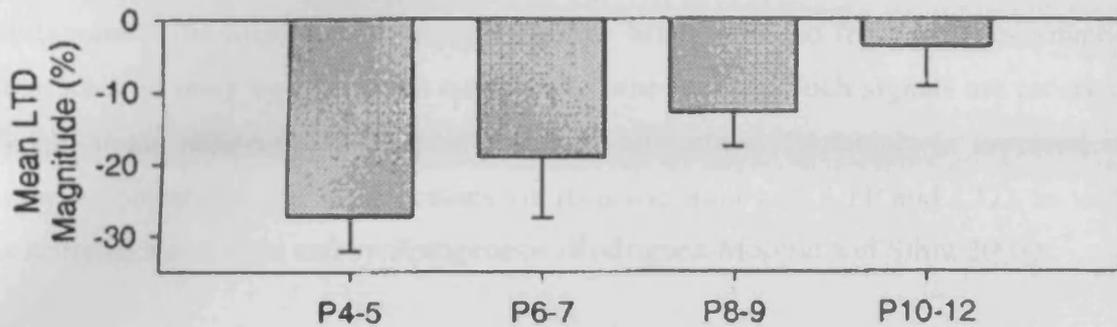


Figure 1.11: Representation of the decrease of long-term depression (LTD) throughout development. Error bars represent the standard errors of the mean ($n = 11, 8, 10$ and 14 cells for the age groups presented, from left to right). 0% indicates lack of depression. LTD is at its maximum levels at P4-5 and it gradually decreases with time, until it reaches minimal (close to zero) levels at P10-12 (Feldman *et al.* 1999).

As a pre-synaptic associated property, depression is associated with a number of processes such as inactivation of pre-synaptic calcium channels and negative feedback loops through pre-synaptic metabotropic receptors (Kiehl and Heggelund 2002). It has been suggested that depression is also involved post-synaptically (Rozov *et al.* 2001) but experiments on AMPA and NMDA receptors have shown that it is mainly a pre-synaptic event (Kiehl and Heggelund 2002).

Potentiation is the strengthening of synaptic efficacy (Gaiarsa *et al.* 2002). The long lasting increase of neuronal response to stimulation is referred to as long-term potentiation (Bliss and Lomo 1973). Signs of the first LTP experiments take us back to the sixties (for review see (Lomo 2003). There are two phases of LTP; early and late (Sweatt 1999). The early LTP (E-LTP) lasts about 60 minutes, whilst late LTP (L-LTP) is protein synthesis dependent (Sweatt 1999; Malenka and Bear 2004). Each phase - early and late - is characterised by involvement of mediating molecules, proteins and enzymes, which respond to chemical reactions and signals outside the

cell and within the cell. These molecules also inhibit the succession of one phase to the other (Malenka and Bear 2004).

1.13. Receptors of the nervous system

1.13.1. Glutamate receptors

Glutamate is the major neurotransmitter in the brain, released from the pre-synaptic membrane to relay signals across synapses to other nerves. Such signals are received by glutamate receptors on the post-synaptic cell-surface. Glutamate is involved in main molecular and cellular processes via its association with LTP and LTD, as well as neuronal maturation and synaptogenesis (Rodriguez-Moreno and Sihra 2007).

Glutamate receptors fall into two main categories, classified by their structural type. **Ionotropic glutamate receptors (iGluRs)** are ion channels, whilst the **metabotropic receptors (mGluRs)** are G protein-coupled receptors (Headley & Grillner 1990; Sladeczek *et al.* 1985). The iGluRs are sub-divided into three further groups, based on their selective agonists. These are *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and Kainate (Krogsgaard-Larsen 1980; Watkins and Evans 1981). A number of genes have been identified and cloned for each of the above groups.

1.13.2. NMDA receptors

The ion channel NMDA receptors (NMDARs) allow free passage of cations such as Na^+ , K^+ and Ca^{2+} (Mayer and Westbrook 1987; Ascher and Nowak 1988) under activation by glutamate (Figure 1.12), with calcium and sodium ions entering the cell whilst potassium ions diffuse out (Ascher and Nowak 1988; Monaghan *et al.* 1989). Critically, NMDA receptors are only able to conduct current at depolarized membrane potentials, as they are distinctively blocked by Mg^{2+} in a voltage dependent manner (Mayer *et al.* 1984).

When magnesium enters the ion pore, it blocks synaptic transmission. Johnson & Ascher (1990) suggested that there might be two Mg^{2+} binding sites facilitating the

interference of intracellular and extracellular magnesium ions with the receptor, leading to the blockage of NMDARs. Other compounds can also act selectively on NMDA receptors, including the anaesthetics ketamine and phencyclidine (Anis *et al.* 1983). These act upon the receptors by blocking the influx of cations through the NMDA channel (Mayer and Westbrook 1987).

Live-imaging studies have shown that NMDARs are transported onto developing synapses gradually (Washbourne *et al.* 2002), Bresler *et al.* 2004). This delivery of NMDARs into the postsynaptic membrane is a PKC-dependent process (Lan *et al.* 2001). There are a number of different NMDAR subtypes (table 1.1). NR2B receptors are recruited by the synapses, early in development, in an activity-dependent manner. Later in development these NR2B receptors are replaced by NR2A, inducing synaptic plasticity (Barria and Malinow 2002). In the past, it was believed that NMDA receptors were not as dynamic as AMPA receptors (see below), and were all regulated in an activity-dependent manner (Allison *et al.* 1998). In recent studies, however, it has been shown that NMDA receptors are not immobilised in the post-synaptic membrane (Heynen *et al.* 2000; Montgomery and Madison 2002; Montgomery *et al.* 2005).

Ionotropic Receptors (Ion Channels)		
NMDAR	AMPA	KainateR
NR1	GluR1	GluR5
NR2A	GluR2	GluR6
NR2B	GluR3	GluR7
NR2C	GluR4	KA1
NR2D		KA2
NR3A		

Table 1.1: representation of the glutamate receptor subunits

NMDARs are involved in the recognition of pre-synaptic glutamate release and the post-synaptic calcium increase (Debanne *et al.* 2003; Lynch 2004). NMDARs are also implicated with long-lasting plasticity, directly related to normal neuronal function in the hippocampus, neocortex and cerebellum (Debanne *et al.* 2003). NMDAR-induced plasticity has also been seen in the amygdala and the visual cortex, making the activation of these receptors vital for long and short-lasting effects in learning and memory (Lynch 2004).

1.13.3. AMPA receptors

AMPA receptors (AMPA receptors) are a subtype of glutamate receptors expressed in excitatory synapses (Figure 1.12) (Genoux and Montgomery 2007). Like NMDARs, AMPARs transmit most current at depolarised membrane potentials (Sommer *et al.* 1992). The majority of AMPARs are tetramers comprised of subtypes GluR1, GluR2, GluR3 and GluR4 (table 1.1) (Kornau *et al.* 1997), although the actual composition of the AMPAR is dependent upon its location within the brain. For example, hippocampal synapses mostly contain GluR1/GluR2 or GluR2/GluR3 dimers (Song and Huganir 2002). Trafficking of the AMPARs to the post-synaptic membrane relies upon their interactions with post-synaptic density proteins (Kornau *et al.* 1997; Montgomery *et al.* 2004). Synthesis of AMPARs can also take place in the dendrites (Ju *et al.* 2004).

Synaptic insertion of AMPARs is stargazin-mediated, involving the interaction of a number of proteins (Chen *et al.* 2000). Firstly, AMPARs are transferred to the synaptic membrane by Stargazin, where they are recruited by the membrane via the interaction of the phosphorylating enzyme protein kinase A (PKA) and the post-synaptic density protein PSD95 (Chetkovich *et al.* 2002; Schnell *et al.* 2002). Stargazer-knockout mice have been created; these lack AMPARs in cerebellar granule cells (Chen *et al.* 2000; Tomita *et al.* 2004).

Neuronal function is highly associated with protein phosphorylation as most of the pathways involved include the protein kinases and phosphatases (Greengard 2001; Malinow 2003). AMPA receptors are found to be phosphorylated in multiple sites on their C-terminal domains by a number of kinases (Roche *et al.* 1996; Matsuda *et al.*

1999; Chung *et al.* 2000; McDonald *et al.* 2001). It has been shown that induction of LTP causes Ca^{++} influx via the NMDA receptors, activating CaMKII (Calcium/calmodulin-dependent protein kinase II) which in turn phosphorylated GluR1 at Ser845 and Ser831 (Song and Huganir 2002). During long-term depression, when GluR1 is dephosphorylated, GluR2 phosphorylation at Ser880 occurs; leading to further interaction of this AMPAR subunit with other proteins and regulation of LTD (Song and Huganir 2002). Further studies have shown that the C-terminus of GluR2, GluR3 and GluR4 interact with a few kinases and their variances, regulating membrane fusion events (Dong *et al.* 1999; Song and Huganir 2002).

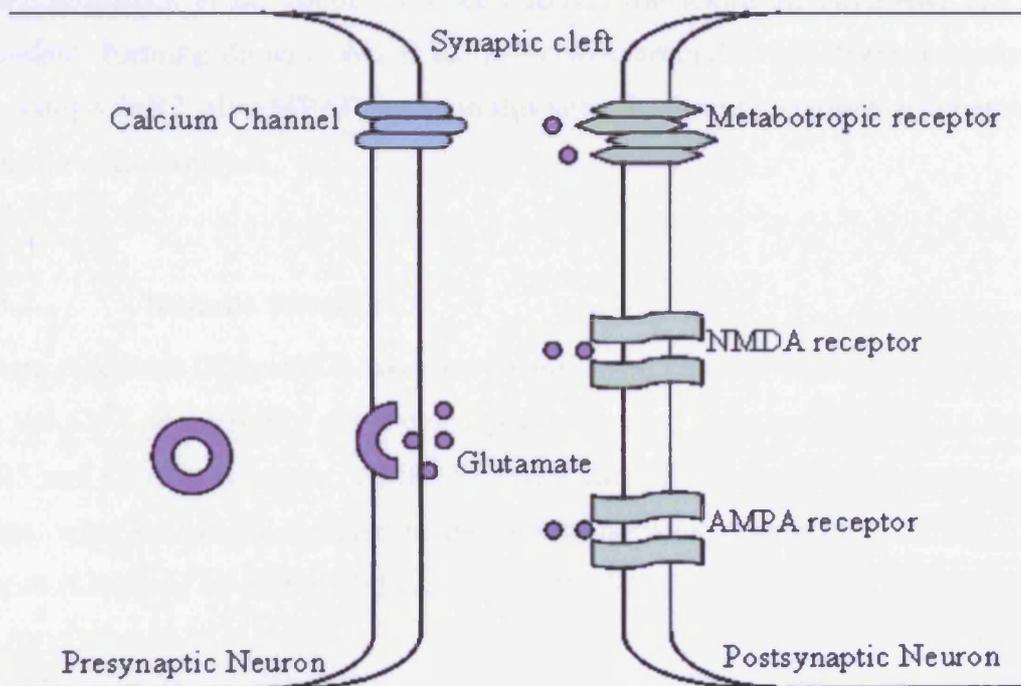


Figure 1.12: Graphic representation of the glutamate receptors on the post-synaptic neuron. They are activated by glutamate (among other molecules) which is released from the pre-synaptic neuron. This figure is a modification from (Genoux and Montgomery 2007).

The removal of AMPARs from synapses is a rapid process, leading to weakening of the synapses in a matter of a few minutes (Daw *et al.* 2000; Carroll *et al.* 2001; Lee *et al.* 2002). This AMPAR removal involves clathrin-mediated endocytosis via the AP-2

adaptor of the GluR2 subunit, stimulating endocytosis and long-term potentiation (LTP) (Chung *et al.* 2000; Carroll *et al.* 2001; Lee *et al.* 2002).

The AMPARs are evident throughout the central nervous system (Belachew & Gallo 2004; Wisden & Seeburg 1993), with the majority containing GluR2 (Wenthold *et al.* 1996; Greger *et al.* 2002). The expression levels of GluR2 during early post-natal stages are significantly lower than those of GluR1, with a gradual increase during the first week (Monyer *et al.* 1991; Wisden and Seeburg 1993). The function and properties of most mammalian AMPARs is determined by the GluR2 subunit; indeed it is one of the most critical subunits for normal brain function, with profound phenotypic changes occurring when it is genetically manipulated (Shimshek *et al.* 2006a; Shimshek *et al.* 2006b). As seen above, trafficking of AMPARs is GluR2-dependent, forming dimers (Wu *et al.* 1996; Rosenmund *et al.* 1998). In cells over-expressing GluR2, all AMPARs contain this subunit, forming symmetrical heteromers (Mansour *et al.* 2001).

1.13.4. Kainate receptors

Kainate receptors (KainateRs) have very similar ion channel properties to AMPARs, with the Ca²⁺ permeability dependent upon glutamate receptor subunits; in this case GluR5 and GluR6. However, AMPARs and KainateRs do belong to separate protein groups, with KainateRs detected in the central nervous system on both sides of the synapse (Lerma *et al.* 2001); Kidd & Isaac 1999; Wilding & Huettner 1995).

The most effective agonist for this type of receptor is ATPA ((*RS*)-2-amino-3-(hydroxy-5-*tert*-butylisoxazol-4yl)propanoic acid) which, incidentally, was used as an AMPAR agonist (Lauridsen *et al.* 1985) until it was shown that it is more efficient against KainateRs containing the GluR5 subunit (Clarke *et al.* 1997). Notably, ATPA is also a weak agonist for GluR7 and KA2 receptor subunits, whilst being completely inactive towards GluR6 (Clarke *et al.* 1997).

The kainate group of receptors (table 1.1) can be further divided into two categories: the high affinity subunits – KA1 and KA2 – and the low affinity subunits – GluR5, GluR6 and GluR7. Functional groups cannot be formed by the high affinity subunits

alone (Werner *et al.* 1991; Herb *et al.* 1992), unless they form dimers with the low affinity subunits (Hollmann and Heinemann 1994; Bettler and Mülle 1995).

Although the differentiation of KainateRs from the AMPARs was difficult in the beginning due to the lack of definite agonists, the involvement of KainateRs in pre and post-synaptic events (Figure 1.13) and induction of synaptic plasticity is now confirmed (Rodriguez-Moreno and Sihra 2007). Studies have been carried out in the hippocampus, where it was found that GluR6 activation can cause long-lasting depression, requiring G proteins and protein kinase C (PKC, (Melyan *et al.* 2002; Debanne *et al.* 2003).

KainateRs show metabotropic and ionotropic activity (Rodriguez-Moreno and Lerma 1998; Schmitz *et al.* 2001; Lerma 2006; Pinheiro and Mülle 2006). As ion channels they are involved in post-synaptic neuron depolarization and neurotransmitter release; whereas as metabotropic receptors they are involved in the activation of G proteins leading to PKC and PKA stimulation, which are closely related with synaptic transmission (Rodriguez-Moreno and Sihra 2007).

KainateRs have been studied in the somatosensory cortex of rats. Post-synaptic kainate receptors are found present in thalamocortical synapses during early stages of development, more specifically up to 8 day-old rats; results obtained from layer IV recordings (Huettner 2003). Similar results were obtained when pre-synaptic activity of KainateRs was studied in brain slices obtained from young and older animals, suggesting that changes of these receptors occur in early developmental stages (reviewed in Huettner 2003).

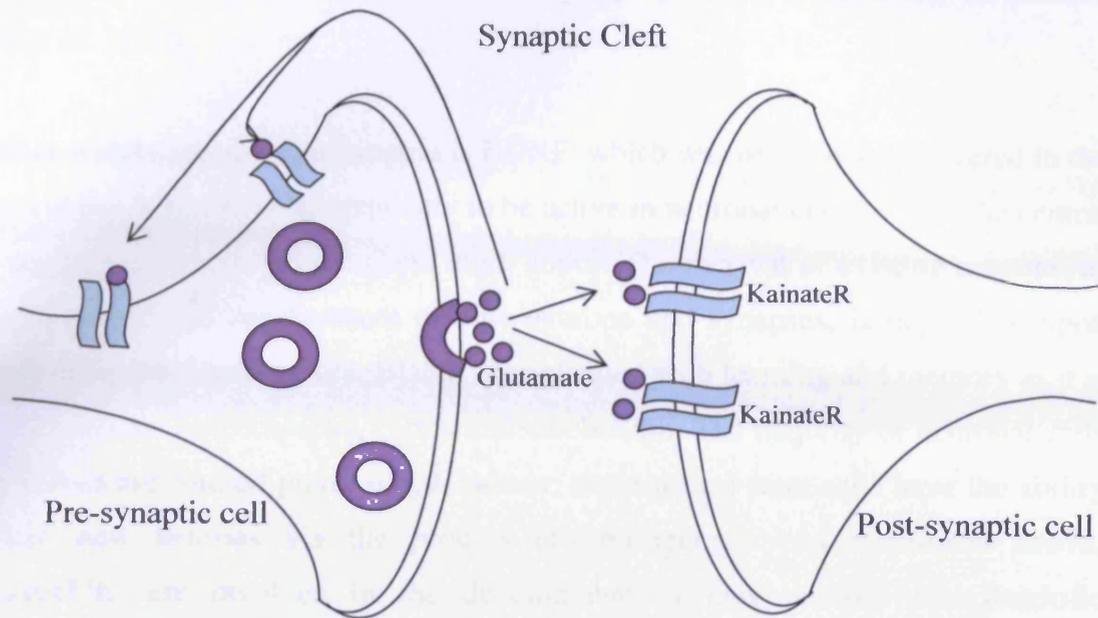


Figure 1.13: schematic representation of post-synaptic and pre-synaptic KainateRs. During synaptic release of glutamate, KainateRs are activated in both pre and post-synaptic cells. (Lerma 2003)

1.14. Action of neurotrophins on plasticity

The neurotrophin family of proteins are responsible for neuronal survival (Lein *et al.* 2000). Neurotrophins are secreted by target tissue and send signals to cells to prevent initiation of programmed cell death. Moreover, they induce differentiation of progenitor cells in order to form neurons. Family members are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and Neurotrophin1, 3 and 4 (NT1, NT3 and NT4). Neurotrophins bind to two receptor types: p75 and tyrosine kinase (Trk) receptors. Binding to p75 is of low affinity but it is common to all the members of the neurotrophin family. In contrast, only specific members bind to the Trk receptors with higher affinity (Lein *et al.* 2000).

The progenitor of the neurotrophin family is NGF. This growth factor is important for the survival and maintenance of sensory neurons. It binds to tyrosine kinase A (TrkA) in order to activate it, and results in migration of this NGF/TrkA protein complex into

the cell body. Indeed, the movement of NGF from the axon to the soma via the formation of a protein complex is involved in long-distance signalling of neurons (Lein *et al.* 2000).

Another widely studied neurotrophin is BDNF, which was originally discovered in the brain but has been found subsequently to be active in neuronal cells of both the central and peripheral nervous system (Lein *et al.* 2000). The survival of existing neurons, as well as growth and development of new neurons and synapses, is dependent upon BDNF activation. Furthermore, BDNF is implicated with learning and memory as it is found within the hippocampus, cortex and cerebellum. The majority of neuronal cells in mammals are formed prenatally. However, some neural stem cells have the ability to form new neurons via the process of neurogenesis. As mentioned above, neurotrophins are involved in the development of new neurons and dendritic connections, making BDNF very important for the mammalian brain. For example, rodents lacking BDNF show developmental defects and die soon after birth indicating that BDNF is a vital protein for neural development (Lein *et al.* 2000).

Neurotrophin-1 (NNT1), also known as β cell stimulating factor 3 (BSF-3), is a recent entry to the interleukin-6 family of cytokines (Senaldi *et al.* 1999; Uemura *et al.* 2002; Vlotides *et al.* 2004). The IL-6 family of cytokines have a protective role towards neurons, which manifests through their binding ability to the signal transducing receptor subunit glycoprotein 130 (gp130) resulting in the activation of signal transducer and activator of transcription factor 3 (STAT3; Seidel *et al.* 2000). Neuroprotective properties of NNT-1 on retinal ganglion cell (RGC) loss *in vivo* have been investigated. It was demonstrated that in models of retinal neuronal damage, NNT-1 significantly protected RGCs from degeneration (Schuettauf *et al.* 2005).

Neurotrophin-3 (NT3) was the third family member to be discovered, after NGF and BDNF. It is found in neuronal cells of the peripheral and the central nervous system. Similar to other neurotrophins, NT3 is important for the survival of neurons, as well as the regrowth and differentiation of new ones. It has been shown that NT3 mRNA (along side BDNF) is expressed in cat thalamocortical areas during development of

the visual system, with expression levels dropping after the first week after birth (Lein *et al.* 2000).

Neurotrophin-4 (NT4) is another member of this family and has been shown to bind to TrkB – the tyrosine kinase receptor. It is equally important for the development of the nervous system. It has also been found to be implicated in a number of neurological pathologies, such as Parkinson's, depression and eating disorders (Arevalo and Wu 2006).

Secretion of neurotrophins in an activity-dependent manner is involved in the activity-dependent refinement of synaptic connections (Cabelli *et al.* 1995, 1997). Canossa *et al.* (1997) have shown that exogenous application of neurotrophins induces Trks-mediated (tyrosine kinase neurotrophin receptors) secretion of neurotrophins. Neurotrophin induced neurotrophin secretion requires intracellular calcium; a process similar to activity-dependent secretion of neurotrophins. Neurotrophin-induced neurotrophin release is thought to reinforce and stabilise synaptic connections (Canossa *et al.* 1997). Survival and differentiation of population of neurons in the peripheral nervous system is dependent upon the availability of neurotrophins (Lewin & Barde 1996). As far the central nervous system is concerned, the survival of a given population of neurons is dependent upon multiple neurotrophic factors (Lindholm *et al.* 1996).

Neurotrophins modulate synaptic transmission by pre and post synaptic effects (Lohof *et al.* 1993, Kang & Schuman 1995, Levine *et al.* 1995, Suen *et al.* 1997). Presynaptically, neurotrophins enhance neurotransmitter release. In cultured neurons (Marsh & Palfrey 1996) BDNF and NT-3 induce the increase of intracellular calcium; a similar effect is observed in the neuromuscular junctions of *Xenopus* (Stoop & Poo 1996). Postsynaptically, neurotrophins act through NMDA receptors (Levine *et al.* 1995) promoting the phosphorylation of NMDA receptor subunit 1 (Suen *et al.* 1997). Another function of neurotrophins has been observed in the BDNF^{-/-} mice (Korte *et al.* 1995, Patterson *et al.* 1996). These mice are showing impaired LTP at Schaffer collateral/CA1 synapses.

Neurotrophins have also been shown to be associated with neurodegeneration; not only during development of the brain but also during adulthood (see Hennigan *et al.* 2007 for review). Cell loss has been associated with decreased expression of Trk receptors; whereas the expression of p75NTR is induced in the occasion of injury while it is found in decreased levels during adulthood. In particular, p75NTP (p75 neurotrophin receptor) expression is increased in adult rat motor neurons following sciatic nerve lesion and in the hippocampus following seizure (Roux *et al.* 1999). In addition, p75NTR expression is associated with neuronal degeneration after experimentally induced ischaemia (Greferath *et al.* 2002).

Almost every area of neuroscience research has identified a novel role for one or more neurotrophins and their receptors. Researchers are putting their tools to the test to investigate the pathways that neurotrophins are involved and how their actions connect them to plasticity, neurodegeneration and neuroprotection. Key insights have been published in almost every major journal advancing our knowledge of their cellular mechanisms and their neuronal function.

1.15. How CREB relates to gene transcription

1.15.1. Overview

cAMP Responsive Element Binding Protein (CREB) is a member of a family of transcription factors that mediate transcriptional activation, DNA binding and dimerization through binding to specific promoter cAMP response element (CRE) sites (Johannessen *et al.* 2004). Genome analysis has revealed that both the mouse and human CREB genes are composed of 11 exons (Hoeffler *et al.* 1990; Waeber *et al.* 1991; Cole *et al.* 1992). Similar analysis on CRE sites has located 1349 hits and 1663 hits in the mouse and human genomes, respectively (Conkright *et al.* 2003). The above figures correspond to response elements for genes encoding a large variety of proteins. This diversity implicates CREB in a number of cellular processes.

In mammals, the CREB-family consists of three principal members; CREB itself, the cAMP response element modulator (CREM) and the activating transcription factor-1

(ATF-1) (Foulkes *et al.* 1991; Molina *et al.* 1993). All three show high sequence homology and are conserved throughout evolution (Mayr and Montminy 2001).

Montminy & Bilezikjian (1987) described the CRE site of the somatostatin promoter (5' to 3' prime end) palindromic sequence as the first CRE site. Before binding to the CRE site, CREB forms a dimer (Richards *et al.* 1996). Notably, the CRE sequence may occur multiple times in one promoter region, and the length of the nucleotide sequence of the CRE site has a considerable effect on CREB binding (Richards *et al.* 1996).

The CRE binding factors have also been distinguished by their ability to interact with CREB (Kerppola and Curran 1995; Shaywitz and Greenberg 1999). The first group, consisting of CREB, CREM and ATF-1 (Hai and Curran 1991), can form homodimers or heterodimers with CREB. All three utilise their leucine zipper domains to facilitate dimerization (Foulkes *et al.* 1991; Hoeffler *et al.* 1991; Hurst *et al.* 1991). In contrast, the second group of factors do not dimerise with CREB. These proteins include the proto-oncogene c-Jun, other ATFs and members of the CAAT/enhancer binding protein gene family (Yun *et al.* 1990; Hai and Curran 1991; Hummler *et al.* 1994).

CREB – the main member of the CREB/CREM/ATF family – has two transcription activation domains: a glutamine rich (Q2) domain and a kinase-inducible transactivation domain (KID) (Figure 1.14). The interaction of Q2 with the TATA-binding protein-associated factor is necessary for the transcriptional activity of CREB and the recruitment of the RNA polymerase II transcriptional complex. Mutations in this domain can severely affect its role (Matsumoto *et al.* 1998; Shaywitz and Greenberg 1999; Quinn 2002; Swarthout *et al.* 2002; Conkright *et al.* 2003), leading to possible transcription failure. What is more, the KID domain acts in combination with the Q2 domain, promoting CREB-mediated transcription. This is mediated via phosphorylation of Ser133 within KID, which facilitates the interaction of the architectural CREB-binding protein (CBP) and the recruitment of the RNA polymerase II complex (Pugazhenti *et al.* 1999).

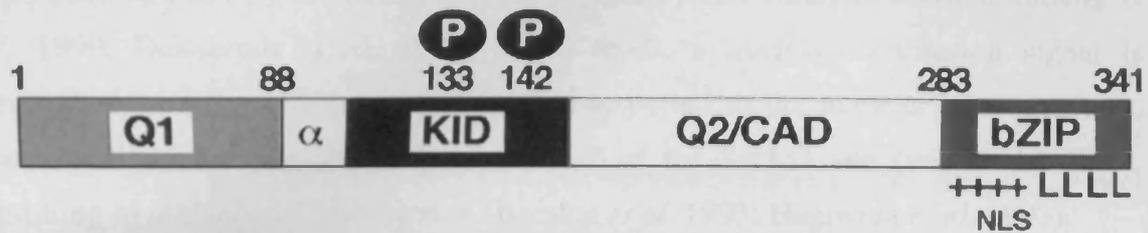


Figure 1.14: representation of the principal domains of CREB. The phosphorylation sites are represented with black circles (ser133 and ser142) (Shaywitz and Greenberg 1999).

Another family member - CREM - contributes to the Ser133 phosphorylation due to the presence of Q2 and KID domains, which facilitate the functional interaction of this molecule with the TATA binding protein associated factor (Liu *et al.* 2002).

In addition to Ser133, as shown in Figure 1.14, the KID domain of CREB contains several other phosphorylation sites for a number of kinases (Gonzalez *et al.* 1991). Research has revealed the necessity of KID for CREB activation. Deletion of some regions of KID can completely eliminate CREB activation, whereas the lack of other domains within CREB does not affect signal-induced transcription (Gonzalez *et al.* 1991; Brindle *et al.* 1993; Quinn 1993).

The search for a protein that binds to serine-133 site of CREB started with a human thyroid library and led to the identification of CREB binding protein (CBP). Sequence analysis of CBP revealed several calmodulin kinase II (CaMKII) phosphorylation sites, one PKA phosphorylation site, two zinc-finger regions and a glutamine-rich domain (Chrivia *et al.* 1993).

1.15.2. Transcriptional activation pathways

There are three main pathways involved in the transcriptional activation of CREB. Each involves a number of steps, leading to the phosphorylation of CREB and the initiation of transcription (Figure 1.15).

The first pathway starts with increased levels of intracellular cAMP, leading to activation of PKA by dissociation of the regulatory and catalytic subunits (Sheng *et al.* 1990; Deisseroth *et al.* 1998). As a result, a nuclear localisation signal is unmasked, and the catalytic subunits are transported to the nucleus where, in turn, they facilitate the phosphorylation of CREB at the Ser133 site (within the KID), resulting in initiation of transcription (Bacskai *et al.* 1993; Hagiwara *et al.* 1993).

Increased intracellular calcium ion concentrations might also be responsible for CREB activation, bringing up a second potential pathway. Deisseroth (1996) suggested that increased intranuclear Ca^{2+} levels alone are insufficient for CREB activation (Deisseroth *et al.* 1996). However, raised intracellular Ca^{2+} concentrations do trigger a pathway involving calmodulin which, in turn, activates the Calmodulin (CaM) kinases (Bito *et al.* 1996, 1997). In particular, CaMKIV phosphorylates CREB at Ser133, leading to membrane depolarisation of neuronal cells (Bito *et al.* 1996). Experiments on CaMKIV-deficient mice have shown decreased levels of the immediate early transcription factor, c-fos known to be an immediate early gene and to have CRE sequences on its promoters (Ho *et al.* 2000). The evidence suggests that calcium released in the synapse enters the cell through the synaptic cleft, activating calmodulin and other protein kinases which, in turn, enter the nucleus and initiate transcription via the phosphorylation of CREB at Ser133 (Deisseroth *et al.* 1996).

Another potential transcriptional CREB activation pathway is via a cascade of kinase activity initiated by nerve growth factor (NGF). When NGF binds, NGF receptors stimulate guanine-nucleotide exchange factors (GEFs) which, in turn, activate Ras – a small G protein. This triggers the activation of MEK, and thus the cascade of mitogen-activated protein kinases (MAPKs) (Blenis *et al.* 1991). Translocation of downstream molecules into the nucleus once again leads to phosphorylation of CREB at the serine-133 site (Chen *et al.* 1992).

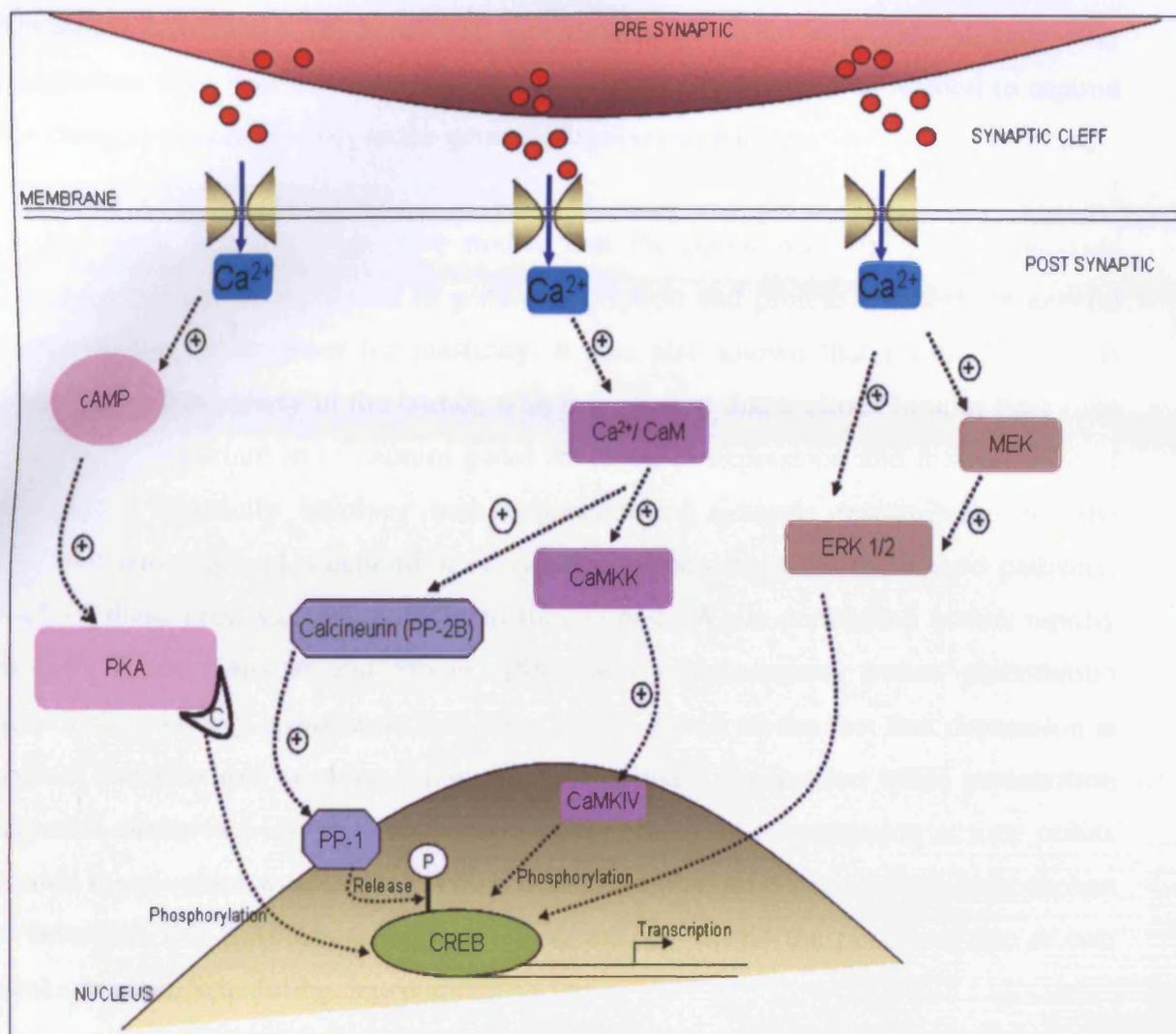


Figure 1.15: representation of the three pathways putatively involved with CREB phosphorylation and activation and regulation of gene transcription. Modified from (Shaywitz and Greenberg 1999).

1.16. Aims

The aim of this study is to identify differentially expressed genes in the mouse barrel cortex via whisker deprivation in order to induce plasticity. The design of this study is partly based on the observation that gene expression changes in the barrel cortex following whisker deprivation. It is known that CRE-mediated gene expression is present 24 hours post whisker deprivation (Barth et al, 2000). Several immediate early genes are also expressed within 24 hours such as c-fos and JunB (1 hour), ICER 6

hours) and Krox (24 hours) (Bisler et al., 2002). An initial pilot study by Dr Abrahams (Abrahams 2003) showed that more genes were expressed at very early time points than later ones. For this reason an early time point was needed to capture the changes occurring early in the gene expression mechanism.

It had been apparent from pilot studies that the initial wave of gene expression involved transcripts implicated in gene transcription and protein synthesis in general rather than effector genes for plasticity. It was also known that plasticity itself is expressed rather slowly in the cortex, which suggested that a closer look at later time points was important in to capture genes involved in expression and maintenance of plasticity. Plasticity involves both depression of synaptic transmission for the deprived pathway and potentiation of synaptic transmission for the spared pathway. Each of these processes has a different time course. While depression occurs rapidly in both visual (Mioche and Singer, 1989) and somatosensory cortex potentiation occurs more slowly (Glazewski and Fox., 1996). Based on the fact that depression is present and maximal at about 7 days with chessboard deprivation while potentiation saturates closer to 14 days (Hardingham et al., 2008), an investigation at time points around these values was decided. The actual time points of 8 and 16 days were chosen to be within this time frame without falling exactly within the period of one or two weeks to make scheduling deprivations easier.

Deprivation could affect gene expression simply by altering the level of sensory drive to the cortex without necessarily causing plasticity. For example, Cytochrome oxidase is a mitochondrial enzyme that changes its expression based on levels of sensory activity to the cortex, but is not thought to be involved in pathways leading to synaptic plasticity. Presumably the levels of cytochrome oxidase activity are related to the amount of energy production required by a cell and this decreases when the sensory input is decreased. In order to distinguish between changes in gene expression caused by a reduction in activity versus those involved in plasticity processes three conditions of whisker deprivation were compared, control animals which have not been subjected to any whiskers deprivation (denoted ALLs); animals completely deprived of their whiskers (denoted DEPs) and a group deprived in a chessboard pattern (denoted CBs). If a gene altered due to changes in activity its expression was expected to be greatest in the ALL group, less in the CB group and lowest in the DEP group.

Conversely, if the gene was involved in potentiation its expression would have been greatest in the CB group and lower in the ALL and DEP group. One ambiguity arises when considering genes involved in depression because we would expect these genes to show the same profile as those affected by activity. However, two further comparisons are useful here; first, the time series is of some use because we would not expect full expression of depression at Day 1. Therefore activity dependent genes might be affected at the initial time point while depression genes later on. Second, the GluR1 knockout animals do not show depression in layers II/III and IV (Wright et al., 2008) and would therefore show a difference in depression genes but not activity dependent genes. A comparison of the DEP group between wild-type and GluR1 knockouts should therefore separate activity dependent genes from depression effector genes.

During potentiation (LTP), GluR1 is inserted into the synapse via phosphorylation of S818, S831 and S845 sites (Lee et al., 2000). Conversely, during depression, the above sites are dephosphorylated and GluR1 is internalised. Thus, there is a differential requirement for GluR1 (and GluR1/GluR2 heteromeric subunits) during different processes. After the insertion of GluR1 or GluR1/2, GluR2/3 heteromers take their place via an autonomous insertion mechanism (Shi et al., 2001; Zhu, 2009), highlighting the importance of GluR1 in activity-dependent processes. GluR1 is required for experience-dependent depression as this mechanism could not be induced in knockouts following whisker deprivation, although surround whisker potentiation was not abolished (Wright et al., 2008). Knowing the importance of GluR1 for experience dependent depression, whisker deprivation will be used in the present study to identify molecules important for this mechanism and their course of action. Although potentiation requires phosphorylation events of GluR1, the finding that experience-dependent potentiation can occur in its absence is consistent with other studies finding that LTP can occur in the GluR1 knockout both in the hippocampus and the barrel cortex (Hardingham and Fox, 2006; Hoffman et al., 2002). GluR1-independent plasticity processes are as yet unclear; using the GluR1^{-/-} animals in this project we will try to identify the transcripts and their pathways involved in GluR1 independent processes. One major signalling pathway to be implicated in this mechanism is nitric oxide (Hardingham and Fox, 2006; Chapters 6 and 7). Indeed, all plasticity in the GluR1 knockout was completely abolished following inhibition of

NOS; but how is NOS acting in the absence of GluR1 compared to wild type subjects will be investigated in this thesis. The activation of NOS in the GluR1 knockout was however linked to synaptic activity and calcium influx, as similar to the NOS antagonism, LTP was completely blocked by application of the NMDA receptor antagonist APV and MK-801 (Hardingham and Fox, 2006). Calcium influx has been shown to activate NOS to result in potentiation, so the manner under which NOS is regulated by deprivation will be studied in this thesis and is analysed in subsequent chapters. It is likely that calcium will affect numerous other synaptic processes. Calcium dependent mechanisms could be up or down regulated with deprivation and could potentially be identified in this microarray study.

Nitric oxide was traditionally thought as a presynaptic modulator (Garthwaite et al., 1988), and in particular GluR1 knockout have a presynaptic locus of plasticity that is associated with nitric oxide (Hardingham and Fox, 2006). If NOS is responsible for causing presynaptic plasticity, it is probable that other molecules are required in addition to modify the presynapse for the enhanced release probability (Hardingham and Fox, 2006). Although presynaptic plasticity remains a controversial topic, some molecules associated with presynaptic modification are known, such as *bassoon* and *synapsin*, and the regulation of these genes will be investigated during plasticity.

The above questions are all related generally to molecular plasticity processes. However, the evolution of plasticity and depression occurs over different time courses (Glazewski and Fox, 1996). Thus, how these receptors and molecules are regulated over time is likely to vary. Finding correlations between deprivation time and genes known to be required for synaptic modifications would provide further evidence for processes that have been postulated by LTP/EDP studies.

The above will be achieved through the following steps:

- Reanalysis of a global microarray experiment, performed by Dr Richard Abraham, aimed to investigate transcriptional effects of experience dependent plasticity.
- Optimization of the design, printing and interpretation of a targeted microarray.

- Identification of confounding factors affecting neuronal plasticity.
- Investigation of the temporal deprivation induced changes in gene expression.
- Identify the relationship of the previously investigated transcripts with the GluR1 subunit of AMPA receptors in GluR1 knockout mice.

CHAPTER 2

MATERIALS & METHODS

MATERIALS

2.1 Reagents

The sources of reagents used in this thesis are provided in the table below. Reagents were of molecular biology grade, unless otherwise stated.

REAGENT	SUPPLIER
Oligonucleotide primers (see Table 2.6)	<i>MWG-Biotech, Ebersberg Germany</i>
Ethanol, Isopropanol	<i>Fisher, Loughborough, Leicestershire.</i>
RNA ^{later} [®] , 5-(3-aminoallyl)-dUTP	<i>Ambion, Huntingdon, Cambs., UK</i>
mRNA Purification Kit, CyScribe [™] GFX [™] Purification Kit, Universal ScoreCard, Universal ScoreCard DNA, CyDye Post-Labeling Reactive Dye Packs	<i>Amersham, Bucks., UK</i>
UVettes	<i>Eppendorf UK Ltd., Cambridge, UK.</i>
THERMO-FAST [®] 96-well plates (Skirted, Semi-skirted, Detection), adhesive PCR foil seals, gas permeable adhesive seals, 1ml, 200µl, 100µl, 10µl, 1µl filter pipette tips	<i>AbGene, Surrey, UK.</i>
Tris	<i>Boehringer-Mannheim Ltd., Lewes, East Sussex</i>
dNTPs (Ultra Pure)	<i>Clontech UK, Basingstoke, UK</i>
Acetic acid, EDTA, Glycerol, SDS, Sodium chloride, Sodium hydroxide	<i>Fisons Scientific Equipment UK Ltd., Loughborough, UK.</i>
Disposable Sterile Universal Tubes, 1.5ml microcentrifuge tubes	<i>Greiner, Stonehouse, UK</i>
Mach1 [™] T1 Phage-Resistant (T1R) E. coli, IPTG, 10x TAE, SuperScript [™] II Reverse Transcriptase, First-strand reverse transcriptase buffer (5X), 0.1M	<i>Invitrogen Ltd., Paisley, UK.</i>

DTT	
M13 sequencing oligonucleotides, Custom Synthesised oligonucleotides	<i>MWG Biotech UK., Milton Keynes, UK.</i>
Restriction enzymes and their buffers	<i>New England Biolabs, Beverly, Massachusetts, USA</i>
ABI Prism™ Dye Terminator Cycle Sequencing Reaction Ready Kit	<i>Applied Biosystems, Foster City, CA, USA</i>
dNTPs (dATP, dCTP, dGTP, dTTP), T4 DNA ligase, oligonucleotides, random hexamer oligonucleotides	<i>Pharmacia Biotech Ltd., St. Albans, UK.</i>
Agarose powder	<i>Bioline Ltd., London, UK.</i>
50ml Falcon tubes, 1ml pipette tips	<i>Alpha Laboratories, Eastleigh, Hants, UK.</i>
100bp, 1kb DNA ladder	<i>New England Biolabs (UK) Ltd. Herts., UK.</i>
loading dye, MgCl ₂ (25mM), 10X Mg- free buffer, MMLV reverse transcriptase, RNAsin, 5X RT buffer, molecular mass markers (ΦX174 DNA/Hae III and Lambda DNA/EcoR I + Hind III) pGEM®-T Vector System, Wizard plus SV Minipreps kit.	<i>Promega Ltd., Southampton, UK.</i>
QIAquick gel purification kit, RNeasy Mini Kit	<i>Qiagen Ltd., Crawley, West Sussex, UK.</i>
ampicillin, BSA, Chloroform, CHROMASOLV® Plus water for HPLC, LB agar, LB broth, Mineral oil, Phenol, 20X SSC, Tri® Reagent, sodium acetate, β-mercaptoethanol, Ethidium bromide	<i>Sigma-Aldrich, Gillingham, Dorset, UK.</i>
Ultra-GAPs	<i>Corning, Koolhovenlaan, Schiphol-Rijk The Netherlands</i>
0.22µm Nucleopore™ filters	<i>Whatman International Ltd., Maidstone Kent, UK</i>

Table 2.1: Reagent supplier

2.2 Buffers and Solutions

The composition of the major buffers and solution used in the work presented are provided in Table 2.2 below. All routine laboratory solutions were prepared using ddH₂O. Sterilisation was achieved by autoclaving (120°C at 15 psi for 20 minutes) where required. Heat sensitive components were passed through 0.22µm Nucleopore™ (Whatman International Ltd., Maidstone Kent, UK) filters and added

separately following autoclaving. Where required β -mercaptoethanol and DTT were always added fresh to any solution. EDTA, TAE and TE were made according to the protocol of Sambrook *et al.*, (Sambrook 1989).

Procedure and method reference	Buffer name and acronym	Recipe	Storage/ Notes
2.14.1 Brain Removal	ACSF 10x	72.5g NaCl 21.83g NaHCO ₃ 1.71g KCl 1.72g KH ₂ PO ₄ 2.46g MgSO ₄ 1 litre Sterile Filtered water Dilute 1:10 before use Add 1.8g glucose per litre	Bubble before adding 2ml 1M CaCl. Store for 2 months in 4°C
2.14.4 Histological analysis	0.1M Phosphate Buffer (PBS) pH 7 – 7.2	23.7g Dibasic Anhydrous Na ₂ HPO ₄ 3.96g Monobasic Anhydrous NaH ₂ PO ₄ 8.2g NaCl 1 Litre of Distilled water	4°C
	Fixative	Heat 1L dH ₂ O at 55 – 60°C Add 40g of paraformaldehyde Add dilute NaOH slowly until clear Stop heating. Add: 23.7g Dibasic Na ₂ HPO ₄ 3.96g Monobasic NaH ₂ PO ₄ and 8.2g NaCl Stir to dissolve and filter	Store 4°C and use within 7 days
	DAB reaction mix	Dissolve 8g of sucrose in 180ml of 0.1M PBS. Add 123mg of cytochrome C and mix. Then, add 100mg DAB and mix until dissolved	Use immediately
	Subbing solution	heat 500ml of H ₂ O to 55°C, add 5g of bovine gelatine and completely dissolve before adding 0.5g of chromium potassium sulphate and let to dissolve fully	Keep in 4°C but it is best to use fresh
2.16.2 Reverse Transcription	5x First Strand Buffer	250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl ₂	Keep aliquots in -20°C
	aa-dUTP/dNTP mix	Make ratio 3:2:5, U:T:ACG	-80°C
		0.72g NaOH in 20mls sterile	RT

2.21.1 Modified Reverse Transcription	1M NaOH	filtered water	
	0.5M EDTA pH 8	Add 2.92g EDTA in 15ml sterile filtered water. Add NaOH pellets until pH 8. Make up to 20mls with sterile filtered water.	RT
	1M HEPES pH 7	4.77g HEPES in 20ml sterile filtered water	RT
2.22.2 Precipitation and cDNA recovery	3M Sodium Acetate pH4.8 - 5.5	Add 24.6g NaOAc in small amount of filtered water. Add glacial acetic acid until pH 4.8-5.5. Make up to 100ml with filtered water. Autoclave to sterilize	RT
	0.3M Sodium Bicarbonate pH9.0 (NaHCO ₃)	0.5g NaHCO ₃ 20ml Sterile water Adjust to pH9.0 with 2.5M NAOH	Do not autoclave, it degrades at 50°C. Make fresh
2.22.3 Coupling reaction	4M Hydroxylamine Hydrochloride	Add 5.56g of powder in 20mls of sterile filtered water	RT
2.24.2 Preparation of substrate	Blocking Buffer	Mix 50ml of 20xSSC, 1ml 20% SDS and 2g BSA. Make up to 200ml with sterile filtered water	Make fresh Prewarm at 42°C
2.24.3 Hybridisation of microarray	20% SDS	Make 2g SDS up to 100ml with HPLC water	RT
	Hybridization Buffer	Mix 500µl formamide, 500µl 20xSSC and 10µl 20%SDS	Make fresh Prewarm at 42°C
2.24.4. Washing the microarray	Wash Buffer 1	50ml 20xSSC 10ml 20% SDS 940ml Sterile filtered water	Make fresh
	Wash Buffer 2	5ml 20xSSC 5ml 20% SDS 990ml Sterile filtered water	Make fresh
	Wash Buffer 3	5ml 20xSSC 995ml Sterile filtered water	Make fresh

Table 2.2: Recipes of buffers and solutions

2.3 Water

Several distinct grades of water were used. Where the grade of water is not specified double distilled water (ddiH₂O) was used. For all applications involving DNA manipulations autoclaved HPLC grade water was utilised.

2.4 Media

LB (Luria-Bertani Broth) media and LB agar were prepared with ddH₂O from capsules according to manufacturer's instructions (Bio101, Vista, CA, USA) and autoclaved at 120°C at 15 psi for 20 minutes prior to use. Following sterilisation, the medium was left to cool to 55°C and where required, ampicillin (Sigma-Aldrich, Gillingham, Dorset, UK) was added at a concentration of 100µg/ml.

2.5 Antibiotics

Stock solutions of ampicillin (100mg/ml) in sterile ddH₂O were passed through a 0.22µM Nucleopore™ (Whatman International Ltd., Maidstone Kent, UK) filter and stored at -20 °C.

2.6 DNA Markers

The DNA markers used were lambda DNA digested with HindIII (λDNA/HindIII) and φX174 digested with HaeIII (φX174/HaeIII) available from Promega, Southampton, UK and the 100bp and 1kb ladders available from New England Biolabs (UK) Ltd. Herts., UK. The fragment sizes of these markers (bp) are given in Table 2.1.

DNA Markers	Fragment Sizes (bp)	Supplier
λ/Hind III	23130, 9416, 6557, 4361, 2322, 2027, 564, 125	Promega, Southampton, UK
φX174/Hae III	1353, 1078, 872, 603, 310, 281, 271, 234, 194, 148, 72	Promega, Southampton, UK
100bp	1517 (45ng), 1200 (35ng), 1000 (95ng), 900 (27ng), 800 (24ng), 700 (21ng), 600 (18ng), 517 (97ng), 500 (97ng), 400, (38ng), 300, (29ng), 200 (25ng), 100 (48ng).	New England Biolabs (UK) Ltd. Herts., UK
1kb	10002 (42ng), 8001 (42ng), 6001 (50ng), 5001 (42ng), 4001 (33ng), 3001 (125ng), 2000 (48ng), 1500 (36ng), 1000 (42ng), 517 (42ng), 500 (42ng).	New England Biolabs (UK) Ltd. Herts., UK

Table 2.1: DNA marker fragment sizes (bp)

2.7 Vectors

The vectors used throughout this work are shown in Table 2.4 and full vector maps are provide in Appendix 1.

Vector	Selective Marker	Source
pGEM-T	Amp	Promega, Southampton, UK
pSPORT	Amp	Invitrogen Ltd., Paisley, UK.

Table 2.4: Cloning vectors

2.8 Bacterial Strains

The genotypes of the *E. coli* strains used during this study are detailed in Table 2.5.

<i>E. COLI</i> Strain	Genotype
Mach1™	F ⁻ ϕ 80(<i>lacZ</i>) Δ M15 Δ <i>lacX74</i> <i>hsdR</i> (r _K ⁻ m _K ⁺) Δ <i>recA1398</i> <i>endA1 tonA</i>

Table 2.5: Bacterial genotypes

2.9 Genetic background of experimental animals

All mice used in the experiments were the offspring of C57BL/6J01aHsd crosses for the targeted plasticity microarray experiment and *GluR1*^{-/-} homozygote knockouts for the additional array experiments. The breeding was done in house. At the start of both experiments all animals were between 28-32 days old.

METHODS

2.10 Sterilisation

To ensure sterilisation, reagents and consumables were autoclaved at 120°C at 15 psi for 20 minutes prior to use. Reagents and consumables that were to be used for RNA applications were autoclaved twice to ensure nuclease-free contents. All autoclaved materials were dried in an oven. Reagents and chemicals that could not be autoclaved were filtered in order to be sterilised.

2.11 Animal husbandry

Animals were used in agreement with the guidelines in the Animal Act 1986. The place of work was specified by a Project Licence and it was carried out by the Personal Licence holder.

2.12 Vibrissal deprivation

Vibrissal deprivation was carried out as described in Schedule 2a of the Animals Act 1986. The selected animals were placed in a clear chamber in order to be anaesthetised with isoflurane/O₂ (supplied by Astrazeneca, UK). A pair of forceps was used to remove the vibrissae. After the plucking, the animal was allowed to recover before returning to its cage.

In total three conditions were used for this study. The first treatment (referred to as ALL) did not include any deprivation. However, the animals were anaesthetised and checked to ensure that all vibrissae were intact. The second (indicated as DEP) involved the removal of all the vibrissae and the third (CB – chessboard) removed every other whisker. Figure 2.1 shows all three conditions and indicates the exact whiskers deprived in the case of chessboard pattern.

Regular checks were performed every second day to check for vibrissal re-growth and when necessary re-grown vibrissae were removed. The animals in the control

condition (ALL) were also anaesthetised every other day. All deprivations and checks were performed at the same time of the day and the deprivations were done for 1 day, 8 days and 16 days.

2.13 Animal sacrifice

Animals were sacrificed under Schedule 1 of the Home Office Animals Act 1986 by cervical dislocation.

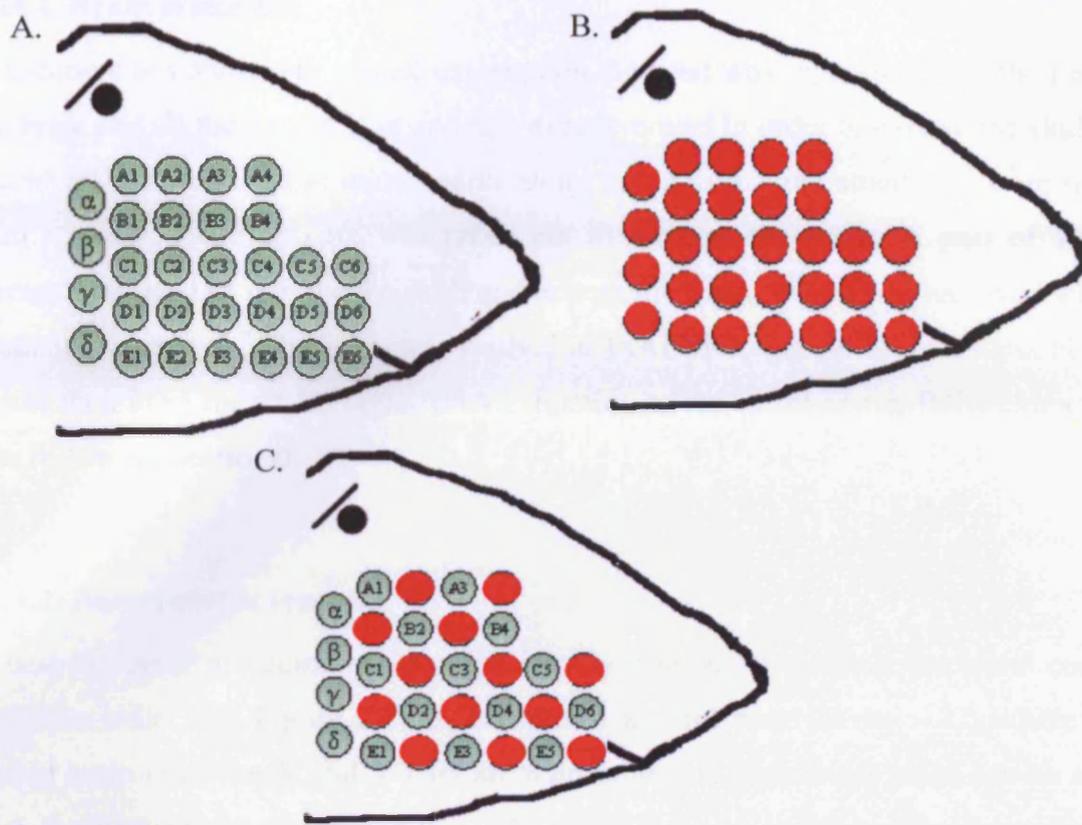


Figure 2.1: Representation of the whiskers and the three patterns of deprivation.

Panel A: Illustrates the condition where all whiskers are present (condition designated ALL). As shown, the vibrissae are arranged in columns (1-6) and rows (A-E). The vibrissae labelled α , β , γ and δ do not belong to any row. For this condition whiskers were checked to ensure that they were all intact.

Panel B: Illustrates the condition where all whiskers are deprived (condition designated DEP). For this condition, all the whiskers were deprived, indicated with the red circles.

Panel C: Illustrates the condition where whiskers have been removed in a chessboard pattern (condition designated CB). Here, every other whisker was deprived, namely A2, A4, B1, B3, C2, C4, C6, D1, D3, D5, E2, E4 and E6. The external whiskers named α , β , γ and δ were not deprived in this case. The red circles indicate the deprived whiskers.

2.14 Manipulation and histological analysis of the murine brain

2.14.1 Brain removal

Subsequent to confirming animal extenuation the head was separated from the rest of the body and all the excess skin and fair were removed in order to expose the skull. A sterile scalpel was used to make marks along the mid-line and small cuts were made with scissors. Extreme care was taken not to damage the brain. A pair of sterile forceps was used to remove the skull and expose the brain, which was removed with a small sterile spatula. The brain was washed in 1xACSF to get rid of the excess blood. It was then used for whole-brain-RNA extraction or for barrel-cortex-RNA extraction (see following sections).

2.14.2 Barrel cortex removal

A bespoke piece of equipment was specifically designed to remove the barrel cortex from the brain (see Figure 2.2). It comprised a solid base (cavity – C) where the mouse brain sits. The X and Y bars are adjustable and their exact place can be seen with the help of the attached rulers. The 2mm diameter stainless steel borer has a sharp end in order to facilitate tissue penetration.

The X and Y bars are used to position the brain so that the borer (B) was placed above the landmark (see Figure 2.3) where it was gently pressed against the tissue to leave a visible mark. The barrel cortex was extracted by placing the borer 2.1mm anterior to the landmark and 2.8mm laterally. Once in place, the borer was lowered carefully towards the brain and the barrel cortex was withdrawn. The tissue was then removed with a pair of sterile forceps and preserved in RNAlater (Ambion, Austin, TX, USA) at -20°C or -80°C prior to RNA extraction (Section 2.15.2). The remaining brain was kept in fixative prior to sectioning and staining (Section 2.14.4).

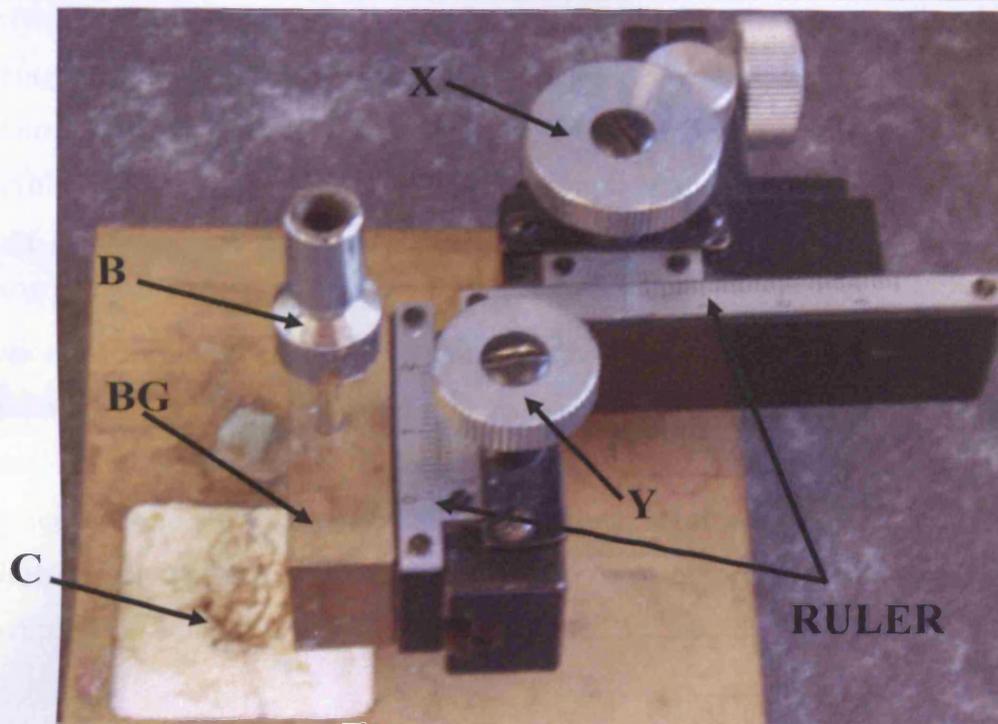


Figure 2.2: Apparatus for mouse barrel cortex extraction.

The brain is carefully placed on the cavity (C). The required adjustments are made so that the long edge borer guide is parallel to the mid-line of the brain. X and Y are used to move the borer (B) at all directions so that the desirable area of cortex is extracted.

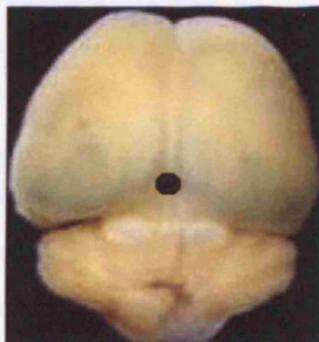


Figure 2.3 Indication of the landmark

The point indicated by the black dot is the landmark made by the borer, by applying pressure on the brain. The landmark was used as a reference for all the measurements necessary.

2.14.3 Sectioning the remaining brain

After the brain was removed from the skull and the barrel cortex was safely removed using the previously described apparatus, the remaining brain was kept in fixative overnight at 4°C. The following day, the cerebellum was removed and the brain was divided in half along the mid-line. The anterior of the cortex was removed from each half of the brain. Each hemisphere was flattened between two microscope slides (VWR;

Leicestershire, UK) and secured using plasticine. The flattened hemispheres were then returned to fixative for overnight incubation. Flattening the brains helped to place the barrel field in the horizontal plane, which made visualisation of the barrel field more straightforward when sectioned and stained. Following overnight fixation, the flattened brains were placed into 20%w/v sucrose in phosphate buffer for another overnight incubation.

Subsequent to the overnight incubation the brains were cut into 35µm sections using the Leica SM2000R microtome (Leica, Germany). A flat bed was formed on the freezing stage of the microtome using small amounts of 20%w/v sucrose in PBS (Section 2.2). The cortex was placed on the flat sucrose bed and once frozen, the tissue was cut and the sections were transferred into individual wells of 24-well culture plates (VWR) which contained PBS without sucrose. In order to remove all traces of sucrose, the sections were kept on a shaking platform for 2 hours during which time the PBS was replaced twice to remove any remaining sucrose.

2.14.4 Histological analysis

The staining protocol involved the usage of diaminobenzidine (DAB: Sigma-Aldrich, Dorset, UK). DAB is a potent carcinogen that requires a comprehensive risk assessment prior to experimental procedures being undertaken. All the work described below was carried out in fume hoods and ovens specifically for DAB work. Protective clothing was worn in all times and gloves were used when handling the sections. The DAB was inactivated with bleach and the same was used to treat any glassware used.

PBS was removed from the culture plates containing the sections and replaced with DAB reaction mix. The plates were incubated at 37°C until slices were brown (around

6 hours). Once the reaction was complete, the DAB was replaced with phosphate buffer and the sections were rinsed to remove excess DAB.

2.14.5 Creation of gelatine coated slides

Prior to use new slides were immersed in nitric acid for 20 minutes and then rinsed in distilled water for one hour. The slides are then rinsed in deionised H₂O and allowed to drain. Once dry the slides were coated in gelatine by dipped in subbing solution (Section 2.2) for one minute. Then, they are left to dry in a 37°C oven overnight. The following day they are carefully removed and placed back in their packs.

2.14.6 Section Mounting

The desired tissue sections were transferred onto gelatine-coated microscope slides using a paint brush. The slides were left overnight to dry at room temperature. The following day they were dipped in xylene (Sigma – Aldrich, Dorset, UK) for 5 minutes and covered with DPX mounting medium (Raymond A Lamb - Laboratory Supplies, Eastbourne, East Sussex, UK). The slides were carefully covered with a cover slip (VWR; Leicestershire, UK) and left to dry overnight. Once dried the slides could be studied using a light microscope.

2.15 Procedures for purifying and manipulating RNA

2.15.1 General guidelines

Due to the prevalence of RNase in the environment together with the enzymes inherent stability RNA is very sensitive to degradation. During this study, all precautions reasonably possible were taken in order to minimize the risk of RNA degradation. Work surfaces were treated by wiping with RNase-free wipes (Ambion, Austin, TX, USA) and the same approach being applied to all the equipment, such as pipettes, forceps, scalpels etc. Consumables, such as pipette tips (Starlab, Milton Keynes, UK) and eppendorff tubes (Eppendorf, Germany) were autoclaved twice at 120°C at 15 psi for 20 minutes and subsequently dried in an oven. Gloves were worn at all times and changed regularly to avoid cross contamination. Sterile filtered tips were used in most of the micro-volume liquid transfers (Bioline, London, UK). RNA

samples were kept on ice as much as possible to reduce the activity of RNases and all centrifugations were performed at +4°C. Furthermore all RNA samples were stored in -80°C between manipulations.

2.15.2 Total RNA extraction from the barrel cortex

Extraction of RNA was performed using the RiboPure – *Isolation of high quality total RNA* – kit from Ambion (Austin, TX, USA) as it is the only extraction kit compatible with the RNAlater solution used to store the tissue.

As described in 2.14.2, the barrel cortex was removed from the brain and was kept in RNAlater in -20°C till further use. At this point, the samples were thawed at room temperature and excess solution was removed. The samples were weighed and transferred in to Bijou bottles (Sterilin, Staffs., UK) and 10-20 volumes of TRI@Reagent was added (supplied in the kit). The tissue was homogenized to disrupt cells using the Ultraturrax T25 homogenator (IKA Labortechnik, Staufen, Germany). Between each homogenization operation, the machine was washed with 4M NaOH followed by 2 rinses with autoclaved sterile water and a final rinse with 100% ethanol. The homogenates were incubated for 5 minutes at room temperature. This incubation allows nucleoprotein complexes to completely dissociate. The samples were then centrifuged for 10 minutes at 4°C to remove insoluble materials that contain high amounts of protein, fat, polysaccharide, high molecular weight DNA or extracellular material, such as muscle. The supernatant, which included the RNA, was transferred into a new, sterile 1.5ml microcentrifuge tube (Eppendorf, Germany). Bromochloropropane (BCP - 100µl, Sigma-Aldrich, Dorset, UK) was added and the samples were centrifuged for 15 seconds. Chloroform could have been used for this step, but BCP was preferred as it is less toxic and it reduces the risk of contaminating RNA with DNA. The mixture was incubated at room temperature for 5 minutes and then centrifuged for 10 minutes at 4°C to achieve separation into three phasing including; a lower (red) organic phase containing the proteins; a DNA containing interphase; and an upper colourless aqueous phase containing the RNA. The volume of the aqueous phase is about 60% of the volume of the TRI reagent employed for the initial homogenisation. The aqueous phase was transferred into a new microcentrifuge

tube using extreme care not to transfer any of the interphase layers. 100% ethanol (200µl) was added and subsequent to vigorous shake for 5 seconds to avoid RNA precipitation, the samples was transferred into a Filter Cartridge-Collection Tube (provided in the kit) and centrifuged at room temperature for 30 seconds at 13,000g. The RNA is now bound to the filter and the flow-through can be discarded. Wash Solution (provided in the kit - 500µl) was added in the sample and centrifuged at room temperature for 30 seconds at 13,000g. The flow-through was discarded and the step repeated one more time. The RNA was still bound on the filter, thus the Filter Cartridge was transferred into a new collection tube. Elution Buffer (provided in the kit - 100µl) was added. The sample was incubated at room temperature for 2 minutes and then centrifuged at room temperature for 30 seconds at 13,000g. The elute, containing the RNA, was collected and the RNA stored at -80°C prior to further study.

2.15.3 RNA extraction from whole brain

The brain was removed from the mouse skull as described in Section 2.13.1 and it was washed in 1xACSF (Section 2.2) to get rid of excess blood. Then it was divided in half and each hemisphere was kept in a Bijou bottle (Sterilin, Staffs., UK) with 1ml of Tri@Reagent (Sigma – Aldrich, Dorset, UK). The same procedure as explained in Section 2.14.2 was followed to homogenise the tissues.

The homogenates were incubated at room temperature for 10 minutes and transferred into a fresh tube, where mixed with 500µl of chloroform (Sigma – Aldrich, Dorset, UK). The samples were mixed vigorously and let to stand for 15 minutes at room temperature. Then they were centrifuged at 4°C for 15 minutes 13,000g. The top clean area was transferred into a fresh tube with 500µl of isopropanol (Sigma-Aldrich, Dorset, UK). The mix was shaken and let to stand for 10 minutes. It was then centrifuged at 4°C for an additional 10 minutes at 13,000g to precipitate collect the RNA. The pellet was washed with 70% ethanol and centrifuged at 4°C for an additional 10 minutes at 13,000g. All the liquid was removed and let to air – dry for 3 minutes. It was then dissolved in pre-warm sterile water (100µl). The dissolved pellet was cleaned using the RNeasy Mini Kit (Qiagen, West Sussex, UK) following the protocol provided.

The purified RNA was incubated overnight at -20°C mixed with 1/5 volume of sodium acetate pH 5.2 and 2.5 volumes of ice cold 100% Ethanol. The next day it was centrifuged at 4°C for 10 minutes at 13,000g. The supernatant was discarded and 1ml of ice cold 75% ethanol was added to the pellet. The sample was centrifuged at 4°C for 7 minutes at 13,000g. Once the supernatant was discarded, the pellet was let to air-dry and then dissolved in $30\mu\text{l}$ of pre-warm water. The resulting sample was stored in -80°C for further study.

2.15.4 Analysis of the integrity and purity of recovered RNA

The quantity and quality of RNA was measured using UV light spectroscopy. A diluted sample was prepared (commonly using a 1 in 50 or 1 in 10 dilution) and absorption spectra obtained using an Ultraspec 2100pro spectrophotometer (Amersham pharmacia biotech, Germany). Absorption at 260nm was used to calculate the concentration of the RNA (extinction coefficient for RNA 1 OD unit = $40\mu\text{g}/\text{ml}$). Furthermore deviation of 260/280 and 260/230 ratios below 2 was used to indicate the presence of protein/phenol or salt/ethanol contamination respectively. RNA concentration was adjusted to $0.5\mu\text{g}/\text{ml}$ and aliquots of $10\mu\text{g}$ were stored in -80°C . In order to assess the integrity of the RNA approximately $2\mu\text{l}$ of the sample (equivalent to $1\mu\text{g}$) was assessed by agarose gel (1.5%) electrophoresis (Section 2.20.2).

2.16 Protocols for purifying and manipulating DNA

2.16.1 General guidelines

DNA is less susceptible to degradation than RNA but certain precautions need to be taken. Work was done on ice, whenever possible, gloves were worn at all times and different surface area, set of pipettes and consumables were used, from those used for RNA work. All consumables, should be purchased as RNAase/DNAase free or autoclaved (120°C at 15 psi for 20 minutes) prior to use. Solutions should be autoclaved (as above) prior to use or where components are heat instable they should be made from sterile components and passed through a $0.22\mu\text{m}$ Nucleopore™ (Whatman International Ltd., Maidstone Kent, UK) filter before use.

2.16.2 Reverse transcription (RT)

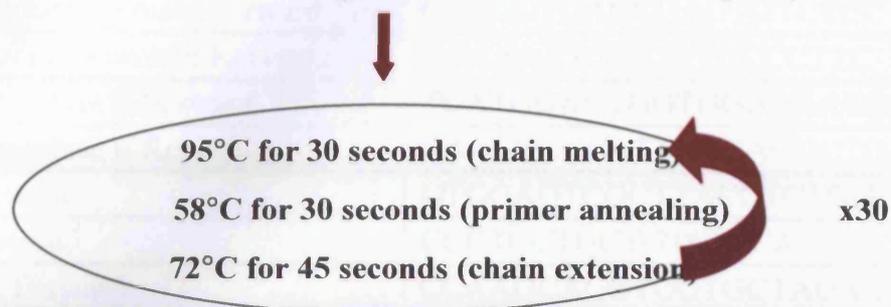
The generation of a complementary DNA strand from a single stranded RNA template molecule is achieved by providing appropriate priming sites and dideoxynucleotides to a reverse transcriptase enzyme. In practical terms we combine and mix 2µg purified total RNA (dissolved in not more than 10µl of sterile DiH₂O) (Section 2.15.2), 1µl 50-250ng/µl random hexamers, 1µl of 10mM dNTPs and autoclaved water to a final total volume of 12µl within a nuclease-free microcentrifuge tube. The primers are annealed by heating the mixture at 65°C for 5 minutes after which it is quickly transferred onto ice. The contents of the tube were collected at the bottom with a brief centrifugation. A 5xFirst-Strand Buffer concentrate (4µl; Section 2.2) was added which provide optimal environment for enzyme synthesis to which 2µl of 100mM DDT was added to reduce RNA secondary structure and together they were combined with 1µl of 40units/µl RNaseOUT Recombinant Ribonuclease Inhibitor to reduce the risk of RNA degradation. This mixture was then incubated at 25°C for 2 minutes. Subsequently, the enzyme reaction was initiated by adding 1µl of 200units/µl of Superscript™ II Reverse Transcriptase and the mixture was incubated at 25°C for 10 minutes for efficient random hexamer annealing, followed by 50 minutes at 42°C. The reaction was inactivated at 70°C for 15 minutes. The generated cDNA was stored at -20°C for further study. The source of all reagents used and the recipe of relevant buffers are provided in Sections 2.1 & 2.2.

2.16.3 Polymerase chain reaction (PCR)

Logarithmic amplification of DNA was achieved by harnessing the thermal stability of Taq DNA polymerase (Mullis and Faloona, 1987). Appropriate template DNA was combined with specific primers designed to flank the target amplicon site DNA sequences were generated, directed by primers, using the enzyme Taq Polymerase All PCR reactions were carried out in thin-walled PCR tubes (ABgene, Epsom, UK). The reaction mix contained the template DNA (2µl), 3 units Taq Polymerase (Promega, Southampton, UK), 1µl 10mM dNTPs and 1µl of each 10µM primer and 10xBuffer (supplied with the Taq).

The cyclic conditions used for the reactions were:

95°C for 5 minutes (initial denaturation of template)



72°C for 10 minutes (final extension)

4°C HOLD

2.17 Primer Design

All primers were designed using the Primer3 on-line software developed by Whitehead Institute for Biomedical Research and is located at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.

All primers were between 9-40bp in length, had a GC content of between 20-80% and their resulting product was between 450-600bp. Also, they were all purchased from MWG (MWG, Milton Keynes, UK) and were HPLC purified. Table 2.1 shows a list of the primers used and their sequences.

Name	Sequence
Bassoon Forward	AGCCACAGACACAACAGCAG
Bassoon Reverse	GAGCCCTTCTGGACACAATC
Bcl2 – associated protein Forward	GAAGCTGAGCGAGTGTCTCC
Bcl2 – associated protein Reverse	GAAAAATGCCTTTCCCCTTC
Carboxypeptidase E Forward	TGATGGAGTGGTGGAAAATG
Carboxypeptidase E Reverse	GAAGTGGCATTAAACAGGCTGA
Citron Forward	GTGGAGTCGCTTACCTCTGG
Citron Reverse	CCCTGCTGCTGTCTTCAAAC
Cortistatin Forward	CCAAGCAGGTGGTGCTAGAG
Cortistatin Reverse	GCTGATTGACAGTCTTTATTCAGGT
Grin2a Forward	GCTTCCCAACAATGACCAGT
Grin2a Reverse	CTCCTCTTGCTGTCCTCCAG
Netrin 1 Forward	GATGTGCCAAAGGCTACCAG
Netrin 1 Reverse	TTCTTGCACTTGCCCTTCTT
Neurogenic Differentiation 2 Forward	CGACCCCTTCTTTTTCTTTG
Neurogenic Differentiation 2 Reverse	GGCTTGGCTCTCTCTTTCCT
Neurotrophin 3 Forward	AGTGAGAGCCTGTGGGTGAC
Neurotrophin 3 Reverse	TTACAGAAGGGTTCCCGAGAG
Nitric Oxide Synthase 1 Forward	CTCCTGGCTCAACCGAATAC
Nitric Oxide Synthase 1 Reverse	GAACACACCAGCATCCTCCT
Nitric Oxide Synthase 3 Forward	GCACCCAGAGCTTTTCTTTG
Nitric Oxide Synthase 3 Reverse	GAGGTGTCTGGGACTCACTGT
Paxillin Forward	TTCAAGGAGCAGAACGACAA
Paxillin Reverse	CTCTGGGAAACTGGGTGGT
Plasticity Related Gene 1 Forward	AACCCAAGCTGCAGTATTTGA
Plasticity Related Gene 1 Reverse	TCAGTTTGGAAAACATTGCAT
Quiescin Q6 Forward	CCCATTCCTGCTGAAGTCTC
Quiescin Q6 Reverse	CTAAACCCAGCACCTTCCAC
Soat Forward	GCTTCGGTGGTATGATGCTT
Soat Reverse	AACAGCAAAGCCCTTCTGAG
Spectrin <i>beta</i> Forward	TCAGAGCCCAGATGAGTGTG
Spectrin <i>beta</i> Reverse	CGACAGACAATGGTGTGCGAG
Synaptopodin Forward	GGGGTGCTGGAGTTAGATGA
Synaptopodin Reverse	AAGAGGCACAAGGCAGGATA
VgluT2 Forward	TGAAACTCATGCCACAAAGC
VgluT2 Reverse	TGCAGTAAATTGGGATGTGC

Table 2.6: Representation of the primer sequences used to clone the corresponding genes.

2.18 Cloning

2.18.1 Ligation reaction

The gene products generated by PCR (section 2.7.3), using the primers shown in table 2.1, were used for further cloning. They were ligated with the pGEM®-T easy vector (Promega, Southampton, UK) that is 3015bp in size. This vector is a convenient and efficient system for cloning of PCR products. The vector's insertion site has 3' thymidine overhangs at both ends. This allows the vector to immediately ligate with the PCR product, which has a single deoxyadenosine at its 5' terminal, preventing recirculation of the vector and improving the efficiency of the reaction. The reaction can be performed in one hour in room temperature or overnight in the fridge (for greater recovery). The ratio insert: vector was always 3:1.

The desired PCR product was mixed with 50ng pGEM®-T vector, 2x Rapid Ligation Buffer and 3 units T4 Ligase (all provided with the vector) in a 10µl total volume reaction. The reaction was incubated at 4°C overnight.

2.18.2 Transformation

Following the ligation reaction, the plasmids were transformed into chemically competent *E. coli* Mach1-T1 cells (Invitrogen). Aliquots of the competent cells were stored in -80°C and their efficiency was 1×10^9 cfu/µg plasmid DNA. These cells have a faster doubling time compared to other standard strains, hence colonies could be visualised 8 hours after plating on ampicillin plates. Also plasmid DNA can be prepared 4 hours after inoculating a single colony.

The cells were thawed on ice and 2µl of ligated plasmid DNA was added. The reaction was mixed by gently flicking the tube and incubated on ice for 30 minutes. Then, it was heat shocked for 30 seconds at 42°C and transferred immediately on ice for 2 minutes. S.O.C. medium (250µl, supplied in the kit, it contains 2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄ and 20mM Glucose) was added and the reaction was shaken horizontally for 1 hour at

37°C at 225rpm in an orbital incubator. In the meanwhile, ampicillin LB plates were being incubated in a cell culture oven at 37°C. A sterile spreader was used to plate out 100µl, 50µl and 25µl of each sample. This would give sequential dilutions and well spread colonies on the plates, which were easily picked up. The plates were incubated at 37°C for 8 hours.

2.18.3 Selecting and screening the successful transformants

Following the 8 hour incubation of the plates, individual colonies were inoculated in 5ml of liquid broth for further growth in an orbital incubator at 37°C for 9 hours. The resulting cultures were amplified and cleaned using the Wizard® *Plus* SV Miniprep DNA Purification System (Promega, Southampton, UK). The supplied protocol was followed and the resulting DNA plasmid was stored in 50µl of 10xTE (supplied) buffer at -20°C.

The size of the insert, of the resulting plasmid, was checked via PCR (section 2.7.3) using the appropriate primers from table 2.1. Once happy with the size of the product, the plasmid DNA was subjected to sequencing.

2.19 Sequencing

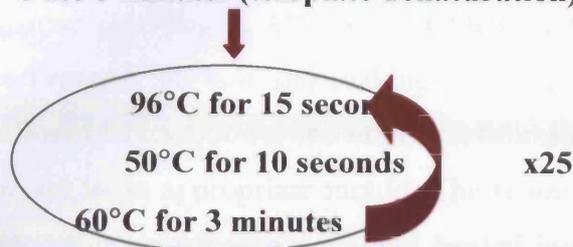
2.19.1 The Reaction

Once the cloning had been completed, sequencing of the resulting plasmid was required to ensure that no mistakes have been incorporated during the initial PCR reaction.

Sequencing of the plasmid DNA was carried out using the ABI Prism Dye™ Terminator cycle sequencing, using a combination of BigDye V3 and BigDye Terminator buffer (Applied Biosystems, CA, USA). To each reaction mixture was added 1 µl of ABI BigDye V3 and 5 µl of big dye terminator buffer, 2.4 pmols of primer, 290 ng of PCR product and sterile water to a final volume of 15 µl.

The reactions were placed in a Techne Flexigene PCR thermo cycler and the following thermo cycle was performed:

96°C for 3 minutes (template denaturation)



Excess BigDye was removed by precipitating the DNA in 4 volumes of 90% isopropanol. The contents were mixed by gentle inversion and then centrifuged at 12000 x g for 30 minutes. The supernatant was removed and the pellet was washed in 70% isopropanol. The DNA was pelleted by centrifugation at 12000 x g for 15 minutes, the supernatant removed and the pellet allowed to air dry in the dark. The resulting amplified fluorescently labelled DNA fragments were separated, detected and sequence determined using an ABI 3100 Prism[®] DNA sequencer (Cheshire, UK).

2.19.2 Sequence Analysis

A selection of computer generated programmes was used to analyse the nucleic acid sequences. The main purpose of such software was to identify homologies of any given sequence with known genes from the genomes of other model organisms.

Thus, the generated sequences were compared with international database entries using the Basic Local Alignment Search Tool (commonly known as BLAST). Derivatives of BLAST were used to check different formats of data; i.e. the nucleotide sequences were checked through BLASTn whereas the protein sequences were checked through BLASTx. In cases where the desired sequences were already available, the BLAST 2 Sequences tool was used to find similarities with the sequenced fragment. All software were accessible through the National Centre for Biotechnology Information (commonly known as NCBI) homepage <http://www.ncbi.nlm.nih.gov/>.

2.20 Agarose Gel Electrophoresis

2.20.1 Gel electrophoresis of DNA samples

Gel electrophoresis was used to assess the quality of the samples. The gel was prepared by melting agarose (Invitrogen, UK) in 1xTAE buffer (Invitrogen, UK), usually 1.5% w/v. Once brought to the boil and making sure that all the agarose has dissolved, the gel was allowed to cool down and ethidium bromide (Sigma, UK) was added. The gel was left to set in an appropriate mould. The required amount of DNA was mixed with one fifth volume of loading dye and loaded into the gel wells. To check the size of the samples, an appropriate marker (λ or ϕ , Promega, Southampton, UK) was loaded in the gel as well. Electrophoresis was carried out at 110volts for approximately 40 minutes in 1xTAE buffer. The DNA bands were visualized by UV light using a Syngene Gene Genius Bioimaging System (Syngene, Cambridge, UK).

2.20.2 Gel electrophoresis of RNA samples

Gel electrophoresis in this case was carried out similarly to the DNA electrophoresis. The main difference was that all the glassware and buffers were autoclave prior to use and the gel tank, mould and combs were soaked in 0.1% SDS overnight to prevent RNase degradation.

2.20.3 Gel electrophoresis of fluorescently labelled DNA

Gel electrophoresis was also used to assess the quality of incorporated fluorophore into the cDNA generated during the modified reverse transcription of barrel cortex RNA (section 2.11.1). A specially designed gel tray was used, which could only accommodate a reduced amount of agarose. The 1.5% gel was made without ethidium bromide and was moulded on a glass microscope slide. It was then placed into a tank, which was previously rinsed twice with 1x TAE to remove any traces of ethidium bromide. Only 1 μ l of sample was loaded, combined with 1 μ l of 50% glycerol (Fisher Scientific International, USA), and run for 30 minutes at 100V. The gel was scanned in the Perkin Elmer life sciences LSIV carousel scanner at 550nm and 650nm wavelength for Cy3 and Cy5 respectively. At this stage a label is assessed as good if it has a large size distribution through the gel and is not degraded. The amount of

protein can be assessed as it appears to be around the well. If there is a significant amount of protein in the sample, it is suggested to clean up the RNA further before labelling again.

2.21 Quantification of nucleic acids

The quantity of the nucleic acids was checked by measuring their absorbance of UV light using the Ultrospec 2100pro (Amersham pharmacia biotech, Germany). For RNA and DNA samples, reading were taken for 260nm and 280nm wavelength. Then, the spectrophotometer converted the measurement into a concentration and calculated the OD 260/ 280 ratio.

2.22 Preparing for the arrays

2.22.1 Modified reverse transcription

This is a modified version of the reverse transcription described in section 2.16.2, requiring the use of 5-(3-aminoallyl)-dUTP. Generation of complementary DNA involved 10µg of RNA (this sample was extracted from the Barrel Cortex only described in section 2.15.2), 3µl 100mM random primer, 1µl Universal ScoreCard and sterile DiH₂O to a total volume of 17.2 being incubated at 70°C for 10 minutes. Meanwhile, the reverse transcription enzyme mix was being prepared using 5xFirst-Strand Buffer (6µl; Invitrogen Ltd, Paisley, UK), 0.1M DTT (3µl; Invitrogen; Ltd, Paisley, UK) and aa-dUTP/dNTP mix (1.2µl; ratio 3/2) per reaction. 10.2µl of the reverse transcription mix was added to the mixture and it was incubated at 25°C for 2 minutes for optimum random hexamer binding to the RNA molecules. Then, 2µl of 400units/µl of SuperscriptTM II Reverse Transcriptase was added to the reaction followed by a 3 hour incubation at 42°C. The reaction was inactivated by the addition of 10µl 1M NaOH and 10µl 0.5M EDTA (pH8.0), which interact with the activity of the enzyme, and incubation at 65°C for 15 minutes. In order to recover the pH of the mixture 25µl of 1M HEPES (pH7.0) was added along with 225µl HPLC grade water to make the sample up to 300µl.

2.22.2 Precipitation and cDNA recovery

For this step, 33 μ l of 3M sodium acetate and 500 μ l of pre-chilled ethanol were added to the sample. The mixture was vortex and incubated overnight at -80°C. The following day, the mixture was centrifuged for 30 minutes at 4°C and a pellet was formed at the bottom of the tube. This pellet should be take care of cause is very difficult to see. It was washed twice with ice-cold 70% ethanol and air-dried. Once dry, it was completely resuspend in 5 μ l of filtered sterile HPLC water. If it is too difficult to resuspend the pellet, it could be placed at 65°C for 30 seconds and returned immediately on ice. Sodium bicarbonate (3 μ l of 0.3M, pH9, Section 2.2) was added to the mixture, which was vigorously shaken and briefly centrifuged.

2.22.3 Coupling reaction

The Cy dye (Amersham, Germany) and DMSO (Sigma – Aldrich, Dorset, UK) was allowed to reach room temperature. Immediately before adding to the cDNA, 4 μ l of DMSO were added to a Cy dye pack and the powder was carefully resuspended. The Cy dye (2 μ l) was added to the cDNA, which was mixed and centrifuged to collect everything at the bottom of the tube. The sample was not vortexed at this point to avoid the creation of bubbles which may decrease the efficiency of the labelling reaction. Incubation in the dark at room temperature for one hour took place. To stop the reaction 4M Hydroxylamine Hydrochloride (5 μ l; take extreme care, it is explosive when heated above 110°C) was used. The sample was, once again, incubated in the dark at room temperature for 15 minutes. The final volume was adjusted at 50 μ l with HPLC water (35 μ l).

2.22.4 Clean up of the labelled oligonucleotides

For every cDNA labelling reaction to be purified, one GFX column (CyScribe GFX Purification Kit; Amersham, Germany) was placed into a clean collection tube and capture buffer was added (500 μ l). The labelling reaction was added to the column and mixed gently (avoiding the creation of bubbles) by pipetting up and down. The probes should not be left in capture buffer for more than 10 minutes cause the yield could be reduced. For the same reason, dealing with small numbers of samples is advised at

this stage. The sample was centrifuged for 30 seconds and the flow-through was discarded. Wash buffer (made up with ethanol) was added to the column (600µl) and the sample was once again centrifuged at maximum speed for a minute. This wash step was repeated for a total of 3 sequential washes and the flow-through was discarded every time. To ensure the complete removal of ethanol, the sample was centrifuged one more time without the addition of wash buffer. The column was then transferred into a new, autoclaved (make sure it is nuclease free) collection tube and 60µl of pre-warm (at 65°C) elution buffer was added. The column was incubated for 5 minutes, to increase yield, and then centrifuged for 1 minute. The elute was now the clean label and was kept for further microarray studies.

2.22.5 Calculation of CyDye frequency of incorporation (FOI)

Gel electrophoresis was used in order to assess the quality of the fluorescence incorporated into the cDNA (see section 2.9.3). The quantity of the cDNA, Cy3 and Cy5 was assessed with the use of the Ultraspec 2100pro (Amersham pharmacia biotech, Germany). Readings were taken at 260nm to calculate the amount of cDNA present in the sample and at 550nm and 650nm to calculate the amount of labelled cDNA in the sample for Cy3 and Cy5 respectively. Using the values from the three different wavelength readings the required volume of sample to be added to the hybridization was calculated in order to obtain 20pmols of label per hybridisation.

2.23 Creation of microarray slides

2.23.1 Printing the microarray slides

Selected bacterial clones were amplified by PCR and the generated cDNA was printed onto CMT – GAPSTM coated glass slides (Corning, NY, USA). The microarrays were printed using Perkin Elmer SpotArray 72 printer (Perkin Elmer, MA, USA). The clones were arranged in 384 well plates which were given a unique ID.

Prior to printing, a number of parameters had to be specified into the computer software that controlled the printing process, some of them being: the number of slides, number of replicates of each spot per slide, number of 384 plates to be used,

the use of control spots (blanks and landmarks) etc. The above information was used by the software not only to print the slides but also to generate a file that linked each spot on the slide to a specific clone. Each cDNA clone was printed in triplicate on the microarray slide. The titanium pin head would pick up the PCR product from the appropriate well and deposit it on a specific position on the slide. After completing the spotting of the triplicates, the pins would proceed with the Wash and Dry Pins option of the software. After getting rid of all the excess material of the cDNA clone already used, the pins would continue with the printing of the next clone. Sequential events of spotting and cleaning would complete the printing process.

2.23.2 Design of the slides

Each slide was designed to have 2 arrays, one printed on the top and the other on the bottom. Each array was comprised of (4x4) 16 subarrays, each consisting of (16x21) 336 cDNA spots. In total, each array had 5376 cDNA spots. This number of spots included the blanks, the controls and the cDNA clones printed in triplicate (see Figure 2.4).

2.23.3 Stabilization of the cDNA clones on the CMT – GAPSTM coated glass slides

Immobilization of the cDNA onto the CMT-GAPSTM coated glass slides was achieved by means of baking and UV crosslinking. The printed slides were initially immobilized by placing them in a UV StratalinkerTM 2400 bench top transilluminator (Stratagene Ltd) for five minutes, in order to initially immobilise the cDNA. The slides were then placed in a lightproof container and baked in an oven at 80°C for 2 hours. The slides were then stored in a lightproof desiccator at room temperature, until further use.

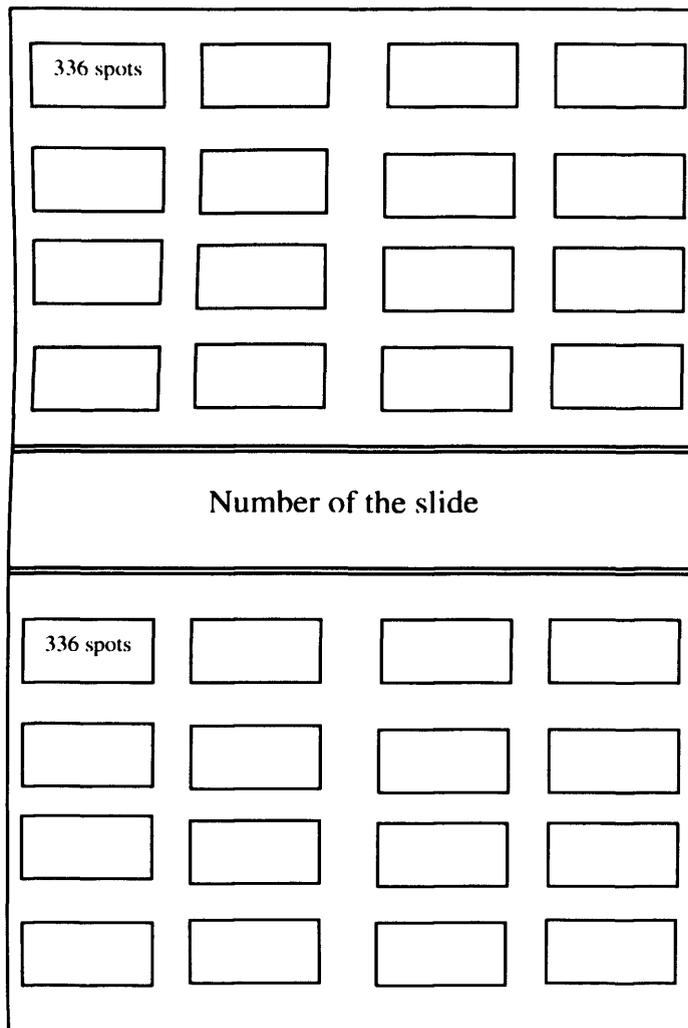


Figure 2.4: Array Design.

Each slide contained 2 arrays of 5376 genes. During the printing process, the pins spotted the cDNA clones on the top array, firstly, with a 4.5mm distance from the top and 3.5mm distance from the sides. On the completion of the printing protocol, the pins returned to “start” position. Then, the user turned the slides 180 degrees. Now the bottom of the slide, which was still not spotted, became the top. The printing protocol was run once again with the exact same parameters. By the end of the whole process, the slide had 2 complete arrays separated by two lines and the number of the slide, which were engraved in the middle

2.23.4 Quality control of the printing

The printing was done in batches of 30 at a time. One slide from each batch was taken randomly and was tested using SYBR Green, which was diluted in TE buffer. The slide was incubated in the above solution for 2-3 minutes while shaking. It was then rinsed in TE buffer, followed by distilled water. The slide was then scanned using a ScanArrayTM Express HT Microarray Scanner (Perkin Elmer, MA, USA) at 600nm.

2.24 Hybridization and washing of the microarray

2.24.1 Preparation of the probe

The calculated amount of Cy3 labelled cDNA probe was pipetted into a new sterile tube and dried down to 17 μ l in a speed vac at 60°C in the dark. If volume of the dried sample was less than 17 μ l, HPLC water was used to reach the desired volume. Then, 1 μ l of polyA (100 μ M, blocking DNA), 1 μ l of pSport Cy5 labelled probe (sigma) and 1 μ l of pGEMT Cy5 labelled probe (sigma) were added. The company manufactured probes were used as an additional control measure. They were carefully designed to bind on every single cDNA clone by identifying a ~60base fragment immediately before the cloning site of those vectors (see table 2.2).

Probe Name	Oligo Sequence 5' to 3' end (Cy5 added on the 5' end)	No. of bases
pSport1FCY5	GACGGCCAGTGAATTGAATTTAGGTGACAC TATAGAAGAGCTATGACGTCGCATGCACGC	60
pGEMTFCY5	GCCAGTGAATTGTAATACGACTCACTATAGGG CGAATTGGGCCCGACGTCGCATGCTCCCGGCC	64

Table 2.7: Details of the company synthesised Cy5 labelled oligos.

2.24.2 Preparation of substrate

The slides were incubated in freshly prepared blocking buffer, for 45 minutes at 42°C. The slides were washed by dipping them in four changes of sterile water, each time for 1 minute and then quickly dipping them in 100% Isopropanol. They were then dried quickly using compressed air and stored in the dark for up to an hour until hybridization.

2.24.3 Hybridization of the microarray

The combined labelled probes were preheated at 95°C for 3 minutes and mixed with 20µl of pre-warm (at 37°C until the SDS is dissolved) hybridization buffer. The sample (40µl in total) was then pipetted along the top half of the blocked microarray slide which was then covered with a 22x32mm cover slip. Extra care was taken to prevent the excess liquid travelling beyond the engraved middle lines. The same was done for the lower part of the slides, as well. The sandwich – slide was now carefully transferred and placed in a humidity chamber at 42°C for 46 – 48 hours.

2.24.4 Washing the microarray

Before the washing process, the slides needed to be separated in wash buffer 1 at room temperature. Once separated, the microarray slide was incubated in a fresh, pre-warm aliquot of wash buffer 1 for 10 minutes at 55°C. The slide was then transferred in 3 sequential Coplin jars (VWR; Leicestershire, UK) for 10 minutes in each at 55°C containing wash buffer 2, followed by 2 incubations, each 1 minute long, in wash buffer 3 at room temperature. The slide was dried using compressed air.

2.25 Signal detection

The dried hybridised microarray slides were stored in the dark until they were scanned, otherwise the fluorescence would diminish in ambient light. When scanning, the Cy5 channel was scanned first, followed by the Cy3 channel, because it is more light-sensitive. Scanning was done with a ScanArrayTM Express HT Microarray Scanner

(Perkin Elmer, MA, USA) at the appropriate wavelength for each channel, i.e. at 550nm for Cy3 and at 650nm for Cy5. The images for each channel were saved as *.tiff files.

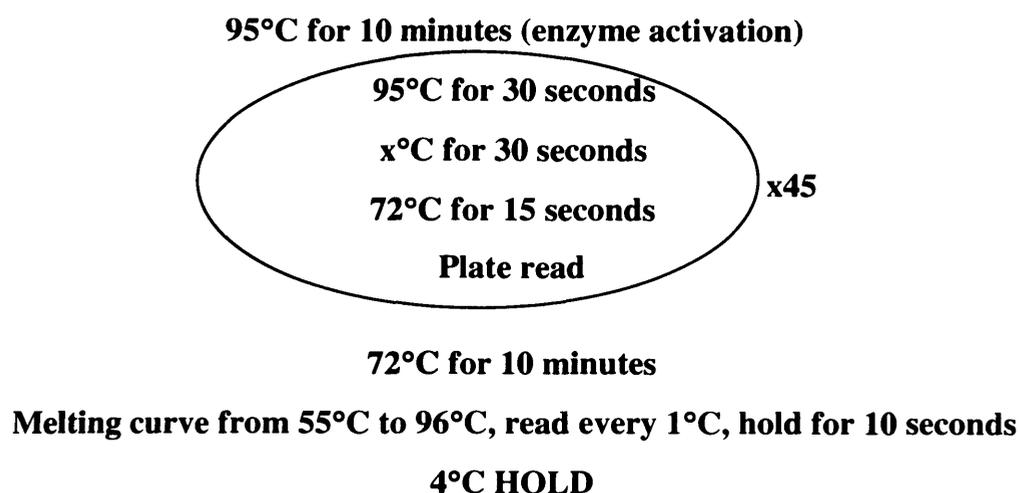
2.26 ImaGene microarray analysis

The scanned .tiff images were analysed using Imagene™ (Biodiscovery, USA) microarray analysis software. It allowed the user to identify spots and quantify the amount of fluorescence of each spot. This initial analysis generated two .txt files and one .sst file, which were used for further study.

2.27 Real Time PCR

2.27.1 SYBR Green

QPCR validation of some genes was performed using the SYBR Green chemistry. The kit of preference was the SensiMix *Plus* SYBR (Quantance, London UK). The kit provided the master mix and extra magnesium (MgCl₂) if it was considered necessary for the reaction. The master mix was a 2x Mix containing reaction buffer, heat-activated Taq DNA polymerase, dNTPs, MgCl₂ (6mM), internal reference dye, stabilisers and SYBR® Green I. Manufacturers guidelines were followed in the preparation of the reaction mix with 2x master mix, 10ng of cDNA and the primers of choice in a final concentration of 200nM in a final reaction volume of 25µl. The machine used was the Opticon II GRI Cyclor and the following protocol was employed for every reaction:



In the above protocol “x” indicates the desirable primer annealing temperature. All QPCR reactions included experimental samples (cDNA), blanks (only water) and standards (plasmid DNA). The latter was used in known concentrations ranging from 1ng to 1fg in order to create a standard curve.

2.27.2 Quantitative Analysis

All QPCR reactions included experimental samples (cDNA), blanks (only water) and standards (plasmid DNA). Calibration standards were generated using plasmid DNA from cloned and sequence verified amplicons of each target diluted to a concentration range from 1 ng/μl to 1 fg/μl. A standard curve was obtained and a regression line generated by plotting the cycle number required to attain a threshold (Ct value) fluorescence pertaining to the logarithmic portion of the amplification against log₁₀ [molecules of target gene]. Sample amplifications giving rise to a Ct value outside the standard range were not employed in subsequent calculations.

2.27.3 Validation of QPCR amplifications

Product formation was monitored at the end of each extension step by measuring the fluorescence emitted from SYBR green molecules intercalated with double stranded DNA. The chain melting analysis allowed the specificity of end products to be

assessed. Data was only assessed as valid if the melting curve analysis of the amplicon represented a single peak with the sample dissociation temperature as that observed with products generated using the calibration standards.

CHAPTER 3

GLOBAL MICROARRAY

3.1. Overview

The rodent's facial vibrissae are represented by the barrel field, found within the brain's cerebral cortex (Section 1.6, Figure 1.5). The whiskers transmit essential information to the barrel field, where the majority of neurons in a single barrel respond to stimuli from a single whisker, usually termed the principal whisker (Welker 1976).

Over the years, a number of whisker deprivation experiments have been performed with the objective of studying the induction of neuronal plasticity in the mouse barrel cortex. Two main effects have been observed when all the whiskers, with the exception of one (D1 spared; Section 1.8), are deprived for a given period of time. Neurons corresponding to the deprived barrels, immediately surrounding the spared barrel, now respond to stimulation of the spared whisker; a phenomenon known as potentiation. Furthermore, neurons corresponding to the deprived whiskers respond weakly to stimulation of their re-grown principal whisker; an action called depression. Potentiation and depression occur mainly in layer 2/3 neurons of four to eight week old rodents (Fox 1992).

Protein synthesis and mRNA transcription are related to memory and synaptic transmission underpinned by long-term potentiation (Section 1.9.4; Frey *et al.* 1988; Nguyen *et al.* 1994). The connection between synaptic activity and long-term potentiation lies in the fact that gene transcription is affected by synaptic activity via the phosphorylation of CREB (Sheng *et al.* 1991; Yin *et al.* 1995), which can be activated by a number of pathways involving signalling molecules and transmission of calcium (Montminy *et al.* 1990; Bito *et al.* 1997; Figure 1.15). Bourtchuladze 1994, has investigated the relation between CREB, synaptic plasticity and learning, showing that long-term potentiation does not last for more than 90 minutes in hippocampal brain slices acquired from an animal lacking key CREB isoforms (Bourtchuladze *et al.* 1994). In order to investigate the implications of CREB in plasticity in the barrel

cortex whisker deprivation experiments on transgenic mice carrying a *Cre-LacZ* reporter gene have been carried out (Barth *et al.* 2000). A significant upregulation of CRE-mediated gene transcription was observed during whisker deprivation patterns that induce plasticity; all whiskers being deprived but one. A considerable increase of the *Cre-LacZ* reporter gene was seen within layer IV of the barrel corresponding to the spared whisker (Barth *et al.* 2000). In contrast, mice with all their whiskers present or all their whiskers deprived do not show considerable changes in CRE-mediated gene transcription and have no potentiation (Glazewski *et al.* 1998).

CRE-mediated gene transcription has been studied in order to understand long term facilitation in *Aplysia* (Mohamed *et al.* 2005; Kim *et al.* 2006), ocular dominance plasticity in cats (Desai *et al.* 2002) and long term potentiation in hippocampus (Lynch 2004).

3.2. Introduction

In this chapter results from a microarray study exploiting a comprehensive set of mouse cDNAs, performed prior to this project, will be outlined and re-analysed in order to introduce and inform subsequent investigations presented in this thesis. This experiment, denoted the “*Global Microarray*” study, was performed by Dr Richard Abraham (Abraham 2005; please refer to his thesis for a full list of his data and genes) and aimed to further understand the genetics behind neuronal plasticity and experience-dependent gene expression in the mouse brain. This work focussed on the barrel cortex and included a comprehensive mouse cDNA microarray (almost 15,000 clones) supplied from National Institute of Health (NIA Mouse cDNA Project; <http://lgsun.grc.nia.nih.gov/cDNA/>). The initial study was based on knowledge acquired by other research groups on the importance of visual cortex organisation and its significance to responses of the plastic brain to external stimuli. The methods employed by Dr. Abraham to extract tissue and RNA from the mouse brain are analogous to those outlined in Chapter 2, and utilise the same treatments incorporating animals with all whiskers present (ALL), all whiskers deprived (DEP) and chessboard deprivation (CB) over a time course between day 1 and 12. Findings may have important implications in the design of future experiments. Reanalysis of the data generated may be used to inform subsequent targeted studies.

3.3. Results

Dr Abraham measured the relative expression levels for 15,000 reporters over a time course (1, 2, 4, 8 and 12 days) of whisker treatment (see above). However, due to the number of animals required, it was only feasible to have three biological replicates representing each time point and treatment group, thereby restricting the power of statistical data analysis. These analyses were additionally hindered by the level of functional gene annotation available at that time. Continual improvement and development of associated bioinformatics software has enabled this Global Microarray data to be reanalysed. During the development of further plasticity experiments, analysis of the Global Microarray set was deemed necessary in order to ascertain all relevant information about gene expression profiles, functional groups and most importantly the design of the experiment itself.

The experiment design used for this Global Microarray made the assumption that deprivation patterns altered by brain plasticity would display no significant difference between genders (Section 1.8). For the above reason, individuals were picked for the study randomly from available litters bred in-house, with no specific selection or record of the sex of the experimental subjects being made. The microarray data itself was introduced to Genespring in which further data analysis was performed. Initially the samples were divided into two groups; the control (ALL) and experimental animals (CB and DEP). The effect of anaesthetic induction was studied in the control animals. This is an important issue as isoflurane has been described as being influential on plastic responses (Kaech *et al.* 1999; Nikizad *et al.* 2007). In order to study the anaesthetic effect, the control data (from undeprived animals only) was normalised using Global Lowess and expressed as the mean of the cohorts of animals sacrificed at each of the different time points used (1, 2, 4, 8 and 12). Expression level of each gene was then expressed relative to the median expression of that gene within the day 1 cohort.

3.3.1. Isoflurane

The number of transcripts influenced by the introduction of isoflurane to the control animals for each time point is shown in Figure 3.1. It is evident that the lowest number of genes affected by the anaesthetic are after the second and the fourth day of

the experiment; indicating that maybe short term exposure to the volatile gaseous anaesthetic does not cause major changes in the rodent's brain. However, the number of transcripts is considerably increased after the first week of exposure (Day 8) onwards; leading to the assumption that continuous exposure might have a long term effect on neurological function including plasticity-dependent gene expression.

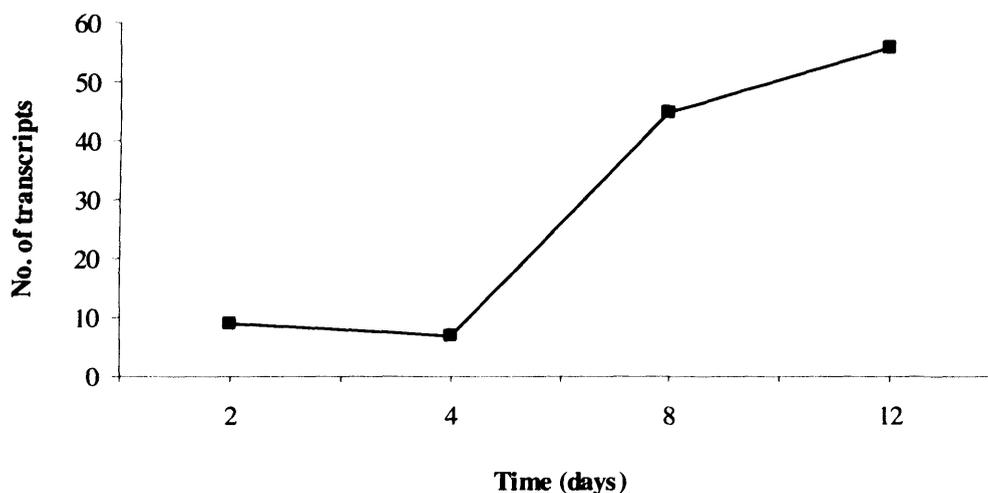


Figure 3.1: Graphical representation of the number of genes affected by the introduction of isoflurane to the control animals in the Global Microarray experiment. The numbers of transcripts were identified after applying Global Lowess normalisation followed and expressing values relative expression at Day 1. Affected genes were defined as those displaying a 2 fold change in expression at a significance of $p < 0.05$. This selection was performed using a Volcano plot within Genespring.

Although the trend in the number of genes influenced by isoflurane is clear, the total number of genes even at the final time point, day 12, is relatively small at 56. The reason underlying the small number of genes comply with the parameters of the filter, 2 fold change and p value < 0.05 , when compared to day 1 expression levels is most probably due to the low number (three) of biological replicates used at each time point of the control un-deprived animals. However, this may have been confounded by additional sources of variance, i.e. sex and health status of the organism.

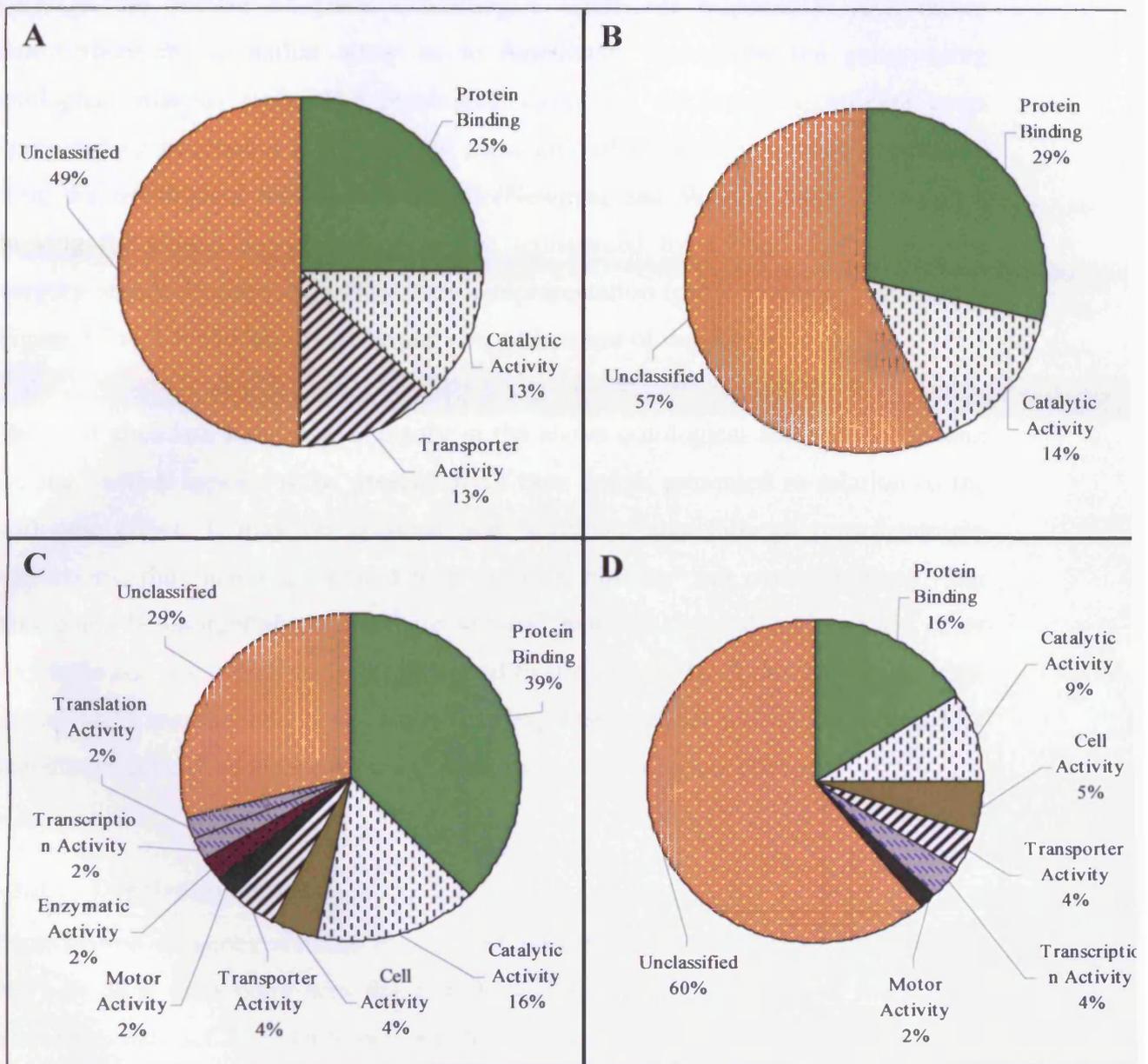


Figure 3.2 Pie charts representing ontological analysis of the genes affected by isoflurane. Ontological bias analysis was performed on gene exhibiting significant changes (2 fold and $p < 0.05$) within the control (undeprived – ALL) animals at day 2 (Panel A; $n=8$), day 4 (Panel B; $n=7$), day 8 (Panel C; $n=45$) and day 12 (Panel D; $n=50$). The proportion of genes which are not represented within a significantly over-represented ontological category is denoted “Unclassified”. Where “n” represents the number of genes. For full list of genes refer to Appendix 3.

Although the number of genes exhibiting a significant response is low, recent improvement in annotation allow us to functionally categorise the genes using ontological analysis tools. The ontological categories displaying significant over-representation in relation to the original population of 15,000 genes were determined using the ontological bias software L2L (Newman and Weiner 2005). Pie charts showing the proportion of each gene list represented by a particular ontological category which displays a significant over-representation ($p < 0.05$) were constructed (Figure 3.2) for better visualisation and comprehension of the data.

The most abundant functional category in the above ontological analysis is “protein binding” which appears to be present in all time points presented in relation to the isoflurane effect. It may be assumed that isoflurane acts through protein-protein interactions, thus genes associated with “protein binding” are over-expressed. The same could be thought about “catalytic activity” which may involve kinase and other enzymatic activity within the cell to respond to stimuli. At day 8 onwards, transcripts related to “transcription activity” are over-expressed which allows us to consider anaesthetic-dependent gene expression and prolonged isoflurane effects.

3.3.2. Deprivation effects

Identification of genes affected by the two different types of deprivation (CB and DEP) in each time point was the next logical step in the analysis of the Global Microarray data set. In order to perform this analysis, the experiment was divided into the 5 different days and each day was separated into the three conditions; one was the undeprived control animals (ALL) and the other two were the whisker deprivation treatments (CB and DEP). The data was normalised using the Global Lowess method (same as before) followed by relative expression to the time-matched control animals for every day. This way, the isoflurane effect is believed to be taken under account during analysis of the results.

The two experimental conditions were compared to the control animals of each time point and the genes which successfully displayed a 2 fold change at a significance of $p < 0.05$ have been encountered as the ones affected by deprivation. This process was done for all the time points and the graph presented in Figure 3.3 illustrates the

results. The most distinct feature of this graph is the amount of genes affected on Day 1 and Day 8 of the experiment, leading to the hypothesis of two plasticity mechanisms; an early response and a late response.

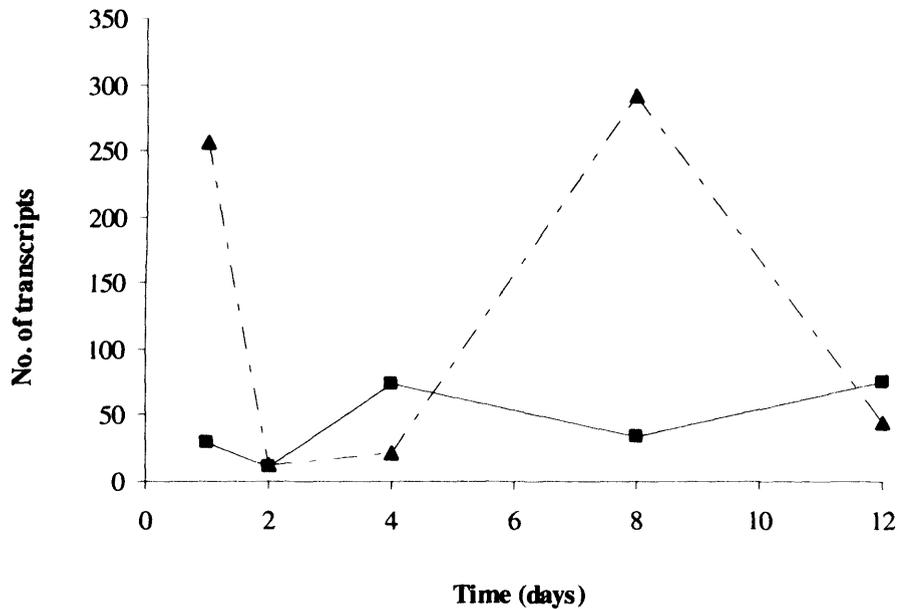


Figure 3.3: Graphical representation of the effect of deprivation in chessboardly deprived animals (CB) and totally deprived animals (DEP) throughout time in comparison with the time-matched control (undeprived) animals for every time point. The continuous black line represents the CB and the dashed black line represents the DEP. Major changes are noticeable after the first and the eighth day of experiment. The numbers were obtained after applying Global Lowess normalisation with a 2 fold expression change at a significance of $p < 0.05$.

To obtain a better insight on the functional importance of those genes, ontology tools had to be used once again. Gene ontology was performed online and the results are illustrated here as pie charts for better visualisation of the data. The first analysis tool to be used was the online version of the software “L2L” Microarray Analysis Tool (Newman and Weiner 2005). Due to the stringency of the settings, the gene lists were not being separated into any functional categories. Different software was employed known as “Onto Express” (Draghici *et al.* 2003) to analyse the above gene lists obtained from Genespring. The results were presented by the software in the form of an online table which was then used to produce the pie charts presented below, for a better understanding.

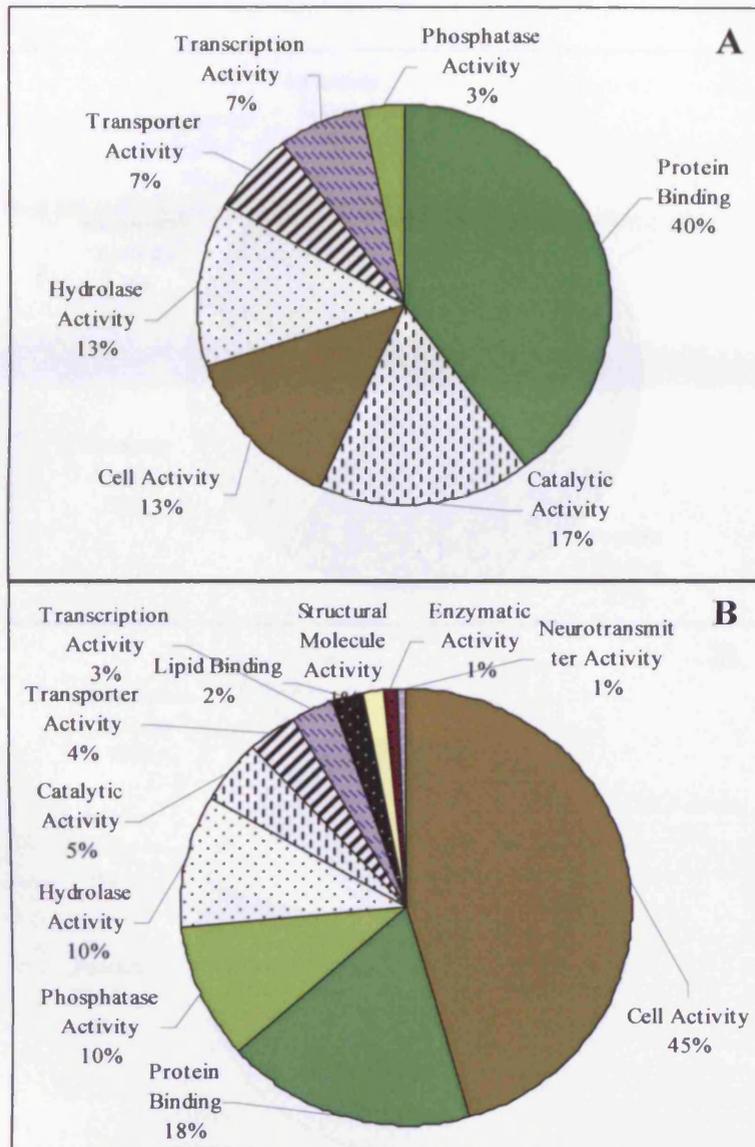


Figure 3.4: Pie charts representing ontological analysis of the genes affected due to deprivation on Day 1 of the experiment; chessboard (CB; n=25) is Panel A and total deprivation (DEP; n=255) is Panel B. Ontological bias analysis was performed on genes exhibiting significant changes (2 fold and $p < 0.05$). For full list of genes refer to Appendix 3

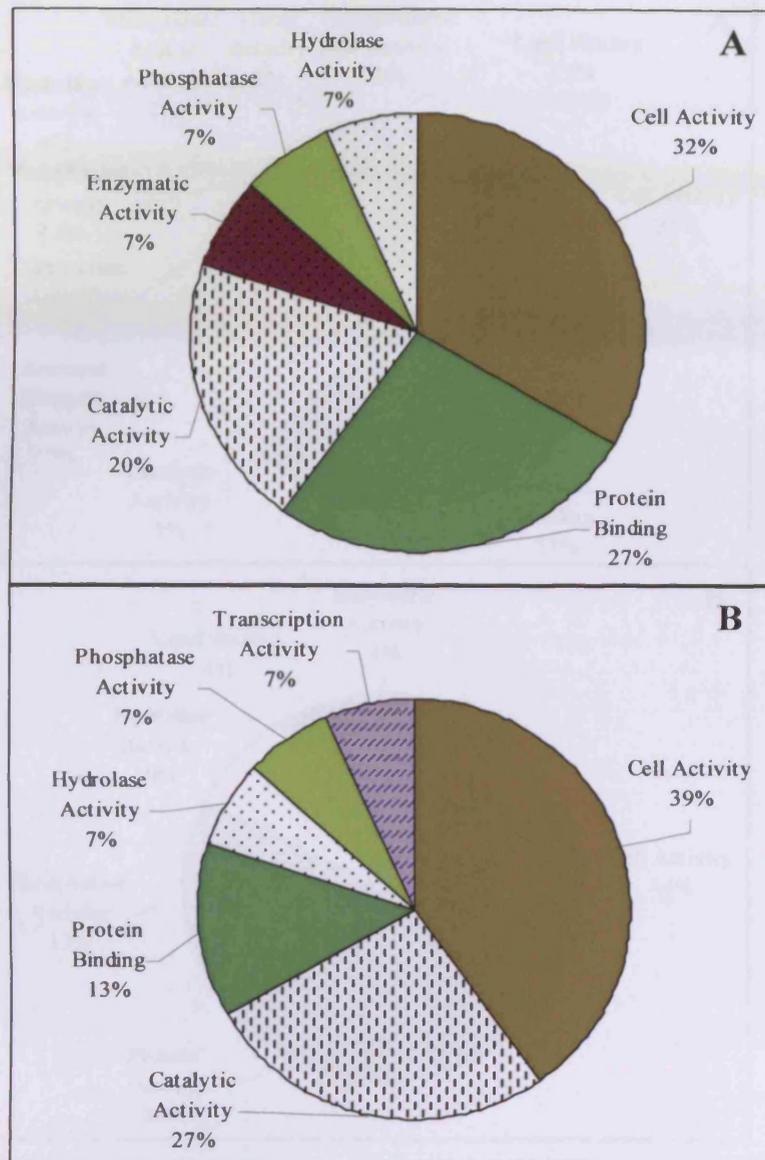


Figure 3.5: Pie charts representing ontological analysis of the genes affected due to deprivation on Day 2 of the experiment. Chessboard (CB; n=10) is represented in Panel A and total deprivation (DEP; n=12) is represented in Panel B. Ontological bias analysis was performed on genes exhibiting significant changes (2 fold and $p < 0.05$). For full list of genes refer to Appendix 3.

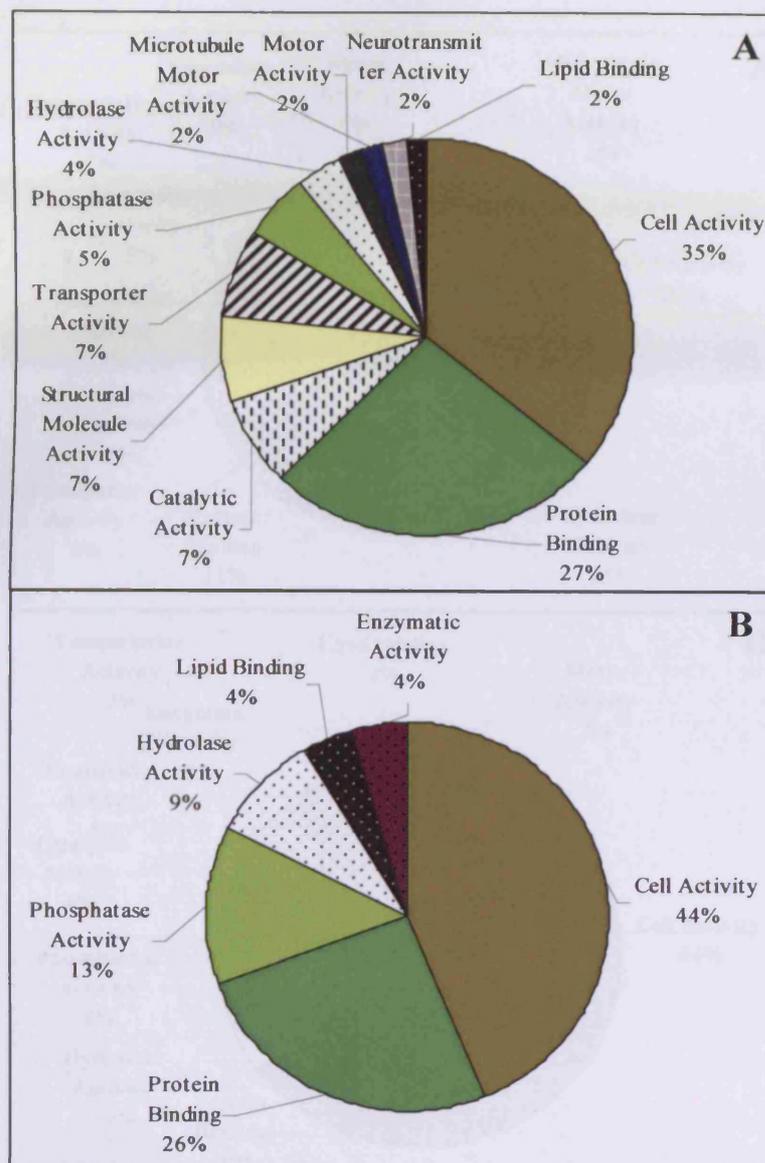


Figure 3.6: Pie charts representing ontological analysis of the genes affected due to deprivation on Day 4 of the experiment. Chessboard (CB; n=74) is represented by Panel A and total deprivation (DEP; n=20) is represented in Panel B. Ontological bias analysis was performed on genes exhibiting significant changes (2 fold and $p < 0.05$). For full list of genes refer to Appendix 3.

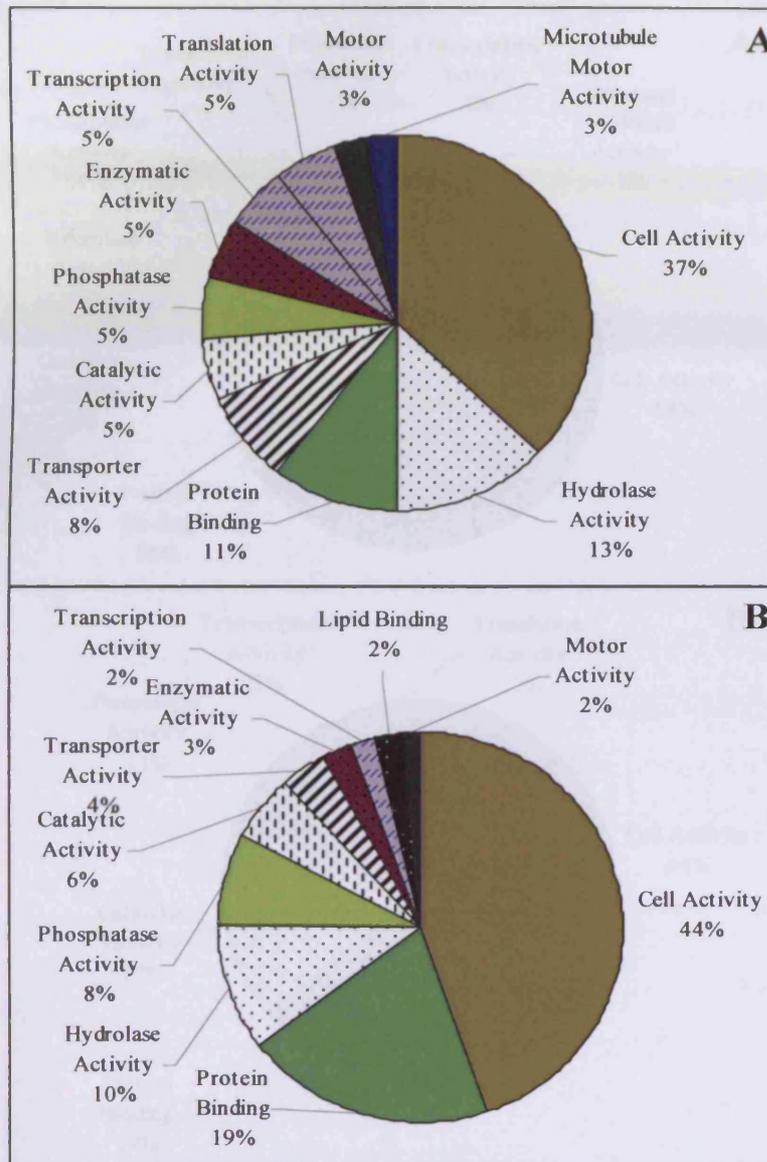


Figure 3.7: Pie charts representing ontological analysis of the genes affected due to deprivation at Day 8 of the experiment. Chessboard (CB; n=34) is represented in Panel A and total deprivation (DEP; n=291) is represented in Panel B. Ontological bias analysis was performed on genes exhibiting significant changes (2 fold and $p < 0.05$). For full list of genes refer to Appendix 3.

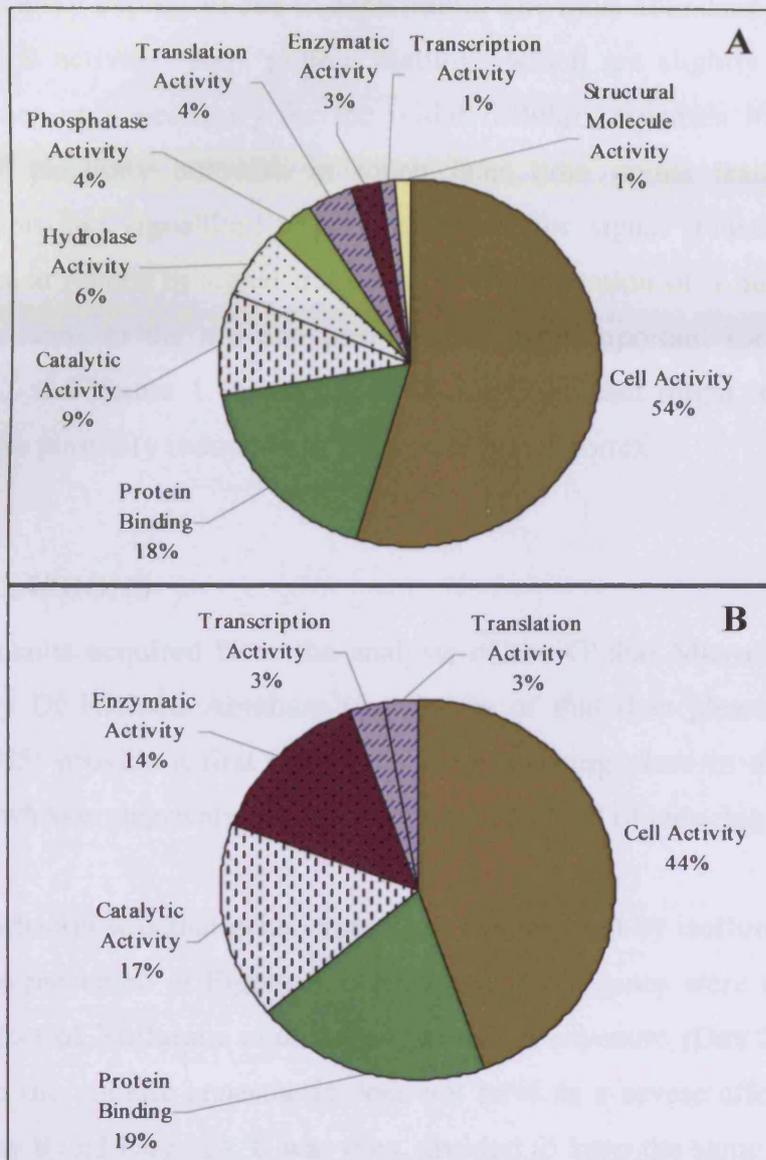


Figure 3.8: Pie charts representing ontological analysis of the genes affected due to deprivation at Day 12 of the experiment. Chessboard (CB; n=74) is represented in Panel A and total deprivation (DEP; n=43) is represented in Panel B. Ontological bias analysis was performed on genes exhibiting significant changes (2 fold and $p < 0.05$). For full list of genes refer to Appendix 3.

The pie charts illustrated in Figures 3.4 – 3.8 summarise the functional analysis of genes significantly expressed due to deprivation. The most abundant groups from this study are “cell activity” and “protein binding” which are slightly expected. “Cell activity” is not only necessary for the initial cellular responses immediately after induction of plasticity but also in much later time points leading to cell-cell communication and signalling; important factors for signal transduction. “Protein binding” is also related to signal transduction via activation of a number of kinases and other proteins in the pre and post-synaptic cell important for creb activation (Section 1.12 and Figure 1.15) and gene transcription that might underline genetic changes due to plasticity induction in the mouse barrel cortex.

3.4. CONCLUSIONS

The above results acquired from the analysis of the Global Microarray experiment performed by Dr Richard Abraham (for details of that data please refer to thesis Abraham 2005) provide a first insight to what is taking place in the mouse barrel cortex when whisker deprivation is employed as a method of inducing plasticity.

The first conclusion was that gene expression was affected by isoflurane as shown in the pie charts presented in Figure 3.4. Although many genes were unclassified, the long term effect of isoflurane is distinct. Short term exposure (Day 2 and Day 4) of the animal to the volatile anaesthetic does not have as a severe effect as long term exposure (Day 8 and Day 12). It was thus, decided to keep the same method for any future studies for consistency.

A different aspect of the experiment was studied in Section 3.3.2, where the effect of deprivation was investigated making it clear that induction of plasticity via whisker removal has an early and a later effect, in particular after Day 1 and Day 8 (Figure 3.3). The pie charts presented in Figures 3.4 – 3.8 indicate that both early and later responses involve key functional categories such as “cell activity” and “protein binding” being responsible for cell-cell signalling involved in protein activation and signal transduction, the latter being important in plastic responses and memory

mechanisms such as LTP and LTD. Day 12 shows gene categorisation in less functional groups; a result probably because of low biological replication producing non-significant results or an indication that the already acquired neuronal changes (from earlier time points) have sufficiently influenced gene expression, forcing the experimental subjects back to a relatively normal state. To further investigate transcriptional changes in later time points, plasticity will be induced for longer with greater biological and technical replication. It was also considered important, for further plasticity studies, to use whisker deprivation in a time course of choice starting always from Day 1 in order to investigate the early effects. Day 8 was also considered important due to the numerous functional categories present and the fact that some of them are indicative of plastic responses in the barrel cortex, such as “transporter activity”, “motor activity” and “protein binding”.

Reanalysis of this data allowed us to conclude that it was necessary to use greater biological replication in future plasticity studies in order to achieve improved statistical confidence in the data and allow for more robust findings. It was also decided that the global microarray experiment would assist the cautious selection of a refined set of genes potentially associated with plasticity in order to create a targeted microarray. Changes in the expression of the selected targets should exploit the two types of deprivation patterns (CB and DEP) used previously to focus on the critical periods identified by the global microarray study (days 1, 8 and 16). The temporal anaesthetic effect must also be further investigated using undeprived control animals to establish the best method of compensating for this major confounder.



CHAPTER 4

DESIGN AND VALIDATION OF A TARGETED PLASTICITY ARRAY

4.1. Aim

In order to further explore the molecular pathways underlying the neurological processes of potentiation and depression within the mouse barrel cortices distinct patterns of facial vibrissae deprivation were employed; involving control animals (ALL), which have been subjected to anaesthesia but where no whisker deprivation has been performed, an experimental cohort that have had their whiskers totally deprived (DEP) or where whiskers have been removed in a chessboard manner (CB). To dissect the temporal gene expression profiles these conditions were maintained over 1, 8 and 16 days at which time-points the animals were sacrificed and the barrel field removed for molecular analysis (Section 2.14.2). These time points were selected to explore short and long term transcriptional effects (at days 1 and 16 respectively) together with investigating the critical point within the process when plasticity dependent genes expression should be observed (day 8). The selection was informed by a previous study using a Global Microarray (Chapter 3). The major disadvantage of this previous work was due to the statistical limitation caused by the low biological replication. The current experimental design would address these issues by exploiting a targeted microarray design that contained both technical replication and allowed increased biological replication. As with previous studies the proposed experiments utilised mixed sex animals based on the assumption that there was no previous overt evidence from neurological studies that the two genders exhibited differential plastic responses. Critical to the successful conversion of this investigation was the absolute design (i.e. reporter selection and array fabrication) and validation of the arrays used. This chapter documents the steps undertaken to generate a series of array data that could be further examined to determine the transcriptional changes involved in plasticity.

4.2. Microarray Design

Prior to selecting the reporters that would comprise the targeted plasticity array three things need to be considered: the physical constraint of the array, technical replication and exogenous elements that needed to be introduced to allow for quality control and validation. To allow for increased biological replication within the experiment we engineered a print protocol that enabled the fabrication of independent array at the top and bottom of a single slide with the identifier engraved in the middle to create “dead” space and avoid liquid travelling from one side to the other. Using the printing Spotarray 72 platform fitted with 100 μ M split pins allowed us to print a metagrid of 4 rows by 4 columns with a maximal spot dimension of 16x21 (rows versus columns), providing for a total of 5376 elements (Figure 4.1). A minimal technical replication of three spots for each element reduced our possible reporter number to 1792. It was decided to incorporate the 23 Lucidia Score Card reporters (GE LifeSciences) that represented a set of reporters complementary to externally validated spikes that would be added to each target prior to labelling providing a calibration series to evaluate a linear response of the array together with appropriate negative controls. Taking into account these factors the final design could incorporate a maximum of 1769 genes.

The genes selected for the present study are highly related to mechanisms associated with learning and memory, such as potentiation, depression and other plastic responses of the rodent’s brain. The selected genes from the previous study have been found to be associated with functional categories related to cell-cell signalling and communication, an important component for signal transduction. An average number of about 70 genes with a 2 fold change in their expression profiles were selected from each time point (1, 2, 4, 8 and 12 days) of the Global Microarray experiment. Moreover, the targeted microarray set included house keeping genes, considered the non-changing standards of the experiment. The desired genes were carefully selected after an initial indication of their expression profiles in the Global Microarray (Chapter 3) study followed by an extensive literature research in order to investigate their documented relationship to neuronal plasticity employing Pubmed (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed&itool=toolbar>).

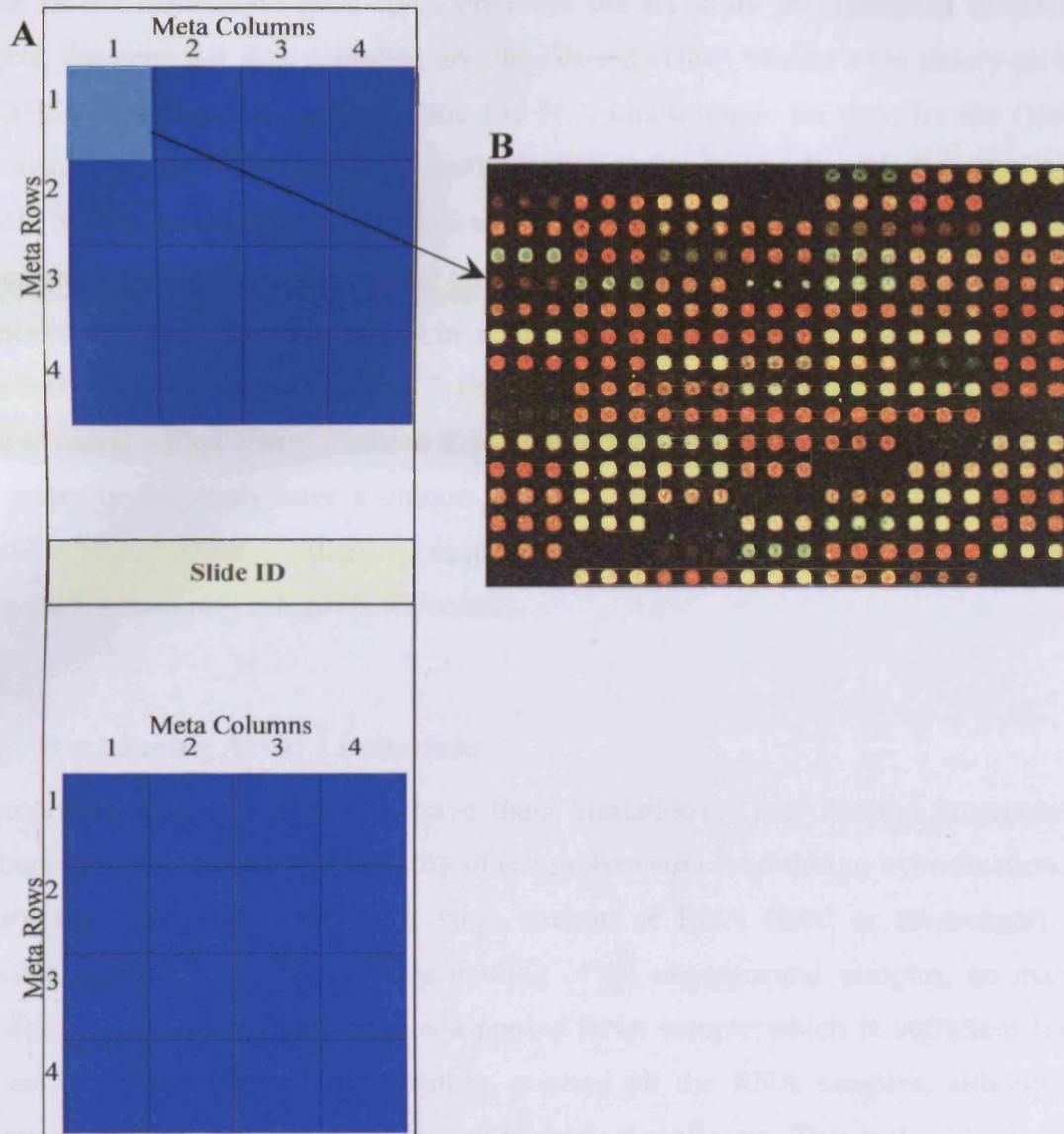


Figure 4.1: A) Graphic representation of the microarray glass slides used in the targeted plasticity experiment. The genes were printed on top and bottom sides of the slide and the ID was engraved in the middle to create cavities preventing the liquid from travelling from one side to the other, resulting to contamination of the experiment. B) Magnification of an actual meta-grid of a portion of the microarray glass slide after being hybridised with Cy3 and Cy5 labelled probes. Different gene expressions are evident already due to the colour differences between the printed dots.

Once all the limitations were considered and the literature has indicated preferable targets, the gene list was complete and the desired cDNA clones were cherry-picked from 384-well plates – containing the 15k NIA mouse clone set used for the Global Microarray study – to create 96-well plates, which later comprised the targeted plasticity microarray set. In addition to the cDNA NIA set of targets, a few more genes were cloned (the primers for these genes can be found in Table 2.6) as their function indicated an implication in neuronal plasticity and learning mechanisms (Section 1.9.4). Cloning (Section 2.18) was performed using cDNA derived from mouse barrel cortex tissue (Section 2.14.2) into the pGEM-T vector (Table 2.4). All the genes in this study have a unique ID related to a unique entry, which could be used to obtain their sequences from ENTREZ-NUCLEOTIDE (<http://www.ncbi.nlm.nih.gov/sites/entrez>).

4.3. Overcoming Array Limitations

Microarrays, although powerful, have their limitations. Their success is primarily dependent on the quality and quantity of the probe being used during hybridization. A microarray experiment requires a large amount of RNA (total or messenger). A possible solution to this demand is pooling of all experimental samples, no matter condition or treatment. This creates a pooled RNA sample which is sufficient for a successful hybridization. Unfortunately, pooling all the RNA samples, although it creates enough material, leads to loss of biological replicates. This lack of biological replication causes a reduction in the power of the statistical analysis that can be used for the resulted data. For the above reason no pooled RNA samples were used in this study. On the contrary, every mouse barrel cortex gave an approximate 0.4µg of RNA (Section 2.15.2), which was used for cDNA synthesis (Section 2.22.1). A variety of enzymes is commercially available for cDNA synthesis but during this study, Superscript II (Invitrogen Ltd., Witham, Essex, UK) was found to be the most reliable and efficient. The stability of the enzyme (3 hours at 42°C) increased the efficiency of the reverse transcription reaction considerably, producing up to 3300ng of cDNA in average. This amount of material was sufficient for the production of a fluorescent probe for a microarray hybridization and template for real time PCR for results validation.

The two-colour microarray approach is well established where two different samples (usually corresponding to two different experimental conditions) are labelled with two different fluorescent dyes hybridising the same microarray slide, followed by a dye swap. Genes might appear to be differentially expressed during the analysis of a two-colour experiment. This observation might be an experimental result or due to differential Cy dye incorporation. The Cy dyes used are the Cy5 and Cy3 (artificially denoted red and green respectively). These two fluorescent molecules are not the same molecular size leading the Reverse Transcriptase enzyme to exhibit differential incorporation. This is primarily addressed by exploiting an indirect method for label incorporation in which the Reverse Transcriptase enzyme incorporates an Amino-allyl dUTP which is subsequently chemically coupled to either Cy3 or Cy5-ester. This reduces any dye bias substantially; however, the two dyes also display minor differences in their intensity dependent response profiles. This problem may be overcome with dye swaps, leading to a doubling of the number of experiments, as each animal needs its RNA hybridised twice. Consequentially there is a need for greater quantity of RNA, something which is not possible with the small amount of tissue used in this study.

In the targeted plasticity experiment described here, all the experimental samples were labelled with Cy3 whereas the Cy5 channel was hybridized with a custom-made primer to avoid technical variations (Table 2.7). This 60bp long oligonucleotide was specially designed to anneal to the vector ends just before the PCR fragment representing the gene of interest in every spot of the array. Hybridizing with a universal primer, which was able to recognise every clone on an array slide, made it easier to identify a successful array and provided a good positive control and a reasonably clean background.

The possibility of getting false results – positives or negatives – in an array experiment is always great but it can be eliminated. To avoid the incorporation of false results, biological replication needs to be considered when designing a microarray experiment. The number of replicates needed depends on the experiment itself. In the present study, a high number of biological replicates is used to confidently determine the fold change in the expression levels of the genes in

question. Sufficient biological replicates were used in order to generalise the effects of the experiment to the population as a whole.

Technical replicates are usually as important as biological replicates for high-throughput experiments. Their importance is based upon the fact that there might be variability within the experiment. In theory, high quality experiments do not need technical replicates. However, their usage can define differences during printing and hybridisation. Printing variability is checked with the presence of three replicates for every spot, whereas differences during the hybridisation step are avoided by hybridizing different slides with the same experimental sample. Technical replicates are not accounted as different experimental samples; on the contrary their results are averaged into a single measurement for every biological sample decreasing variability and making the array data more powerful.

4.4. Image Abnormalities

The raw data of an array experiment is represented by a spotted image (Figure 4.2) generated by scanning the printed and hybridised glass slide. Software such as *Image*TM and *GeneSpringGX 7.3* have the ability of converting the raw image data into numerical algorithms, which represent raw gene expression. The first step of analysing the data is its transformation into numbers representing the measurements of hybridisation intensities of each channel (Cy3 and Cy5).

Sometimes abnormalities could occur during the printing process of a glass array. The first problem could be a curve within the grid and uneven spot spacing (Figure 4.2), due to a non-horizontally positioned glass slide or due to misalignment of the printing pins. Another problem is uneven spot sizes (Figure 4.2). This is simply the result of disposition of unequal amounts of liquid on the glass slide during printing. Such abnormalities were minimal in the present study due to the close observation and care taken during the generation of the glass slides.

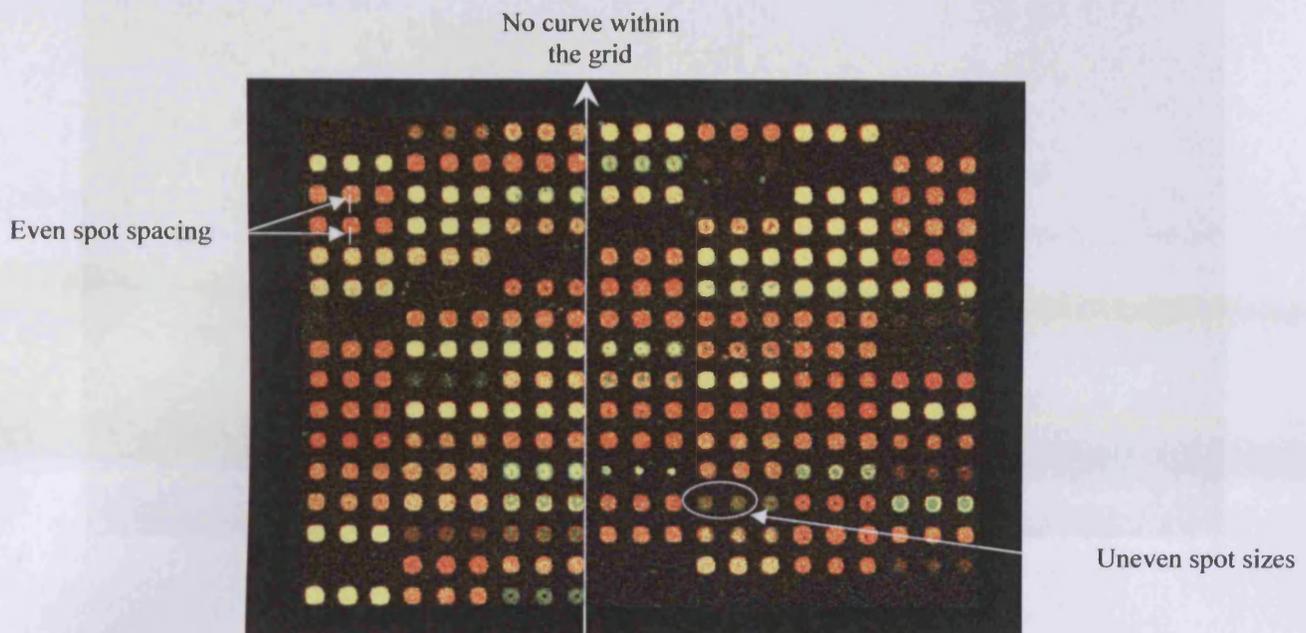


Figure 4.2: Representation of potential problems of spotted arrays; a curve could be present; spots could be unevenly spaced and the spots could be unevenly sized. The first two problems are the results of a non-horizontal glass slide or not properly aligned pins on the printing head, whilst the third problem could occur because the pins are not displacing equal amounts of liquid on the top of the slide. Fortunately, the two first problems did not occur in this experiment, whereas the third one could not be avoided.

4.5. Scanning and segmentation

Following hybridisation the glass slide was scanned at the appropriate wavelengths for Cy3 and Cy5 detection and a colour is applied to each dye – red for Cy5 and green for Cy3 – for better identification and a facilitated study (Yang *et al.* 2001). The picture is broken down into pixels and a quick analysis of the spot intensity and background is performed. The 16-bit image is then saved as a TIFF file and uploaded onto ImagenTM, which in turn quantifies the amount of fluorescence and identifies the location of each spot (Yang *et al.* 2001).

The software places grids and metagrids on the array image (Figure 4.3). The grids and metagrids are placed on a predicted position which is not precisely on top of every single spot (due to inconsistencies throughout the array) requiring every sub-array to be readjusted (Figure 4.3c).

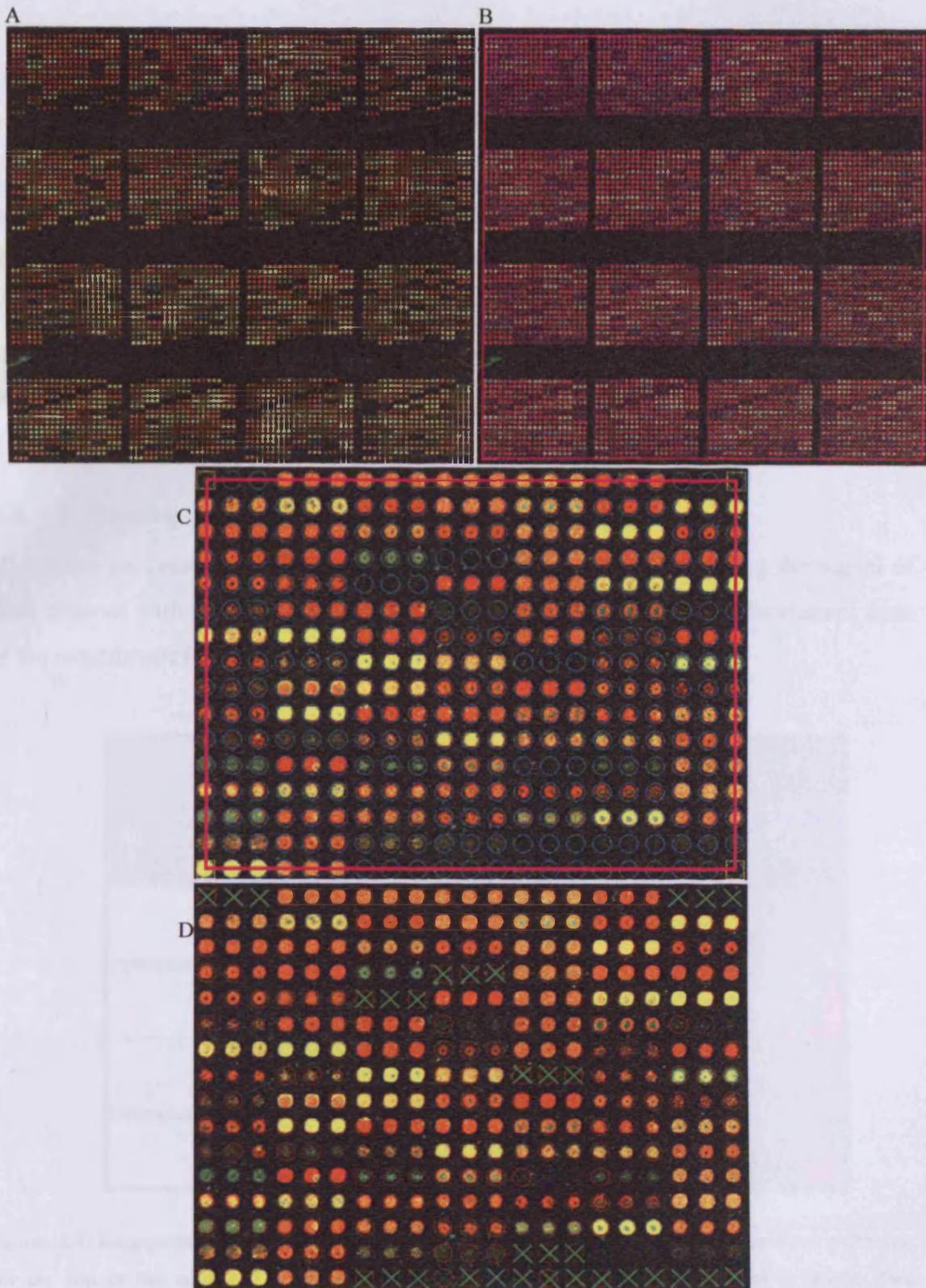


Figure 4.3: Identifying the spots on the glass array image. A) Initial image as seen in Imogene. B) A metagrid has been added on the image, which is broken down into sub-arrays (4x4). C) A close-up of one of the sub-arrays with the circles placed around the spots. The metagrid has been adjusted so that every circle is around each spot. D) After the perfection of the metagrid, empty spots are crossed out by the software (seen as green x).

Once the grid has been placed and checked, the segmentation is used to divide the image into pixels and distinguish the signal from the background or contamination. The fluorescent intensity for each spot is calculated from the total pixel intensity from both channels (Cy3 and Cy5). Artefacts, such as dust, could be easily removed from the analysis by excluding very high and very low pixel intensities. The removal of these pixels could either be done manually or by using an automated tool of the software's drop-down menu. Manual segmentation is more efficient as the automated parameters are not as stringent.

4.6. Extracting the information

Histogram analysis of all the spots is provided by Imagene™ showing the signal of each channel with red and green lines representing the two different fluorescent dyes of the experiment (Figure 4.4).

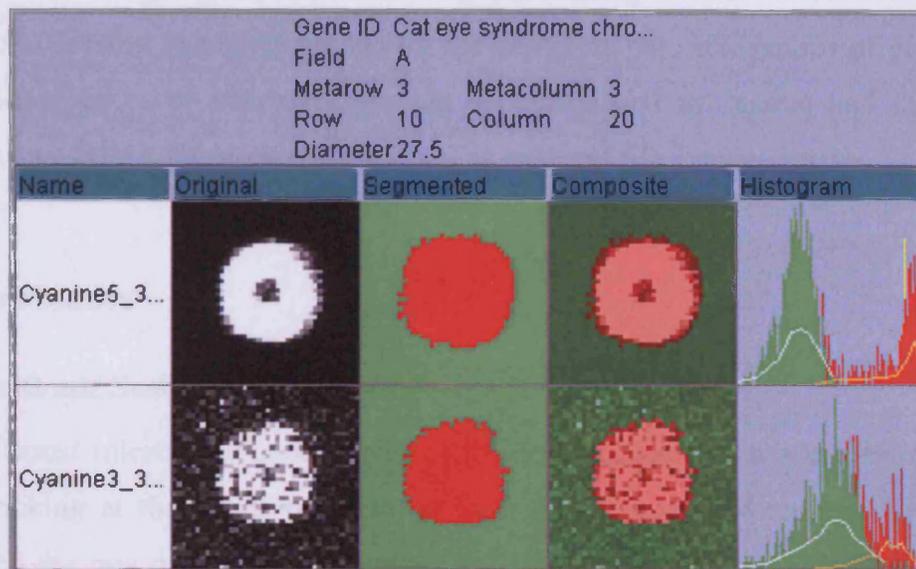


Figure 4.4: Representation of a selected spot as seen on Imagene™ microarray analysis software. On the top of the captured window the name and unique position of the spot is found. Two sections are seen; one is for Cy5 and the other for Cy3. The spot is broken down to pixels and analysed for any abnormalities or contamination. Every spot is represented by a histogram, with red representing signal intensity and green representing background intensity. The very few black bars are the unused pixels. Because of the selected spot's shape irregularity, a better understanding of its hybridisation and signal is obtained by looking at the histogram.

The next step on image analysis is to obtain a table created by Imagene™ with all the important values corresponding to readings of signal intensities and background for both channels for every spot in the microarray. The features are listed below:

- Signal mean and median: the mean and median values of the signal, respectively
- Background mean and median: the mean and the median values of the background around the spot, respectively
- Signal and background standard deviation: the standard deviation of the signal and the background, respectively
- Diameter: the size of the spot
- Flags: numerical values indicating the quality of the spot

At the end of the analysis the work was saved as two txt files – one for Cy3 and one for Cy5. Further analysis was performed using GeneSpring GX 7.3, which recognises the above txt files and generates graphs for the complete data set, groups of genes or even individual genes of interest. Data can be normalised as desired and the \log_2 values for every cDNA clone can be exported as an excel file (Stekel 2003).

4.7. Normalisation

4.7.1. Logarithmic Scale

The aim of most microarray experiments is to identify changes in gene expression levels by looking at thousands of genes at the same time. These changes are best studied when the raw data has been normalised and low-quality measurements have been excluded. The intensities of the genes are therefore compared to each other and significant changes are identified (Quackenbush 2002).

In most cases microarray experiments study differences between expression patterns of biological samples. It seems that a simple ratio of the Cy5 value over the Cy3 value for a given gene would give the desired feature:

$T_i = \frac{R_i}{G_i}$, where T is the ratio of the desired feature, R is the experimental value for Cy5 (the red channel) and G is the reference value for Cy3 (the green channel), as quoted in Quackenbush 2002.

Although representing differences in expression by a ratio seems logical, it is not the preferred method due to the fact that it does not feature upregulated genes in the same way as downregulated ones. Thus, the data is transformed into logarithmic values in the base of 2 (\log_2), providing a symmetry (Table 4.1). This grants an even spread across the data and a bell-shaped distribution of intensities.

Log₂ value	Numeric value
Log ₂ (1)	0
Log ₂ (2)	1
Log ₂ ($\frac{1}{2}$)	-1
Log ₂ (4)	2
Log ₂ ($\frac{1}{4}$)	-2

Table 4.1: Representation of the symmetry the logarithmic scale provides.

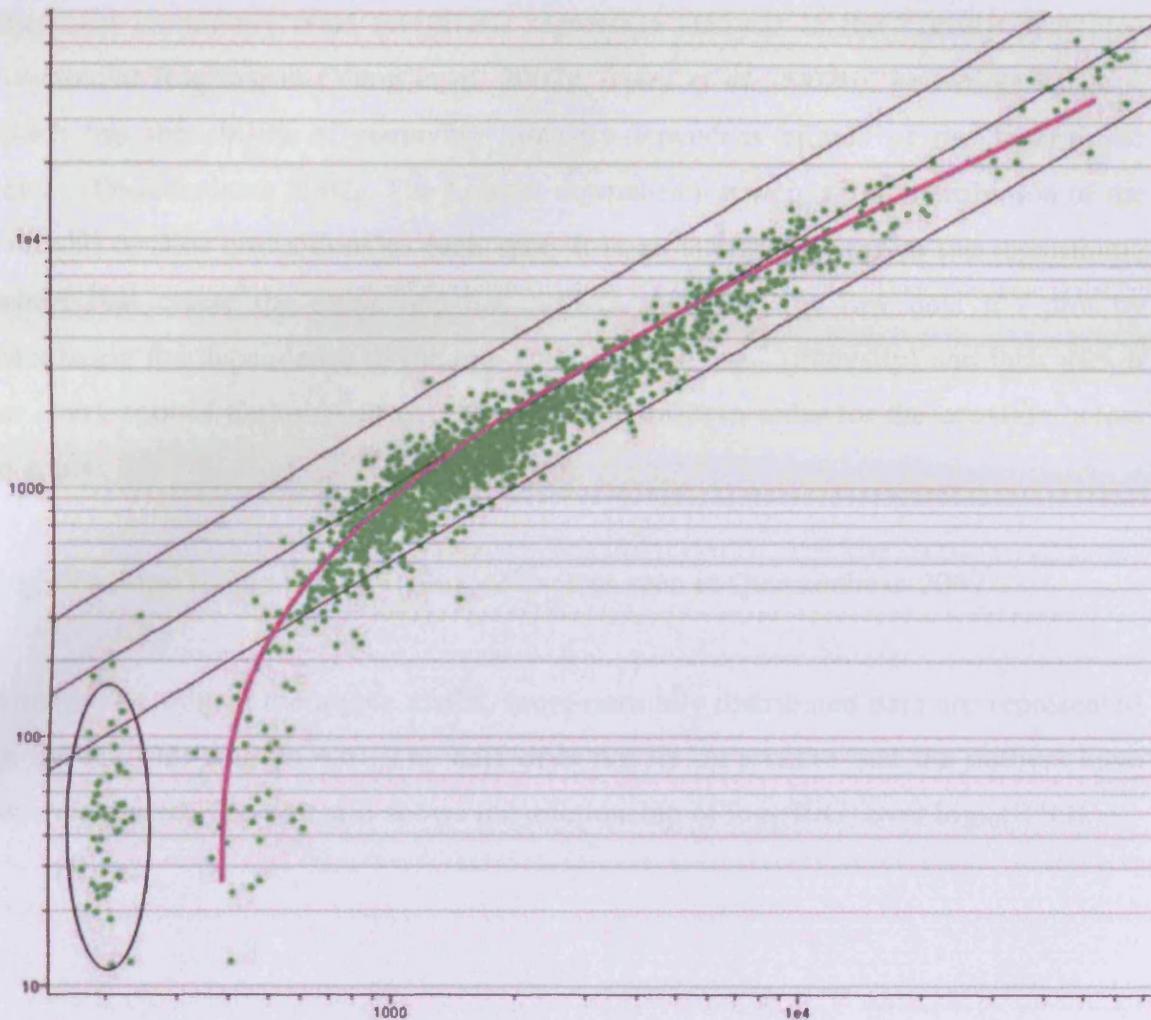


Figure 4.5: Scatterplot (or R-I plot) representing the raw microarray data (GeneSpring GX 7.3). The Y axis represents the \log_2 (Cy3 intensity / Cy5 intensity) or else R and the X axis represents the \log_{10} (Cy3 intensity * Cy5 intensity) or else I for each spot on the graph. Every dot is a gene, making up to the total number of genes studied in this experiment (1666). A straight line has been drawn, along with the confidence level lines. A perfect fit line has been drawn (in purple) but it is not a straight line. Also, a few of the spots (indicated by the circle) are outliers.

4.7.2. Lowess Normalisation

A common feature of microarray data is the lack of linearity between the two channels (red and green or Cy3 and Cy5) as shown in Figure 4.5. The R-I plot (ratio-intensity) of this targeted plasticity microarray experiment shows the raw data and the relation between the intensities of the two dyes. The lack of linearity is obvious and a non-linear regression is in need.

The most commonly used non-linear regression analysis is the Locally Weighted Polynomial Regression (Yang *et al.* 2002a; Yang *et al.* 2002b), known as Lowess, which has the ability of removing intensity-dependent effects of the logarithmic values (Quackenbush 2002). The Lowess normalisation weighs the distribution of the data and applies corrections to each spot. It is an attempt to correct the logarithmic values that create the curve and the outliers shown in the raw data R-I plot by calculating the dependence of the \log_2 (ratio) on the \log_{10} (intensity) and then uses it for every spot of the microarray (Quackenbush 2002) in order for the equation below to apply:

$$\text{Log}_2(T_i) = \log_2(T_i) - y(x_i) = \log_2(T_i) - \log_2(2^{y(x_i)}), \text{ as seen in Quackenbush 2002.}$$

After application of the above maths, more normally distributed data are represented on the R-I plot (Figure 4.6). The data does not lie on a curve and the outliers have been dismissed. The plot still shows the relationship of $\log_2(R/G)$ over $\log_{10}(R*G)$.

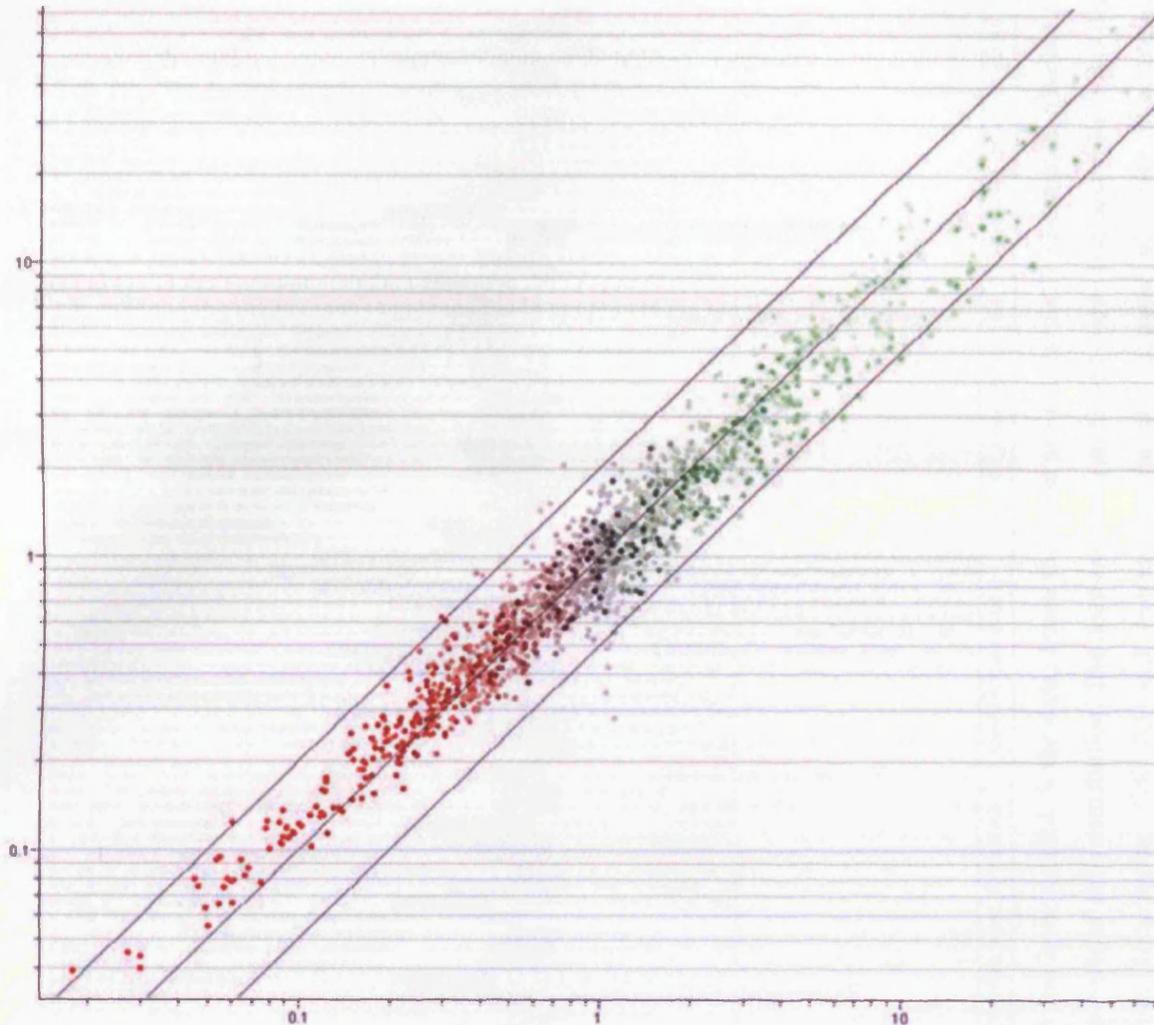


Figure 4.6: Scatterplot (or R-I plot) representation of the normalised targeted microarray data, as plotted by GeneSpring GX 7.3, after Lowess has been applied. The Y axis represents the \log_2 (Cy3 intensity / Cy5 intensity) or else R and the X axis represents the \log_{10} (Cy3 intensity * Cy5 intensity) or else I for each spot on the graph. Every dot is a gene, and the same as before the graph contains the total number of genes studied in this experiment (1666).

4.8. Visualising the data

One way of visualising microarray data is in box plots, which show the distributions of log ratios of genes in different microarrays (Figure 4.7b). In these plots data are shown as boxes, hence the name “box plots”, representing the standard deviation of the distribution or the median absolute deviation. The line at the centre of each square indicates the mean or the median and the lines above and below the boxes are the extreme values of each distribution (Kooperberg *et al.* 2002; Quackenbush 2002).

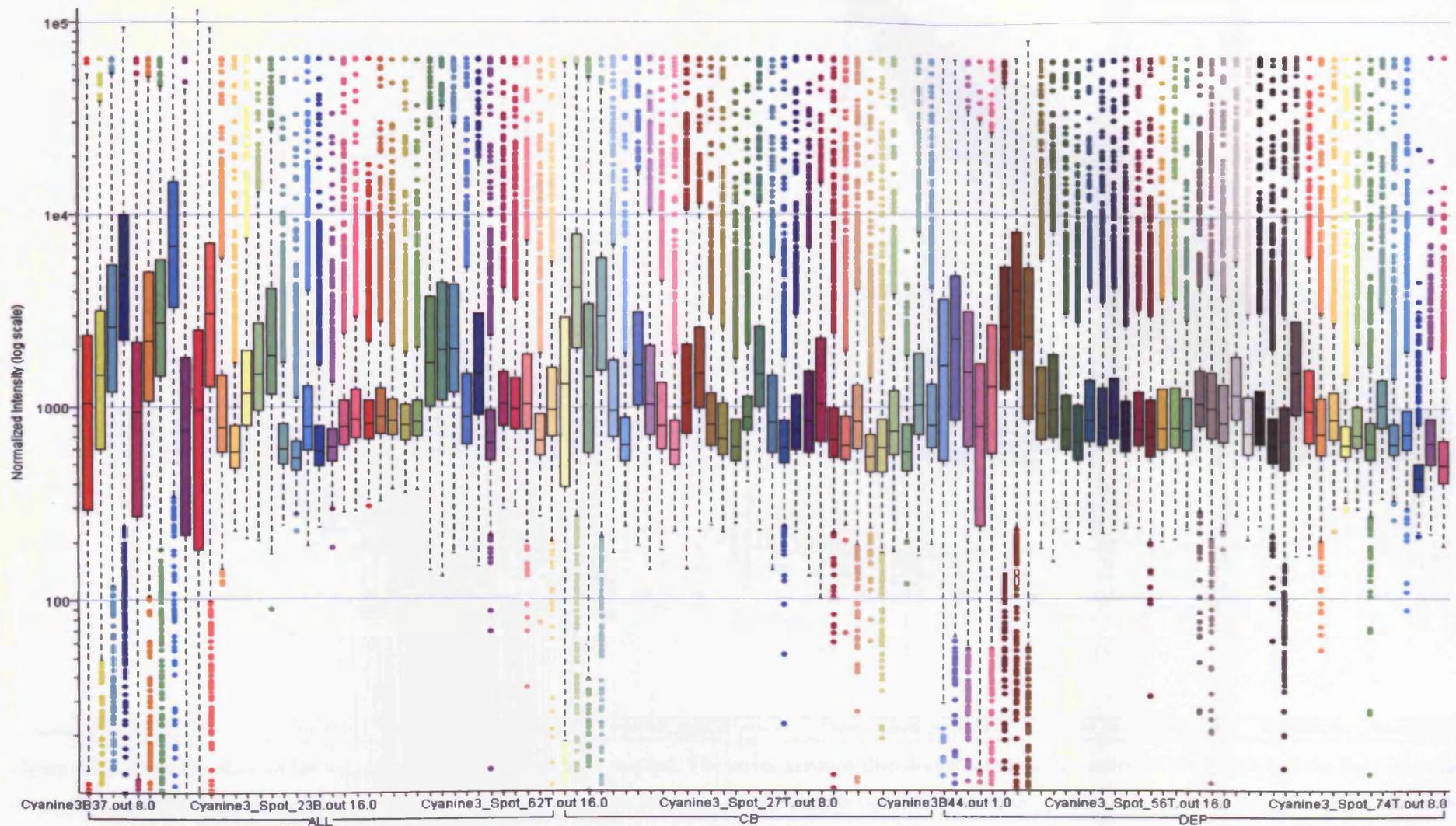


Figure 4.7a: \log_2 data represented in box plots. No further normalisation has been applied to the data; hence the boxes are not normally distributed. The boxes show the median absolute deviation and the line of each box is the median value of each distribution. The lines and dots positioned above and below the boxes are the extreme values of each distribution. The picture is taken from GeneSpring GX 7.3 and corresponds to the targeted plasticity experiment.

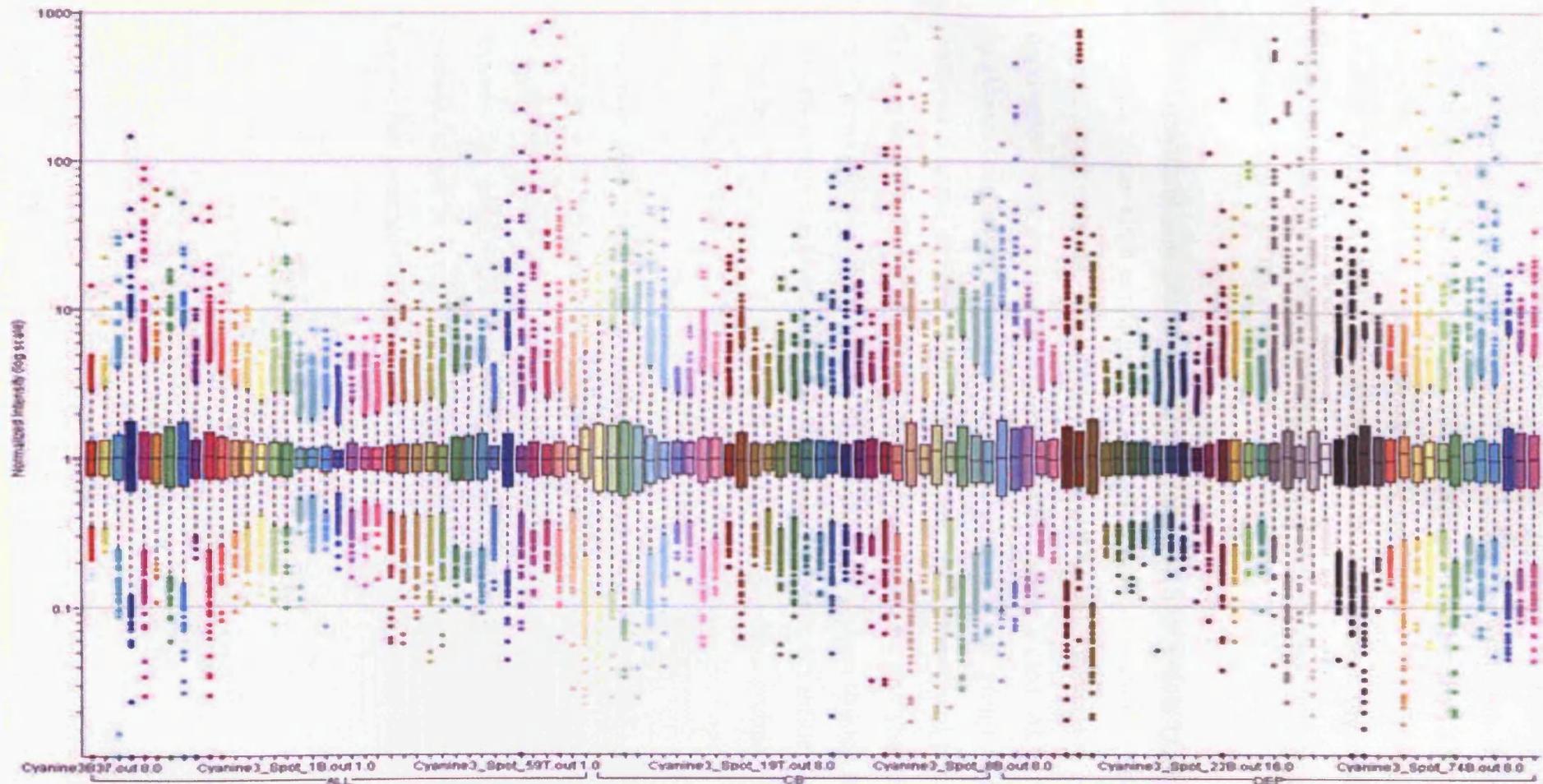


Figure 4.7b: Representation of the \log_2 data after scaling has been applied. The boxes are now distributed evenly at the centre of the graph and the lines (showing the median values of each array) are centred. The \log_2 of the medians for every distribution is equal to zero, as the medians are equal to one. The graph shows the normalised \log_2 intensities (on the Y axis) against the different arrays (on the X axis). Every coloured box is a different array (represented by a different colour). The arrays are shown in order of treatment; first are all the whiskers present mice (ALL), followed by the chessboardly deprived mice (CB) and in the end are the mice that have totally deprived of their whiskers (DEP). This picture has been taken from GeneSpring GX 7.3.

The necessity for scaling microarray data is tested by forming a hypothesis. We hypothesise that variation in the distribution between the arrays is a result of experimental conditions and not due to biological variability. If the assumption is not true then the method is inappropriate. By scaling the data (Figure 4.7b) the medians become equal, showing no biological variation, whilst the distributions are still different. The method of normalisation seems appropriate so the hypothesis is accepted (Stekel 2003).

The experiment was performed over a series of time points (1, 8 and 16 days) and different deprivation conditions were used to induce plasticity (CB and DEP). There were control animals, which were subjected to anaesthesia for consistency but no deprivation was performed – these animals were called ALL. The experimental conditions consisted of two groups of animals. One group had all its whiskers deprived (known as DEP) and the other one had a chessboard pattern of deprivation (known as CB). The DEP condition is a good model for studying depression whereas the chessboard deprivation gives the researcher a chance to look at the up-regulated and down-regulated genes, whose expression patterns are influenced by induction of plasticity. The data was submitted to scaling and then grouped according to time points (Figure 4.8).

The box plot shows equal medians for all groups, but the distributions are still variable, with different extreme values for every group. Every time point was treated as a different experiment and normalisation was performed within the groups and not between the groups. Each group is normalised in reference to its own control animals, which in every case are the undeprived animals: ALL1, ALL8 and ALL16, the number indicates the number of day (1, 8 and 16 days respectively).

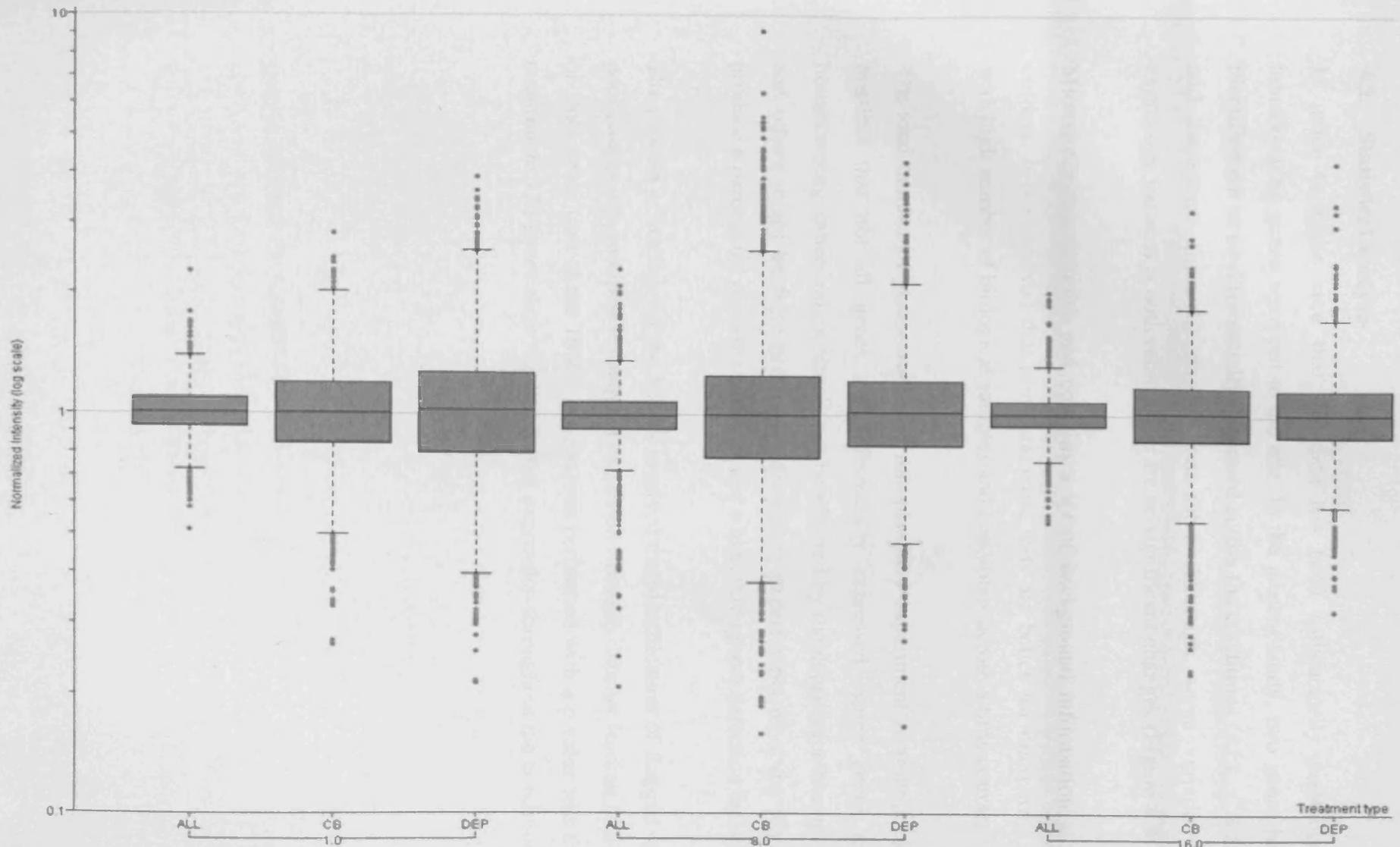


Figure 4.8: Box plot of the data grouped according to time points. The graph shows the normalised-scaled \log_2 data over treatment type and time. Where ALL is all whiskers present, CB corresponds to chessboardly deprived mice and DEP refers to total deprivation. The image was obtained from GeneSpring GX 7.3.

4.9. Statistical analysis

In order to make sure that the data has been satisfactorily normalised, two housekeeping genes were put to the test. In the present study, two genes have been identified not to be differentially expressed across the conditions (ALL, CB and DEP) and throughout time; Gapdh and beta-actin. Although, some variation in their expression patterns is noticeable, there are no significant changes (Figure 4.9).

Microarray data is noisy and contains a lot of background information along with outliers. It is conceived that non-parametric tests are better for microarray analysis with high number of biological samples and conditions across a time course.

The total number of genes studied in this plasticity experiment is around 1666. It is possible that not all genes are differentially expressed. Some genes might be housekeeping, others might simply not be affected by the deprivation treatments used and others might be false positives. Microarray experiments have the tendency to produce a percentage of false results, as it is a high throughput detection technique.

The process of sorting out the results involved the identification of flagged spots and poor data points and their subsequent exclusion from the data set leading to a new list of 1593 genes (out of the 1666). A t-test was performed with a p value less than 0.05 resulting to 736 genes showing differential expression throughout the conditions.

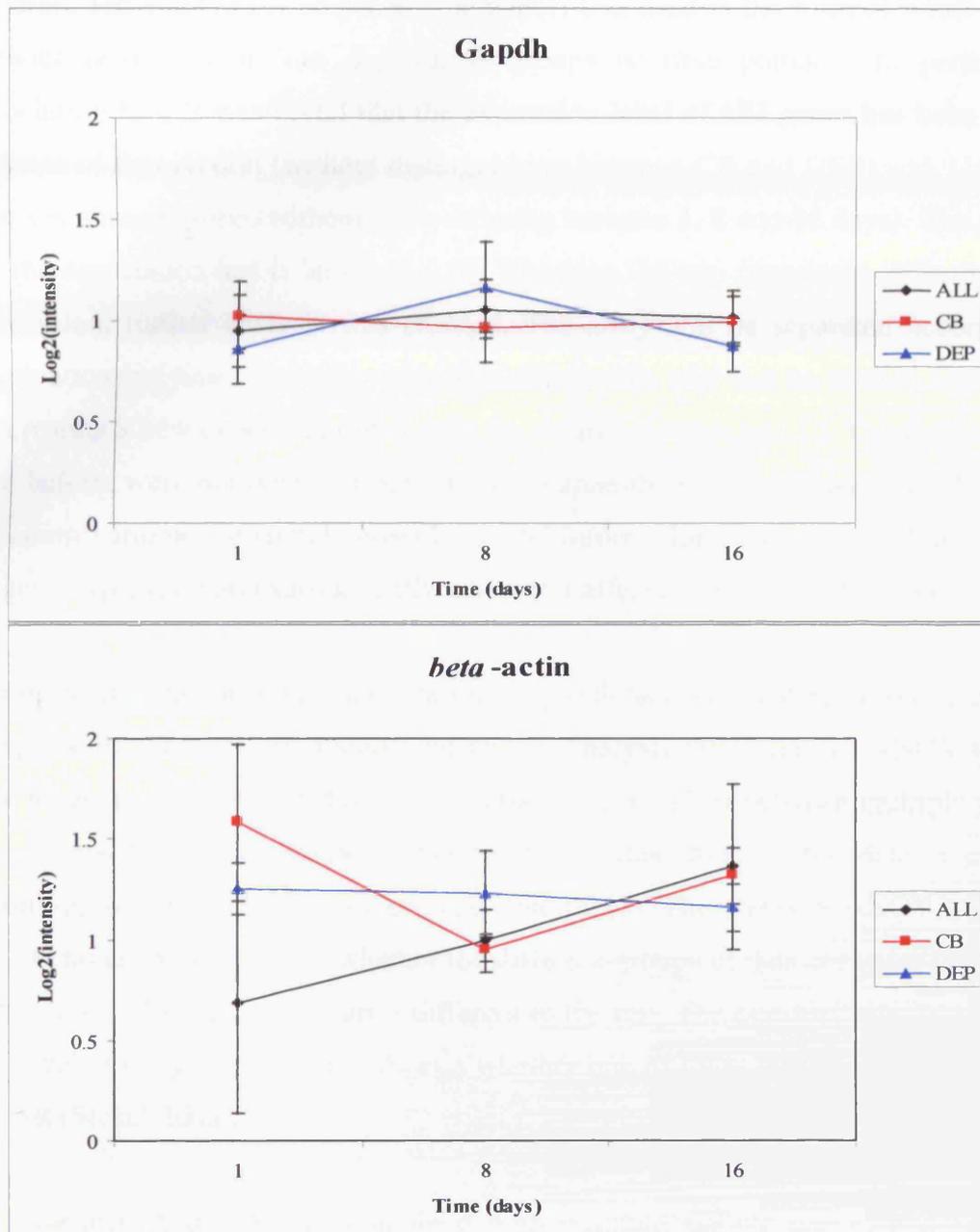


Figure 4.9: Microarray graphs of the two most commonly used housekeeping genes, Gapdh and beta-actin, showing their expression patterns throughout the chosen time course, after two different types of deprivation. Where ALL is data from undeprived mice, CB corresponds to chessboard deprivations and DEP refers to total deprivations.

The present study involves many parameters and attributes, so it was considered important to identify which of the parameters are significantly affecting expression patterns. The filtered list of genes (736 genes) was used in the form of a full array – without separating it into deprivation groups or time points – to perform an association test. It was found that the expression level of 682 genes has been altered because of deprivation (without distinguishing between CB and DEP) and 724 genes changed through time (without differentiating between 1, 8 and 16 days). The p value for the association test is less than 0.05. Knowing the two parameters affecting gene expression, further analysis was enabled. The array will be separated according to deprivation and time. The effect of lack of deprivation will also be studied across time by creating a new experiment with only the control samples (ALL). These animals, as said before, were not deprived but they were anaesthetised for consistency. Studying the control animals separately provides useful information on the effect of anaesthesia on gene expression and subsequently whether it affects plasticity in the mouse brain.

During microarray analysis there are many questions asked that need multiple group comparisons. The preferred statistical test is Analysis Of Variance (ANOVA). It is used to test the presence of differences in the average values between multiple groups, simultaneously, which makes it appropriate for this study as the data is grouped according to treatment (deprivation) and time (days). The one-way ANOVA was the test of choice in order to see whether the different groups of data are independent and if the mean value of each group is different to the rest. The one-way ANOVA returns one p-value per group, which indicates whether one or more groups is different from the rest (Stekel 2003).

The one-way ANOVA was combined with multiple sample correction. This was considered necessary in order to remove false results. The Bonferroni adjustment was chosen to perform multiple tests in parallel. It is a commonly used statistical test for microarrays with not equal distributions between the groups of data and it offers the correct amount of stringency. At first, p-values for the experiment were calculated using one-way ANOVA (described above) and then they were all multiplied by the number of tests performed. These tests were performed on 736 genes, which came through the flagging filter and t-test, giving a final number of 108 genes.

4.10. Conclusions

Investigation of the effect of anaesthesia on the control (undeprived) animals, termed ALL, throughout the three time points is crucially needed before any other analysis looking into plasticity effects on the data. In order to do that the study was separated into the different conditions and a new experiment was created consisting of the control animals in the three time points (1, 8 and 16 days). Global Lowess normalisation was performed in this data set followed by relative expression of the three time points (ALL1, ALL8 and ALL16) to the earliest one (ALL1); based on the hypothesis that these animals have been subjected to the gaseous anaesthetic only once making the effect of isoflurane minimal compared to the rest of the animals that have been subjected to anaesthesia regularly in order to check that their whiskers were still intact.

Principal Component Analysis (PCA) plots (discussed in later chapters) gave an indication of a more complex data set than the one initially considered, making further separation of the data considerably important for a deeper and more detailed approach. Later chapters will deal with the anaesthetic effect on gene expression and sexual differences within the population. Analysis of the control animals will be provided, in order to study the effects of isoflurane in plasticity. Differences in the data sets within the same time point will be addressed by studying female and male differences. Finally the effect of induction of plasticity on gene expression will be discussed provided the associated functional analysis.

CHAPTER 5

ANAESTHETIC AND GENDER EFFECT

5.1. Overview

Surgical manipulation of vibrissae requires sedation of experimental animals. It is therefore imperative that the transcriptional changes associated with this treatment are evaluated, in order to distinguish these alterations from those caused by various patterns of whisker deprivation. To investigate neuronal plasticity induced transcriptional responses two patterns of deprivation were exploited; chessboard (CB) and complete (DEP), in addition a control group was included within the study where all whiskers remained intact (ALL) (Section 2.12). The latter group of control animals was, for consistency, concurrently anaesthetized during regular whisker checks performed on the test animals. In this chapter the data from control animals is considered separately from the rest of the transcriptomic experiment in order for the effect of isoflurane (the anaesthetic of choice for this study) to be investigated. Principal Component Analysis (PCA) is used to provide a better insight as to the discrete transcriptional profiles associated with different amounts of repeated anaesthesia. Inconsistencies in the data set will be further evaluated by considering other parameters that might have affected gene expression. The manner and extent of the volatile gaseous anaesthetic effects on neuronal proteins, ion channels and neurotransmitters are unravelled in this chapter.

5.2. Genes affected by isoflurane

General anaesthesia with isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane; the gas of choice for this study) causes muscle relaxation, an inability to react to commands, followed by quick recovery allowing painless, rapid procedures to be undertaken (Jia *et al.* 2008). Animals were anesthetized at the start of the experiment, manipulated (to remove vibrissae where appropriate) and allowed to recover for 24 hours (day 1) at which time the anaesthesia was repeated to allow for confirmation of whisker removal. Subsequent evaluation of whisker absence was performed every 48

hours (days 3, 5, 7, 9, 11, 13 & 15). Samples were harvested for transcriptomic studies on day 1, 8 and 16. In all cases 24 hours recovery from the anaesthesia was allowed before samples were taken. The barrel field (Section 1.5) was carefully selected and removed from each brain (Section 2.14.2), RNA was purified (Section 2.15.2) and used as template for cDNA synthesis and subsequent probe labelling (Section 2.22) which was later utilized for hybridizations onto specially designed targeted plasticity microarray glass slides (Section 2.24). Normalization of the data exploited tip-Lowess (Section 4.7.2) and data was filtered to remove spots which did not conform to strict quality control criteria. An average of the signal intensity for technical replicates was determined which returned 1178 genes (70% of genes represented on the array). Statistical analysis employing non-parametric approaches, since the targeted array data is not normally distributed, revealed the transcripts affected by the introduction of the gaseous anaesthetic. Transcripts exhibiting a 2-fold change when compared to day 1 at a significance of $p < 0.05$ revealed 202 genes to be affected by the administration of isoflurane at day 8 and 110 genes at day 16. The reduction of total number of responsive transcripts over time may be indicative of familiarization of the mice to the stress caused by the gas or even their handling by the researcher. The distribution of genes impacted can be illustrated by volcano plots (Figure 5.1) indicating the transcripts passing defined filters (fold change and $p < 0.05$).

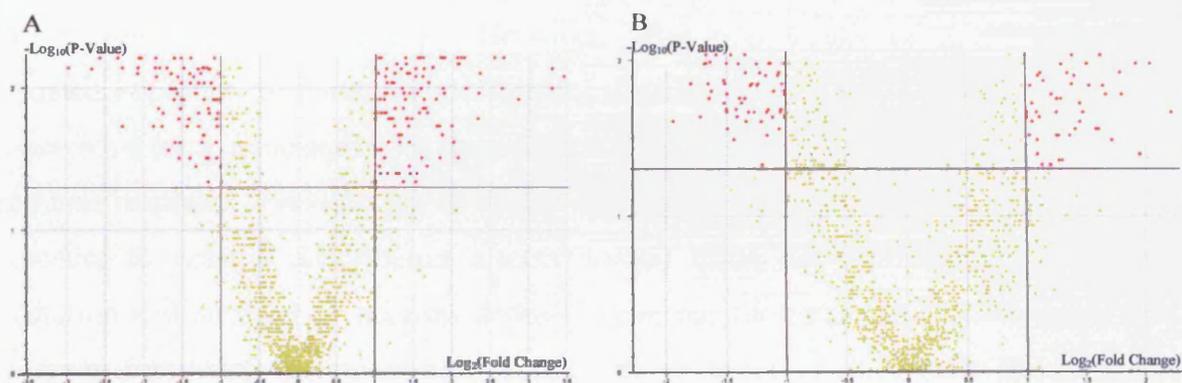


Figure 5.1: Volcano plots from Genespring indicating the genes whose expression profiles have been affected by the introduction of isoflurane. A non-parametric test, multiple sample correction, 2-fold change in expression and p value < 0.05 were applied. Statistically changing genes are indicated by the red dots and un-changing ones are shown in yellow. Plot A is the comparison between Day 1 and Day 8 data whereas plot B is the comparison between Day 1 and 16 data.

5.3. Ontological Bias Analysis

In an attempt to understand the function of the genes differentially expressed at day 8 and 16 (Figure 5.1; Appendix 4), the statistical over-representation of genes belonging to particular functional categories was assessed. The enrichment calculation evaluated the frequency of occurrence of a specific GO ontology term within a defined gene list when compared to its representation within the complete 15K NIA array. This process was greatly streamlined by an online version of the pathway analysis software known as “L2L” Microarray Analysis Tool (<http://depts.washington.edu/l2l/>). Analysis was performed to investigate the over-representation ($p < 0.05$) of Gene Ontology terms associate with “*Biological Process*” and “*Molecular Function*” categories within the genes displaying significant changes in response to anaesthesia between day 1 and days 8 and 16 (Figure 5.2; Panel A and B respectively). Only terms associated with “*Molecular Function*” exhibited significant over-representation ($p < 0.05$). A direct comparison between the functional groups over-represented at these two time points reveals limited overlap with only two terms, “*DNA Bending Activity*” and “*Microtubule Motor Activity*” appearing at both time points with the latter term showing a substantial increase in frequency from 4% to 23% within the 8 and 16 days gene lists. Inspection of the functional groups represented after the first week of experimentation (Figure 5.2; Panel A) reveals genes that may be associated with an increase in metabolic activity, denoted by increased “*Oxydative Phosphylation*” and major reorganization events (“*Structural Molecular Activity*”, “*Kinase Activity*” and “*Microtubule Motor Activity*”). However, after two weeks of non-continuous repeated exposure to isoflurane the functional category of “*Calmodulin Binding*” is observed; a term associated with short-term and long-term memory, nerve growth and immune response. Previous observations have shown that long term mammalian exposure to volatile anaesthetics affects normal brain development and that the formation and survival of neurons depends upon the formation and maintenance of synapses for which Calmodulin is responsible (Nikizad *et al.* 2007). Hence, the appearance of this functional group over-represented at day 16 might indicate the necessity for neuronal survival and growth in response to repeated exposure to anaesthetic.

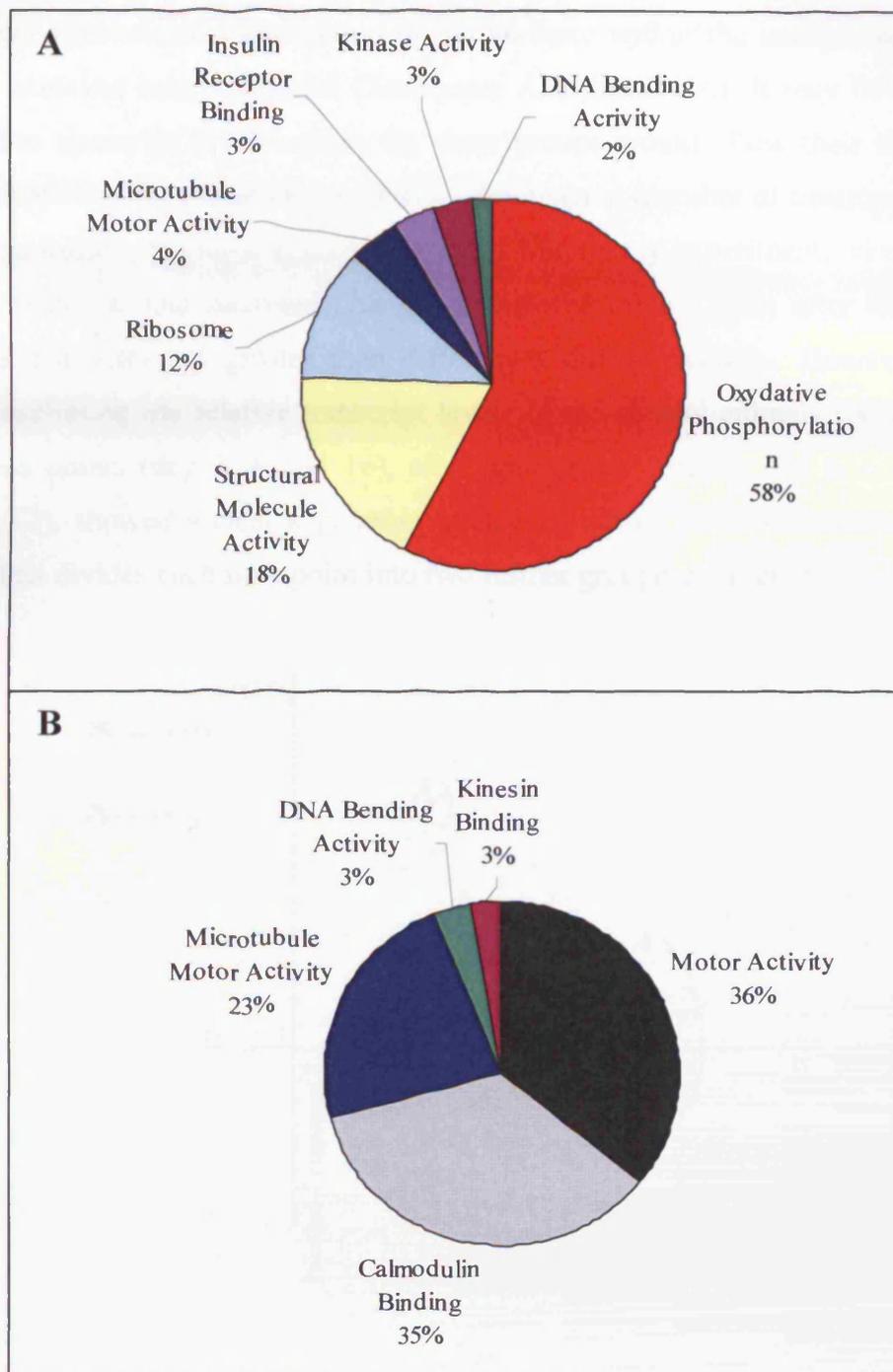


Figure 5.2: Pie charts A and B show the pathway analysis using L2L Microarray Analysis Tools (<http://depts.washington.edu/l2l/>). Panel A (n=307) represents the genes which were differentially expressed between Day 1 all whiskers present group (ALL 1) and Day 8 all whiskers present group (ALL 8); whereas Panel B (n=191) is the comparison between Day 1 all whiskers present (ALL 1) and Day 16 all whiskers present (ALL 16). Ontological bias analysis was performed on genes exhibiting significant changes (2 fold change and $p < 0.05$). For full list of genes refer to Appendix 4.

5.4. Multivariate analyses of anaesthetic induced transcript changes

Precise two-dimensional visualisation of the variance within the undeprived control data was achieved using Principal Component Analysis (PCA). It may be expected that relative transcript levels within the three groups would allow their separation being indicative of the extent of anaesthetic administered (number of treatments). This assumption follows the logic behind the Global Microarray experiment, where it was generally believed that neuronal changes within the mouse brain after experience dependent plasticity are greater than differences due to gender. However, PCA analysis exploiting the relative transcript levels of the control animals (ALL) at the three time points (day 1, 8 and 16), after appropriate filtering and normalisation (Section 5.2), showed a clear separation of day 16 but an additional component of variance that divides each time point into two further groupings (Figure 5.3).

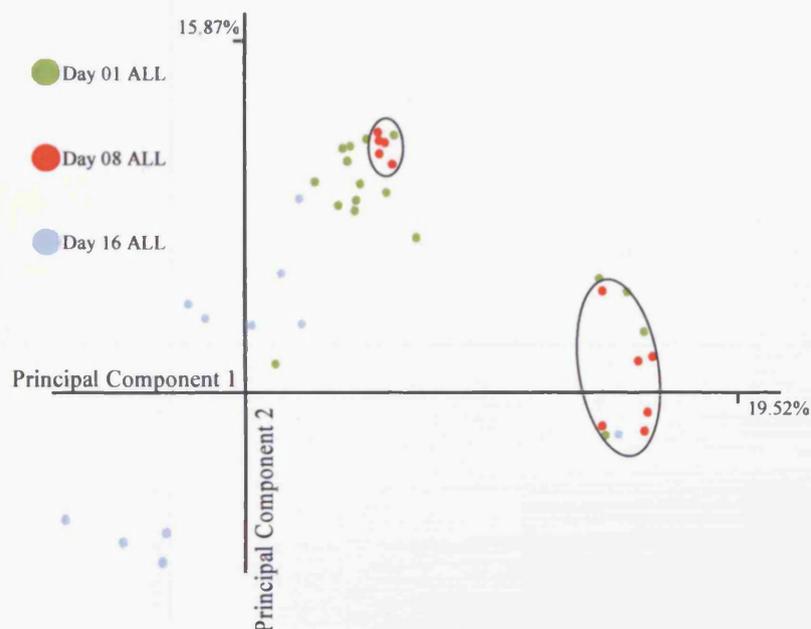


Figure 5.3: Principal Component Analysis (PCA) of the undeprived control data over time. Relative transcript levels for reporters passing quality control parameters were used to analyse the variance within the data using PCA. The major contributors to the variance, Principal Component 1 and Principal Component 2 representing 19.52% and 15.87% respectively, are displayed on the horizontal and vertical axes. The data points represent relative transcript levels at Day 1 (green dots), 8 (red dots) and 16 (blue dots). Black circles highlight the two distinct clusters of day 8 samples indicating that they do not separate into a single group.

This global analysis has identified the confounding effect of both gender and anaesthesia; however, the data set provides an ideal platform to explore the molecular changes occurring within the nervous system in response to anaesthetic.

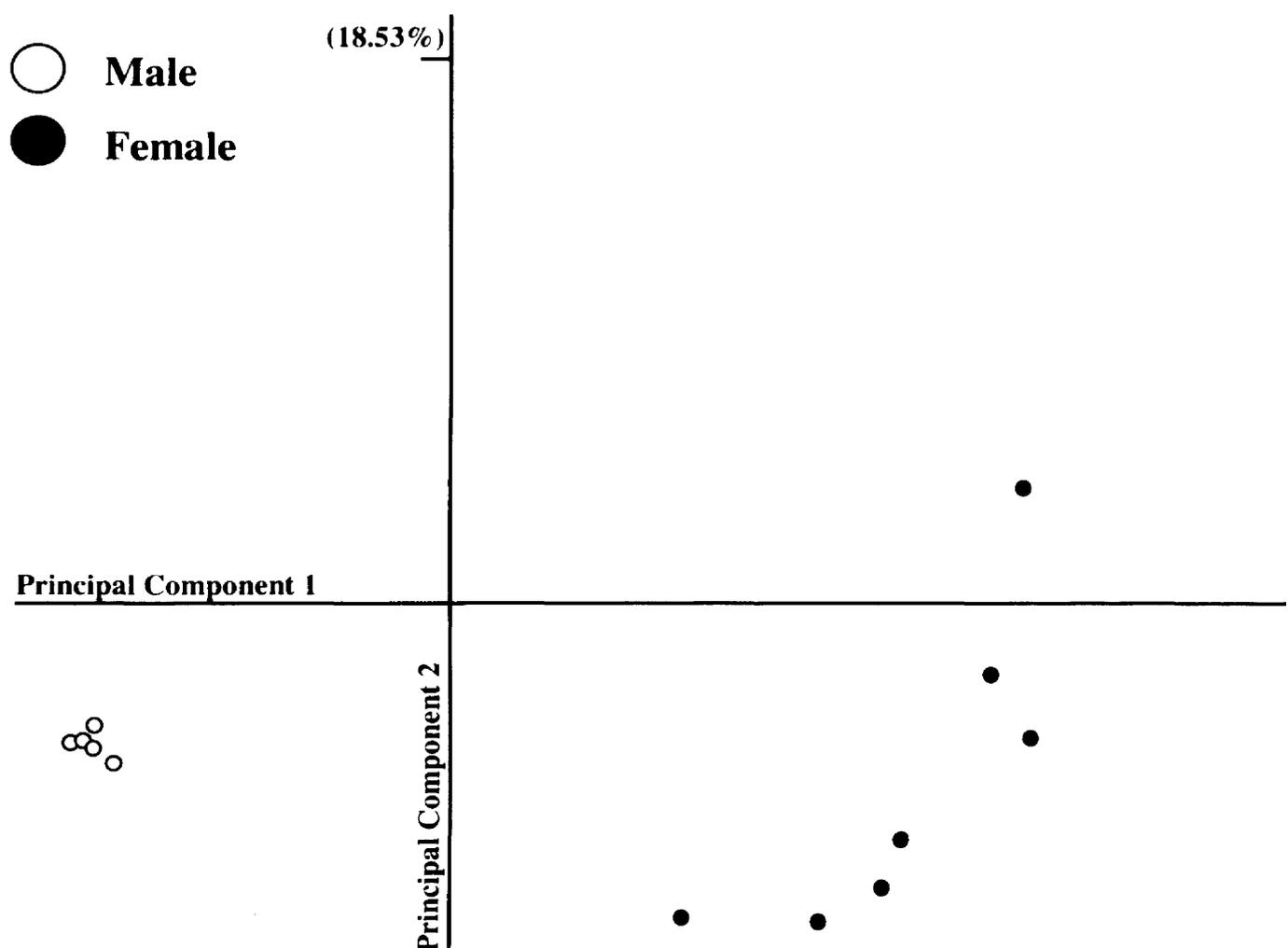


Figure 5.4: Principal Component Analysis (PCA) of the undeprived control data. Relative transcript levels for reporters passing quality control parameters were used to explain the variance within the data using PCA. The major contributors to the variance were Principal Component 1 and Principal Component 2 representing 36.22% and 18.53% respectively. The data points are displayed on the horizontal and vertical axes. The data points represent relative transcript levels for males (white dots) and females (black dots). The separation between the two groups on Day 8 is clear.

5.5. Effect of volatile anaesthetics on synaptic transmission

Approximately three decades ago, it was believed that volatile anaesthetics, such as isoflurane, dissolve in the lipid neuronal plasma membrane (Seeman 1972; Miller 1985), whereas more recent studies have revealed that the anaesthetics bind onto neuronal proteins, ion channels and neurotransmitters (Scholz *et al.* 1998). There is a theory suggesting that anaesthetics have several neuronal targets; based on the idea that they are associated with the hydrophobic binding sites of neuronal proteins which are directly linked to dendritic spines (Fischer *et al.* 1998). Volatile anaesthetics may block actin-dependent fibroblast motility (Kaech *et al.* 1997), suggesting that these compounds cause anaesthesia by acting at a variety of sites simultaneously (Harrison 1998). In the central nervous system, actin is concentrated at the dendritic spines, where the postsynaptic site is located (Kaech *et al.* 1999), indicating the interaction of the anaesthetic with the excitatory synapses.

Although, the effects of volatile anaesthetics have not been completely understood and analyzed, it is hypothesized that anaesthetic-induced neuronal plasticity and memory loss is somehow related to NMDA-mediated excitatory synapses (Pocock and Richards 1993); NMDA (Section 1.10.2) being another type of neurotransmitter receptor ((Rosenmund and Westbrook 1993) that play a vital role in the central nervous system of mammals by regulating excitatory synaptic transmission (Hollmann and Heinemann 1994). The effect is such that it may alter spine shape and lead to changed excitatory transmission between the spine head and the dendrite (Kaech *et al.* 1999). The effect of general anaesthesia on spine mobility indicates the sensitivity of the latter suggesting that morphological changes could lead to short-term memory and minor brain function alterations (Kaech *et al.* 1999).

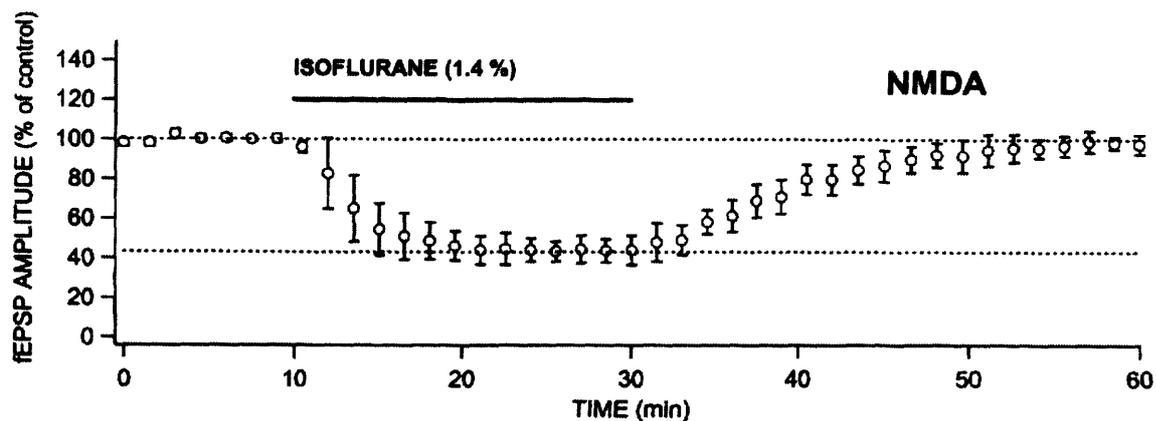


Figure 5.5: Representation of the effects of isoflurane on NMDA receptors. NMDA receptors show great sensitivity to the application of this gaseous compound (Nishikawa and MacIver 2000).

The effect of isoflurane on glutamate receptors and excitatory postsynaptic potentials (EPSPs) recorded with intracellular electrodes is illustrated below. Figure 5.5 shows the extent of depression of field EPSPs mediated by NMDARs (ion channel receptors; Section 1.10.2) leading to the assumption that depolarization of the post-synaptic membrane is greatly depressed affecting the flow of positive ions into the post-synaptic cell (Nishikawa and MacIver 2000).

5.6. Glutamate receptors and isoflurane

Changes in synaptic plasticity, and subsequent changes in the expression profiles of ion channel receptors associated with the two main mechanisms of plasticity; LTP and LTD (Section 1.10), underline the ability of the brain to learn and memorize events as well as stimuli changes.

The expression of NMDA receptors in relation to anaesthesia was investigated in the targeted plasticity microarray experiment (Figure 5.6). A total of 3 clones were identified in the data set; NMDA receptor 2, NMDA2A and NMDA receptor 1, the numerical values of which were normalised to Day 1 following the assumption that the first time point is the one with the least changes due to the minimum non-continuous exposure of the subjects to the anaesthetic.

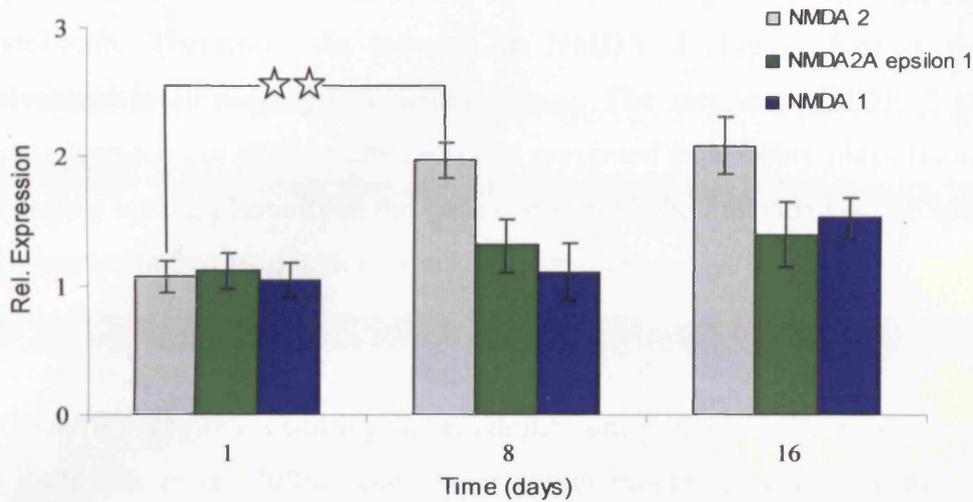


Figure 5.6: Representation of the relative expression of the three NMDAR clones identified in the targeted plasticity microarray in control undeprived animals (ALL; all whiskers present) at Days 1, 8 and 16. The grey bar represents NMDA 2, the green bar shows NMDA2A epsilon 1 and the blue bar indicates NMDA 1. The data was normalised with Lowess and was expressed in relation to the day 1 data under the assumption that Day 1 control animals have the least changes in gene expression due to short and non-continuous exposure to the anaesthetic. The standard deviation displayed represents variance within true biological replications (n=11, 12 and 16 for Day 1, 8 and 16 respectively). The double star indicates significance with $p < 0.03$.

Although the effects of isoflurane are not fully understood, it is believed that it interacts with receptors and synaptic transmission (Tachibana *et al.* 2007) leading to the assumption that cell-cell communication was compromised in this plasticity experiment, especially when NMDA receptor 2 was involved due to the 2-fold increase observed after 2 weeks of regular isoflurane exposure (Figure 5.6). Thalamocortical connections, requiring NMDA receptors for ion exchange, have been identified as important during sedation because of their role in processing sensory information and maintaining activity (Alkire and Miller 2005).

In principle, microarray experiments, like the one presented in this study, can not distinguish between the post-synaptic activity of a gene or neurotransmitter and its pre-synaptic properties. As discussed in Section 1.10.2, NMDA receptors act pre-

synaptically on glutamate release and post-synaptically on calcium increase (Debanne *et al.* 2003; Lynch 2004). Isoflurane facilitates the depression of pre-synaptic glutamate release in order to successfully relax the subject via the lack of neuronal transmission. Therefore, the increase in NMDA 2 (Figure 5.6) is due to its involvement in post-synaptic calcium release. The fact that NMDA 2 shows the greatest increase out of the three receptors presented it probably plays the most vital role in long lasting plasticity in the cortex or it might be indicative of the specific part of the transcript involved in neurotransmitter responses.

Isoflurane is also associated with potassium channels whose openings are stimulated by GABAB receptors, resulting in an equilibrium potential of potassium within the cell itself (Jia *et al.* 2008). The above action blocks all neurotransmitter release (Manev and Dimitrijevic 2004) in the neurons facilitating the properties of the anaesthetic. The targeted plasticity experiment has shown an increase in the expression levels of GABAB receptor 1 (Figure 5.7 and Figure 5.8). Three clones were identified in the array data set; however the receptor with the most significant changes over time was GABA-B1, which agrees with the previous theory that requires GABA-B to facilitate the effects of isoflurane resulting to an effective anaesthesia stopping the majority of neurotransmitter release.

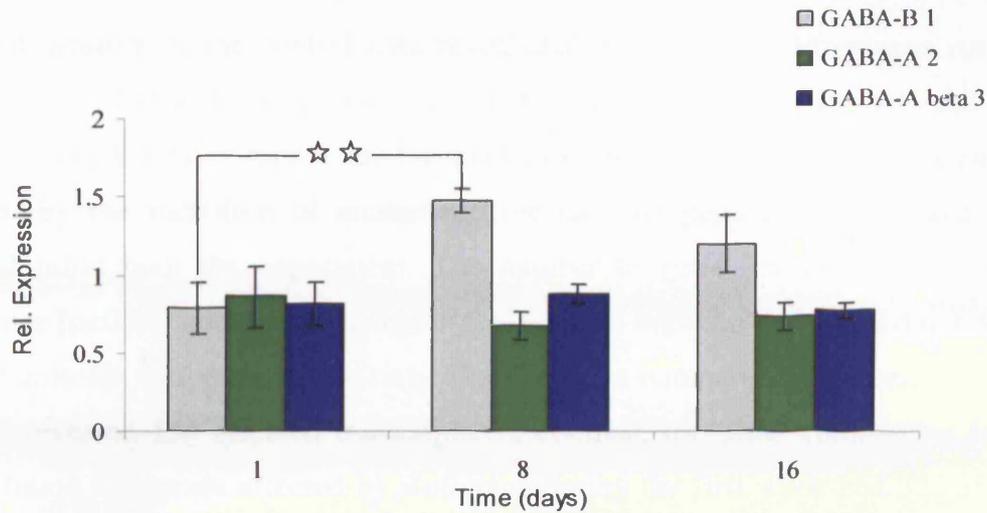


Figure 5.7: Representation of the relative expression of the three GABAR clones identified in the targeted plasticity microarray in control undeprived animals (ALLs; all whiskers present) at Days 1, 8 and 16. The grey bar represents GABA-B1, the green bar shows GABA-A2 and the blue bar indicates GABA-A beta 3. The data was normalised with Lowess and was expressed in relation to the day 1 data under the assumption that Day 1 control animals have the least changes in gene expression due to short and non-continuous exposure to the anaesthetic. The standard deviation displayed represent variance within true biological replications (n=11, 12 and 16 for Day 1, 8 and 16 respectively). The double stars indicate significance of $p < 0.03$.

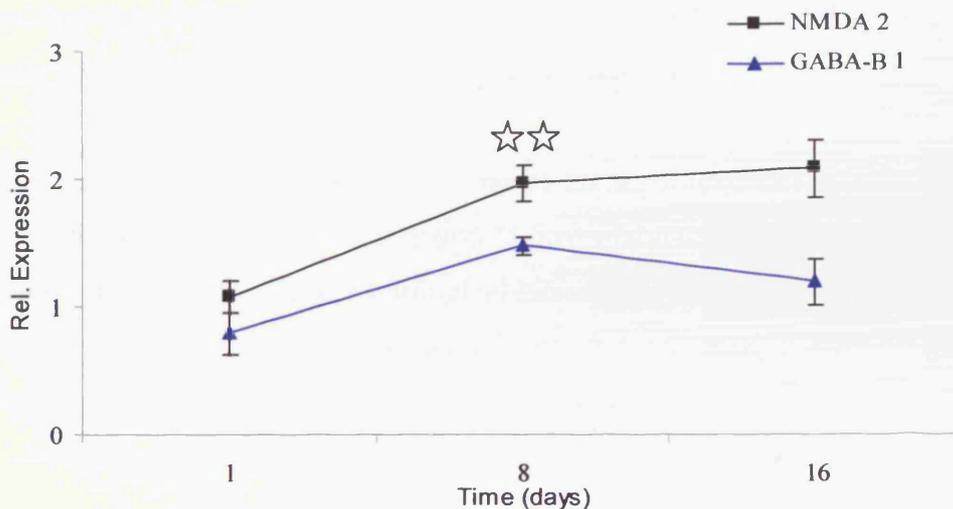


Figure 5.8: Summary of the two glutamate receptors mostly affected in control undeprived animals by the induction of the gaseous anaesthetic over time (Day 1, 8 and 16). NMDA receptor 2 is represented by the black line and GABA-B receptor 1 is represented by the blue line. The double stars indicate significance with $p < 0.03$.

5.7. Differential responses of male and female mice to anaesthesia

Detailed scrutiny of the control data revealed that significant differences could be observed in transcriptional responses between gender groups. At the most fundamental level we observed that the quantity of genes (for list refer to Appendix 4) affected by the induction of anaesthetic for the two genders (female and male) differed throughout the experiment. The number of genes exhibiting a statistical difference ($p < 0.05$) and 2 fold change in female mice between day 1 and day 8 for the control animals was shown to be only 77, whereas a comparison between day 1 and day 16 revealed 134 affected transcripts. In contrast, the same comparison for the males found 330 genes affected by isoflurane during the first week and 321 after 16 days of repeated, short and non-continuous exposure. One possible explanation for our observations is that biological replicates within the female population show slightly different expression patterns i.e. increased biological variation when compared to the male cohort. This is clearly illustrated in Figure 5.4 where the female data shows increased variance compared to the male counterpart. A plausible explanation for the biological differences observed within the female population and the inconsistency in the results is the fact that the females undergo sexual maturation and ovarian cycles every 4 days. Their behaviour is hormone-dependent and can be altered by steroid exposure in early developmental stages (Kudwa *et al.* 2006). This increased variation impacts significantly on the analysis and the use of grouped data may lead to a reduction in the power of the analysis.

The increased analytic power provided by the separation of the genders also enhances our ability to perform secondary ontological analysis and provide a level of functional interpretation of the data. Ontological bias analysis, using a comparative approach to that employed with the group data (Section 5.3), using the sex specific gene lists showed significant ($p < 0.05$) over-representation of various terms within “*Molecular Function*” categories for both days and sexes.

The male data presented below (Figure 5.9 and 5.10, Panel B in both figures) show significant conservation of terms between Days 8 and 16, indicating a level of consistency in males in some of the over-expressed categories. A number of ontological categories disappear by Day 16, indicating abolition of some responses

within the male population. The female data presented in the same figures (Panel A in both figures) support the findings of the male data ontological analysis with categorical overlap. However, the female data lacks the consistency seen in the males, leading to the assumption of hormonally affected transcripts due to their menstrual cycle every 4 days.

It is clear that the sexes differ in their global response to the anaesthetic and in subtle changes in their functional response. It is intriguing to interrogate the full data set to explore whether these sex differences also impact the treatments designed to examine plasticity. Therefore the variance of the complete data set was explained to provide a deeper understanding of the implications of sex specific effects on studies considering potentiation and depression.

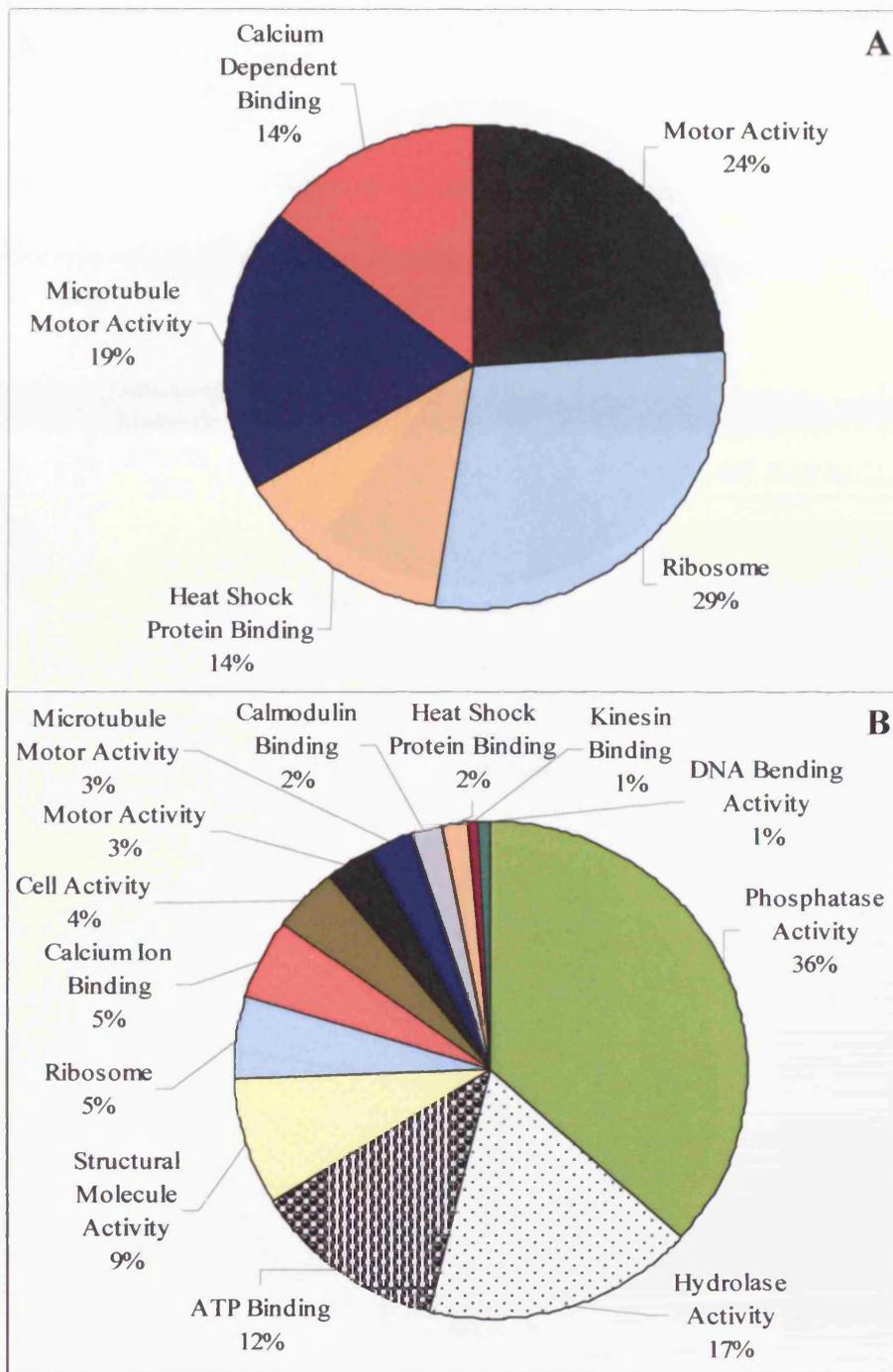


Figure 5.9: Pie chart representing functional analysis of the female (A; n=77) and male (B; n=330) data as two separate sets for the undeprived (control) animals exhibiting significant changes (multiple sample corrections, 2 fold and $p < 0.05$) when Day 1 and Day 8 were compared for the control animals in the two genders, separately, in order to identify the number of genes affected by the introduction of isoflurane.

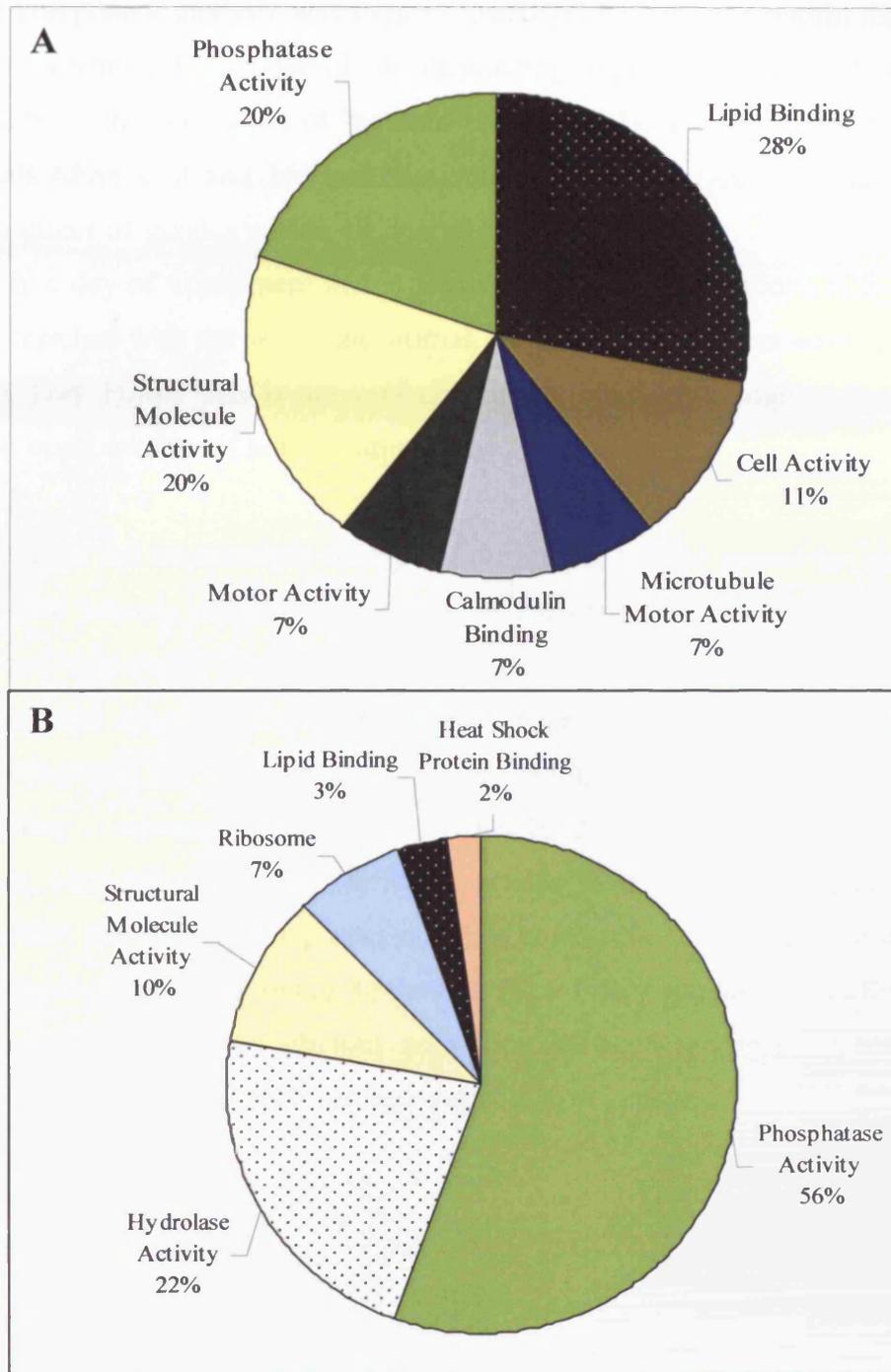


Figure 5.10: Pie chart representing functional analysis of the female (A; n=134) and male (B; n=321) data as two separate sets for the undeprived (control) animals exhibiting significant changes (multiple sample corrections, 2 fold and $p < 0.05$) when Day 1 and Day 16 were compared for the control animals in the two genders, separately, in order to identify the number of genes affected by the introduction of isoflurane. For full list of genes refer to Appendix 4.

5.8. Multivariate analyses of plasticity induced transcript changes

Principle component analysis was used to visualize the variance within the data and investigate whether the gender of the originating experimental animal contributed significantly to the separation of the data (Figure 5.11). The data was divided into time points (days 1, 8 and 16) and deprivation conditions (ALL, CB and DEP) to study the effect of gender within all conditions. The separation observed in the data after the first day of experiment under the two deprivation conditions (CB and DEP) does not correlate with the sex of the animal. When all the whiskers were spared for 1 day (ALL Day 1), the data is not very conclusive, although a slight pattern is seen, due to the small number of female samples (n=2).

With respect to the intermediate time point during the critical period for plasticity (8 days) we can observe definite segregation of the data. As indicated by the orange (female) and blue (male) circles around the data, in the all whiskers spared for 8 days (ALL8) and the all whiskers deprived for 8 days (DEP8) females and males are clearly separated showing that they are being affected differently by the corresponding conditions (Figure 5.11). After 16 days of non-continuous repeated anaesthesia (ALL16) animals of different gender maintain their separate profiles. Unfortunately, the DEP16 group does not give conclusive results because of the low replicates for the female set (n=2). In the chessboard deprived animals (CB), for both 8 and 16 days, there is no obvious separation between the sexes indicating that induction of plasticity is a much stronger variable than gender.

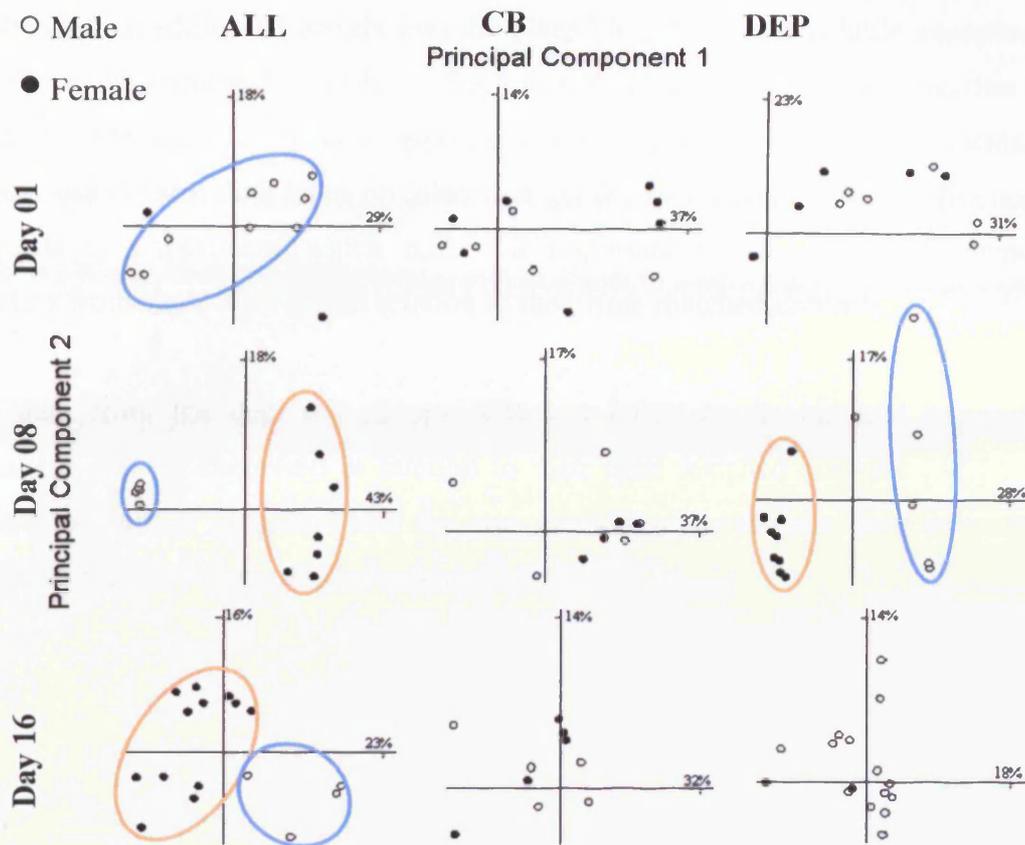


Figure 5.11: PCA analysis of the microarray data separated into females and males. There is no obvious gender separation after Day 1 of the experiment but there are certain differences at Day 8 and Day 16 of deprivation. However, the two genders seem to be overlapping at the chessboard conditions (CB); an indication that gene expression is driven by plasticity and not gender. The percentages indicate the proportion of each variant. The clear dots are male whereas the black dots are female mice. The blue circles surround the male grouped data and the orange circles surround the female grouped data for a more obvious separation of the genders.

5.9. Conclusions

The observations stated in this chapter clearly indicate that in addition to the extent of anaesthesia providing a significant contribution to expression profiles there is a significant difference between the response of female and male animals. Therefore all further analysis should be expressed in respect to appropriate time matched control animals and all data should be separated into gender specific groups.

The anaesthetic effect on the data was indicated with the NMDA receptor study which has shown a significant change in expression of the receptors under study (as shown

in Figures 5.6, 5.7 and 5.8). The study performed on the control anaesthetised animals has provided an additional insight into the plausible effects that volatile anaesthetics can have on experimental procedures. Such a study requires further investigation and will most certainly provide new opportunities in plasticity related experiments. In addition, our control data have revealed that gender has a confounding influence in our plasticity experiment which makes it important to study the two genders separately from each other and in relation to their time matched controls.

Thus, data from the deprived groups (CB and DEP) in the targeted microarray experiment will be expressed in relation to their time matched controls (ALL) and they will be further separated into the two genders to investigate gene expression profiles altered by experience dependent plasticity.

CHAPTER 6

TARGETED PLASTICITY

6.1. Overview

Analysis of the control samples used for the targeted plasticity microarray experiment (Chapter 5) has led to the discovery of two major confounding factors affecting plasticity in the rodent's brain, extent of anaesthesia and gender. Whereas, the initial hypothesis was to study plasticity induction using whisker deprivation, it soon became apparent that transcription within the barrel cortex was influenced substantially by the anaesthesia mandatory for the surgical procedure. Furthermore, substantial differential responses were observed between the responses of male and female subjects. Genes affected by plasticity may be distinguished from the influences of the confounding factors by performing specific normalisation using time matched controls together with gender separation. Therefore each time point (1, 8 and 16) will now be considered as an independent experiment and its data expressed in relation to its time point controls (ALL 1, ALL 8 and ALL 16 respectively). In addition, the data from each time point will be further separated into the two genders allowing us to study the effect of deprivation in relation to sex. This chapter aims to provide a better insight on how mechanisms (such as deprivation) which can induce plasticity, affect gene expression throughout time taking into account gender differences. Ontological analysis of the affected transcripts will be provided in order to better visualise the results and obtain a better understanding of the pathways involved in processes related to depression and potentiation, the two main mechanisms for memory and learning (Section 1.9.4).

6.2. Genes affected by deprivation

For the targeted plasticity experiment (for list of genes refer to Appendix 5), the data was separated into the three different time points (1, 8 and 16 days) and every time point was further divided into the three deprivation conditions (ALL, CB and DEP). Data from each time point was normalised using Global Lowess and it was expressed

in relation to the time-matched control animals (ALL), with the genders treated separately where appropriate. Following normalisation, the deprived animals (CB and DEP) from each time point were investigated further to identify the number of genes affected by each deprivation type (CB or DEP) for each day (Figure 6.3 indicates the layout of this step).

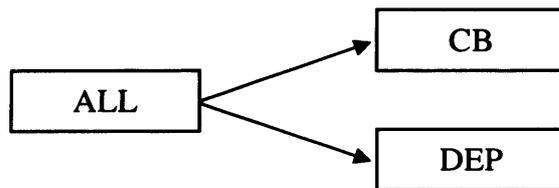


Figure 6.1: Schematic representation of the comparison employed in order to identify affected transcripts which were later subjected to functional analysis. Every time point was subjected into the same method of comparing; ALL with CB and ALL with DEP.

Identification of genes differentially expressed between the three types of treatment, undeprived (ALL), chessboard (CB) and the removal of all whiskers (DEP), throughout the chosen time course employed both the relative level of gene expression, the fold change, and the statistical confidence (p-value) that the gene expression was different to time matched control animals (Figure 6.1). These parameters were used to generate a subset of genes acquired from the full data set, initially without any gender differentiation, where $p\text{-value} < 0.05$ and expression change > 2 fold. The number of transcripts thus differentially expressed at each time point was thereby determined (Figure 6.2). It is clear that the least number of differentially expressed genes is found after 1 day of deprivation rising to a peak after 8 days with the amount of genes falling significantly at day 16.

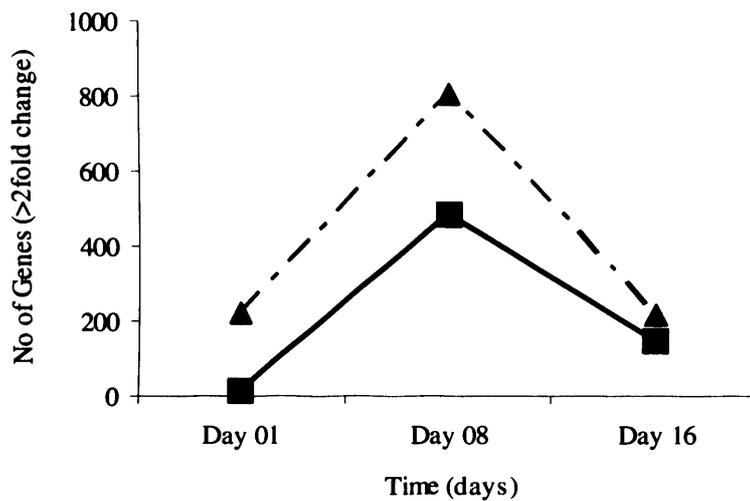


Figure 6.2: Time course of number of differentially expressed transcripts within the barrel cortex upon various patterns of whisker deprivations. Differential expression is defined by a >2 fold change in expression exhibiting a significance of $p < 0.05$ between time matched control (undeprived) (ALL), chessboard (CB, solid line and square) and fully deprived animals (DEP, dashed line and triangle). It is rather interesting that both types of whiskers deprivation show a peak of gene expression after 8 days, which is consistent with the critical period referred to in the literature.

When similar analysis are performed with separate genders very different observations are made (Figure 6.3 A and B) with the two sexes showing different temporal profiles in the number of differentially expressed genes detected under the two facial hair deprivation treatments. In particular far fewer transcripts have been affected in the males by the chessboard deprivation throughout the time course, whereas the females show a similar peak for both treatments after 8 days. One can argue that during the period of the experiment the males are passing through adolescence, which can affect their gene expression. On the other hand the females are initiating their oestrogen cycle which has a four days period, which makes it possible that hormonal levels are not the same through out the study. Detailed analysis into the female population has shown greater variation in gene expression, which makes further investigation crucial.

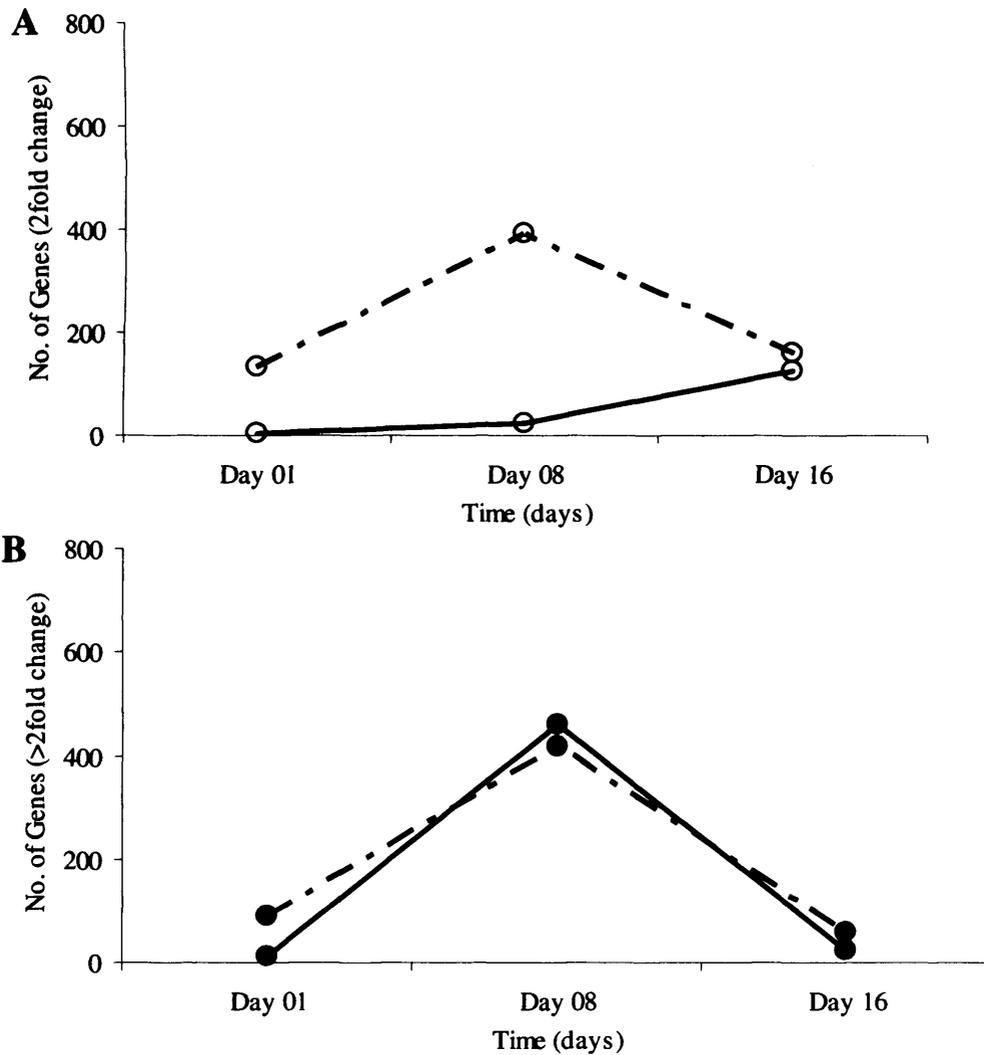


Figure 6.3: Gender specific analysis of number of differentially expressed transcripts within the barrel cortex upon various patterns of whisker deprivations. Differential expression is defined by a >2 fold change in expression exhibiting a significance of $p < 0.05$ between time matched control (undeprived) (ALL), chessboard (CB, solid line) and fully deprived animals (DEP, dashed line). Genes differentially expressed in males (Panel A, open circles) and females (Panel B, closed circles) were analysed separately. This data indicates that there is a gender difference in the population of mice used for the study.

6.3. Ontological Bias Analysis

In order to better understand the processes of plasticity and the effect of deprivation, functional analysis was performed on differentially expressed genes, exhibiting >2 fold differential expression at a statistical confidence $p < 0.05$ (Section 6.2), to assess the statistical over-representation of genes belonging to particular functional categories. This was achieved by using the online version of “L2L” Microarray Analysis software which was configured with the ontological descriptors of the NIA clones which made up the majority of the genes on our targeted plasticity microarray. The proportion of effected transcript assigned to specific ontological categories is represented using pie charts to aid visualisation and allow for analysis of the comparative distribution of the functional impact of a given treatment at a specific time.

The purpose of the above functional analysis is not only to investigate different responses between the two genders but also to understand the genomics behind the plasticity paradigm. After the first day of experiment, the cellular balance is disturbed and cells (neurons, in this case) undergo major changes and reorganisation. Hence, functional categories such as “*cell activity*” and “*structural molecule activity*” are over-represented (Figure 6.4). Other ontological categories such as “*binding*” indicate cellular communication and protein activation in order for the subject to respond to the changes in external stimuli.

Most of the molecules represented by the functional category “*structural molecule activity*” (Figure 6.4) are ribosomal proteins which are involved in cellular processes of translation. This was expected from data at Day 1 as immediate responses to the introduction of procedure were detected. Another molecule detected at the Day 1 data was fibronectin 1 which is involved in adhesive and migratory processes of cells (Muro *et al.* 2003), indicating cellular communication at Day 1. Another molecule mediating cellular adhesion and communication is catenin beta 1 which was included in the Day 1 data (Figure 6.4) as a member of the “*structural molecule activity*” functional category. Research has also shown that beta-catenins function during mammalian neuronal development and are involved in neural precursor cells

generation (Chenn and Walsh 2002); indicating a possible migration of immature neurons, outgrowth of axons or even initial changes in the synapses.

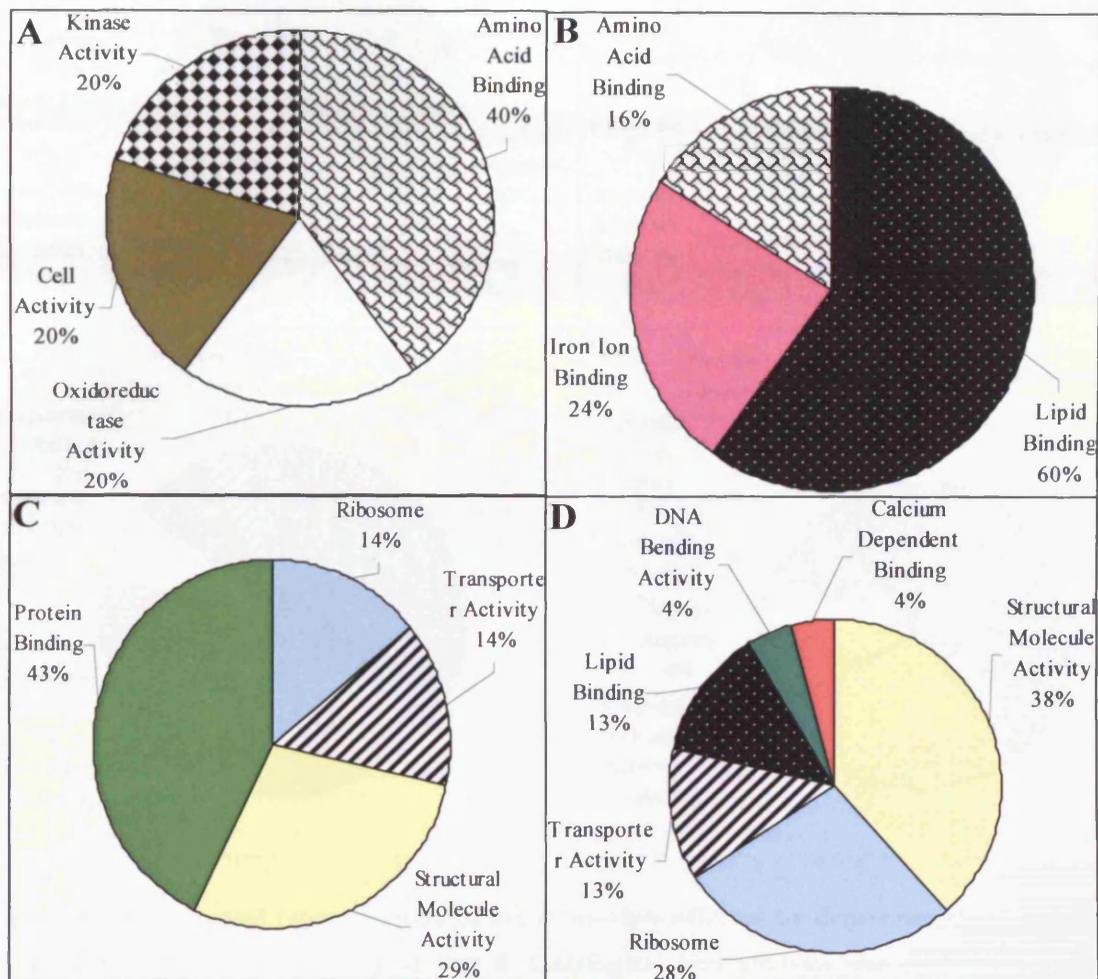


Figure 6.4: Ontological representation of the transcripts affected by deprivation (CB and DEP) in females (A&B) and males (C&D) at Day 1. Ontological bias analysis was performed on gene exhibiting significant changes (2 fold and $p < 0.05$). Panels A ($n=11$) and B ($n=91$) represent the data for the female population whereas Panels C ($n=5$) and D ($n=133$) represent the male data. Panels A and C show the effect of chessboard deprivation whereas Panels B and D show the effect of complete deprivation. The proportion of genes represented within the over-expressed ontological categories exhibit significant changes (2 fold and $p < 0.05$) between the control and experimental subjects. For full list of genes refer to Appendix 5.

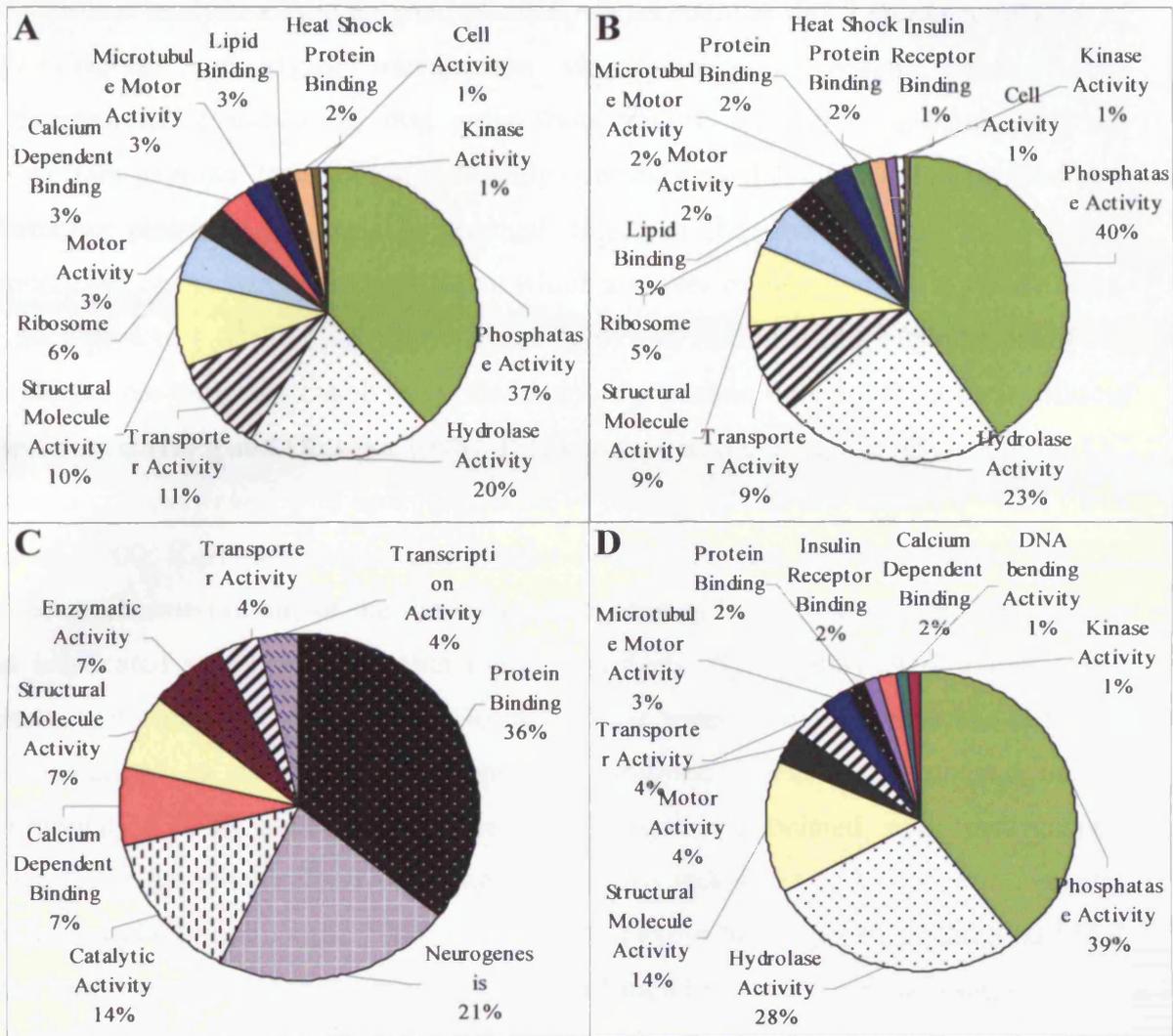


Figure 6.5: Ontological representation of the transcripts affected by deprivation (CB and DEP) in females and males separately at Day 8. Ontological bias analysis was performed on gene exhibiting significant changes (2 fold and $p < 0.05$). Panels A ($n=461$) and B ($n=416$) represent the data for the female population whereas Panels C ($n=23$) and D ($n=391$) represent the male data. Panels A and C show the effect of chessboard deprivation whereas Panels B and D show the effect of complete deprivation. The proportion of genes represented within the over-expressed ontological categories exhibit significant changes (2 fold and $p < 0.05$). For full list of genes refer to Appendix 5.

Functional analysis of the targeted plasticity experiment at day 8 shows regulation of transcription and signal transduction via “*calcium dependent binding*” and “*neurogenesis*”; indicating that gene transcription and cell communication are important neuronal functions significantly over-expressed ($p < 0.05$) after one week of inducing plasticity via whisker removal. Some of the transcripts included in the functional categories mentioned above which are over-represented at Day 8 are Heat-Shock protein 1 (HSPF1), Synaptophysin (Syp) and Taxilin Alpha (TxlnA). HSPF1 is a chaperone molecule involved in disassembling protein complexes, protein folding and export (Hata and Ohtsuka 1998). It is known that the 5' region of the gene is GC rich; a context linked with neurodegenerative diseases (Hata and Ohtsuka 1998; Chan *et al.* 2000; Kazemi-Esfarjani and Benzer 2000). The fact that this *HSPF1* gene is over-represented in one of the functional categories at Day 8, makes it possible that it is implicated in mechanisms that induce plasticity. Synaptophysin is a membrane protein of small synaptic vesicles located in the rodent's brain which has not been characterised as essential for synaptic transmission unless it is knocked out in combination with another molecule; synaptogyrin, associated with pre-synaptic vesicles in neuronal cells. Double knockout mice lack short and long term synaptic plasticity (Felkl and Leube 2008). The role of Taxilin has to yet be understood but it has been found that it is the binding partner of members of the syntaxin family in the rodent's central nervous system where it is highly expressed in motor neurons and sensory neurons. It has been suggested that through its interactions with syntaxins it plays a strong role in the maintenance and generation of neurons (Sakakibara *et al.* 2008) making its over-representation at Day 8 very important especially after deprivation.

The fact that molecules involved in “*calcium dependent binding*” leading to protein activation via phosphorylation are seen in both day 1 and day 8 (Figure 6.4 and 6.5) indicates that there are two kinds of changes; immediate that require early protein expression and prolonged that require maintenance or/and abolition of the early changes depending on the ability of the rodent's brain to adapt to the altered stimuli and the introduction of genes involves in long-term memory and synaptic strength. Prolonged changes are observed not only at Day 8 but also at Day 16 of the targeted plasticity microarray experiment. Analysis of Day 16 (Figure 6.6) has revealed that “*channel activity*” is the abundant GO category indicating synaptic plasticity and cell-

cell signalling. “*Channel activity*” might involve any type of channel, from potassium to calcium, even GABA or NMDA receptors. This suggests that the brain is working towards the maintenance of plastic changes that have occurred during the first two weeks of experiment in an attempt to respond to the continuous altered external stimulus. Some of the genes involved in “*channel activity*” are the calcium channel, voltage-dependent, L type, alpha 1D subunit (*Cacna1d*), the gap junction protein, alpha 1 (*Gjal*), the potassium channel, subfamily K, member 6 (*Kcnk6*) and the potassium voltage-gated channel, subfamily H (eag-related), member 3 (*Kcnh3*). Voltage-gated L-type Ca^{2+} channels (such as *Cacna1d*) are expressed in neurons and appear to be essential for normal auditory function and cardiac activity (Platzner *et al.* 2000). The gap junction protein is one of the two members of the connexin gene family, connexins 43 and 32, and it is mainly present in sympathetic and sensory nerve fibres as well as in perivascular nerve terminals in the rodent (Li *et al.* 2002; Hobara *et al.* 2006). It has also been found to be axonally transported in sensory axons (Knyihar-Csillik *et al.* 2001; Hobara *et al.* 2006) an immediate link to plasticity.

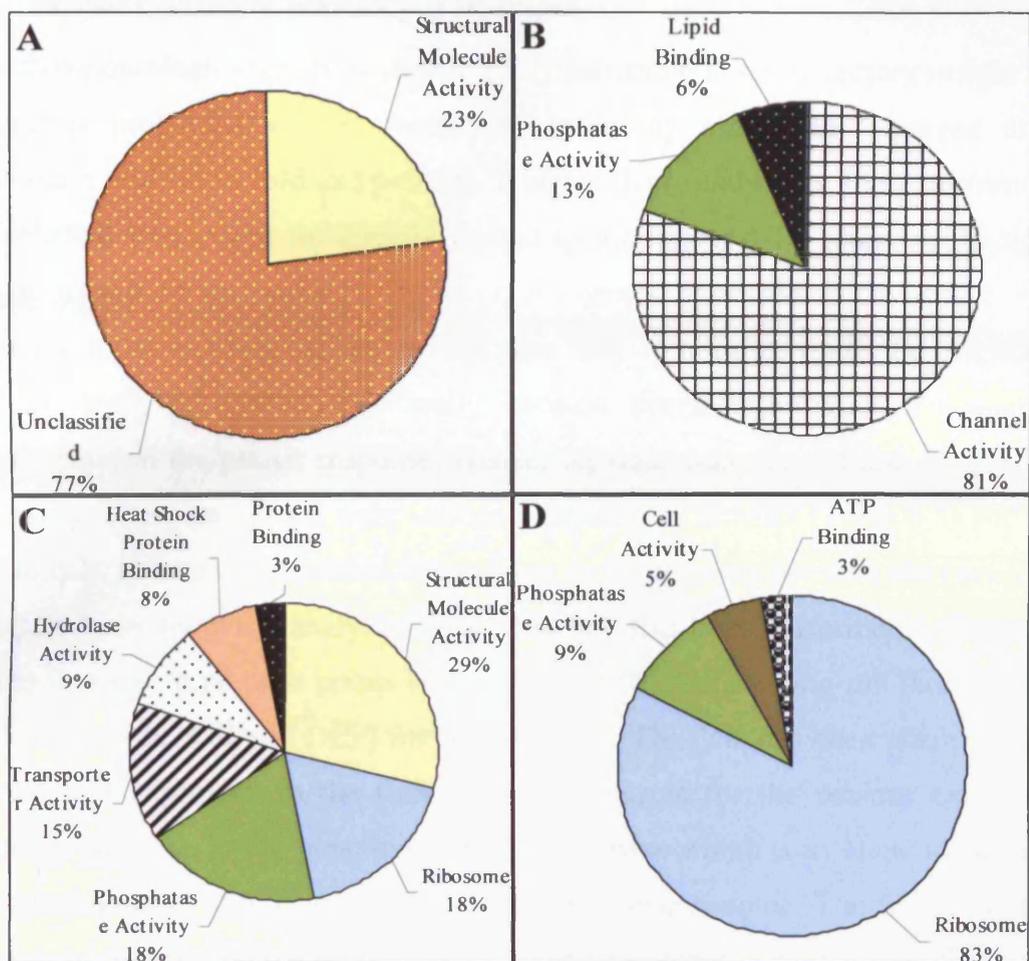


Figure 6.6: Ontological representation of the transcripts affected by deprivation (CB and DEP) in females and males separately at Day 16. Ontological bias analysis was performed on gene exhibiting significant changes (2 fold and $p < 0.05$). Panels A ($n=22$) and B ($n=57$) represent the data for the female population whereas Panels C ($n=126$) and D ($n=161$) represent the male data. Panels A and C show the effect of chessboard deprivation whereas Panels B and D show the effect of complete deprivation. The proportion of genes represented within the over-expressed ontological categories exhibit significant changes (2 fold and $p < 0.05$). For full list of genes refer to Appendix 5).

6.4. Bespoke putative plasticity transcripts

The above ontological analysis (Section 6.3) may provide a satisfactory insight on the functional properties of the transcripts exhibiting significant changes at their expression profiles (2 fold and $p < 0.05$). When such an analysis is being performed, all the selected transcripts are directly related to the mouse NIA clone set using their unique identification numbers; however, the genes presented in Table 2.6 are not included in that ontological analysis because they do not map on to the original NIA set and were introduced specifically because previous research indicated their involvement in the plastic response. Hence, separate analysis of these 53 genes was necessary. These transcripts were studied separately in females (Table 6.1) and males (Table 6.2), since expression has been shown to be different between the two genders (Chapter 5). A temporal analysis of the data has also been performed separating the results into the three time points (Day 1, 8 and 16) representing the three treatment conditions (ALL, CB and DEP) for both genders. The data has been normalised with Lowess and expressed to the time matched controls for the reasons explained in presiding chapters. Every gene has a systematic name which is its clone identifier and a unique identifier (MGI) which links it to the mouse genome. The female and male normalised data are presented in Tables 6.1 and 6.2, where significantly different (>2 fold change and $p < 0.05$) values are appropriately highlighted by colouration.

Gender independent clustering was performed (Figure 6.7) to better visualise the results depending on time and treatment. The two resultant cluster trees are presented; female and male (Figure 6.7). The sequence of the genes in the cluster trees follows their sequence in Tables 6.1 and 6.2 for females and males respectively. Clusters of genes are obvious throughout the trees; with genes showing a distinct up-regulation throughout the conditions, or an up-regulation at one time point over the others.

One cluster (Figure 6.7) in the female tree which exhibits significant down-regulation after 8 days of whisker deprivation (CB and DEP) includes glutamate receptors 1 and 3 (Section 1.10, Table 1.1), which show similar down-regulation in the male tree. Excitatory neurotransmission in the mammalian brain is mediated by glutamate receptors which also affect synaptic plasticity as well as LTP and LTD (Rodriguez-Moreno and Sihra 2007); Section 1.9.4).

Another interesting transcript exhibiting down-regulation at Day 8 after both types of deprivation (CB and DEP) is *nerve growth factor beta* (NGF), involved in the regulation of growth and differentiation of sensory neurons. The necessity of nerve growth factor beta was revealed when adult rats with injured sensory axons were treated with NGF, resulting in selective regrowth of damaged axons (Ramer *et al.* 2000), providing a possible functional relationship between NGF and neuronal plasticity. NGF is also associated with members of the neurotrophin family (Section 1.11), important for survival and maintenance of sensory neurons (He and Garcia 2004), strengthening its link with neuronal plasticity in the barrel cortex.

Deprivation has affected another transcript involved in synaptic processes; *Bassoon*. It seems to exhibit significant up-regulation in both genders after 8 days of chessboard (CB) and total (DEP) deprivation. Bassoon is an important pre-synaptic neuronal component (Winter *et al.* 1999) showing differential expression at the critical time point for plasticity. As indicated by the ontological analysis presented in Figure 6.5, day 8 seems to be mostly affected by deprivation, a result which agrees with the significant up-regulation of *Bassoon* as well as the down-regulation of some of the transcripts described above.

Treatment Time	Systematic	MGI ID	MGI symbol	FEMALE					
				CB	DEP	CB	DEP	CB	DEP
				1		8		16	
RICH_G3	MGI:97320	Klk1b4		0.7	1.5	6.5	6.3	1.1	2.2
ELE1	MGI:1277955	Bsn		1.1	0.7	3.1	4.4	1.5	1.9
ELE8	MGI:107755	Neurod2		0.8	1	2	3.2	1	3.1
ELE7	MGI:105088	Ntn1		1.6	1.7	1.7	1.6	1.3	1.1
ELE9	MGI:97380	Ntf3		1.1	1.2	1.5	1.9	1.5	0.9
RICH_B4	MGI:95739	Glul		1.3	1	1.2	1.2	0.9	1.4
RICH_F2	MGI:97912	Rheb		1.5	1.2	0.9	1.1	1.1	1.7
RICH_C3	MGI:96568	Ina		1.1	1.5	0.9	1.5	1	1.5
ELE4	MGI:105313	Citron		1.1	1	1	1.4	1.3	2.7
ELE10	MGI:97360	Nos1		1.5	1.6	1	1.2	1.9	1.8
ELE13	MGI:106530	PRG1		0.6	0.7	1.2	1	1	1.1
RICH_C2	MGI:108470	St6gal1		0.8	0.9	0.8	0.9	1.5	1.2
ELE6	MGI:95820	Grin2a		1.1	0.7	0.7	0.9	1.2	1
ELE11	MGI:97362	Nos3		0.9	0.8	0.7	1	2.1	1.7
RICH_D6	MGI:95811	Gria4		0.9	0.7	0.6	1.4	1.2	1.8
ELE17	MGI:1099446	Synpo		1.3	0.8	1	0.6	0.7	1.3
ELE5	MGI:109538	Cort		1.9	0.7	0.7	0.7	0.7	1.4
ELE3	MGI:101932	Cpe		1.1	0.9	0.7	0.9	0.8	0.6
ELE15	MGI:1923000	Slc10a6		1.2	0.8	0.7	0.7	1.1	0.7
RICH_G4	MGI:95524	FHF3		0.5	1.3	1	1.2	1.1	2.3
RICH_E3	MGI:1298366	Atf1		0.5	1.3	0.7	0.9	0.9	1.9
RICH_F3	MGI:88145	Bdnf		0.6	1.5	0.6	0.9	1.1	1.8
RICH_G1	MGI:1860487	Pcdh7		0.6	1.2	0.6	1.2	1.6	1.5
RICH_D2	MGI:88495	Crem		0.7	1.1	0.5	0.6	1.3	1.3
RICH_C4	MGI:99261	Ptgds		0.6	1.3	0.5	0.5	1.1	1.8
RICH_F4	MGI:95390	Engrailed 2		0.4	1.6	0.5	0.5	1.1	1.3
ELE18	MGI:2156052	Slc17a6		1	0.6	0.7	0.5	0.9	2.7
RICH_B6	MGI:95809	Gria2		1	0.9	0.5	0.6	1.1	2.1
ELE14	MGI:1330818	Qsox1		0.6	0.6	0.5	0.5	1.2	1.8
RICH_B1	MGI:108092	Bin1		0.5	0.8	0.4	0.4	1.2	1.5
ELE12	MGI:108295	Pxn		0.7	0.4	0.5	0.4	1	1.2
RICH_E2	MGI:2151253	Calca		0.6	0.4	0.4	0.6	1.2	1.2
ELE16	MGI:98387	Specb1		0.8	0.5	0.3	0.3	1	1.3
RICH_H4	MGI:107384	Dynamin 1		0.8	0.7	0.2	0.3	1.3	1.3
RICH_E4	MGI:108025	Prkar2a		0.6	1.1	0.3	0.3	1.1	2
RICH_A3	MGI:1194506	Serpini1		1.1	1.7	0.3	0.4	1.1	1.2
RICH_A2	MGI:98796	Tph1		2.1	0.9	0.2	0.4	1.3	1.5
RICH_H6	MGI:97175	Mtap 2		0.8	0.8	0.3	0.4	1.1	5.8
RICH_F1	MGI:98397	Src		0.5	0.6	1.1	1.4	4.1	2
ELE2	MGI:99702	Bax		0.7	0.6	0.8	1.2	3.8	2.5
RICH_B3	MGI:95634	Gad2		0.1	0.2	0.5	0.4	1.4	1.1
RICH_D4	MGI:1306778	Mtap1b		0.2	0.3	0.3	0.4	1.2	1.4
RICH_C1	MGI:1855700	Pcdh12		0.4	0.2	0.5	0.6	1.6	1.9
RICH_E1	MGI:97610	Plat		5.4	3	2.9	2.8	1.3	1.2
RICH_D3	MGI:109349	Atf2		6.8	7.7	1	0.9	1.1	1.1
RICH_H1	MGI:96224	Hras1		0.1	0	0.8	1.1	1.6	1.1
RICH_H3	MGI:97321	Ngfb		0	0	0.2	0.3	1.2	3.2
RICH_H2	MGI:2389091	Rps15a		0.6	0.8	0	0	0.9	45.9
RICH_D1	MGI:95660	Gas6		0.8	1.2	0	0	1	1.1
RICH_A6	MGI:95808	Gria1		1.3	1	0	0	1.1	1.9
RICH_C6	MGI:95810	Gria3		0.5	0.9	0	0	1.3	1.9
RICH_B2	MGI:108173	Prkg2		0.3	0.6	0	0	1.4	1.2

Table 6.1: Relative temporal expression levels of bespoke plasticity transcripts resulting from chessboard and total whisker deprivation in female mice. The local systematic name, the MGI ID and MGI gene symbols are presented along with the normalised values of each transcript. Values highlighted in “orange” exhibit a 2 fold up regulation whereas values highlighted in “green” exhibit a 2 fold down regulation with significance at $p < 0.05$. Genes are provided in the order they appear when the expression profiles are clustered using a distance algorithm (Figure 6.7).

Treatment Time	CB	DEP	MALE					
			CB	DEP	CB	DEP	CB	DEP
	1		8		16			
Systematic	MGI ID	MGI symbol						
RICH_G3	MGI:97320	Klk1b4	1.3	0.8	1.1	0.1	0.1	0
RICH_E1	MGI:97610	Plat	1.3	0.9	0.9	0	0.2	0.1
RICH_H2	MGI:2389091	Rps15a	0.9	0.4	1.2	0	0.9	0.8
ELE1	MGI:1277955	Bsn	0.8	0.8	3.8	2.5	0.7	1.4
RICH_F1	MGI:98397	Src	1.2	1	1	3.6	0.9	1.3
RICH_F4	MGI:95390	Engrailed 2	1.1	1.2	1.1	4.9	1.1	0.9
RICH_G1	MGI:1860487	Pcdh7	1.1	0.6	1.2	3.9	1	0.8
ELE5	MGI:109538	Cort	1.3	2.1	1.1	3.8	1.2	1.2
ELE18	MGI:2156052	Slc17a6	1.2	2	1.3	4.6	1.5	1.1
ELE4	MGI:105313	Citron	0.7	0.9	1.9	1.1	0.9	1.2
ELE3	MGI:101932	Cpe	0.7	1.4	2	1.3	1	0.8
ELE2	MGI:99702	Bax	0.8	0.8	1.6	1.8	0.5	1
ELE10	MGI:97360	Nos1	1.2	1	1.5	2.1	1	1.4
ELE15	MGI:1923000	Slc10a6	1.2	1.6	1.2	1.4	0.6	0.9
ELE14	MGI:1330818	Qsox1	1.2	1.2	1.2	1.6	0.7	0.8
RICH_B3	MGI:95634	Gad2	1	1.2	0.9	1.5	1	0.9
ELE6	MGI:95820	Grin2a	1.3	1.8	1.2	2.7	1.1	0.7
RICH_A3	MGI:1194506	Serpini1	1.1	1.4	1.3	2	1.3	0.9
RICH_H3	MGI:97321	Ngfb	0.5	1.4	1.2	2.5	1.1	0.9
RICH_D6	MGI:95811	Gria4	0.9	1.2	1.1	2.6	0.5	0.4
RICH_D2	MGI:88495	Crem	0.9	0.9	1.2	2.3	0.6	0.5
ELE16	MGI:98387	Specb1	0.8	1	1.2	1.7	0.6	0.5
RICH_B1	MGI:108092	Bin1	0.8	0.8	1	1.3	0.6	0.6
RICH_C1	MGI:1855700	Pcdh12	0.9	0.6	1.1	4.1	0.5	0.4
ELE11	MGI:97362	Nos3	1.2	1.4	1.3	1.9	0.3	0.7
RICH_C2	MGI:108470	St6gal1	1.9	0.5	1.2	2.1	0.5	0.6
ELE8	MGI:107755	Neurod2	0.7	1	2.4	1	3.2	3.8
ELE7	MGI:105088	Ntn1	1.5	1.1	1.3	1.1	2.9	1.5
ELE17	MGI:1099446	Synpo	1.2	1.5	1.4	1.6	1.8	1.4
ELE13	MGI:106530	PRG1	0.9	0.9	1.3	0.9	1.7	1.5
ELE9	MGI:97380	Ntf3	1.3	0.9	1.2	0.7	2.7	3.1
RICH_E4	MGI:108025	Prkar2a	1.1	1.2	1.2	2.1	1.9	3.5
ELE12	MGI:108295	Pxn	0.8	1.3	1.3	1.2	1.3	2.8
RICH_B4	MGI:95739	Glul	1	0.6	1.1	2	6.5	1.8
RICH_F3	MGI:88145	Bdnf	1.1	1.2	1.1	3	3.4	2.9
RICH_D4	MGI:1306778	Mtap1b	1.1	0.8	1.3	2.1	3.3	3.9
RICH_C3	MGI:96568	Ina	1.4	0.5	1	0.4	3.7	2.1
RICH_F2	MGI:97912	Rheb	0.9	1.5	1.3	11.9	0.6	0.6
RICH_E2	MGI:2151253	Calca	0.5	1.6	1.3	8.5	0.5	0.8
RICH_H1	MGI:96224	Hras1	1.2	0.6	1.2	13.8	0.4	0.4
RICH_D1	MGI:95660	Gas6	1	0.8	0.9	15.3	0.6	0.5
RICH_C4	MGI:99261	Ptgds	0.8	0.9	1.2	19.1	1	0.8
RICH_D3	MGI:109349	Atf2	0.9	1.7	1.1	0.2	0.3	0.3
RICH_G4	MGI:95524	FHF3	0.9	0.4	1	0.3	1.1	0.9
RICH_E3	MGI:1298366	Atf1	0.9	1.1	1	0.2	1.2	1.1
RICH_A6	MGI:95808	Gria1	1.2	0.8	1	0.2	1.1	1.2
RICH_B2	MGI:108173	Prkg2	1	0.9	0.9	0.4	1.1	1
RICH_H6	MGI:97175	Mtap 2	1.2	1.4	1.3	0.2	0.9	0.8
RICH_C6	MGI:95810	Gria3	1.3	1.7	1.1	0.2	0.6	0.7
RICH_B6	MGI:95809	Gria2	0.4	1.4	0.8	0.7	0.6	0.5
RICH_H4	MGI:107384	Dynamin 1	0.4	0.7	0.9	0.5	0.6	0.5
RICH_A2	MGI:98796	Tph1	0.4	1.2	1.2	0.6	0.1	0.2

Table 6.2: Relative temporal expression levels of bespoke plasticity transcripts resulting from chessboard and total whisker deprivation in male mice. The local systematic name, the MGI ID and MGI gene symbols are presented along with the normalised values of each transcript. Values highlighted in “orange” exhibit a 2 fold up regulation whereas values highlighted in “green” exhibit a 2 fold down regulation with significance at $p < 0.05$. Genes are provided in the order they appear when the expression profiles are clustered using a distance algorithm (Figure 6.7). Cross illustrates missing data.

Three transcripts are of particular consequence due to their considered involvement in neurogenesis and calcium dependent binding, *Nos1*, *Nos2* and *Bassoon* (highlighted in Figure 6.7). *Bassoon* has been previously shown to be involved in pre-synaptic connections, neurotransmission and axon formation (Bresler *et al.* 2004; Angenstein *et al.* 2008), whereas nitric oxide production by either NOS1 or NOS3 has been implicated in learning and memory (Chen and Popel 2007). Significant up-regulation of *Bassoon* at day 8 is observed during chessboard (CB) and complete whisker deprivation (DEP) within both female and male animals suggesting that deprivation at the critical point of day 8 has a much more profound effect than gender. Similarities can be seen at the expression profile of *Nos1* for both genders, suggesting once again that whisker deprivation and induction of plasticity is independent of gender. On the other hand, *Nos3* exhibits a gender specific different expression profile, an indication of a clear differentiation between males and females. Another possible explanation might be the presence of *Nos3* in endothelial cells making it difficult to replicate the results due to tissue variation between samples.

These three selected transcripts were selected for further analysis using quantitative PCR (QPCR) to provide validation of the microarray array, gain a better understanding of their implications in synaptic plasticity and also to understand how their expression is altered by whisker deprivation. Choosing a pre-synaptic molecule, such as *Bassoon*, might help us unravel pre-synaptic connectivity and signal transduction. On the other hand, studying nitric oxide synthase 1, involved with such a controversial molecule as nitric oxide, might provide a better idea of the function of this post-synaptic molecule with pre-synaptic effects.

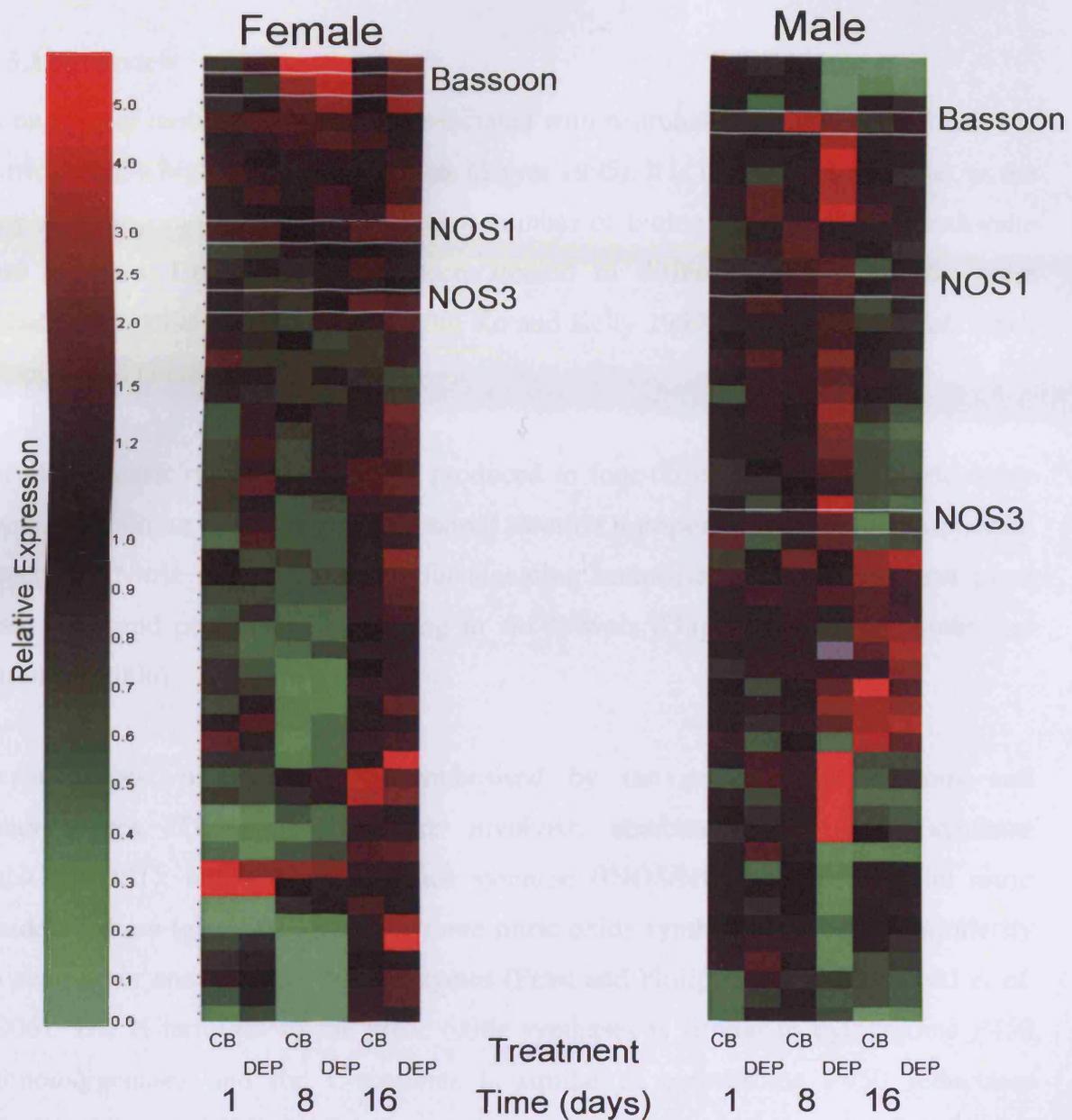


Figure 6.7: Hierarchical clustering of the expression profile of bespoke plasticity genes. A distance algorithm was used to cluster the genes within the bespoke set which displayed a significant change in expression at any time point, treatment condition or in either gender. The tree indicates relative expression profiles of genes listed in Tables 6.1 and 6.2 representing females (left) and males (right) respectively and listed in order they appear within the gender specific trees. Every column represents a different deprivation condition (CB and DEP) over time (Day 1, 8 and 16) as indicated at the bottom of the figure. The expression profiles of the three genes to be studied further are indicated on each tree.

6.5. Nitric Oxide

6.5.1. Overview

A number of molecules have been associated with neuronal plasticity; one of which is nitric oxide, a highly reactive molecule (Stryer 1995). It is considered, by some, as the key signaling molecule important for a number of biological processes (Garthwaite and Boulton 1995) and it has been studied in different regions of the brain (Garthwaite 1995; Arancio *et al.* 1996; Ko and Kelly 1999; Weitzdoerfer *et al.* 2004; Hopper and Garthwaite 2006).

In plants, nitric oxide is said to be produced in four different ways; by nitric oxide synthases, nitrate reductase, mitochondrial electron transport chain and non-enzymatic reactions. Nitric oxide, this powerful signaling molecule, has a role against plant pathogens and prevents early wilting in cut flowers (Gupta *et al.* 2005; Stohr and Stremmlau 2006).

In mammals, nitric oxide is synthesised by the endothelium, neurons and macrophages. Three enzymes are involved; neuronal nitric oxide synthase (nNOS/NOS1); inducible nitric oxide synthase (iNOS/NOS2) or endothelial nitric oxide synthase (eNOS/NOS3). The three nitric oxide synthases share great similarity to each other and to some p450 enzymes (Prast and Philippu 2001; Dudzinski *et al.* 2006). The N-terminus of the nitric oxide synthases is similar to cytochrome P450 monooxygenases and the C-terminus is similar to cytochrome P450 reductases (Dudzinski *et al.* 2006). In order to synthesize nitric oxide, NOS3 and NOS1 require the presence of calcium, in contrast to NOS2 (Dudzinski *et al.* 2006). The link of NOS3 and NOS1 to calcium is via an amino acid loop within the FMN binding domain of the C-terminus, which destabilizes the binding of calmodulin at low calcium levels (Stuehr 1997). Production of nitric oxide is impeded in the case where calmodulin is not bound to the C-terminus causing the lack of electron transfer from NADPH (Stuehr 1997).

In humans, similar to mice, the same three nitric oxide synthase isoforms have been identified (Hall *et al.* 1994). The three human isoforms are located throughout the

genome at 17qcen-q12 (NOS2) (Marsden *et al.* 1994), 7q35-36 (NOS3) (Robinson *et al.* 1994) and 12q24.2 (NOS1) (Marsden *et al.* 1993). Physiological studies have revealed the diverse role of neuronal nitric oxide synthase (reviewed in Hall *et al.* 1994). It has been described as a neurotransmitter (Peunova and Enikolopov 1993), a fluid homeostasis regulator (Balligand *et al.* 1993) and it is also involved in sexual function (Lee *et al.* 1994). As revealed by sequence analysis and Southern blot, the human NOS1 is a 160kb long complex gene (Figure 6.8) consisting of 29 exons and 28 introns (Hall *et al.* 1994).

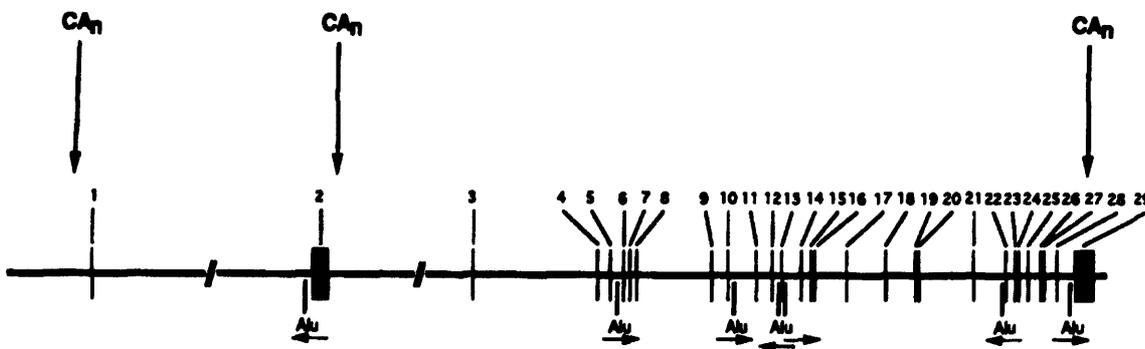


Figure 6.8: Representation of the structural organization of the human neuronal nitric oxide synthase gene. The exons (29 in total) of the gene are numbered and represented by black vertical boxes on the sequence. CA_n indicates dinucleotide repeat sequences (aC-dA) $_n$ (adapted from Hall *et al.* 1994).

Homozygous knockout mice show minimal enzymatic activity in some brain areas, such as the hippocampus, originating from the neuronally expressed NOS3 (Dinerman *et al.* 1994). Comparison between the three human isoforms of the nitric oxide synthase gene has revealed a high degree of conservation, indicating a common ancestral gene and a probable origination from gene duplication (Hall *et al.* 1994). It has recently been found that NOS1 has a domain of 250 amino acids more than NOS3 and NOS2, which links the enzyme to the synaptic membrane (Brenman *et al.* 1997) and interacts with the C-terminus of the NMDA receptors. It has been proposed that calcium entry through the NMDA receptors could activate nitric oxide generation by increasing calcium concentration and calmodulin binding (Sasaki *et al.* 2000).

Pharmacological methods have been used in the past to inhibit nitric oxide synthase activity, due to the lack of knockout mice. The use of NO-Arg, a known nitric oxide synthase blocker, has been found to block long-term potentiation (LTP) in hippocampal CA1 in guinea pigs, which was later rescued by the addition of L-arginine in high concentration (O'Dell *et al.* 1991). Use of other inhibitors, such as L-NAME, in order to study learning and memory tasks while NOS is inhibited showed that only some forms of memory were impaired whereas others were preserved (Knepper and Kurylo 1998).

Nitric oxide is one of the most controversial molecules in neuroscience. Evidence from different research groups on its properties and implications in plasticity has not reached a consensus. In an attempt to understand the importance of this molecule in learning and memory mechanisms, NOS3 was studied in combination with NOS1. Double mutants were studied in order to observe the effect the mutations have on LTP (Son *et al.* 1996); concluding that Nitric Oxide Synthase is involved in LTP. They suggested that the variability on the published NOS data is due to the fact that although NOS is involved in LTP, it is not required. Furthermore observations have been further confounded due to the fact that most inhibitors used in electrophysiological recordings are not specific. Son *et al.* (1996) provided evidence of compensation between the two forms of NOS (Neuronal and Endothelial) suggesting that LTP is reduced in double but not in single mutants (Son *et al.* 1996) which is in contrast with the impairment of LTP found in NOS3 mutant mice identified by other researchers (Haul *et al.* 1999).

6.5.2. NO involvement in learning and memory

Long-term potentiation (LTP) and long-term depression (LTD) are the two most important components of synaptic plasticity (Section 1.9.4). Postsynaptic injections of NO synthase inhibitors, such as L-NAME, on rat hippocampal slices have prevented the induction of LTP; indicating the postsynaptic location of NO synthases (Figure 6.9) and their signaling to the pre-synaptic neurons (O'Dell *et al.* 1991; Holscher 1997; Calabrese *et al.* 2007).

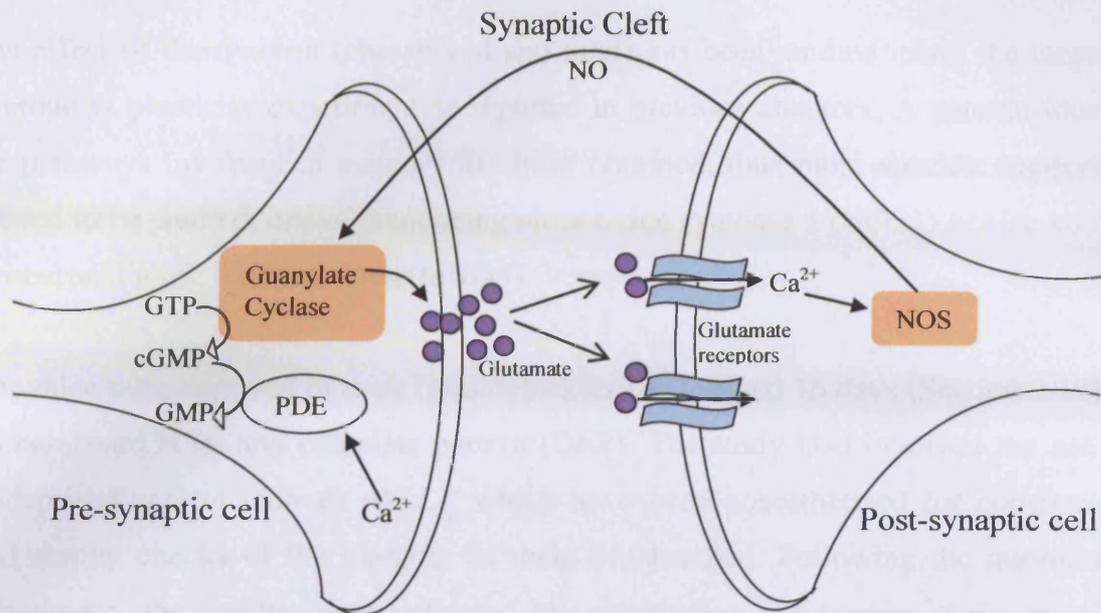


Figure 6.9: Representation of post-synaptically located nitric oxide synthase and its pre-synaptic signalling. Glutamate receptors on the post-synaptic cell are activated by the glutamate release from the pre-synaptic cell, causing increased levels of intracellular calcium which activates nitric oxide synthases (NOS). Nitric oxide (NO) travels through the cellular membranes and is being absorbed by Guanylate Cyclase in the pre-synaptic cell. Guanylate Cyclase is activated via the production of cGMP and due to NO binding, leading to neurotransmitter release. The calcium-activated phosphodiesterase (PDE) helps to decrease cGMP levels once Guanylate Cyclase has been activated (Holscher 1997).

Blocking NO has an inhibiting effect on LTP. Since LTP is thought to be related to memory, this inhibition could in turn affect learning in animal models. This was confirmed by behavioural experiments in rats using a water maze. Chapman *et al.* has observed amnesia after the administration of NOS inhibitors (Chapman *et al.* 1992; Holscher 1997). Spatial memory deficits have been observed in similar behavioural experiments where rats have been tested in a radial-arm maze after the injection of L-NARG (Holscher 1997). However, not all forms of learning are NO-dependent as shock-avoidance learning has been unaffected. On the other hand, there has been evidence that spatial memory may not be affected by the inhibitors, after pre-trained rats in water maze have prevented learning impairment (Holscher 1997).

6.6. Results

6.6.1. Overview

The effect of deprivation (chessboard and total) has been studied using the targeted microarray plasticity experiment as reported in previous chapters. A general idea of the pathways involved in memory has been obtained, thus more specific transcripts needed to be studied, one of them being nitric oxide synthase 1 (NOS1) or else known as neuronal nitric oxide synthase (nNOS).

The mice were deprived of their facial whiskers for 1, 8 and 16 days (Section 2.12) in a chessboard (CB) and complete pattern (DEP). The study also involved the use of undeprived control animals (ALL), which have been anaesthetized for consistency and regular checks of the integrity of their whisker pad. Following the microarray experiment, the results were validated by quantitative polymerase chain reaction (QPCR; Section 2.27).

6.6.2. NOS1 / nNOS

Lowess normalisation followed by expression of the results relative to their time-matched controls was employed. Thus, the data from the three different time points (Day 1, 8 and 16) were normalised to the undeprived animals of the matched time point. This way each group of data from the three different time points was considered a different independent experiment.

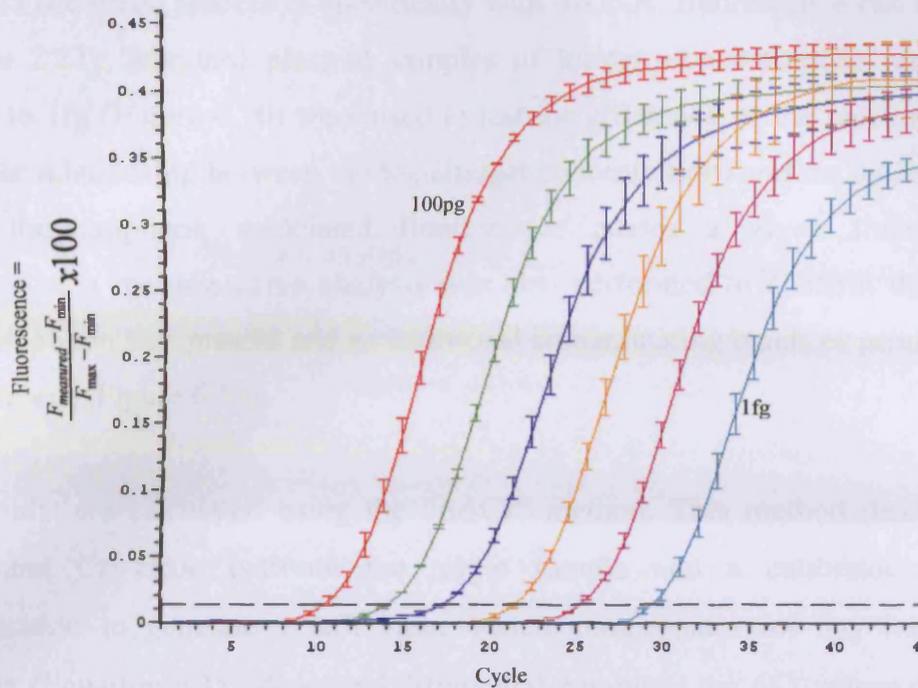


Figure 6.10: Exemplar *NosI* QPCR calibrator amplifications. The differently colored lines correspond to a 10 fold dilution series of *NosI* target gene ranging from 100 pg to 1 fg appearing from left to right on the graph. Calibrators were prepared using a spectrophotometrically quantified plasmid preparation for a sequence verified cloned of the *NosI* gene.

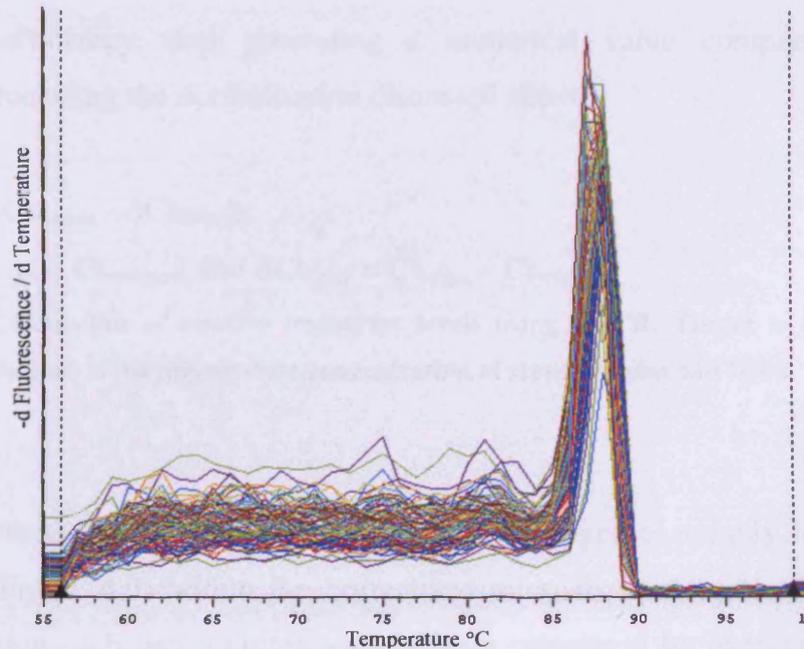


Figure 6.11: Dissociation curve (melting curve) of *NosI* amplicon. Temperature was raised from 55°C to 99°C and fluorescence recorded every 1°C in order to determine the rate of the decrease in fluorescence. Secondary or art-factual products exhibit a dissociation temperature distinct from the target amplicon.

When SYBR-green associates specifically with dsDNA, fluorescence can be detected (Section 2.27). Standard plasmid samples of known concentrations ranging from 100pgs to 1fg (Figure 6.10) were used to test the efficiency of the reaction verifying the linear relationship between the $\log_{10}(\text{target concentration})$ and the cycle number at which the amplicon associated fluorescence passes a given threshold (Ct). Furthermore a melting curve analysis was also performed to confirm that only the target amplicon was present and no additional contaminating bands or primer artifacts were present (Figure 6.11).

The results are calculated using the “ $\Delta\Delta\text{Ct}$ ” method. This method determines the differential Ct value between the target sample and a calibrator of known concentration to generate a ΔCt value which compensates for any inter-analysis variation (Equation 6.1). A second differential combines the ΔCt values of the gene of interest with the equivalent ΔCt value for the housekeeping gene to generate the final $\Delta\Delta\text{Ct}$ (Equation 6.1). This allows us to express the expression of our target gene relative to the house-keeping gene providing appropriate normalization accounting for minor differences in cDNA concentrations resulting from factors, such as reverse transcription efficiency, and generating a numerical value comparable to the microarray value using the normalisation discussed above.

$$\Delta\Delta\text{Ct} = 2^{-(\Delta\text{Ct}_{\text{Gapdh}} - \Delta\text{Ct}_{\text{Gene}})},$$

$$\Delta\text{Ct}_{\text{Gapdh}} = \text{Ct}_{\text{target}} - \text{Ct}_{\text{reference}} \text{ and } \Delta\text{Ct}_{\text{Gene}} = \text{Ct}_{\text{target}} - \text{Ct}_{\text{reference}}$$

Equation 6.1: Calculation of relative transcript levels using QPCR. Target is the sample in question and reference is the appropriate concentration of standard plasmid DNA.

Gapdh was selected as a reference gene since it displayed extremely low variation (Section 4.9, Figure 4.9) within the normalized microarray data. *Nos1* and *Gapdh* expression within each barrel cortex sample were calculated by averaging the three technical replicates to obtain a single numerical Ct value. A mean $\Delta\Delta\text{Ct}$ (i.e. the relative changes in transcript level) was calculated as a mean of three independent animals experiencing identical treatment at specific time points.

It is apparent that after one day of deprivation, total or chessboard, there is no significant changes in the expression levels of *Nos1* compared to the control undeprived mice (Figure 6.12). However, after eight days of deprivation, there is a two-fold increase of *Nos1* in the chessboardly deprived animals (CB 8) which increases even more in the totally deprived animals (DEP 8). After two weeks (Day 16) of deprivation, though, the levels of *Nos1* in the mouse barrel cortex decrease again, this being the case for both types of deprivation (CB and DEP).

T-tests were performed on the microarray and QPCR data which compared within the time points but not between them, as each time point is considered an independent experiment. Due to the method of normalisation (Lowess and relative expression to the time-matched control animals) every time point is considered a different experiment so no t-test was performed between groups at different days. T-tests revealed a significant change in expression with p value < 0.01 for both deprivation conditions at day 8.

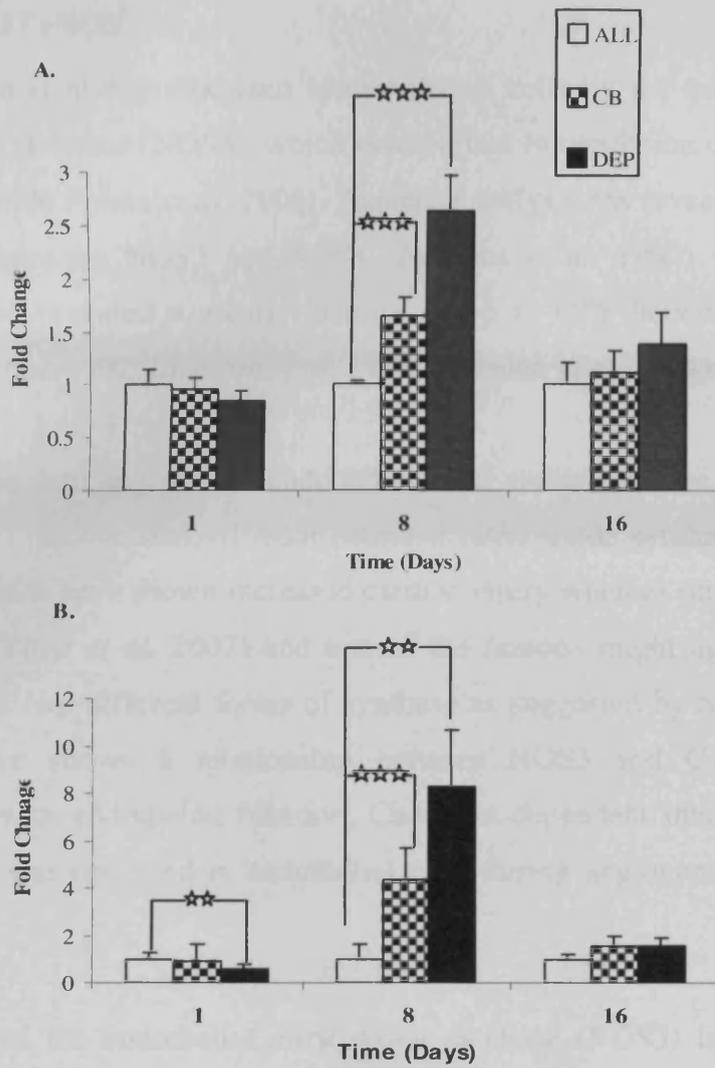


Figure 6.12: Relative *Nos1* gene expression at various time points and under different pattern of whisker deprivation measured by microarray (Panel A) and QPCR (Panel B). Double stars indicate significance with p value<0.03. Triple star indicates significance with p value<0.01.

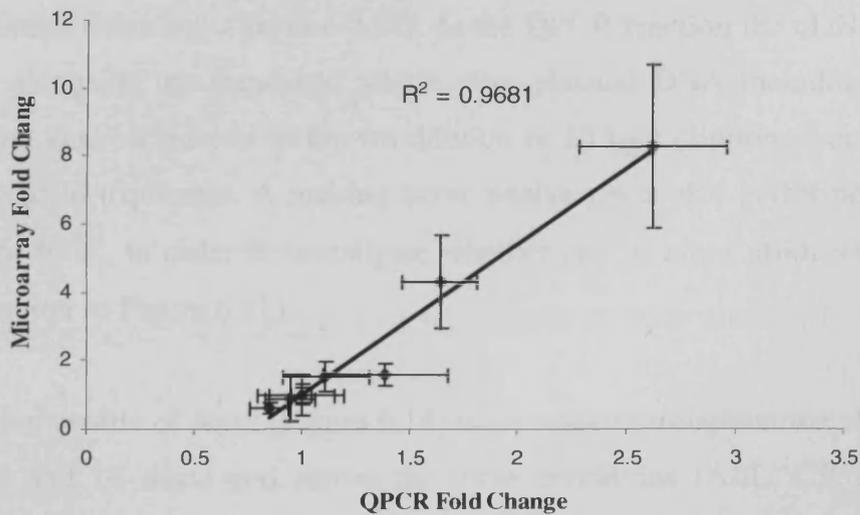


Figure 6.13: Correlation between relative *Nos1* expression levels measured by microarray and QPCR.

6.6.3. NOS3 / eNOS

Nitric oxide is also synthesized in endothelial cells by the endothelial form of the nitric oxide synthase (NOS3), which is important in regulation of vasomotor tone and blood flow (De Palma *et al.* 2008). Sequence analysis has revealed a 52% amino acid similarity between NOS3 and NOS1 (Janssens *et al.* 1992) while other sequence studies have revealed a greater identity of up to 60% between the two synthases (Marsden *et al.* 1992; Marsden *et al.* 1993; Marsden *et al.* 1994).

Nitric oxide derived from the endothelial nitric oxide synthase, is as a controversial molecule as the one derived from neuronal nitric oxide synthase. Studies on NOS3 knockout mice have shown increased cardiac injury whereas others failed to replicate the result (Zhao *et al.* 2007) and one of the reasons might again be compensation between the two different forms of synthase as suggested by Son *et al.* 1996. Other studies have shown a relationship between NOS3 and Cadmium; a molecule associated with endothelial function. Cadmium-dependent inhibition of nitric oxide production was observed in endothelial cells during angiogenesis (Majumder *et al.* 2008).

The study of the endothelial nitric oxide synthase (NOS3) is quite similar to the neuronal nitric oxide synthase (NOS1; Section 6.6.2). The microarray experiment is the same for every gene studied as well as the analysis and normalisation methods (Section 6.6.2). The validation of the microarray data was once again performed using the SYBR Green chemistry (Section 2.27). In the QPCR reaction the cDNA samples were tested alongside the standards, which were plasmid DNA including the PCR product of the gene of interest in known dilution in 10 fold dilutions from 100pg to 1fg represented in triplicates. A melting curve analysis was also performed, ranging from 55°C to 99°C, in order to investigate whether one or more products are being produced (similar to Figure 6.11).

The expression profile of *Nos3* (Figure 6.14) is presented throughout the chosen time course (1, 8 and 16 days) and across the three conditions (ALL, CB and DEP). Significance is represented with stars (single, double and triple) depending on the p value. The *Nos3* microarray data (Figure 6.14, Panel A) indicated a trend in

expression similar to that of *Nos1* (Figure 6.12, Panel A); suggesting that they might be affected by deprivation in a similar manner which is in agreement with the literature that wants these two forms of NOS closely related to each other (Son *et al.* 1996).

QPCR validation (Figure 6.14, Panel B) was not as successful this time due to the fact that it was performed on a new population of wild type mice. Because of the limited amount of material, not enough cDNA was produced for QPCR validation from the initial subjects, making it important for the experiment to be repeated with new animals. Although the same method was used to extract the barrel cortex, RNA and synthesis of cDNA, the validation of *Nos3* was not as successful. Main reason is believed to be the fact that *Nos3* is endothelial and the amount of NOS3 amplified will vary depending on the amount of blood vessels carried in the sample. However a similar trend with the microarray can be detected in Day 1 of the experiment, whereas the chessboard animals are not in agreement for the other two time points.

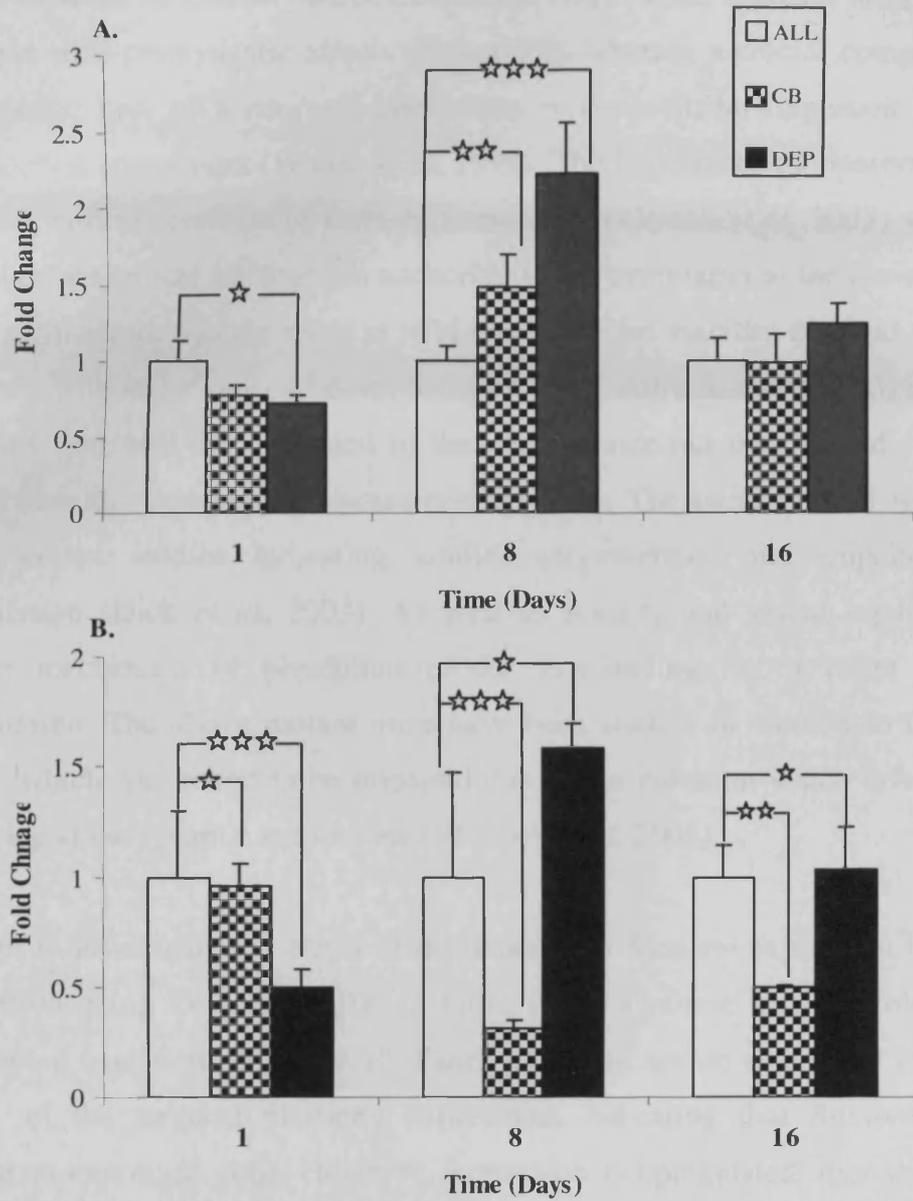


Figure 6.14: Relative *Nos3* gene expression at various time points and under different pattern of whisker deprivation measured by microarray (Panel A) and QPCR (Panel B). Single stars indicate significance with p value <0.05. Double stars indicate significance with p value <0.03. Triple star indicates significance with p value <0.01.

6.6.4. Bassoon

At the formation of synapses a number of pre-synaptic and post-synaptic molecules are involved for successful neurotransmission. Nitric oxide synthase is a post-synaptic molecule with pre-synaptic effects (Figure 6.9), whereas a crucial component of the pre-synaptic part of a neuronal connection is Bassoon; an important pre-synaptic cytoskeleton component (Winter *et al.* 1999). The importance of Bassoon (Bsn) was revealed with the creation of BSN-deficient mice (Altrock *et al.* 2003) which lacked central exons critical for Bassoon anchoring to the cytomatrix at the pre-synaptic site. Brain architecture was the same as wild type mice but viability dropped to 50% after 6 months with major cause of death being epilepsy. Altrock *et al.* (2003) revealed that synapses were still being formed in the mutant mice but they lacked regulation of neurotransmitter release at glutamatergic synapses. The same animals were used for visual cortex studies indicating similar abnormalities and impaired synaptic transmission (Dick *et al.* 2003). As well as sensory and visual input, hearing is another mechanism of perception of the surroundings as it relies in synaptic transmission. The above mutant mice have been studied in relation to their hearing ability, which was found to be impaired due to the mutation which affected protein anchoring at the synaptic active zone (Khimich *et al.* 2005).

In order to investigate the effect of deprivation on *Bassoon* expression the data was normalised using Lowess similar to nitric oxide synthase and the fold change is represented over time (Figure 6.15, Panel A). There are no significant changes after Day 1 of the targeted plasticity experiment, indicating that *Bassoon* is not an immediate expressed gene. However, expression is upregulated after the first week (Day 8) of chessboard deprivation (CB; almost three fold), a response linked to the severity of deprivation as a greater up-regulation is observed when total deprivation is applied for eight days (DEP 8). Expression seems to decrease after the second week (Day 16) of deprivation indicating that the observed changes are not prolonged ones.

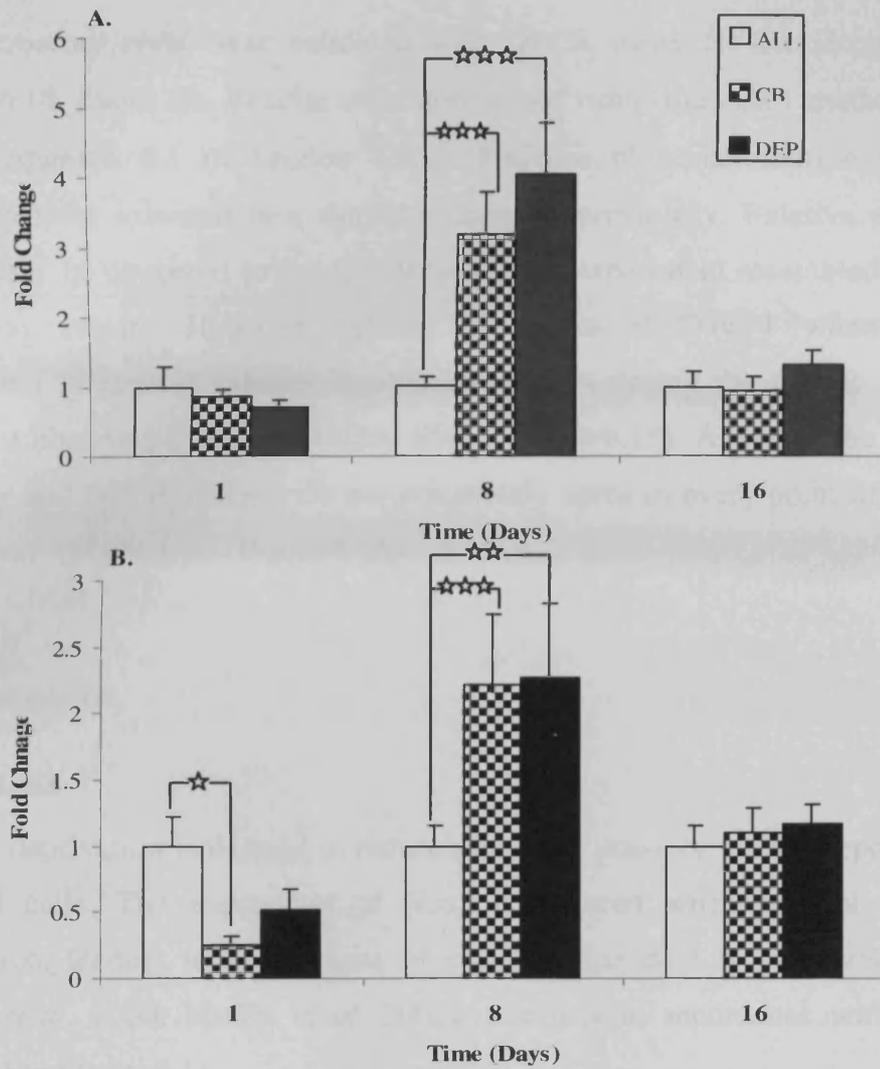


Figure 6.15: Relative *Bassoon* gene expression at various time points and under different pattern of whisker deprivation measured by microarray (Panel A) and QPCR (Panel B). Single stars indicate significance with p value <0.05. Double stars indicate significance with p value <0.03. Triple star indicates significance with p value <0.01.

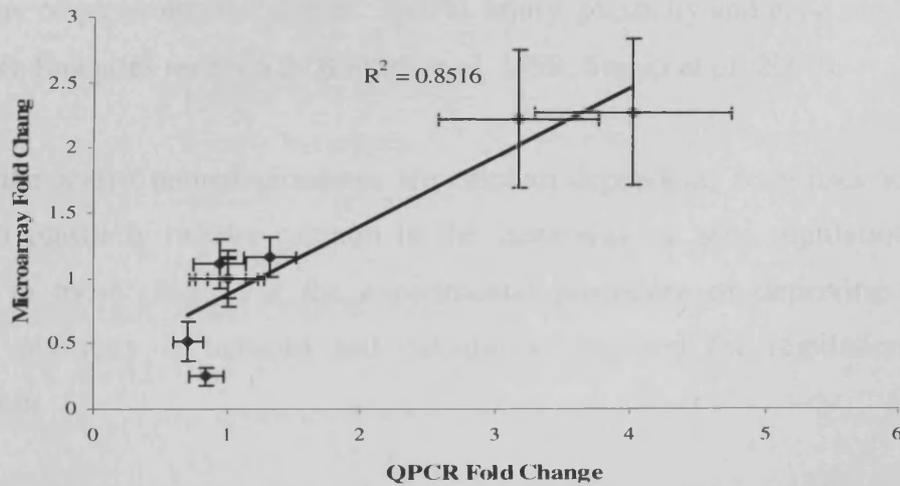


Figure 6.16: Correlation between relative *Bassoon* expression levels measured by microarray and QPCR.

The microarray result was validated with QPCR using SYBR Green Chemistry (Figure 6.15, Panel B). Results were normalised using the $\Delta\Delta C_t$ method explained earlier (equation 6.1 in Section 6.6.2). Presence of contamination and reaction efficiency were assessed in a similar manner as previously. Relative expression at Days 8 and 16 observed in the QPCR validation experiment resembled the targeted microarray results. However, relative expression at Day 1 when chessboard deprivation is applied exhibits significant changes during the QPCR validation; a result not observed in the microarray study (Figure 6.15). Although the results from the array and QPCR analysis do not completely agree in every point of the study, a correlation was obtained (Figure 6.16).

6.7. Discussion

6.7.1. NOS1

Whisker deprivation is thought to induce plasticity, possibly via the depolarization of neuronal cells. The expression of *Nos1* is induced when cortical neurons are depolarized, leading to an increase of calcium-dependent NOS1 catalytic activity (Sasaki *et al.* 2000; Maffei *et al.* 2003), which is in accordance with the results presented in Figure 6.11.

It was believed that NOS1 was a constitutively expressed enzyme (Bredt and Snyder 1992), however during the last decade research has revealed that NOS1 is responding to a number of physiological stimuli, such as injury, plasticity and gene regulation, via its CRE binding sites on exon 2 (Boissel *et al.* 1998; Sasaki *et al.* 2000).

Many of the above named processes are calcium dependent; responses to neuronal injury and plasticity require calcium in the same way as gene regulation is CRE-mediated in most cases. Via the experimental procedure of depriving the facial whiskers, plasticity is induced and calcium is required for regulation of gene transcription.

An increase in the expression of *Nos1* is observed after one week, which coincides with the critical period described by Fox in 1992. The fact that no increase is seen

after the first day of facial hair loss indicates that *Nos1* is not one of the early expressed genes, such as heat shock proteins and kinases which respond to physiological stimuli and pathological conditions at once. The fact that NOS1 is up-regulated after one week of induction of plasticity coincides with the fact that late-phase LTP is a long lasting form of synaptic transmission (Section 1.9.4), hence NOS1 is implicated in prolonged brain changes in the mouse barrel cortex associated with long term memory and learning (Lu *et al.* 1999).

As mentioned previously (Hall *et al.* 1994), the NOS1 gene comprises of 29 exons and 28 introns making a very long transcript. Alternative splicing of exon 1 to exon 2 results in a similar functioning protein (Wang *et al.* 1999a; Wang *et al.* 1999b). Other alternative splicing events give rise to more identical transcripts (reviewed in Sasaki *et al.* 2000). It is believed that the existence of so many diverse splice variants is important for the regulation of NOS1 expression in mammals, especially during the disruption of synaptic plasticity and in response to neuronal development and injury (reviewed in Sasaki *et al.* 2000).

6.7.2. NOS3

Nitric oxide has been associated with cell-cell signalling and cell communication (Calabrese *et al.* 2007) due to its properties to travel between post and pre-synaptic cells (Figure 6.9). Apart from the NOS1 isoforms, discussed above, NOS3 is also responsible for NO production and it has been found significantly over-expressed in a number of pathological conditions, such as nerve injury and other neurodegenerative diseases (De Palma *et al.* 2008). Although, NOS3 is the endothelial form of nitric oxide synthase, it has been identified in neuronal cells and high enzyme concentrations are associated with neuronal conditions (Kashiwado *et al.* 2002). The relationship between the neuronally expressed NOS3 and neuronal damage was studied by De Palma *et al.* (2008) using human neuroblastoma cells to specifically investigate Amyotrophic Lateral Sclerosis (ALS). It was shown that NOS3 is over-expressed in the motor neurons of the spinal cord in ALS patients which associated the presence of this enzyme with the presence of toxic stimuli – a common sign of ALS – which in turn might indicate a protective role of NOS3 on the neurons.

The targeted microarray results have shown that *Nos3* expression was considerably increased in deprived animals (CB and DEP) after 8 days of deprivation and it seems that the observed increase was relative to the severity of deprivation with total deprivation (DEP) having a greater effect (Figure 6.14, Panel A). The targeted microarray results indicated an enzymatic response to the whisker plucking and possibly a neuronal protection function. Due to the lack of stimuli, one might assume that neuronal connections are being weakened, thus over-expression of NOS3 is crucial for the protection of neurons forming synaptic connections within and between the barrels. The result, however, was not totally validated by the QPCR (Figure 6.14, Panel B). In order to draw any valid conclusions, the experiment will have to be repeated with a larger number of biological replicates.

6.7.3. Bassoon / BSN

Synapses are the sites of contact between neurons establishing cell-cell communication and signal transduction. Synapses have pre-synaptic and post-synaptic terminals as well as an active zone, which is the part of a synapse where neurotransmitter release takes place (tom Dieck *et al.* 1998). Bassoon was found to be one of the pre-synaptic proteins acting on protein docking and regulation of neurotransmitter release on the active zone of a synapse (tom Dieck *et al.* 1998). It co-localises with Piccolo (another pre-synaptic protein) and it is predominately found on synaptic junctions making it a crucial molecule for regulation of synaptic release in the brain. It has been associated with neurodegenerative diseases, which is not a surprise given its position in a synapse (tom Dieck *et al.* 1998; Dresbach *et al.* 2006). A recent spectroscopy and histochemical study has revealed that mice lacking Bassoon exhibit altered cortical structures (Angenstein *et al.* 2008). It was revealed that only a subset of synapses is affected by the mutation but this is sufficient for the functional and morphological reorganisation observed (Angenstein *et al.* 2008).

In the targeted microarray results, an increase of *Bassoon* expression was observed after Day 8 of chessboard deprivation (CB), which was even greater in the case of total deprivation (DEP) at the same time point. Interestingly, expression showed a considerable decrease after the second week of experiment (Day 16). Based on previous research, as discussed above, it is logical to assume that *Bassoon* is affected

by the change of external stimulus due to whisker removal. Its role in morphology of the cortex and its position on the pre-synaptic site of a synapse leads to the conclusion that over-expression of *Bassoon* is important when deprivation patterns that induce plasticity are applied in order to facilitate signal transduction and cell-cell communication. The same pattern of expression is validated by the QPCR as well, apart from one case (CB 1), which might require further investigation.

6.8. Conclusions

In an attempt to unravel the mysteries of synaptic plasticity, signal transduction and gene expression in the mouse brain, the targeted microarray discussed above has been designed (Appendix 5). Throughout the chapter the effect of the induction of plasticity in the mouse brain has been studied and functional ontological analysis of the differentially expressed transcripts has been presented. It has been shown that initial gene changes occur at Day 1 which might be preparing the system for the major neuronal changes to come in later time points. It has also been revealed (Figure 6.5) that Day 8 is of great importance when studying whisker deprivation as most gene changes occur at transcripts related to functions associated with synaptic transmission, signal transduction and cell-cell communication, as indicated by the GO terms presented in the pie charts. It has also been shown that after two weeks (Day 16) things return to baseline, suggesting that less neuronal activity is required to maintain the acquired changes. Looking into individual genes (Section 6.4) associated with the pre and post-synaptic site of an active synapse has led to the discovery of specific molecules important for signal transmission which are obviously affected by any changes in sensory input.

Having identified molecules differentially expressed in the barrel cortex of whisker deprived wild type mice, it was considered important to investigate alterations in their expression profiles in the GuR1 knockout strain and their relationship with the GluR1 subunit of AMPA receptor to comprehend the plastic responses in the barrel cortex of this mouse.

CHAPTER 7: PLASTICITY IN GLUR1 -/-

7.1. Overview

To gain a better insight into the changes in gene expression during experience dependent plasticity in the mouse barrel cortex a transgenic mouse line was exploited which lacks the AMPA receptor subunit glutamate receptor 1 (GluR1) due to a targeted gene mutation. Plasticity has been studied in the resultant homozygote GluR1 -/- mouse strain exhibiting little or no post-synaptic component and lower levels of potentiation when compared to wild types (Hardingham and Fox 2006), thus revealing the necessity of GluR1 subunit of the AMPA channel for post-synaptic plasticity in the rodent's barrel cortex. Furthermore, it has been shown that GluR1-/- exhibit reference memory but not working memory suggesting that reference memory depends upon mechanisms independent of post-synaptic GluR1 (Schmitt *et al.* 2005).

Synaptic connections of the thalamus and the cortex with the amygdala (Section 1.4, Figure 1.3), a group of neurons within the medial temporal lobes playing an important role in the processing and memory of emotional reactions (Amunts *et al.* 2005), have also been studied in the GluR1 -/- mouse. In the GluR1 knockout mice these connections were found to have impaired LTP and fear memory (Humeau *et al.* 2007). This led to the conclusion that GluR1-dependent plasticity has a principal role in synaptic connections of the thalamus and the cortex with the amygdala.

On the basis that facial vibrissae deprivation leads to potentiation of spared whisker responses and depression of deprived ones in the cortex (Fox 2002), whisker deprivation experiments, carried out in our lab using the GluR1-/- mice, have identified the necessity of AMPA subunit GluR1 for experience dependent depression and long term depression (LTD; Section 1.9.4) in the mouse barrel cortex (Wright *et al.* 2008). Thus it may be postulated that probing the transcriptional responses to different patterns of whisker deprivation and comparing to the wild type mice will show a lack of responses in post-synaptically located molecules; indicating a lack of synaptic signal transmission from the pre-synaptic cell to the post-synaptic cell. However, we shall not discard the fact that other synaptic receptors located on pre-

synaptic neurons might intervene and compensate for the lack of GluR1; thus facilitating synaptic plasticity.

7.2. Introduction

Neuronal plasticity induced transcriptional responses were investigated in the GluR1 knockout mice using the same two patterns of deprivation described previously in this thesis, chessboard (CB) and complete (DEP), in addition a control group was included within the study where all whiskers remained intact (ALL) (Section 2.12). The control undeprived animals (ALL) have been subjected to anaesthesia using isoflurane for consistency and regular checks of their facial hair. Animals were 4 weeks old on the first day of the experiment, same as the wild type population documented previously. Due to limited resources and time, only two time points were studied, day 8 and 16 based on findings discussed in previous chapters. The effect of isoflurane was removed by using time matched controls which ensured the transcript changes are associated with deprivation rather than the confounding influence of anaesthesia. This was achieved by separating the data into two independent sets; Day 8 and Day 16 and normalising using a similar approach as with the targeted plasticity microarray, a within-chip TIP-Lowess followed by expression of the data relative to the time matched control undeprived animals (ALL). Due to difficulties with litter size only three biological replicates were available per condition per time point which did not make gender separation possible within this experiment.

7.3. Temporal analysis of transcripts affected by differential patterns of whisker deprivation in the GluR1-/- mouse

Data from each time point was separated into the three different deprivation conditions (ALL, CB and DEP) similar to the process described in Section 6.2. A direct comparison of each deprivation data set to the control time matched undeprived mice was performed (as described in Figure 6.1). Analysis was performed to identify differentially expressed transcripts which exhibited a 2 fold change in expression with a statistical confidence of $p < 0.05$, no gender separation was performed due to the small size of litters available. A gradual increase in the number of transcripts affected

by chessboard deprivation is illustrated (Figure 7.1), whereas the opposite effect is observed for those animals where all the whiskers had been removed.

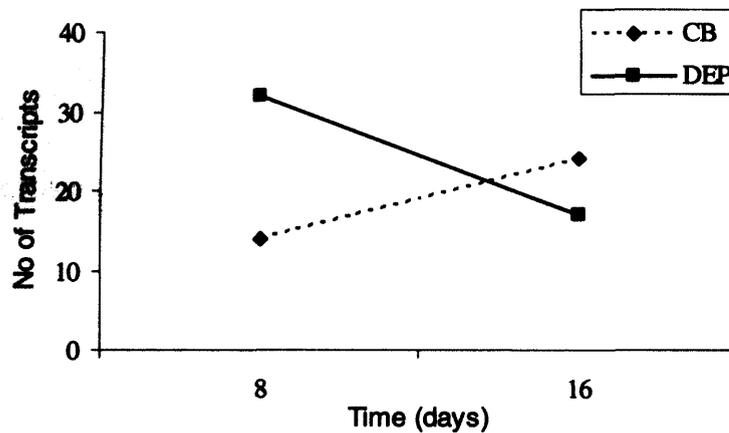


Figure 7.1: Time course of number of differentially expressed transcripts within the barrel cortex upon various patterns of whisker deprivation. Differential expression is defined by a >2 fold change in expression exhibiting a significance of $p < 0.05$ between time matched control (undeprived) (ALL), chessboard (CB, dashed line and \diamond) and fully deprived animals (DEP, solid line and \blacksquare). For full gene list refer to Appendix 6.

7.4. Ontological bias analysis of transcripts affected by differential patterns of whisker deprivation in the GluR1-/- mouse

To reveal the functional processes underlying the transcripts differentially expressed at each time point and under the different deprivation patterns (Section 7.3) an ontological bias analysis was employed on the cohorts of genes exhibiting a 2 fold change at significance $p < 0.05$ derived in Section 7.3. This was performed using the online software “L2L” Microarray Analysis Tool (<http://depts.washington.edu/l2l>), which utilised the most recent annotation for the NIA clone set and could be used to determine the overrepresentation of genes belonging to specific functional categories. Although this revealed over-representation ($p < 0.05$) of a number of categories associated with “*Biological Process*” for each time point and deprivation treatment (Figure 7.3) no significant terms were identified belonging to “*Molecular Function*”

or “*Cellular Component*”, probably due to the restricted number of genes associated with each individual condition.

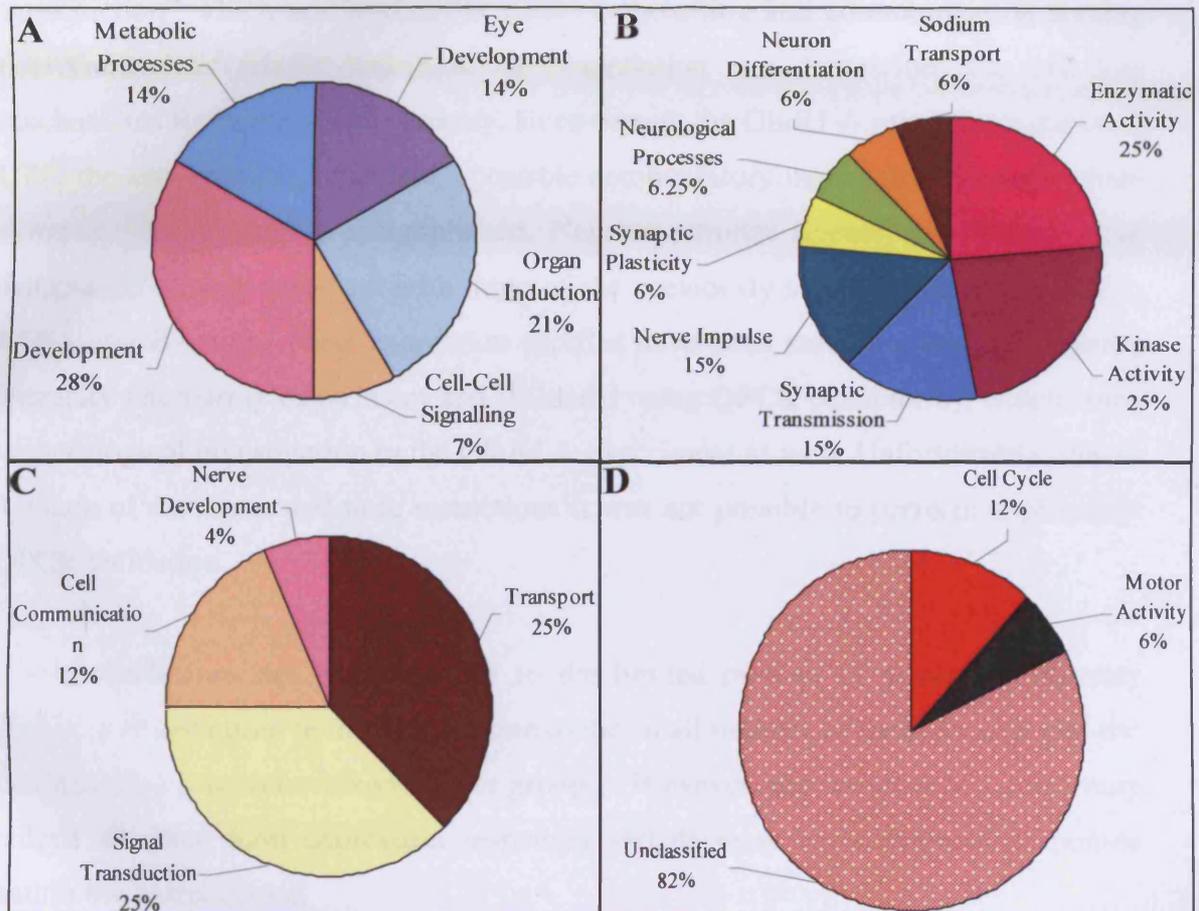


Figure 7.2: Ontological representation of the transcripts affected by deprivation (CB and DEP) at Day 8 (Panels A and B) and Day 16 (Panels C and D). Ontological bias analysis was performed on gene exhibiting significant changes (2 fold and $p < 0.05$). Panels A ($n=14$) and C ($n=24$) represent chessboard deprivation (CB at Day 8 and 16 respectively), whereas Panels B ($n=32$) and D ($n=17$) represent total deprivation (DEP at Day 8 and 16 respectively). For full list of genes refer to Appendix 6.

Ontological analysis of the two different deprivation patterns at time points 8 and 16 revealed the majority of differentially expressed genes from complete whisker deprivation at day 16 were intriguing ontologically “Unclassified” is hindering the functional analysis, however further conclusions can be drawn for other treatments

and time points. Functional categories such as “*synaptic plasticity*” and “*neuron differentiation*” are observed after 8 days in the totally deprived animals (Figure 7.2, Panel B) which could be preparing the barrel cortex of these knockout mice for the neuronal changes observed in later time points. Functional analysis (Figure 7.2; Panel C) has also identified ontological categories “*nerve development*” and “*signal transduction*” which are involved in cell-cell signalling and communication serving neurotransmitter release important for potentiation and depression; the two key mechanisms for learning and memory. Even though the GluR1^{-/-} mice show impaired LTP, the above findings indicate a possible compensatory mechanism through which synaptic transmission is accomplished. Neurotransmitter release and formation of synapses is closely associated with three of the previously studied molecules; NOS1, NOS3 and Bassoon. Their expression profiles have been studied using the targeted plasticity microarray experiment and validated using QPCR (Chapter 6), which leads to their logical investigation in the GluR1^{-/-} experiment as well. Unfortunately, due to the lack of resources and time restrictions it was not possible to perform appropriate QPCR validation.

These conclusions are restricted due to the limited number of genes significantly changing at each time point possibly due to the small number of animals used and the fact that they represent mixed gender groups. However, the removal of GluR1 may reduce the long term depression responses and decrease transcriptional responses within the barrel cortex.

7.5. Pattern independent transcriptional changes induced by whisker deprivation in the GluR1^{-/-} mouse

To attempt to compensate for the small number of individuals analysis was performed combining the data for both chessboard (CB) and complete deprivation (DEP) at day 8 and day 16. Transcripts exhibiting a 2 fold differential expression at significance $p < 0.05$ were determined revealing that at day 8 and subsequently at day 16 480 and 530 genes were differentially regulated respectively. A number of genes (discussed below) can be identified at both time points that have been previously implicated with regulation of synaptic plasticity.

Ontological bias analysis was performed to investigate the overrepresentation ($p < 0.05$) of specific Gene Ontology terms associated with “*Biological Processes*” using the online version of the “L2L” Microarray Analysis Tools (Figure 7.3). From this analysis, it became obvious that some functional categories were associated with plasticity, such as “*neuron recognition*”, “*neuron maturation*” and “*signal transduction*”. Results (Figure 7.3) illustrate plasticity induction in the knockout mice after the first week of experiment (Day 8), an effect that seems to persist even after the second week (Day 16) of deprivation.

The GO term “*signal transduction*” detected at Day 8 after deprivation (Figure 7.3, Panel A) comprises of around 96 transcripts associated with plastic responses in the mouse brain. Some transcripts are associated with calcium intake and protein phosphorylation which can be linked to signal transduction and cell-cell signalling. One of the transcripts encodes the neuronal PAS domain protein 2 (NPAS2) which was identified in neurons and its expression pattern was temporally matched with the ontogeny of learning and memory (Zhou *et al.* 1997). Mutant mice, exhibiting a targeted insertion of a beta-galactosidase reporter gene (*lacZ*) resulting to an altered form of NPAS2 lacking the basic helix-loop-helix (bHLH) domain, were studied and found to express *Npas2-lacZ* in the cortex, hippocampus, striatum, amygdala, and thalamus and were found to exhibit deficits in long-term memory (Garcia *et al.* 2000). The representation of the *Npas2* transcript in the “*signal transduction*” functional group after 8 days of deprivation suggests that it is affected by the experimental procedure and combined with the fact that it is implicated with learning and memory leads to the conclusion that it affects plasticity in the rodent’s barrel cortex. Another transcript is *integrin beta 1* which has been described as a receptor mediated neuronal adhesion and migration. *Integrin beta 1* is co-expressed with *Dabl* (Drosophila gene “disabled”) in cortical neurons, both transcripts implicated in neuronal development (Dulabon *et al.* 2000). The expression pattern of *integrin beta 1* and its association with neuronal development in combination with its significant representation after deprivation at Day 8 suggests that it might be implicated with cortical organisation and neuronal connections affected by alterations at the external stimuli. *Neurotrophic tyrosine kinase receptor 1* (*Ntrk1*) is yet another interesting transcript detected at Day 8 in the “*signal transduction*” functional category and it belongs to the neurotrophin family which play a key role in the development of central and peripheral nervous

system (Bibel and Barde 2000). *NTRK1* is implicated in survival and differentiation of the nervous system and its significant over-representation at Day 8 indicates that it is affected by deprivation and it is linked with mechanisms that induce plasticity such LTP and LTD (Section 1.9.4).

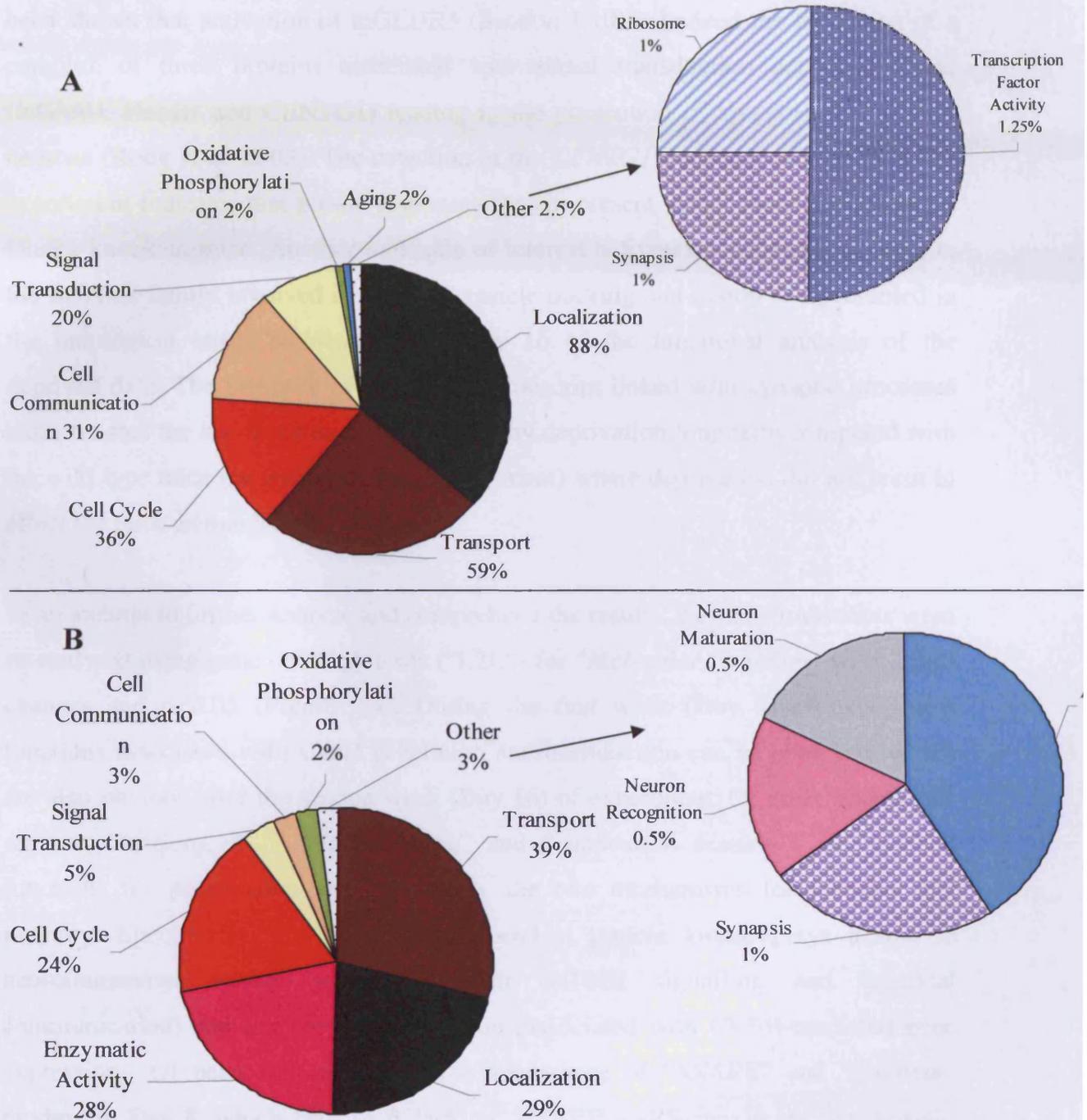


Figure 7.3: Ontological representation associated with “*Biological Processes*” of the transcripts affected by deprivation (CB and DEP) at Day 8 (Panel A) and Day 16 (Panel B), exhibiting 2 fold change at significance $p < 0.05$. The above categories show the effect of any deprivation condition per time point, without differentiation between CB and DEP.

One of the transcripts exhibiting differential expression after 16 days (Figure 7.3, Panel B) of whisker deprivation is centaurin, gamma 3 (*Centg1*); dominant mutant of which prevented nerve growth factor involved in the regulation of growth and differentiation of sympathetic and certain sensory neurons (Ye *et al.* 2000). It has been shown that activation of mGLUR5 (Section 1.10) enhanced the formation of a complex of three proteins associated with signal transduction across synapses (mGluRI, Homer and CENTG1) leading to the prevention of apoptosis in cultured neurons (Rong *et al.* 2003). The detection of the *CENTG1* transcript at Day 16 of the experiment indicates that plastic responses remain present in the barrel cortex of the GluR1 knockout mice. Another molecule of interest is Syntaxin 16 which belongs to the SNARE family involved in synaptic vesicle docking and fusion is represented in the ontological term “localisation” at Day 16 of the functional analysis of the deprived data. The presence of yet another transcript linked with synaptic processes indicates that the knockout mice are affected by deprivation long term compared with the wild type mice (targeted plasticity experiment) where deprivation did not seem to affect the mice as much after 16 days.

In an attempt to further analyze and comprehend the results, the same transcripts were re-analysed using gene ontology tools (“L2L”) for “Molecular Function” with 2 fold changes and $p < 0.05$ (Figure 7.4). During the first week (Day 8) of experiment functions associated with signal generation and transduction can be observed, which are also obvious after the second week (Day 16) of experiment. Of great importance are the functions of “calcium binding” and “calmodulin binding”; two distinct functions for potentiation and depression, the two mechanisms for learning and memory. Specifically, Ca^{2+} /calmodulin-dependent protein kinase plays a role in neurotransmitter release (associated with cell-cell signalling and neuronal communication) and transcription regulation (associated with CREB-mediated gene expression). Of particular interest is the appearance of “SNARE” and “syntaxin” binding in Day 8, which Day 16 is lacking. SNARE and Syntaxin are pre-synaptic proteins acting on the pre-synaptic active zone of a synapse. Their presence indicates that although these mice are lacking glutamate receptor 1 they are over expressing other pre-synaptic proteins important for signal transmission and successful formation of synapses; leading to the proposal of some compensatory mechanism.

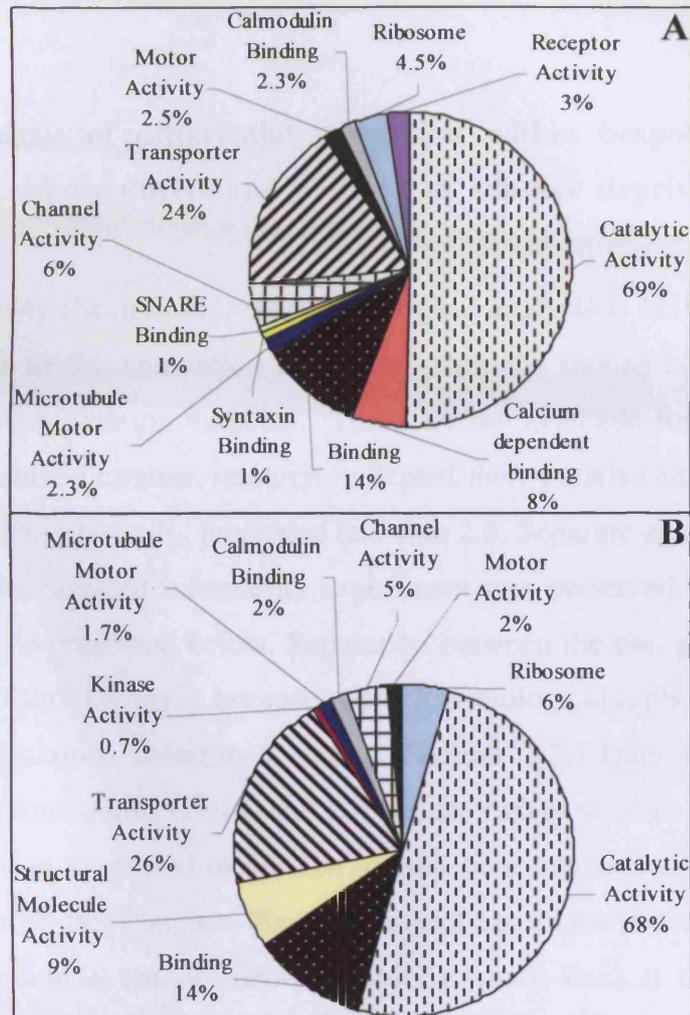


Figure 7.4: Ontological representation associated with “*Molecular Component*” of the transcripts affected by deprivation (CB and DEP) at Day 8 (Panel A) and Day 16 (Panel B). The above categories show the effect of any deprivation condition per time point, without differentiation between CB and DEP. Genes exhibited 2 fold change and $p < 0.05$.

To expand our knowledge on the pathways induced in the GluR1^{-/-} mice when plasticity is induced, ontological analysis was performed again using “*Cellular Components*”. The most intriguing functional categories were “*axon/dendrite growth*”, “*neuron projection*” and “*channel activity*”; all indicative of synaptic plasticity mechanisms, which are indicative for days 8 and 16. This might indicate that induction of plasticity is still affecting gene expression the barrel cortex of the

GluR1-/- knockout mice in such a way that genes associated with “*neuromuscular junctions*”, “*dendrite development*”, “*cell projection*” and “*channel activity*” are still active and differentially expressed.

7.6. Temporal analysis of differential expression within bespoke plasticity transcripts induced by differential patterns of whisker deprivation in the GluR1-/- mouse.

As mentioned previously (Section 6.4) when ontological analysis is being performed the analysis is linked to the annotation associated with the mouse NIA clone set through their unique identification numbers. These are not available for the bespoke genes specifically because literature research indicated their putative involvement in the experience dependent plasticity, presented in Table 2.6. Separate analysis of these genes in relation to the targeted microarray experiment was presented (Section 6.4) and a similar analysis is presented below. Separation between the two genders could not be possible in the GluR1-/- array because of the low biological replicates used for this study due to limitations mentioned earlier (Section 7.2). Data was however separated into the two time points (Day 8 and 16) and the two experimental conditions (CB and DEP). Data was expressed in relation to their time matched controls for the reasons explained in presiding chapters. Every transcript has a systematic name which is its clone identifier and a unique identifier (MGI) which links it to the mouse genome. The normalised data are presented in Table 7.1, where significantly different (>2 fold change and $p < 0.05$) values are appropriately highlighted by different colours. A hierarchal cluster of the normalised data of the bespoke gene list displaying differential expression changes is presented in Figure 7.5.

Treatment Time			CB	DEP	CB	DEP
	Systematic	MGI ID	8		16	
		MGI Symbol				
RICH_G3	MGI:97320	Klk1b4	1.3	1.1	0.5	1.5
RICH_C1	MGI:1855700	Pcdh12	1.1	1.7	0.4	1.3
ELE7	MGI:105088	Ntn1	1.0	1.5	0.9	1.8
RICH_B1	MGI:108092	Bin1	1.1	1.7	0.8	1.7
RICH_D6	MGI:95811	Gria4	0.9	2.5	0.9	1.4
RICH_B4	MGI:95739	Glul	1.5	2.4	1.1	1.3
RICH_A2	MGI:98796	Tph1	1.3	2.1	1.0	1.3
RICH_B6	MGI:95809	Gria2	1.6	1.9	0.9	1.5
ELE18	MGI:2156052	Slc17a6	1.7	1.9	0.8	1.2
RICH_B2	MGI:108173	Prkg2	1.9	1.6	0.7	1.1
RICH_H6	MGI:97175	Mtap2	1.1	1.6	1.1	1.1
RICH_B3	MGI:95634	Gad2	1.2	1.2	0.7	1.1
RICH_H3	MGI:97321	Ngfb	1.1	1.3	0.8	1.0
RICH_D4	MGI:1306778	Mtap1b	1.1	1.2	1.1	0.8
RICH_H1	MGI:96224	Hras1	1.9	1.3	1.4	0.6
RICH_F3	MGI:88145	Bdnf	1.8	1.6	1.3	0.8
ELE15	MGI:1923000	Slc10a6	0.5	1.0	0.4	0.9
RICH_D2	MGI:88495	Crem	0.5	1.3	0.5	1.2
ELE16	MGI:98387	Specb1	0.6	0.9	0.9	1.6
RICH_H2	MGI:2389091	Rps15a	0.8	1.1	0.5	1.3
RICH_D1	MGI:95660	Gas6	0.6	1.5	0.7	1.6
RICH_E1	MGI:97610	Plat	0.7	0.8	0.5	0.5
RICH_G1	MGI:1860487	Pcdh7	0.6	0.7	0.5	0.6
RICH_F2	MGI:97912	Rheb	0.7	1.3	0.5	0.5
ELE9	MGI:97380	Ntf3	1.0	0.7	0.8	0.4
ELE13	MGI:106530	PRG1	1.0	1.4	0.8	0.7
RICH_E2	MGI:2151253	Calca	0.9	1.9	0.7	0.7
RICH_C3	MGI:96568	Ina	1.3	2.2	0.8	0.6
RICH_F1	MGI:98397	Src	0.8	1.3	1.0	0.5
RICH_D3	MGI:109349	Atf2	0.8	1.2	1.0	0.6
RICH_A3	MGI:1194506	Serpini1	0.7	1.7	1.0	0.7
RICH_G4	MGI:95524	FHF3	1.0	1.2	1.1	0.4
ELE1	MGI:1277955	Bsn	0.6	1.0	1.6	1.0
RICH_E3	MGI:1298366	Atf1	0.7	1.1	1.2	1.2
ELE2	MGI:99702	Bax	0.5	0.6	1.3	1.4
RICH_C4	MGI:99261	Ptgds	1.2	0.8	1.4	1.5
ELE10	MGI:97360	Nos1	1.1	0.4	0.9	0.7
RICH_H4	MGI:107384	Dynamin1	1.7	0.5	1.3	0.7
ELE17	MGI:1099446	Synpo	1.0	0.6	1.6	0.6
ELE3	MGI:101932	Cpe	0.7	0.5	1.8	0.6
RICH_C2	MGI:108470	St6gal1	0.8	0.7	1.2	0.9
RICH_E4	MGI:108025	Prkar2a	0.7	0.7	0.9	0.9
ELE11	MGI:97362	Nos3	1.0	0.7	0.9	0.7
ELE4	MGI:105313	Citron	2.6	3.5	1.8	1.3
ELE14	MGI:1330818	Qsox1	1.5	1.7	1.4	3.3
RICH_A6	MGI:95808	Gria1	2.0	2.1	0.8	2.3
ELE8	MGI:107755	Neurod2	0.6	1.1	2.7	0.5
ELE6	MGI:95820	Grin2a	0.5	1.2	3.8	1.0
ELE12	MGI:108295	Pxn	0.6	0.9	4.1	0.9
RICH_F4	MGI:95390	Engrailed2	1.3	1.0	3.1	1.9

Table 7.1: Relative temporal expression levels of bespoke plasticity transcripts resulting from chessboard and total whisker deprivation in GluR1^{-/-} mice. The local systematic name, the MGI ID and MGI gene symbols are presented along with the normalised values of each transcript. Values highlighted in “orange” exhibit a 2 fold up regulation whereas values highlighted in “green” exhibit a 2 fold down regulation with significance at $p < 0.05$. Genes are provided in the order they appear when the expression profiles are clustered using a distance algorithm (Figure 7.5).

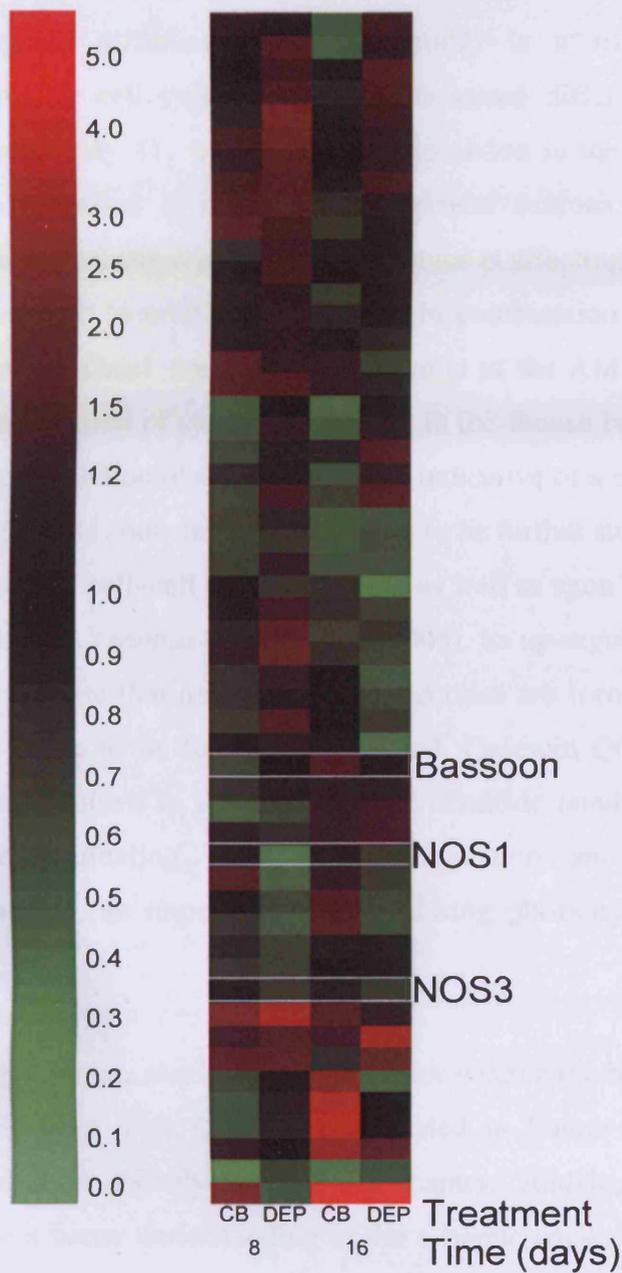


Figure 7.5: Hierarchical clustering of the expression profile of bespoke plasticity genes in the GluR1^{-/-} microarray experiment. A distance algorithm was used to cluster the genes within the bespoke set which displayed a significant change in expression at any time point, treatment condition or either gender. The tree indicates relative expression profiles of genes listed in Table 7.1 and listed in order they appear within the tree. Every column represents a different deprivation condition (CB and DEP) over time (Day 8 and 16) as indicated at the bottom of the figure. The expression profiles of the three genes to be studied further are indicated on the tree.

One distinct cluster (Figure 7.5) showing up-regulation after 16 days of whisker deprivation includes the transcripts of *Neurod2*, *Grin2a*, *Engrailed2*, *Qsox1* and *Gria1*. Neurogenic differentiation 2 (*Neurod2*) is a transcription factor involved in determining cell type. It mediates neuronal differentiation, initially expressed at embryonic day 11, with persistent expression in the adult nervous system. Its post-natal expression is necessary for cortical neurons survival (Mattar *et al.* 2008). Whisker deprivation of the knockout mice is affecting cortical neurons as indicated by the observed *Neurod2* up-regulation in combination with its functional importance. *Grin2a* and *Gria1* are glutamate subunits of the AMPA channel receptors important for transmission of synaptic plasticity in the mouse barrel cortex (Chung *et al.* 2000). The up-regulation of *Grin2a* might be indicative of a compensatory mechanism for the lack of *GluR1*, but such a result needs to be further studied and confirmed. *Engrailed2* has a role in cell–cell communication as well as axon guidance in the visual system as described in *Xenopus* (Brunet *et al.* 2005). Its up-regulation when plasticity is induced might indicate that new cortical connections are forming in the mouse barrel cortex, which needs to be further investigated. Quiescin Q6 sulfhydryl oxidase 1 (*Qsox1*) may be involved in the formation of disulfide bonds within the cell or on the cell surface facilitating cell-cell communication and channel activity for signal transduction, an important function during plasticity induction (Chakravarthi *et al.* 2007).

The three genes, *Nos1*, *Nos3* and *Bassoon* that have been further studied in Chapter 6 and validated with QPCR are indicated in Figure 7.5. Their detailed results are discussed extensively later in the chapter. Studying these three transcripts might provide a better understanding of the synaptic connections in the barrel cortex of the *GluR1*^{-/-} mice and might reveal a new mechanism compensating for the lack of *GluR1*. Although, QPCR validation is not provided, due to reasons mentioned earlier, initial conclusions and a basic understanding can be presented.

7.7. Plasticity related genes

7.7.1. Nitric Oxide Synthase I (NOS1)

Nitric oxide is a controversial molecule (Section 6.4) associated with plasticity and memory (Hopper and Garthwaite 2006) that has caused some confusion to the scientific world of neuroscience; however, its role in synaptic transmission has yet to be defined. NOS1 is a post-synaptic molecule with pre-synaptic effects as NO has the ability to travel between cells and act on the pre-synaptic cell (O'Dell *et al.* 1991; Holscher 1997; Calabrese *et al.* 2007). The targeted plasticity microarray experiment (Chapter 6) has indicated an increase in the expression of NOS1 (Figure 6.12) after the first week of experiment which is dependent upon the severity of the deprivation. However, after the second week of the experiment NOS1 levels decrease to similar levels as day 1; a result validated by QPCR (Figure 6.12 and 6.13).

When the same transcript was studied in the GluR1 knockout microarray experiment, the same primers were used, as already explained, for two time points; 8 and 16 days. No significant changes occurred in the expression of NOS1 after the week of deprivation (Figure 7.5). On the other hand, when the experiment was continued up to 16 days, the effect of chessboard deprivation (CB) is obvious and significantly different ($p < 0.05$, as indicated by the single star in Figure 7.5) from the control undeprived animals at that given time point.

There is a clear difference in the expression patterns between the wild type mice used for the targeted plasticity microarray experiment and the GluR1 knockout mice used for the knockout experiment. The up-regulation observed in this microarray experiment is delayed by almost a week (Figure 7.5); leading to the suggestion that the lack of the AMPA receptor subunit glutamate receptor 1 might have some implications to the post and pre synaptic actions of NOS1. However, even though the GluR1^{-/-} mice have no post synaptic plasticity the results suggest that synaptic transmission is eventually succeeded probably through alternative routes or receptors located at the synaptic cleft. Unfortunately, due to the lack of resources and time, no QPCR validation was plausible. Additionally, no gender differences can be discussed at this point due to the low n of the experiment. More definite conclusions about the actions of NOS1 and its relationship with GluR1 can be drawn if the above

experiment is repeated with a larger biological sample and with the appropriate QPCR validation.

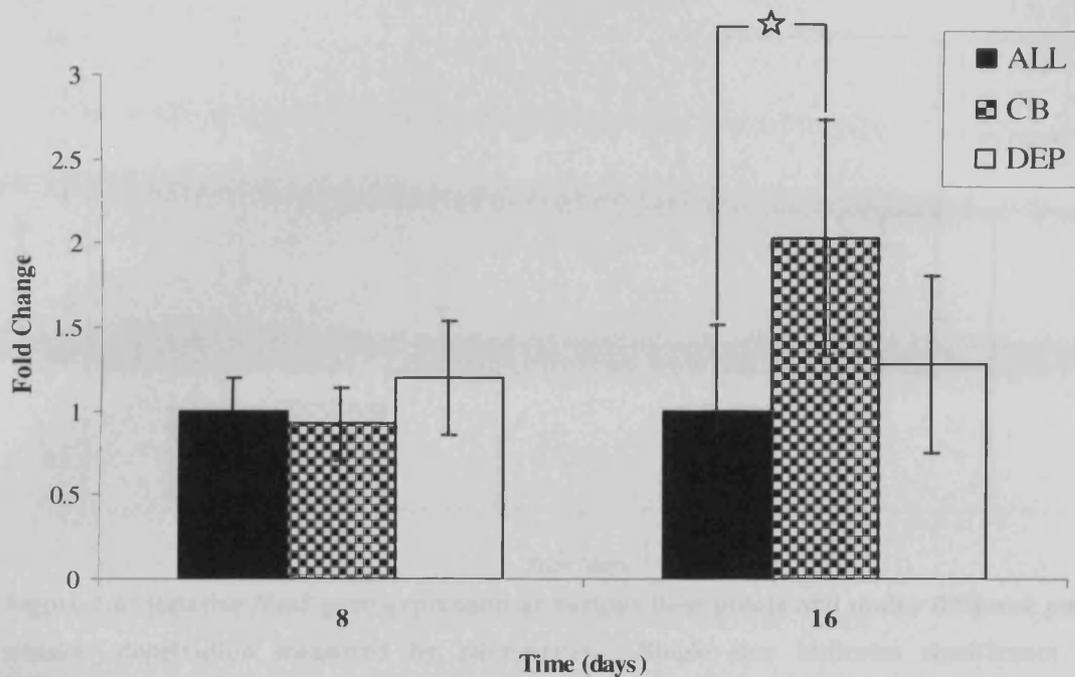


Figure 7.5: Relative *Nos1* gene expression at various time points and under different pattern of whisker deprivation measured by microarray. Single star indicates significance with p value < 0.05 .

7.7.2. Nitric Oxide Synthase 3 (NOS3)

Nitric oxide is also synthesised in endothelial cells by eNOS (NOS3) (De Palma *et al.* 2008). The endothelial function of eNOS (Section 6.4.3) is related with Cadmium, a calcium dependent molecule (Majumder *et al.* 2008).

A significant increase in the expression levels of the transcript was observed after the first week of deprivation in the targeted microarray experiment which is dependent up on the severity of the facial hair removal. Although, clear conclusions can not be drawn as the QPCR validation was not successful for the targeted plasticity experiment, a small but yet significant change after the first day of total deprivation (DEP) in the targeted microarray (Figure 7.6) as well as after 8 days of deprivation

(CB and DEP) were observed. However, no significant changes were illustrated after the second week of deprivation (Figure 7.6).

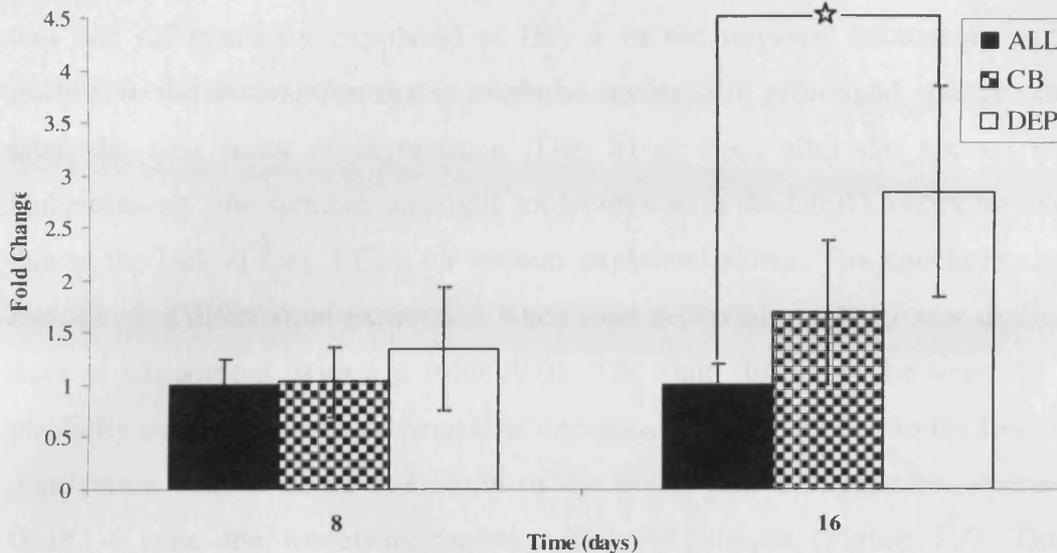


Figure 7.6: Relative *Nos3* gene expression at various time points and under different pattern of whisker deprivation measured by microarray. Single star indicates significance with p value < 0.05.

The results of the targeted plasticity microarray experiment (Chapter 6) are not confirmed by the GluR1 knockout microarray experiment. Due to the lack of Day 1 data (Figure 7.6), no comparison can be done for this early time point. After 8 days of deprivation in the knockout animals no significant changes are observed whereas there is a significant up-regulation after 16 days of total deprivation (DEP 16). The lack of up or down regulation after 8 days in the knockout experiment can be explained as a delayed response which is manifested after 16 days; however such a result needs a more intense investigation with a greater n . The second hypothesis could be based on the fact that NOS3 is mainly detected in endothelial cells. The two experiments were performed on different animals (wild type and knockout) thus the tissue extracted could have included different amounts of blood vessels. The experiments should be repeated and especially the knockout study where more animals should be included for greater biological representation. QPCR validation is necessary in order to identify any false positives or negatives of this microarray study and to further validate any results obtained so far.

7.7.3. Bassoon (Bsn)

Bassoon is an important pre-synaptic component (Section 6.6.4), found on synaptic junctions and it is involved in synaptic release (tom Dieck *et al.* 1998). Bassoon has been revealed (Figure 6.15) not to be an immediately expressed gene as its expression was not differentially expressed at Day 1 in the targeted microarray experiment, leading to the assumption that it might be involved in prolonged changes that occur after the first week of deprivation (Day 8) or even after the second (Day 16). Unfortunately, the same result could not be seen with the GluR1 knockout experiment due to the lack of Day 1 data for reasons explained above. The knockout experiment has shown a differential expression when total deprivation (DEP) was applied after 8 days of experiment, with a p value < 0.05 . The main difference between the targeted plasticity experiment and the knockout experiment is the fact that in the first case total deprivation caused an up-regulation to the given gene's expression whereas in the GluR1-/- case the treatment caused a down-regulation (Figure 7.7). This major difference might be due to the genetic difference between the two mice populations. The GluR1-/- mice lack the AMPA receptor subunit glutamate receptor 1 (GluR1) due to a targeted gene mutation, as mentioned in the beginning of this chapter, which most certainly is influencing the expression profile of Bassoon in this experiment. It is interesting that after 16 days the expression of this pre-synaptic component is the same in both experiments (Figures 7.7 and 6.15). Unfortunately, there is no QPCR validation which in this case would have helped to clarify Bassoon's expression. It seems that the two strains of mice are being influenced by deprivation in different ways. This might lead to the hypothesis that the mutation introduced to the GluR1-/- mice is influencing synaptic connectivity and signal transduction.

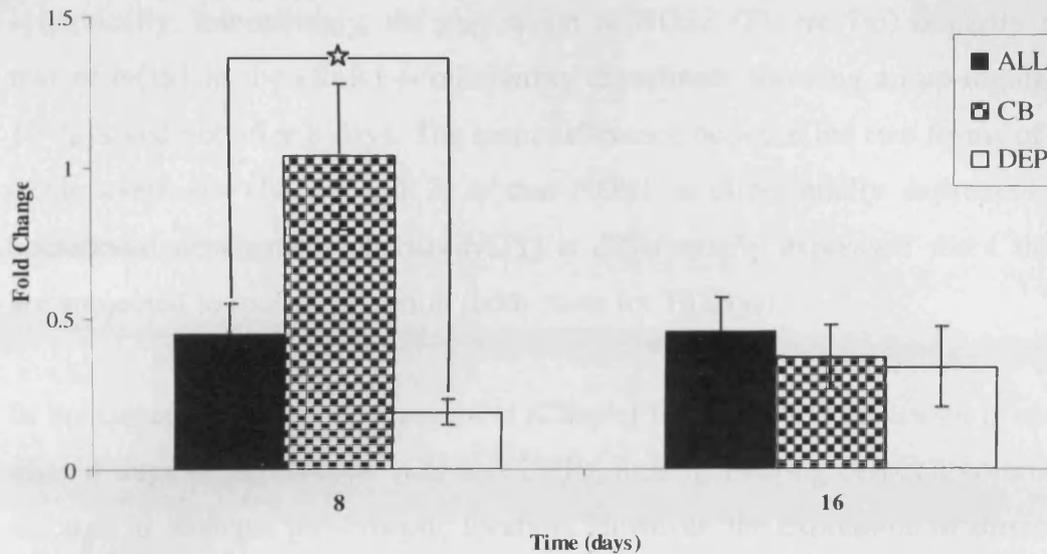


Figure 7.7: Relative *Bassoon* gene expression at various time points and under different pattern of whisker deprivation measured by microarray. Single star indicates significance with p value < 0.05 .

7.8. Conclusions

In an attempt to further understand brain plasticity, the GluR1 knockout experiment (for full gene list refer to Appendix 6) was performed with the same methodology as the targeted plasticity microarray study. Although there is no QPCR validation and low biological replication, some conclusions can be drawn.

Comparing the two experiments (GluR1^{-/-} and Wild Type), a delay in the effect of deprivation on gene expression can be observed in the knockout study. In the case of NOS1, the up-regulation seen after 8 days of deprivation (CB and DEP) in the targeted plasticity microarray study, which is eliminated after 16 days, is not apparent in the knockout study. On the contrary, the mutation introduced into the mouse genome has caused a delayed response in the NOS1 expression. Knowing that NO is a post-synaptic molecule with pre-synaptic effects, it can be hypothesised that the lack of GluR1 receptor is affecting the binding of glutamate to the receptor, preventing the rapid depolarisation of the post-synaptic site (Figure 6.9). Thus, the lack of calcium influx would therefore prevent activation of nitric oxide synthase and the subsequent

release of nitric oxide which needs to travel through the cell membranes and act pre-synaptically. Interestingly, the expression of NOS3 (Figure 7.6) is partly similar to that of NOS1 in the GluR1-/- microarray experiment showing an up-regulation after 16 days and not after 8 days. The main difference between the two forms of the nitric oxide synthases (NOS1 and 3) is that NOS1 is differentially expressed after the chessboard deprivation whereas NOS3 is differentially expressed when the animals are subjected to total deprivation (both cases for 16 days).

In the targeted microarray experiment (Chapter 6, Figure 6.15) *Bassoon* is upregulated after 8 days of deprivation (CB and DEP), thus facilitating cell-cell communication through its strategic pre-synaptic location. However, the expression of *Bassoon* in the GluR1 knockout microarray experiment is down-regulated after 8 days of complete deprivation (DEP 8). It can be assumed that depression (caused by the complete facial hair deprivation and the lack of input) is negatively affecting Bassoon; however such a result needs to be confirmed with other means of molecular biology such as QPCR.

The GluR1-/- study has indicated important molecules affected by facial hair removal which need further investigation and close analysis in order to better understand synaptic transmission in the absence of one of the most important synaptic receptors GluR1. Changes in sensory input affect the molecules referred to above and these effects manifest themselves with either an up-regulation or a down-regulation of their expression profiles. All results need to be confirmed with a higher number of biological replicates and real time PCR validation. The main finding is that although GluR1-/- mice have been found not to exhibit post-synaptic plasticity and our research has shown that GluR1 is necessary for experience dependent depression and potentiation (Wright *et al.* 2008), the present study has revealed that synaptic plasticity can be accomplished through alternate routes and involves molecules that might not have been associated directly with GluR1 in the wild type mice.

CHAPTER 8: GENERAL DISCUSSION

8.1. Overview

The work presented in this thesis was aimed at understanding the molecular mechanisms underlying plasticity in the rodent's somatosensory cortex using the mouse as the model system and whisker deprivation as the experimental procedure. These studies were a natural progression from the first Global Microarray experiment on mouse barrel cortex performed by Dr R. Abraham (Abraham 2005; refer to thesis for more details about genes included in his study), which included almost 15000 transcripts, as described in Chapter 3 and investigated the effect of alterations of sensory input in the mouse barrel cortex in a vast microarray study. Analysis of the global microarray experiment made the design of the present targeted microarray experiment possible and allowed us to further investigate molecular modifications brought about by sensory deprivation. In the following sections, the effect of anaesthesia and the temporal effect of deprivation on gene expression in relation to gender will be discussed. In addition, studies on the GluR1 knockout mice that have been studied to investigate the role of glutamate receptor 1 in gene expression will be discussed.

8.2. Wild Type Mice

8.2.1. Isoflurane effect in control animals in Global and Targeted array

The global microarray experiment directed our attention to a confounding factor modulating gene expression; isoflurane. A simplified overview of the isoflurane results from the global microarray is provided here (Figure 8.1), with the red bars indicating ontological categories present in the corresponding time points.

Two molecular functions appear throughout the chosen time course in the global microarray experiment: "*catalytic activity*" and "*protein binding*" associated with enzymatic activity necessary for cellular processes, suggesting that these alterations are taking place within the neuronal cells throughout time. Many of the functional categories that appear on Day 8 persist after Day 12, which suggests that longevity

of this response may indicate that the subjects are responding to anaesthetic with long term effects in gene expression.

Molecular Function	Day 2	Day 4	Day 8	Day 12
Catalytic Activity	Red	Red	Red	Red
Cell Activity	Pink	Pink	Red	Red
Enzymatic Activity	Red	Red	Red	Pink
Motor Activity	Red	Red	Red	Red
Protein Binding	Red	Red	Red	Red
Transcription Activity	Pink	Pink	Red	Red
Translation Activity	Red	Red	Red	Pink
Transporter Activity	Red	Pink	Red	Red

Figure 8.1: Overview of the temporal isoflurane effect in the global microarray study, with the ontological terms shown on the left. Red colour indicates the presence of a functional category at a specific time point and pink shows its absence. This is the simplified form of Figure 3.2.

The targeted microarray has shown an isoflurane effect (Appendix 4), presented here in the simplified form of a table (Figure 8.2). In the targeted microarray experiment Day 8 and Day 16 were compared to Day 1 under the hypothesis that Day 1 has had the least exposure to isoflurane hence it has undergone the least transcriptional changes. It is apparent that gene expression was mostly affected at Day 8; a result similar to the global microarray findings (Figure 8.1).

Molecular Function	Day 8	Day 16
Calmodulin Binding	Pink	Red
DNA Bending Activity	Red	Red
Insulin Receptor Binding	Red	Pink
Kinase Activity	Red	Red
Kinesin Binding	Pink	Red
Microtubule Motor Activity	Red	Red
Motor Activity	Pink	Red
Oxydative Phosphorylation	Red	Pink
Ribosome	Red	Pink
Structural Molecule Activity	Red	Pink

Figure 8.2: Overview of the temporal isoflurane effect (Day 8 and 16) in the targeted microarray study, with the ontological terms shown on the left. The red colour shows the presence of a category at a specific time point and pink shows its absence. This is the simplified form of Figure 5.2.

Most of the functional categories observed at Day 8 are absent at Day 16; whereas three new ones appear: “*calmodulin binding*”, “*kinesin binding*” and “*motor activity*”. Calmodulin is a calcium binding protein regulated by calcium entry via L-type calcium channels and NMDA receptors, triggering transcription factors implicated in plasticity (Mori *et al.* 2004) and CaMKII implicated in synaptic plasticity. This study has revealed significant changes in the expression of calmodulin in the control undeprived animals at Day 16, demonstrating the possibility that pathways implicated in long term mechanisms for plasticity are affected by the induction of isoflurane; however a more in-depth investigation needs to be performed before final conclusions are made.

Kinesins are cytoplasmic proteins that facilitate the transport of organelles within cells and move chromosomes along microtubules during cell division. Their over-representation at Day 16 (Figure 8.2) indicates changes on a cellular level after 2 weeks of repeated but non-continuous isoflurane induction which might indicate a possible interaction with calmodulin and a plausible action on further gene transcription affecting pathways associated with learning and memory. However, the fact that two factors are affected at the same time, needs to be further investigated and studied to make a more solid conclusion on what is exactly happening and how. “*Motor activity*”, over-represented in the undeprived control animals at Day 16, is another functional category with transcripts that may indicate intra-cellular changes concurrent with regulation of transcription a key feature for long term potentiation and long term depression; the proposed mechanisms of plasticity.

8.2.2. Gender differences in control animals of the targeted array

Analysis of the isoflurane effect (Chapter 5) revealed a number of outliers which when investigated further, revealed significant differences between the two genders used throughout the study which made it crucial to study the isoflurane effect in the two genders separately (Figures 5.9 and 5.10).

In particular, the female mice were less affected by isoflurane after the first week of experiment compared to the males (Figure 8.3). On the other hand, the male mice exhibited less difference in gene expression associated with specific ontological

categories after 16 days of exposure to the anaesthetic (Appendix 5). It is important to point out that there are only two female animals present at Day 1 – all whiskers present (ALL 1). Variation of the responses of the two genders to the induction of isoflurane is apparent (Chapter 5) but a clear assumption cannot be made due to the unequal numbers of biological replicates. Further experiments with larger number of replicates would be required to fully determine the extent of the different responses to isoflurane in males and females. It could be suggested that future plasticity experiments should use single sex approaches or large enough samples of both sexes.

Nevertheless, data suggests that “*calcium binding*” is over-represented in both sexes leading to the assumption that isoflurane induces calcium binding which is crucial for cell communication and cell signalling independent of gender. Consistency can be observed between the two genders and throughout time as far as “*microtubule motor activity*” and “*motor activity*” are concerned, which are related with ATPase and Kinesin activity linking the results with energy production and consumption. However, microtubule and motor activity may very well be associated with trafficking of AMPA receptors and spine dynamics. Such an assumption needs to be further investigated and carefully studied before claiming that anaesthetised subjects have altered spine dynamics.

Molecular Function	FEMALE		MALE	
	Day 8	Day 16	Day 8	Day 16
ATP Binding				
Calcium Ion Binding				
Calcium Dependent Binding				
Calmodulin Binding				
Cell Activity				
DNA Bending Activity				
Heat Shock Protein Binding				
Hydrolase Activity				
Kinesin Binding				
Lipid Binding				
Microtubule Motor Activity				
Motor Activity				
Phosphatase Activity				
Ribosome				
Structural Molecule Activity				

Figure 8.3: Overview of the isoflurane effect throughout time in the targeted microarray study presented separately in the two genders, with the ontological terms shown on the left. Red colour shows the presence of a functional category at a specific time point and the faint pink colour shows its absence. This is the simplified form of Figures 5.9 and 5.10.

It is very important to take under consideration hormonal differences between the two genders (see section 8.4) as well as hormonal variation within the females depending on the time of their cycle as this might have a crucial effect on their gene expression profiles and their response to any given treatment or experiment. Variations should be studied in depth and greater biological replication should be considered for future synaptic plasticity investigations. Results presented in this thesis need to be further confirmed with equal numbers of females and males in order to obtain a more informed understanding of how plasticity is affected in the mouse brain.

8.2.3. Temporal deprivation effect in females and males

The greatest majority of genes are regulated by deprivation at Day 8 (Figures 6.2 and 6.3) making it a crucial time point for plasticity induction and should be considered in future experimental studies of brain plasticity. Ontological analysis of the results from both deprivation types (CB and DEP) at Day 16 (CB and DEP) revealed fewer functional categories were significantly different (Figures 6.6) than at Day 8. The

above result is presented in this chapter in a more simplified form (Figures 8.4 and 8.5).

Gene ontological analysis of the deprived male mice (Figures 8.4) indicated the presence of the “*calcium dependent binding*” category, which includes molecules that bind any protein or protein complex in the presence of calcium. This is a rather general category and includes molecules with very different functions. For example, one of the set of calcium binding proteins are those that initiate neurotransmitter release and therefore could modulate synaptic transmission (Catterall and Few 2008) following sensory deprivation. An example of this subcategory is Piccolo (differentially expressed in this study), a presynaptic cytoskeletal matrix component, which co-localises with Bassoon (Dresbach *et al.* 2006). However, the annexins, of which two are present in the male data set at Day 8 in the deprived (DEP) mice (annexin A6 and annexin A11) are also included in this category and are members of a protein family that bind to the membrane and the cytoskeleton in a calcium dependent manner. In particular, annexin A6 modulates calcium and potassium conductance. The appearance of the “*calcium dependent binding*” functional category at Day 1 and its maintenance in Day 8 along side the over-expression of the related transcripts could indicate that synaptic changes may have been altered in the barrel cortex of the mouse brain by deprivation. Such an argument needs, however, to be further investigated and studied in order to identify the specific synaptic changes occurring in the barrel cortex of the subjects used in such an experiment.

The above suggested changes might have disappeared by Day 16; however the presence of different ontological categories indicates that alternative mechanisms are now activated to respond to the continuous lack of sensory input. Neuronal changes in the mouse brain are indicated in the functional category “*structural molecule activity*” which shows adhesion molecules. Catenin beta 1 is an adhesion junction protein responsible for mediating adhesion between cells (Lee *et al.* 2004). The presence of a molecule like this indicates further cellular communication in order to maintain already acquired neuronal changes or create new ones. The fact that neuronal connections might be structurally altered cannot be overlooked (Cheetham *et al.* 2008). Categories such as “*structural molecule activity*” and “*transporter activity*” are

over-represented through the chosen time course in the male data for the totally deprived mice (Figure 8.4), indicating altered neuronal circuitry associated with synaptic plasticity in barrel cortex.

The effect of complete deprivation (DEP) at Day 8 in female mice (Figure 8.4) is more profound than the effect of deprivation at the same time point in the male mice, indicating that females may have a quicker neuronal re-organisation than males, which may lead to earlier onset of detectable plasticity in electrophysiological studies. Such a suggestion should be further analysed and investigated before final conclusions could be drawn. Results from females indicated regulation of genes in structural categories that underpin structural changes within the cell or between neuronal cells affecting their communication and linkage, such as “*structural molecule activity*”, “*transporter activity*”, “*protein binding*” and “*kinase activity*”. “*Kinase activity*” is of particular interest as a number of kinases are involved in the CREB activation (Figure 1.15) and subsequently influence gene transcription which is important for plasticity. Inducing gene transcription after 8 days of total deprivation (apparent in both males and females) via the activation of protein kinases indicates that Day 8 is of great importance when plasticity induction is studied and it is independent of gender.

Molecular Function	FEMALE			MALE		
	Day 1 ALL_DEP	Day 8 ALL_DEP	Day 16 ALL_DEP	Day 1 ALL_DEP	Day 8 ALL_DEP	Day 16 ALL_DEP
Amino Acid Binding	Red	Pink	Pink	Pink	Pink	Pink
Calcium Dependent Binding	Pink	Pink	Pink	Red	Pink	Pink
Cell Activity	Pink	Red	Pink	Pink	Pink	Pink
Channel Activity	Pink	Pink	Red	Pink	Pink	Pink
DNA Bending Activity	Pink	Pink	Pink	Red	Pink	Pink
Heat Shock Protein Binding	Pink	Red	Pink	Pink	Red	Red
Hydrolase Activity	Pink	Red	Pink	Pink	Red	Red
Insulin Receptor Binding	Pink	Red	Pink	Pink	Red	Pink
Iron Ion Binding	Red	Pink	Pink	Pink	Pink	Pink
Kinase Activity	Pink	Red	Pink	Pink	Red	Pink
Lipid Binding	Red	Red	Red	Red	Pink	Pink
Microtubule Motor Activity	Pink	Red	Pink	Pink	Red	Pink
Motor Activity	Pink	Red	Pink	Pink	Red	Pink
Phosphatase Activity	Pink	Red	Red	Pink	Pink	Red
Protein Binding	Pink	Red	Pink	Pink	Red	Red
Ribosome	Pink	Red	Pink	Red	Pink	Red
Structural Molecule Activity	Pink	Red	Pink	Pink	Red	Red
Transporter Activity	Pink	Red	Pink	Red	Pink	Red

Figure 8.4: Overview of the temporal total deprivation effect (DEP only) in the targeted microarray study presented separately in the two genders, with the ontological terms shown on the left. Red colour shows the presence of a functional category at a specific time point and the faint pink colour shows its absence. The first three columns are the female data followed by the male data. This is the simplified form of Figures 6.4, 6.5 and 6.6. Appendix 5.

Molecular Function	FEMALE			MALE		
	Day 1 ALL_CB	Day 8 ALL_CB	Day 16 ALL_CB	Day 1 ALL_CB	Day 8 ALL_CB	Day 16 ALL_CB
Amino Acid Binding	Red	Faint Pink	Faint Pink	Faint Pink	Faint Pink	Faint Pink
ATP Binding	Faint Pink	Faint Pink	Faint Pink	Faint Pink	Faint Pink	Red
Calcium Dependent Binding	Faint Pink	Red	Faint Pink	Faint Pink	Red	Faint Pink
Catalytic Activity	Faint Pink	Faint Pink	Faint Pink	Faint Pink	Red	Faint Pink
Cell Activity	Red	Faint Pink	Faint Pink	Faint Pink	Faint Pink	Red
Enzymatic Activity	Faint Pink	Faint Pink	Faint Pink	Faint Pink	Red	Faint Pink
Heat Shock Protein Binding	Faint Pink	Red	Faint Pink	Faint Pink	Faint Pink	Faint Pink
Hydrolase Activity	Faint Pink	Red	Faint Pink	Faint Pink	Faint Pink	Faint Pink
Kinase Activity	Red	Faint Pink	Faint Pink	Faint Pink	Faint Pink	Faint Pink
Lipid Binding	Faint Pink	Red	Faint Pink	Faint Pink	Faint Pink	Faint Pink
Microtubule Motor Activity	Faint Pink	Red	Faint Pink	Faint Pink	Faint Pink	Faint Pink
Motor Activity	Faint Pink	Red	Faint Pink	Faint Pink	Faint Pink	Faint Pink
Neurogenesis	Faint Pink	Faint Pink	Faint Pink	Faint Pink	Red	Faint Pink
Oxidoreductase Activity	Red	Faint Pink	Faint Pink	Faint Pink	Faint Pink	Faint Pink
Phosphatase Activity	Faint Pink	Red	Faint Pink	Faint Pink	Faint Pink	Red
Protein Binding	Faint Pink	Faint Pink	Faint Pink	Red	Red	Faint Pink
Ribosome	Faint Pink	Red	Faint Pink	Faint Pink	Faint Pink	Red
Structural Molecule Activity	Faint Pink	Red	Red	Faint Pink	Red	Faint Pink
Transcription Activity	Faint Pink	Red	Faint Pink	Faint Pink	Red	Faint Pink
Transporter Activity	Faint Pink	Red	Faint Pink	Red	Faint Pink	Faint Pink

Figure 8.5: Overview of the temporal chessboard deprivation effect (CB only) in the targeted microarray study presented separately in the two genders, with the ontological terms shown on the left. Red colour shows the presence of a functional category at a specific time point and the faint pink colour shows its absence. The first three columns are the female data followed by the male data. This is the simplified form of Figures 6.4, 6.5 and 6.6. Appendix 5.

Day 8 was found to be the time point of greatest changes in gene expression, when ontological bias analysis was performed for the subject deprived in a chessboard manner (CBs), indicating the importance of this time point irrespectively to deprivation type and gender (Figure 8.5). Similar functional categories are present in both genders indicating a parallel effect of the chessboard deprivation pattern in the barrel cortex of males and females. However, “*heat shock protein binding*”, “*hydrolase activity*”, “*kinase activity*”, “*lipid binding*”, “*microtubule motor activity*” and “*motor activity*” were over-represented in the results from the female mice only (Figure 8.5). The fact that female mice undergo ovarian cycle every 4 days which influences not only their hormone levels but also their hormonal-dependent responses (Fernandez *et al* 2003) should not be over-looked.

Both sexes can display plasticity, yet surprisingly not many published experiments clearly state that both males and females were used or one sex was preferred over the other for whatever reason. Therefore the question of how plasticity occurs in males and females is not always considered, with some papers using single sex studies or not documenting the gender of their subjects.

To those hoping to prise apart the different mechanisms of plasticity between the genders, these results provide indications to where future experiments can be driven and provide a general warning that plasticity is not a uniform mechanism. In other terms, this study has given us clues on two alternatives possibilities; the first being that the same set of molecular changes could occur in both genders but within a different time course and the second is that different changes occur in the two sexes which happen to follow a separate time course.

8.3. GluR1 knockout mice

8.3.1. Plasticity in the GluR1^{-/-} mice

Understanding plasticity in the GluR1 knockouts was of particular interest as they do not show depression of synaptic transmission in layer II/III and IV following chessboard deprivation but do show potentiation. Since wild-types show both depression and potentiation comparing GluR1 knockouts and wild-types could potentially allow us a way of discovering

those genes associated with depression. To investigate the role of GluR1 in plasticity in the barrel cortex, gene expression (Appendix 6) was investigated in mutants versus wild types after whisker deprivation for the previously chosen time course. The GluR1 knockout study was designed using the same experimental protocol as the one used in the targeted microarray experiment. Unfortunately, ontological analysis revealed that 82% of the genes over-expressed were unclassified transcripts after 16 days of complete deprivation (Figure 7.2, Panel D). Nevertheless some conclusions can be drawn and are presented in Figure 8.6.

Gene ontology has revealed some functional categories of particular interest: “*neurological processes*”, “*neuron differentiation*”, “*sodium transport*”, “*synaptic plasticity*” and “*synaptic transmission*” which modulate the ability of synapses to change in response to altered sensory input. These functional groups are only present after 8 days of complete deprivation (DEP 8), which suggests that total lack of facial vibrissae has the ability to severely affect gene expression in the GluR1 mice.

GluR1 -/-				
Biological Processes	Day 8 ALL CB	Day 8 ALL DEP	Day 16 ALL CB	DAY 16 ALL DEP
Cell Communication				
Cell Cycle				
Cell-Cell Signalling				
Development				
Enzymatic Activity				
Eye Development				
Kinase Activity				
Metabolic Processes				
Motor Activity				
Nerve Development				
Nerve Impulse				
Neurological Processes				
Neuron Differentiation				
Organ Induction				
Signal Transduction				
Sodium Transport				
Synaptic Plasticity				
Synaptic Transmission				
Transport				

Figure 8.6: Overview of the temporal deprivation (CB and DEP) effect on the GluR1 knockout mice. This is the simplified form of Figure 7.2. Appendix 6.

The transcript which is most over-expressed in all the above functional categories is tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide (known as protein 14-3-3), which is a protein kinase-dependent activator of tyrosine hydroxylase, an

adaptor protein and an endogenous inhibitor of protein kinase C. Tyrosine hydroxylase is implicated with neurological diseases (Bodeau-Pean *et al.* 1999) such as Parkinson's Disease but its more known function is in the synthesis pathway for dopamine. Its association with protein kinase C might relate this molecule with glutamate receptors and synaptic efficacy, hence its relevance with LTP. The 14-3-3 protein family consists of seven highly homologous isoforms (γ , ϵ , β , ζ , η , σ and τ), involved in neuronal development, apoptosis, cell cycle control, and signal transduction (Berg *et al.* 2003). A knockout mouse strain of protein 14-3-3 isoform γ was created to test the role of these proteins in neurological disorders and specifically in sporadic Creutzfeldt-Jakob (Steinacker *et al.* 2005). This isoform was chosen because of its abundance in the brain and in the cerebrospinal fluid of Creutzfeldt-Jakob (CJD) patients (Wiltfang *et al.* 1999). Steinacker *et al.* (2005) revealed that the mutation in the 14-3-3- γ isoform has not affected the expression levels of the rest 14-3-3 isoforms, suggesting that endogenous levels of other 14-3-3 isoforms can compensate for the loss of the γ isoform. Also, adapter protein 14-3-3 is required for a presynaptic form of LTP in the cerebellum (Simsek-Duran *et al.* 2004). The consistency with which protein 14-3-3 maps in the above functional categories reveals the extent of total deprivation effect in gene expression and its interaction with a cascade of molecules, including kinases and glutamate receptors, might be an attempt to react to total whisker deprivation.

Comparing functional analysis of Day 8 with Day 16 for both deprivation treatments (Figure 8.6), it is apparent that few gene changes occur at Day 16; confirming the results observed in the targeted microarray experiment of the wild type population. It was indicated that the critical time for induction of plasticity is Day 8 in the GluR1 knockout study as well as the targeted microarray experiment. However, it is noticeable that the number of transcripts in the GluR1^{-/-} experiment showing differential expression is considerably lower than in the targeted array study, explained by the low number of biological replicates used for this study (Chapter 7). In an attempt to unravel the secrets of the GluR1 mutants, transcripts with 2 fold changes at significance $p < 0.05$ in any of the two deprivation treatments were ontologically analysed for “*biological processes*” and “*molecular function*” (Figure 7.3 and 7.4). The above results are presented in this chapter in a more simplified form (Figures 8.7 and 8.8).

When data from chessboard and total deprivations are pooled together and analysed throughout time for “*biological processes*” and “*molecular function*” (Figures 8.7 and 8.8), it is revealed that gene expression in Day 16 is as affected by deprivation as in Day 8; a results that is not in agreement with the targeted microarray where Day 8 is crucial for induction of plasticity (Chapter 6). Most of the functional categories over-represented in Day 8 (in both ontological analyses: “*biological processes*” and “*molecular function*”) are also over-represented at Day 16; suggesting that the occurring changes are not absent from the GluR1-/- barrel cortex after the second week of experiment. On the contrary, these mice seem to undergo more plasticity related changes involving “*neuron maturation*”, “*transporter activity*” and “*signal transductions*” to name a few. One of the transcripts associated with the above functional categories is *neuroD2* which is a member of the neuroD family of neurogenic basic helix-loop-helix (bHLH) proteins that are responsible for the induction of transcription from the neuron-specific promoters, which contain a specific DNA sequence (the E-box). The products of the genes of this family may have a role in determination and maintenance of neuronal cell fates (Shibata *et al.* 1999). Another set of molecules identified in the above molecular function groups is the annexins which provide membrane scaffolding and facilitate the trafficking of vesicles and the formation of calcium channels (Gerke *et al* 2005). The above may provide a possible sign of longer term effects and possible compensatory mechanisms in the brain of GluR1-/- mice leading to the conclusion that alternate routes are employed to exhibit synaptic connections even in the absence of one of the most important synaptic receptors, the GluR1.

GluR1 ^{-/-}		
Biological Processes	Day 8	Day 16
Aging		
Cell Communication		
Cell Cycle		
Enzymatic Activity		
Localization		
Neuron Maturation		
Neuron Recognition		
Oxidative Phosphorylation		
Ribosome		
Signal Transduction		
Synapsis		
Transcription Factor Activity		
Transport		

Figure 8.7: Overview of the temporal effect of deprivation without treatment separation between CB and DEP using Biological Processes. This is the simplified form of Figure 7.3.

GluR1 ^{-/-}		
Molecular Function	Day 8	Day 16
Binding		
Calcium dependent binding		
Calmodulin Binding		
Catalytic Activity		
Channel Activity		
Kinase Activity		
Microtubule Motor Activity		
Motor Activity		
Receptor Activity		
Ribosome		
SNARE Binding		
Structural Molecule Activity		
Syntaxin Binding		
Transporter Activity		

Figure 8.8: Overview of the temporal effect of deprivation without treatment separation between CB and DEP using Molecular Function. This is the simplified form of Figure 7.4.

8.3.2. GluR1^{-/-} and wild type mice similarities

GluR1^{-/-} mice (Appendix 6 for the data on all the genes from the GluR1^{-/-} study) shared no similarity with male wild type mice (Appendix 8 for the data on all the genes from the wild type study) at Day 8 after chessboard deprivation. However, there was a 29% similarity with the female mice at the same time point and condition. This may suggest that the depression present at this age in the wild type subjects requires certain expression changes or that these specific expression changes require depression. The gender of the GluR1^{-/-} mice was investigated, but it was found that group CB 8 contained 3 female and 2 male mice, making it clear that the similarity of GluR1^{-/-} data with the female wild type data at CB 8 is unlikely to be a gender dependant.

Of particular interest is transcript BDNF (Section 1.11), which is only significantly changes in the GluR1^{-/-} mice but neither in the female nor the male wild type mice at Day 8 after chessboard deprivation. BDNF is member of the neurotrophin family, necessary for neuronal survival as well as growth and development of new neurons and synapses (Liu *et al.* 2005). BDNF is thought to be involved in conversion of silent synapses to active synapses since they are more abundant in BDNF knockout mice (itami et al 2003). GluR1 is missing from the GluR1 knockouts and is not able to contribute to the conversion of silent synapses either with or without BDNF expression. It is therefore not clear whether the over-expression of BDNF is a futile compensation mechanism that cannot lead to conversion of silent synapses because of absence of BDNF, or whether silent synapses can actually be formed by other AMPA receptor subunit insertion in its absence. It may also be suggested that the wild type mice do not necessarily need immediate activation of BDNF after 8 days of deprivation for expression of potentiation.

One of the transcripts that were differentially expressed in both GluR1^{-/-} and wild type female mice at Day 8 after chessboard deprivation was the fibroblast growth factor receptor substrate 2 (Frs2), which is an important plasticity and neuronal fate modulator (Jordan *et al.* 2008). It has been found that the activation of tyrosine receptor kinase B (TrkB) receptors by BDNF can lead to the phosphorylation of tyrosine residues, which can in turn create docking sites for proteins such as src homology 2 domain containing transforming protein (Shc) and FGF receptor substrate 2 (FRS-2) that subsequently activate downstream effectors. An example of such an effector is cAMP response element-binding protein (CREB), which

couples to the transcriptional machinery and controls the expression of a variety of genes linked to long-term changes in neuronal plasticity (Rivera *et al* 2004). The fact that Frs2 appears only to be expressed in the female wild type mice might be an indication that its expression profile is gender dependant. To obtain a better understanding the experiment needs to be repeated with enough biological replication to make gender separation possible.

When a similar comparison was performed with the totally deprived data at Day 8 from both the GluR1^{-/-} and wild type mice, more similarities were identified. In this case, the GluR1^{-/-} mice exhibit 16% similarity with the female wild type and 13% similarity with the male wild type mice. One of the transcripts expressed in the GluR1^{-/-} mice but not in either female or male wild type mice at DEP 8 is nucleoporin 155 which is involved in bidirectional trafficking of mRNAs and proteins between the nucleus and the cytoplasm in eukaryotic cells (Gorlich and Mattaj 1996); indicating cellular activity which may contribute to induction of depression. This might a contradicting result, as GluR1^{-/-} mice do not show depression and seeks further investigation. It can always be explained as a false positive by the microarray or as a result to the more vigorous response of the GluR1^{-/-} cells to the lack of sensory input as they are unable to depress. Whichever is the case, it still needs to be investigated.

Ontological analysis of the differentially expressed transcripts of the GluR1 data which are not found in the wild type plasticity microarray data for total deprivation at day 8 was performed using DAVID Functional Annotation Tool (available at <http://david.abcc.ncifcrf.gov/summary.jsp>). It revealed a number of functional categories some of which are “*transport*”, “*regulation of transcription*”, “*regulation of synaptic plasticity*”, “*regulation of synapse structure and activity*”, “*regulation of neuron apoptosis*” and “*neuron generation and differentiation*”. Their presence indicates regulation of synaptic plasticity via generation of new neurons or establishment of connections between existing ones and regulation of gene transcription via the appropriate protein transport within or between neuronal cells. The above ontological categories or similar ones have been detected in similar analysis performed for data derived from the wild type animals; however the detection of unique transcripts in the knockout experiment indicates the necessity of different pathway activation to respond to the continuous lack of sensory input. It could be suggested that the transcripts expressed in the knockout animals are positioned closer to the beginning of the cascades of proteins taking part in regulation of plasticity. The fact that the GluR1

knockout mice are lacking an important synaptic receptor might necessitate the activation of different pathways than the one activated in the wild type mice. Further investigations are of course needed before final conclusions can be drawn.

8.4. Hormonal effects on data set

8.4.1. Background information

Steroid hormones are implicated in sexual maturation of mammals; testosterone (T) and estradiol (E2), which are necessary in order to obtain a masculine-like behaviour in castrated mice (Burns-Cusato *et al.* 2004). Testosterone is produced in the testes and it is converted to estradiol in both the testes and the brain (Baum *et al.* 1982), which can then bind to estrogen receptors ER α and ER β (Kuiper *et al.* 1996; Merchenthaler *et al.* 2004). The two receptors share great sequence homology apart from the fact that in the ligand-binding pockets ER α has a leucine amino acid instead of methionine which is found in ER β (Moras and Gronemeyer 1998). Mutant mice for both receptors have been created and are being used to identify important properties of ER α and ER β (Burns-Cusato *et al.* 2004). Although, ER β knockout male mice are fertile, compared to ER α mutants (Krege *et al.* 1998), they still show delayed puberty (Burns-Cusato *et al.* 2004). Further studies have been performed on double mutant males (ER $\alpha\beta$ KO) but no sexual behaviour was found. It was, though, hypothesised that these mice have a female phenotype (Ogawa *et al.* 2000).

8.4.2. Sexual maturation

Sexual maturation of female and male mice is a hormone dependent process and it is developmentally different driving the two sexes to diverse behaviours. Male adult behaviour is affected by masculinisation and defeminization, the former controls masculine behavioural patterns (Morris *et al.* 2004) whether the latter diminishes female-like behaviour in the males (Kudwa *et al.* 2006). ER α is involved in masculinisation as shown by studies performed on the corresponding knockout mice, whilst ER β is involved in defeminization. Due to differences of the specificity of these receptors to estradiol, masculinisation and defeminization has different critical periods during development (Todd *et al.* 2005; Kudwa *et al.* 2006).

Female sexual maturity and behaviour is also hormone-dependent and can be altered by steroid exposure in early developmental stages (Kudwa *et al.* 2006). Similar knockout mice study, as above, have indicated that ER α is necessary for normal female sexual maturation and fertility (Couse and Korach 1999). The use of ER α knockout mice does not allow us to identify the critical time for the expression of this receptor in female mice, something that could be achieved with appropriate pharmacological applications (Kudwa and Rissman 2003). However, although ER β knockout female mice could be infertile, they still have signs of estrous cycles (Kudwa *et al.* 2004; Hewitt *et al.* 2005).

8.4.3. Estrogen receptors in non-reproductive systems

Estrogen is vital during brain development and neuronal differentiation. It is closely related to synaptic plasticity and neuronal cell survival (Harris 2007). Estrogen has indicative functions against brain injury via two mechanisms; genomic, which involves the two estrogen receptors ER α and ER β , and non-genomic that is independent of estrogen receptors activity (D'Astous *et al.* 2004; Bodo and Rissman 2006). In adult rodents, estrogen has been found in numerous parts of the brain (Harris 2007), but mostly in those implicated to mood, cognition and memory (Wise *et al.* 2001; D'Astous *et al.* 2004).

The association of estrogen receptors and memory is due to their presence in hippocampus and amygdala. In double knockout mice (ER $\alpha\beta$ KO), lack of c-fos induction has been demonstrated in the hippocampus implicating the two receptors in rapid c-fos expression (Bodo and Rissman 2006). Day and colleagues (2005) have investigated long-term potentiation deficits in ER β knockout mice when put through fear conditioning tests. Similar behavioural studies have shown significant impairment for both male and female ER β knockouts that could not associated learning with shock and box content throughout the experiment (Harris 2007). Such impairments show interaction of the estrogen receptors with memory thus hippocampal activity (Harris 2007). Morphological abnormalities have been identified in the brain of the above single knockout mice indicating that ER β is necessary for maintenance of synaptic connections (Wang *et al.* 2001). Experimental studies on ER β knockout mice have identified a close relationship of this receptor with CREB-mediated gene expression (Day *et al.* 2005). In the above single mutant mice, reduced glutamate binding on NMDARs is observed leading to decreased Ca²⁺ influx that in turn affects the activation of protein kinases in a number of pathways leading all the way to CREB and gene transcription

(Murphy and Segal 1997; Day *et al.* 2005). This alteration in CREB-mediated gene transcription is associated with LTP deficits of these mutant mice and may lead to impaired or even unstable synaptic connections (Day *et al.* 2005).

Both receptors are associated with gene transcription and kinase-mediated CREB activation, but it was found that they have different binding efficiencies to estradiol with ER β only 30% as efficient as ER α (Pettersson and Gustafsson 2001; D'Astous *et al.* 2004). Their difference in efficiency is mostly because of their diversity in the C-terminal ligand binding-domain and the N-terminal transactivation domain (Mendez *et al.* 2003), although they share great sequence homology (D'Astous *et al.* 2004). Due to this variation in the sequence of the two receptors, only ER α is suitably reacting with insulin-like growth factor I (IGF-I) that activates subsequent kinase signalling pathways implicated with neuroprotection (D'Astous *et al.* 2004). ER α was found to be upregulated after a stroke, whilst downregulation of the ER β was observed in rodent and primate models (Blurton-Jones and Tuszynski 2001; D'Astous *et al.* 2004); leading to the assumption that the two receptors react and interact differently in order to induce neuronal survival after brain injury and assist required gene transcription (D'Astous *et al.* 2004).

To the above estrogen-mediated neuronal-excitability mechanism might be the basis or another plausible explanation for the differences seen in gene expression and the grouping of gene profiles as represented in the PCAs summarising the array experiments performed in this study and presented in preceding chapters. Male and female mice undergo similar but yet different pathways throughout sexual maturation. Some researchers could argue that mice at P28 (28 days old; the age of the study) are mature enough but cortical changes can be introduced in their barrel cortices and alterations can occur in their synaptic connections. The behaviour or even the response of the two genders can be driven differently due to the altered hormonal influences they are subjected to. The male mice are subjects to sexual maturation, whereas, at the same time, the females are subjects of their ovarian cycle every four days, which possibly means that hormonal alterations affect gene transcription once, twice or even four times throughout the complete time course of the experiment. The summary of the results can be visualised in the appropriate chapters with data pooled together or separated into the two genders (Chapter 6). The graphs representing the number of transcripts affected by the different types of deprivation in males and females show that the same numbers of

transcripts is changed due to both types of deprivation in the females whereas the males seem to be more affected by the loss of all their whiskers. Once again we might be able to explain this response by looking at their hormonal levels. However, due to the fact that sexual differences were identified once the experiment was complete, it was impossible to identify at which stage of the ovarian cycle every female was at the time of the experiment. Have been able to obtain that information it would have been much easier to identify specific hormonal responses.

To further investigate the differences within our population and try and understand what role do sexual maturity and different age stages have in our data, another ontological analysis was performed, using <http://amigo.geneontology.org>. A group of GO categories is unique to the female population at day 8 which appears on the second week (Day 16) of this study in the male data. The categories involve “**antioxidant activity**”, “**chemoattractant activity**” and “**nutrient reservoir activity**”. Antioxidants are easy targets for oxidation reactions substituting other molecules or substances. The product of an oxidation reaction is free radicals which may lead to further chain reactions that damage the cells. The role of the antioxidants is to capture those free radicals and stop or slow down the cell-damaging reactions by being oxidised. A balance between harmful and beneficial effects of free radical is achieved by the presence of antioxidants in appropriate concentrations. Low levels of antioxidants cause oxidative stress which may lead to stroke incidents, generation of neurodegenerative diseases and aging (Valko *et al.* 2007). Chemoattractants are another type of inorganic or organic substances associated with normal cellular function. Their activity is involved in the movement of a motile cell towards high concentrations of a signal (<http://amigo.geneontology.org>); coinciding with high concentrations of antioxidants described above. Nutrient reservoir activity, the last GO term seen in the female data at day 8 of this study, is associated with storage of nutritious substrates (<http://amigo.geneontology.org>) valuable for cellular and neuronal survival.

The above GO categories appearing in the female data on the 8th day of the experiment are associated with cellular, in this case neuronal, survival, protection against toxicity, responses to signal transmission and protein storage. Signal transduction and extracellular stimuli trigger the above responses, enabling cell-to-cell communication (Valko *et al.* 2007). Signals are, supposedly, sent to the transcription machinery activating biological pathways leading gene expression associated with processes like nerve transmission (Thannickal and Fanburg

2000). Activation of gene-expression related pathways might be a plasticity-stimulated response. The fact that the males show latency in their response might indicate a gender-dependent response.

8.5. Association of array studies in visual and barrel cortex

During the development of the visual system, activity is important for the organisation of connections that can be influenced by changes in the visual input over a specific time period (the critical period). For example, monocular deprivation during the critical period has the ability to create a shift of cortical neurons in favour of the eye that has been left open and to alter the width of the ocular columns corresponding to the open eye in the expense of those corresponding to the closed eye. The shift is created via the weakening of synapses in the closed eye and the subsequent strengthening of the synapses in the open eye. Dark rearing delays the activity dependent development of the visual cortex and delays the critical period for ocular dominance plasticity. Dark rearing has the extreme effect of changing synaptic strength and structure as well as synaptic potentiation and depression (Chapter 1).

During the past few years, scientists have taken a different approach in order to investigate the factors required for the above processes via the use of strong genetic tools, such as microarrays, which have the ability to report gene expression in depth and uncover candidate genes and signalling pathways involved in plasticity. Two main studies have been performed in order to investigate the molecular pathways mediating plasticity in the visual cortex (Majdan & Shatz 2006, Tropea *et al.* 2006) and more recently a study focusing on the critical period (Lyckman *et al.*, 2008). The main aim of these studies lies in the identification of genes whose expression is regulated by visual deprivation. It is therefore worth discussing the findings of these papers in relation to the findings reported here as all of these studies share a common aim. However, it should be noted that two factors complicate a direct comparison; the first relates to technical differences in the way the experiments were performed and the second relates to the fact that the visual cortex shows a clear critical period at a relative mature stage of development (P23-32) whereas the somatosensory cortex shows a much earlier critical period between P0 and P4 (Fox *et al.*, 1992), which was not studied in the present set of experiments. Furthermore, the somatosensory cortex shows plasticity throughout life in layers II/III (Fox *et al.*, 1992) and while a similar phenomenon has recently been described in the visual cortex, it is only tangentially studied by Madjan and Shatz (2006)

in trying to detect changes in gene expression at the end of the critical period for visual cortex (Sawtel et al., 2003)

The second major difference between the two sets of studies is the fact that in the visual cortex, studies were aimed at understanding the regulation of gene expression in and around the critical period, while in our study, we looked at gene expression after the layer IV critical period during the layer II/III adult plasticity phase. The visual cortex critical period comes after a period of intense synaptogenesis between P7 and 14 (Micheva and Beaulieu, 1996; Li et al., 2009). Lykman et al (2008) show several gene sets that track this period of development and are strongly up regulated at these ages, such as genes involved in “process outgrowth”, “inhibitory transmission”, “myelination” and “synaptogenesis” itself (Figure 4). Very large changes occur between P0 and P14 in all groups except “myelination”, which continues to show changes up to P28 (Lykman et al., 2008). The changes beyond P14 and during the critical period are far more diverse and smaller in magnitude by comparison.

The Majdan & Shatz study (2006) also demonstrated age specific gene alterations; although they tend to limit their analysis to those genes that show developmental profiles that depend on visual experience. To do this they compare gene expression patterns in two cortical hemispheres, one receiving contralateral input from a normal open eye and the other receiving no contralateral input due to eye enucleation. This methodology is not as easy to interpret as simply studying differences between ages. For one thing the experienced hemisphere contralateral to the intact eye is missing the ipsilateral input from the enucleated side and therefore all the binocular interactions that normally accompany binocular receptive field formation during the critical period is missing. Nevertheless, there are a number of consistencies with the Lykman study. For example, BDNF is strongly up regulated during natural development between the ages of P0 and P28 (Lykman et al. 2008), and BDNF was part of the common regulated gene set of that was regulated by visual input (Madjan and Shatz, 2006). Similarly, alpha-synuclein was up-regulated strongly between P0 and P28 (Lykman et al., 2008) and depended on visual input for expression between P20 and P24 (Madjan and Shatz, 2006). Unfortunately, alpha-synuclein was not included on our gene array; although BDNF was included it showed up-regulation that did not reach our criterion for significance (alpha 0.05).

The table below (Table 8.1) summarises the numbers of genes that are unique for every time point. In this study we obviously only looked at a very short period of development and one that occurs after most of the major development of the somatosensory cortex is finished (Section 1.9). We assumed that gene expression did not change appreciably between P28 and P44 during normal development and in any case each comparison of a deprived animal was made relative to an age matched undeprived animal (Chapter 2; Methods)

Time Point	Age of Subject	No of genes
Day 1	P28	157
Day 8	P36	406
Day 16	P44	86

Table 8.1: Representation of number of transcripts that are unique to the time points referred.

The visual cortex study has identified a specific age when major changes are taking place (4 days after enucleation). In a similar manner, we identified greater number of transcripts affected by whisker deprivation at Day 8 where the age of our subjects is around P36. Our observation is significantly different from theirs, but this could be based on the fact that the two studies use different paradigms to study plasticity and certain variations and dissimilarities are to be expected. However, one should concentrate on the fact that both studies have identified a time point (P46 for the visual study and P36 for the barrel cortex study) when crucial plasticity induced changes are observed. However, the obvious difference within the above similarity is the fact that the visual study identified a crucial time point just after critical period and we identified a crucial time point several weeks after the equivalent critical period.

Unique genes, represented in Appendix 7 (file name: Molecular Function_GO_UniqueGenes), have been analysed ontologically for Molecular Function. The number of transcripts in each GO category, the percentage of these transcripts in every category and their p values are included in the file, which is divided into three spreadsheets according to the time point (Day 1, Day 8 and Day 16). The same Appendix (7) also represents the lists of transcripts with their annotations (file name: Unique Gene Lists For

Time Points). Examples (only a few; for full list please consult the Appendix 7) of some transcripts that are unique to specific time points (amongst others) are summarised in the table below (Table 8.2).

Time Point	Age of Subject	Names of Transcripts
Day 1	P28	Ca ²⁺ /CaM, Grin2a, Protein Kinase C Isoforms Delta/Iota/Nu
Day 8	P36	Netrin 1, Bcl2, Quienscin Q6, Paxillin, Vglut2, Spectrin beta 1, Protein Kinase C Isoform Zeta
Day 16	P44	Neuro 3, NOS 1

Table 8.2: Representation of an example of transcripts that are unique to the time points referred. A complete list of the transcripts is situated in Appendix 7; file name Unique Gene Lists For Time Points.

As mentioned above, a number of transcripts are found to be unique for each time point and the complete list of those genes is presented in Appendix 7. However, what is of particular interest is the fact that different isoforms of Protein Kinase C are over represented in different time points; with Protein Kinase C isoform zeta (ζ) found in Day 8 in particular.

Kinases, and in particular CaMKII, have been candidate molecular mechanisms for memory storage due to its ability to autophosphorylate (Lisman 1985; Miller and Kennedy, 1986). We did not find CaMKII alpha up regulated in the present study, in concert with Tropea et al (2006) who only found it up regulated during dark rearing and not monocular deprivation. Interestingly PKC can also mimic the effect of autophosphorylating by protecting itself from phosphatase activity (Sweatt et al., 1998) and different isoforms of Protein Kinase C are overexpressed in different time points in our study; with Protein Kinase C isoform zeta (ζ) found in Day 8 in particular. Protein kinase C was experimentally shown to be critical for maintaining long term memory (Pastalkova *et al.* 2006) and in particular phosphorylation by the zeta isoform of PKC was proven to be required for maintaining LTP in the hippocampus and for sustaining hippocampus-dependent spatial memory (Pastalkova *et al.* 2006). Persistent PKC ζ activity is critical for maintaining enhanced synaptic plasticity (Hernandez *et al.* 2003, Serrano *et al.* 2008). Possibly, the overexpression of the zeta isoform of PKC is

indicative of a plasticity mechanism employed by the mice after long term whisker deprivation or even a machinery where PKCzeta maintains the late phase of this memory mechanism through the possible action of glutamate-dependent AMPA receptors trafficking to the synapse. The fact that the zeta isoform of PKC is not actually over-expressed until day 8 of our study, might indicate the presence of a “window” that is needed for this molecule to come to action. However, such a model would need further investigations in order to establish whether other elements are involved in this machinery and how they achieve their roles.

Furthermore, the visual cortex microarray study supplies us with the information of a specific group of genes, which is commonly regulated in all ages of their study suggesting a universal mechanism regulating OD plasticity. A similar analysis of the barrel cortex array data has provided a list of transcripts (90) that are regulated by whisker deprivation throughout the experiment (Appendix 7; file name: Genes Common to All Time Points; Spreadsheet: Common Genes). A look at the ontology (Appendix 7; file name: Genes Common to All Time Points; Spreadsheet: Gene Ontology) of the transcripts involved throughout the experiment independent of deprivation reveals ontological categories such as receptor activity, signalling activity and binding. One of the prominent signalling pathways up regulated at 8 days were molecules generally belonging to the Ras super family. These molecules bind GDP and GTP and interconversion, under the control of GTP-GDP exchange factors (GEFs), control their activation. At the 8 day time point we found 2 Rho related factors (Rho-GEF 11 and SLIT ROBO), 13 Ras and 5 ADP ribosylation (ARF) factors to be regulated by deprivation. Regulation of the ARF pathway is a common finding with the visual cortex studies were they were found to be regulated by monocular deprivation (Tropea et al., 2006). There are a number of notable members of this family known to play a role in cytoskeletal rearrangements including spine rearrangements (Yoshihara et al., 2009). Spine dynamics are known to be affected by whisker deprivation (Trachtenberg et al., 2002) and are likely to be important for rearrangements in synaptic connectivity during deprivation induced plasticity in both visual and somatosensory cortex (Fox and Wong, 2005).

A number of other genes were regulated by whisker deprivation in common with visual cortex including CaMKII delta (Tropea et al., 2006), which is not known to be involved in plasticity, but is regulated by BDNF (Slonimsky et al., 2006), which is implicated (Kaneko et al., 2008). BDNF message levels appeared to be elevated in our study (approximately 2-fold)

but this did not reach significance ($\alpha = 0.05$). Other enigmatic factors included DEAD-box, which were regulated in the present study (14 cases at 8 days including Ddx 3y, 5, 10, 18, 26, 27, 31 and 55) and by monocular deprivation Ddx6 (Tropea et al., 2006). These DEAD box proteins are helicases that are involved in transcription and translation. Their involvement in cortical plasticity is unknown, but is evident that continued control of transcription and translational processes are required during plasticity in the visual and somatosensory cortices.

Identifying common and unique genes within the experiments, has certainly given a better insight into the plasticity paradigm studied. The next step into the analysis that would enhance our understanding was to identify a mechanism unique for the wild type mice, which is not present in the GluR1 knockouts used in the study. Such a comparison revealed one of the members of the homer family of proteins, which are the principal component of glutamatergic postsynaptic density protein complexes (Shiraishi *et al.* 1999, 2003). The identified transcript is *Homer 2*, which is mainly localised in the postsynaptic density in neurons acting as an adaptor for other proteins (Shiraishi-Yamaguchi & Furuichi 2007). During postnatal mouse brain development, *Homer 2* is observed in the cortex and one of its interacting partners is glutamate receptor 1 (Yoko Shiraishi-Yamaguchi & Teiichi Furuichi 2007). Recent research has indicated that *homer 2* is critically involved in synaptic morphology (Shiraishi *et al.* 1999, 2003). In particular, it binds to actin binding and remodelling proteins in dendritic spines giving us the chance to better understand dendritic clustering and synaptic targeting. The fact that this transcript is unique to the wild types, it indicates a possible mechanism for plasticity that requires glutamate receptor 1 subunit that is obviously missing from *Glur1^{-/-}*. Further investigations, should make possible the detection of mechanisms that require glutamate receptors and help us obtain a better insight of the pathways involved and action and interaction of a variety of molecules.

8.6. Final Conclusions

Although, whisker deprivation induced plasticity has been studied since the 1960's to detect long lasting synaptic strength changes (LTP and LTD) underpinning learning and memory, this thesis studied experience dependent plasticity from a molecular aspect and has revealed a

number of contributing genes underlying synaptic plasticity in the barrel cortex in a greater manner than considered before.

Analysis of the global and targeted microarray experiments has shown that isoflurane regulates plasticity associated genes in the mouse barrel cortex when applied repeatedly but not continuously over time. The implication of calmodulin as documented in the Day 16 data indicates that mechanisms potentially implicated in learning and memory are affected by isoflurane. Thus, it is suggested by this work that similar future studies should take into account the anaesthetic effect when investigating changes in neuronal strength by applying similar normalisation protocols as the one suggested in this thesis (Chapter 5). It is crucial to separate isoflurane induced changes from deprivation induced ones.

Control data has revealed a profound gender effect. Targeted microarray study has indicated different profiles between females and males and has also revealed a lack of consistency within the females. Steroids are implicated in sexual maturation of mammals and they have definitely shown their effects in the present study. Sexual maturation drove the two genders to diverse hormone-dependent behaviours. This study reveals to future researchers the necessity for a more careful, considered and well documented selection of subjects for their studies. Equal numbers of both genders has been shown to be important in order to obtain a better representation of the population under investigation.

Analysis of the deprivation effect on gene expression has revealed important information on time points of great interest for potentiation and depression. Early changes in gene expression were identified at Day 1, whereas the most critical time point was found to be Day 8 which opens new field opportunities. This thesis suggests more intense investigation of day 8 using whisker deprivation as well as other plasticity paradigms such as monocular or binocular deprivations.

The discovery of “*calcium dependent binding*” during gene ontological analysis confirmed the documented importance of calcium at synaptic formation and regulation of synaptic transmission as well as the importance of molecules associated with calcium or activated by its entry into the neuronal cell, such as NMDARs, AMPARs and NOS. Calcium mediated neurotransmitter release was shown to be of significant importance when plasticity was induced in the wild type mice. A significant difference between females and males was

indicated by the presence of GO categories only in the female data associated with cellular changes affecting cell communication, which were no longer over-represented after two weeks suggesting that the already obtained (during the first week) neuronal changes were enough to alter synaptic structure and strength in the female barrel cortex.

Studying the GluR1^{-/-} mice has been found to be more intriguing than initially anticipated. Results suggested a compensatory mechanism providing support for synaptic changes in an attempt to respond to deprivation. Although, GluR1^{-/-} mice have been found, in the past, to exhibit impaired LTD and LTP, which are an artificial way of detecting synaptic changes, they were found in this study to exhibit differential expression of proteins associated with memory and plasticity, such as PKC, BDNF and Frs2. The present result indicated that synapses in the GluR1 knockouts can show potentiation or depression which underpin activation of molecules, other than the GluR1, leading to synaptic alterations. This array provided more reasons for the GluR1 to be studied in depth.

Concluding, the presented thesis has revealed that anaesthetic, gender and deprivation regulate a number of genes after deprivation. It has also suggested that the critical point for plasticity related gene changes is day 8 and should be studied further. To disambiguate the contribution of genes to synaptic changes with deprivation, future array experiments are needed to determine gene associated with transcription factors discussed in the thesis. Lastly, compensatory mechanisms could be activated in the absence of GluR1 receptor, which need further investigating.

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APPENDIX 1

VECTOR SEQUENCES

pSPORT 1 Sequence, 4110 bp

CCATTGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAG
 GGGGATGTGCTGCAAGGCGATTAAGTTGGGTAAACGCCAGGGTTTCCAGTACGACGTTGTAAAACGACGGCCAGTGAA
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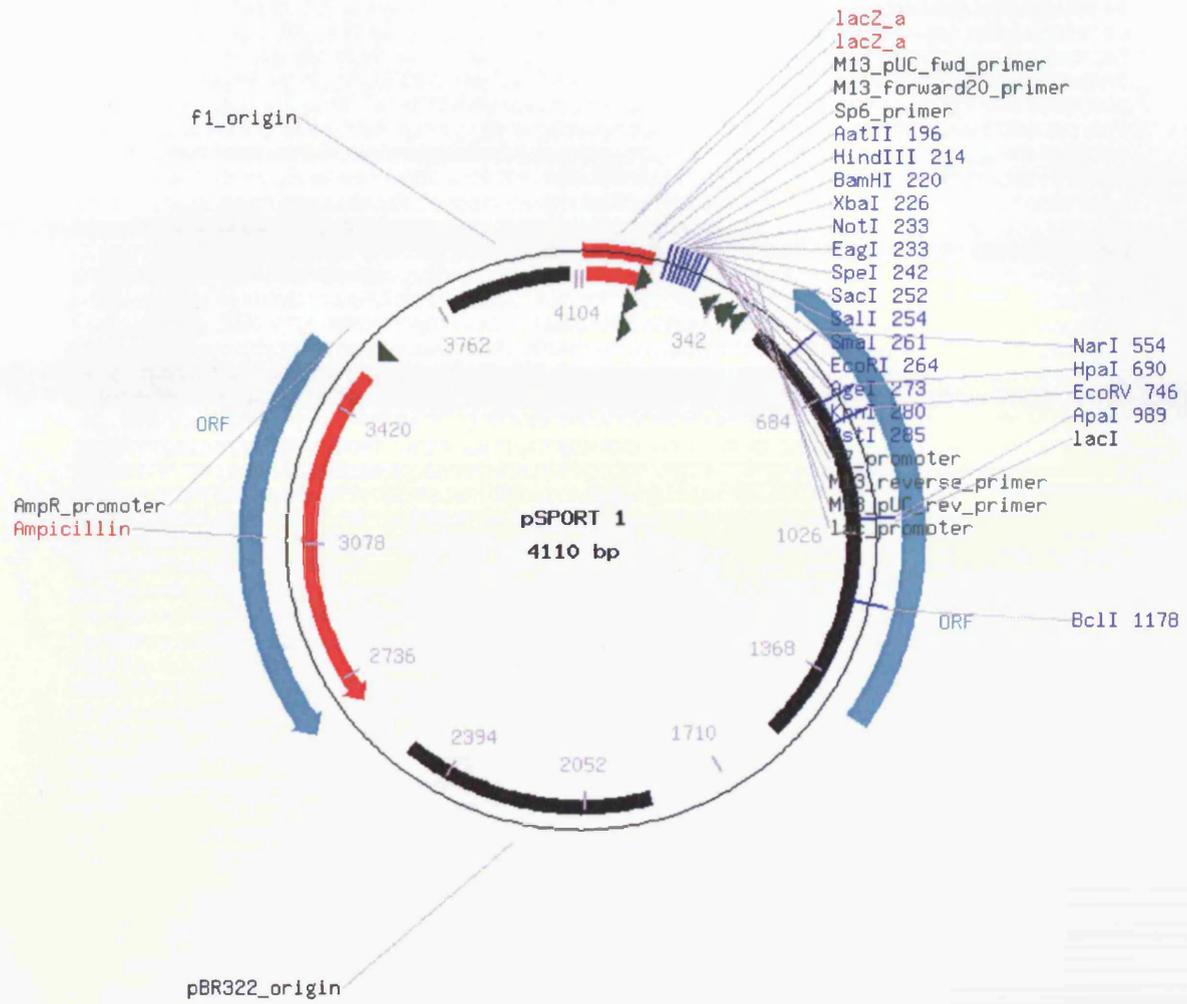


Figure 1: Graphical representation of the pSport 1 vector as obtained from <http://www.addgene.org/pgvec1?vectorid=99&f=v&cmd=showvecinfo>.

pGEM-T vector Sequence, 3000 bp

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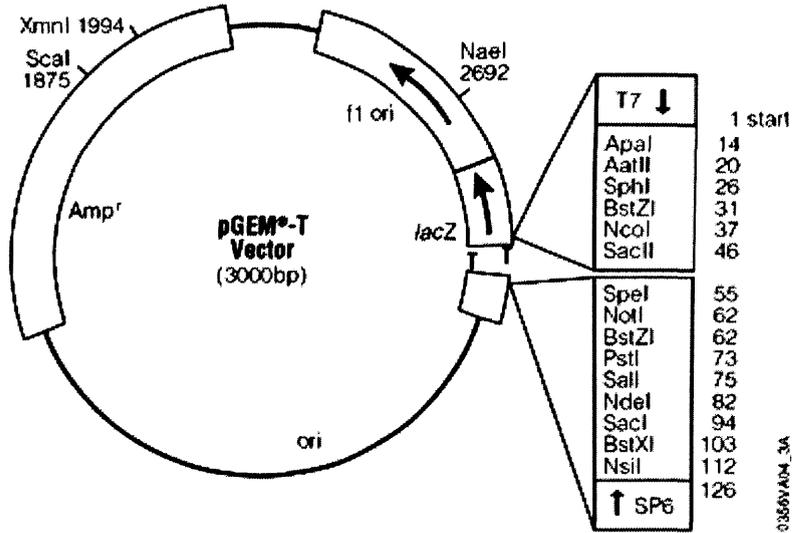


Figure 2: Graphical representation of the pGemT vector as obtained from <https://www.addgene.org/pglabs?f=v&cmd=showvecinfo&vectorid=153>.

APPENDIX 2

Gene Name	Common Name	Forward	Position of For.	Reverse	Position of Rev.	Amplicon Size	Unigene ID
Bassoon	ELE 1	AGCCACAGACACAACAGCAG	11351	GAGCCCTTCTGGACACAATC	11847	497	Mm.20425
Bcl2 – associated X protein	ELE 2	GAAGCTGAGCGAGTGTCTCC	239	GAAAAATGCCTTTCCCCTTC	734	496	Mm.19904
Carboxypeptidase E	ELE 3	TGATGGAGTGGTGGAAAATG	1479	GAAGTGGCATTAAACAGGCTGA	2025	547	Mm.31395
Citron	ELE 4	GTGGAGTCGCTTACCTCTGG	6282	CCCTGCTGCTGTCTTCAAAC	6826	545	Mm.8321
Cortistatin	ELE 5	CCAAGCAGGTGGTGCTAGAG	19	GCTGATTGACAGTCTTTATTAGGT	500	482	Mm.6204
Grin2a	ELE 6	GCTTCCCAACAATGACCAGT	3531	CTCCTCTTGCTGTCCTCCAG	4058	528	Mm.2953
Netrin 1	ELE 7	GATGTGCCAAAGGCTACCAG	1304	TTCTTGCACTTGCCCTTCTT	1805	502	Mm.39095
Neurogenic Differentiation 2	ELE 8	CGACCCCTTCTTTTTCTTTG	1392	GGCTTGGCTCTCTCTTTCCT	1941	550	Mm.4814
Neurotrophin 3	ELE 9	AGTGAGAGCCTGTGGGTGAC	676	TTACAGAAGGGTTCCCGAGAG	1225	550	Mm.267570
Nitric oxide synthase 1	ELE 10	CTCCTGGCTCAACCGAATAC	3734	GAACACACCAGCATCCTCCT	4238	505	Mm.44249
Nitric oxide synthase 3	ELE 11	GCACCCAGAGCTTTTCTTTG	3539	GAGGTGTCTGGGACTCACTGT	4078	540	Mm.258415

Paxillin	ELE 12	TTCAAGGAGCAGAACGACAA	1720	CTCTGGGAAACTGGGTGGT	2254	535	Mm.18714
Plasticity Related Gene 1	ELE 13	AACCCAAGCTGCAGTATTTGA	5052	TCAGTTTGGAAAACATTGCAT	5584	533	Mm.461245
Quiescin Q6	ELE 14	CCCATTCCTGCTGAAGTCTC	2682	CTAAACCCAGCACCTTCCAC	3219	538	Mm.27035
Soat	ELE 15	GCTTCGGTGGTATGATGCTT	1524	AACAGCAAAGCCCTTCTGAG	2038	515	Mm.7446
Spectrin <i>beta</i> 1	ELE 16	TCAGAGCCCAGATGAGTGTG	7433	CGACAGACAATGGTGTCGAG	7976	544	Mm.32881
Synaptopodin	ELE 17	GGGGTGCTGGAGTTAGATGA	1899	AAGAGGCACAAGGCAGGATA	2436	538	Mm.252321
VgluT2	ELE 18	TGAAACTCATGCCACAAAGC	3298	TGCAGTAAATTGGGATGTGC	3798	501	Mm.256618

APPENDIX 3

This appendix is provided electronically and it provides information on gene lists used to ontologically re-analyse the results from the Global Microarray study (Chapter 3), performed by Dr Richard Abraham. The gene lists are provided in the form of tables in Microsoft Excel.

ALL Time Course: not deprived control animals throughout the chosen time course

CB Time Course: CB deprived animals throughout the chosen time course

DEP Time Course: DEP deprived animals throughout the chosen time course

APPENDIX 4

This appendix is also provided electronically and it provides information on gene lists used to ontologically analyse the transcripts affected by the two confounding factors identified in Chapter 5, isoflurane and gender. The gene lists are provided in the form of tables in Microsoft Excel.

Undeprived Controls Final: contains a number of spread-sheets which include genes for not deprived control animals for female and male animals throughout the chosen time course as well as the genes in the complete group of animals, without gender separation.

APPENDIX 5

This appendix is provided electronically as well. It provides information on gene lists used to ontologically analyse the transcripts affected by whisker deprivation (CB and DEP) throughout the chosen time course (Day 1, 8 and 16) in both female and male wild type mice in the targeted microarray study (Chapter 6).

Two files; Female and Male deprived giving the genes affected by the two types of deprivation throughout time for the two different genders.

APPENDIX 6

This appendix is provided electronically as well. It provides information on gene lists used to ontologically analyse the transcripts affected by whisker deprivation throughout the chosen time course (Day 8 and 16) in GluR1-/- mice (Chapter 7). The gene lists are provided in the form of tables in Microsoft Excel.

One file under the name GluR Deprived CB DEP: giving the details on differentially expressed genes used throughout the knockout array study.

DAY08 and DAY16 files give the numerical values of all the genes used in the study whether they were differentially expressed or not.

APPENDIX 7

This appendix gives the details of Unique genes which were used in section 8.5 in chapter 8. This appendix contains 3 files with self explanatory names.

APPENDIX 8

This contains the GEO submissions we have created. These submission files give the numerical values of all the data and genes looked at in the array experiments performed. In the final version, these files will be replaced with a GEO submission number which has not become available to us yet. With this number, the reader can have a look at all the data of the experiment online.