The Role of the AMPA Receptor Subunit GluR1 and Nitric Oxide in Experience-Dependent Plasticity and Memory Formation.

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Abstract

Cortical maps undergo experience-dependent reorganisation throughout life and this type of synaptic plasticity is thought to underlie memory storage mechanisms. Neocortical and hippocampal synaptic long-term potentiation (LTP) initially requires the AMPA receptor subunit GluR1, while late-phase LTP depends upon nitric oxide synthase (NOS) signalling. To investigate whether this was relevant to *in vivo* experience-dependent (ED) potentiation, mice deficient in GluR1 and/or NOS were deprived of all whiskers but the D1 to induce barrel cortex synaptic potentiation, which was quantified by single unit recordings. In deprived cortex, D1 whisker responses potentiated approximately 40% less in GluR1 and NOS3 knockout mice than wild-type mice. Potentiation in the NOS1 knockout was influenced by gender; female NOS1 knockout potentiation was similar to wild-types, yet was absent in male NOS1 knockout mice. The barrel cortex ED potentiation in GluR1 knockout mice was dependent upon NOS, supporting LTP studies. However, NOS1 was more important for potentiation. Thus, while potentiation occurred in the GluR1/NOS3 double knockout mice, it was completely absent in the GluR1/NOS1 double knockout. To determine the interaction between GluR1 and NO activity in memory, behavioural studies examined their impact on spatial and contextual memory. The results partly confirmed earlier findings that retention of contextual fear conditioning was sensitive to GluR1 deletion. However, this was only the case in male GluR1 knockout mice. Female GluR1 KO mice were unimpaired. In a spatial radial arm watermaze task, GluR1 knockout mice acquired the location of a submerged platform more slowly than wild-types. Nevertheless, spatial reference memory was comparable to wild-type mice at the end of training and was not influenced by gender. In contrast to predictions, GluR1-independent reference memory was not dependent upon NOS. Therefore while emotional learning requires GluR1 in male mice, spatial reference memory can form in its absence in both genders and is insensitive to NOS antagonism. In conclusion, although GluR1-independent synaptic plasticity is supported by NO in the barrel cortex, this mechanism is not responsible for GluR1-independent spatial memory formation.

Acknowledgements

All of the work presented within this thesis is my own, except where clearly stated in Chapter 4. As such, I would like to thank Stanislaw Glazewski and Nick Wright for performing the early experiments required to launch this project. I would also like to thank Kevin Fox for taking a chance on me so I could achieve this thesis. Many thanks are owed to Mark Good for putting in a good word to Kevin so that the opportunity would exist at all. I am also appreciative to Nick Wright for spending such a great amount of time teaching me how to perform the experiments when I was new in the lab. I hope I have repaid this kindness in the way I have mentored new students in the lab.

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Abbreviations and Units of Measurements

ACSF	Artificial cerebrospinal fluid
αCaMKII	Alpha calcium/calmodulin-dependent protein kinase II
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AMPAR	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor
ANOVA	Analysis of variance
Brl	Barrelless phenotype mouse
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
CaMK	Calcium/calmodulin kinase
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
cm	Centimeter
CS	Contextual stimuli
D-APV or AP5	NMDA receptor antagonist
D1	D1 barrel/whisker
DAB	3,3′-diaminobenzidine tetrahydrochloride
DG	Dentate Gyrus
E	Embryonic day
EC	Entorhinal cortex
EDD	Experience-dependent depression
EDP	Experience-dependent plasticity
EPSP	Excitatory postsynaptic potential
GABA	Gamma-aminobutyric acid
GluR	Glutamate receptor
Hz	Hertz
icv	Intracerebroventricular

IP	Intraperitoneal
ISI	Inter-shock interval
KO	Knockout
LBD	Ligand binding domain
LTD	Long-tern depression
LTP	Long-term potentiation
M	Molar
mGluR	Metabotropic glutamate receptor
μA	Micro amp
μm	Micrometer
min	Minute
mm	Millimeter
mOsm	Milliosmoles
ms	Millisecond
NMDA	N-methyl-d-aspartate
NMDAR	N-methyl-d-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS1 or nNOS	Neuronal nitric oxide synthase
NOS2 or iNOS	Inducible nitric oxide synthase
NOS3 or eNOS	Endothelial nitric oxide synthase
ODI	Occular dominance index
ODP	Ocular dominance plasticity
P	Postnatal day
PBS	Phosphate buffered saline
PKA	Protein kinase A
POM	Posterior medial nucleus
PSD	Postsynaptic density
PSTH	Post-stimulus time histogram

PW	Principle whisker
s	Second
S1	Primary somatosensory cortex
S2	Secondary somatosensory cortex
SEM	Standard error of the mean
SRF	Surround receptive field
SW	Surround whisker
TBS	Theta burst stimulation
TMD	Transmembrane domain
US	Unsignalled stimuli
US	Unsignalled footshock
V1	Primary visual cortex
VDI	Vibrissae dominance index
VPM	Ventroposteriomedial nucleus
vs	Versus
WT	Wild-type
WVDI	Weighted vibrissae dominance index

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Chapter 1:

General Introduction

1.1. Thesis Aims

The first demonstration of sensory map plasticity came in a landmark publication from Hubel and Wiesel (1963). They showed that monocular deprivation of a kitten resulted in neocortical plasticity. Cortical cells would be preferentially driven by the non-deprived eye but not influenced by the deprived eye (Wiesel and Hubel, 1963). These types of manipulations led to the development of a new field of research interested in experience-dependent plasticity (EDP). Although visual cortex EDP has a defined critical period of onset (Gordon and Stryker, 1996), other neocortical structures are modifiable well into adulthood. Modification of the receptive fields were first demonstrated by relatively major manipulations of the sensory apparatus. Transection of nerves to the front paw in adult cats caused a cortical reorganisation in somatosensory cortex 1 (S1) whereby cortical cells now responded to stimulation of sensory areas or nerves that did not previously, under normal conditions, produce a strong response (Kalaska and Pomeranz, 1979). Similar results have been observed in adult monkeys where either nerve transection or digit amputation alters the receptive field such that the adjacent sensory apparatus (either skin or digit) expands its influence into the now deprived sensory cortical area (Merzenich et al., 1983, 1984; Clark et al., 1988). Such results have also been replicated in lower animals by digit amputation in the raccoon, suggesting this process is not restricted to just higher mammals (Rasmusson, 1982).

The rodent somatosensory cortex, or barrel cortex, also undergoes similar EDP in response to sensory experience while remaining plastic throughout adult life (Glazewski and Fox, 1996). The barrel cortex organization and synaptic physiology shares many similarities to the visual cortex (Fox and Wong, 2005), suggesting that common mechanisms for plasticity exist across brain regions. EDP measured in the barrel cortex therefore represents an excellent model system to investigate synaptic plasticity in relation to experience in adult subjects.

The barrel cortex processes the tactile stimuli that arises from whisking behaviour. Mice sense their surroundings using whiskers located on the snout and moving these whiskers in the air enables mice to form a representation of the local surroundings. Under control conditions, each whisker is represented by a single neocortical barrel, and responses evoked by stimulating a whisker are generally confined to its

topographically related barrel column (Armstrong-James et al., 1992). However intracortical connections between the barrel columns do not remain fixed; in fact they are highly plastic in response to manipulations made to the whiskers and this process will continue throughout the life of the animal (Glazewski and Fox, 1996). Similar to the monocular deprivation experiments performed by Weisel and Hubel (1963), depriving the whiskers of a rodent leads to changes in the cortical receptive fields within the barrel cortex by the expansion of the spared sensory input (Fox, 1992).

There are several advantages to performing EDP experiments using the barrel cortex as a model system. (1) Rodents are relatively inexpensive to house. (2) Genetic manipulations are now possible in mice that allow receptors to be manipulated where previously no pharmacological inhibitors were available. (3) The anatomical organization of the barrel cortex is less complicated than the visual cortex and is well-characterised (Fox, 2002). (4) Performing *in vivo* extracellular single unit recordings is not limited by the age of the subject, unlike *in vitro* intracellular techniques, allowing EDP to be examined at any age. (5) The stimulus is a naturalistic manipulation of the sensory experience. The EDP that subsequently occurs following whisker deprivation is a natural process. This is not the case for artificial long-term potentiation (LTP) protocols where it is not definitively known what plasticity induction paradigm is likely to be most relevant to 'natural' potentiation during learning. Taken together, whisker deprivation allows whole animal neocortical synaptic plasticity to be studied at ages rarely attempted *in vitro*, unlike LTP that requires young tissue and induction is highly dependent upon the chosen induction method (see Feldman, 2009).

The molecular components required for experience-dependent potentiation are beginning to be documented. It is known that neocortical and hippocampal LTP requires the GluR1 subunit of the AMPA receptor (Hardingham and Fox, 2006; Phillips et al., 2008; Frey et al., 2009; Romberg et al., 2009; Hoffman et al., 2002). GluR1 also appears necessary for barrel cortex EDP (Clem and Barth, 2006). Despite this, it is not known how the genetic removal of GluR1 affects response magnitude following experience-dependent potentiation. LTP studies have indicated the potentiation can occur in the GluR1 knockout (KO), although only if a spike-timing dependent plasticity (STDP) protocol is used (Hardingham and Fox, 2006). Therefore

this thesis will examine whether the AMPA subunit GluR1 is necessary and/or sufficient for barrel cortex experience-dependent potentiation. LTP studies have also demonstrated that barrel cortex LTP in the GluR1 KO is sensitive to nitric oxide synthase (NOS) antagonism (Hardingham and Fox, 2006), although the NOS isoform responsible for the spared plasticity has yet to be characterised. Therefore should EDP be possible to establish in the GluR1 KO, its susceptibility to NOS antagonism will be investigated. Individual NOS KO mice will be bred with GluR1 KOs to determine whether any single NOS isoform is more important for neocortical plasticity. Developmental analysis will also be conducted in undeprived control (whiskers are not deprived) mice of all genotypes to determine whether any one molecule is required for neocortical development that could potentially undermine abnormalities observed in EDP.

It is thought that synaptic plasticity within the neocortex is not only important for recovery from major neurological disease states (such as ischemia; Fox, 2009) and the processing of sensory experience, but also underlies long-term memory (see Alvarev and Squire, 1994; Bontempi et al., 1999). LTP in the hippocampus and EDP in the barrel cortex share many of the molecular components required for cortical synaptic plasticity. For example, synaptic potentiation in both regions requires NMDA receptors, AMPA receptors and CaMKII (Feldman, 2009; Fox, 2002). It is therefore likely that deficits in experience-dependent potentiation found in the barrel cortex reflect synaptic plasticity deficits in other brain regions. Indeed, hippocampal LTP also requires GluR1, with late-phase LTP being sensitive to NOS antagonism (Phillips et al., 2008; Romberg et al., 2009), similar to the barrel cortex (Hardingham and Fox, 2006). Hippocampal-dependent learning and memory also shows sensitivity to GluR1 deletion. The GluR1 KO has a deficit in spatial working memory/short term habituation, while spatial reference memory/long-term habituation is unaffected (Schmitt et al., 2003, Sanderson et al., 2009). It is possible that this dissociation reflects the deficit in early-phase LTP but not late-phase LTP in the GluR1 KO. Hence, the synaptic plasticity that mediates the reference memory in the GluR1 KO could depend upon nitric oxide (NO) signalling. Therefore the final aim of this thesis will be to test whether the spatial learning that can occur in the GluR1 KO is sensitive to NOS antagonism, similar to the findings from barrel cortex and hippocampal LTP studies (Hardingham and Fox, 2006; Phillips et al., 2008).

Within this chapter I will review the development of the barrel cortex, the plasticity of layer II/III connections and the molecular mechanisms that mediate this plasticity, with particular reference to GluR1-dependent and independent processes. I will also review the hippocampus' role in memory and it's anatomy, along with how the absence of GluR1 and NO affects memory formation, drawing parallels with LTP studies.

1.2. The Barrel Cortex

1.2.1. From Whisker to Cortex: Inputs to the Cortex

This section provides an overview of the anatomy of the barrel cortex. Each whisker follicle has a dense connection of nerves that relays the whisker's sensory input (Figure 1.1) (Ebara et al., 2002). The nerves from the follicles eventually form part of the maxillary nerve, which becomes a subdivision of the trigeminal nerve (see Petersen, 2007). Inputs from the trigeminal nerve project to the thalamus. For this reason, the thalamus is a critical component of cortical plasticity circuits.

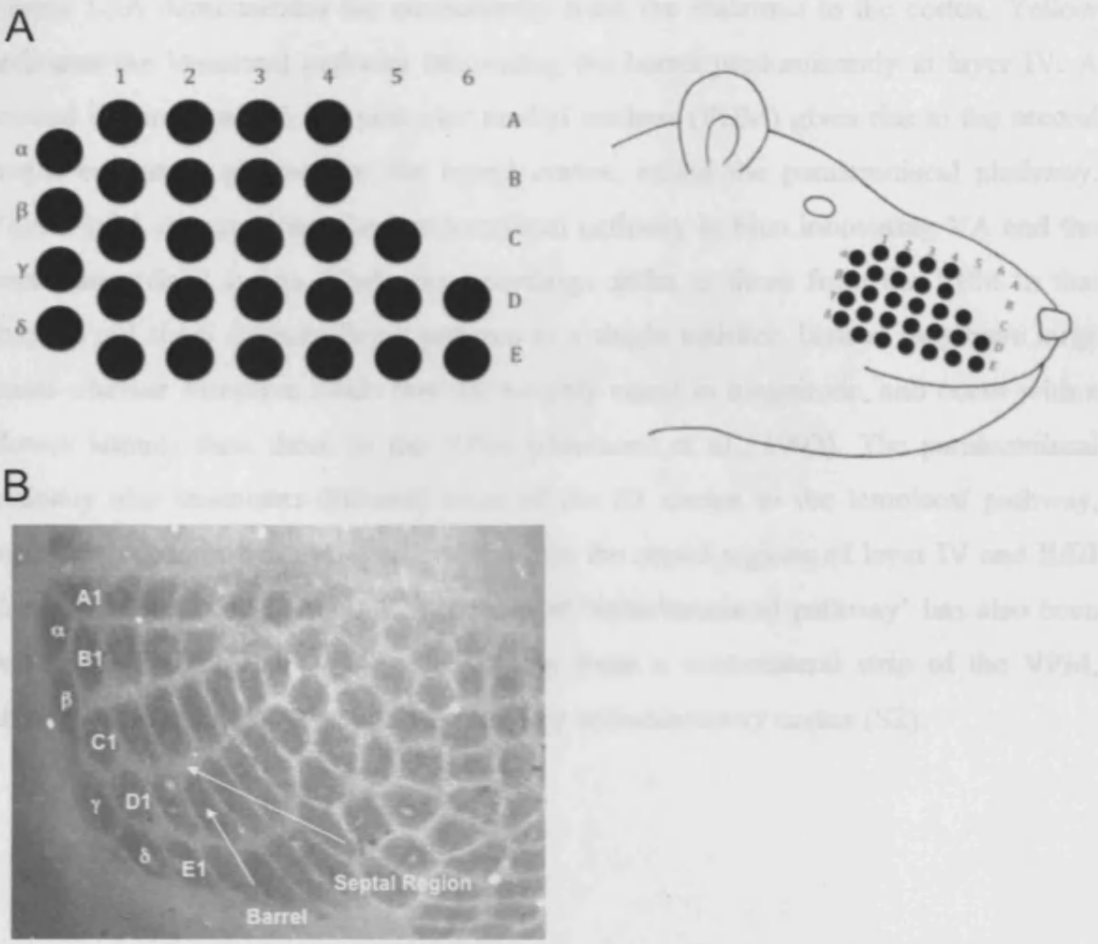


Figure 1.1. *The structure of the whisker/mystacial pad and its cortical representation.* ***A.*** *The major whiskers are found on the snout of the animal and arranged in rows (letters) and arcs (numbers).* ***B.*** *The whiskers are represented in the cortex in a barrel formation, with each barrel representing its topographically related whisker. The arrows indicate either the barrel or the septa surrounding the barrel, innovated by the lemniscal and paralemniscal pathway, respectively. The example barrel field is adult layer IV stained for cytochrome oxidase.*

The entire mystacial pad is mapped onto the ventroposteriomedial nucleus (VPM) of the dorsal thalamus, similar to the barrel field, in a 'barreloid' formation. Neurons in the VPM respond predominantly to a single whisker (that being the topographically related principal whisker; Diamond et al., 1992; Petersen, 2007; Simons and Carvell, 1989). The lemniscal pathway arises from the VPM and innovates mainly layer IV barrels (the barrel proper and not the septal regions surrounding the barrel) and more weakly layer VB and VI (for review of thalamocortical pathways see Brecht, 2007).

Figure 1.2A demonstrates the connectivity from the thalamus to the cortex. Yellow indicates the lemniscal pathway innovating the barrel predominantly at layer IV. A second thalamic nuclei, the posterior medial nucleus (POM) gives rise to the second major excitatory pathway to the barrel cortex, called the paralemniscal plathway. Figure 1.2A demonstrates the paralemniscal pathway in blue innovating VA and the septal superficial layers. Thalamic recordings differ to those from the VPM in that they do not show discrete firing patterns to a single whisker. Instead they have large multi-whisker receptive fields that are roughly equal in magnitude, and occur with a slower latency than those in the VPM (Diamond et al., 1992). The paralemniscal pathway also innovates different areas of the S1 cortex to the lemniscal pathway, innovating predominantly layer VA and also the septal regions of layer IV and II/III (Brecht, 2007; Bureau et al., 2006). A third 'extralemniscal pathway' has also been described (Pierret et al., 2000) originating from a ventrolateral strip of the VPM, although this mostly innovates the secondary somatosensory cortex (S2).

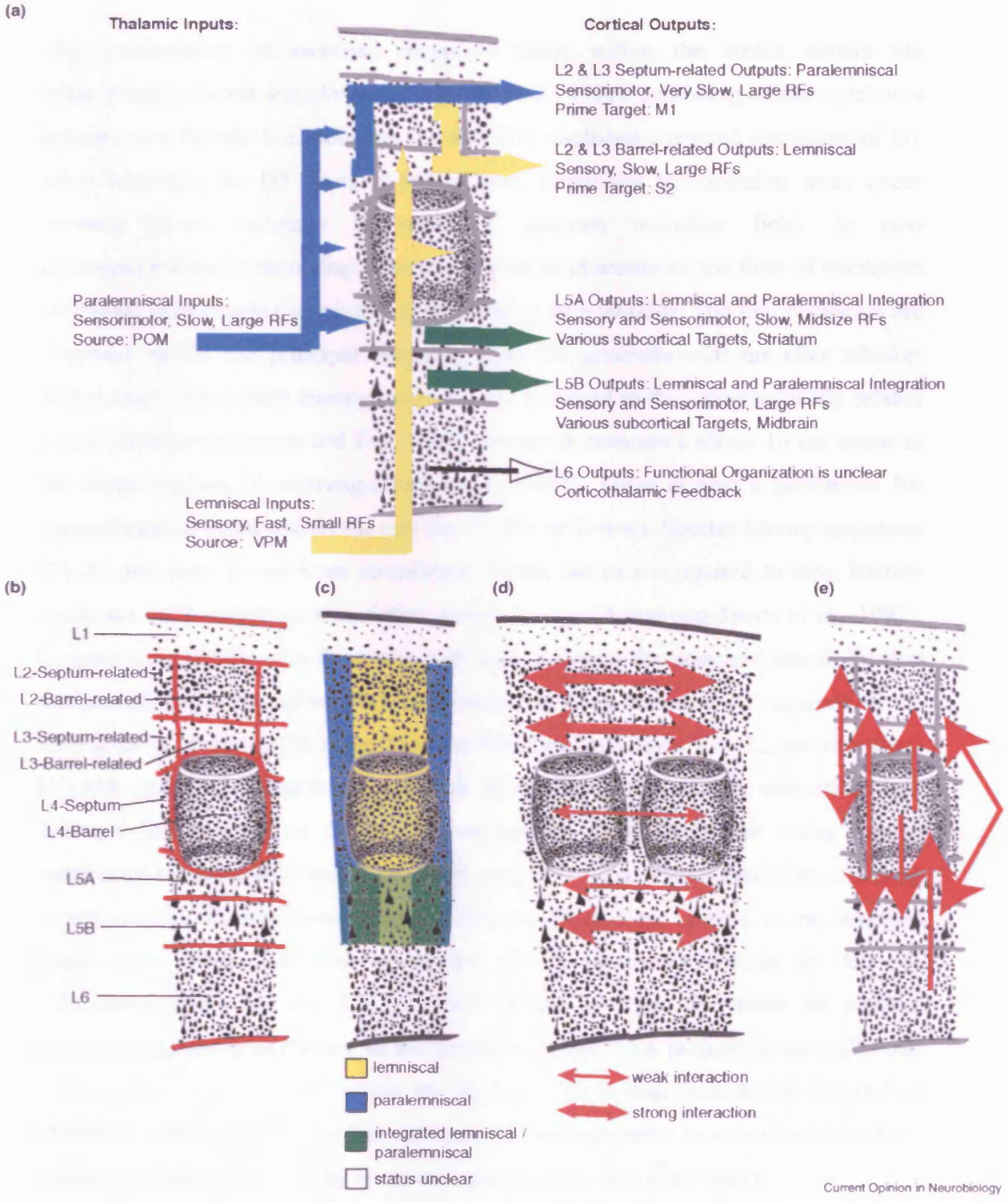


Figure 1.2. *Innervation of the cortex from the thalamus by the lemniscal and paralemniscal pathways. The relative size of the arrows represents strength.* ***A.*** *The innervation of a barrel by both thalamic pathways.* ***B.*** *The architecture of a barrel.* ***C.*** *The lemniscal and paralemniscal pathways within a barrel column.* ***D and E.*** *Interactions with other columns and within a column. Taken from Brecht (2007).*

The development of surround receptive fields within the cortex occurs via intracortical transmission (Armstrong-James et al., 1991). Lesioning of the septal area between two barrels (i.e. between D1 and D2) abolishes surround responses of D1 when recording in D2 (Fox, 1994). Hence, horizontal transmission must occur between barrel columns to refine a neurons receptive field. *In vivo* electrophysiological recordings have been used to characterise the flow of excitation within the barrel cortex in relation to the striking of a whisker. The first responses are observed within the principal barrel in layer IV generally <10 ms after whisker stimulation. These short latency responses are confined to the topographically related barrel (Armstrong-James and Fox, 1987). However responses above 10 ms occur in the septal regions (Armstrong-James et al., 1992). There is also a preference for transmission to occur within the row (i.e. C row or D row). Shorter latency responses (11-20 ms) were found from stimulation within the row compared to long latency responses (>21 ms) from stimulation across the arc (Armstrong-James et al., 1992). Response magnitude also decreases with distance from the principal barrel. Further analysis of the latency of vertical transmission revealed that layer III responds ~2 ms after layer IV, followed by layer II (~1 ms after layer III), layer VA (3.2 ms after layer IV) and layer VI (Armstrong-James et al., 1992). Some 93% of cells respond to their principal whisker before that of the surrounds, highlighting that under control conditions, transmission occurs within the principal barrel before that of the surround receptive field. Connections in surrounding barrels that are nearest to the principal barrel occur faster than those on the far side of the barrel (3.9 ms vs 11.6 ms) (Armstrong-James et al., 1992). These results provide the basis of cortical transmission, firstly occurring in the principal barrel, then projecting vertically and finally emanating outward across the barrels. Given that cross-barrel transfer is abolished with septal lesions but without affecting responses in either barrel hollow, communication must occur by horizontal transmission (see Fox, 2002).

Layer II/III receives input based on their location in relation to the barrel/septal region. Layer II/III cells that are located directly above the layer IV barrel receive the majority of their input from that barrel, although a subset of neighbouring barrels do provide an input (Shepherd et al., 2003). Layer II/III cells that are located above the septa receive a non-uniform input from layer IV that do not reflect distinct barrel boundaries (Shepherd et al., 2003). In many cases, transmission was stronger from

other layer II/III cells than it was for layer IV (Shepherd et al., 2003). The predominant excitatory cell type in layer II/III is pyramidal, whereas in layer spiny stellate and star pyramidal neurons dominate (see Feldmeyer and Sakmann, 2000).

1.3. Barrel Cortex Development

1.3.1. Prenatal Patterning

The major input to the barrel cortex arises from its related thalamic nuclei. Sensory transmission through thalamocortical afferents (TCA) is vital for the perception of 'sense' via whisker activation and failure for these connections to occur normally will undoubtedly impede normal sensory detection and co-ordination. Therefore the ability of these projections to locate and innervate the correct cortical area is critical. Full maturation of the barrel cortex is underpinned by interplay between intrinsic genetic mechanisms governing development and extrinsic sensory signals. Taking each in turn, the next section will establish the nature of the genetic factors that contribute to barrel cortex development.

Development of the barrel cortex occurs before birth at around embryonic day (E) 10 to 17 (Inan and Crair, 2007). Until recently, the genetic information regarding neuronal migration was lacking. However, a series of transcription factors and morphogens have been implicated (see Inan and Crair, 2007). The process by which the cortex is separated into its functional areas is called arealisation, of which there are four (Figure 1.3): visual (V1), somatosensory (S1), auditory (A1) and motor (M1). The majority of cortical neurons are glutamategic, which are formed by progenitors in the ventricular zone and the subventricular zone of the dorsal telencephalon. Projections from the ventricular zone form layer 5 and 6 and the subventricular zone is the source of layers 2, 3 and 4. GABAergic inhibitory interneurons are generated in the ganglionic eminences of the ventral telencephalon (O'Leary and Sahara, 2008). *Fgf8* and *17* have been implicated in the formation of the commissural plate, where their major role is to form the concentration gradients that define transcription factor expression in the cortical progenitors (Fukuchi-Shimogori and Grove, 2001 and 2003; Storm et al., 2006). In particular, *Emx2*, *Pax6*, *COUP-TFI* and *Sp8* have all been heavily implicated in the area patterning process (Bishop et al., 2000 and 2002; Hamasaki et al., 2004; Pinon et al., 2008).

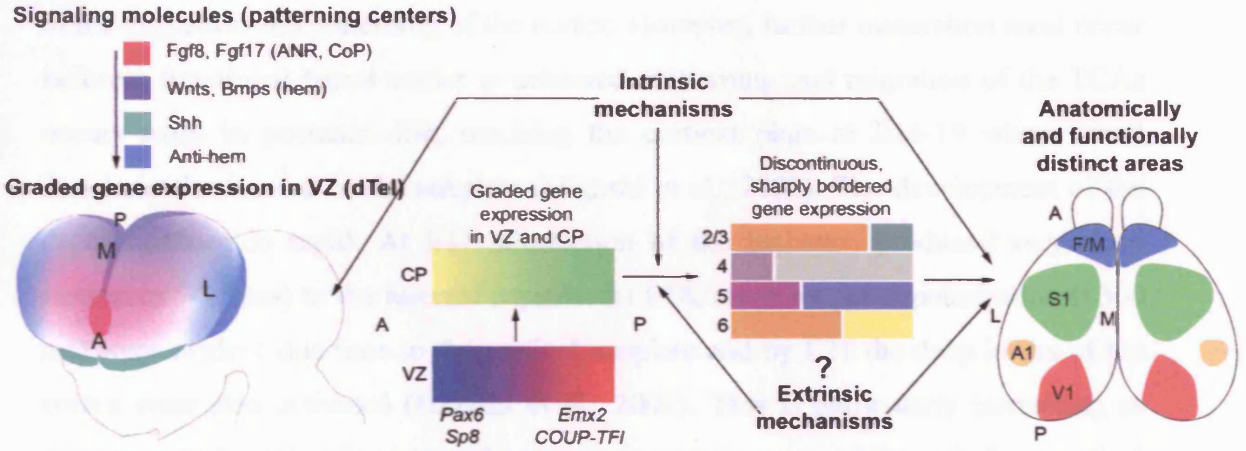


Figure 1.3. *Morphogens and transcription factors involved in arealisation. Gradients of factors form the medial to lateral and anterior to posterior axis of the cortex. VZ = ventricular zone, CP = cortical plate. Taken from O'Leary and Sahara, 2008.*

In addition to the patterning of the cortical areas, guidance of the thalamocortical afferents (TCA) is critical to a functional cortex. The process of arealisation is independent of TCA migration (for example Garel et al., 2003; Pinon et al., 2008). Guidance of the TCAs is mediated by a set of transcription factors, in particular *neurogenin2* (*Ngn2*). It has been shown that within the projecting axon, *Ngn2* is responsible for controlling the effectiveness of cues within the intermediate target zone, the ventral telencephalon, that affect the patterning of the topography of thalamocortical projections (Seibt et al., 2003).

Ngn2 is an important factor for development, as it seemingly has two roles. As discussed above, it is involved in TCA guidance. It also has a role in proneural functions, along with *Ngn1* and *Mash1*. Single and double mutants (of either *Ngn1* and/or *Mash1*) all show some degrees of neurogenesis defects with a loss of the ability to initiate the Notch signalling processes (Bertrand et al., 2002). Other critical functions include the regulation of cell cycle, neuronal subtype differentiation and differentiation between glial and neuronal cells (for review see Bertrand et al., 2002).

1.3.2. Development Mediated by Activity

The previous section provides a small insight into the various factors that are involved in the migration and patterning of the cortex. However, further maturation must occur before a functional barrel cortex is achieved. Patterning and migration of the TCAs occurs early in postnatal life, reaching the cortical plate at E16-19 where small depolarisations occur in the subplate (Higashi et al., 2002). The development of the depolarisations is rapid. At E17 stimulation of the thalamus produced very short responses (<15 ms) to the internal capsule. At E18, much longer depolarisation (<300 ms) were evident this time to the cortical subplate and by E21 the deep layers of the cortex were also activated (Higashi et al., 2002). This is particularly interesting as patterning and arealisation is based on intrinsic genetic cues and these thalamocortical connections become active before birth, both facts suggesting that barrel cortex cortical formation occurs before birth. TCAs continue to develop postnatal, and by P4 a recognisable structure to layer IV can be detected (Erzurumlu and Jhaveri, 1990). Development continues after this period, and by P12 each barrel has the classic one-to-one topography with its thalamic barrelliod, cells bodies are arranged into the barrel walls with dendrites orientated to the barrel hollow (Inan and Crair, 2007). Postnatal development and the increased dependence on synaptic potentials to guide formation are theoretically interesting as it provides the opportunity to determine whether pharmacological inhibition of synaptic activity could disrupt barrel field formation.

NMDA was a candidate receptor that could mediate developmental synaptic activity, especially as GluR1 is not present until P4 (Watson et al., 2006). The NMDA receptor (NMDAR) antagonist D-2-amino-5-phosphonopentanoic acid (D-APV) was applied to the barrel cortex from birth in an attempt to inhibit NMDAR activation. Surprisingly, the barrel field pattern was not disrupted, although plasticity induced by manipulations of the sensory apparatus was inhibited (Schlaggar et al., 1993). A follow up study confirmed a phenotype that could not be visualised by staining. *In vivo* recordings from layer IV revealed that instead of the expected one-to-one topographic organisation (by short latency responses), TCAs spread across several surrounding barrels and short latency responses were found some considerable distance from the topographically related barrel (Fox et al., 1996). The results of this study provide two lines of evidence. Firstly, activity is required for topographic

refinement and secondly, the confirmation that basic patterning of the barrel cortex occurs prenatal and can take place despite postnatal activity.

While NMDA has been implicated in full development, antagonists can only be applied after birth. The advent of genetically modified animals has allowed the investigation of other receptors and molecules in development during pre and postnatal stages. Serendipity provided the first rodent lacking a barrel field (Welker et al., 1996). Aptly, the mutation was called *barrelless* (*brl*) and they were found to have much broader TCA arbors with whisker responses found in the non-topographically related barrel (Welker et al., 1996). Detailed investigation of the mutant revealed that the adenylyl cyclase type 1 gene (*Adcy1*) was disrupted. This inhibited the cAMP pathway cascade following calcium influx (Abdel-Majid et al., 1998). From this initial work, further steps in the signalling cascade have been identified as critical to barrel development. In no particular order, mutants of the PKA RIIβ (Watson et al., 2006), NR1 subunit of the NMDAR (Iwasato et al., 2000), metabotropic glutamate receptor 5 (mGluR5) and phospholipase C-β (PLCβ) (Hannan et al., 2001), 5-hydroxytryptomine transporters (5HTT) and monoamine oxidase A (MAOA) (Salichon et al., 2001) and finally growth associated protein 43 (GAP43) (Maier et al., 1999) all result in some form of barrel cortex abnormalities. One notable feature of all of these is that they are activated or are related to responses to synaptic activity, underlining that activity is required in addition to intrinsic signalling.

1.3.3. The Critical Period

As discussed in the previous section, activity is one factor that guides cortical development. The activity that drives the barrel cortex comes from deflections of the whiskers, much like sight drives the visual cortex. Therefore, if one were to disrupt this activity by manipulations of the sensory apparatus, this could retard development? This suggestion receives support from inhibition of NMDARs, which causes aberrant TCA layer IV connections (Fox et al., 1996).

The critical period was first proposed from studies conducted in the visual cortex. The visual cortex is a cortical structure that like the barrel cortex can be manipulated by

experience; that is deprivation of sight by suturing of the eyelid. Monocular deprivation (suturing of one eyelid) produced shifts of the receptive field properties in the visual cortex (area 17) towards the open eye and there was reduced response to the closed eye (Wiesel and Hubel, 1965). Further experiments by Hubel and Weisel revealed that these shifts were age dependent. Molecular deprivation initiated up to 12 weeks could caused dominance shifts away from the closed eye, yet beyond 3 months there was a sharp decline in these receptive field shifts and were absent in adults (Hubel and Wiesel, 1970). Also, 3 months of deprivation from birth followed by up to 5 years of normal sight produced very limited recovery (Hubel and Wiesel, 1970). Therefore, neocortical plasticity can occur but its initiation and magnitude is governed by a critical period.

An analogous mechanism exists in the rodent barrel cortex. Sensory activity in the barrel cortex is driven by whisker activity. Manipulation of sensory activity found that cortical modifications could take place if whisker deprivation was conducted soon after birth. Removal of a row of whiskers by follicle cauterisation directly after birth resulted in the absence of barrels in S1 (Van der Loos and Woolsey, 1973). Yet if this treatment was conducted only 3 days later, no obvious effect on layer IV could be seen (Schlaggar et al., 1993). Similar results were obtained following transection of the infraorbital nerve after birth (Figure 1.4; Killackey et al., 1994).

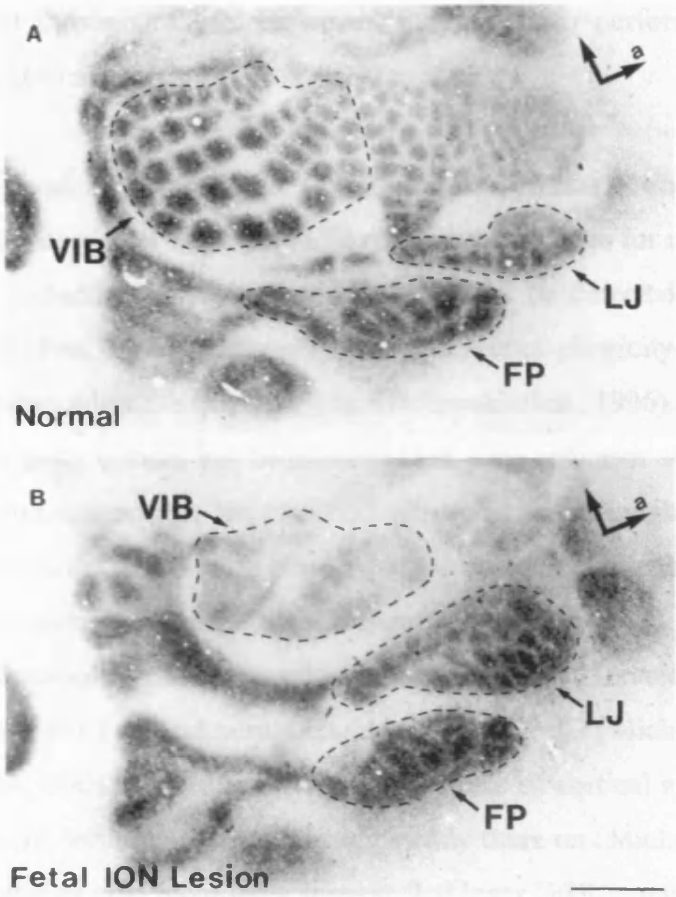


Figure 1.4. *Transection of the infraorbital nerve at birth causes the absence of layer IV barrel patterning. There is now an overwhelming body of evidence that links extrinsic activity to barrel cortex formation. Taken from Killackey et al., 1994.*

Cauterisation and nerve transection are major and irreversible manipulations. To investigate if and when these manipulations no longer affected patterning, Fox (1992) pulled the whiskers from their follicles to initiate sensory deprivation and measured the sensory responses *in vivo*. The results indicated clear time windows that were not similar to other cortical regions. Plasticity decreased remarkably rapidly in layer IV if the deprivation was initiated after P4. Interestingly, short latency responses to D1 stimulation could be detected outside of the D1 barrel column, a condition that is not normal in controls (Fox, 1992). This rather narrow time window fits remarkably well to TCA modifications. The first barrel can be observed at P4 due to the TCA clustering into the barrel shape (Erzurumlu and Jhaveri, 1990). The obvious conclusion is that early activity disruption caused aberrant TCA connections and that

these are present throughout life. However, manipulations performed after P4 are relatively resistant to modification (Fox, 1992).

Layer II/III is also an important factor in barrel cortex critical period. While layer IV plasticity all but ceases after P4, layer II/III plasticity continues for much longer (Fox, 1992). A small reduction in plasticity magnitude can be detected if deprivation is initiated after P2 (Fox, 1992), although the magnitude of plasticity from P2 remains fairly consistent into adult life (for example, Glazewski et al., 1996). A more thorough analysis of layer II/III critical has been conducted. One criticism of the Fox study is that recordings were made in 'adult' (P30-90) animals, although deprivation was initiated close to birth. This leaves a long period where modifications might occur. Also, single unit recordings only provide a measure when a cell spikes. Therefore significant subthreshold activity is missed from analysis. Developmental analysis suggests that layer II/III critical period could be later than P2 (Micheva and Beaulieu, 1996; Stern et al., 2001). There is a two-step increase of cortical synapse number at P10-15 and P20-30, with numbers remaining steady there on (Micheva and Beaulieu, 1996). Intracellular *in vivo* recordings suggest that layer II/III is mature by P14 yet if deprivation occurs before this period, receptive field maps are disrupted (Stern et al., 2001).

Development of the barrel cortex is a delicate balance of intrinsic genetic and extrinsic activity signals. Disruption of each step can lead to developmental abnormalities, some more striking than others. Even once patterning has occurred, manipulations of the sensory signals if initiated at the correct time can lead to lasting plastic changes in cortical connections. Considering that experience ultimately underlies how we learn and is the factor that governs barrel cortical modifications, understanding the mechanisms underlying these processes can inform our understanding of the role of plasticity mechanisms in encoding and storing information.

1.4. Barrel Cortex Plasticity

1.4.1. Plasticity of Layer II/III Connections

Although plasticity in layer IV is restricted to very early postnatal days, layer II/III remains plasticity well into adulthood. Deprivation of all but a single whisker resulted in the expansion of that whisker into surrounding barrel columns while weakening and contracting the representation of the deprived barrel columns (Fox, 1992; Diamond et al., 1993; Glazewski and Fox, 1996). The depression of the deprived input and the expansion of the spared input are time- and age-dependent. That is, the time course of potentiation and depression are separable and the occurrence of depression is dependent on the age of the animal.

Using the single whisker paradigm, it was found that after 7 days deprivation there was an increase in the proportion of layer II/III cells that responded to the single whisker (Glazewski and Fox, 1996). However this effect was related to the depression of the deprived input. Surround whisker potentiation occurred more quickly (between 7 days and maximal at 14 days) in subjects that underwent chessboard deprivation (deprivation of every other whisker – each whisker has 4 out of 8 surrounding whiskers removed; Hardingham et al., 2008). Depression continued for up to 20 days, which was also accompanied by the expansion of the spared whisker representation (Glazewski and Fox, 1996). Spared whisker responses continued to expand up to 60 days, although at this time point the depression effect was less pronounced (Glazewski and Fox, 1996). Recent studies have found that around 20% of layer II/III cells show some potentiation in the spared barrel column after 16 hours of single whisker deprivation (Barth et al., 2000). Yet after 20 days deprivation, no significant principal whisker potentiation or deprived whisker depression was observed in the spared whisker column (Glazewski and Fox, 1996). It should be noted that depression is stronger if there are competitive inputs, i.e. activity from a subset of whisker(s) as opposed to a total deprivation (Glazewski et al., 1998). Indeed depression is strongest if only one whisker is removed (Glazewski et al., 1998). Whisker deprivation has also been shown to decrease the amplitude of EPSPs in the deprived but not undeprived row of whisker barrels, facilitate subsequent *in vitro* LTP and occlude LTD (Allen et al., 2003). Spike firing in layer II/III and IV were also altered following deprivation by 7 ms, such that in the deprived barrel layer II/III spikes occurred before layer IV, which theoretically would favour the induction of LTD (Allen et al., 2003).

Decorrelation of spikes trains from layer IV to II/III would also favour LTD (Celikel et al., 2004). Modelling of spike-timing-dependent plasticity (STDP) from data collected *in vivo* found that by cutting the PW, spike timing alterations were sufficient to predict LTD opposed to LTP in layer IV to II and layer IV to III cells (Celikel et al., 2004). The potential depression in the deprived barrel column would also facilate subsequent stronger LTP than in non-deprived barrel columns (Allen et al., 2003). Although this suggests that map plasticity can be driven by depression and LTD-like processes, adult S1 plasticity has yet to find an experience-dependent depression component (Glazewski and Fox, 1996). The Allen et al. 2003 study only used rats up until ~P28 and the Celikel et al. 2004 study between P30 and 41, which is below the ~P60 threshold for the cessation of depression (Glazewski and Fox, 1996). Hence, this mechanism might represent map plasticity in juvenile subjects. Whether spike timing changes following deprivation that would favour STDP and LTD-like processes would need to be examined in adult models to determine whether this process is general or age specific.

The majority of studies that have investigated the process of plasticity use deprivation protocols on the contralateral snout to the target barrel field. Colossal inputs also affect receptive field dynamics. Plasticity of the spared whisker expansion and spared column itself can be exaggerated if, in addition to the standard single whisker experience, all ipsilateral whiskers are also deprived (Glazewski et al., 2007).

Taken together these results suggest that in adolescent rodents, depression of the deprived input occurs first, followed by the expansion of the spared input. Interestingly depression related to the deprived input was lost between two and six months of age beyond which only potentiation was possible (Glazewski et al., 1996). In both cases, the plasticity is intracortical in nature, as recordings from thalamic VPM neurons showed no change in firing pattern during deprivation (spared or deprive whisker) (Armstrong-James et al., 1994; Glazewski and Fox, 1996; Wallace and Fox, 1999). The potentiation component remained present to 18 months (Glazewski et al., 1996), and it is seems unlikely that it can be abolished.

The potentiation and depression mechanisms are also separable genetically. For example, adult mice deficient of αCaMKII show abolished potentiation with no effect

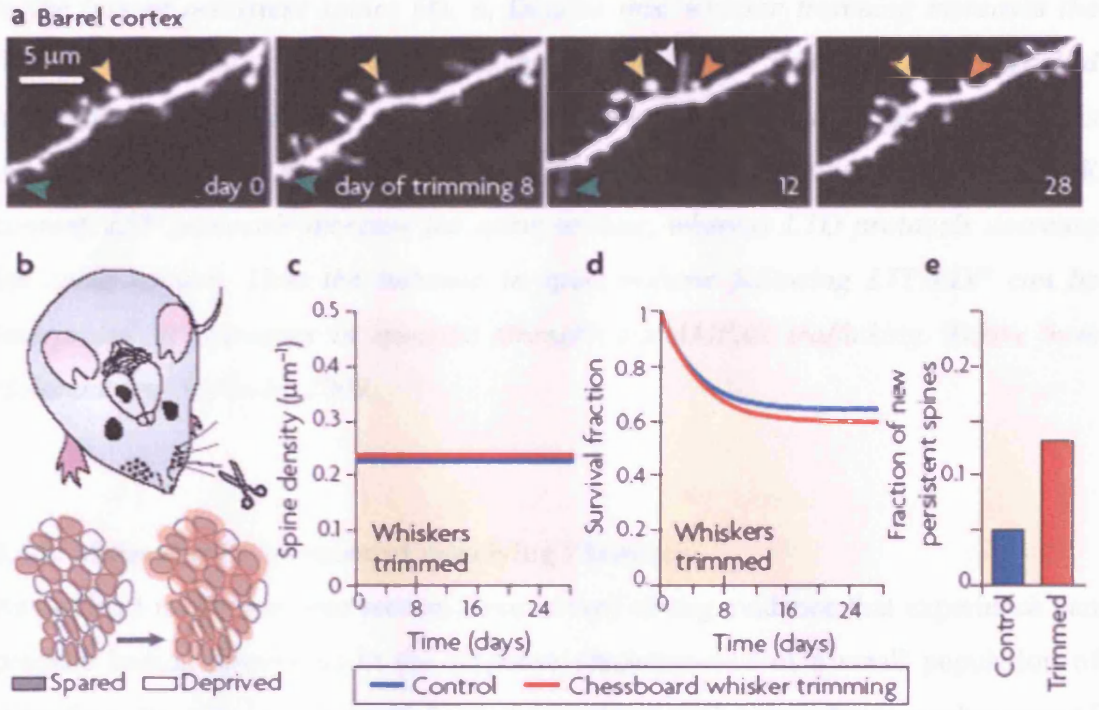
on depression, whereas adolescent mice were comparable to WTs (Glazewski et al., 1996). Conversely, in mice carrying a point mutation for T286A that disables the autophosphorylation ability of αCaMKII, plasticity was abolished at all ages (Glazewski et al., 2000). The fact that αCaMKII activity is required for potentiation of the spared whisker input shows that plasticity in the barrel cortex shares common mechanisms to hippocampal LTP (Giese et al., 1998; Silva et al., 1992a).

Structural modifications have also been found in response to changes in sensory stimuli. The phrase 'neurons that fire together, wire together' (Hebb, 1949; the phrase was proposed by Lowel & Singer, 1992, p. 211) is quoted many times in neuroscience. However research into the wiring aspect has only recently accelerated. One possible mechanism that could alter connections is remodelling of either axons or dendrites, although the evidence for large scale changes as a result of experience is limited (for reviews see Alvarez and Sabatini, 2007; Holtmaat and Svoboda, 2009). It has been shown that following deprivation there is a re-wiring of local excitatory connections (Cheetham et al., 2007). This involves lengthening of the presynaptic axon allowing more regions of proximity to postsynaptic dendrites in existing paired layer II/III neurons (Cheetham et al., 2008).

While only minor changes to axonal and dendritic arbors might occur, other mechanisms could still facilitate changes in efficacy. Dendritic spine turnover is another mechanism that with the advent of 2-photon technology has gained recent prominence. Layer II/III of the barrel cortex lends itself well to this type of study due to its proximity to the skull and the narrow width of the layer. Trimming whiskers in a chessboard pattern leads to a higher proportion of persistent spines (Figure 1.5A) (Holtmaat and Svoboda, 2009; Trachtenberg et al., 2002). Furthermore, paired recordings provide direct evidence that following whisker trimming, there is an increase in connection probability (Cheetham et al., 2007, 2008). Similar results have also been proposed for the visual cortex in response to monocular deprivation. Spine density increased following deprivation and remained after vision was resorted, and it has been proposed that spine formation could provide a basis for functional shifts should sensory manipulations be performed again (Hofer et al., 2009). Spine turnover, in particular spines that become stable, and spine volume is strongly related to the

area of the postsynaptic density 95, which in turn is proportional to its AMPA receptor content (Figure 1.5B, Holtmann and Svoboda, 2009). AMPA receptor activity is widely accepted as being required for LTP induction and expression (for review see Malinow and Malenka, 2002) and for EDP (Clem and Barth, 2006). This mechanism is also relevant to learning protocols. Recently, it has been shown that newly synthesised GFP tagged GluR1 receptors are recruited to mushroom spines in response to learning in a fear conditioning trial (Matsuo et al., 2008).

A.



B.

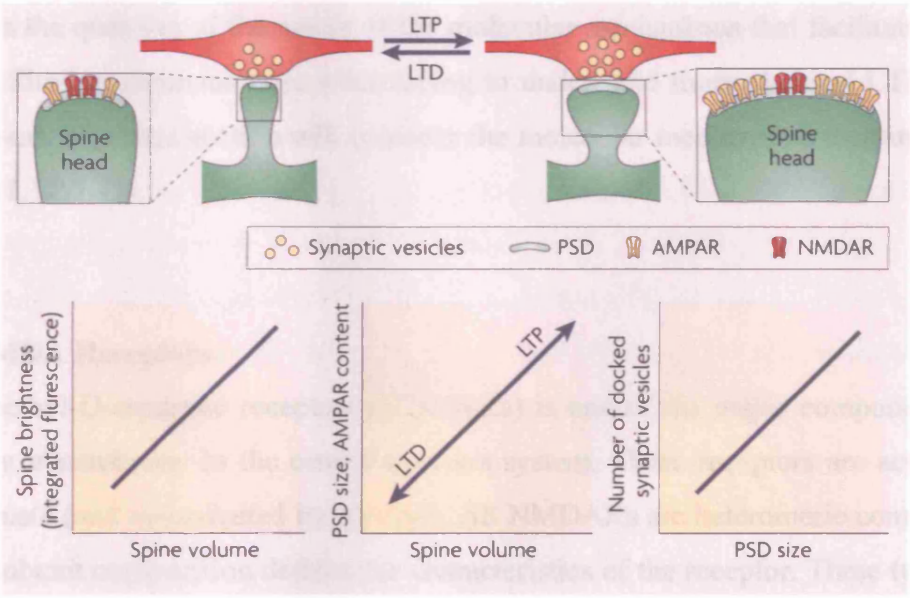


Figure 1.5. *Dendritic spines modify in response to whisker deprivation protocols to induce EDP.* ***A. a.*** *Chessboard deprivation* ***(b)*** *results in the formation of new spines (orange arrowhead) while some spines remain stable (yellow arrowhead) and others disappear (green arrowhead).* ***c.*** *Chessboard deprivation does not increase spine*

(continued from previous page) *density although the survival fraction decreases due to the loss of persistent spines (**d**). **e.** Despite this, whisker trimming increases the fraction of newly formed persistent spines. **B.** Spine volume can be tracked longitudinally in vivo by measuring the spine fluorescence. Spine volume is proportional to the area of the PSD, which is also proportional to the AMPAR content. LTP protocols increase the spine volume, whereas LTD protocols decrease the spine volume. Thus the increase in spine volume following LTP/EDP can be interpreted as increases in synaptic strength via AMPAR trafficking. Taken from Holtmaat and Svoboda, 2009.*

1.4.2. Molecular Mechanisms Underlying Plasticity

As outlined in the previous section there is very strong evidence that experience can produce lasting alterations in the response characteristics of a small population of synaptic pathways. In this sense, EDP shares many of the same basic mechanisms of LTP/LTD. While it is obvious that the majority of these changes occur from synaptic activity relating to the permitted level of sensory activity conveyed via the whiskers, this raises the question of the nature of the molecular mechanisms that facilitate these changes. Similar questions arise when trying to understand more classical LTP/LTD mechanisms. The next section will consider the molecular mechanisms that underpin EDP and LTP/LTD.

1.4.3. NMDA Receptors

The *N*-methyl-D-aspartate receptors (NMDARs) is one of the major components of excitatory transmission in the central nervous system. These receptors are activated by glutamate (and co-activated by glycine). All NMDARs are heteromeric complexes and the subunit composition defines the characteristics of the receptor. Three types of subunit that compose the NMDAR have been discovered; NR1, NR2 and NR3. In addition, there are four types of NR2 subunit (NR2A, B, C and D) and all subunits (except NR2A) can have different splice variants. NMDARs are formed (usually) of two NR1 subunits and either two NR2 or NR3 subunits (for review see Cull-Candy et al., 2001). Measuring the EPSC of NMDARs reveals the subunit composition. Those containing NR2A have the quickest decay constant, followed by NR2B and NR2C (at

roughly equal decay constants) and NR2D the slowest (Vicini et al., 1998). Recombinant NR1/NR2A has a deactivation time constant of ~50 ms, the quickest for NMDARs. NR1/NR2D are the longest at ~1.7 s (Vicini et al., 1998). The different subunit combinations are not only related to decay kinetics. Different NMDAR subunits provide either a 'high-conductance' channel opening with a high sensitivity to extracellular magnesium (Mg^{2+}) blockade or a 'low-conductance' channel opening with a low sensitivity to extracellular magnesium (Mg^{2+}). These subunits are NR2A or NR2B for high-conductance and NR2C or NR2D for low-conductance (Cull-Candy et al., 2001; Michaelis, 1998; Misra et al., 2000; Wyllie et al., 1996). As such, the sensitivity to Mg^{2+} conveys the channels sensitivity to Ca^{2+} influx. In spite of the variety of NMDAR subunit composition, not all are uniformly expressed in the mammalian brain. As a general rule, NR2B (widely expressed in brain) and NR2D (expressed in diencephalon and brainstem) are at their highest expression levels in the neonatal brain, whereas later in development NR2A becomes most prevalent (various brain expression), along with NR2C (cerebellum) (Cull-Candy et al., 2001; Liu et al., 2004; Monyer et al., 1994).

Our understanding the role of NMDARs in *in vivo* synaptic plasticity processes is limited due to technical considerations. NMDARs are involved in normal baseline transmission (that is, the transmission of a sensory signal from the whisker to the cortex) in the neocortex (Fox et al., 1989). Should NMDARs be blocked/removed, sensory responses are also decreased. The problem is avoided *in vitro* because synaptic activity is minimal with the exception of the LTP protocol. Hence *in vitro*, baseline activity would not be substantially affected (for example Hardingham et al., 2003). *In vivo* there would be a constantly high level of synaptic activity predominantly from whisking behaviour but also from spontaneous network activity. Any manipulation that affects baseline transmission or transmission from the whisker to the cortex is likely to affect the ability of those synapses to undergo activity dependent plasticity, where intracortical activity is required (see Fox, 2009; Armstrong-James et al., 1985). This would be independent of any direct effect on potentiation.

Given that NMDARs are required for LTP (for example Banerjee et al., 2009; Bender et al., 2006; Hardingham et al., 2003) in the IV to II/III synapse, it seems likely that

they could have a role in EDP. EDP is often, at least in this thesis, referring to the effect of sensory modifications on neocortical connectivity and responses. EDP can also be driven by learnt experiences and in a conceptual sense behavioural experiments are a form of EDP (the experience of learning associations and the plasticity that results from this). Hippocampal NMDAR inhibition plays a specific role in learning and memory, selectively impairing spatial working memory (Bannerman et al., 1995; Engelhardt et al., 2008; Nakazawa et al., 2002; Niewoehner et al., 2007). The requirement of NMDARs for LTP and learning make it likely that they are required for plasticity, although until a more specific method of manipulation is discovered their exact role in barrel cortex EDP will remain elusive. As such, NMDARs will not be investigated by the work in this thesis.

1.4.4. AMPA Receptors

α-amino-3-hydroxyl-5-methyl-4-isoxazole propionate receptors (AMPARs) are the second major excitatory subunit that are activated by glutamate. AMPARs consist of four closely related subunits; GluR1 (or GluRA[1]), GluR2 (or GluRB), GluR3 (or GluRC), GluR4 (or GluRD) and are formed from the *GluR1-4/gria1-4* genes that share up to 73% homology (Hollmann and Heinemann, 1994). AMPARs are formed of combinations of these subunits and similar to NMDARs, different subunits convey different properties. GluR4 expression is highest in the postnatal brain (Rossner et al., 1993; Wenthold et al., 1996; Zhu et al., 2000; Zhu, 2009), which means that the majority of AMPARs in the adult brain are formed from GluR1-3. The subunit combinations are most commonly GluR1 homomers, GluR1/GluR2 heteromers and GluR2/GluR3 heteromers (Wenthold et al., 1996). Unlike the NMDARs, AMPARs mediate fast synaptic transmission. GluR1 homomers have a decay time constant of ~6 ms, GluR1/2 heteromers ~17 ms and the longest, GluR2 at ~36 ms (Verdoorn et al., 1991). This is compared with the shortest and longest NMDAR decay time constant at 50 ms and 1.7 s, respectively.

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[1] *This thesis will use the numeric notation of AMPA subunits, although the two are interchangeable in the literature.*

A number of structural modifications to the subunits themselves also promote a diversity of AMPA function. All subunits contain four transmembrane domains (TMD) and a ligand binding domain (LBD) that is homologous to glutamate. The LBD is split into S1 and S2 segments. Due to alternative splicing, S2 can be expressed in either a 'flip' or 'flop' configuration. The difference is only a few amino acids but this can convey significant differences to synaptic function (for review see Santos et al., 2009; Sommer et al., 1990). GluR1-flip/GluR2-flip and GluR1-flop/GluR2-flip are considerably more sensitive to glutamate than GluR1-flop/GluR2-flop and GluR1-flip/GluR2-flop (Sommer et al., 1990). Flip/flop configurations are differentially regulated, with expression of the flip module evident at embryonic and postnatal ages, and the flop module starting at a low level and increasing between P9-12 (Monyer et al., 1991). Alternative splicing can also form two types of GluR2 subunit; GluR2-short or GluR2-long. The main difference between the two is the association with other GluRs and their subsequent delivery to the synapse. GluR2-short associates with GluR3 and is continuously cycled in and out the synapse, whereas GluR2-long associates with GluR1 and is inserted in response to activity (Shi et al., 2001). GluR2 also has an important function in dissociating AMPAR function. Its inclusion into the receptor renders the channel less permeable to Ca^{2+} (Hollmann et al., 1991). In particular, the dominance of GluR2 in AMPAR Ca^{2+} permeability was found to reside in an arginine (R) residue in the TMD2 region instead of a glutamine residue (Q) (Verdoorn et al., 1991). This was not observed in any other GluR subunit. The process by which this occurs is thought to be mRNA editing (or specifically in this case Q/R editing) (Higuchi et al., 1993; Sommer et al., 1991). This editing process is substantial, and up to 99% of GluR2 exists in the adult brain as the edited form (R) (see Hollmann and Heinemann, 1994; Santos et al., 2009). Edited forms of GluR2 are Ca^{2+} impermeable and compared to GluR2-lacking AMPARs, have either a linear or outwardly rectifying current/voltage relationship (Verdoorn et al., 1991). Edited GluR2 is preferentially retained unassembled in the endoplasmic reticulum (ER) and may form a limit to the formation of tetradimers and also infer preference to the formation of heterodimers (Greger et al., 2003).

1.4.5. GluR1 and Plasticity

One of the main aims of this thesis is to characterise the role of GluR1 in synaptic plasticity processes. As such, a major review of GluR2, GluR3 and GluR4 will not be reported, although some focus throughout the text will be given to GluR2 due to its heteromeric association with GluR1. The GluR1 subunit of the AMPA channel has received particular scrutiny for its role in plasticity and learning and memory. The following section will therefore consider the role of GluR1 in plasticity.

The occurrence of plasticity (with a postsynaptic loci), be it potentiation or depression, is strongly associated with the addition or removal of AMPA receptors (AMPARs) respectively (see Malinow and Malenka, 2002). Most AMPA channels exist as heterodimers of GluR1/2 or GluR2/3 (Wenthold et al., 1996), although it is possible that as many as 10% exist as homomeric GluR1 (Wenthold et al., 1996; Clem and Barth, 2006).

A large body of evidence has confirmed that GluR1 trafficking occurs in response to a plasticity protocol. A seminal study into the role of specific AMPARs in plasticity was undertaken by Hayashi (2000). This study used the fluorescent marker GFP to visualise trafficking of AMPARs in response to electrophysiological stimuli. In this study, GFP was link to GluR1 and overexpressed in hippocampal neuron cultures. Tagged GluR1 alone had no effect on the rectification properties, yet when co-expressed with CaMKII (tCaMKII) or following LTP, rectification was increased (Hayashi et al., 2000). Rectification is a property that can be used to reveal the GluR content of AMPA channels. Receptors that contain GluR1 are inwardly rectifying (at positive membrane potentials there is little outward current) whereas those that contain the calcium impermeable GluR2 are not. Hence, inward rectification is likely to be indicative of GluR1 insertion. Surprisingly the role of CaMKII in delivery was not simply to phosphorylate GluR1, as mutation of GluR1 at the S831 site did not prohibit delivery (Hayashi et al., 2000). The PDZ domain is the likely cofactor between CaMKII and delivery, as mutation at the COOH terminus completely blocked rectification in the presence of the overexpressed tCaMKII that had earlier enhanced transmission (Hayashi et al., 2000).

Homomeric GluR1 receptors are not the most commonly found subunit; these are either GluR1/2 or GluR2/3 (Wenthold et al., 1996). Once again, in response to tCaMKII, GluR1/2 receptors are trafficked to the synapse and is dependent on interactions with group I PDZ domains (Shi et al., 2001). AMPA insertion was specific to GluR1, as GluR2/3 were continuously cycled only at synapses that previously contained AMPARs. This cycling required group II PDZ domains and N-ethylmaleimide-sensitive factor (NSF) (Shi et al., 2001).

The requirement for interactions of GluR1 and group I PDZ domains to illicit delivery and enhance transmission was confirmed by examination of a point mutation on the group I PDZ domain on GluR1. This was found to prevent accumulation in the dendrite; GluR1-GFP remained in the dendritic shaft (Piccini and Malinow, 2002). Normally, tCaMKII will cause accumulation of GluR1 in the dendritic spine (Piccini and Malinow, 2002). Therefore, interaction of GluR1 and other proteins are not only a factor in synaptic delivery, but also translocation within the dendrite. A more recent study has further investigated the movement of GluR1 in activity. Previous studies have shown that simply overexpression of GluR1 alone does not enhance transmission (Hayashi et al., 2000). Accumulation of GluR1 was found in the cell bodies, yet if phosphorylation of S818, S831 and S845 was mimicked, dendritic GluR1 was increased (Kessels et al., 2009). Importantly, surface expression was still not enhanced. The TARP protein stargazin was found to be responsible for increasing the dendritic component of GluR1 while decreasing the somatic content, similar to GluR1 required phosphorylation by PKA and/or CaMKII (Kessels et al., 2009). Stargazin has differential roles between AMPARs. GluR1 surface expression was not affected by stargazing phosphorylation, while GluR2 surface expression did increase (Kessels et al., 2009). Movement of AMPARs following LTP has also been examined. Two distinct processes seem relevant. First, lateral movement of GluR1 receptors takes place from extrasynaptic sites on the dendrite (Makino and Malinow, 2009). Second, exocytosis of receptors from intracellular pools replenishes the local extracellular pool of receptors – these are not incorporated into the synapse and could provide AMPARs for future potentiation (Makino and Malinow, 2009).

Many studies have investigated how phosphorylation affects GluR1. Phosphorylation and dephosphorylation of GluR1 results in bidirectional plasticity. Overexpression of

tCaMKII caused phosphorylation of GluR1 at the S831 site and promoted delivery into the synapse (Hayashi et al., 2000; Shi et al., 2001). Phosphorylation has also been demonstrated in naïve synapses upon induction of LTP (Lee et al., 2000), suggesting that this mechanism is not an artefact of a genetic manipulation. Of particular interest is that when LTD is induced in already potentiated synapses, the CaMKII site is dephosphorylated (Lee et al., 2000). One can therefore envisage a situation where potentiation causes phosphorylation, and depression results in dephosphorylation, depending on the initial state of the synapse. Hardingham et al., (2008) showed that in cortex following whisker deprivation, application of PKA activator (Sp-cAMP-S) potentiated synapses, whereas there was no effect in control (activity permitting) cortex (presumably because phosphorylation of S845 is saturated). This was independent of CaMKII, as potentiation could be achieved in T286 mutants of αCaMKII (which do not have CaMKII dependent potentiation) and was abolished by PKA inhibition (Rp-cAMP-S) (Hardingham et al., 2008). In deprived wild-types, LTP could be partially reduced by inhibition of CaMKII or PKA, and was only fully blocked by both (Hardingham et al., 2008). Therefore, depending on whether there had previously been whisker deprivation, which in turn modulates the phosphorylation of GluR1 sites, parallel mechanisms of GluR1 plasticity can be induced.

Convergent evidence in support of the role played by CaMKII in GluR1 delivery has been obtained from the study of mice deficient in αCaMKII, and subsequently of specifically the T286 site that mediates autophosphorylation. These mutants show deficient LTP (Giese et al., 1998; Hardingham et al., 2003; Silva et al., 1992) and EDP (Glazewski et al., 1996 and 2000; Hardingham et al., 2003). This strongly suggests that CaMKII activity is required for full expression of plasticity, at least in part by S831 phosphorylation.

PKA phosphorylation of GluR1 has also been strongly implicated in delivery. PKA alone is not sufficient for GluR1 insertion, and blockade of the PKA S845 site prevented the rectification increases normally observed with tCaMKII (Esteban et al., 2003). It is likely that the role of PKA phosphorylation is to prevent internalisation of GluR1, hence stabilising insertion following plasticity (Esteban et al., 2003; Lee et al.,

2003). PKA also phosphorylates GluR4 (Esteban et al., 2003), although this is unlikely to be a major mechanism for synaptic change in adult rodents as GluR4 expression is highest postnatal (Rossner et al., 1993; Zhu et al., 2000; Zhu, 2009). LTD induction in naïve synapses is associated with dephosphorylation of S845, while conversely in the depressed synapse potentiation resulted in phosphorylation of this site (Lee et al., 2000). 'Phospho free' mice have been developed where the S831 and S845 sites have been mutated to loss of function. In adult mutants, it was found that LTP was reduced and LTD was completely abolished. In addition dephosphorylation and internalisation via LTD was prevented in these mutants (Lee et al., 2003). Therefore, PKA and CaMKII are both required for plasticity, although single 'phospho free' mutants only result in partial reductions of LTP compared to the double mutant (Boehm et al., 2006).

Recently PKC phosphorylation at S818 on GluR1 has also been implicated in trafficking. LTP in the hippocampus was found to increase S818 phosphorylation and was required for full LTP expression (Boehm et al., 2006). Rectification increases were also blocked. The S818 is located on the membrane proximal region on GluR1 and considering that a number of other proteins interact with this region (4.1N (Shen et al., 2000), AP2 (Lee et al., 2002) and PI3-kinse (Man et al., 2003)), phosphorylation by PKC could promote stabilisation or delivery by protein interaction (Boehm et al., 2006).

As alluded to earlier, these mechanisms have been investigated and proven significant to *in vivo* mechanisms. Visual cortex ocular dominance plasticity along with *in vitro* LTD has is sensitive to mutation of PKA, in particular the RIIβ subunit of PKA (Fischer et al., 2004). Several studies have used barrel cortex as their preparation. Overexpression of PSD-95-GFP was achieved by injection of sindbis virus into the barrel cortex. Rats were then either subjected to total whisker deprivation (activity lacking) or all whiskers were left in tact (activity permitting). Where activity was allowed, the PSD-95 occluded experience driven AMPA insertion. However, in deprived rats, where there should have been little activity trafficking, AMPAR transmission was enhanced in comparison with that seen in controls (Ehrlich and Malinow, 2004). Very similar results were observed in the hippocampus *in vitro* where PSD-95 delivery mimicked LTP by insertion of GluR1 and occluded further

LTP (Ehrlich and Malinow, 2004). A more direct link between GluR1 was shown where the cytoplasmic tail of GluR1 (GluR1-ct; to inhibit delivery of endogenous GluR1) was injected into the barrel cortex. Activity was shown to increase rectification yet was blocked when whiskers were trimmed (Clem and Barth, 2006; Takahashi et al., 2003). The GluR1-ct construct as expected blocked the enhanced transmission that results from activity dependent trafficking. However independent of activity, GluR2 was shown to continually cycle in and out of the synapse (Takahashi et al., 2003). Recently, it was found in knockouts of GluR1 that *in vivo* experience-dependent depression was inhibited although surround potentiation was not (Wright et al., 2008). Considering LTD is dependent on the phosphorylation states of the S831 and S845 sites (Lee et al., 2000), it is conceivable that without GluR1, depression cannot occur and is similar to results seen in the 'phospho-free' mice (Lee et al., 2003).

Together, associations of GluR1 with PDZ domains and PSD-95 are required for insertion and this in turn depends on phosphorylation states of S818, S831 and S845 by PKC, CaMKII and PKA respectively. As a result of phosphorylation, GluR1 homomeric and GluR1/2 heteromeric insertion and internalisation are strongly linked to both *in vitro* LTP and LTD or *in vivo* EDP or EDD plasticity processes. In addition, there is an activity independent cycling of GluR2/3 heteromers into the synapse that can replace previously delivered GluR1 AMPARs.

1.4.6. GluR1-Independent Potentiation

The research detailed above has used c terminal constructs of GluR1 and 'phospho-free' mutants to determine the role of the GluR1 subunit in plasticity processes. However a different approach to this question has been running concurrently. Zamanillo et al. (1999) were the first group to create a knockout of the GluR1 subunit and used this to investigate plasticity. This approach has potential advantages over very specific phosphorylation site mutants. Since all of the subunit is lacking, the risk of interactions with proteins/phosphorylation sites that are currently undiscovered is removed, leaving one with a 'pure' study of plasticity processes that occur in the absence of GluR1.

In this mutant, no significant upregulation of GluR2-4 or the NMDA subunits NR1 and NR2A-C in the hippocampus and NMDA currents were found. However, as expected, the AMPA current was strongly reduced. Field EPSPs taken from the Schaeffer-collaterals (CA3-CA1) were not significantly different from WTs but sensitive to AMPA antagonists (CNQX), suggesting that stimulation elicited potentials are GluR2/3 dependent (Zamanillo et al., 1999). Surprisingly, LTP was completely absent in the GluR1 KO mice when induced with a 100 Hz stimulation protocol (Zamanillo et al., 1999). This was surprising considering that no differences were observed in transmission kinetics or dendritic formation and calcium transients. Yet, generally 'phospho mutants' have produced deficits that were variable but rarely complete (Boehm et al., 2006; Lee et al., 2000). This was the first suggestion that the GluR1 subunit was critical to the establishment of LTP.

The proposition that LTP is abolished in the GluR1 KO mice has required modification in the light of more recent evidence. Hoffman et al. (2002) used protocols other than 100 Hz to determine whether plasticity was dependent on the frequency of induction. In adult hippocampal preparations (CA3-CA1), theta burst stimulation (TBS) with postsynaptic pairing was found to induce LTP in mutant mice. 100 Hz stimulation did not produce any early plasticity, similar to previous studies (Zamanillo et al., 1999), yet TBS given to the same cells did produce LTP thereafter very similar to TBS alone (Hoffman et al., 2002; Phillips et al., 2008). However, TBS LTP in mutant mice did have features that were different to WT. Specifically, early phases of LTP were impaired compared to WTs, yet a slow rising potentiation was evident that eventually brought LTP to the same magnitude as WTs 50 minutes post induction (see Figure 1.6) (Hoffman et al., 2002).

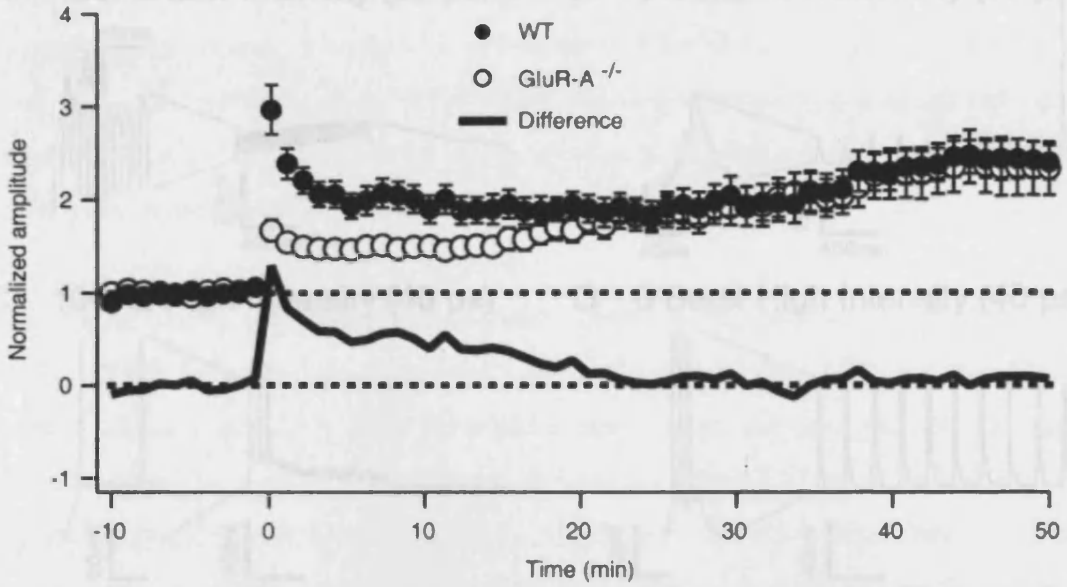


Figure 1.6. *LTP expression in the GluR1 KO mice. TBS stimulation produced a slow rising form of LTP that was not observed using 100 Hz protocols. Plasticity in the GluR1 KO is therefore induction dependent, and thus GluR1 dependent and independent plasticity exists in the hippocampal formation. Figure from Hoffman et al., 2002.*

This raised the question of why one type of induction protocol should produce LTP whereas the other fails in GluR1 KO mice? During TBS, more complex spiking was observed. Those cells that underwent complex spiking during induction resulted in a larger EPSP magnitude than those cells that did not (Hoffman et al., 2002). Further studies have confirmed that the probability of spiking is larger in orthodromic TBS with a 40 μs pulse width than 100 Hz stimulation, suggesting that spiking is critical to LTP expression in GluR1 KO mice (Figure 1.7) (Phillips et al., 2008). The importance of the Hoffman study is that GluR1 deletion reveals that there are GluR1 dependent and independent forms of LTP that requires a pairing TBS protocol.

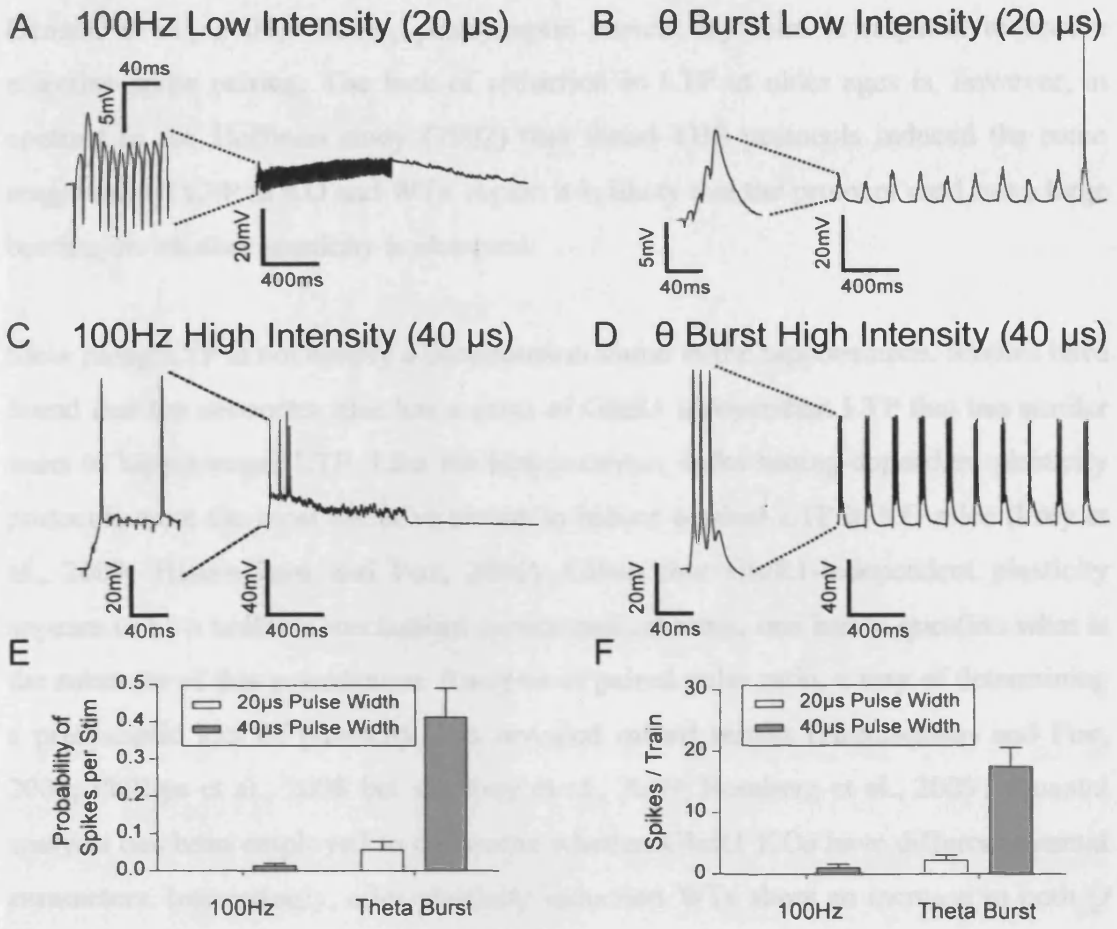


Figure 1.7. *Bursting is dependent on the stimulation protocol used. Previous studies have revealed that 100 Hz stimulation is ineffective in inducing LTP in the GluR1 KO. However, TBS (both spike-timing dependent and extracellular) can induce LTP. Spiking observed between protocols is markedly different. Virtually no spiking is encountered when stimulating by 100 Hz or 20 µs pulse width TBS (**B and C**), but the probability is much higher with 40 µs pulse width TBS is used (**D, E and F**). Spiking is therefore critical for synaptic plasticity in GluR1 KO mice (Hoffman et al., 2002; Phillips et al., 2008). Taken from Phillips et al., 2008.*

Other studies have replicated the existence of the slow rising LTP, albeit in an age dependent manner. Using the 100 Hz protocol that previously did not elicit LTP, at P14 GluR1 did have a magnitude of LTP that was NMDA-dependent yet reduced to baseline levels by P42 (Jensen et al., 2003). A pairing induction protocol also produced LTP at P14 in KOs, but this was reduced although not abolished at P42

(Jenson et al., 2003). Hence, postsynaptic current injection is required to ensure effective spike pairing. The lack of reduction in LTP at older ages is, however, in contrast to the Hoffman study (2002) that found TBS protocols induced the same magnitude of LTP in KO and WTs. Again it is likely that the protocol used has a large bearing on whether plasticity is observed.

Slow rising LTP is not simply a phenomenon found in the hippocampus. Studies have found that the neocortex also has a form of GluR1 independent LTP that has similar traits to hippocampal LTP. Like the hippocampus, spike-timing-dependent plasticity protocols were the most effective means to induce cortical LTP in KO mice (Frey et al., 2009; Hardingham and Fox, 2006). Given that GluR1-independent plasticity appears to be a uniform mechanism across cortical areas, one has to question what is the substrate of this potentiation. Analysis of paired pulse ratio, a way of determining a presynaptic loci of plasticity, has revealed mixed results (Hardingham and Fox, 2006; Phillips et al., 2008 but see Frey et al., 2009; Romberg et al., 2009). Quantal analysis has been employed to determine whether GluR1 KOs have different quantal parameters. Interestingly, after plasticity induction WTs show an increase in both Q (quantal amplitude recorded at the soma) and NP_r (transmitter release), whereas GluR1 KOs only had an increase in NP_r (Hardingham and Fox, 2006). Given that increases to Q are associated with postsynaptic mechanisms and NP_r presynaptic, it is plausible that the WTs have a mixed loci of potentiation whereas GluR1 KOs are reliant upon presynaptic plasticity processes. Further analysis using the change in EPSP variance versus the change in mean amplitude (also called $1/CV^{-2}$) (Malinow and Tsien, 1990) has been used to identify the loci. Put simply, depending on what quantal parameter is changing, $1/CV^{-2}$ will behave in accordance to that parameter. $1/CV^{-2}$ is non-linearly related to P_r (probability of release), linearly related to N (number of release sites) and independent of Q. Therefore on a graph with a line of x=y, a trajectory steeper than the line is associated with increases to P_r, along the line if only N increases and below if Q increases. Increases of more than one parameter would cause an intermediate shift that was dependent on the relative weighting of the parameters. For example an equal increase in N and P_r would restrict changes to the x=y line.

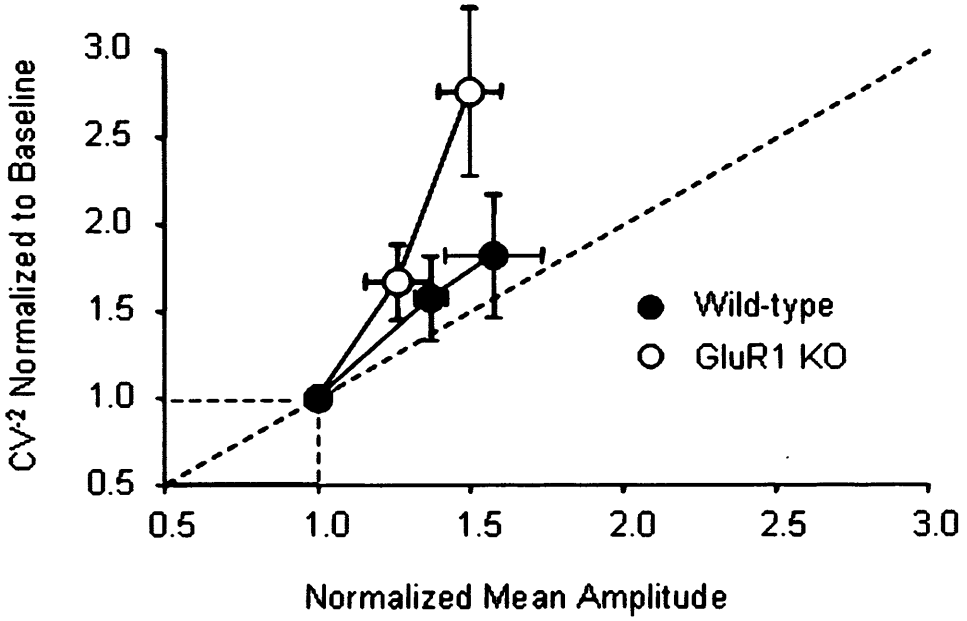


Figure 1.8. *GluR1 KOs have a presynaptic loci of plasticity one hour following pairing. Increases in P_r cause a trajectory shift above the x=y line, N along the x=y line and Q below. Increases in two or more parameters will produce intermediate effects (i.e. increases to P_r and Q will cause the trajectory to remain on the x=y, assuming the increase in Q and P_r are the same). Considering GluR1 KOs have a trajectory above the line, it is likely that there is an increase in P_r which is associated with enhanced presynaptic function. WTs however remain close to the line, suggesting a mixed loci. Taken from Hardingham and Fox, 2006.*

As predicted from the NP_r increase observed in GluR1 KOs, the mean response versus $1/CV^2$ trajectory was higher for than the x=y line, which was most pronounced at one hour after pairing (Figure 1.8). WTs on the other hand followed the x=y line (Hardingham and Fox, 2006; Phillips et al., 2008; Sjöström et al., 2007). NOS inhibition in WTs has also been shown to drive the loci to the post-synapse (Sjöström et al., 2007). Taken together, it is likely that the loci of plasticity in GluR1 KOs is more presynaptic whereas WTs have a mixed loci. A recent study by Frey et al. (2009) has challenged the idea of a presynaptic loci in the GluR1 KO, although the experimental design was different. Firstly, genotypic differences were only convincingly observed at one hour post-pairing in the Hardingham (2006) and the

Phillips (2008) study, yet the Frey study measured between 20-40 minutes (Frey et al., 2009); a time frame where the GluR1-independent LTP was still forming. Secondly the Hardingham and the Phillips study used minimal stimulation whereas the Frey study used non-minimal stimulation. The ramifications on results are uncertain but with non-minimal stimulation the certainty of recruiting multiple release sites becomes more likely, making the separation of peaks in quantal amplitude (indicative of single quantal events) more difficult.

Much progress has been made in uncovering the cellular mechanisms are responsible for GluR1-independent LTP. Many lines of evidence converge towards the same signalling cascade. The first discovery that GluR1 KOs had LTP that was differentially expressed compared to WTs (temporal and protocol dependent) also revealed that the late phase LTP was sensitive to postsynaptic intracellular calcium. The calcium buffer BAPTA loaded in the patch pipette similarly affected potentiation in both WTs and GluR1 KOs (Hoffman et al., 2002). This suggests that the same calcium signalling cascade is responsible for the late-phase LTP, which is in turn divergent from the calcium mediated early LTP (Hoffman et al., 2002). Calcium mediated transmission in the absence of the GluR1 subunit is likely to require NMDARs. Therefore, one would presume that the plasticity observed in the GluR1 KO could be sensitive to NMDA agonists. As expected, inhibition of the NMDARs by pharmacological antagonists produces a complete abolition of LTP (Hardingham and Fox, 2006; Hoffman et al., 2002; Jenson et al., 2003; Romberg et al., 2009). Furthermore, inhibition of CaM or CaMKII in the postsynapse also inhibits GluR1 KO LTP, suggesting that the postsynaptic calcium is likely affecting CaMKII cascades (Hardingham and Fox, 2006; Romberg et al., 2009). Very recently, inhibition of PKC was also found to impair GluR1 KO LTP (Romberg et al., 2009), suggesting a role for GluR2 insertion whereby PKC phosphorylation increases surface expression (Daw et al., 2000; Gardner et al., 2005). Consistent with the idea that GluR2 could be involved, inhibition of exocytotic membrane fusion by the intracellular inhibitor BoNT/B blocked potentiation, presumably by impairing the delivery of GluR2 to the synapse (Frey et al., 2009).

While these data suggest a postsynaptic loci, we must remember that quantal analysis in the GluR1 KO mice suggested that there was a presynaptic loci. It is not

unreasonable to propose that that the signalling cascade may begin postsynaptically and then travel back across the synaptic cleft and alter presynaptic release probability via a retrograde factor. Presynaptic forms of plasticity have been suggested before (Malinow and Tsien, 1990). However a leading candidate molecule that could be the retrograde signal is nitric oxide (NO). The role of NO in plasticity has been long proposed and remains highly controversial (reviewed later but see Hölscher, 1997 for review). Yet considering that there is a presynaptic loci in the GluR1 KO, it is highly likely that NO could mediate it. In fact, inhibition of postsynaptic plasticity mechanisms (GluR1) could be the best way to reveal the full effects of presynaptic NO, as postsynaptic plasticity could be sufficient for LTP in WTs considering that they have more of a mixed pre and postsynaptic loci (Hardingham et al., 2007; Hardingham and Fox, 2006).

Application of the general nitric oxide synthase (NOS) inhibitor L-NNA and L-NAME to GluR1 KO mice both completely inhibited late-phase LTP in the barrel cortex. This compares to a 50% reduction in LTP in WTs (Hardingham and Fox, 2006). Of particular interest is that WTs with NOS inhibitor show a mean response versus CV^{-2} trajectory below the x=y line so that Q is now most affected. This suggests that inhibition of NOS produces a predominantly postsynaptic form of LTP (Hardingham and Fox, 2006). Similar dependence on NO has been suggested for LTP in the hippocampus. Non-specific antagonism of NOS produces a profound, albeit not complete block of hippocampal LTP (Phillips et al., 2008). There has been further research to determine which NOS isoform(s) is more important to plasticity. NOS1 KOs and NOS3 KOs have been crossed with GluR1 KOs and then tested for LTP. Surprisingly there was a 50% reduction in late-phase LTP magnitude for both GluR1/NOS1 KOs and GluR1/NOS3 KOs, both of which were sensitive to a further reduction of LTP with L-NNA application. This resulted in LTP of a similar magnitude to GluR1 KOs with L-NNA (Figure 1.9) (Phillips et al., 2008). This indicates that NO produced by both isoforms of NOS are important for LTP (see also Hopper and Garthwaite, 2006; Son et al., 1996). Similar dependence of GluR1-independent LTP on NOS1 has been confirmed by pharmacological inhibition using two NOS1 specific inhibitors (Romberg et al., 2009), although there can be no guarantees that the inhibitors were not also affecting the other NOS isoforms.

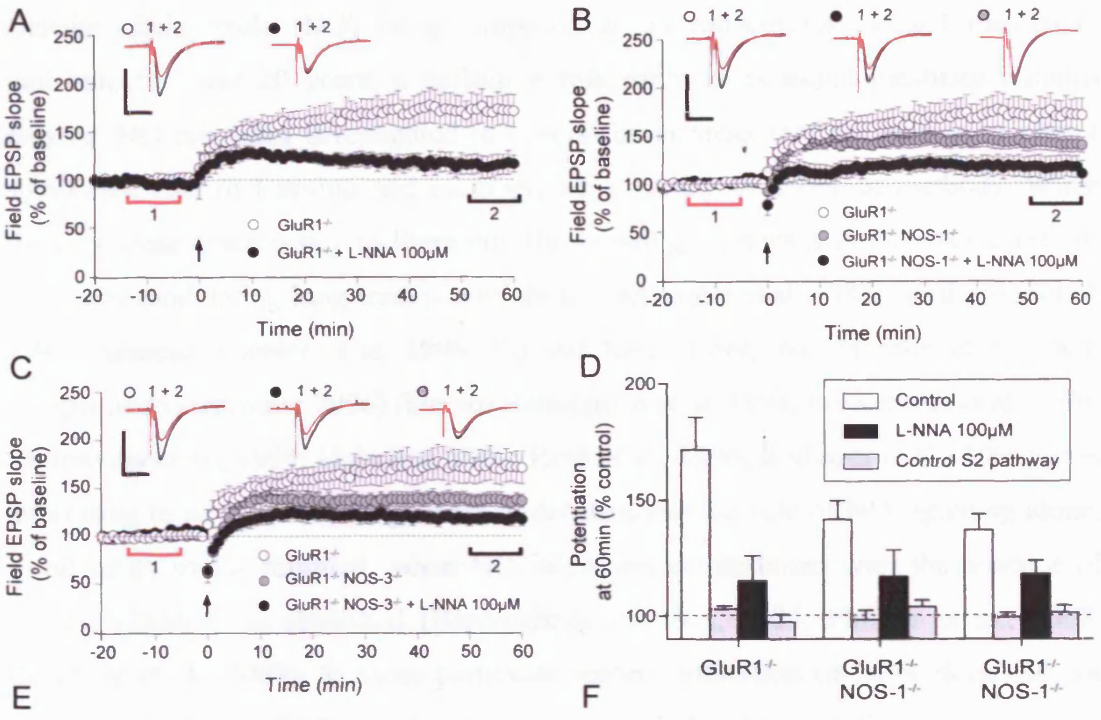


Figure 1.9. *The dependence of GluR1-independent hippocampal LTP on NO. Application of the non-specific NOS inhibitor reduces late phase LTP* ***(A)****. Inhibition of NOS1 or NOS3 produces a 50% reduction in that LTP, which is sensitive to further reduction by application of L-NNA, presumably which is inhibiting the other in tact isoform* ***(B and C)****. This suggests that both NOS1 and NOS3 are important for LTP in the hippocampus. Note in* ***D*** *the middle column is the LTP magnitude of GluR1/NOS1 KOs and the right columns is the LTP magnitude for GluR1/NOS3 KOs.*

In conclusion, GluR1 KOs are able to undergo LTP but induction is protocol dependent. The LTP expressed in both the hippocampus and the barrel cortex has an early-phase impairment compared to WTs, yet by one hour is virtually identical. This slow rising form of LTP is sensitive to NMDAR, CaMKII and PKC inhibition and although results vary across labs largely depending on the analysis method employed, it has a presynaptic locus. This presynaptic plasticity is sensitive to NO inhibition in the barrel cortex and the hippocampus, with both NOS1 and NOS3 playing equal roles.

1.4.7. Nitric Oxide

Despite nitric oxide (NO) being proposed as an intracellular second messenger molecule for over 20 years, a definitive role for it in neuronal plasticity remains elusive. NO has been investigated in several brain areas that are known to play a significant role in learning and memory, by a variety of different methods. While there is some discrepancy in literature, the growing consensus is that NO exerts its affects by modulating long-term potentiation (Garthwaite et al. 1988; Garthwaite et al. 1995; Arancio, Kiebler et al. 1996; Ko and Kelly 1999; Weitzdoerfer et al. 2004; Hopper and Garthwaite 2006) (but see Bannerman et al. 1994; Bannerman et al. 1994; Cummings et al., 1994; Holscher, 1997; Reid et al., 1996; Ruthazer et al., 1996). It is interesting to note that although there is debate about the role of NO signaling alone, in all cases so far reported, when NO inhibition is combined with the absence of GluR1, plasticity is abolished (Hardingham and Fox, 2006; Phillips et al., 2008; Romberg et al., 2009). In those particular reports, inhibition of NOS alone did not result in abolition of LTP. It has been suggested that the inability to consistently reproduce results could be linked to specific experimental conditions within each lab (see Hölscher 1997 for review).

NO in the mammalian organism is formed in a variety of tissues, mainly the endothelium, neurons and macrophages by three nitric oxide synthase (NOS) enzymes: endothelial NOS (NOS3/eNOS), neuronal NOS (NOS1/nNOS) and inducible NOS (NOS2/iNOS) respectively (see Blackshaw et al., 2003). The NOS family shows homology to each other and with certain P450 enzymes. The N terminus has similarity to cytochrome P450 monooxygenase enzymes and the C terminal domain to various cytochrome P450 reductases (Bredt et al., 1991; Dudzinski et al., 2006). Within the N terminus, there are binding sites for iron protoporpyrin IX (heme), tetrahydrobiopterin (H4biopterin) and in the C terminus for FAD, FMN, L-arginine (l-Arg), calmodulin and NADPH (Stuehr, 1997). NO is produced by oxidising a guanidine nitrogen of L-arg with molecular oxygen directly associated with the ferrous iron of heme via electron transfer from NADPH (for review see Stuehr, 1997).

NOS3 and NOS1 are similar in that they require the presence of calcium via the CaM site in order to synthesis NO (Bredt and Snyder, 1990). NOS2 is different, as even in

suprathreshold levels of intracellular calcium, calmodulin is fully bound (Cho et al., 1992). Hence, calcium levels do not affect it in the same way as the other two forms. NOS1 and NOS3 are linked to calcium concentrations by an amino acid loop within the FMN binding domain. In the presence of low physiological calcium levels, this will destabilise the binding of calmodulin. If calmodulin is not bound, the electron transfer from NADPH is impeded and catalytic activity ceases (see Bredt and Snyder, 1992; Stuehr, 1997). This provides a mechanism whereby the influx of calcium would activate the generation of NO.

It is interesting to note that NOS1 has an 'extra' 250 amino acid domain contained within the N terminus that is not found on either NOS3 or NOS2 (Brenman et al., 1996). Experiments were performed to test whether this domain provided NOS1 with the ability to form interactions with other proteins. It was found to include a PDZ domain which interacts with PSD-95 and PSD-93, hence forming a protein-protein interaction linking the enzyme to the synaptic membrane (Brenman et al., 1996; Brenman and Bredt, 1997). It is known that the PDZ domain can interact with the C terminus of the NMDA receptor (Kornau et al. 1995). This represents a functional model where calcium entry through the glutamatatergic activation of NMDA receptors could activate NOS1 by increasing calcium concentration and calmodulin binding. Despite this hypothesis, a clear role has yet to be fully understood (Doyle et al., 1996; O'Dell et al, 1994; Phillips et al., 2008; Romberg et al., 2009; Son et al., 1996). Pharmacological manipulation *in vitro* and *in vivo* have also produced a wide diversity of results, from complete inhibition (Doyle et al., 1996; O'Dell et al., 1991; Schuman and Madison, 1991), partial inhibition (Chetkovich et al., 1993; Hardingham and Fox, 2006; O'Dell et al., 1994; Son et al., 1996) to no effect (Bannerman et al., 1994; Cummings et al, 1994; Reid et al, 1996; Ruthazer et al, 1996).

NOS3 has been less well individually investigated compared to complete NOS antagonism or NOS1 KO studies. NOS3 has been shown to be confined to endothelial cells in both rat and mouse brain (Blackshaw et al., 2003), dispelling earlier theories that it was also found in pyramidal cells (Dinerman et al., 1994; O'Dell et al., 1994). Despite the theoretical difficulty of linking activity-dependent release that is possible by NOS1, the importance of NOS3 in LTP has been reported. It has been

demonstrated that NOS3 KOs have fully inhibited LTP (O'Dell et al., 1994), while other suggest only a transient deficit (Wilson et al., 1999; Doreulee et al., 2001). Interestingly, inhibiting both NOS1 and NOS3 by either mutant with inhibitor or complete genetic knockout produces a stronger inhibition of potentiation (O'Dell et al., 1994; Son et al., 1996).

NOs primary action is to modulate cyclic 3', 5' guanosine monophosphate (cGMP) levels by the metalloprotein guanylate cyclase (GC), leading to downstream changes in synaptic strength (for review see Hofmann et al., 2006; Bredt and Snyder, 1989; O'Dell et al., 1991). An inhibitor of cGMP-dependent protein kinases injected into the presynapse blocked the induction of LTP, whereas the postsynapse had no effect (Arancio et al., 2001). These modifications have traditionally been hypothesized to be of presynaptic loci. It should be noted that some studies have indicated that there might be a postsynaptic element to NO signaling (Huang et al., 2005; Serulle et al., 2007). For example, it has been shown that NO release can also affect the trafficking of AMPA receptors containing GluR1 and GluR2 into the synapse (Huang et al., 2005; Serulle et al., 2007). This would provide a mechanism whereby NO has a more direct effect on synaptic strengthening in addition to presynaptic modifications to change release probability.

1.5. The Hippocampus and Memory

Donald Hebb (1949) made the postulation of how pre- and post-synaptic events could be linked temporally and spatially. He suggested that *"when an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process of metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased"* (Hebb, 1949, p. 62). This is more often known in the colloquial term as "neurons that fire together, wire together" (Lowel and Singer, 1992, p. 211). While this hypothesis has perhaps proven to be an over simplification of synaptic plasticity processes, it has provided the theoretical cornerstone for subsequent work. One of the most commonly used tools used to evaluate the plasticity and memory hypothesis is the induction of LTP/LTD - these stimulation patterns are, of course, artificial. To obtain a more comprehensive understanding of plasticity and its function, molecular pathways important for LTP-

induced synaptic plasticity must be tested in more naturalistic paradigms such as EDP and learning and memory.

While the barrel cortex serves as an excellent model system to study cortical plasticity, robust behavioural paradigms have not evolved in tandem with increasingly elegant electrophysiological techniques. Part of the problem is that very little is known about barrel cortex function during awake behaviour (for review see Petersen, 2007 and O'Connor, 2010 for recent work). Although it is possible to teach rodents tactile discrimination tasks, it is not clear whether this requires plasticity in the barrel cortex to occur (Krupa et al., 2004). As such, relating deficits in EDP following whisker deprivation to tactile learning in mutant mice remains difficult (see Section 5.1 for detailed discussion). The hippocampus requires the same molecular mechanisms to support synaptic plasticity as the barrel cortex (for example Hardingham and Fox, 2006; Phillips et al., 2008) and it is known that learning and memory requires hippocampal synaptic plasticity (Silva et al., 1992a and b). Therefore assessing molecular contribution to learning and memory formation remains best undertaking within the hippocampus, which in turn can inform assessment of barrel cortex function.

This section will consider the role of the hippocampus in learning and memory. The hippocampus has long been proposed essential for memory mechanisms, in particular episodic memory. A serendipitous complication to a neurosurgical technique was to spawn a field of research. Patient HM suffered severe and frequent seizures and it was decided that removal of the medial temporal lobe represented the best means to improve the quality of his life. Indeed, HM's epilepsy was drastically reduced by bilateral medial temporal lobe resection. However, the resection also resulted in severe anterograde memory (declarative memory) along with deficiencies in spatial memory (Corkin, 2002; Scoville and Milner, 1957). This finding raised two interesting questions. What is the neurological basis that underlies these memories and could a lesion pattern be duplicated to replicate these impairments in a rodent model under laboratory conditions?

Episodic memory is an integral component of declarative memory (Squire, 1986; Tulving, 1984). The data from HM suggested that the hippocampus is pivotal to

episodic memory. The presence of episodic memory, as defined by Tulving (1983) in animals remains controversial. In particular, the ability to access evidence of 'mental time-travel' to retrieve past experiences and consciously recollect is hard to probe in non-verbal species (Clayton et al., 2001). Nevertheless, evidence has emerged that animals are able to form episodic-like memories, that is, memories that bind time, place and a target cue together (Clayton et al., 2001). One common feature shared by the hippocampus in humans and other animals is its role in spatial memory and navigation (O'Keefe & Nadel, 1978). Spatial memory is dependent on normal hippocampal function and evidence to support this view in animals has been provided largely by lesion and unit recording (place-cell; see section 1.5.3 for further details) studies (for example, Morris et al., 1982; O'Keefe and Dostrovsky, 1971). Developments in fMRI technology have confirmed a key role for human hippocampus in spatial memory processes (Maguire et al., 2000). Indeed, a landmark study by Maguire et al. (2000) found a correlation between increased hippocampal size and time spent as a London taxi driver. This finding suggests that plasticity processes in this structure are highly sensitive to spatial information (Maguire et al., 2000).

1.5.1. The Structure of the Hippocampus

The major input to the hippocampus arises from the entorhinal cortex. Figure 1.10 shows the major connectivity of the hippocampus. There are number of pathways which convey information to the hippocampus. The major output of entorhinal layer II cells to the hippocampus is via the perforant pathway to the dentate gyrus or to the CA3 sub-region. A separate pathway also supplies fibres from entorhinal layer III directly to the CA1 subregion. A recent study used anterograde tracers to determine how the separate pathways innovated CA1. The tracer was injected either into the layer III of the entorhinal cortext or the CA3 sub-region. It was found that all pyramidal cells and most interneurons received input from both the perforant pathway (entorhinal cortex) and the Schaffer collaterals (CA3), although some interneurons only received an input from the perforant pathway (Kajiwara et al., 2008). These pathways seem conserved between rodent models, although in mice there is not a large projection to the contralateral dentate gyrus (van Groen et al., 2002). This result

suggests that although there are dual inputs to the CA1 region (direct and indirect), both pathways converge on to the same target; that is principal cells or interneurons.

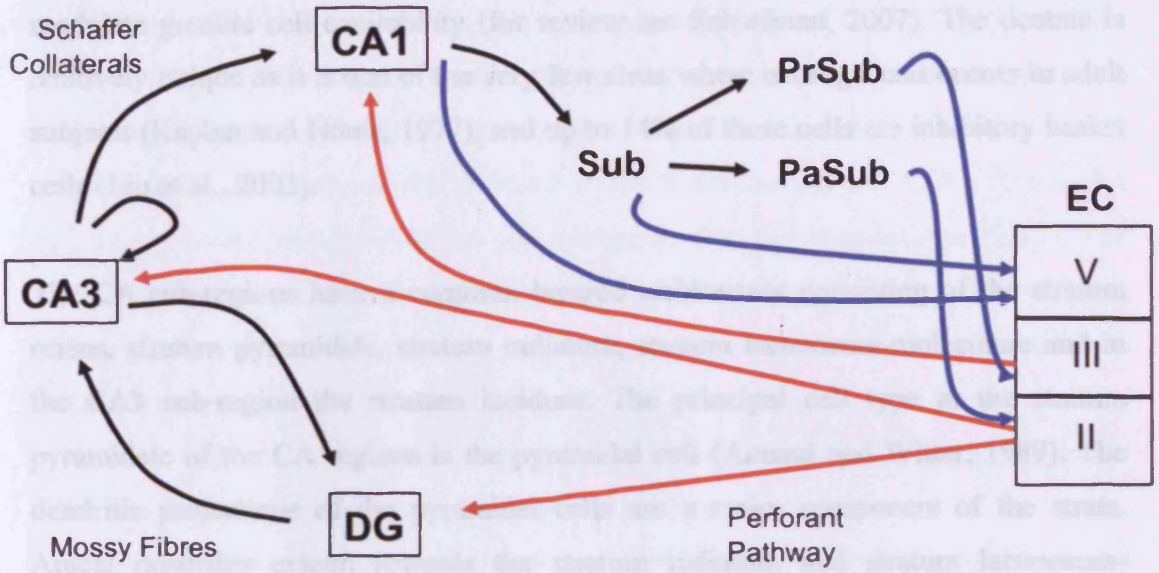


Figure 1.10. *A simplified cartoon showing the connectivity of the hippocampus. From right to left. Major output occurs from the entorhinal cortex (EC) to the dentate gyrus (DG) and the CA sub-regions via the perforant pathway (red lines). Information flow within the hippocampus occurs (black lines) through the dentate via the mossy fibre pathway to the CA3. CA3 projects to the CA1 via the Schaffer collaterals, then to the subiculum (sub), the pre or parasubiculum (PreSub and PaSub) and back to the deep layers of the entorhinal cortex (blue lines).*

As previously described, the entorhinal cortex innervates, among other areas, the dentate gyrus via the perforant pathway. The dentate gyrus is divided into three layers; the molecular, the granule and the polymorphic layer. The molecular layer receives the input from the entorhinal cortex. The granule layer contains the granule cells (principal cell type of the dentate gyrus), which gives rise to the axons (mossy fibres) that collateralise in the polymorphic layer before entering the CA3 sub-region (Amaral and Witter, 1989; Blackstad et al., 1970). Transmission from the granule cell layer via the mossy fibres to the CA3 region is predominantly glutamatergic (Yamamoto et al., 1983), although it has been demonstrated that GABAergic transmission at $GABA_A$ receptors reduces mossy fibre excitability (Ruiz et al., 2003).

Indeed, the inhibitory circuits within the hippocampus may play an important role in the flow of information through the structure. It has been suggested that a back-projection from CA3 to dentate gyrus influences GABAergic neurons in the dentate to modulate granule cell excitability (for review see Scharfman, 2007). The dentate is relatively unique as it is one of the very few areas where neurogenesis occurs in adult subjects (Kaplan and Hinds, 1977), and up to 14% of these cells are inhibitory basket cells (Liu et al., 2003).

The CA sub-regions have a common layered architecture consisting of the stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum-moleculare and in the CA3 sub-region the stratum lucidum. The principal cell type in the stratum pyramidale of the CA regions is the pyramidal cell (Amaral and Witter, 1989). The dendritic projections of the pyramidal cells are a major component of the strata. Apical dendrites extend towards the stratum radiatum and stratum lacunosum-moleculare whereas the basal dendrites project to the stratum oriens (for review see Amaral and Witter, 1989). The stratum lucidum is located proximal to the stratum pyramidale and contains the mossy fibres. The main input to CA3 arises from the mossy fibre projections from the dentate region (Cajal, 1911; de Nó, 1934). CA3 pyramidals have highly collateralised axons that contribute to projections from the CA3 cells that either loop back to the CA3 (recurrent collaterals termed by Lorente de Nó) or transverse to the CA1. The collaterals that project from CA3 to CA1 are called the Schaffer collaterals (Schaffer, 1892). A separate branch of collaterals leaves via the fornix (commissural pathway) for the contralateral hippocampus (Anderson et al., 1966). Tracing studies have confirmed that projections from the CA3 region extend throughout the hippocampus proper, to the dentate molecular layer and hilus and to the subiculum and it has been proposed that one CA3 pyramidal can make synapses with up to 60,000 neurones in just the ipsilateral hippocampus (Ishizuka et al., 1990; Li et al., 1994). It is easy to appreciate how single cells in the CA3 can provoke an extensive modulation of sensory information. The Schaffer collaterals project mainly to the stratum oriens and radiatum but not to the stratum lacunosum-moleculare Ishizuka et al., 1990). Interestingly these connections are not uniformly distributed to CA1. Fibres projecting from near the dentate gyrus distribute preferentially to the distal CA1 (nearest to the subiculum) whereas projections from CA3 nearest the CA1 boarder project to CA1 nearest to the CA3 boarder (hence a short range projection)

(Ishizuka et al., 1990). Despite the prominence of the pyramidal cell type (stratum pyramidale) there is a strong inhibitory presence within CA1 that provides both feed-forward and feed-back inhibition of up to 1000 pyramidal cells (Buhl et al., 1995; Freund and Buzsáki, 1996). Unlike the recurrent collaterals of CA3, CA1 pyramidals do not connect with other CA1 cells. Instead they project in a columnar fashion to either the subiculum (Finch and Babb, 1981; Finch et al., 1983) or weakly to the entorhinal cortex (Amaral and Witter, 1989; Swanson et al., 1978). From the subiculum, axons project to the pre- and para-subiculum and then to layer III and II of the entorhinal cortex, respectively (Amaral and Witter, 1989; Kohler, 1985; Shipley, 1975).

1.5.2. Lesions of the Hippocampus

Lesion studies were traditionally performed with electrical current to 'burn' the surrounding tissue indiscriminately; an electrolytic lesion. The fact that all tissue, including neural and vasculature, was destroyed by this lesion method was not ideal. A more focal lesion method was developed using excytotoxic toxins that removed cells but left axons *en passage* and vasculature relatively intact (Jarrard, 1989). It was known from the original Morris water maze study (Morris et al., 1982) that spatial navigation was dependent on the hippocampus. Rats that underwent total hippocampal lesions did not learn the location of the hidden platform (Morris et al., 1982). This does not provide any further information as to which sub-region, if any one, is involved in spatial learning. To address this point, Moser (1993) produced lesions of various sizes in the rodent dorsal and ventral hippocampus and then tested their performance on the Morris water maze. Only relatively small dorsal hippocampal lesions (~20%) were required to produced a profound impairment in spatial learning, whereas nearly the whole ventral hippocampus had to be removed before an effect was observed (Moser et al., 1993, 1995). Other labs have replicated the dorsal hippocampal deficit in spatial learning in the water maze (e.g. Bannerman et al., 1999). The above experiments have mainly focused on spatial reference memory. This is defined by the fact the platform location remains stable across successive trials and days of training. Spatial working memory is also impaired with dorsal hippocampal lesions in the water maze and T-maze, but not with ventral lesions (Bannerman et al., 1999, 2002). Spatial working memory tasks require the

animal to learn a new item of spatial information on each trial of the procedure. Importantly, the same study documented that hippocampal lesioned animals exhibit hyperactivity, a potential confounding factor when considering tasks relying upon exploratory activity.

Taken together, these results highlight the importance of the dorsal hippocampus in spatial learning. This raises the question of what role the ventral hippocampus plays in memory. The ventral hippocampus, in contrast to the dorsal hippocampus, has strong connections to the amygdala (for review see Moser and Moser, 1998). One of the major roles of the amygdala in learning is in the modulation of emotional responses. A task that has been frequently employed to study this is fear conditioning. Lesions of the amygdala impair conditioned freezing elicited by a tone and a context paired with a foot shock unconditioned stimulus (US). In contrast, a number of studies have shown that hippocampal lesions impair only contextual freezing (Kim and Fanselow, 1992; Phillips and LeDoux, 1992); although this continues to be an area of controversy. It should be noted however that specific lesions to sub-regions of the amygdala have differential effects on emotional responses. Thus, lesions of the basolateral amygdaloid nuclei impair contextual conditioning whereas lesions of the lateral amygdaloid nuclei disrupt tone conditioning (Calandreau et al., 2005 but see Goosens and Maren, 2001). One again, sub-region hippocampal involvement remains controversial. Some studies have found that ventral but not dorsal hippocampal lesions produce freezing deficits to the context (Richmond et al., 1999). Indeed the proximity of the ventral hippocampus to the amygdala does not rule out the possibility that damage could also be occurring to the amygdala (Anagnostaras et al., 2002; Maren, 1999). More selective inhibition with muscimol infused into the ventral hippocampus also reduced freezing response (Rudy and Matus-Amat, 2005). Despite this, many studies have found that dorsal lesions do in fact impair contextual freezing (for example Anagnostaras et al., 1999; Kim and Fanselow, 1992; Maren et al., 1997). Similarly, muscimol infusion into the dorsal hippocampus has also been found to impair freezing (Matus-Amat et al., 2004). Given the mixed nature of the results there are suggestions that contextual fear conditioning is expressed throughout both regions of the hippocampus (Rudy and Matus-Amat, 2005). Despite this, a dissociation of hippocampal function still exists, with the dorsal hippocampus required for spatial

navigation and contextual learning and perhaps both the dorsal and ventral sub-regions required for emotional learning.

1.5.3. Hippocampal Place Cells

A truly remarkable study was published in 1971 that demonstrated cells in the hippocampus would fire selectively when the rat was positioned in an enclosed environment (O'Keefe and Dostrovsky, 1971). It's remarkable by the fact that not only did they provide the founding evidence for one of the most influential theories of hippocampal function (O'Keefe & Nadel, 1978), but also the discovery predated LTP (Bliss and Lomo, 1973). This, along with visual cortex ocular dominance plasticity documented in 1963 (Weisel and Hubel, 1963) highlight that research into EDP was being conducted prior to synaptic plasticity via LTP.

Hippocampal place cells were selective to a specific location, and rarely fired outside of that position, although in a different environment they would also contribute in a distinct, separate manner (O'Keefe, 1976). It was postulated that the contributions of a number of place cells could summate across the hippocampus to form a representation of the environment (Wilson and McNaughton, 1993). As such, place cells were thought to provide the fundamental mechanism for the formation of an allocentric representation spatial layout of the animal's environment and position within that space (Moser et al., 2008; O'Keefe and Nadal, 1978). That is not to suggest that hippocampal place cells solely mediate spatial information. Non-spatial stimuli (odour, texture etc) have also been shown to cause place cell activation (Wood et al., 1999), and there is evidence that spatial/non-spatial representations are represented independently via changes to the firing location and the firing rate, respectively (Leutgeb et al., 2005; Moser et al., 2008).

The topic of place cells and hippocampal lesion studies are worthy of detailed reviews individually, and indeed many are produced on a yearly basis. However, a detailed characterisation of hippocampal function is not the main goal of this thesis. The two lines of evidence do confirm that the hippocampus is required for spatial learning, and in turn that such procedures can be used to assess manipulations of hippocampal synaptic function. With the potential difficulties and lack of progress in designing

barrel cortex paradigms (see Chapter 5, Section 5.1 for a detailed review), assessing molecular mechanisms required for learning is still best carried out using procedures sensitive to hippocampal dysfunction.

1.6. Molecular Mechanisms for Hippocampal Spatial Learning

This section considers evidence related to the major molecular events underpinning LTP and EDP (in particular AMPA and NMDA receptors) and how their manipulation affects learning. This is not an exhaustive survey of the substrates of plasticity and this discussion will therefore focus on those areas related to the aims of this thesis.

1.6.1. NMDA Receptors and Hippocampal Function

It has been somewhat difficult to determine the role of NMDARs in barrel cortex EDP for one technical reason. Any manipulation that affects NMDARs *in vivo* will also affect transmission (Fox et al., 1989). Thus, reduced synaptic transmission makes it difficult to probe the contribution of these receptors in EDP (see Fox, 2008).

However, in an *in vitro* preparation, NMDA receptors are not substantially activated, except during the plasticity protocol (*in vivo* preparations contain considerable spontaneous activity even during anaesthesia and synaptic activity during exploration of the environment via whisking). In the layer IV to II/III synapse, antagonism of NMDARs by AP-5 prohibits LTP until it is washed off, after which time LTP can be induced via STDP protocols (Banerjee et al., 2009; Bender et al., 2006; Hardingham et al., 2003). During the recording period, EPSP size does not decrease, suggesting that the drug itself does not alter baseline transmission as it would do *in vivo* (Bender et al., 2006; Hardingham et al., 2003). These finding suggest that NMDARs are involved in neocortical plasticity, although their role *in vivo* remains elusive.

Despite the technical difficulties associated with the barrel cortex, efforts have been made to characterise the role of NMDARs in the hippocampus *in vivo*. In the study by Morris (1986), spatial learning and *in vivo* LTP were assessed in the presence of pharmacological inhibition of NMDARs by AP5 (D, L-AP5) directly administered by

intracerebroventricular (icv) infusion. The AP5 treated rats were slower to escape from the water during training and had showed no preference for the hidden platform location during a transfer test (Morris et al., 1986; Morris, 1989). However, when a rat that had previously acquired the task was administered AP5, the drug did not disrupt performance on the task (Morris, 1989). Perforant pathway LTP was assessed at the end of spatial training and it was found that in those rats that had constant infusion of AP5, *in vivo* LTP was abolished (Morris et al., 1986; Morris, 1989).

The effects of AP5 have remained controversial to some extent, as some authors (Cain et al., 1996) argued that the learning impairments were associated with peripheral sensory/motor confounds (NMDAR blockade *in vivo* does have the effect of dampening transmission which could affect other cortices such as the motor cortex). Indeed, other studies showed that NMDAR antagonists did not impair spatial learning following pretraining in a similar spatial task in a different environment (only marginal improvement was found with non-spatial pretraining (Bannerman et al., 1995). The important point from these studies was that spatial learning occurred despite the absence of perforant path LTP in AP5 treated rats. These findings were clearly inconsistent with a simple plasticity=memory hypothesis. Indeed more recent work has highlighted that hippocampal NMDA receptors are critical for certain properties of spatial memory. That is, impairments in hippocampal LTP lead to deficits in spatial working memory tasks (e.g., Steele and Morris, 1999; Bannerman et al., 2006).

Much of the contemporary work on synaptic plasticity and memory has made use of genetically modified mice. Mutants with specific KOs of a NMDAR subunit (NR1 (this renders the NMDAR inactive as NR2 associate with NR1 as a complex), NR2A or NR2B) at specific brain regions have been tested for spatial learning and synaptic plasticity (LTP). Mice that were deficient of NR1 in the CA3 region were unimpaired in a standard Morris water maze reference memory task despite the absence of commissural/associational LTP (Nakazawa et al., 2002) but were impaired in spatial working memory (similar design to the Steele and Morris (1999) study) (Nakazawa et al., 2003). A remarkably similar phenotype has been found in selective dentate NR1 KOs. Spatial reference memory was unaffected in a radial arm maze task modified to prevent working memory errors, although on the standard radial arm maze, spatial

working memory errors were above those of WTs (Niewoehner et al., 2007). LTP unsurprisingly was abolished in the perforant path to dentate synapse (Niewoehner et al., 2007). Again in mice deficient of NR2A, spatial reference memory was intact whereas spatial working memory was impaired (Bannerman et al., 2008). Finally, mice that are deficient of NR2B subunits in the hippocampus were also unimpaired at spatial reference memory task (Morris watermaze) but were impaired in spatial reversal learning and spatial working memory tasks (spontaneous alteration T-maze) (von Engelhardt et al., 2008). Taken together, NMDAR mutants have provided strong evidence that spatial working memory is dependent on NMDARs and spatial reference memory is independent of NMDAR plasticity.

1.6.2. GluR1 and Hippocampal Function

GluR1 has been shown important for the induction of plasticity in LTP and LTD processes, but is also relevant to EDP. CaMKII, PKA and PKC (Hardingham et al., 2008; Lee et al., 2000; Silva et al., 1992) all act to alter the phosphorylation state of GluR1, which in turn promotes its synaptic insertion or internalisation. Despite the characterisation of GluR1 in plasticity, it is important to understand whether GluR1 is required for learning.

The original characterisation of the GluR1 KO found that LTP was abolished (Schaffer-collateral). Nevertheless, spatial reference memory in the Morris watermaze was not impaired in KO mice (Zamanillo et al., 1999). This led to the proposal that "LTP, although not critical for the type of reference memory used here, could be important in spatial tasks that involve only episodic or working memory" (Zamanillo et al., 1999, p. 1809). Indeed spatial reference memory exists in rodents that have had hippocampal LTP abolished with NMDARs antagonists (see Bannerman et al., 2006). While the spatial memory phenotype of the GluR1 KO has been replicated, the LTP deficit has not. LTP has been shown possible using STDP protocols (Hoffman et al., 2002), and recently using standard orthodromic protocols (Phillips et al., 2008), although early phase LTP was still impaired. Induction is highly dependent on the protocol chosen. As such, the link of learning and LTP remains. Given that early but not late phase LTP is impaired, it is possible that specific learning impairments

(working vs reference memory) could be related to the phases of LTP in the GluR1 KO.

The absence of GluR1 results in a similar phenotype to rodents that undergo hippocampal NMDA antagonism or specific NMDAR knockout where spatial working memory is impaired but spatial reference memory is not (Bannerman et al., 1995; Nakazawa et al., 2003; Niewoehner et al., 2007). Reisel et al. (2002) found that while water maze learning was unaffected in the GluR1 KO, if the dorsal hippocampus of the KO was lesioned then spatial reference memory was similar to WT lesioned mice. This strongly suggests that in the GluR1 KO, spatial reference memory is at least in part dependent upon the hippocampus. Given that synaptic plasticity is required for memory formation (for example Silva et al., 1992), it seems likely that other molecules/receptors are sufficient to support spatial reference memory in the GluR1 KO.

Although spatial reference memory is unaffected in the GluR1 KO, spatial working memory has been shown sensitive to its deletion. Spatial working memory was impaired in the GluR1 KO as assessed by the T-maze, where they made significantly more working memory errors (entries to previously visited arms) compared to WTs (Reisel et al., 2002). Schimtt et al. (2003) provided data that would further the dissociable nature of memory mechanisms in GluR1 KO mice. A radial arm maze similar to that of Olton (1979) was used to investigate spatial working and reference memory. When spatial working memory errors were allowed to occur (i.e. to revisit arms that were already visited), GluR1 KOs consistently made more errors than WTs, while spatial reference memory never formed (Schmitt et al., 2003). However when the task was modified so that spatial working errors could not be made (once an arm was visited, doors prevented that arm being chosen again), spatial reference memory was acquired at a similar rate to WTs (Schmitt et al., 2003). Once again this task was dependent upon the hippocampus, as lesioned KO mice were unable to acquire the reference memory version, remaining at floor throughout the acquisition training (Schmitt et al., 2003).

Another type of procedure that requires encoding of the experimental context is contextual-fear conditioning (Kim and Fanselow, 1992). Following conditioning to

tone and context, GluR1 KOs do not increase their freezing behaviour to either (Humeau et al., 2007). Considering that GluR1 KOs have LTP deficits in the thalmo-LA, cortico-LA and BLA pathways (Humeau et al., 2007), along with specific induction related LTP deficits in the hippocampus (Phillips et al., 2008), it is difficult to determine where the source of this impairment arises. Also, the protocols used to induce LTP in the Humeau (2007) study were either not well enough defined or not inline with other studies that have been able to induce GluR1-independent LTP. This, therefore, limits the possible extrapolations of the LTP deficits to the behavioural impairments. However, the LTP deficits to and within the amygdaloid nuclei would suggest that deficit was in part associated with the amygdala (Phillips and LeDoux, 1992). However, short-term memory processes are disrupted in the GluR1 KO (Riesel et al., 2002), suggesting that the lack of freezing to the context could be hippocampal in origin. One trial learning that requires rapid encoding of trial specific memories may be insufficient to form a spatial reference-type memory in the KO; water maze or radial arm training requires many training trials before the memory is well-established.

Others have confirmed that GluR1 is required for contextual fear conditioning. GFP tagged GluR1 containing AMPA subunits are recruited to mushroom spines in CA1 hippocampal neurons up to 24 hours following fear conditioning (Matsuo and Mayford, 2008). Fear conditioning also increases synaptic incorporation of GluR1-GFP in the amygdala, while injections of a dominant-negative GluR1-ct construct (which blocks incorporation of endogenous GluR1) impair freezing when tested to the tone (Rumpel et al., 2005). It seems likely that GluR1 is required for hippocampal dependent encoding of contextual fear memory. There remains ambiguity as to the whether the deficit in fear memory represents a phenotype similar to that of spatial working memory. However, recent work has proposed that the GluR1 spatial phenotype reflects a deficiency in spatial habituation, which could in part account for the fear deficit. Deficiencies in spatial habituation could reflect an impaired formation of a representation of the spatial/contextual information, which could result in a lack of habituation in exploratory activity.

Recent works have postulated that the spatial working memory deficit in the GluR1 KO may actually reflect a deficiency in short-term habituation (for review see

Sanderson et al., 2010). Wagner's Sometimes Opponent Process (SOP) (1981) model proposed that there are three states in which stimulus elements can reside in, and these stimulus elements are made up of all the elements in the context that are co-existing at any one time, and those elements can only exist in one state at any one time. The three states are termed A1 (primary active), A2 (secondary active) and I (inactive). Transitions can occur from A1 to A2, A2 to I, I to A2 and I to A1 (the latter represents initial contact with a novel stimulus). The transition between states depends on 1) the surprise/novelty of the stimulus elements and 2) whether the stimulus elements have associative strength. Elements in the A1 state are considered to be the focus of attention and thus command behavioural responding.

On introduction to a novel (or surprising) set of stimuli, these elements are represented in the A1 state from the I state. The elements rapidly decay to the A2 state. When an element has been presented recently (non-associative priming) or associatively activated (associative priming) it is primed into the A2 state (termed self-generated priming and associative priming respectively). Elements will then transfer to the I state. The accumulation of elements in the A2 state following self-generated priming is thought to mediate short-term habituation; that is following repeated exposure to the stimulus, fewer elements of its representation are available for activation in the A1 state (as most have been primed into the A2 state) and thus fail to prompt a strong unconditioned response. When a stimulus is predicted (i.e., it has gained associative strength) its elements are directly primed to the A2 state by a process called retrieval-generated priming. In the case of long-term habituation, it is thought that the context becomes associated with the target cue over trials and thus the context primes the target cues representation into the A2 state (retrieval-generated priming). In other words, "whereas self-generated priming is dependent on the recency of a stimulus presentation, retrieval-generated priming is dependent on the strength of the prior association formed between the context and the target stimulus" (Sanderson et al., 2010, p. 2308).

Sanderson (2009) found that modulation of the interval between tests of novelty preference produced separable short-term and long-term habituation. WT and GluR1 KO mice were exposed a Y-maze of which one arm was always blocked (the novel arm) but the other two were available for exploration. Exposure lasted for 2 minutes

either 5 times with a 1 minute inter-trial interval (ITI) or 5 times with a 24 hour (ITI), after which all arms were made available for exploration and novelty preference was assessed. GluR1 KO mice showed much stronger novelty preference when the ITI was set at 24 hours than when the ITI was set at 1 minute (Sanderson et al., 2009). This result is similar to previous findings that varying the length of the interstimulus interval influences short and long-term habituation to startle responses in the rat (Davis, 1970). When the ITI is short, memory is regarded as short-term (i.e. performance reflects short-term habituation to the arm visited 1 minute ago), opposed to if the interval is long (i.e. a long-term habituation). According to Sanderson et al. (2009), GluR1 plays an important role in self-generated priming underlying short-term habituation, whereas retrieval-generated priming reflects a GluR1-independent process. Transferring this theory to spatial memory, short-term habituation deficiency would mean that the recent memory of visiting an arm would be impaired, thus compromising habituation to that arm (in a radial arm maze for example), and so at the choice point all arms would remain effectively novel. However, associative memory can form between the goal (i.e. a food reward) and the arm location within the context, so that the contextual elements can gain associative strength. Upon subsequent exposure to the context, the context primes retrieval of the stimulus target (the goal). It is possible that this process requires GluR1-independent synaptic plasticity.

Taken together, the data are clear in that GluR1 KO mice have impairments in short-term habituation and spatial working memory, while long-term habituation and spatial reference memory is intact. This could correlate to the two phases of LTP in the GluR1 KO. A deficit in early LTP could contribute to working memory/short-term habituation deficits. In contrast, more protracted acquisition of reference memory tasks and long-term habituation could be supported by the late phase of LTP that is similar in magnitude between the GluR1 KO and WTs after one hour (Hoffman et al., 2002). The recent findings that GluR1-independent LTP is supported by NO in both the neocortex (Hardingham and Fox, 2006) and hippocampus (Phillips et al., 2008) suggests that NO-dependent plasticity could mediate long-term habituation and spatial reference memory in the GluR1 KO.

1.6.3. GluR2 and Hippocampal Function

As has previously been described, GluR2 can form associations with GluR1 and GluR3. The difference in function between the two associations is significant. Whereas around 8% of the total population of GluR1 exists as a homomeric receptor, ~70% of GluR1 is associated with GluR2 and ~50% of all AMPARs exist as GluR2/3 heteromers (Wenthold et al., 1996). Functionally, GluR1/2 (like GluR1 homomers) have been suggested to traffic into synapse following activity whereas GluR2/3 replace existing receptors in the synapse continuously (Shi et al., 2001; Zhu, 2009).

Considering the wide-ranging role of GluR2, it could be reasonably expected that its absence would affect learning and plasticity. Mice with GluR2 deletion restricted to the forebrain exhibit decreased excitatory transmission but increased excitability in CA1. Nevertheless, LTP was largely comparable to WTs and was NMDAR dependent (Shimshek et al., 2006). However, GluR2 mice were impaired in acquisition of a reward alternation T-maze task (spatial working memory) and in a spatial reference memory Y-maze once extramaze cues were partially removed (Shimshek et al., 2006). This deficit in spatial reference memory is seemingly different to that of the GluR1 KO (Reisel et al., 2002). The reference memory deficit once spatial cues were partially removed suggests that there is a specific memory impairment in the GluR2 KO. This deficit is seemingly similar to the impairment in pattern completion to the NR1 CA3 KO mice used in Nakazawa (2002). The memory deficits documented in the Shimshek et al. (2006) study were found using a forebrain specific GluR2 deletion and are in striking contrast to that of the global GluR2 KO, where peripheral motor, object exploration, locomotion abnormalities could confound any hippocampal dependent task (Jia et al., 1996; Gerlai et al., 1998). Given the large fraction of AMPA receptors that contain GluR2, it is perhaps not surprising that its absence in motor, visual, cerebellar etc areas would cause substantial effects on the animal's performance.

1.6.4. The Role of Nitric Oxide in Hippocampal Function

As discussed in section 1.4.6. (p. 46), NO may act as the mediator of GluR1-independent plasticity (Hardingham et al., 2006; Phillips et al., 2008; Romberg et al., 2009). Given that GluR1 KOs are able to learn spatial reference memory tasks, it is a

logical hypothesis that NO-mediated forms of plasticity may be responsible for this. However, no experiment has investigated this specific idea. There have been a number of studies that have looked at the role of NO alone on learning and the following section provides a brief overview of the major findings.

Much like the disputed role for NO in synaptic plasticity protocols, a definitive function in learning and memory remains elusive. Much of the research that has been conducted using NOS inhibition has investigated spatial reference memory. Chapman et al. (1992) found that an IP dose of 75 mg/kg of L-NAME impaired the acquisition the hidden platform version of the water maze but did not affect the recall of a previously learnt platform location (trained before drug treatment). Another NOS inhibitor, 7-NI which is more selective towards NOS1, found an initial deficit during acquisition training in the water maze although by the end of training, the latency to find the platform was similar to control rats. 7-NI treated rats were unaffected at learning the new location of a platform (Hölscher et al., 1996). Somewhat surprisingly, during a transfer test 7-NI rats performed statically poorer than control rats, spending roughly equal time in each quadrant (Hölscher et al., 1996).

ICV delivery of L-NAME at a dose of 5 μM impaired the reference memory water maze task, whereas at lower doses, the rats were unaffected (Qiang et al., 1997). In a version of the water maze where the platform always contained a visual cue, rats performed similar to controls, suggesting that the deficit was not related to peripheral confounds (Qiang et al., 1997). A recent study using central infusion of L-NAME to the CA1 region in rats found that at doses of 100 and 200 μg/0.5 μl, latency to find the escape platform was higher and rats spent less time in the target quadrant than control animals (Majlessi et al., 2008). Nevertheless, the drug treated rats showed a preference for the target quadrant (~42% quadrant entries in controls vs ~33% in 200 μg animals; Majlessi et al., 2008). Although the authors did not disclose whether goal arm entry was significantly above chance in the 200 μg L-NAME group, the graph (Figure 4; Majlessi et al., 2008) shows a clear preference for the target quadrant compared to the other three quadrants. This therefore suggests that some degree of learning occurred in drug treated animals.

In the 8-arm radial maze, 7-NI rats also made more spatial working and reference memory errors during the initial phase of training, whereas by the end of training performance was comparable to control rats. There was also no significant effect of NOS antagonism on learning a new set of baited arms (Hölscher et al., 1996). This therefore suggests that although NOS inhibitors slow the acquisition of spatial navigation tasks, after sufficient training spatial working and reference memory formation is unaffected. In another experiment with a radial arm maze, the effects of 7-NI and L-NAME delivered IP were compared. Both 7-NI and L-NAME affected the acquisition of spatial reference memory. However, by the end of training the drug groups performed at a level similar to controls (Zou et al., 1998). Spatial working memory was only statistically affected in the L-NAME treated rats (Zou et al., 1998). In another study by Böhme et al., (1993), spatial memory was affected by the administration of the non-specific inhibitor Arg(NO_2). High doses, resulted in greater memory errors up until the final two days of training, at which point performance was comparable to controls at concentrations that impaired learning, LTP was also impaired in the CA1, albeit not abolished (Böhme et al., 1993).

Studies have however often produced contradictory reports to the role of NOS in memory. Using the same test, two separate labs have been unable to derive the same conclusion. These assessed spatial working and reference memory. When L-NAME was infused into bilaterally into the dorsal hippocampus, more spatial working memory errors were made at all drug concentrations, whereas spatial reference memory was unaffected (Ohno et al., 1993). Conversely, delivery of 7-NI via IP injection caused no impairment of spatial working memory but did reduce reference memory performance, again at all drug concentrations used (Yildiz Akar et al., 2009). This suggests that compound, delivery or laboratory specific conditions could confound the assessment of NO in memory formation.

Other studies have found little effect of NO inhibitors in spatial learning. Bannerman et al. (1994) used L-NAME at a does that inhibited 90% of NOS activity, found that although rats had minor deficits during acquisition training and the probe tests in a Morris water maze task, no deficit was found if the rats were pretrained in a water maze in a separate environment. Although not exactly the same, the deficit in reversal learning could be similar to that of pretraining AP5 infused rats (Bannerman et al.,

1995). There was also no deficit in learning a new platform location or the retention of the platform location (Bannerman et al., 1994). The authors therefore propose that inhibitor-induced behavioural deficits could reflect a peripheral confound. Indeed, this explanation has been proposed elsewhere in relation to a dose dependent impairment in the Morris water maze (Prendergast et al., 1997a) and a delayed-matching-to-sample task (Prendergast et al., 1997b). It is highly like that the contraindications of gastrointestinal malaise and blood pressure fluctuations with L-NAME injections could contribute to an altered state of the subject. Therefore, caution should be exerted when interpreting the findings of studies that have used non-region specific NOS inhibitor delivery. Another study has also found that spatial learning in the water maze was unaffected by central delivery of L-NA into the dorsal hippocampus. While acquisition was mildly retarded, preference to the platform location during a transfer task was unaffected (Blokland et al., 1999). L-NA also had no effect at reversal learning (Blokland et al., 1999).

NOS mutant animals have also been tested in behavioural paradigms. NOS1 KO mice acquired an 8-arm radial maze task at a similar rate to WT mice (Tanda et al., 2009). This suggests that spatial working memory was unaffected by the mutation. However acquisition in a water maze was slower than WT controls, although performance was comparable at the end of training and during the probe test. Preference towards the platform quadrant had diminished when a second probe test was conducted 7 days following training (6 days after the first probe test) (Tanda et al., 2009). This result has also been shown in a separate study that tested platform location preference up to 14 days post-training (Weitzdoerfer et al., 2004). In contrast, testing on a multiple T-maze was unaffected (Weitzdoerfer et al., 2004). This led the authors to suggest that NO involvement in learning is dependent on whether the experimental condition is associated with stress (Weitzdoerfer et al., 2004). NOS3 KO mice showed similar acquisition and preference for the platform quadrant to WTs in the water maze. However, spatial memory for the goal location was much greater in the NOS3 KO 5 days following the probe (Frisch et al., 2000). Spatial reversal learning was also marginally better for NOS3 KO mice (Frisch et al., 2000).

These results highlight that no clear phenotype has been identified in response to NOS antagonism. It is likely that the experimental conditions (that being whether the test is

stressful, the delivery method of the NOS antagonist or the type of antagonist used) could strongly influence the behavioural outcomes. Despite this, many labs have reported specific memory impairments following NOS manipulation, making it probable that NO mediates some type of memory process.

1.7. Conclusion

Taken together, these data suggests that NO plays some role in memory processes supported by the hippocampus. Research into whether NO supports a specific memory type, for example spatial working or reference memory, remains ambiguous.

However the role mediated by GluR1 in learning and memory is clearer. GluR1 is required for the processing of short-term or working memory, whereas spatial reference memory does not require GluR1. This dissociation of memory processes is strikingly similar to temporally distinct phases of LTP in the GluR1 KO. Early-phase LTP in the neocortex and the hippocampus is GluR1-dependent, whereas late-phase LTP is GluR1-independent. It is possible that working memory may be supported by early-phase LTP while reference memory is supported by late-phase LTP. The question remains as to the nature of what synaptic plasticity supports GluR1-independent plasticity mechanisms. It has been recently suggested that NOS-dependent plasticity completely supports neocortical GluR1-independent potentiation and partially supports hippocampal GluR1-independent potentiation. Hence, spatial reference memory could be mediated by NOS signalling in the GluR1 KO. Despite this, it is currently not known whether GluR1 is required for *in vivo* synaptic plasticity following synaptic potentiation in rodents that are of adult ages. Therefore the dependency of GluR1-containing AMPARs in EDP will be tested in the barrel cortex and should potentiation occur, then its abolition will be attempted by using NOS mutant mice. Having established that NOS signalling mediates the residual synaptic potentiation in the GluR1 KO, the intact reference memory in the GluR1 KO will challenged by NOS antagonism.

Chapter 2:

Materials and Methods

All experiments were conducted in accordance with UK Home Office approved Project and Personal licences and the ethical review process of Cardiff University.

2.1. Animals

2.1.1. Subjects

All mice were housed under conditions controlled for light (12 hour light/dark cycle), temperature (22°c) and humidity. Food and water was available ad libitum for the duration of the housing.

The colony was maintained as heterozygotes of the targeted mutations of the NOS3 (B6.129P2-Nos3tm1Unc/J) (The Jackson Laboratory, Maine, USA), NOS1 (B6;129S4-Nos1tm1Plh/J) (The Jackson Laboratory, Maine, USA) and GluR1 genes (supplied originally by the Rawlins lab, Oxford, UK), extensively bred into a C57/BL6JOlaHSD background (Harlan, Oxon, UK). Experimental null mutants and wild-type littermates were bred from heterozygote crosses (cousin mating). The double knockouts contained mutations of either the NOS1 and GluR1 gene or the NOS3 and GluR1 gene. Again, cousin mating from heterozygous parents of both genes derived these animals. Occasionally due to the difficulty in producing a double knockout, it was necessary to mate one parent that was homozygous and heterozygous with another parent that was homozygous and heterozygous. For example, to produce a GluR1/NOS1 knockout a heterozygous/homozygous male was bred with a heterozygous/homozygous female.

The NOS1 mutant animal has evoked strong debate within the scientific community regarding the effectiveness of the blockade of neuronal nitric oxide, albeit unpublished. A clarification of this controversy is provided.

Paul Huang created this mutant by disrupting the first protein coding exon, exon 2, which contains the ATG initiation codon. Exon 2 encodes the PDZ domain that associates with PSD-95 of the NMDA receptor (personal communication, Huang 2008). Hence, the αNOS1 splice variant is knocked out, but not the β and λ splice variants. These do not contain the PDZ domain, so while β and λ variants remain, they cannot associate with NMDA activation so should not be neuronally activated (Eliasson et al., 1997). There is also considerable work from our own studies

indicating that the animal develops normally, and has significantly reduced NO release during NMDA activation (Hardingham et al., 2010), which is consistent with the association of NMDA and NOS1 via PSD-95 being lost.

2.1.2. Animal Housing

The mice were housed with their single sex littermates up until the time of the experiment. Housing during the testing phase was determined by the specific requirements of the experiment. This is discussed in each experimental procedure section.

Deprived mice were sometimes housed with a reduced number of littermates. During the course of the deprivation fighting became a problem, causing the remaining single whisker to be lost. It was found that reducing the number of littermates, especially the dominant animals, helped. Littermates were always kept with the deprived animal wherever possible for enrichment purposes. Undeprived mice were always kept with their littermates until recording.

2.2. Solutions

2.2.1. Phosphate Buffered Saline

Phosphate buffered saline (PBS) was created from the following:
1 litre distilled water,
3.25 g sodium mono phosphate (Sigma-Aldrich, Dorset, UK),
10.35 g sodium dibasic phosphate (Sigma-Aldrich, Dorset, UK),
8.2 g sodium chloride (Sigma-Aldrich, Dorset, UK).
This was then chilled to 4°c and stored for up to 2 weeks.

To make 20 % sucrose PBS solution, 40 g of sucrose (Sigma-Aldrich, Dorset, UK) was added to 200 ml of PBS and then stored at 4°c.

2.2.2. Paraformaldehyde

40 g of paraformaldehyde (Sigma-Aldrich, Dorset, UK) was dissolved in 1 litre of distilled water heated to 60°c. 1 M sodium hydroxide (Sigma-Aldrich, Dorset, UK) was added drop-wise until the solution had cleared, with care not to cause precipitation. The chemicals used for PBS was added, the solution filtered and stored at 4°c.

To make 20 % sucrose, 4 % paraformaldehyde solution, 40 g of sucrose (Sigma-Aldrich, Dorset, UK) was added to 200 ml of paraformaldehyde and then stored at 4°c.

2.2.3. Urethane

1.5 g of urethane (Sigma-Aldrich, Dorset, UK) was dissolved in 10 ml of distilled water, providing a solution of 15 % urethane. This was supplemented by an addition of approximately 10% acepromazine maleate (Novartis, Herts, UK) mixed into the urethane filled syringe to provide rapid sedative action. This was then stored at room temperature in darkness, to protect against photo degradation. The dose of acepromazine maleate was reduced by 50% for experiments with the NOS3 mutant animals as it was discovered that they are more susceptible to the respiratory complications associated with the depressive side effects of the drug. A total dose of 1.5 g/kg was calculated and 70% of this was injected intrapertioneally (IP). Supplemental dose were administered up to the total dose to total dose if a surgical level of anaesthesia had not been initially achieved.

2.2.4. Isoflurane Anaesthetic

To provide short-term anaesthesia for deprivation of whiskers, mice were placed into a chamber where isoflurane (Baxter, Berkshire, UK) inhalation anaesthesia (5% isoflurane 1.5 l/min^{-1} O_2) was induced. Following the procedure, the mice were transferred to a homeothermic heat pad.

2.2.5. Avertin Anaesthetic

Avertin anaesthetic was chosen to implant minipumps due to its relatively short acting duration (1 hour) and preferable depth stability as compared to isoflurane. The anaesthetic was made using the following procedure.

Stock:

10g 2,2,2-tribromoethanol (Sigma-Aldrich, Dorset, UK) fully mixed into 10ml of tert-amyl alcohol (Sigma-Aldrich, Dorset, UK). This stock solution was light protected and stored for up to 6 months.

Working:

Working solutions were created by dissolving 1 ml of stock solution in 39 ml of dH_2O by vortexing. Suitable volumes were aliquoted and light protected and stored at 4°c. 150 µl/g was injected IP to provide a surgical level of anaesthesia.

2.2.6. Sterile Artificial Cerebrospinal Fluid (ACSF)

For solutions to fill the minipumps, artificial cerebrospinal fluid was made under aseptic conditions. Extra care was taken to ensure conditions remained sterile as failure to do so can affect pumping rates and potentially risks postoperative infection. ACSF was created using the following procedure.

Solution A:

To 500ml sterile dH_2O, the following was fully dissolved:

8.66 g sodium chloride (NaCl), 0.224 g potassium chloride (KCl), 0.206 g calcium chloride ($CaCl_2$), 0.163 g magnesium chloride ($MgCl_2$).

Solution B:

To 500ml sterile dH_2O, the following was fully dissolved:

0.214 g sodium phosphate dibasic (Na_2HPO_4), 0.027 g sodium phosphate monobasic (NaH_2PO_4).

All chemicals were sourced from Sigma-Aldrich, Dorset, UK.

Solution A was added to solution B in a 1:1 ratio. No bicarbonate was added as HCO_3 converts to CO_2, which can form bubble in the pump that could potentially affect pumping. This conversion can cause shifts in pH in the solution.

2.2.7. Cytochrome Oxidase Staining

8 g of sucrose was dissolved in 180 ml of PBS (solution A). Once fully dissolved, 123 mg of cytochrome C (Sigma-Aldrich, Dorset, UK) was added to solution A. At the same time, 5 ml was syringed into an ISOPAC bottle containing 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, Dorset, UK). The fully dissolved solution was then added to solution A and thoroughly mixed.

2.3. Barrel Cortex Recordings

2.3.1. Deprivation Method

Experience-dependent plasticity was induced by using the D1 spared protocol. All mice were allowed to reach adulthood (average age 5 months; youngest animal 2 months, oldest animal 12 months) before recording. This age range is consistent with previous experience-dependent plasticity studies (Galzewski et al., 2000).

Deprivation was performed under isoflurane anaesthesia. On average, the animal was anaesthetised for no more than 5 minutes, allowing enough time to painlessly removed the whiskers. During the deprivation procedure, the mouse would be taken from its littermates for a short period (approximately 10 minutes) and then returned.

Deprived animals had all whiskers unilaterally removed from their follicles except for the D1 whisker (Figure 2.1). This method has been previously described, and is preferred to trimming, which requires daily attention and is likely to cause the animal more stress.

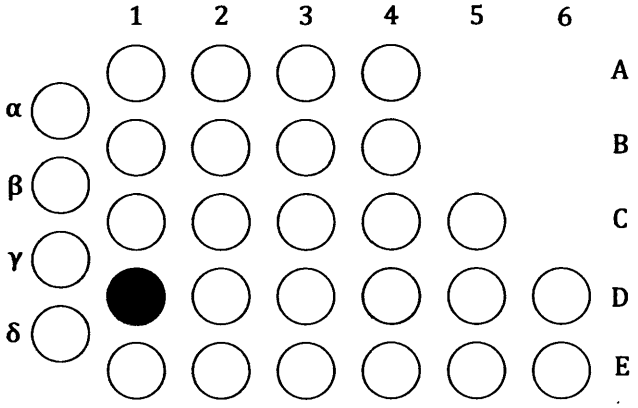


Figure 2.1. *The method of deprivation used to induce plasticity. White circles represent all the deprived whiskers and the black circle represents the spared D1 whisker.*

Removal of the whiskers was performed under microscopic lens by applying slow and steady tension until the whisker was released from the follicle. This is highly important, as a 'yanking' action can damage the follicle, which may make the animal scratch the whisker pad and remove the remaining whisker. Gentle plucking has been shown to cause no damage to the follicle or innovation to the barrel cortex (Li et al., 1995).

After the first plucking, further removal was performed 3 days later and then every other day for 18 days. After the 18 days deprivation, a regrowth period of between 6 and 11 days allowed the whiskers to grow to suitable size (~1 cm) for stimulation. Any whiskers that had not regrown to a suitable size were avoided during the experiment.

2.3.2. Recording Anaesthesia and Surgery

On the day of recording, anaesthesia was induced by isoflurane (5% isoflurane 1.5 l/min^{-1} O_2). An IP injection of 15% urethane (1.5 mg/g) mixed with acepromazine maleate was then given to induce a long-term surgical level of anaesthesia. Anaesthesia depth was measured by regular checks of the hind limb withdrawal reflex (present but sluggish), breath rate and spontaneous neuronal firing at around 2 Hz.

Correct anaesthesia depth created an environment where most of the cortical cells were spontaneously active, which is equivalent to anaesthesia stage III-2. If at any point the animal's state became too light, an addition urethane supplement of 10% of the total does was given. Likewise, if the anaesthesia state became too deep, the animal was rested until such time that the correct conditions (see above) had been reached again. If disruptive spindle activity occurred, the recording was temporarily interrupted until the cortical activity had calmed. EEG and respiratory rates were not recorded during the experiments. Due to the technical limitations of the experimental setup, the activity of up/down states were unknown during the recordings.

It is known that receptive field properties can be dramatically altered in relation to anaesthetic depth. Specifically, urethane anaesthesia from stage III-2 to III-3 reduces the receptive field size from six whiskers to two (Friedberg et al., 1999). While only visual checks of anaesthesia depth were performed throughout the experiment, the receptive field recorded under control conditions within the experiments contained within this thesis (Figure 3.7, p.121) was consistently higher than the two whiskers that would be indicative of anaesthesia depth III-3. For the same reason, it was unlikely that the depth of anaesthesia affected the up/down states induced by urethane. Although slow oscillations are readily induced under urethane anaesthesia and the depth is likely to modify the excitatory/inhibitory balance via $GABA_{A-B}$ receptors (Steriade et al., 1993), the transitions between states are likely to be stable throughout my recordings. The consistent magnitude of the receptive field (see Chapter 3) suggests that stage III-2 was achieved; hence the oscillation activity would have been consistent for all of the genotypes.

Once the initial injection of urethane had been given, the mouse was placed onto a heating pad. After a steady state of anaesthesia had been achieved, the mouse was given a liquid lignocaine (C-Vet Vetinary Products, Lancashire, UK) injection subcutaneously under the incision site and a lignocaine gel (Biorex Laboratories Ltd, Enfield, UK) was applied to the ear canal. The former reduces bleeding caused by the incision and the latter removes the discomfort from the ear restraint bars.

After a short delay to allow the lignocaine to take effect, the ear bars were carefully applied and the mouse transferred to a stereotaxic apparatus (Narashige, Tokyo,

Japan). Body temperature was maintained at 35-37°c by placing the animal on a heating pad (Harvard Apparatus, Kent, UK) controlled by a rectal thermometer.

An incision was made along the centreline of the scalp to expose the skull. Connective tissue was carefully removed and a square over the barrel field highlighted for the craniotomy. The skull was thinned by careful drilling between 2 – 6 mm lateral of the midline and 1 – 4 mm posterior of bregma. Drilling ceased when the surface of the brain was visible, although a small layer of skull remained. In the mouse, removing a large area of skull causes drying of the superficial cortical layers, and thus damage. Consequently, small holes through the skull were made with a fine needle tip (27 gauge, 0.5" length) through the remaining skull and dura for each penetration through which an electrode could be inserted.

After all cells had been recorded from the penetration, an approximate 50 μm lesion was placed at 300 μm by passing a 1.0 μA DC current (tip negative) through the electrode for 10 seconds. The reason for this is two-fold; it provides visual evidence for the exact location of the penetration and the depth at which all the recordings were taken. Both of these can be used to modify results should the penetration be at a different location or depth to what was thought.

2.3.3. Carbon Fibre Electrodes

The recording electrode used for the barrel cortex experiments was a single barreled carbon fibre microelectrode for extracellular recording (Armstrong-James et al., 1980). The construction and functional characteristics have previously been described in detail (Armstrong-James et al., 1980). Carbon fibre electrodes were chosen because of their particular suitability for extracellular recordings. Their tip impedance is typically between 200 kΩ and 2 MΩ, although most commonly 1 MΩ. When comparing to more conventional glass microelectrodes, they exhibit considerably lower noise levels (Fox et al., 1980), meaning that a larger population of activity can be sampled accurately. Hence, smaller spikes that might otherwise be lost to background noise or higher tip impedance can be recorded (see Figure 2.2). This is significant as large, more obvious spikes might not be driven by D1 stimulation or might be an irregular spiking cell. The small diameter of the electrode minimises damage when penetrating through the cortex, unlike the much larger diameter of

tungsten electrodes. This could be key to recording plasticity, as cortical connections are lost in far fewer numbers.

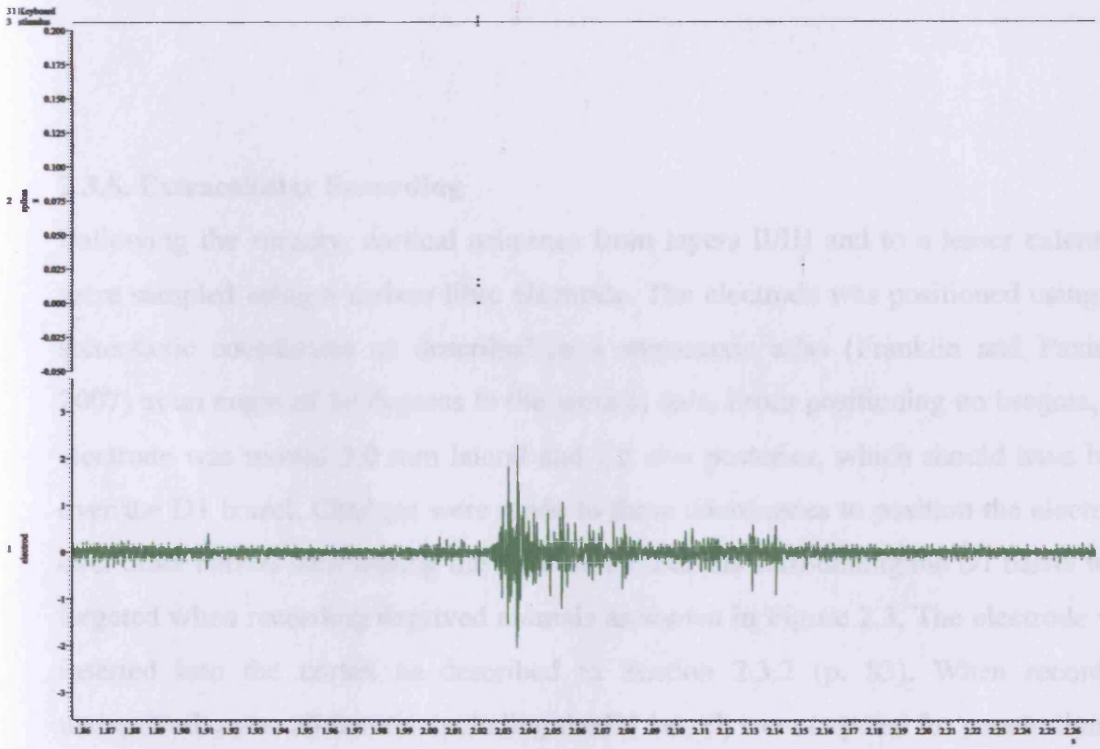


Figure 2.2. *The recorded spiking activity from layer II/III following single whisker deflection. The electrical trace (green) highlights that carbon fibre electrodes have low noise properties, which enables the spiking activity to be clearly observed and thus discriminated.*

2.3.4. Construction

The electrodes were constructed by selecting a single carbon fibre, using a number 5 forceps (Fine Science Tools, Germany), and inserting it into a 2.0 mm diameter glass capillary (Harvard Apparatus, Kent, UK). The capillary was filled with absolute ethanol so the fibre could be threaded through the length of the tube. Once inserted, the ethanol was drained. This construct was then pulled using a vertical electrode puller (Narashige, Tokyo, Japan), which formed a fine tapering glass tip with the carbon fibre projecting through. A wire (RS Components, UK) was constructed with a gold pin connector (Digitimer Ltd, Welwyn Garden City, UK). Electrical contact was achieved with the carbon fibre by coating the wire with silver paint (RS Components,

UK) and inserting it into the capillary. A bead of chromic acid with a small current was used to etch the tip to the desired shape and length (approx 15 µm) under microscope control.

2.3.5. Extracellular Recording

Following the surgery, cortical neurones from layers II/III and to a lesser extent IV were sampled using a carbon fibre electrode. The electrode was positioned using the stereotaxic coordinates as described in a stereotaxic atlas (Franklin and Paxinos, 2007) at an angle of 10 degrees to the vertical axis. From positioning on bregma, the electrode was moved 3.0 mm lateral and 1.5 mm posterior, which should have been over the D1 barrel. Changes were made to these coordinates to position the electrode over other barrels surrounding the D1 column. Barrels surrounding the D1 barrel were targeted when recording deprived animals as shown in Figure 2.3. The electrode was inserted into the cortex as described in Section 2.3.2 (p. 83). When recording undeprived cases, all barrels, including the D1 barrel, were targeted for penetration.

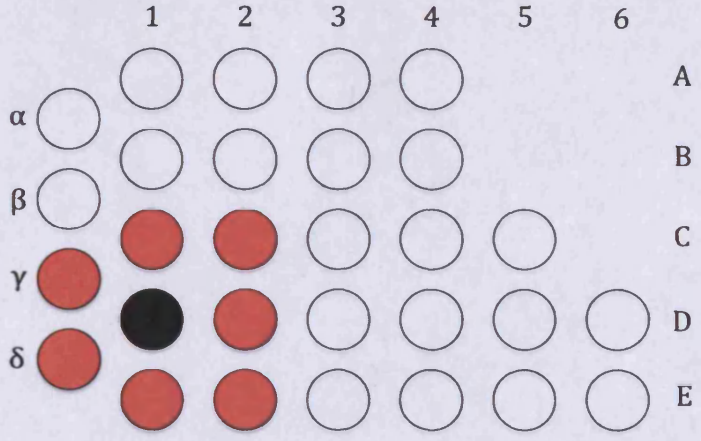
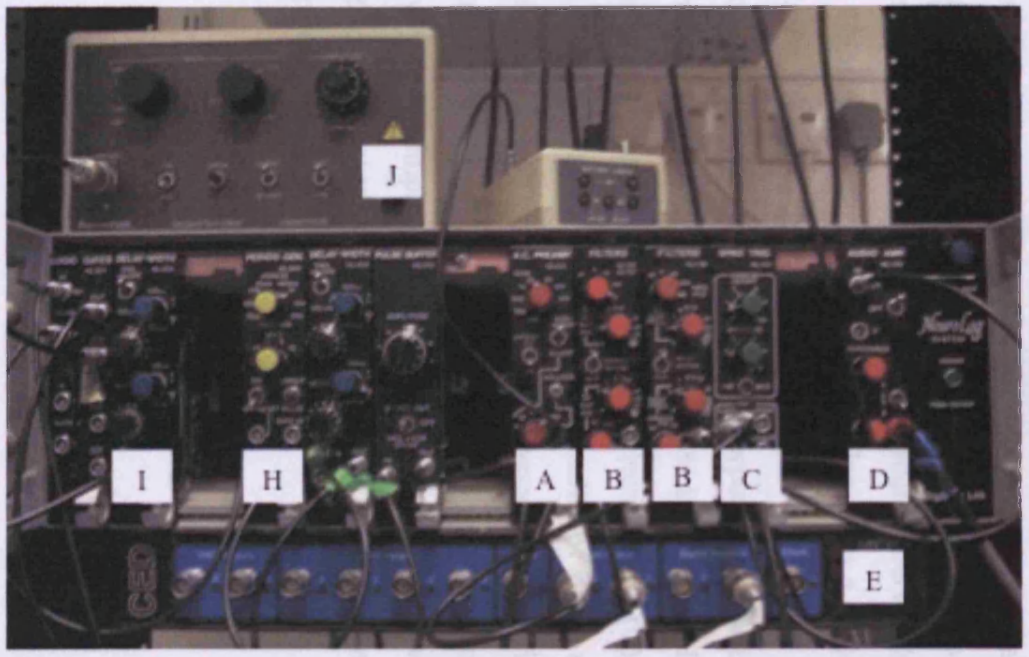
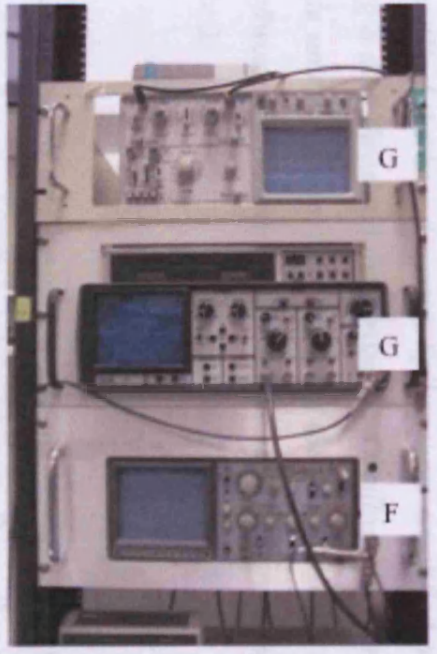
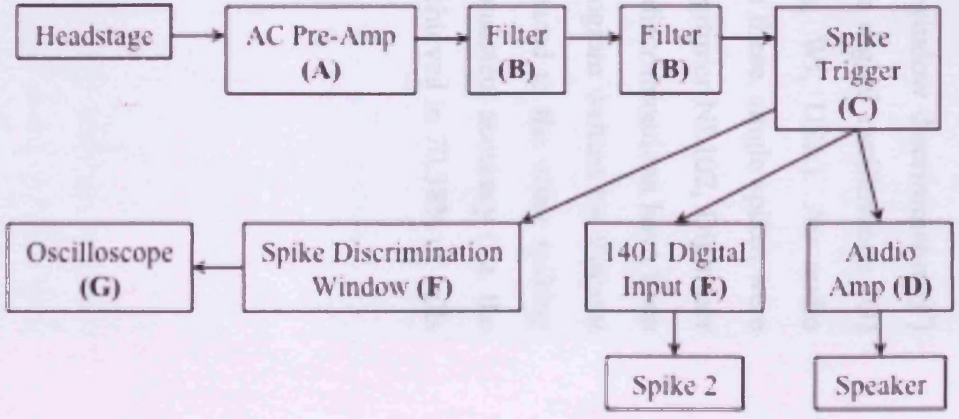


Figure 2.3. *The targeted barrels during recording. Red circles representing the barrels surrounding the D1 barrel were targeted during the deprived experiment. The black circle represents the spared whisker, which was avoided. However, the black circle was targeted during the control experiments. The white circles represent barrel columns that were avoided during both deprived and control experiments.*





A. The Recoding of Spikes.



B. Stimulation Activation.

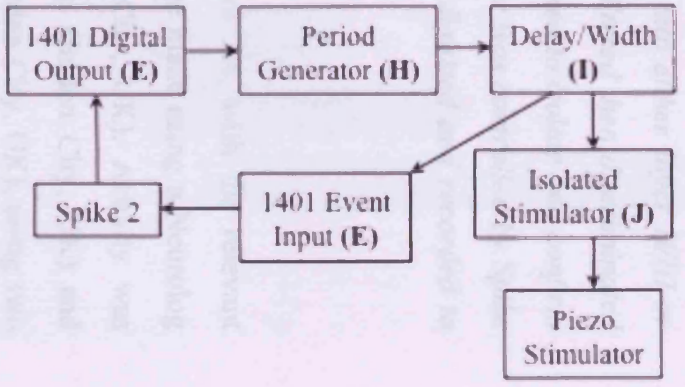


Figure 2.4 (previous page). *The Neurology system used to record single unit neocortical spikes. A carbon fibre electrode was lowered into either layer II/III or layer IV of the barrel cortex. Spikes were amplified and filtered then discriminated using a dual threshold discriminator. Oscilloscopes were used to isolate and confirm single unit responses. Stimulation of the piezo electric wafer was controlled by Spike 2 software via a Micro 1401. Single unit responses were digitized and recorded to Spike 2.*

A schematic of the recording system is shown in Figure 2.4, with the relevant apparatus described with bracketed letters. Recordings were made using a Neurolog headstage (NL100AK, Digitimer Ltd, Welwyn Garden City, UK). Activity was amplified (A) (Digitimer NL104, Digitimer Ltd, Welwyn Garden City, UK) and filtered (B) (Digitimer NL126, Digitimer Ltd, Welwyn Garden City, UK), using two notch filters set on 50 Hz and fourth order Butterworth band-pass filters set between 600 Hz and 6 KHz. Spikes were displayed on a voltage window discriminator (F) (Hitachi VC-6023, Japan) and the waveform viewed on a digital oscilloscope (G) (Nicolet 310, Nicolet Instrument Corporation, Maidison, WI, USA). An audio monitor also allowed neuronal activity to be detected. From these, single spikes were isolated using a dual threshold spike discriminator (C) (Digitimer NL102, Digitimer Ltd, Welwyn Garden City, UK). The quality of single unit discriminations have been confirmed previously by a computer generated analysis program written by Vincent Jacob, where the shape of isolated waveforms were compared to the other spiking activity. In a sample of 736 cells, single unit activity of consistent accuracy (i.e. the length of the recording) at a rate of greater than 80% was achieved in 70.38% of cells (Figure 2.5).

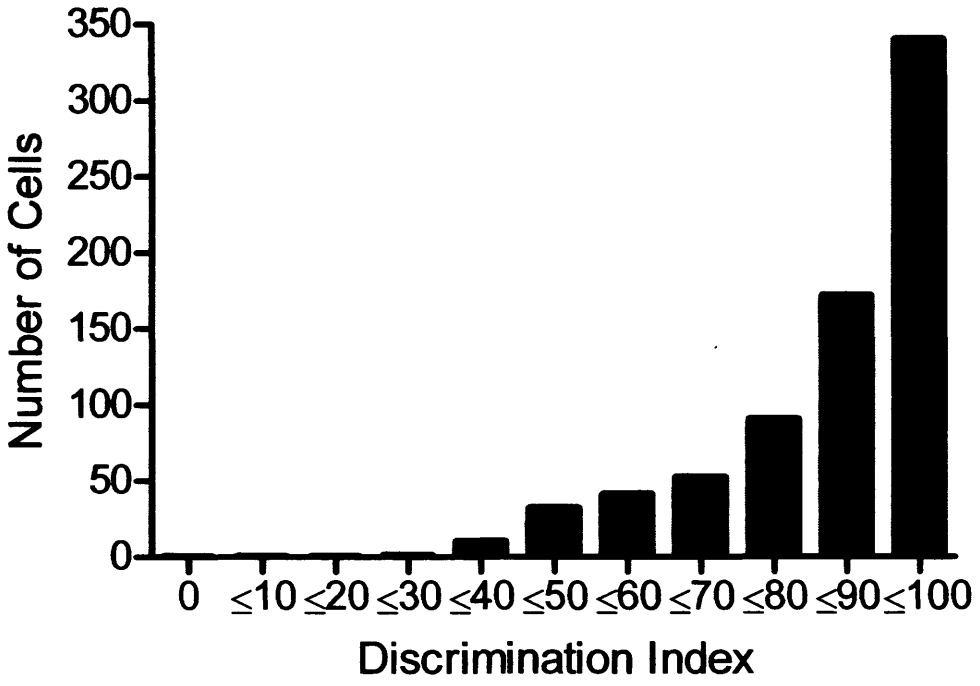


Figure 2.5. *The quality of single unit recordings. Waveform comparison of discriminated spikes to spontaneous spikes enabled an estimation of the percentage of cells that were either single unit or multi-unit. 70.38% of cells achieved a discrimination index (single unit discrimination) of greater than 80% for the length of the recording.*

During the recording, single unit activity was digitised using a CED Micro 2 1401 (E) (Cambridge Electronic Design, Cambridge, UK) connected to a computer running Spike2 Version 3 or Spike2 Version 6 (Cambridge Electronic Design, Cambridge, UK), which generated online post-stimulus time histograms (PSTH) and latency raster plots that could be analysed offline. Both versions of Spike2 produced the same data using the same window discrimination method, however Version 6 provided the ability to record the full spike trace during the stimulus train.

2.3.6. Whisker Stimulation

A schematic of the stimulating system is shown in Figure 2.4, with the relevant apparatus described with bracketed letters. Whisker stimulation was provided by a

pointed glass capillary (Harvard Apparatus, Kent, UK) attached to a fast piezoelectric bimorph wafer, driven by a DS2 high-capacity isolated voltage stimulator (J) (Digitimer Ltd, Welwyn Garden City, UK). Stimulus pulses were delivered at 1 Hz for 50 seconds, causing a 200 μm whisker deflection. Pulses were generated by a Digitimer Neurolog NL304 period generator module (H) (Digitimer Ltd, Welwyn Garden City, UK) fed into a Digitimer Neurolog NL403 delay/width module (I) (Digitimer Ltd, Welwyn Garden City, UK), activating the isolated stimulator. During the stimulus train, the activation of the isolated stimulator was controlled by the Spike 2 software triggering from the Micro 1401. The capillary was placed on the whisker around 10 mm from the base. This is consistent with all previous studies from the lab, allowing useful comparisons to be made.

2.3.8. Cell Sampling

After a small hole was made in the skull and dura, the electrode was lowered into the cortex. This was achieved by careful monitoring of the audio activity, listening to when spikes first appeared. The electrode was then lowered very slowly and carefully to layer IV (approximately 400 μm). At this point, due to the direct innovation, it is easiest to determine the location of the penetration within the barrel field by gentle tapping of each vibrissa. With the principle whisker identified, a short period of time (~ 5 minutes) was given for the penetration to stabilise.

Single neurons were recorded from around 400 μm (layer IV) and then around every 50 μm to 50 μm (layer II/III). Cells were identified and isolated preferably using spontaneous activity, but failing that a couple of taps of the principle whisker. Discrimination was aided by making small movements of the recording electrode (10-20 μm) to reduce multi-unit activity. If during the recording a larger and/or more easily discriminated cell was spotted, then the previous cell was abandoned and the new one used.

2.3.9. Spike Analysis

The modal latency and the magnitude of the response to the stimulation protocol were monitored online and analysed using PSTH and latency raster plots offline. The

magnitude of the response was defined as the number of spikes per stimuli (1 Hz 200 μm deflection every second) from 3 to 53 ms after onset, with the spontaneous activity subtracted. Responses beyond 53 ms were ignored. The modal latency plot was defined as a 1 ms bin that required a minimum of 3 counts after the 50 stimuli train.

2.3.10. Perfusion Fixation and Histology

At the completion of recording, the animal was injected with a lethal overdose of euthatal (pentobarbital sodium) and left until all reflexes were abolished. 0.1 M PBS was perfused through the heart until all blood had been flushed through, followed by 4°c 4% paraformaldehyde in 0.1 M PBS. The brain was carefully excised, the cerebellum removed and the brain divided down the midline. The diencephelon was removed from the left hemisphere and the remaining brain was flattened between two slides. This was left in 20% sucrose in 4% paraformaldehyde in 0.1 M PBS for 24 hours at 4°c, when it was transferred to 20% sucrose in 0.1 M PBS for a minimum of 24 hours, again at 4°c.

Sections of 35 μm were cut on a freezing microtome and reacted for cytochrome oxidase. The sections cut on the microtome where placed into wells filled with the cytochrome / DAB solution and transferred to a 37°c incubator for 6 hours or until the barrels were visualised by the naked eye. The sections were then transferred back to PBS prior to mounting. Cytochrome oxidase histology allows the barrels (layer IV) to be visualised. The sections were mounted onto histology slides with 1% gelatin, cleaned with xylene (Fisher Scientific, UK) and cover slipped using DPX (RA Lamb, UK).

2.3.11. Barrel Morphology

After the sections had been mounted out on slides, they were put under the microscope at either x2 or more commonly at x4 magnification. At this magnification, the barrels and lesions were easily visualised. The barrel field was drawn out via a camera lucida system (Leica Microsystems GmbH, Wetzlar, Germany). The depths

and locations of all recording penetrations were compared to the positions thought during the experiment and modified accordingly.

The drawn barrel field was scanned into a computer and analysed using the ImageTool (UTHSCSA, USA) software. This software measured the length of the far edge to the D1 to the far edge of the D3 barrel and area of the D1 to D5 individual barrels using a calibrated scale drawn from the microscope.

2.4. Data Analysis

Response levels for the principle whisker (PW), D1 whisker and surround whiskers were defined as the number of spikes evoked during the 50 whisker deflections. The total cells for each whisker in either layer II/III or IV for each animal was averaged. These averaged responses were then average across all animals within the condition to obtain a mean response. Surround whiskers (those not the PW) were calculated in order from highest to lowest responding for each cell, and termed surround 1 (S1) to surround 8 (S8). All mean figures were expressed with ± standard error of the mean (SEM). This methodology was used to calculate the receptive fields of layer II/III and IV in Chapter 3 and the PW and D1 response magnitudes of Chapter 4.

2.4.1. D1 Short Latency Domains

In undeprived control mice, cells within layer IV were discriminated and the D1 whisker was stimulated. Cells were recorded from penetrations both within the D1 barrel column and surrounding the D1 barrel column. The spike recordings were analysed for latency until the first spike was recorded (3 spikes within a 1 ms bin). If all cells within the penetration responded at ≤10 ms to D1 stimulation, the penetration was coded black. However, if any cell within the penetration responded above 10 ms to D1 stimulation, the penetration was coded white.

2.4.2. Map Plasticity

Penetration locations following electrophysiological recording were confirmed post mortem by cytochrome oxidase staining. Penetration locations were transferred to a

caricature map but were only included if two or more cells were recorded in layer II/III. The D1 response to D1 whisker stimulation was averaged for the penetration and given one of three codes depending on magnitude. If the average response for the penetration was ≤ 25 spikes, the penetration was coded blue; green for 25 to 49 spikes and yellow for ≥50 spikes.

2.4.3. Vibrissae Dominance Index

To investigate response changes to D1 stimulation after deprivation in terms of the PW, the vibrissae dominance index (VDI) was calculated. This can be useful as fluctuations in anaesthetic state can be compensated for since responses from all cells should depress uniformly. This is similar to the ocular dominance index previously used (Ramoa et al., 1988) for visual plasticity experiments.

Responses to D1 stimulation was expressed relative to the PW to obtain a number from each cell termed *F*, where $F = D1/(D1 + PW)$. Cells recorded either where the D1 was the PW or where the PW was absent were excluded from this analysis. This calculation results in *F* numbers between either 0 (where the PW is dominant over D1) to 1.0 (where the D1 is completely dominant). 0.5 would indicate an equal response to both D1 and PW stimulation.

These figures were put into 10 bands as follows. F_0 contained cells with *F* numbers between 0 to 0.099, F_1 contained cells with *F* numbers between 0.1 to 0.199, F_2 contained cells with *F* numbers between 0.2 to 0.299 up to F_9 containing cells with *F* numbers between 0.9 to 1.0. The percentage of cells that fell in each band was calculated from the total number of cells and distributions compared.

A weighted form of the VDI (WVDI) was also calculated from the *F* numbers, this time producing a single figure for each animal, with all WVDI averaged across all animals within the genotype and condition, expressed as mean ± SEM. The WVDI was calculated for each animal, where:

$$WVDI = \frac{(0F_0 + 1F_1 + 2F_2 + 3F_3 + 4F_4 + 5F_5 + 6F_6 + 7F_7 + 8F_8 + 9F_9)}{9N}$$

Where N is the total number of cells in the sample.

2.5. Behavioural Experiments

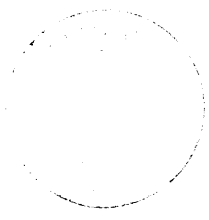
2.5.1. Context Fear Conditioning

2.5.1.1. Subjects

In total 21 wild-type (WT) mice (average age of 3.9 months; oldest 5 months, youngest 2.8 months) and 22 GluR1 KO mice (average age of 6.8 months; oldest 11.9 months, youngest 2.9 months) were used. Of these animals, experimental groups consisted of 11 male and 10 female WT mice and 9 male and 13 GluR1 KO mice. Ad libitum food and water was provided throughout the experiment. All testing took place during the light phase, between 8am and 6pm.

2.5.1.2. Apparatus

Both procedures were performed in a conditioning chamber (Coulbourn Instruments, Whitehall, PA, USA), measuring 18 x 17 x 21 cm, housed inside a sound attenuation box. The front and back of the inner chamber was constructed from clear Perspex, allowing the mouse to observe the surrounding context. A video camera was mounted at the rear of the attenuation box to provide observation of the animal's behaviour. Each conditioning trial and context test was recorded to video for offline analysis. The sidewalls of the chamber were made from metal, and the front panel opened downwards to allow access to the chamber. Four small lamps were located in the ceiling to provide constant and even illumination. The floor consisted of 5 mm diameter steel rods spaced 5 mm apart. This was connected to a Coulbourn precision regulated animal shocker (H13-16, Coulbourn Instruments, Whitehall, PA, USA). All stimuli and responses were under the control of Coulbourn Graphic State software (Coulbourn Instruments, Whitehall, PA, USA) running on a standard PC. Conditioning took place in two identically constructed chambers housed in two separate sound attenuating boxes.



2.5.1.3. Fear Conditioning Procedure

Naïve WT and GluR1 KO mice were transferred from the housing room to the experimental room. The mouse was then placed into the chamber for an initial acclimatisation period of 6 minutes, after which 3 unsignaled foot shocks (2 s, 0.4 mA) were delivered, each separated by 2 minutes. The mice remained in the chamber for 30 seconds following the final shock presentation, after which they were removed and returned to their home cage. The metal floor was removed and cleaned after every session, and shock intensity confirmed before the introduction of the next subject. The rest of the context remained constant throughout the experiment.

2.5.1.4. Context Test

Approximately 24 hours after the conditioning trial, the mice were returned to the chamber to test their response to the context. The chamber and therefore context remained consistent with the conditioning trials. Mice were placed in the chamber for 8 minutes and their activity recorded to videotape. Freezing was then scored offline (see the Scoring Section, 2.5.1.5).

2.5.1.5. Scoring

During the context test the freezing activity from each mouse was assessed. Using a metronome, every 5 seconds the mouse was classified as either freezing or active, which commenced 10 seconds following the closure of the sound attenuation chamber. Freezing was defined by the lack of all movement except that of respiration as has previously been described (Humeau et al., 2007). All scoring was performed blind. A second experimenter scored a sub-selection of mice (n = 26; summer Erasmus student). This individual was blind to the animal and the experimental design. There was an average deviation of 4.89% between the two experimenters. The ability to score reasonably consistently was therefore confirmed.

Two phases of this experiment were scored for freezing. Firstly, the conditioning phase was assessed. All periods were scored; that is the 6 minute acclimatisation period (intershock interval 1, ISI 1), the 2 minutes following the first unsignaled footshock (ISI 2), the 2 minutes following the second unsignaled footshock (ISI 3)

and the final 30 seconds following the third unsignaled footshock (ISI 4). A 'per minute' freezing score was found by dividing the number of times the mouse was found to be freezing by the total scoring opportunities in that minute (12 opportunities per minute or 6 opportunities for the 30 second trial) and multiplying by 100 to obtain a percentage score. Each individual minute was then averaged across animals within the experimental group. This analysis allowed us to determine whether exposure to novel context resulted in differential activity between the groups during the acclimatization period and secondly whether freezing increased with successive presentations of the footshock.

The second phase to be scored was the context test that occurred 24 hours following conditioning. Similar to the conditioning stage, freezing was assessed every 5 seconds. An overall percentage score of freezing was obtained by dividing all events that freezing occurred by the total scoring opportunities in 8 minutes (96) and multiplying by 100. This score was then averaged across all animals within the experimental group.

2.5.2. Radial Arm Water Maze

2.5.2.1. Subjects

Experimental groups consisted of 10 WT and 10 GluR1 KO balanced in a 50:50 ratio of male and female experimentally naïve adult mice aged from 10 weeks to 22 weeks. All testing took place during the light phase cycle. Food and water was available ad libitum throughout the experiment except when testing. For the minipump experiments, a new group of WT and GluR1 KO mice underwent implantation. 8 WT mice (3 male and 5 female) and 8 GluR1 KO mice (4 males and 4 females) had ACSF minpumps inserted. Another 8 WT mice (4 males and 4 females) and 8 GluR1 KO mice (4 males and 4 females) had minipumps containing the NOS inhibitor L-NAME implanted.

2.5.2.2. Apparatus

The radial arm water maze (RAWM) consisted of a 90 cm swim tank and had six identical arms connected to a central arena (Figure 2.6). All arms were of regular

length. Each arm had a width of 19 cm and a length of 30 cm. The maze was located in a well-lit testing room with various extramaze cues attached to the surrounding walls to aid spatial navigation.

During testing, the maze was filled with water kept to approximately 28°c. To hide the submerged platform, the water was made opaque by the addition of milk.

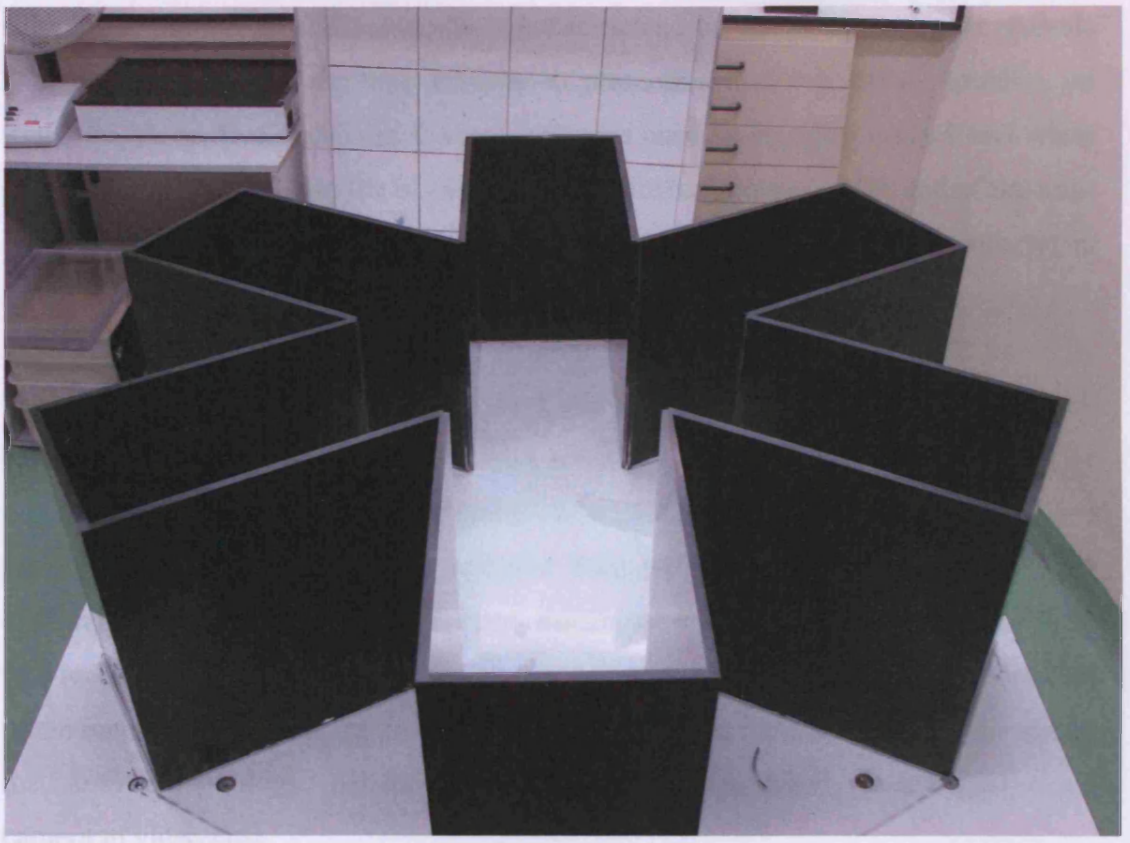


Figure 2.6. *The radial arm water maze (without water) located in the experimental room. Outside of shot were extramaze cues located on the walls around the maze.*

2.5.2.3. Procedure

The experimental cohort was first assigned to one of two goal arms containing a submerged platform, either north or south in a ratio of 50:50 to avoid biasing one spatial location. The start arm varied on each trial and was assigned in a pseudorandom pattern, which was counter balanced across the acquisition training to avoid biasing any one location.

Mice were placed into the water at the end of the appropriate start arm, released and a stopwatch was started. If the mouse swam into an arm that did not contain the submerged platform, the mouse was stopped immediately and retuned to the start location and released again to explore. This was then recorded as a memory error. The trial concluded when either the mouse had found the hidden platform or when one minute had elapsed. In either case, the time was included as the latency measure. When one minute had elapsed, the mouse was picked up by the tail and guided to the submerged platform. In all cases, the mice remained on the platform for 30 seconds after the conclusion of the trial. Initially to promote swimming and exploration, on trials one, three, five and seven the goal arm was cued by inserting a black and white pole (1 cm by 10 cm) onto the platform and a white background at the end of the arm. After the trial, the mouse was dried with a towel and placed in an incubator set to 30°c.

During each trial, the cohort was sequentially tested and returned to the incubator. The testing sequence remained consistent across days. Each subject ran 12 trials per day, which continued for 4 days. A probe was conducted on day 5, where the platform was removed and the mice were released from the adjacent arm to the goal. Free exploration was allowed to occur for one minute, where the time spent in the goal arm was recorded. After one minute, the mouse was removed, dried and allowed to warm in the incubator. A second probe test was run 3 days after the probe test, using exactly the same methodology. All training and testing was recorded via a digital video camera to video tape.

Floating is known to be an issue when running mice in a water maze. During the experiment, it was found that the GluR1 KOs were more susceptible to floating than WTs. However, either temporarily submerging the mouse into the water or lifting by the tail was enough to recommence swimming often for the rest of the trial.

2.5.2.4. Scoring and Analysis

The time taken from being released into the water and reaching the hidden platform or one minute elapsing was recorded and termed the latency.

A memory error was scored whenever the hind legs of the mouse entered the arm. At the point, the mouse was lifted by the tail (although not removed from the water) and returned to the end of the start arm and released to continue exploration. In some cases it was found that the mice would circle the start arm without leaving it (albeit a rare occurrence). This was scored as one memory error.

When scoring the time spent in the goal arm during the probe trials, the same criterion as the memory error was used. In short, the time was started when the hind legs of the mouse entered the goal arm, and stopped when the hind legs exited the arm. If floating occurred, the time was stopped and intervention to promote swimming commenced. As soon as swimming began again, the time was restarted. This was to allow the same amount of swimming exploration time between mice that swam and those that floated.

The latency and memory errors from each trial were averaged into blocks of 4 trials. Data at each block was then averaged across genotypes and/or conditions within the experimental group.

2.5.3. Nitric Oxide Inhibition in the Radial Arm Water Maze

To manipulate NOS, the general nitric oxide inhibitor L-nitro-arginine methyl ester (L-NAME) was infused via minipumps. Control animals also had minipumps implanted by the contents only infused ACSF. The construction and implantation procedure is described.

2.5.3.1. Drugs

100 mM L-NAME was created under sterile conditions. L-NAME (Alexis Biochemicals, Exeter, UK) was dissolved in sterile ACSF. 0.1% trypan blue (Sigma-Aldrich, Dorset, UK) was added to provide visual confirmation of pumping. Control solutions contained only ACSF and 0.1% trypan blue. The high concentration of L-NAME meant that the osmolarity of the drug solution was ~460 mOsm. To match the possible toxic effect that this could have on the adjacent cell population, the control

ACSF solution was made to match the osmolarity by the addition of NaCl. Osmolarity was confirmed by an osmometer.

2.5.3.2. Minipump Construction

The catheter and cannula that attached to the minipump were constructed based on a method previously developed within the Fox Lab. Figure 2.7 provides a technical example of the construction of the cannula.

2.5 mm was measured from the tip of a 30-gauge hypodermic needle and using pliers a 90° bend was created. From this bend a further 5 mm was measured and the needle was cut at this point. Under microscopic control it was confirmed whether the needle shank was still open. To provide mechanical support for the 30-gauge needle, a 3 mm length of a 23 gauge needle (with the sharp tip removed) was cut and opened with pliers. The bent 30 gauge needle was then inserted into this and carefully glued with cyanoacrylate (RS, UK).

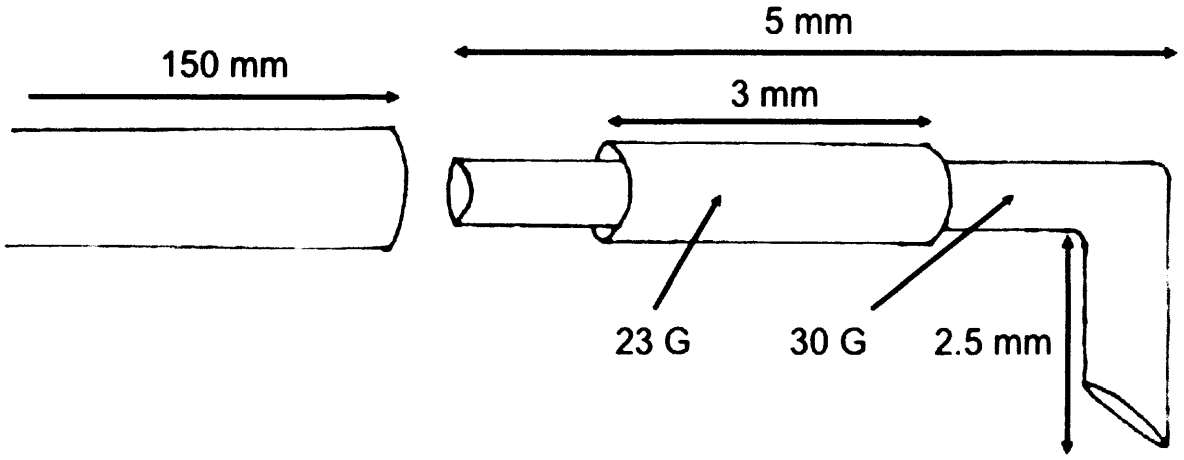


Figure 2.7. *The component parts of the canula construct. A 90° bend was placed into a 30 gauge (30 G) needle 2.5 mm from the tip. The shank of the needle was then cut to give a length of 5 mm. Separately, the tip of a 23 gauge (23 G) needle was cut off and then a 3 mm section was cut. To provide mechanical support, the 30 G needle was placed through the 23 G section and glued into place. Once dried the cannula construct was attached with glue to 150 mm of Alzet 0.69 mm I.D. tubing. When ready for use, the whole assembly was fitted to the minipump.*

The catheter was a length of 1.5 cm tubing (Alzet, Charles River, UK) with the internal diameter (I.D.) of 0.69 mm. A 23-gauge needle was used to splay both ends of the tube and the cannula was inserted and secured in placed with cyanoacrylate. Once dried, the flow through the construct was confirmed by syringing distilled water though and placed in 70% alcohol to sterilise.

The minipumps were filled with either 100 mM L-NAME or ACSF. Both solutions used typan blue for visual identification for diffusion post mortem. The pumps were weighed prior to filling to confirm that once filled the pump did not contain air pockets. Filling was performed under aseptic conditions. The completed pump was incubated in sterile saline overnight to prime the pump for implantation.

2.5.3.3. Minipump Implantation

To infuse nitric oxide inhibitor throughout the length of the radial arm water maze experiment, 1002 model osmotic minipumps (Alzet, Charles River, UK) were implanted. A surgical level of anaesthesia was induced by an IP injection of avertin (see above for details of its creation). After a stable depth had been achieved, the mouse was shaved and betadine (University Hospital Cardiff, UK) applied to the scalp. Lignocaine liquid (C-Vet Vetinary Products, Lancashire, UK) was injected underneath the scalp and auxiliary ear bars applied and the mouse transferred to a stereotaxic frame (Narashige, Tokyo, Japan). Body temperature was maintained at 35-37°c by placing the animal on a heating pad (Harvard Apparatus, Kent, UK) controlled by a rectal thermometer.

Under aseptic conditions, an incision was made along the scalp and the connective tissue removed with number 5 forceps (Fine Science Tools, Germany) and micro scissors (Fine Science Tools, Germany). Micro scissors were also used to remove a small amount of scalp on the contra lateral side to where the cannula was to be inserted to allow more efficient closure of the wound. Blunt-ended scissors were inserted into the space in the mouse's back and opened to form a subcutaneous recess for the pump. Sterile saline was injected before the pump was inserted into the recess. A mark was made -0.45 mm posterior and 1 mm lateral from bregma using a fine stereotaxic manipulator (Narashige, Tokyo, Japan). Rotation of a 30 gauge needle

(Fisher Scientific, UK) was used to create a hole the exact fit of the cannula in the skull. The cannula was then inserted through the burred hole and glued into position with cyanoacrylate (RS, UK) once the skull was fully dried. Dental cement (Durleon, 3M, UK) was applied to the entire exposed skull surface and built up to cover the catheter. One 6-0 absorbable monofilament suture was applied posterior of the surgical site. Cyanoacylate was applied to the dental cement and skin junction to seal the wound and to the suture to increase stability.

The mouse was transferred to a hot box to recover from the anaesthesia. Once the mouse could walk, it was transferred back to its home cage and monitored for weight loss for the next 48 hours. When the glue and cement had fully dried, a topical antibiotic powder was applied onto the surgical site to reduce the risk of post-operative infection. 48 hours of post-operative recovery was allowed before testing began. All surgery and testing cohorts were balanced for genotype and minipump contents.

2.6. Statistical Analysis

All statistical analysis was performed on the specialist software GraphPad Prism version 4 (GraphPad Software, La Jolla, USA) or SPSS version 18 (SPSS, Chicago, IL, USA). Graphs were created using GraphPad Prism or Microsoft Excel.

Since work in this thesis uses two very different techniques (physiological and behavioural), a description of the statistics used for each technique will be provided. In all cases $\alpha = 0.05$. Where significance was not reached, p will represented as $p > 0.05$ except in cases where p was close to significance ($p < 0.1$). In these cases the actual value will be given to highlight trends towards significance.

2.6.1. Physiology

The design of this experiment uses acute recording and morphological data taken from separate control and deprived cohorts. As such, data was not of paired type. All data was expressed as the mean ± standard error of the mean except where clearly stated in the text.

When comparing two groups, an unpaired two-tailed T-test was commonly used. A Mann-Whitney U Test was employed to identify differences in cell population in the vibrissae dominance index analysis after *F* number calculation A chi-squared test was used to indentify bias changes in map plasticity and the response distributions in the D1 short-latency response domain maps. Mutant layer IV modal latency was compared to WTs by a two-sample Kolmogorov-Smirnov test.

To investigate differences between three or more groups, a one, two, three or four-way analysis of variance (ANOVA) was used, depending upon the factoral design, with appropriate post hoc tests used to identify differences between genotypes/conditions. A three-way ANOVA were used to test for plasticity changes between control and deprived conditions across all genotypes. If an interaction between factors was observed then tests of simple main effects was used to investigate differences between groups.

2.6.2. Behavioural Experiments

2.6.2.1. Context Fear Conditioning

All data was expressed as the mean ± the standard error of the mean.

To assess freezing differences during the conditioning of the animals, a repeated measures three-way ANOVA was used with simple main effects analysis used where appropriate. Overall freezing magnitudes were assessed by a two-way ANOVA. Where interactions arose, simple main effects analysis was performed.

2.6.2.2. Radial Arm Water Maze

All data was expressed as the mean ± the standard error of the mean.

The radial arm water maze was designed to investigate longitudinal changes in performance with training within a cohort of animals. As such, the data was of paired origin.

To assess spatial memory formation during water maze training, a repeated measures three-way ANOVA was employed. Where interactions between factors occurred, tests

of simple main effects were conducted. Preference for searching the goal arm during the probe trials was measured using a repeated measures three-way ANOVA.

When testing occurred with osmotic minipumps, spatial memory formation was assessed using a repeated measures four-way ANOVA. Where interactions between factors occurred, tests of simple main effects were conducted. Preference for searching the goal arm during the probe trials was measured using a repeated measures four-way ANOVA.

Chapter 3:

Development of the Barrel Cortex in the Absence of GluR1 and/or Nitric Oxide Synthase

3.1. Introduction

3.1.1. General Introduction

One of the major aims of this thesis is to determine what effect the AMPA receptor subunit GluR1 and nitric oxide (NO) has on experience-dependent plasticity (EDP). To investigate this, knockout mice will be employed. Before differences in plasticity magnitude can be concluded, assessment of baseline conditions in the knockout (KO) mice needs to be investigated. Development of the cortex has been shown to require activity dependent refinement (Chapman and Stryker, 1992; Fox et al., 1996; Shatz, 1990) and the AMPA receptor subunit GluR1 has been shown to be trafficked to the synapse in an experience-dependent manner (Takahashi et al., 2003). Given that GluR1 is required for activity-dependent processes, it was therefore considered important to determine whether the KO influenced the development of the barrel field.

3.1.2. Activity and Development

Experiments have shown that after birth, glutamate receptors are required for the topographical refinement of the thalamocortical afferent connections to layer IV. Schlaggar et al. (1993) slow released the N-methyl-D-aspartate receptor (NMDAR) antagonist D-2-amino-5-phosphonopentanoic acid (D-APV) over the barrel field from birth (P0). They reported that while AP5 had no obvious effect on barrel formation (Schlaggar et al., 1993), subsequent analysis of the receptive fields revealed topographic disruption (Fox et al., 1996). Instead of the normal refined topographic distribution, animals that underwent this treatment had a much larger receptive field, suggesting that the thalamocortical afferents span across a larger area than the intended single barrel (Fox et al., 1996). This provides strong evidence that the development of barrel receptive fields requires sensory activity from the periphery to fine-tune connections. Hence, deletion of synaptic molecules that contribute to synaptic activity could disrupt barrel patterning.

The critical period of layer IV concludes before detectable expression of GluR1 (P4; Watson et al., 2006). Layer II/III development (until P14) occurs at a phase where GluR1 expression (Watson et al., 2006) and synaptic unsilencing via AMPAR insertion increases (Rumpel et al., 2004). Synaptic activity by sensory experience is required for synaptic maturation (Fox et al., 1996), and as such GluR1 insertion might be important

for layer II/III developmental processes. In support of this, mice containing mutation of the regulatory subunit PKARIIβ of PKA, a key phosphorylator of GluR1, were found to have disrupted barrel field topography along with a decrease in GluR1 insertion (Watson et al., 2006). However, cytochrome oxidase staining has revealed that layer IV barrel patterning does exist in the GluR1 KO (Watson et al., 2006; Wright et al., 2008), suggesting that barrel formation occurs independent to the presence of GluR1.

Synaptic transmission in mice that are deficient of the AMPA receptor (AMPAR) GluR1 is not affected in the barrel cortex (Hardingham and Fox, 2006). This suggests that in the absence of GluR1, if synaptic transmission is unaffected then activity-dependent development should take place (unlike in the absence of NMDARs). However, these studies were conducted using slices and the requirement of certain receptors for synaptic transmission is likely to be differ from the *in vitro* preparation compared to *in vivo* preparation (see Feldman, 2009). Receptive field development in layer II/III and IV was also unaffected in young (P28) GluR1 KO mice (Wright et al., 2008), again suggesting that barrel cortex development is not supported by GluR1.

Nitric oxide (NO) has traditionally been associated with changes to synaptic efficacy following plasticity protocols as opposed to neural development. However, subplate neurons have been identified that are NADPH-diaphorase positive (Finney et al., 1998). This suggests that NO could be required for cortical refinement. However, layer IV barrels form normally in NOS1 and NOS3 KOs as revealed by cytochrome oxidase staining (Finney and Shatz, 1998). Of particular note, each mutant also received daily injections of a NOS antagonist to abolish the activity of the remaining isoform (Finney and Shatz, 1998). Synaptic transmission is also unaltered in mutants of NOS. Mice that contained mutations of both GluR1 and one NOS isoform (NOS1 or NOS3) have hippocampal input/output functions that are comparable to WTs (Phillips et al., 2008). So far it is not known how NOS inhibition affects synaptic transmission in the barrel cortex.

The contributions of synaptic molecules to synaptic activity processes might differ between the *in vitro* and *in vivo* preparations. If the absence of GluR1 and NO does affect *in vivo* synaptic transmission during exploratory behaviour, then it is important to characterise what affect this absence has had upon cortical development. This needs to

be done using a variety of techniques (electrophysiological and morphological) as gross layer IV topography can occur independent of normal receptive fields and layer IV targeting (Fox et al., 1996; Schlaggar et al., 1993). This has so far only been attempted in young (P28) GluR1 KOs. Layers II/III and IV were identical to WTs and given that this age is post-critical period for both layers, it is likely that the barrel cortex can develop normally in the absence of GluR1 (Wright et al., 2008). It is therefore predicted that in adult mice, the barrel cortex topography and receptive fields will be normal in the GluR1 KO. The receptive field properties of the cortical layers has so far not been described during NOS antagonism, and given the identification of NADPH-diaphorase positive subplate neurons, there could be a role for NO in fine-scale refinement. Yet given layer IV topography and hippocampal synaptic transmission was normal, it is predicted that the barrel cortex will form normally in KOs or NOS1 and NOS3. The barrel cortex of GluR1/NOSX double KOs has yet to be characterized. However, synaptic transmission in the hippocampus has been studied and was comparable to WTs (Phillips et al., 2008). This, and that neither GluR1 nor NOS antagonism have disrupted barrel cortex, predicts that barrel cortex development will occur normally in the double KO mice.

To test the role of GluR1 and NO in barrel cortex development, mutant mice of GluR1, NOS1 and NOS3 (single and double GluR1/NOSX) will undergo neocortical single unit recordings and histological staining to characterise receptive field formation, response latencies, the confinement of responses to the topographically related barrel and the barrel patterning of layer IV.

3.2. Methods

A full description of the methodology can be found in Chapter 2. In brief, for the electrophysiological recordings undeprived control mice were anesthetised with 15% urethane/acepromazine maleate (1.5 mg/g) and transferred to a stereotaxic frame (Narashige, Tokyo, Japan). The scalp was incised, retracted and the contralateral skull thinned by careful drilling. A 30 gauge needle was used to create a small hole in the thinned skull and a glass insulated carbon fibre was lowered into the cortex. Barrel columns surrounding the D1 barrel column were targeted. Single unit responses were discriminated and the receptive field was mapped for the principle whisker (PW) and all

surrounding whiskers (that is, the whiskers that immediately surround the principle whisker). Stimulation was provided by delivering 50 200 μm deflections of each whisker at 1 Hz. Cells were sampled at 50 to 100 μm intervals from layer IV (400 μm) and layer II/III (up to 50 μm). Responses and latencies were quantified by post stimulus time histograms. At the end of the penetration, a 50 μm lesion was inserted in layer IV. During the recordings, anaesthesia depth was maintained at stage III-2. At the end of the experiment, the animal was fixation perfused, the brain was removed and flattened between two slides.

The post mortem tissue was cut at 35 μm on a freezing microtome and slices were transferred to wells for cytochrome oxidase staining. After the barrel field became visible, the slices were washed in phosphate buffered saline, mounted onto slides and cover slipped. The location and depth of lesions made within layer IV could then be examined and the experimental data modified if necessary. To quantify the barrel field dimensions, the barrel field was drawn via a camera lucida system (Leica Microsystems GmbH, Wetzlar, Germany) and then scanned to computer.

3.2.1. Analysis

3.2.1.1. Morphology

The scanned barrel field morphology was measured using ImageTool (UTHSCSA, USA). A line was drawn from the mid point of the far edge of the D1 barrel to the mid point of the far edge of the D3 barrel. The software was also used to quantify the barrel areas of each individual barrel from D1 to D5. This provided a measure of whether the barrel field size developed normally across all genotypes.

3.2.1.2. Electrophysiological Responses

Single unit spiking was recorded from cells across layers II/III and IV. Spike responses from each cell were recorded from the PW and each of the immediate surrounding whiskers. There are 8 possible whiskers that can surround the PW. The surrounding whiskers were ordered from surround (S) 1 to S8 in relation to the their descending response magnitude. All surrounding whiskers were averaged within the animal, then

averaged across all animals within the genotype. PW responses were averaged within the animal, then averaged across all animals within the genotype.

The latency between striking the PW and first recording a response was also measured. All PW responses within layer IV (where short latency responses (≤ 10 ms) occur) were analysed using a cumulative distribution function and tested for significance using a two-sample Kolmogorov-Smirnov test. Reductions in transmission velocity might be indicative of developmental abnormalities.

The short latency domain of surrounding whiskers was also tested in layer IV. In brief, in layer IV (the input layer to the cortex) short latency responses are confined to the topographically related barrel due to the direct innovation from the thalamus (Armstrong-James et al., 1992; Fox, 1992). Longer latency responses occur in the barrels surrounding the principle barrel due to intracortical transmission. Short latency responses found in the surrounding barrels could represent abnormal TCA targeting (Fox et al., 1996). Since this thesis concerns plasticity shifts to D1 stimulation, the D1 whisker/barrel was chosen to determine whether short latency responses were confined to the D1 barrel during D1 whisker stimulation. If any cell in the penetration responded above 10 ms to D1 stimulation, the penetration was coded white. If any cell in the penetration responded above 10 ms to D1 stimulation, the penetration was coded black. Theoretically black penetrations should be confined to the D1 barrel and white penetrations in barrel columns surrounding the D1 column. Differences between the genotypes were identified by chi-squared analysis.

3.3. Results

3.3.1. The Barrel Field

An example of the barrel field from each genotype following cytochrome oxidase reaction is provided in Figure 3.1. These images provided the quantification for all the anatomical measurements. Micro lesions can be observed in some of the barrel photos (highlighted in 3.1B and D by arrows). These indicate the recording location of each penetration and the depth at which it was placed.

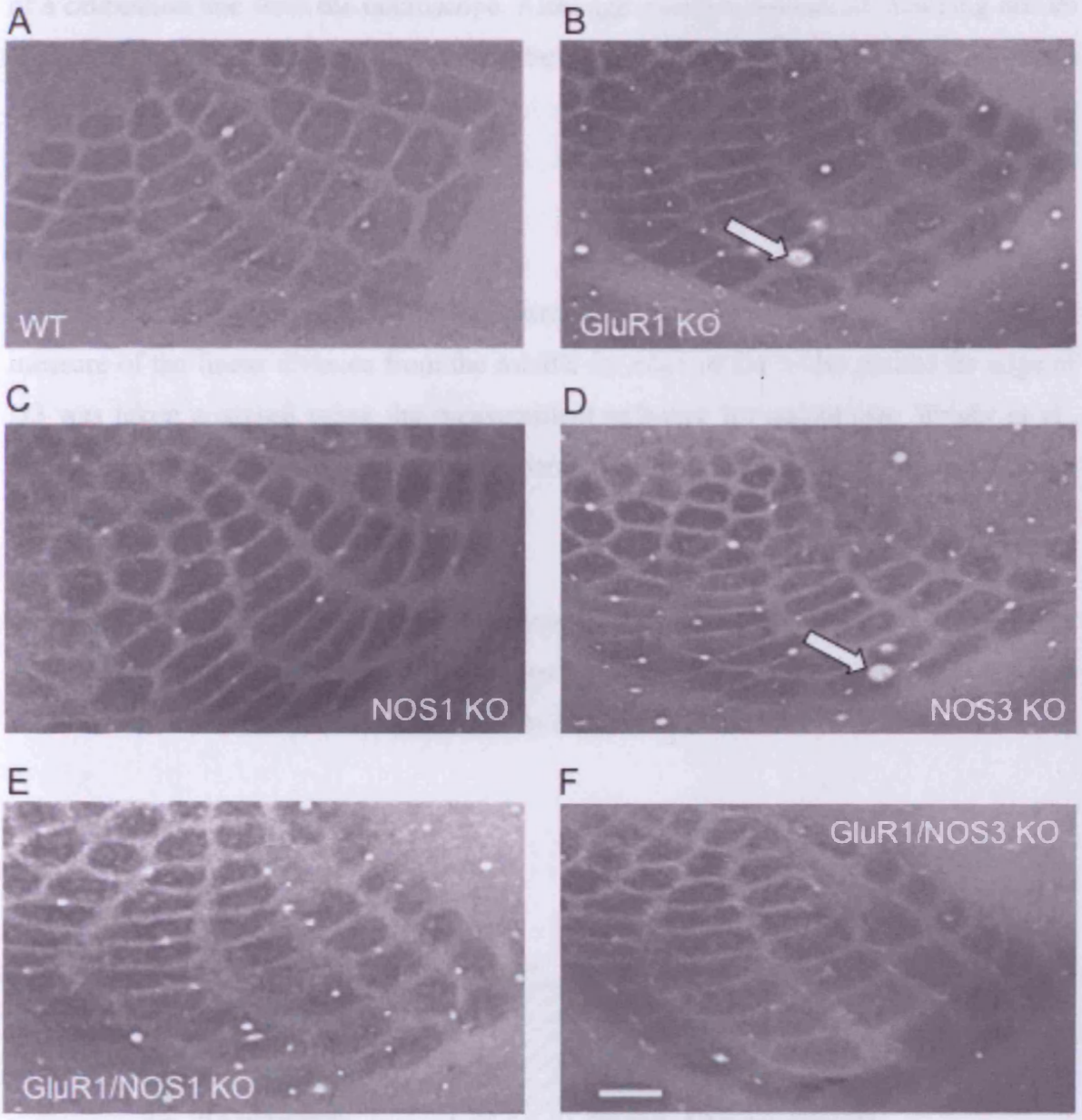


Figure 3.1. *Photomicrographs of the barrel field. The barrel field forms normally in the absence of NOS and GluR1. Layer IV was stained for cytochrome oxidase activity to reveal the barrel architecture. Microlesions can be noticed in* ***B and D*** *highlighted by white arrows. These mark the recording penetration depth and location. Example barrels field of* ***A*** *WT,* ***B*** *GluR1 KOs,* ***C*** *NOS1 KOs,* ***D*** *NOS3 KOs,* ***E*** *GluR1/NOS1 KOs and* ***F*** *GluR1/NOS3 KOs. The scale bar in* ***F*** *= 250 μm.*

3.3.2. Anatomical Measurements

All anatomical analysis was derived from reconstruction of the barrel field using a camera lucida system. Accurate measurement of length and area was allowed by the use

of a calibration line from the microscope. Although a certain amount of shrinking occurs during the histology process, this should be uniform across samples, so the shrinkage should be linear.

3.3.3. Linear Distance

To test whether the overall size of the barrel field was reduced in the null mutants, a measure of the linear distance from the middle far edge of D1 to the middle far edge of D3 was taken analysed using the measurement software Imagetool (see Wright et al., 2008). Lengthening or compacting of the barrel field should be reflected by the distance between the far edges of the barrels.

The mean lengths ± SEM of each genotype are displayed in Figure 3.2. A one-way ANOVA showed no significant effect of genotype ($F_{(5, 48)} = 0.40$, $p > 0.05$). Therefore the null mutants develop a barrel field of similar size to WT controls.

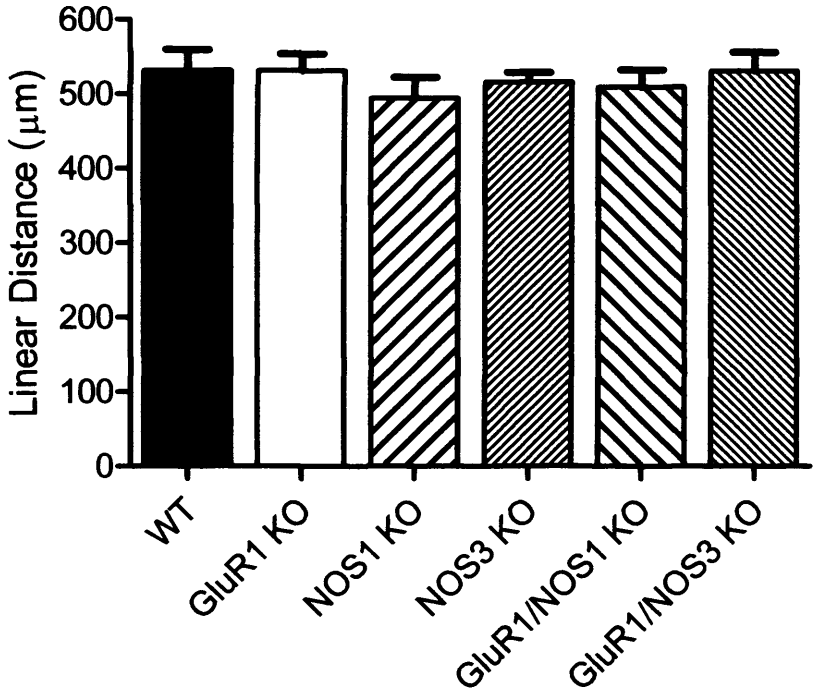


Figure 3.2. *The linear distance between the far edge of D1 to the far edge of the D3 barrel. There were no significant differences between any of the genotypes suggesting that barrel field size develops normally irrespective of the mutations.*

The linear distance of the number one arc (A1 to E1) was also measured for all of the genotypes (Figure 3.3). No significant differences were found between any of the genotypes (one-way ANOVA, $F_{(5, 46)} = 1.014$, $p > 0.05$ (degrees of freedom vary from the D1 to D3 measure as it was not possible to reconstruct the arcs of two animals)).

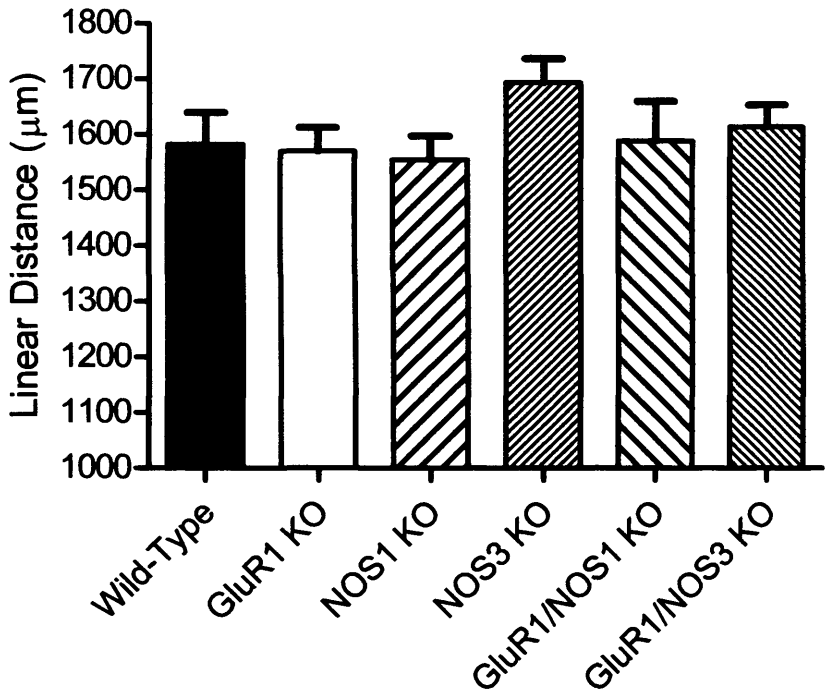


Figure 3.3. *The linear distance of the number one arc from the far edge of A1 to the far edge of E1. There were no significant differences between any of the genotypes suggesting that barrel field size develops normally irrespective of the mutations.*

3.3.4. Barrel Area

A measure was also taken of the area of the individual barrel columns D1 to D5. While no difference was found in the linear distance of the barrel row, indicating normal length development of the barrel field, this analysis does not provide any insight as to the development of individual barrels. For example, while the size of the barrel field might appear normal, barrel columns may be smaller and have a larger septal region.

The results of the area measurement of the D1 to D5 barrel columns are shown in Figure 3.4. A two-way ANOVA using barrel number (i.e. D1, D2 etc) and genotype as factors revealed no main effect of genotype ($F_{(5, 43)} = 1.14$, $p > 0.05$) but a main effect of barrel

number ($F_{(1, 43)} = 41.36$, $p < 0.0001$). There was no interaction between barrel number by genotype ($F_{(5, 43)} = 1.73$, $p > 0.05$). The main effect of barrel number was expected. The whisker diameter decreases moving posterior to anterior along the whisker pad. This therefore means that smaller whiskers represent less area in the barrel cortex (hence smaller barrels). The lack of main effect of genotype and interaction between genotype and barrel number suggests that the barrel area is not affected by the removal of either GluR1 and/or NOS.

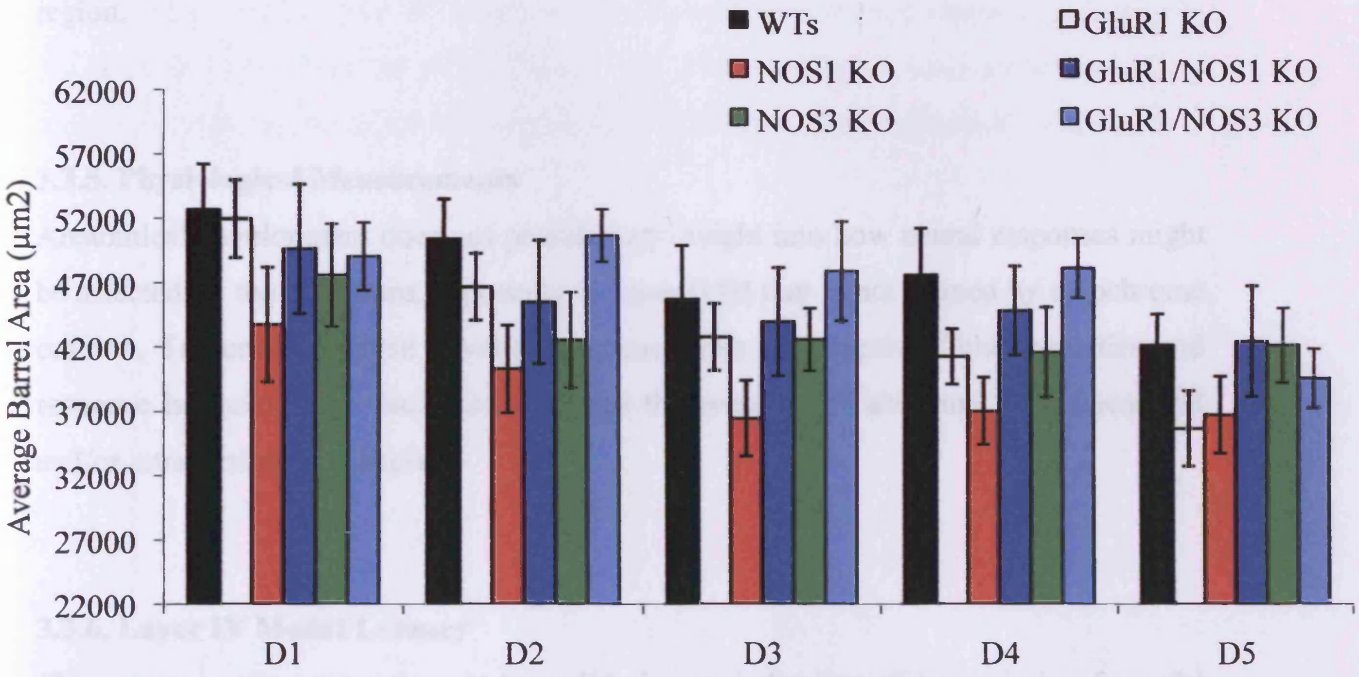


Figure 3.4. *The mean barrel area for the D1 to D5 barrel columns measured in layer IV. No significant differences were found between the genotypes.*

The D1 to D3 barrel column linear distance and the D1 to D5 barrel columns area measurements all show that there are no differences between any of the genotypes. Despite these results, there is a trend towards smaller barrel areas between WTs and NOS1 KOs. To further explore this, a two-way ANOVA was conducted to determine whether NOS1 KOs were different to WTs. There was no main effect of genotype ($F_{(1, 15)} = 2.65$, $p > 0.05$) but was a significant effect of barrel number ($F_{(1, 15)} = 11.29$, $p = 0.004$). There was also no significant interaction between barrel number by genotype ($F_{(1, 15)} = 0.35$, $p > 0.05$). Therefore although the NOS1 KOs appeared to develop with a smaller barrel area, separate statistical analysis failed to confirm a difference between groups.

Therefore, the KO mice are able to develop a barrel field that is of comparable dimensions to WTs. Development of the barrel field in the GluR1 KO (Watson et al., 2006), NOS1 KO and NOS3 KO (Finney and Shatz, 1998) has been previously described and this study is in agreement that development proceeds normally. This analysis also confirmed that barrel field development is not impaired in the combination mutants. Since these are measurements of layer IV, it can also be assumed that thalamocortical afferents are accurately targeting either the septal or barrel column region.

3.3.5. Physiological Measurements

Anatomical development does not provide any insight into how neural responses might be affected by the mutations, especially in layer II/III that is not stained by cytochrome oxidase. To further analyse development, measures of receptive field properties and response latencies were examined to assess the presence of abnormal thalamocortical and/or intracortical connections.

3.3.6. Layer IV Modal Latency

The monosynaptic connections to layer IV give an indication of transmission from the vibrissae to the cortex. Any aberrant development should be reflected in the time course of this transmission, which is referred to the modal latency of layer IV. In short, this is the latency of the response from stimulating the principle whisker to the detection (recording) in the cortex, for a single discriminated cell. For example, any disruption in the latency, and therefore transmission, would shift the distribution to slower values.

The latency of transmission as shown in Figure 3.5 was very similar across all genotypes. Similar to previous studies in the GluR1 KO (Wright et al., 2008), the majority of responses fall below 10 ms. All mutant modal latencies were compared to the modal latency of WT layer IV using a two-sample Kolmogorov-Smirnov test. Results are listed below:

GluR1 KOs $D_{max} = 0.134$, $p > 0.05$.

NOS1 KOs $D_{max} = 0.185$, $p > 0.05$.

NOS3 KOs $D_{max} = 0.076$, $p > 0.05$.

GluR1/NOS1 KOs $D_{max} = 0.74$, $p > 0.05$.

GluR1/NOS3 KOs $D_{max} = 0.181$, $p > 0.05$.

No significant differences were observed between the genotypes, confirming that PW latency was not affected by the mutations. This is consistent with the idea that the predominant thalamic innovation to a barrel column is confined to the topographically related barrel within layer IV, hence why the latency are at their shortest (Armstrong-James et al, 1992). Therefore it is unlikely that there is either an abnormality in sensory transmission to the cortex, or development of the thalamocortical afferents to layer IV.

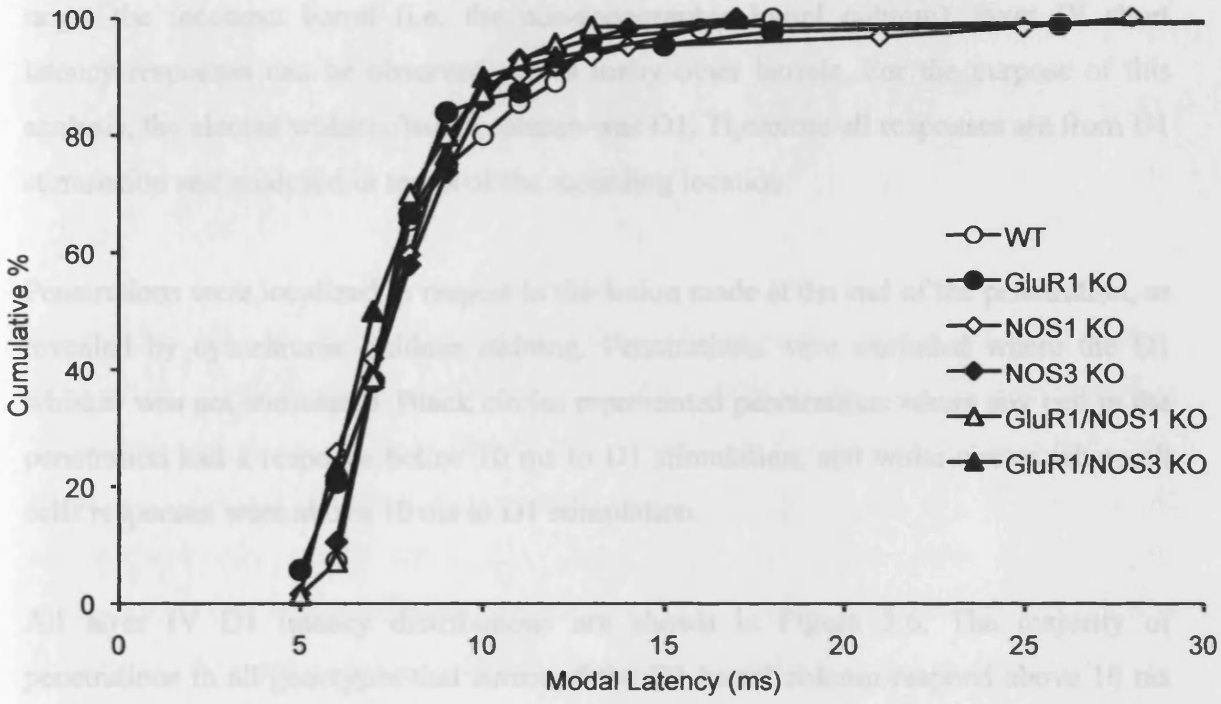


Figure 3.5. *The modal latency of layer IV undeprived principle whisker responses. Responses beyond 30 ms were excluded from the graph to highlight responses in the expected 10 ms range. Stimulation of the principle whisker provides an indication of the monosynaptic connections from whisker to cortex. An increase in the modal latency, i.e. a shift to the right, might be indicative of abnormalities in transmission and/or development. There were no significant differences between the groups.*

3.3.7. D1 Short Latency Response Domains

It is possible to use the modal latency of a given whisker, in this case D1, to determine whether the development of connections in layer IV has arisen normally. In the previous section I demonstrated that PW responses in layer IV for all genotypes were generally below 10 ms, which is consistent with previous work (Armstrong-James et al., 1992; Wright et al., 2008). Similar analysis can be performed on response latencies when recording from a barrel and stimulating a whisker that is not it's topographically related whisker (hence not the principle whisker). When stimulating whiskers surrounding the principle, the latency of the response is longer due to the intracortical connections and horizontal transmission (Armstrong-James et al., 1992; Fox et al., 2003). Developmental abnormalities may also affect the latency of response to surround whisker stimulation (Fox et al., 1996). It has previously been shown that when thalamocortical afferents target the incorrect barrel (i.e. the non-topographic barrel column), layer IV short latency responses can be observed across many other barrels. For the purpose of this analysis, the elected whisker/barrel column was D1. Therefore all responses are from D1 stimulation and analysed in terms of the recording location.

Penetrations were localized in respect to the lesion made at the end of the penetration, as revealed by cytochrome oxidase staining. Penetrations were excluded where the D1 whisker was not stimulated. Black circles represented penetrations where any cell in the penetration had a response below 10 ms to D1 stimulation, and white circles where all cells responses were above 10 ms to D1 stimulation.

All layer IV D1 latency distributions are shown in Figure 3.6. The majority of penetrations in all genotypes that surround the D1 barrel column respond above 10 ms when D1 was stimulated. Some penetrations surrounding the D1 column do have short latency responses, however this was similar to previous studies (Fox, 1992). Penetrations within the D1 barrel column exhibit short latency responses to D1 whisker stimulation, which was expected from layer IV PW stimulation (as shown in Section 3.3.6). No significant differences were found in the latency distributions across genotypes (chi-squared test 5, 3.38, $p > 0.05$). Therefore, the likelihood of finding cells in layer IV responding to D1 stimulation under 10 ms when they surround the D1 barrel column was low. This distribution suggests normal thalamocortical afferent development (Fox, 1992; Fox et al., 1996).

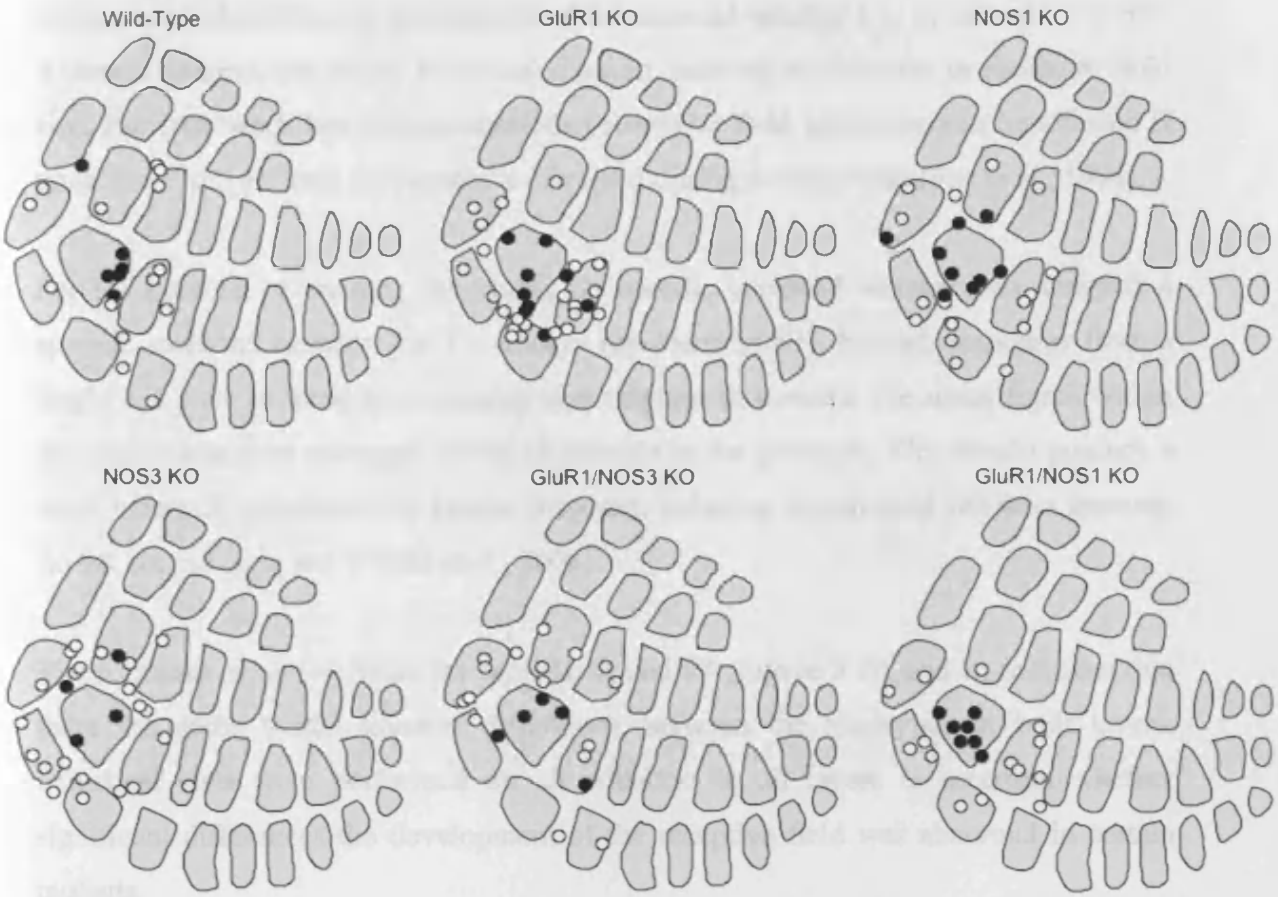


Figure 3.6. *D1 short latency domain maps. Each circle represents a recording penetration as visualized from cytochrome oxidase histology. Where all layer IV cells in the penetration responded over 10 ms to D1 stimulation, penetrations were coded white. However, if a cell in the penetration responded below 10 ms to D1 stimulation, penetrations were coded black. The majority of penetrations show short latency responses in their topographically related barrel (D1), whereas longer latency (>10 ms) responses where found to D1 stimulation from recordings made in barrel columns surrounding the D1 barrel.*

3.3.8. Principle Whisker Response and Surround Receptive Field

Cells respond considerably less when the surround whiskers are stimulated than they do for stimulation of the PW (Armstrong-James et al., 1992). These responses are termed the surround receptive field of the principle barrel and give some indication of the intracortical connections (Armstrong-James and Fox, 1992; Fox et al., 2003). Unlike the D1 short latency domain analysis, this looks at responses from all the surrounding

whiskers, of which there is a maximum of 8 (surround whisker 1 to 8; termed S1 to S8). Aberrant connections might be revealed as an increase or decrease in receptive field size. Previous work has demonstrated that receptive field properties can be affected if thalamocortical afferent refinement is disrupted during development (Fox et al., 1996).

For the purpose of creating the graph, no specific surround whisker was assigned a specific surround number (i.e. C1 always representing S2). Instead, responses from a single cell were ordered in decreasing size (highest to lowest). The mean figure within the animal was then averaged across all animals in the genotype. This should produce a trend where S1 generates the largest response, reducing as surround whiskers increase (to S8; for example see Wright et al., 2008).

The surround receptive fields for layer II/III and IV (Figure 3.7A and B) indicates that there are some trends towards differences between the genotypes in both layers. Statistical tests were performed on all whiskers in all layers to ascertain whether significant differences the development of the receptive field was abnormal in certain mutants.

A two-way ANOVA was performed for layer IV with whisker number as a within-subjects factor and genotype as a between-subjects factor. There was a main effect of whisker number (that is, PW and all surrounding whiskers; $F_{(8, 96)} = 140.20$, $p < 0.0001$) but not of genotype ($F_{(2, 12)} = 2.01$, $p > 0.05$). There was also no interaction between the genotype by whisker number factors ($F_{(16, 96)} < 1$, $p > 0.05$).

A two-way ANOVA was also performed for layer II/III with whisker number as a within-subjects factor and genotype as a between-subjects factor. There was a main effect of whisker number ($F_{(8, 56)} = 196.11$, $p < 0.0001$) but not of genotype ($F_{(1, 7)} = 2.68$, $p > 0.05$). There was also no interaction between the genotype and whisker number factors ($F_{(8, 56)} < 1$, $p > 0.05$).

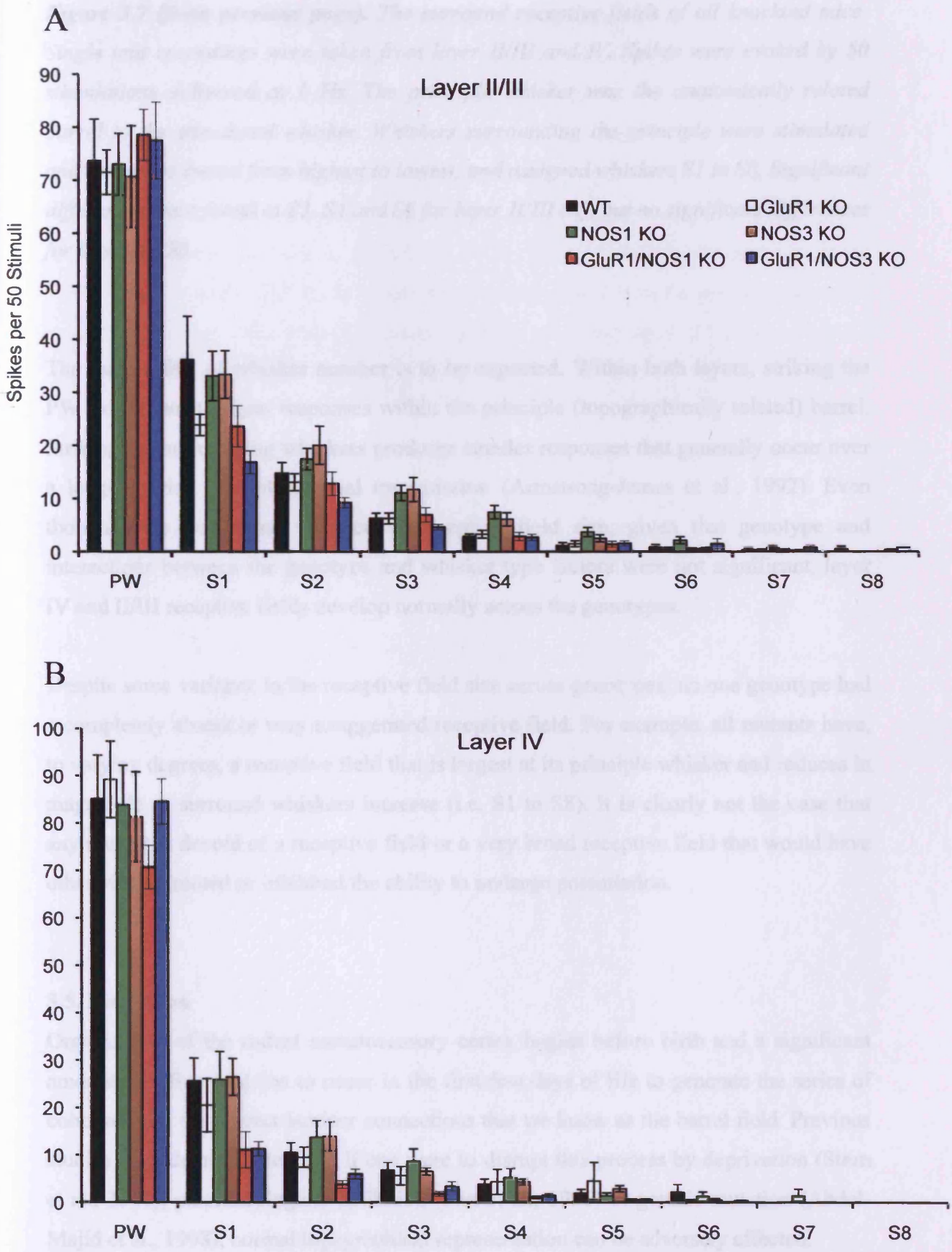

A
Layer II/III
Spikes per 50 Stimuli
90
80
70
60
50
40
30
20
10
0
WT
GluR1 KO
NOS1 KO
NOS3 KO
GluR1/NOS1 KO
GluR1/NOS3 KO
PW
S1
S2
S3
S4
S5
S6
S7
S8
B
Layer IV
100
90
80
70
60
50
40
30
20
10
0
PW
S1
S2
S3
S4
S5
S6
S7
S8

Figure 3.7 (from previous page). *The surround receptive fields of all knockout mice. Single unit recordings were taken from layer II/III and IV. Spikes were evoked by 50 stimulations delivered at 1 Hz. The principle whisker was the anatomically related barrel to the stimulated whisker. Whiskers surrounding the principle were stimulated and responses sorted from highest to lowest, and assigned whiskers S1 to S8. Significant differences were found at S3, S4 and S6 for layer II/III (A), but no significant differences for layer IV (B).*

The main effect of whisker number is to be expected. Within both layers, striking the PW exerts the strongest responses within the principle (topographically related) barrel. Striking the surrounding whiskers produces smaller responses that generally occur over a longer period via intracortical transmission (Armstrong-James et al., 1992). Even though there was some variance in receptive field size, given that genotype and interactions between the genotype and whisker type factors were not significant, layer IV and II/III receptive fields develop normally across the genotypes.

Despite some variance in the receptive field size across genotypes, no one genotype had a completely absent or very exaggerated receptive field. For example, all mutants have, to varying degrees, a receptive field that is largest at its principle whisker and reduces in magnitude as surround whiskers increase (i.e. S1 to S8). It is clearly not the case that any mutant is devoid of a receptive field or a very broad receptive field that would have otherwise promoted or inhibited the ability to undergo potentiation.

3.5. Discussion

Organization of the rodent somatosensory cortex begins before birth and a significant amount of refinement has to occur in the first few days of life to generate the series of columns with the correct laminar connections that we know as the barrel field. Previous studies have demonstrated that if one were to disrupt this process by deprivation (Stern et al., 2001), pharmacological inhibition (Fox et al., 1996) or genetic mutation (Abdel-Majid et al., 1998), normal topographical representation can be adversely affected.

Within this chapter I have confirmed that transmission velocity to the cortex is comparable across all mutants. The majority of short latency responses (< 10 ms) are also confined to the topographically related (D1 short latency domains), suggesting that activity dependent refinement of TCAs targeting occurs normally. Layer IV patterning of the barrel cortex occurs in all mutant mice and the barrel field size and area was comparable across all genotypes. Finally, since there were no significant differences between the receptive fields of layer II/III or IV, intracortical transmission within the layers develops normally. These results were in accordance with the predictions set out in the introduction. Taken together, neither GluR1 nor NOS1 or NOS3 is necessary for barrel cortex formation, development or refinement.

The full implications of these results will be considered in the General Discussion.

Chapter 4:

The Dependence of Barrel Cortex Experience-Dependent Potentiation upon GluR1 and Nitric Oxide

4.1. Introduction

4.1.1. General Introduction

The study of the mechanisms underlying plasticity is a central theme in neuroscience research. However, few candidate molecules associated with plasticity have evoked quite the debate that nitric oxide (NO) has. Despite a flurry of publications in the early to mid 1990s, research into the functions of this molecule has stagnated. Even less well researched is its potential role in neocortical plasticity since independent publications from the Daw and Stryker labs discounted the role of NO in ocular dominance plasticity (ODP) in the visual cortex, an analogous method to EDP. Like most papers published investigating plasticity, it is the hippocampus that is the chosen model system, largely using *in vitro* long-term potentiation (LTP) protocols.

Within the previous chapter I demonstrated that barrel cortex development can proceed despite the absence of both GluR1 and NOS; across all genotypes receptive fields, transmission velocities and barrel field size and area were similar. This is not the case for all synaptic molecules; barrel cortex activity dependent development is impaired if NMDARs are antagonized. Hence, genetic removal of GluR1 and NOS does not adversely affect baseline transmission that could otherwise reduce or advantage that genotype from undergoing EDP. Therefore deficits in EDP that occur due to genetic manipulation are more likely due to impairments in synaptic plasticity as opposed to non-specific synaptic transmission depression.

A widely accepted molecule that is required for potentiation is the AMPA receptor subunit GluR1. Many studies demonstrated the critical role played by GluR1 in activity-dependent synaptic enhancement (for example Shi et al., 2001). Similar to the dependence of GluR1 in hippocampal plasticity, cortical plasticity shares a similar reliance upon GluR1 receptor mechanisms. Short-term potentiation is absent in the GluR1 KO, while LTP can only occur using spike-timing-dependent plasticity protocols (Frey et al., 2009; Hardingham and Fox, 2006). Whisker manipulations have been shown to recruit GluR1 containing AMPARs to the synapse in barrel cortex layer II/III cells (Clem and Barth, 2006). Viral expression of GluR1 in layer II/III of the barrel cortex *in vivo* caused increased rectification only in animals that had their whiskers spared (activity permitting) compared to those that had all deprived (activity blocking), suggesting incorporation (Clem and Barth, 2006, Takahashi et al., 2003).

This strongly suggests that first, GluR1 is required for plasticity in the barrel cortex and second, GluR1-dependent plasticity mechanisms discovered *in vitro* could be relevant to *in vivo* EDP processes. One major criticism of research into GluR1-dependent plasticity is that the chosen model organism is generally juvenile. As highlighted in Appendix 1, the average age of animal used is around P28 and most often the hippocampus is used as the model system. In rodents, adult ages are generally considered to be around 6 months (Fox, 2002). Therefore it is not known whether the GluR1-dependent plasticity mechanisms discovered in juveniles extend throughout life. Indeed it has been suggested that GluR1-dependent plasticity is related to age, and only occurs in subjects younger than P42 (Jensen et al., 2002). However, studies of cognition in the GluR1 knockout animal have used much older subjects (up to one year; Humeau et al., 2007) and suggest that there is a hippocampal deficit that should, theoretically, be related to impairments in plasticity. To what extent the absence of GluR1 has on response magnitude following whisker deprivation is unclear that considering late-phase LTP is comparable to WTs (Hardingham and Fox, 2006), yet GluR1 is required for synaptic insertion during EDP (Clem and Barth, 2006).

4.1.2. Mechanisms Behind GluR1-Independent LTP

Late-phase LTP (~one hour post induction) in the GluR1 KO was comparable in magnitude to WTs in the barrel cortex (Hardingham and Fox, 2006) and showed a similar temporal pattern of plasticity as in the hippocampus (Hoffmann et al., 2002). Inhibition of NO by the application of a NOS antagonist (L-NNA) completely abolished the late-phase LTP in the GluR1 (Hardingham and Fox, 2006). Hence, the presynaptic form of LTP in the GluR1 KO was supported by NO signaling. It is likely that under normal conditions, WT potentiation is formed by pre and postsynaptic modification via NO and AMPARs, respectively (Hardingham and Fox, 2006; Sjöström et al., 2007). Therefore, when the activity-dependent GluR1 containing AMPAR insertion is removed (i.e. the postsynaptic loci of plasticity), this leaves only the presynaptic NO-dependent mechanisms.

Potentiation in WTs has a partial NO dependence whereas LTP is completely NO dependent in GluR1 KOs (Hardingham and Fox, 2006; Phillips et al., 2008; Romberg et

al., 2009). However, nothing is known about whether these mechanisms are required for *in vivo* neocortical EDP. Given that molecular mechanisms previously proposed for both hippocampal and neocortical plasticity have been shown relevant to *in vivo* processes (Clem and Barth, 2006; Hardingham et al., 2003), it is likely that *in vivo* EDP will be reduced or abolished in the absence of GluR1 and NO.

4.1.3. Nitric Oxide in Synaptic Plasticity

Two isoforms of NOS support synaptic potentiation; neuronal nitric oxide synthase (NOS1) and endothelial nitric oxide synthase (NOS3). NOS1 is found in pyramidal cells and contains a PDZ domain that links it to NMDAR activity (Blackshaw et al., 2003; Brenman et al., 1996), while NOS3 is localized to endothelial tissue (Blackshaw et al., 2003).

While clear plasticity impairments have been demonstrated for GluR1 and NOS inhibition in the neocortex, the requirement for NO in WTs in potentiation is unclear. Pharmacological antagonisation *in vitro* and *in vivo* have also produced a wide diversity of results, from complete inhibition (Doyle et al., 1996; O'Dell et al., 1991; Schuman and Madison, 1991), partial inhibition (Chetkovich et al., 1993; Hardingham and Fox, 2006; O'Dell et al., 1994; Son et al., 1996) to no effect (Bannerman et al., 1994; Cummings et al, 1994; Reid et al, 1996; Ruthazer et al, 1996). Discrepancies in the reporting of NO-dependent plasticity deficits has been in part due to lab specific conditions associated with *in vitro* preparation; that is the induction protocol or temperature of the submersion chamber (see Hölscher 1997 for review).

Although NO-dependent plasticity in the hippocampus remains debatable, given that GluR1-independent LTP in the barrel cortex is entirely sensitive to NOS inhibition, it is likely that NO plays some role in neocortical plasticity. In particular, NO seems likely to mediate presynaptic plasticity (Garthwaite et al., 1988; Hardingham and Fox, 2006). It is therefore highly probably that the removal of NOS from the barrel cortex will have some impact on the ability of the cells to undergo full potentiation.

4.1.4. Chapter Aims

Although a role for GluR1 in EDP has been found (Clem and Barth, 2006; Wright et al., 2008), the majority of research has been performed in the hippocampus using brain slices. The magnitude of potentiation has also never been specifically studied in the neocortex *in vivo*. Therefore I will use GluR1 KOs to measure EDP following whisker deprivation. Depriving all but the D1 whisker produces substantial, predictable and measurable increases in the spiking response of units recorded in barrel columns surrounding the D1 barrel column (Fox, 1992). Reductions in potentiation will suggest that GluR1 is required for EDP. Given that GluR1 is inserted into the synapse during EDP and LTP by phosphorylation events, it is probable that its absence will reduce potentiation. Despite this, late phase LTP in the GluR1 KO is not different to WTs, hence potentiation can still occur in the GluR1 KO. It is therefore predicted that in the GluR1 KO, EDP magnitude will either have only a minor reduction or none at all.

The role of gender and age in GluR1 research is little addressed in the literature. Out of 20 studies highlighted in Appendix 1 that have studied GluR1 in plasticity, the average age of subject was P28. This therefore gives little insight into adult plasticity mechanisms. Adult ages are ill-defined in the rodent, although 6 months is generally accepted as adult (Fox, 2002). Silent synapses are rare beyond P28, and is has been proposed that the dependence of GluR1 in plasticity is related to age (Jenson et al., 2003). A similar lack of research has been performed into gender specific differences in potentiation. Again using Appendix 1, only three out of twenty studies disclosed what the gender of the subjects used were. It is therefore possible that (1) what we know about GluR1 in plasticity is based around males and (2) females could have a different dependence on GluR1 signalling than males. Gender specific differences in LTP have been demonstrated in the absence of CaMKK molecules (see Mizuno and Giese, 2010), suggesting that plasticity mechanisms following calcium influx could diverge between the genders. Estrogen can also modulate GluR1 insertion following *de novo* spine formation (Srivastava et al., 2008), highlighting that gender could influence the expression of potentiation. Therefore to extend our knowledge of adult plasticity processes and to determine whether GluR1-depndent plasticity is only required in young (<P42) subjects, adult mice that are approximately 6 months of age will be recorded. Plasticity deficits have been found in behavioural tests in adult knockouts (for example Humeau et al., 2007) and in LTP (Romberg et al., 2009). It is therefore predicted that

there will reductions in experience-dependent potentiation in the GluR1 KO mouse, albeit incomplete, even at adult ages.

Gender will also be a factor for analysis of the EDP recorded. This will highlight any gender specific differences between the ability of the genotypes to undergo potentiation. To the best knowledge of the author, no data currently exists as to whether male and female EDP processes differ within the barrel cortex. A recent report has proposed that NOS1 association with NMDARs is dependent on estrogen (d'Anglemont de Tassigny, 2009), as does GluR1 insertion (Srivastava et al., 2008). This suggests that there could be a different expression of plasticity processes between the genders. It is therefore predicted that females rely more upon NOS1 in plasticity than males, and so EDP will be reduced further in females than males when NOS1 is knocked out. The same prediction is made for GluR1 KO mice.

In vitro studies have also shown that neocortical LTP was partially dependent upon NO (Hardingham and Fox, 2006). No such study has been performed in the barrel cortex for EDP. The dependence of ocular dominance plasticity (ODP) upon NO inhibition has been studied. Visual cortex ODP shares many similar mechanisms to barrel cortex EDP (Fox and Wong, 2005). ODP was found to occur independent of antagonism of NOS (Reid et al., 1996; Ruthazer et al., 1996), which is contradictory to reports that neocortical LTP is reduced after drug application (Hardingham and Fox, 2006). Therefore the role of NOS1 and NOS3 will be tested during EDP in the barrel cortex. Given Hardingham and Fox (2006) found reductions in potentiation after NOS antagonism, it is predicted that the inhibition NOS signaling will reduce EDP.

Finally, the entire late-phase component of LTP in the GluR1 KO was sensitive to NOS inhibition (Hardingham and Fox, 2006). This suggests that in the absence of GluR1 containing AMPARs, NO is the critical plasticity molecule in the barrel cortex. This is not known to be the case for either *in vivo* preparations or EDP protocols. Therefore mutants of GluR1 and NOS will be tested for *in vivo* EDP. Given the LTP deficits, it is to be expected that all D1 potentiation should be abolished. These recordings will be performed on KOs containing mutations of GluR1 and only one type of NOS; this will enable conclusions to be derived of whether one type of NOS is more important for EDP. Considering the literature is mixed as to the importance of one type of NOS over

the other, it is expected that the removal of both types of NOS will reduce plasticity equally.

4.2. Methods

For detailed methods please refer to the Materials and Methods section, Chapter 2.

4.2.1. Animals

For this experiment, the following mice were used.

Genotype	Deprived	Control/Undeprived
Wild-Type	12	7
GluR1 KO	12	13
NOS1 KO	14	10
NOS3 KO	10	8
GluR1/NOS1 KO	5	7
GluR1/NOS3 KO	12	7

4.2.2. Procedure

Mice were either classed as undeprived controls or whisker deprived. Those mice that underwent whisker deprivation were anesthetised by isoflurane and had all whiskers except the D1 gently removed under microscopic control. Whisker deprivation was repeated 3 days after initial deprivation and then every two days. This process continued for 18 days, after which 6-11 days was allowed to pass to permit minimal regrowth of the other whiskers before *in vivo* recording. Undeprived controls had all whisker left intact. The integrity of the whiskers was examined under the microscope before the experiment. If there was any evidence of damage or previous whisker loss, that animal was rejected.

The protocol for recording single units from layer II/III has been described in detailed in the Materials and Methods section (Chapter 2, Section 2.3.5, p. 87). Briefly, mice were

anesthetised with urethane/acepromazine maleate at a dose of 1.5 mg/kg. Once a stable depth was achieved, the mice were transferred to a stereotaxic frame. An incision was made along the centreline of the skull and skin was retracted. The skull was thinned by careful drilling between 2 – 6 mm lateral of the midline and 1 – 4 mm posterior of bregma. Drilling ceased when the surface of the brain was visible, although a small layer of skull remained. Small holes were made with a fine needle tip (30 gauge, 0.5" length) through the remaining skull and dura for each penetration through which a glass insulated carbon fibre electrode could be inserted. A new hole was made for each penetration.

The electrode was lowered to layer IV and the topographic location of the electrode was confirmed by stimulation of the whiskers. Single unit responses were discriminated using a dual threshold spike discriminator and the principle whisker (PW), D1 whisker and immediately surrounding whiskers were stimulated by 50 200 μm deflections delivered at 1 Hz. New cells were sampled in layer II/III every 50 – 100 μm. At the end of the penetration a 50 μm lesion (1.0 μA, tip negative for 10 seconds) was placed in layer IV for post mortem depth and location confirmation. At the end of the experiment, the mouse was given an overdose of sodium pentobarbital and transcardial perfused with phosphate buffered saline followed by paraformaldehyde. The brain was removed, the non-experimental hemisphere discarded, the experimental hemisphere flattened between to slides and cyroprotected for 24 hours. The hemisphere was then cut at 35 μm intervals on a freezing microtome and reacted for cytochrome oxidase activity. This enabled visualisation of the layer IV barrels, which therefore enables the location of the lesions to be confirmed with respect to depth and barrel field location. Results were then modified were appropriate.

4.2.3. Data Analysis

Responses to PW and D1 stimulation were expressed as spikes per 50 stimuli. The layer II/III responses were first averaged within the animal, then across the genotype. All data is expressed as a mean value ± the standard error of the mean (SEM). The exception to this is the vibrissae dominance index (VDI) that was expressed as a ranked distribution.

The VDI was calculated by expressing responses to D1 stimulation relative to the PW to obtain a number from each cell termed *F*, where $F = D1/(D1 + PW)$. These figures were put into 10 bands as follows. F_0 contained cells with *F* numbers between 0 to 0.099, F_1 contained cells with *F* numbers between 0.1 to 0.199, F_2 contained cells with *F* numbers between 0.2 to 0.299 up to F_9 containing cells with *F* numbers between 0.9 to 1.0. The percentage of cells that fell in each band was calculated from the total number of cells and distributions compared.

A weighted form of the VDI (WVDI) was also calculated from the *F* numbers, this time producing a single figure for each animal, with all WVDI averaged across all animals within the genotype and condition, expressed as mean ± SEM. The WVDI was calculated for each animal, where:

$$WVDI = \frac{(0F_0 + 1F_1 + 2F_2 + 3F_3 + 4F_4 + 5F_5 + 6F_6 + 7F_7 + 8F_8 + 9F_9)}{9N}$$

Where *N* is the total number of cells in the sample.

Map plasticity was expressed as responses per penetration. All layer II/III D1 responses within a single penetration were averaged and assigned a colour depending on response magnitude. If the average response for the penetration was ≤ 25 spikes, the penetration was coded blue; green for 25 to 49 spikes and yellow for ≥50 spikes. The penetrations for either a control or deprived genotype were added to a caricature map and chi-squared analysis compared plasticity shifts between the deprived and control conditions.

4.3. Results

4.3.1. Data Collection

Data has been collected by J. Dachtler, S. Glazewski, N.F. Wright and K.D. Fox. All data was analysed by J. Dachtler only.

4.3.2. Experience-Dependent Plasticity

As discussed in the materials and methods section, EDP was induced by depriving all but the D1 whisker on one side of the snout for 18 days, followed by a regrowth period between 6 to11 days. This protocol has been previously shown to induce plasticity in the barrel columns surrounding the D1 barrel during D1 whisker stimulation (Fox, 1992). This plasticity occurs well into adulthood and is localized to layer II/III.

While surround whiskers were stimulated for every cell, due to incomplete whisker regrowth after deprivation (common for a short regrowth period) it was not always possible to record a complete receptive field for every cell unlike control animals. Similarly, some principle whisker responses were not recorded for a penetration due to incomplete regrowth. Since this chapter is studying changes to the D1 spiking response before and after whisker deprivation, only the D1 responses for control and deprived conditions will be examined. Within this chapter, EDP will first be explored in detail with reference to undeprived littermates. Following these within genotype comparisons, all genotypes will be compared via ANOVAs to determine whether genetic deletion of GluR1 and NOS affects the magnitude of EDP following deprivation.

4.3.3. Plasticity in Wild-Type Mice

Plasticity in wild-type (WT) mice has previously been extensively studied and occurs well into adulthood and is predominantly associated with intracortical connections. To investigate the wild-type plasticity in our particular strain of mouse and under our experimental conditions, a total of 81 cells were recorded from 7 undeprived and 242 cells from 12 deprived mice. For undeprived controls, 2 males (16 cells) and 5 females (65 cells) and for deprived WTs 6 males (100 cells) and 7 females (142 cells) were recorded.

4.3.3.1. Wild-Type Controls

In control WTs, responses to 50 D1 stimulations delivered at 1 Hz in the barrel columns surrounding the D1 barrel were 15.71 ± 4.63 single unit spikes. When gender was examined, males responded at 16.17 ± 3.17 and females at 15.53 ± 6.63 (Figure 4.1).

There were no significant differences between males and females (unpaired t-test $t_{(5)} = 0.057$, $p > 0.05$), therefore WT controls will hereon in be discussed as a single group.

Figure 4.1. *Evoked spikes to D1 whisker stimulation in control WT mice. There were no significant differences between baseline male and female D1 responsiveness.*

4.3.3.2. Wild-Type Plasticity

As previously stated, deprivation was induced by sparing all but the D1 whisker for 18 days, followed by a regrowth period of between 6 to 10 days. Previous studies have robustly demonstrated that this produces a large increase in spike responses to D1 stimulation in the barrel columns surrounding the D1 barrel column (Fox, 1992).

Maps showing the recording locations and the average response of all layer II/III cells within the penetration are shown in Figure 4.2. Blue represents less than 25 spikes per 50 stimuli, green 25 to 49 and yellow 50 or above. In control WTs, all but one penetration surrounding the D1 barrel average responses were below 25 spikes per 50 stimuli. However following deprivation over 86% of the penetrations exhibited average responses that were either in the green or yellow band, which compared to controls was statistically significant (chi-squared test = 2, 24.75, $p < 0.0001$).

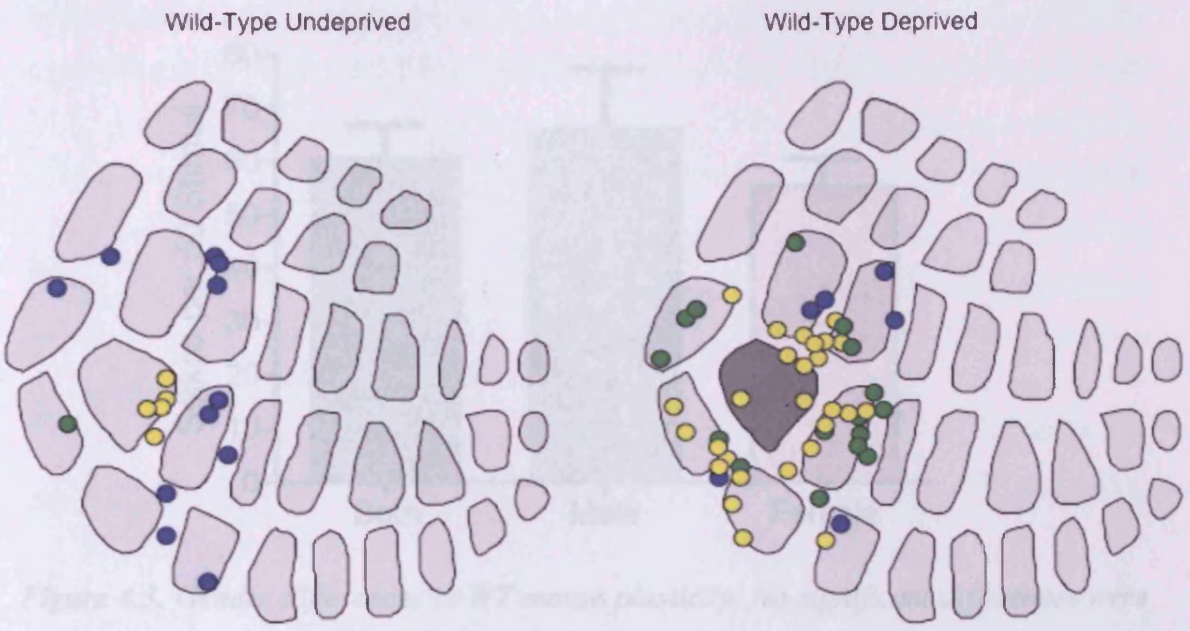


Figure 4.2. *Plasticity in WT mice. Penetration maps show the average responses of cells within barrel columns surrounding D1 column (dark grey) increase following deprivation.*

As expected a 3.86-fold increase was observed in the spiking rate to D1 stimulation when the recording electrode was surrounding the D1 barrel column. Per 50 stimuli train, the number of spikes in control animals increased from 15.71 ± 4.63 to 60.66 ± 6.2 in deprived mice. To investigate whether deprivation induced a differential effect on plasticity magnitude related to gender, the deprived group was separated into males and females. Both males and females had similar increases in response to stimulation (males 65.91 ± 11.49, females 55.40 ± 5.10; Figure 4.3). A two-way ANOVA revealed that there was a significant effect of deprivation ($F_{(1, 15)} = 20.67$, $p = 0.0004$), but not gender ($F_{(1, 15)} = 0.25$, $p > 0.05$) nor an interaction between gender and deprivation ($F_{(1, 15)} = 0.25$, $p > 0.05$). Therefore hereon in the deprived WT group will also be investigated as a single group.

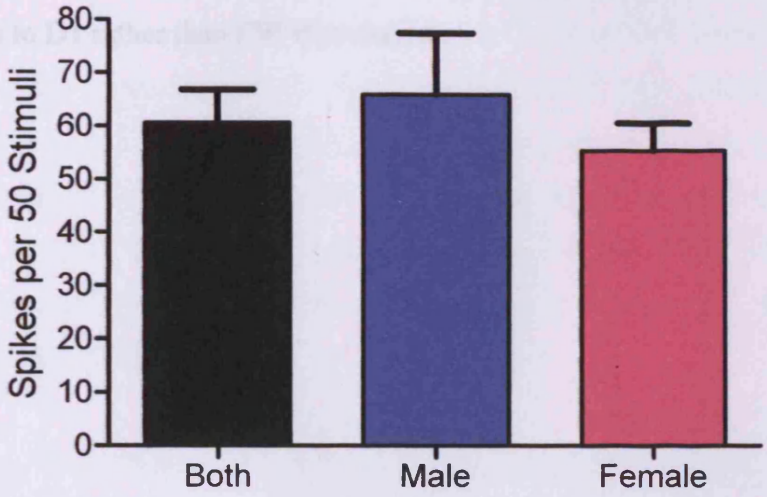


Figure 4.3. *Gender differences in WT mouse plasticity. No significant differences were observed between WT males and females that underwent deprivation.*

4.3.3.3. WT Vibrissae Dominance

Figures 4.2 and 4.3 demonstrate that deprivation induces robust plasticity in adult WTs that is not gender specific. However, it is well known that many other factors associated with the experimental conditions can affect absolute response magnitudes (for example the depth of anesthesia or length of recording). Therefore to limit these possible variables, the vibrissae dominance index (VDI) was calculated (see Materials and Methods, Section 2.4.3, p. 94 for the calculation of VDI), which is similar to the ocular dominance index (ODI) for visual cortex plasticity studies. Since the response of D1 in the VDI calculation is relative to the PW, any generalised depression effects due to poor experimental conditions will be controlled for. Hence, the cell becomes its own control.

Figure 4.4A shows the VDI distribution for both control (white) and deprived (black) WT mice of cells recorded from barrels surrounding the D1 barrel column. For control animals the majority of the values lie towards the left of the graph nearer zero, where most of the cells are driven predominantly by the PW not D1. However, after deprivation the distribution shifts more to the right hand side of the graph (Mann-Whitney test $U = 1702$, $p < 0.0001$). From 0.5 to 1.0 (0.5 being equal response of the D1 and PW and 1.0 being completely driven by D1) cells respond more strongly to D1 whisker stimulation than their own PW. Hence in the WT group deprivation causes

more cells in barrels surrounding the D1 column to respond at equal or higher magnitudes to D1 rather than PW stimulation.

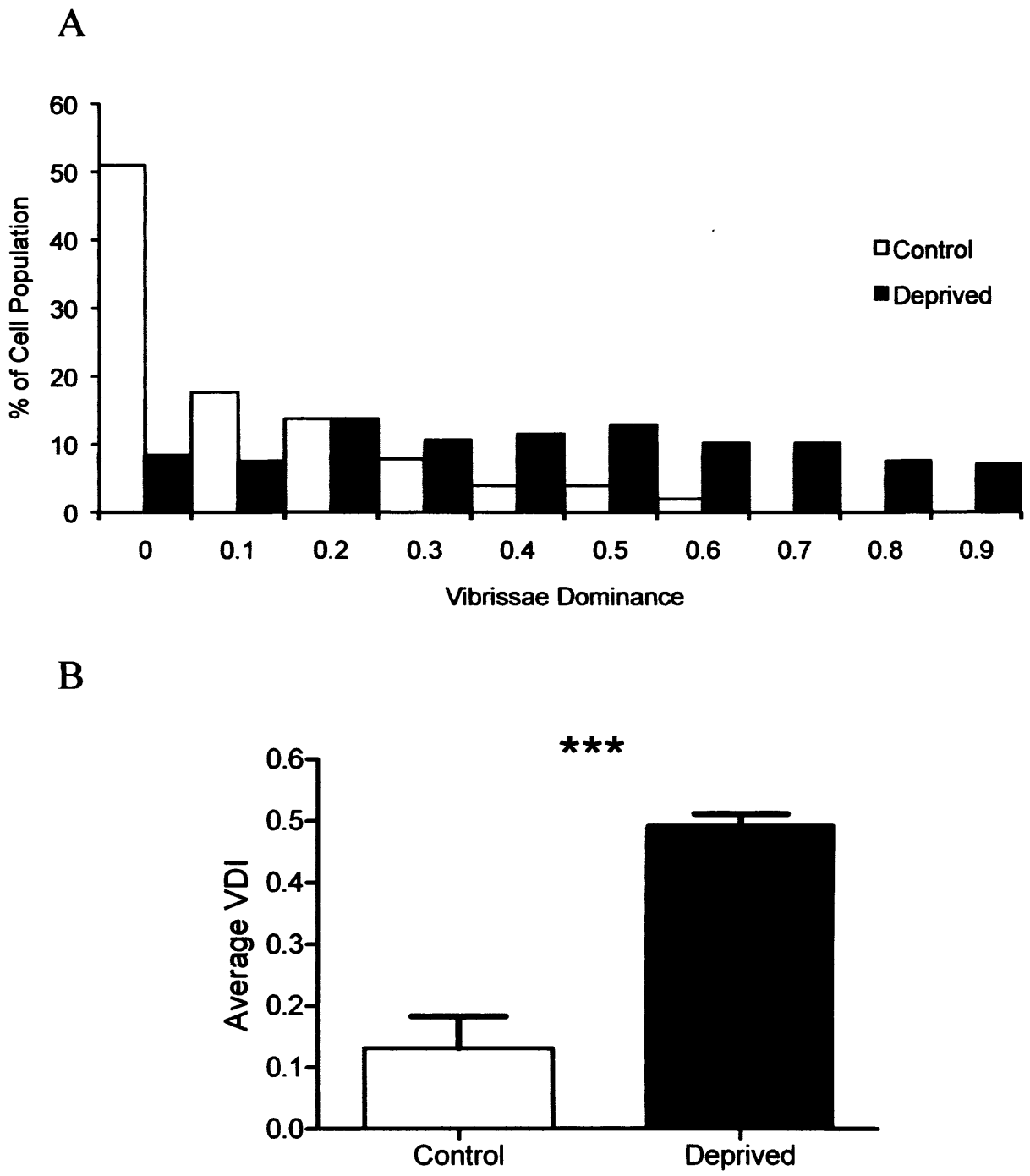


Figure 4.4. *The effect of deprivation on vibrissae dominance in WTs.* ***A.*** *In control animals the majority of cells were driven predominately by their PW (0 to 0.5). However after deprivation more cells were driven by stimulation of D1 rather than their PW (0.5 to 1.0).* ***B.*** *This was confirmed when the WVDI was calculated for each animal, then averaged across the group.*

While the VDI of Figure 4.4A investigates the distribution of all cells within either the control or deprived groups, it is also possible to calculate a VDI for each animal in each group using the method proposed by Daw et al., 1992 (see Material and Methods, Section 2.4.3, p. 94), which provides a mean figure with a standard error of the mean. This is referred to the weighted VDI (WVDI). Similar to the scaling on the previous VDI graph, a figure close to 0 indicates the average cell population within each animal responded predominately to the PW; conversely a figure close to 1 suggests response nearly solely to D1 stimulation.

The average WVDI per animal in the deprived group was significantly larger than in non-deprived controls (0.49 ± 0.02 vs 0.13 ± 0.05, unpaired t test $t_{(17)} = 7.867$, $p < 0.0001$, Figure 4.4B). This, along with the by cell VDI, confirms that deprivation and subsequent plasticity causes a distribution shift where cells adjacent to the D1 barrel respond more strongly to D1 stimulation than under control conditions. Therefore it can be stated that robust plasticity can be induced in mature (average 5 month old) WT mice that is independent of gender.

4.3.4. Plasticity in GluR1 KO Mice

To investigate whether neocortical plasticity in the mature synapse requires the AMPA GluR1 receptor, a total of 244 cells were recorded from 13 undeprived and 286 cells from 12 deprived mice. The average age at time of recording was 5 months. For non-deprived controls, 4 males (61 cells) and 9 females (183 cells) were recorded. For deprived GluR1 KOs, 5 males (114 cells) and 7 females (172 cells) were recorded.

4.3.4.1. GluR1 KO Controls

In control GluR1 KOs, the average response per animal averaged across animals to 50 D1 stimulations in the barrel columns surrounding the D1 barrel was 10.40 ± 1.03. When this split by gender, males responded at 12.30 ± 1.13 and females at 9.56 ± 1.35 (Figure 4.5). While males responded slightly more to D1 stimulation, there was no significant difference between males and females (unpaired t-test $t_{(11)} = 1.249$, $p > 0.05$), therefore GluR1 KO controls will hereon in be discussed as a single group.

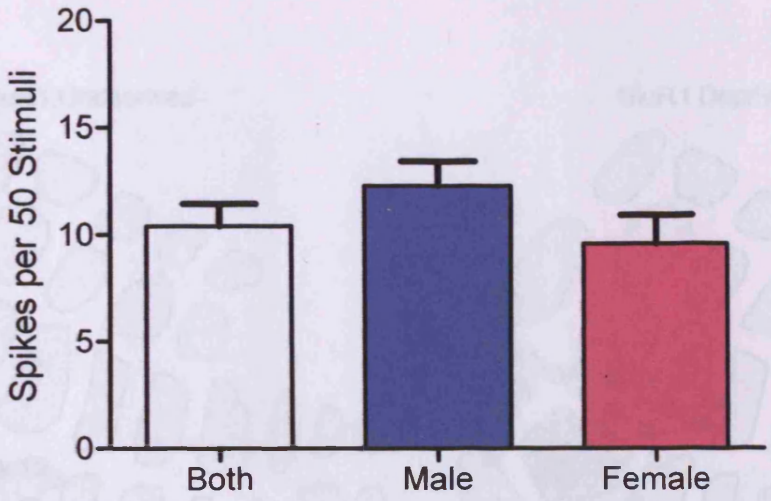


Figure 4.5. *Response magnitude to 50 D1 whisker stimulations in control GluR1 KOs. No significant differences were observed between the genders.*

4.3.4.2. GluR1 KO Plasticity

While plasticity, and specifically neocortical plasticity, has been investigated in GluR1 KOs, it has nearly exclusively been studied using 'young' models (typically 4 to 8 weeks). Since this study uses mice at an average age of 5 months, it will provide an insight of whether GluR1 is required for mature synapse plasticity. Only one study so far has used mature animals (~6 months; Romberg et al., 2009) and this was in the hippocampus. Therefore it is unknown whether GluR1 is required for adult neocortical plasticity.

Following deprivation a 3.74 fold increase in the magnitude of D1 response to D1 stimulation in barrel columns surrounding the D1 barrel column was observed. Per 50 stimuli, the average spikes per animal increased from 10.40 ± 1.03 in control animals to 38.94 ± 2.75 in deprived subjects. Similar to the WT controls, the average penetration responses as shown in the penetration map (Figure 4.6) for controls were consistently in the blue (<25 spikes) band. However, after deprivation a clear change can be seen where there were more penetrations responding in the yellow (>50 spikes) and green (25-49 spikes) bands (33 deprived vs 2 control), which was significant (chi-squared test = 2, 32.10, $p < 0.0001$). Therefore deprivation causes an increase in the response magnitude to D1 stimulation in GluR1 KOs.

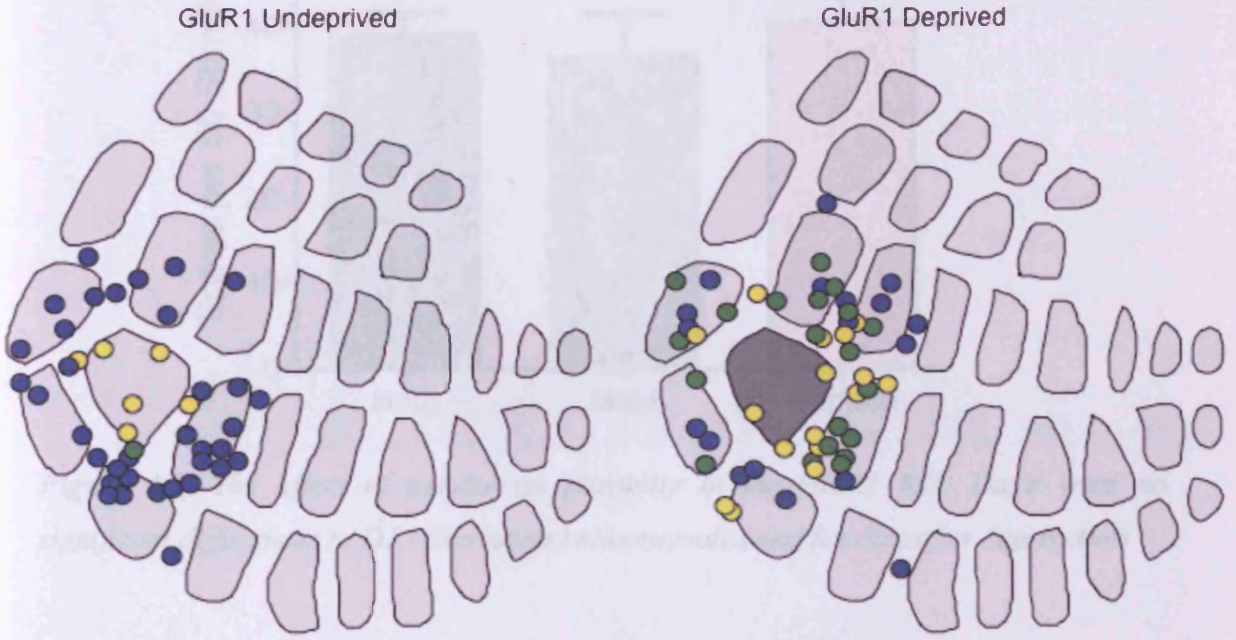


Figure 4.6. *Plasticity in the GluR1 KO following deprivation. Penetration maps showing the average penetration response to D1 stimulation. Following deprivation, in barrel columns surround D1 (dark grey) there were more penetrations responding in the green and yellow bands (>25 spikes) than in controls.*

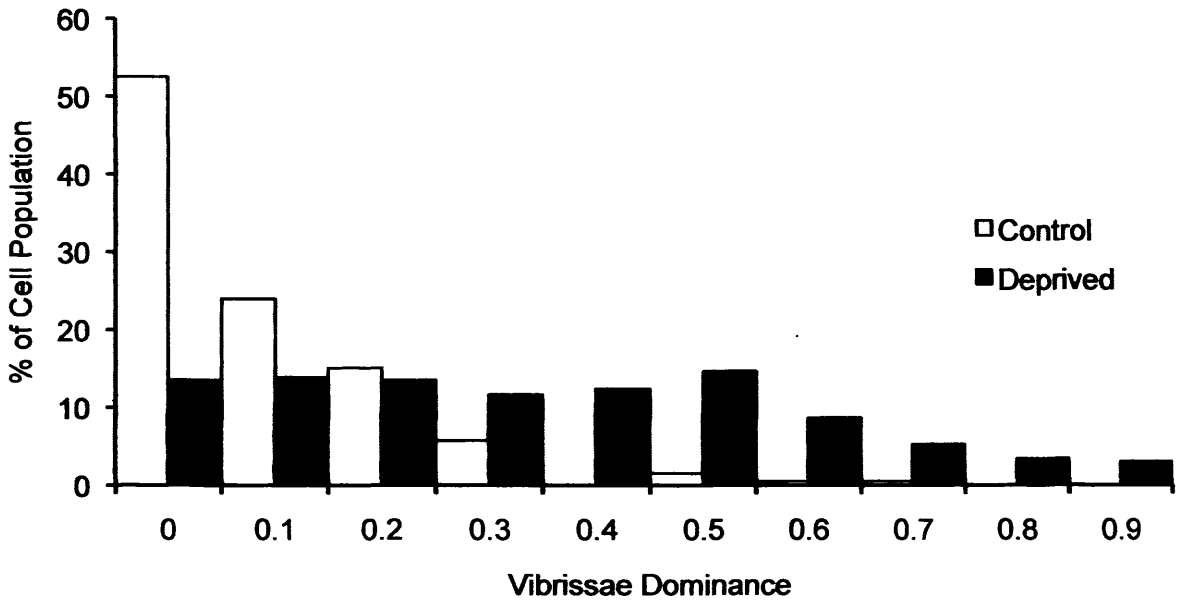
Male and female GluR1 KOs were subdivided and compared (Figure 4.7). The average male response to D1 stimulation recorded from barrels surrounding D1 was 36.48 ± 5.17 and female at 40.70 ± 3.12. A two-way ANOVA revealed that there was a significant effect of deprivation ($F_{(1, 21)} = 83.72$, $p < 0.0001$), but not gender ($F_{(1, 21)} = 0.06$, $p > 0.05$) nor an interaction between gender and deprivation ($F_{(1, 21)} = 1.32$, $p > 0.05$). Therefore hereon in the deprived GluR1 KO group will also be investigated as a single group.

Figure 4.7. *The effect of gender on plasticity in the GluR1 KO. There were no significant differences to D1 stimulation between males and females after deprivation.*

4.3.4.3. GluR1 KO Vibrissae Dominance

Figures 4.6 and 4.7 demonstrate that GluR1 KOs are able to undergo plasticity following deprivation, in terms of the absolute response magnitude to D1 stimulation. It is however, important to investigate whether the VDI bias is altered in these KOs. The total cell distribution for D1 stimulation in barrel columns surrounding the D1 barrel was analysed in Figure 4.8A. For control KOs (white), the majority of cells responded towards the left of the magnitude scale, where the PW responds more strongly to stimulation than to D1. In fact, only 2.6% of cells respond equally to PW and D1 stimulation or more strongly to D1 (≥ 0.5). Following deprivation, there is rightward bias shift where a greater proportion of the cells respond equally or more strongly to D1 stimulation than the PW. Compared to controls, this was significant (Mann-Whitney test $U = 8872$, $p < 0.0001$). A similar trend was observed when the WVDI was analysed by calculating the VDI for each animal, then averaging all VDIs within the group. For control KOs, the average WVDI was closer to 0 (0.10 ± 0.014), indicating that the PW was predominately driving responses from the animal. However, after deprivation, the average WVDI was closer to 0.5 (0.38 ± 0.029), which was significant (unpaired t test $t_{(23)} = 8.861$, $p < 0.0001$, Figure 4.8B). Therefore GluR1 KOs show potentiation following deprivation.

A



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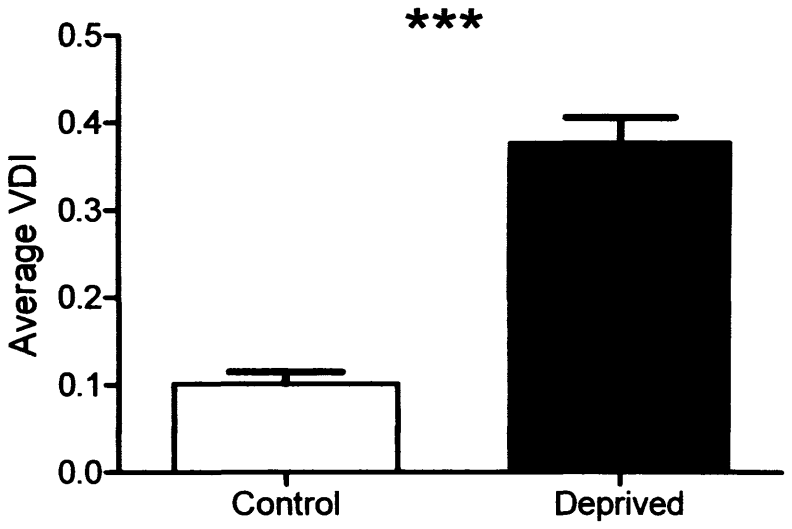


Figure 4.8. *Changes to the vibrissae dominance in GluR1 KOs following deprivation.* ***A and B.*** *Control GluR1 mice respond mainly to the PW (vibrissae dominance closer to 0) whereas after deprivation responses are closer to equal between the PW and D1 (0.5).*

4.3.5. Plasticity in NOS1 KO Mice

Very few studies have looked at the role of NO in neocortical plasticity. In fact, a search on PubMed revealed 5 publications on either barrel or visual cortex plasticity (Reid, Daw et al. 1996; Ruthazer et al. 1996; Finney and Shatz 1998; Kara and Friedlander 1998; Sohn et al. 1999). Only one of these studies (Sohn, Greenberg et al. 1999) used the NOS1 KO mouse and this investigated plasticity in very young subjects (P12) using gross anatomical changes. The remaining studies used pharmacological methods in other species. As such, it is not known whether there is a specific requirement for αNOS1 in adult neocortical EDP. To investigate whether neocortical plasticity in the mature synapse requires the NOS1, a total of 110 cells were recorded from 10 undeprived and 201 cells from 14 deprived mice. The average age at time of recording was 5 months. For non-deprived controls, 6 males and 4 females were recorded. For deprived NOS1 KOs, 9 males and 5 females were recorded.

4.3.5.1. NOS1 KO Controls

In control NOS1 KOs, the average response per animal averaged across animals to 50 D1 stimulations in the barrel columns surrounding the D1 barrel was 15.46 ± 1.90. When this sub-divided by gender, males responded at 15.40 ± 2.03 and females at 15.57 ± 4.11 (Figure 4.9). There was no significant difference between males and females (unpaired t-test $t_{(8)} = 0.041$, $p > 0.05$).

Figure 4.9. *Response magnitude to D1 stimulation in control NOS1 KOs. No significant difference was found between the genders.*

4.3.5.2. NOS1 KO Plasticity

Considering there have been some very influential studies suggesting NOS does not have a role in neocortical plasticity, NOS1 KOs were deprived to confirm this hypothesis. Deprived animals showed a 2.29 fold increase in the magnitude of response to D1 stimulation (35.39 ± 5.17) in recordings made surrounding the D1 barrel compared to controls (15.46 ± 1.90). Surprisingly there is a marked difference between deprived male and female NOS1 KOs (Figure 4.10A). Females are able to demonstrate a plastic increase following deprivation compared to their controls (51.73 ± 7.20 vs 15.57 ± 4.11, Figure 4.10B). Conversely, while males do show an increase in their magnitude of response to D1 stimulation (26.31 ± 4.94 vs 15.40 ± 2.03, Figure 4.10C), it was 49.14% lower than that of the female potentiation (26.31 ± 4.94 vs 51.73 ± 7.20, Figure 4.10D).

A two-way ANOVA revealed a significant main effect of deprivation ($F_{(1, 20)} = 19.55$, $p = 0.0003$), gender ($F_{(1, 20)} = 5.78$, $p < 0.05$) and an interaction between gender and deprivation ($F_{(1, 20)} = 5.63$, $p < 0.05$). Test of simple main effects confirmed that compared to their relative controls, females show significant potentiation ($F_{(1, 20)} = 18.66$, $p < 0.0001$) whereas males do not ($F_{(1, 20)} = 2.75$, $p > 0.05$). D1 response penetration maps were also compared by gender (Figure 4.11). There was no significant

difference between the penetration distributions in non-deprived male and female NOS1 KOs (chi-squared test 2, 0.74, $p > 0.05$) but there was between deprived genders (chi-squared test 2, 8.02, $p < 0.05$).

Interestingly, there was no significant difference between female NOS1 and WT plasticity (unpaired t test $t_{(9)} = 0.426$, $p > 0.05$), suggesting that NOS1 females are able to undergo EDP to the same levels of WTs. Unlike their female counterparts, NOS1 males were unable to reach plasticity levels seen in male WTs (unpaired t test $t_{(13)} = 3.59$, $p = 0.003$). This provides the first indication that EDP via NOS1 in the mouse barrel cortex occurs in a gender specific manner. Unlike the previous genotypes, special consideration of gender will need to be undertaken when interpreting NOS1 findings.

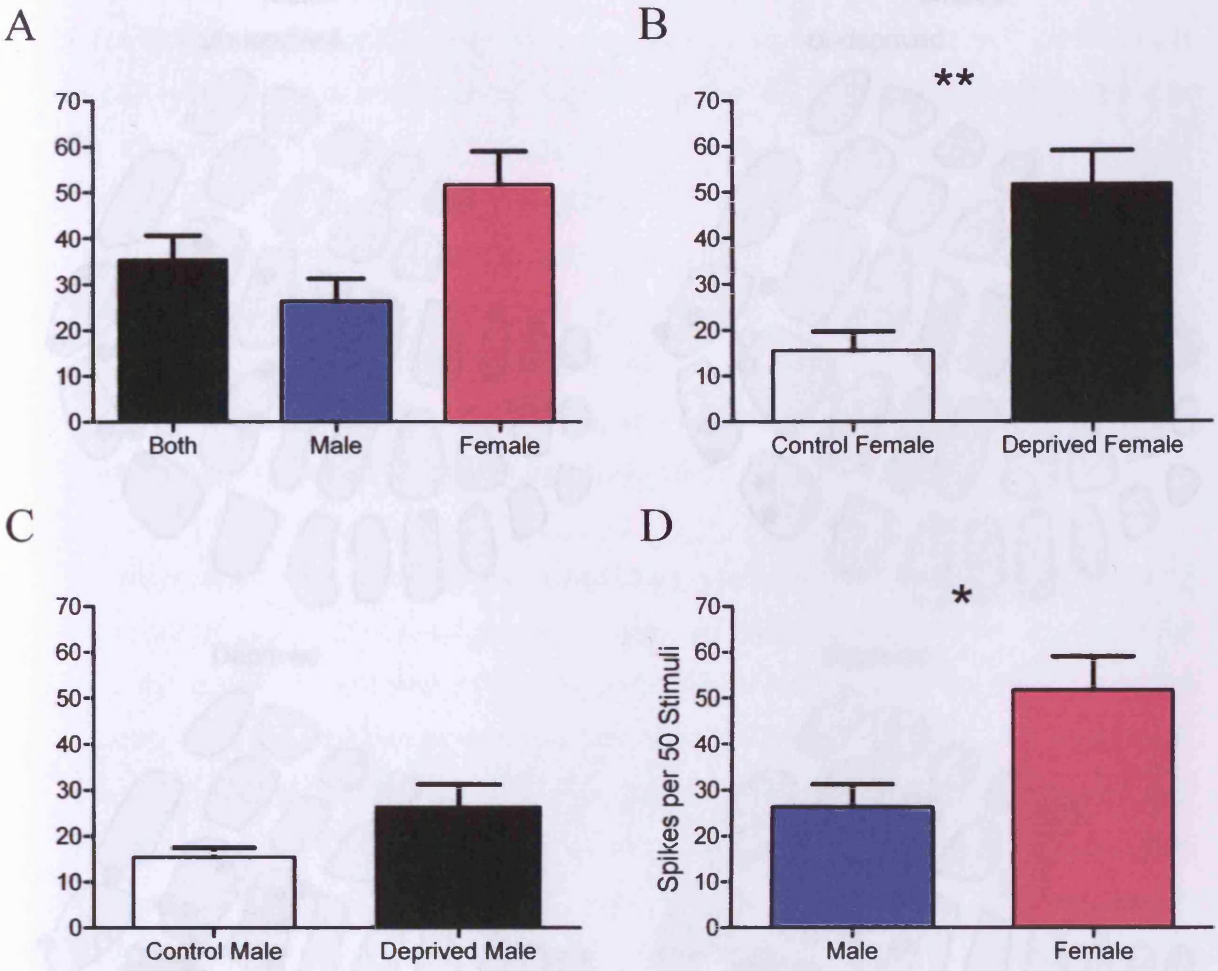


Figure 4.10. *The dependence NOS1 experience-dependent plasticity on gender.* ***A.*** *Comparison of the averaged plasticity (both) with male and female response magnitudes.* ***B.*** *Males showed a non-significant increase in response to D1 stimulation following deprivation.* ***C.*** *Females however demonstrate robust plasticity following deprivation.* ***D.*** *There was a significant difference between the plasticity levels of male and female NOS1 KOs.*

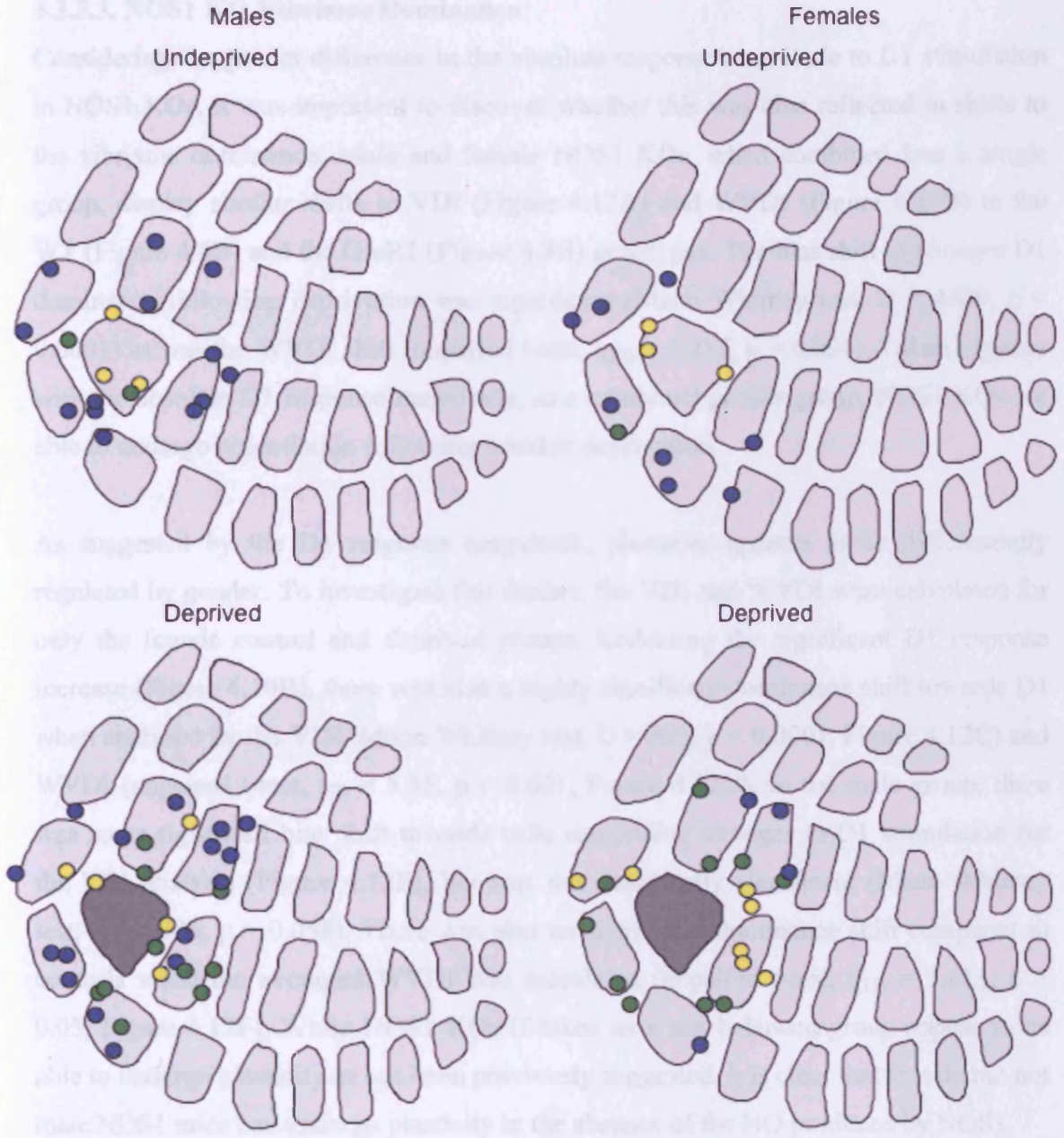


Figure 4.11. *Response penetration maps to D1 stimulation in male and female NOS1 KOs. Undeprived male and female mice have exactly the same distribution of blue and green (< 50 spikes per averaged penetration). In deprived conditions, there is a stronger bias towards higher responding yellow penetrations (>50 spikes) in female KOs with more blue penetrations in males, which was significant.*

4.3.5.3. NOS1 KO Vibrissae Dominance

Considering the gender difference in the absolute response magnitude to D1 stimulation in NOS1 KOs, it was important to discover whether this was also reflected in shifts to the vibrissae dominance. Male and female NOS1 KOs, when combined into a single group, display similar shifts to VDI (Figure 4.12A) and WVDI (Figure 4.12B) to the WT (Figure 4.4B) and the GluR1 (Figure 4.8B) genotypes. The bias shift to stronger D1 dominance following deprivation was significant (Mann Whitney test, $U = 4489$, $p < 0.0001$) as was the WVDI shift (unpaired t-test, $t_{(22)} = 3.215$, $p = 0.004$). Taken together with the absolute D1 response magnitude, as a combined gender group, NOS1 KOs are able to undergo potentiation following whisker deprivation.

As suggested by the D1 response magnitude, plasticity appears to be differentially regulated by gender. To investigate this further, the VDI and WVDI were calculated for only the female control and deprived groups. Reflecting the significant D1 response increase (Figure 4.10B), there was also a highly significant dominance shift towards D1 when analysed by the VDI (Mann Whitney test, $U = 625$, $p < 0.0001$, Figure 4.12C) and WVDI (unpaired t-test, $t_{(7)} = 5.56$, $p < 0.001$, Figure 4.12D). In the male group, there was some rightward bias shift towards cells responding stronger to D1 stimulation for the VDI analysis (Figure 4.12E), but was not statistically significant (Mann Whitney test, $U = 1526$, $p = 0.058$). There was also no significant dominance shift compared to controls when the averaged WVDI was calculated (unpaired t-test, $t_{(13)} = 1.613$, $p > 0.05$, Figure 4.12F). While NOS1 KOs if taken as a sex balanced group appear to be able to undergo plasticity as has been previously suggested, it is clear that female but not male NOS1 mice can undergo plasticity in the absence of the NO produced by NOS1.

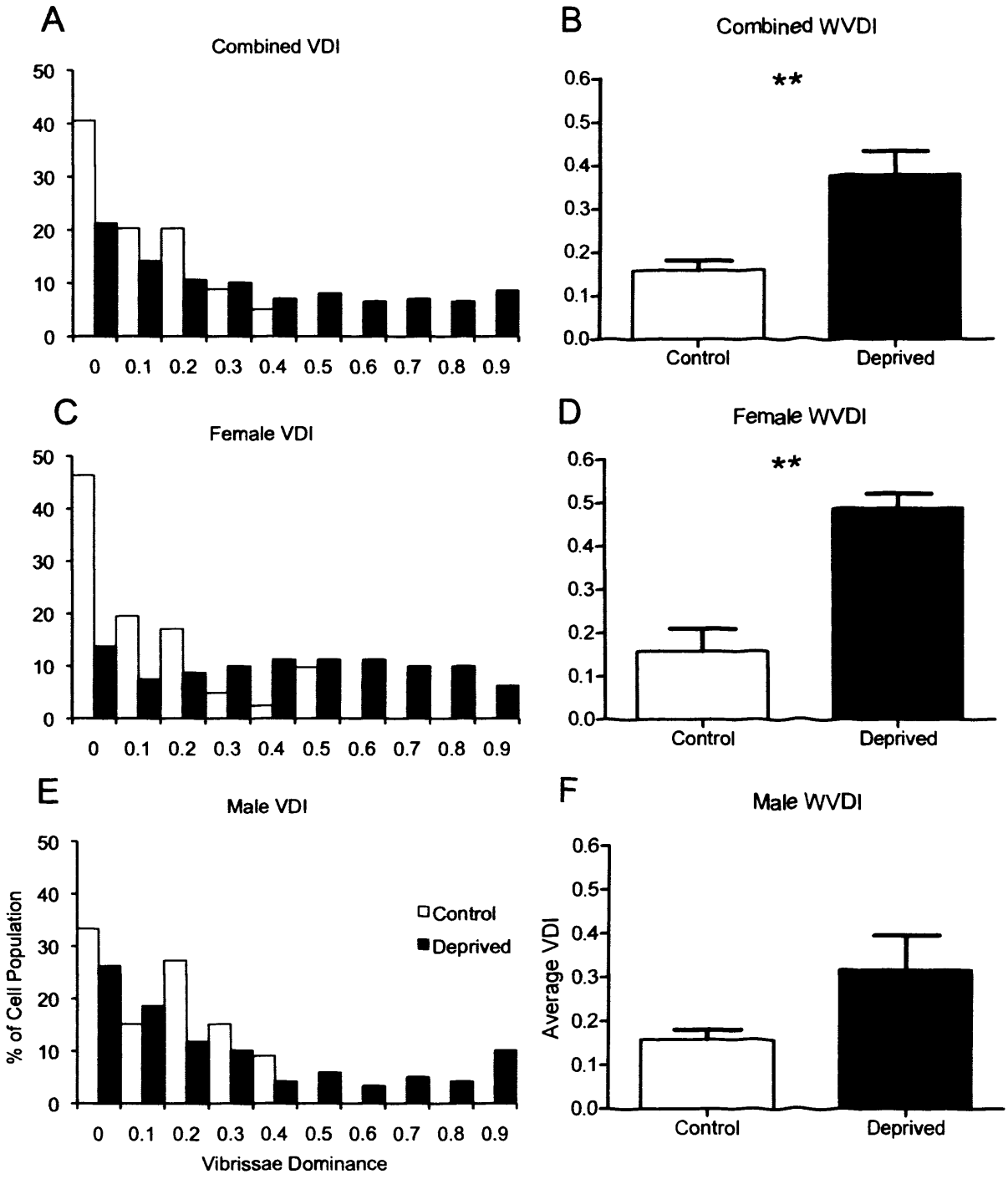


Figure 4.12. *Vibrissae dominance shifts following deprivation in NOS1 KOs.* ***A and B.*** *Total cell and by animal averages of the VDI for male and female control and deprived NOS1 KOs. Following deprivation there was a dominance shift to stronger responses to D1 stimulation, a trend that was mirrored by female NOS1 KOs* ***(C and D).*** *However, although male NOS1 KOs show trends towards dominance shifts following plasticity, neither the total cell VDI* ***(E)*** *or averaged animal VDI* ***(F).***

4.3.6. Plasticity in NOS3 KO Mice

Hardingham and Fox (2006) have documented that LTP in WTs is partially NO dependent and completely NO dependent in the GluR1 KO. While non-specific antagonists have found a role for NO in neocortical potentiation, it is not known which NOS isoform is responsible for this plasticity. The previous chapter has demonstrated that NOS1 is only required for EDP in males. NO is also produced by a similar NOS enzyme, NOS3, the major difference being that NOS3 lacks the PDZ binding domain of NOS1. NOS3 has been shown important for LTP in the hippocampus (Hopper and Garthwaite, 2006; Phillips et al., 2008) and it is possible that the absence of NOS3 in the barrel cortex will reduce EDP. Given reductions in EDP have been found in the absence of NOS1, NOS3 KOs will undergo whisker deprivation to understand whether similar plasticity reductions exist.

4.3.6.1. NOS3 KO Controls

In control NOS3 KOs, the average response to D1 stimulation when recording were made surrounding the D1 barrel was 18.36 ± 5.58 per 50 stimulations. When the control group was spilt into gender, males responded at 20.83 ± 7.24 and females at 14.24 ± 10.08 spikes per 50 stimulations (Figure 4.13). There were no significant differences between male and female NOS3 KOs (unpaired t-test, $t_{(6)} = 0.543$, $p > 0.05$). The NOS3 KO controls will hereon in be discussed as a single group.

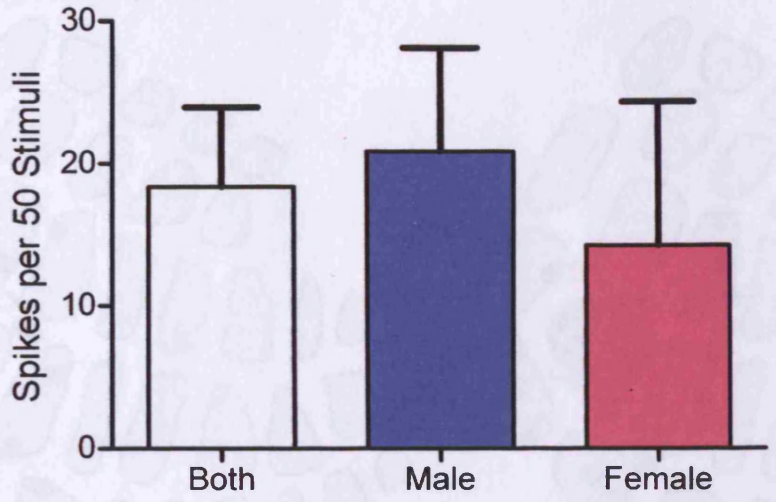


Figure 4.13. *D1 responses from control NOS3 KO mice. No significant differences were found between the genders.*

4.3.6.2. NOS3 KO Plasticity

To further explore the potential role of NOS3 in plasticity, NOS3 KOs underwent deprivation. Deprived animals exhibited a 2.16 fold increase in their response to D1 stimulation compared to controls when recordings were made from barrels adjacent to the D1 barrel. Deprived NOS3 KOs responded at 39.6 ± 5.16 in comparison to controls at 18.36 ± 5.58 spikes per 50 stimuli. A bias shift can also been seen in the response properties of the penetration maps (Figure 4.14). Following deprivation a higher fraction of the penetrations respond in the green and yellow bands that represent higher average spike responses to D1 stimulation. This bias shift compared to controls was statistically significant (chi-square test = 2, 13.06, p = 0.0015).

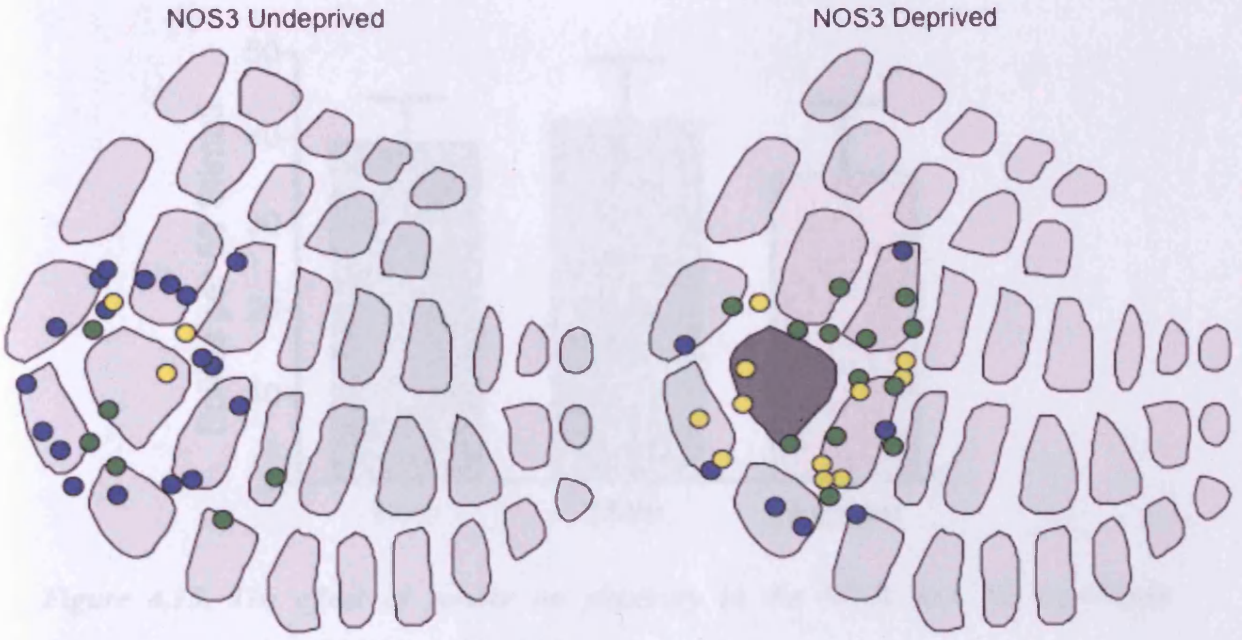


Figure 4.14. *Experience-dependent plasticity in the NOS3 KO. Following deprivation there was a bias shift towards higher responses in penetrations surrounding the D1 barrel.*

The average male response following deprivation was 42.10 ± 7.09 spikes per 50 stimuli. Females responded with slightly fewer spikes per 50 stimuli at 35.85 ± 8.16 (Figure 4.15). A two-way ANOVA revealed a significant effect of deprivation ($F_{(1, 14)} = 6.83$, $p < 0.05$) but not of gender ($F_{(1, 14)} = 0.61$, $p > 0.05$), nor was there an interaction between gender and deprivation ($F_{(1, 14)} < 0.001$, $p > 0.05$). Since no significant differences were observed between deprived male and female NOS3 KO mice, the group hereon in will be referred to a single group.

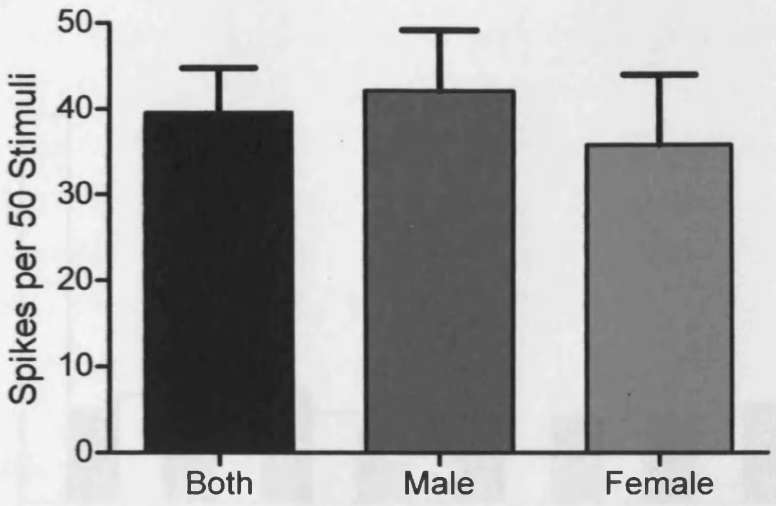
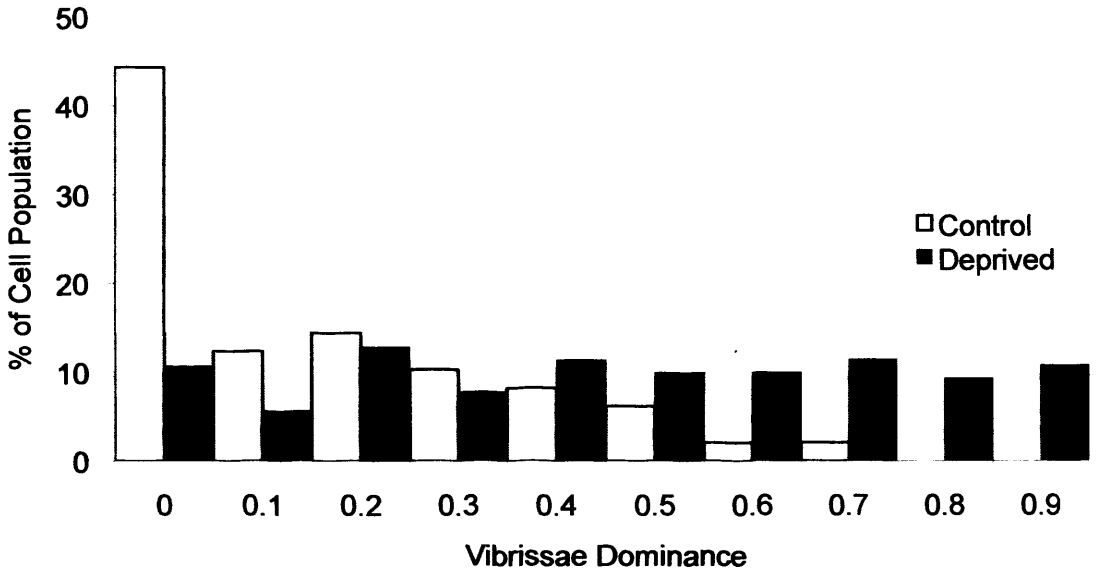


Figure 4.15. *The effect of gender on plasticity in the NOS3 KO. No significant differences were found between the genders.*

4.3.6.3. NOS3 KO Vibrissae Dominance

Figures 4.14 and 4.15 indicated that NOS3 KOs are able to undergo plasticity following deprivation in terms of increases in response magnitude to D1 stimulation following deprivation. The VDI and WVDI were calculated to determine whether this increase was reflected in bias shifts towards stronger D1 responses in relation to the PW. The VDI for control and deprived KOs is shown in Figure 4.16A. Responses from control animals are predominantly towards the left of the scale (nearer 0), indicating that cells respond more strongly to the PW than D1. Following deprivation there is a clear rightward shift towards stronger responses to D1 stimulation than the PW. In fact 51.43% of cells respond either equally to D1 and PW stimulation (0.5) or more strongly to D1 (nearer to 1), compared to 10.31% in controls. This shift was very significant (Mann Whitney test $U = 2763$, $p < 0.0001$). A similar tendency was observed when the WVDI was calculated (Figure 4.16B). The averaged response from control animals was closer to 0 whereas in deprived animals it is closer to 0.5, hence equal responses from D1 and PW, which was significant (unpaired t-test, $t_{(16)} = 4.052$, $p < 0.001$).

A



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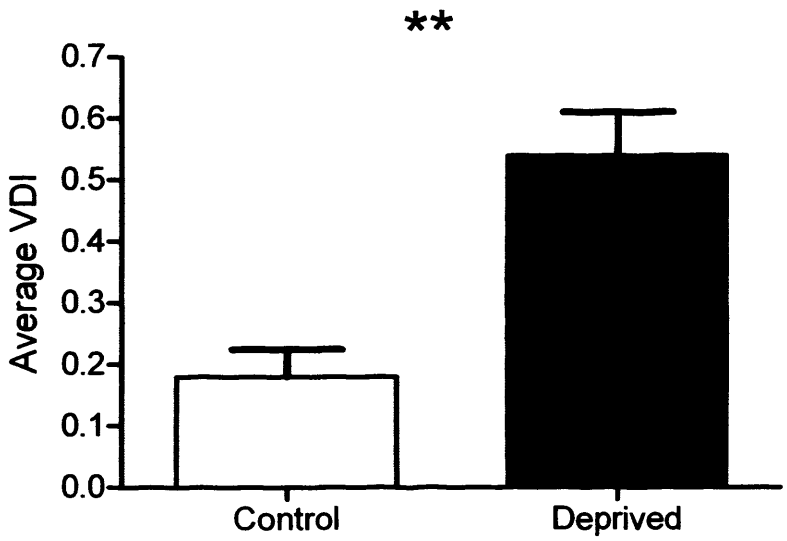


Figure 4.16. *Vibrissae dominance shifts in NOS3 KOs.* ***A and B.*** *Following deprivation there was a shift towards stronger D1 responses to stimulation (0.5 to 1.0) compared to controls that respond largely to the PW.*

4.3.7. Plasticity in Double Knockout Mice

This work has so far demonstrated that EDP is partially mediated by NO. However compared to their non-deprived control KO littermates, GluR1, NOS1 and NOS3 KOs

are all able to undergo plasticity. Similar results have been demonstrated *in vitro*, where GluR1 KOs and WTs with L-NAME (a non-specific NOS inhibitor) all exhibit some degree of LTP (Hardingham and Fox, 2006). However, Hardingham and Fox (2006) showed that the residual plasticity in the GluR1 KO was dependent on NO signaling. Therefore, two hypotheses require investigation; the EDP that has been discovered in the GluR1 KO is entirely dependent on NO signaling and potentiation that is reliant on NO is NOS isoform specific in the GluR1 KO.

4.3.8. Plasticity in GluR1/NOS3 KO Mice

A total of 77 cells were recorded from 7 undeprived and 203 cells from 12 deprived mice. For undeprived controls, 4 males (40 cells) and 3 females (37 cells) were recorded. For deprived GluR1/NOS3 KOs, 8 males (132 cells) and 4 females (71 cells) were recorded.

4.3.8.1. GluR1/NOS3 KO Controls

The average response to D1 stimulation was 8.20 + 1.06 spikes per 50 stimuli in control GluR1/NOS3 KOs. When sub-divided into gender, male KOs responded at 8.44 ± 1.614 and females KOs at 7.88 ± 1.60 spikes per 50 stimuli. No significant differences were observed between control male and female GluR1/NOS3 KOs (unpaired t-test, $t_{(5)} = 0.24$, $p > 0.05$, Figure 4.17). Therefore the control GluR1/NOS3 KO group will be considered as a single group.

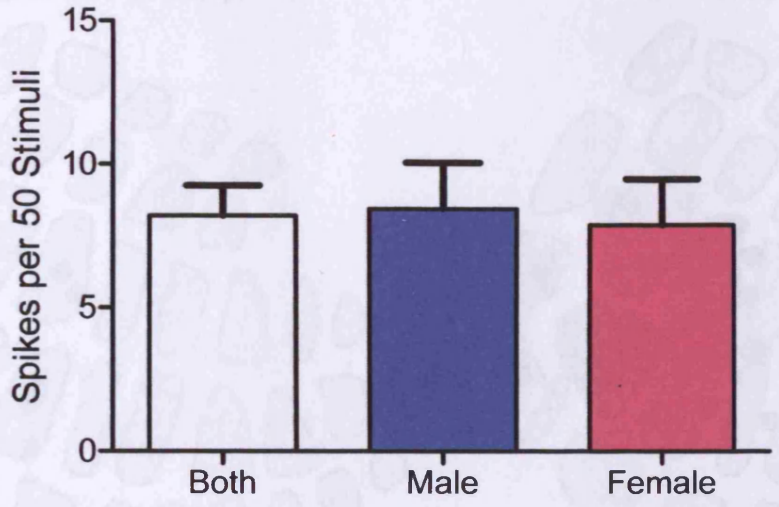


Figure 4.17. Gender differences in GluR1/NOS3 controls. No significant differences were observed between male and female GluR1/NOS3 KOs.

4.3.8.2. GluR1/NOS3 KO Plasticity

To determine whether the residual plasticity in the GluR1 KOs was mediated by NOS3, double KOs of GluR1 and NOS3 were deprived. To D1 stimulation, deprived animals responded at an average of 44.83 ± 5.24 compared to controls that responded at 8.20 ± 1.06 spikes per 50 stimuli. Distribution shifts were also observed in the penetration maps from low responding blue penetrations in controls to higher responding green and yellow in deprived cases (Figure 4.18), which was significant (Chi-square test, 2, 28.18, $p < 0.0001$). Taken together, GluR1/NOS3 KO mice are able to undergo plasticity following deprivation.

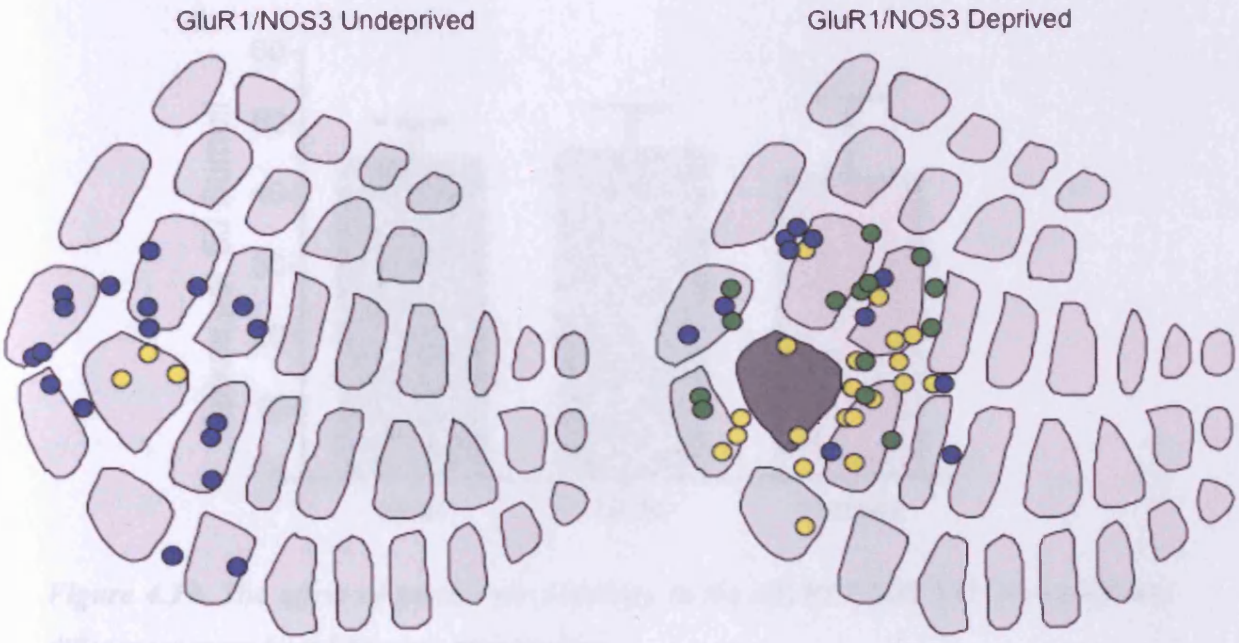


Figure 4.18. *Plasticity in the GluR1/NOS3 KO following deprivation. Penetration maps showing the average penetration response to D1 stimulation. Following deprivation, in barrel columns surround D1 (dark grey) there were more penetrations responding in the green and yellow bands (>25 spikes) than in controls, which was a significant bias shift.*

The group was split to determine whether gender resulted in differential gender expression. The average male response following deprivation to D1 stimulation when recording in adjacent barrels to the D1 barrel was 46.17 ± 6.07 and female was 42.14 ± 11.27 spikes per 50 stimuli (Figure 4.19). A two-way ANOVA revealed a main effect of deprivation ($F_{(1, 15)} = 22.33$, $p = 0.0003$) but not gender ($F_{(1, 15)} = 0.09$, $p > 0.05$) nor an interaction between gender and genotype ($F_{(1, 15)} = 0.05$, $p > 0.05$). Gender is therefore not a factor in determining the magnitude of the plastic response in the GluR1/NOS3 KO.

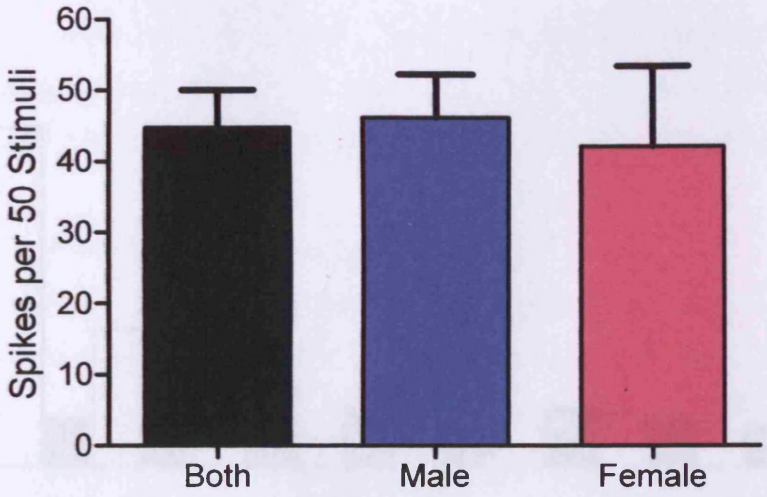
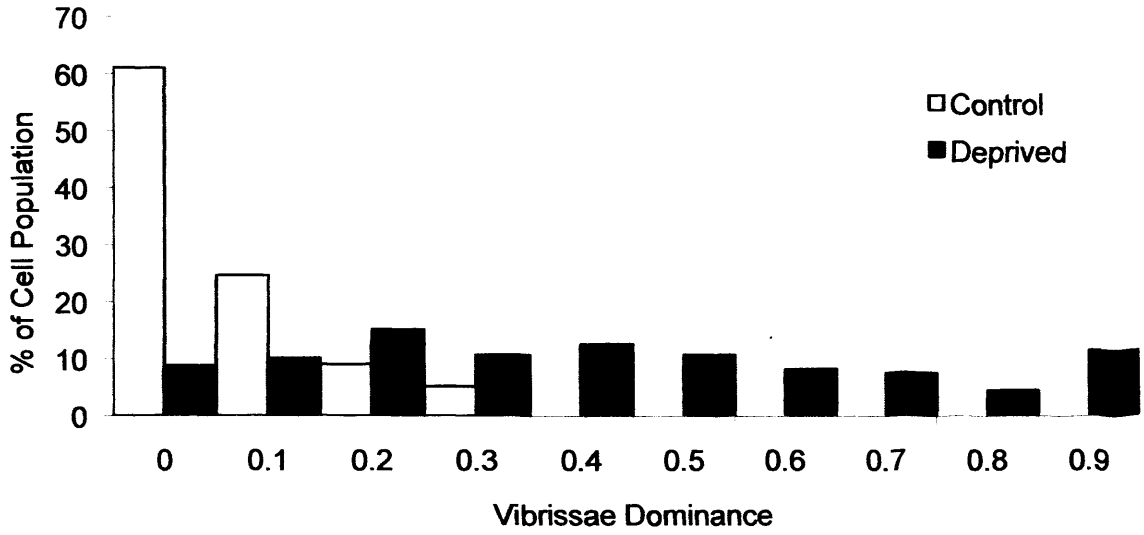


Figure 4.19. *The effect of gender on plasticity in the GluR1/NOS3 KO. No significant differences were found between the genders.*

4.3.8.3. GluR1/NOS3 Vibrissae Dominance

To confirm the plasticity findings from the absolute D1 response magnitude analysis, VDI and WVDI were calculated. The VDI is shown in Figure 4.20A. For the control group, the majority of the responses fall to the left of the graph, indicating dominant responses of the PW instead of the D1 whisker to stimulation. For the GluR1/NOS3 KOs, 0% responses fall at 0.5 or greater, which would have indicated equal or greater response from D1 whisker stimulation in relation to PW stimulation. Following deprivation the bias shifts more towards the right of the graph where responses to D1 stimulation are in many cases equal or dominant. In fact 42.4% of cells responded in this higher band, compared to 0% in controls. This bias shift in response to deprivation significant (Mann Whitney test, $U = 1280$, $p < 0.0001$). Similar results were also found for the WVDI analysis. For control animals, the average WVDI was close to 0, again indicating PW dominance. Yet following deprivation this increased to around 0.5, indicating that each animal responded equally to PW and D1 stimulation (unpaired t-test, $t_{(17)} = 5.89$, $p < 0.0001$, Figure 4.20B). Taken together, GluR1/NOS3 KO mice are able to undergo plasticity following deprivation, which is independent of gender.

A



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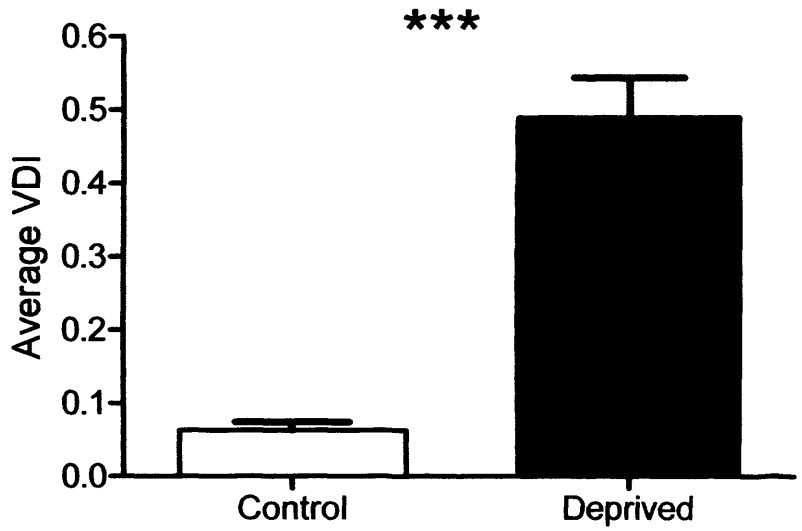


Figure 4.20. *Vibrissae dominance shifts in GluR1/NOS3 KOs.* ***A and B.*** *Following deprivation there was a shift towards stronger D1 responses to stimulation (0.5 to 1.0) compared to controls that respond largely to the PW.*

4.3.9. Plasticity in GluR1/NOS1 KO Mice

A total of 63 cells were recorded from 7 undeprived and 80 cells from 6 deprived mice. For undeprived controls, 4 males (41 cells) and 3 females (22 cells) were recorded. For deprived GluR1/NOS1 KOs, 2 males (23 cells) and 4 females (57 cells) were recorded.

4.3.9.1. GluR1/NOS1 KO Controls

In GluR1/NOS1 KO animals, the average spike response to 50 D1 stimulations when recordings were made in the adjacent barrels was 14.56 ± 3.07. When this was split into gender groups, males responded at 12.20 ± 4.57 and females at 17.70 ± 3.93 spikes per 50 stimuli (Figure 4.21). No significant gender differences were observed (unpaired t-test, $t_{(5)} = 0.87$, $p > 0.05$) so GluR1/NOS1 KO controls will be referred to as a single group.

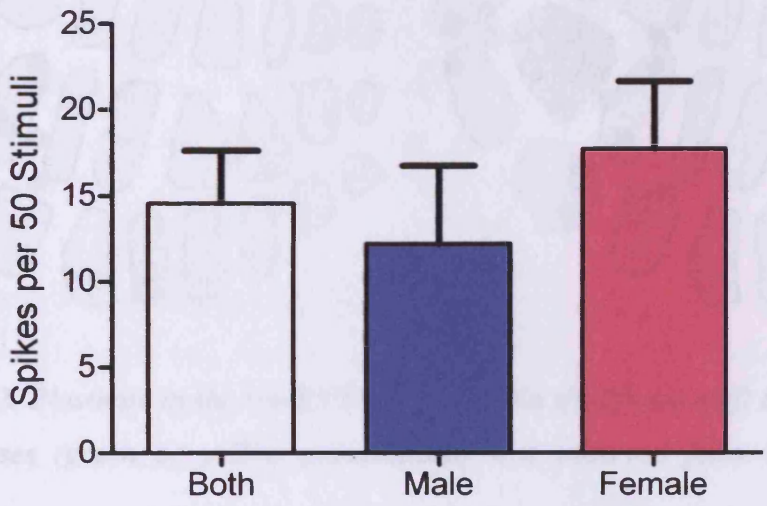


Figure 4.21. *Gender differences in GluR1/NOS1 controls. No significant differences were observed between the genders.*

4.3.9.2. GluR1/NOS1 KO Plasticity

Following deprivation, the mean response to D1 stimulation when recorded from barrels surrounding the D1 barrel was 15.80 ± 3.98. This was almost indistinguishable from their non-deprived control counterparts that responded at 14.56 ± 3.07. There was no bias shift to the penetration map responses. Nearly all penetrations surrounding D1 responded in the blue band (< 25 spikes per 50 stimuli) for both control and deprived

groups (Figure 4.22). No significant change to the distribution was observed (Chi-squared 1, 0.248, $p > 0.05$). This is strikingly different to every other deprived group, which have a higher proportion of yellow (> 50) and green (25 – 50 spikes per 50 stimuli) penetrations. This suggests that plasticity does not occur in the GluR1/NOS1 KO.

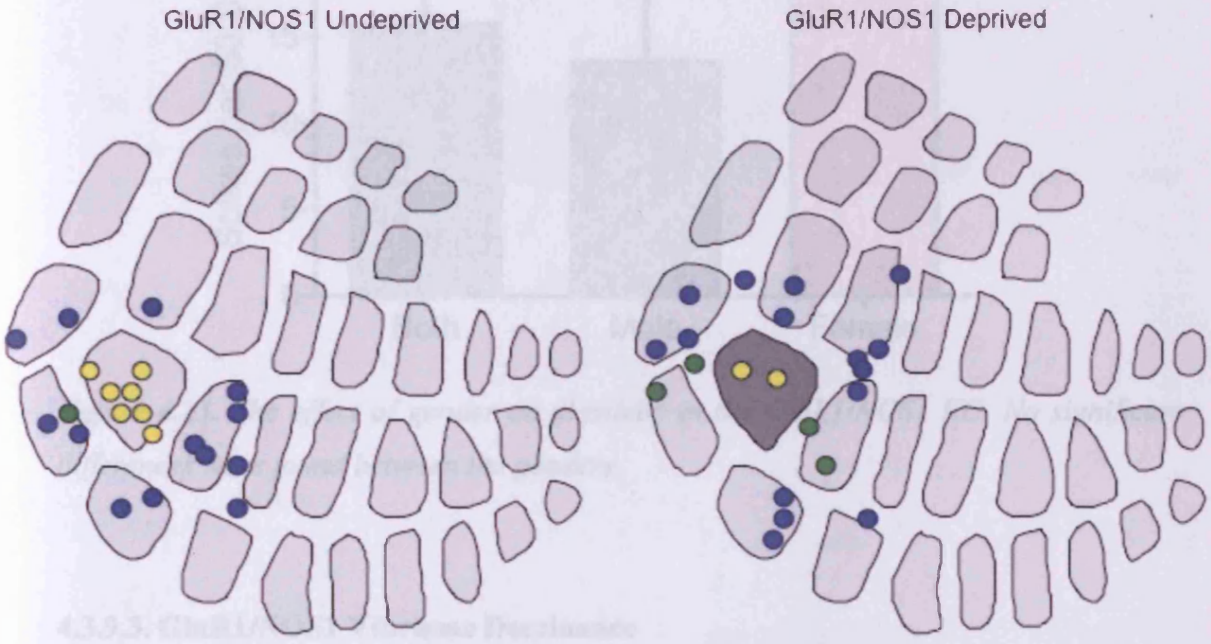


Figure 4.22. *Plasticity in the GluR1/NOS1 KOs.. No significant shift towards stronger D1 responses (green or yellow penetrations) was observed following initiation of plasticity.*

To determine whether that lack of overall plasticity was related to a gender effect, the group was split in their respective groups. To D1 stimulation, males responded at 13.56 ± 5.06 and females at 17.30 ± 6.44 spikes per 50 stimuli (Figure 4.23). A two-way ANOVA revealed no effect of deprivation ($F_{(1, 8)} = 0.01$, $p > 0.05$), gender ($F_{(1, 8)} = 0.075$, $p > 0.05$), nor an interaction between the two ($F_{(1, 8)} = 0.03$, $p > 0.05$). Potentiation of the D1 response therefore does not occur in the GluR1/NOS1 KO and gender is not responsible for the lack of plasticity. Interestingly, female NOS1 KO potentiation (which was not significantly different to WTs) was significantly difference

to female GluR1/NOS1 KOs (unpaired t-test, $t_{(6)} = 3.223$, $p < 0.05$), suggesting that GluR1 is important for female potentiation.

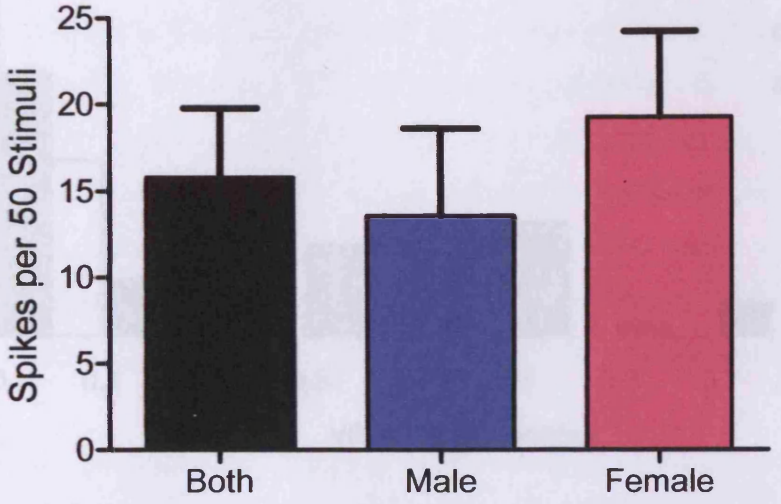
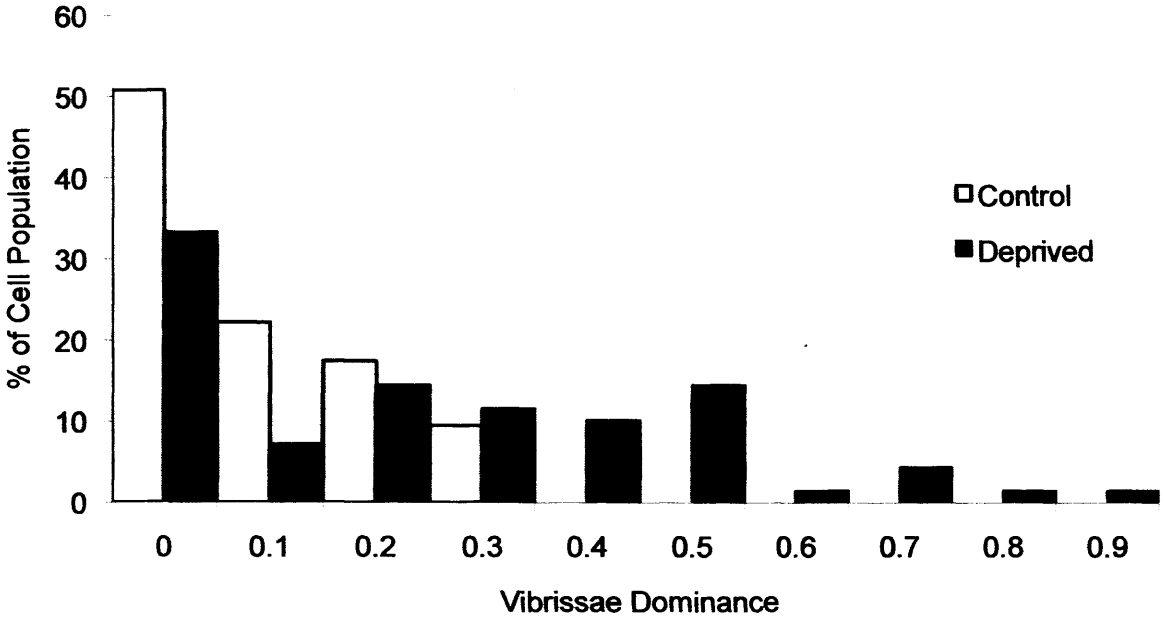


Figure 4.23. *The effect of gender on plasticity in the GluR1/NOS1 KO. No significant differences were found between the genders.*

4.3.9.3. GluR1/NOS1 Vibrissae Dominance

Considering that this is the first genotype where no plasticity has been observed, it was important to confirm this by the VDI analysis. Unexpectedly, following deprivation, bias shifts were observed compared to controls in the GluR1/NOS1 KO mice. Figure 4.24A shows a modest rightward shift towards stronger D1 responses than for the PW cells compared to controls. This shift was statistically significant (Mann Whitney test, $U = 1479$, $p = 0.0016$). A significant difference between deprived and control groups was also found for the WVDI (unpaired t-test, $t_{(10)} = 2.281$, $p < 0.05$, Figure 4.24B). Shifts towards stronger D1 responses relative to the PW following deprivation for both the VDI and WVDI suggest that there is some degree of plasticity within the GluR1/NOS1 KO.

A



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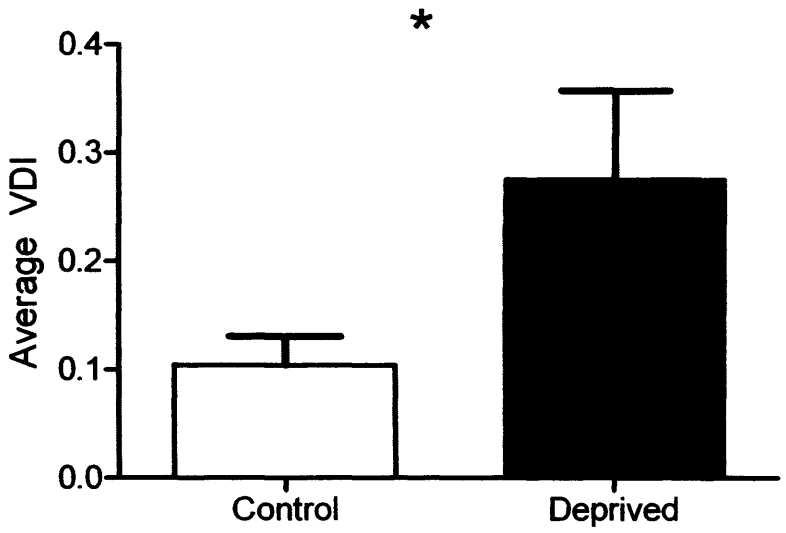


Figure 4.24. *Vibrissae dominance shifts in GluR1/NOS1 KOs.* ***A and B.*** *Following deprivation there was a shift towards stronger D1 responses to stimulation (0.5 to 1.0) compared to controls that respond largely to the PW.*

The results from the VDI and WVDI analysis are somewhat surprising considering there was no indication of any increase in absolute response magnitude to D1 stimulation

(Figure 4.23). The VDI (and WVDI) is designed to use the PW as a within cell control, with changes to the D1 expressed in relation. The rationale behind this is that variance in anaesthesia state will be compensated for, allowing for fairer comparisons. It does not take into account cases where synaptic depression occurs in parallel to the D1 potentiation (or more importantly no potentiation). In this case, following deprivation, D1 responses remain at their baseline level (i.e. no potentiation or depression), whereas the PW is depressed. When the VDI calculation is performed the resulting *F*-number appears to show a shift towards D1 dominance, although no increase in absolute response magnitude was observed. A second calculation method of vibrissae dominance index has been conducted, similar to the 'classic' visual cortex plasticity contrast measure, in an attempt to lessen the impact of the PW on the index shift (Appendix 2), although the deprived GluR1/NOS1 KO group still showed a significant shift.

To investigate this confound further, a comparison between absolute response magnitude to D1 stimulation and weighted vibrissae dominance is provided in Figure 4.25. It is known from the comparable receptive field recordings across layers II/III and IV (Chapter 3, Figure 3.7) that anaesthesia does not generally affect cortical responses in any genotype more than another (with the exception of the higher mortality rate in NOS3 and GluR1/NOS3 KOs) under control conditions. However following deprivation, PW depression was found in the GluR1/NOS1 KO. PW depression was also observed in two other genotypes; NOS3 and GluR1/NOS3 KOs (see Appendix 3). While this would create bias in the VDI calculations (exaggeration by the decrease in PW response magnitude), anaesthesia alone cannot explain the lack of plasticity. Anesthesia depth can cause depression of cortical responses (Armstrong-James and Callahan, 1991; Armstrong-James and George, 1988; Friedberg et al., 1999) but in spite of this potential confound, potentiation was still observed in the NOS3 KO and GluR1/NOS3 KO mice. No such potentiation was observed in the GluR1/NOS1 group.

This dichotomy is visible in Figure 4.25. There is a cluster of groups towards the top right of the graph representing genotypes that can exhibit both potentiation of the D1 response and bias shifts in WVDI. This cluster contains NOS3 and GluR1/NOS3 KOs that also have PW depression. However, the GluR1/NOS1 KO group is located separately to the cluster because even though it has a WVDI shift similar to NOS1 and GluR1 KOs, it fails to demonstrate any increase in D1 response.

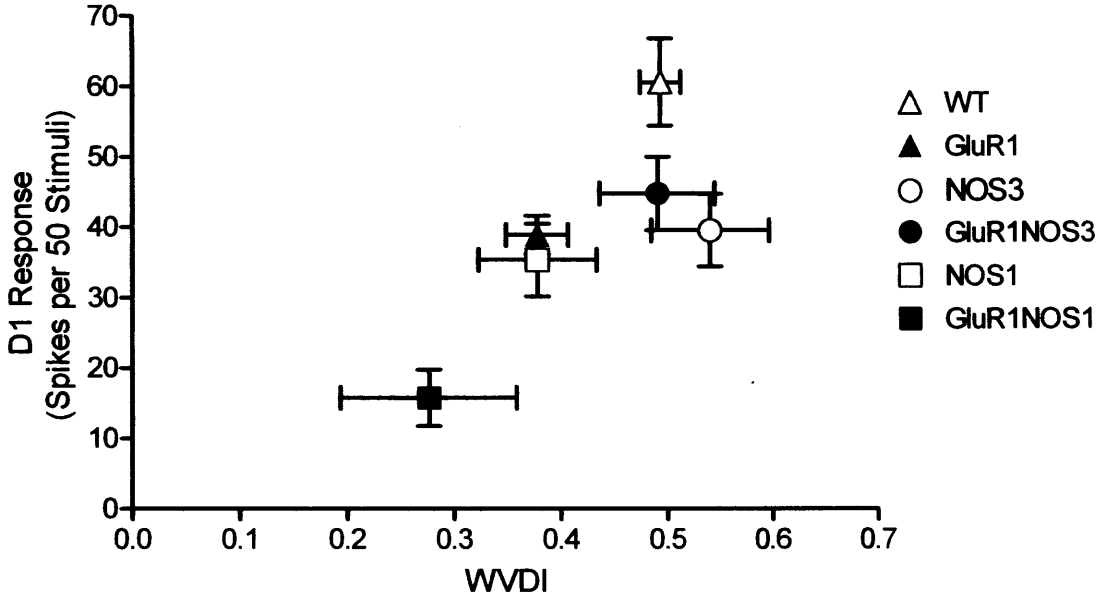


Figure 4.25. *Direct comparison of the absolute D1 response with the WVDI. Genotypes that show both potentiation of D1 response and WVDI bias shifts towards D1 dominance form a cluster at the top right. GluR1/NOS1 KOs while showing some degree of WVDI shift do not potentiate their D1 response, therefore it is located separately.*

Taken together, a state caused by anaesthesia depth cannot fully explain the lack of potentiation in the GluR1/NOS1 KOs as similar conditions are observed in two other genotypes that demonstrate robust plasticity. So despite indications from the VDI and WVDI analysis, GluR1/NOS1 KOs do not show any potentiation.

4.3.10. Plasticity Comparisons Across Genotypes

4.3.10.1. Spared Whisker Potentiation

The first section of this chapter has dealt with comparisons between control and deprived mice within each genotype separately. I specifically wanted to investigate each genotype in isolation so detailed analysis could be undertaken to determine whether potentiation was possible compared to the control littermates within the genotype. This analysis has suggested that compared to control littermates, GluR1/NOS1 KOs and NOS1 male KOs are unable to potentiated their D1 response. Ultimately the predictions set out at the beginning of this chapter are best addressed by comparing whether the

magnitude of plasticity varies across all of the genotypes. Differences in plasticity magnitude between the genotypes are likely to indicate what synaptic molecules are required for the full expression of experience-dependent potentiation.

A three-way ANOVA was conducted to investigate the effects of genotype, gender and deprivation upon D1 potentiation. A significant main effect was found for genotype ($F_{(5, 94)} = 3.79$, $p = 0.004$), deprivation ($F_{(1, 94)} = 87.18$, $p < 0.0001$) but not for gender ($F_{(1, 94)} < 1$, $p > 0.05$). A significant interaction was found for genotype by deprivation ($F_{(5, 94)} = 3.86$, $p = 0.003$) but not for genotype by gender ($F_{(5, 94)} = 1.32$, $p > 0.05$), gender by deprivation ($F_{(1, 94)} < 1$, $p > 0.05$) or genotype by gender by deprivation ($F_{(1, 94)} < 1$, $p > 0.05$). Given that gender was not a factor, male and females NOS1 KOs will not be separated within Figure 4.26.

Following the significant interaction term between genotype and deprivation, tests of simple main effects were conducted to determine the source of the effect. There was a simple main effect of genotype upon the deprived condition ($F_{(5, 94)} = 8.35$, $p < 0.0001$) but not for the control condition ($F_{(5, 94)} < 1$, $p > 0.05$; Figure 4.26A). This highlights that genotypic differences in D1 response occur only following whisker deprivation. Tukey's HSD test was used to further explore differences between the genotypes in the deprived condition. There was a significant difference between WTs and GluR1 KOs ($p = 0.031$), NOS1 KOs ($p = 0.005$) and GluR1/NOS1 KOs ($p < 0.0001$) and between GluR1/NOS1 KOs and GluR1/NOS3 KOs ($p = 0.024$). Figure 4.26B shows the D1 response magnitude from all genotypes following whisker deprivation.

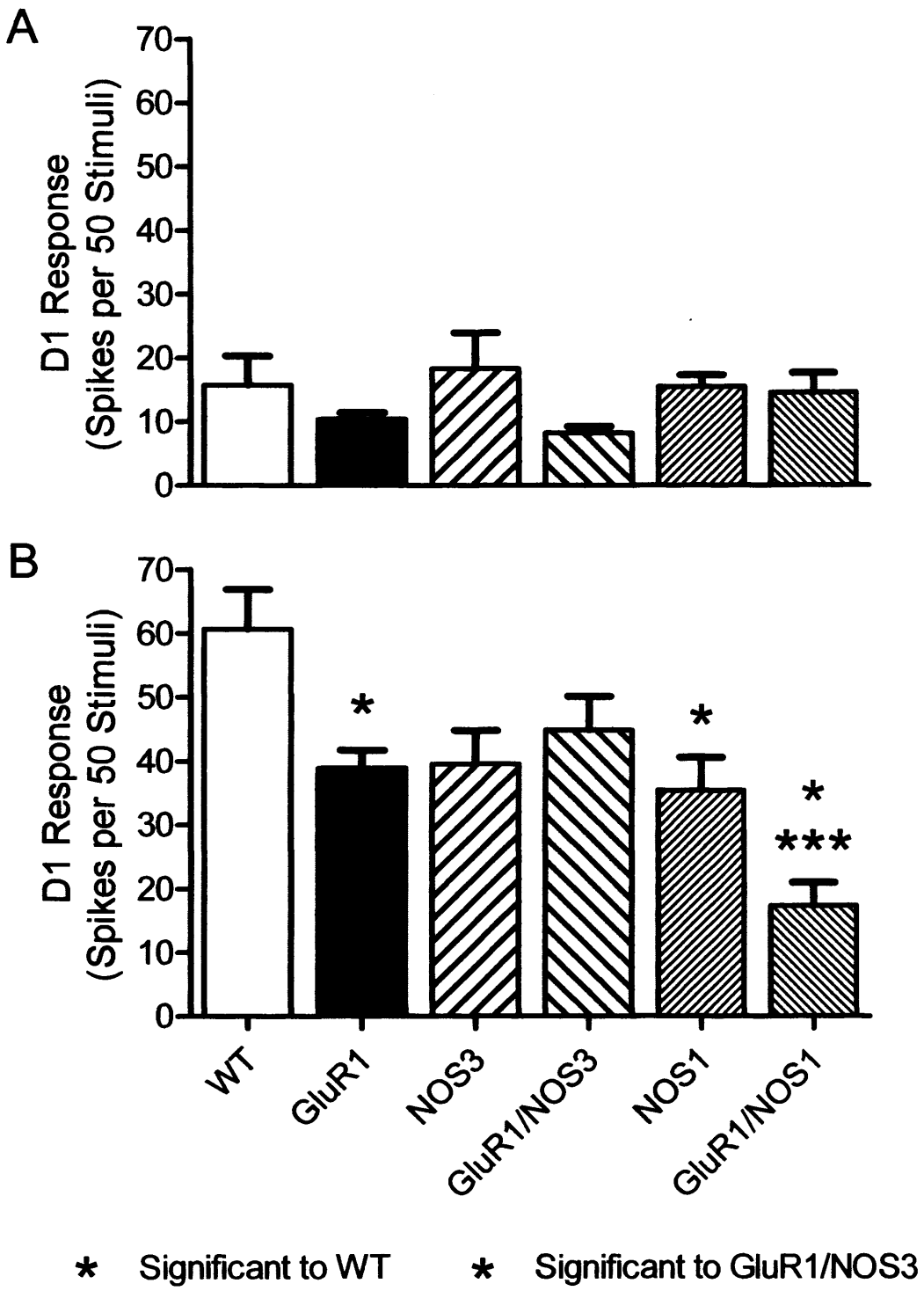


Figure 4.26. *Plasticity across all genotypes following deprivation. D1 response magnitudes from each genotype was compared to all other genotypes. Since gender was not a significant factor, genotypes will not be separated by gender within this figure.*

*(**Continued from previous page**) A significant main effect of genotype and deprivation was found, along with a significant interaction between genotype and deprivation. There was a simple effect of deprivation upon genotype in the deprived but not controls groups. Significance was set at * = $p < 0.05$, ** = $p < 0.001$ and *** = $p < 0.0001$ following Tukey's HSD post analysis.*

4.3.10.2. Vibrissae Dominance

While results from the absolute D1 response magnitude analysis provides compelling evidence for the necessity of certain receptors / enzymes in plasticity, this is less so when analysing the WVDI data. The likely reason for this has been discussed in the GluR1/NOS1 KO plasticity section. Briefly, since GluR1/NOS1 KOs show PW depression as well as no change in response to D1 stimulation following deprivation, it appears to indicate an increase in VDI and WVDI, hence stronger D1 responses. Therefore the divergence between plastic and non-plastic groups was not as strong as predicted.

Figure 4.27A shows the WVDI from all control animals, and Figure 4.27B for all deprived animals. A three-way ANOVA revealed no significant main effect of genotype ($F_{(5, 94)} = 2.03$, $p = 0.08$), gender ($F_{(1, 94)} < 1$, $p > 0.05$) nor interactions between the factors of genotype by deprivation ($F_{(5, 94)} = 1.62$, $p > 0.05$), gender by deprivation ($F_{(1, 94)} < 1$, $p > 0.05$) or genotype by gender by deprivation ($F_{(5, 94)} < 1$, $p > 0.05$). However, there was a main effect of deprivation ($F_{(1, 94)} = 122.59$, $p < 0.0001$) and a significant interaction of genotype by gender ($F_{(5, 94)} = 2.47$, $p < 0.05$). To further explore the interaction, tests of simple main effects of gender upon genotype found significance for the NOS3 KOs only ($F_{(1, 94)} = 7.56$, $p = 0.007$).

The WVDI results are surprising considering the clear differences found for the D1 response magnitude analysis (Figure 4.26B). When directly comparing absolute D1 response to WVDI, a linear correlation is evident that more accurately reflects the distribution of genotypes that range from no plasticity to WT levels ($R^2 = 0.63$; Figure 4.27C).

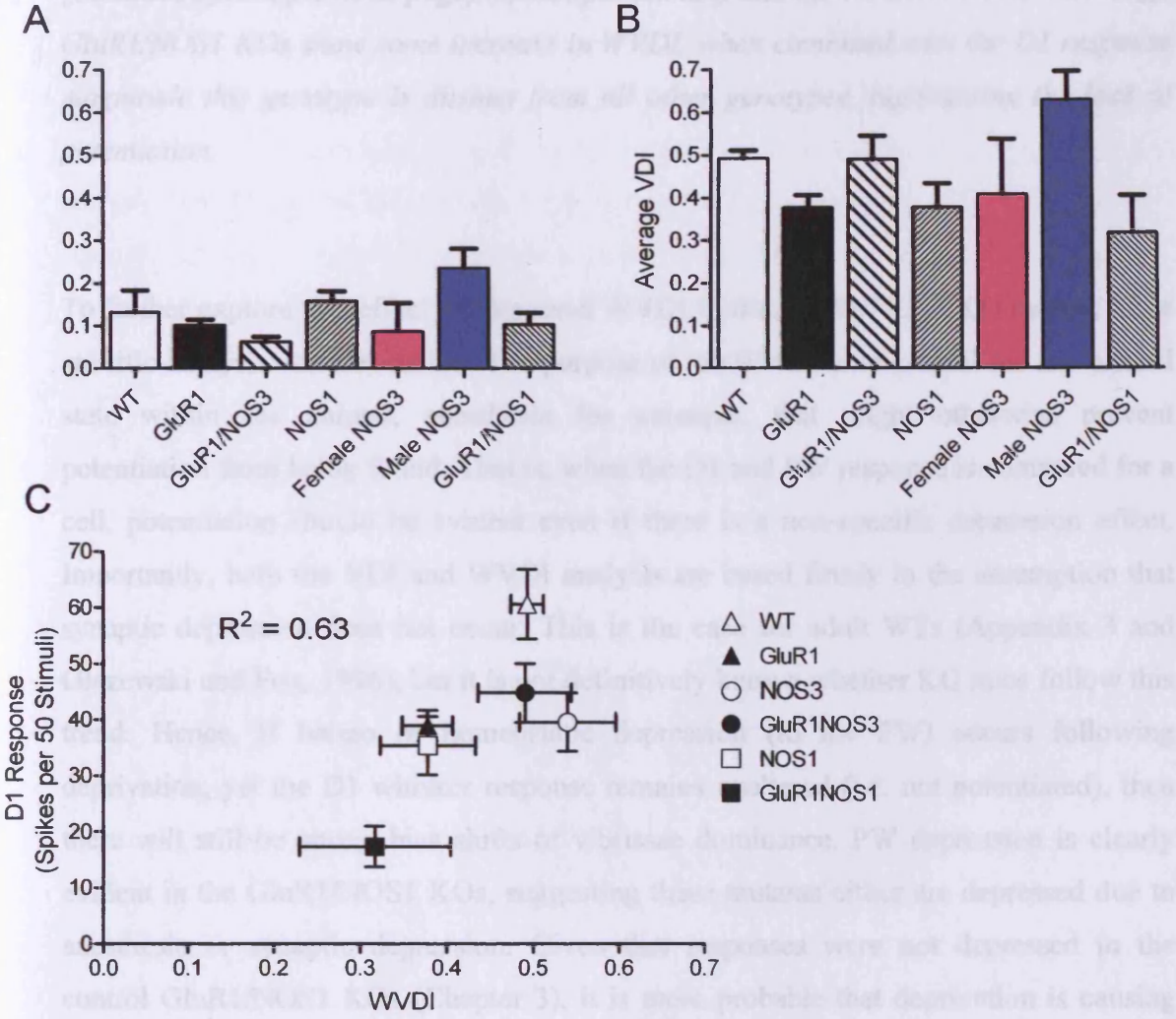


Figure 4.27. *Comparisons of plasticity between genotypes when analysed using the WVDI method.* ***A.*** *No significant differences were observed under control conditions between the genotypes.* ***B.*** *Following deprivation of all but the D1 whisker, there is a bias shift in responses from the PW to the D1 in all genotypes. A main effect of deprivation and an interaction between gender and genotype was found. Tests of simple main effects found that there was an effect of gender upon genotype for the NOS3 KOs only. Therefore the NOS3 genotype has been included within the figure. The significant WVDI bias shift for GluR1/NOS1 KOs is in contradiction to the lack of potentiation observed when analysed for D1 response magnitude.* ***C.*** *Since such clear genotypic differences were not evident using the WVDI analysis as they were for the D1 response magnitude, WVDI and deprived D1 response magnitude were plotted against each other, taken from Figure 4.25. There is a positive correlation between the D1 response*

*(**Continued from previous page**) (spikes per stimuli) and the VWDI. Note that although GluR1/NOS1 KOs show some increase in WVDI, when combined with the D1 response magnitude this genotype is distinct from all other genotypes, highlighting the lack of potentiation.*

To further explore this effect of increased WVDI in the GluR1/NOS1 KO mouse, more specific analysis was conducted. The purpose of the WVDI is to control for any altered state within the animal, anesthesia for example, that might otherwise prevent potentiation from being found. That is, when the D1 and PW response is compared for a cell, potentiation should be evident even if there is a non-specific depression effect. Importantly, both the VDI and WVDI analysis are based firmly in the assumption that synaptic depression does not occur. This is the case for adult WTs (Appendix 3 and Glazewski and Fox, 1996), but it is not definitively known whether KO mice follow this trend. Hence, if hetero or homeostatic depression (to the PW) occurs following deprivation, yet the D1 whisker response remains unaltered (i.e. not potentiated), then there will still be strong bias shifts of vibrissae dominance. PW depression is clearly evident in the GluR1/NOS1 KOs, suggesting these mutants either are depressed due to anesthesia or synaptic depression. Given that responses were not depressed in the control GluR1/NOS1 KOs (Chapter 3), it is more probable that deprivation is causing synaptic depression as opposed to an effect of anesthesia. It is possible that depressed cells biased the lack of potentiation in this genotype (although this is most likely in the case of anesthetic depression) and that cells whose PW responded in the expected range following deprivation (compared to WTs) underwent potentiation. Therefore cells were selected whose PW responses were equal or above 50 spikes per stimulation train (a magnitude that is above PW spiking associated with juvenile depression in the GluR1 KO (~30 spikes per stimuli train; Wright et al., 2008) and similar to all known adult EDP studies). The D1 responses were then averaged for each animal and then within the genotype, and compared to other genotypes. If depression prevented potentiation of the D1 response, then these cells would be ignored and potentiation should be found. Figure 4.28 demonstrates that similar to the total D1 response analysis, WTs have the strongest potentiation (68.62 ± 10.59), followed by the GluR1 KOs (44.17 ± 2.44) and GluR1/NOS3 KOs (41.78 ± 6.65). GluR1/NOS1 KOs potentiation (19.47 ± 7.21) was about half the magnitude of the other KOs and ~70% lower than that of WTs and similar

to GluR1/NOS1 KO undeprived controls. A one-way ANOVA revealed a main effect of genotype ($F_{(3, 34)} = 4.61$, $p = 0.008$), although post hoc analysis only revealed a significant difference between GluR1/NOS1 KOs and WTs ($p < 0.05$). This is due to two reasons. First, this type of analysis increases the variance within the genotype and second, two GluR1/NOS1 KOs only had one PW response above 50 spikes per 50 stimuli, so were excluded. This only left four mutants within the genotype for statistical analysis. Despite this, it does confirm that even in cells that responded robustly to PW stimulation, potentiation was not increased in the GluR1/NOS1 KOs.

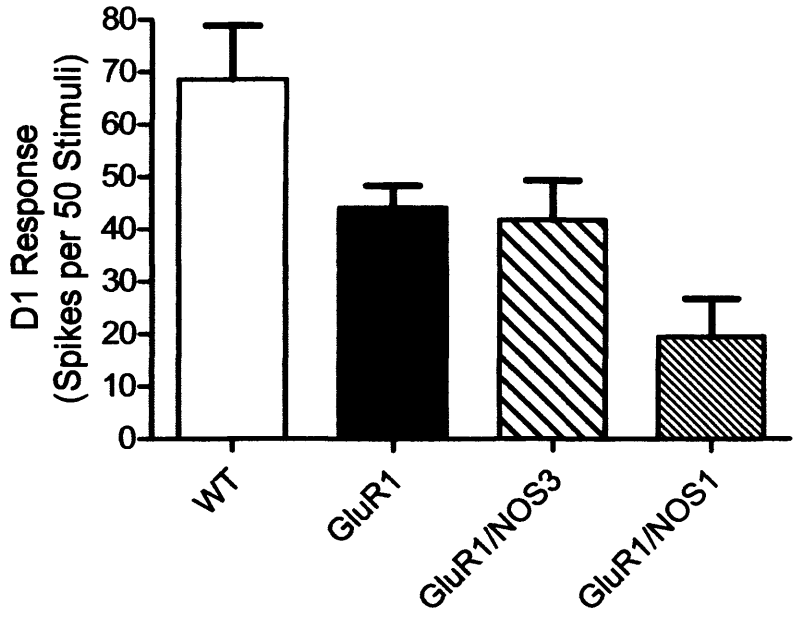


Figure 4.28. *The potentiation of the D1 response when cells that responded to PW stimulation at below 50 spikes per stimulation train were excluded. 50 spikes was chosen as it is well above the value previously associated with juvenile depression in the GluR1 KO (~30 spikes, Wright et al., 2008) but below the control level of ~70 spikes (Chapter 3, Figure 3.7). Theoretically the remaining cells should be the 'healthiest'; not depressed via synaptic or anesthetic confounds and should have the best chance of potentiating. The lack of potentiation in the GluR1/NOS1 KO was not related to a lack of PW response. Cells that responded strongly for the PW did not show D1 potentiation unlike WTs, GluR1 KOs and GluR1/NOS3 KOs.*

4.4. Discussion

Within this chapter I have demonstrated that in the mature synapse, GluR1, NOS1 and NOS3 are required for the full expression of *in vivo* EDP. From the work presented here, three notable results are evident. First, all except the GluR1/NOS3 KOs, NOS3 KOs and the female NOS1 KOs are significantly different from the WT level of plasticity. This suggests that although single GluR1 and NOS KOs are able to demonstrate plastic response compared to their littermate controls, removal impedes the full expression of EDP. Hence, deletion of GluR1 and NOS1 (for males) impairs neocortical barrel cortex synaptic plasticity. Despite this, vibrissae dominance shifts in the GluR1 KO appear comparable to WTs. If the magnitude of the deprived WVDI is compared between WTs and GluR1 KOs, then GluR1 KOs show significantly less potentiation (unpaired t-test, $t = 3.23$, $p = 0.003$). Likewise, there is a significant difference between the distribution of stronger D1 responses from penetrations outside of the D1 barrel (Chi-squared 2, 8.88, $p = 0.012$). These measures, in addition to the significant difference in the absolute D1 response magnitude, suggest that the ability of GluR1 KOs and WT to undergo potentiation differ. Also, it is known that GluR1 is required for LTP (Hardingham and Fox, 2006) as well as EDP (Clem and Barth, 2006), suggesting that electrophysiological measurement of potentiation could detect differences between WTs and KOs. Ultimately, drawing definitive conclusions about 'in-between' reductions (i.e. not all or nothing) in potentiation is difficult for a number of reasons, such as anesthesia, assumptions regarding what synaptic mechanisms should or should not be occurring to the principle whisker following deprivation or compensation mechanisms. To confirm whether GluR1 is required for the full expression of EDP, other techniques such as 2-photon imaging, dendritic spine counting, fMRI or optical imaging, could be employed to compare deprived response magnitudes to that of WTs.

Second, and perhaps most importantly, the GluR1/NOS1 KOs are significantly different from WTs and GluR1/NOS3 KOs. Given that GluR1/NOS1 KOs are different to GluR1/NOS3 KOs, this suggests that NOS3 is mediating a different role to NOS1. Although GluR1, NOS1 and NOS3 are all important for the full expression of EDP, only removal of both GluR1 and NOS1 completely blocks potentiation. Hence, in terms of full neocortical plasticity, it can be stated that NOS1 is the more significant NOS isoform and this finding provides strong evidence for which plasticity mechanism is left intact in the GluR1 KOs. It is also possible that other synaptic mechanisms, aside from a

lack of potentiation, are occurring in the absence of GluR1 and NOS1. PW depression was observed following deprivation, a phenomenon that did not occur during control recordings, making anesthesia an unlikely cause. PW depression should and does not occur in WTs at the ages used in this study (>P60, average age 5 months) and clearly different synaptic mechanisms exist between juveniles and adults (see Glazewski and Fox, 1996; Allen et al., 2003; Celikel et al., 2004). Hence in WTs, potentiation should not be a function of the spiking magnitude of the PW, since PW spiking remains similar between the control and deprived conditions. Yet in the GluR1/NOS1 KO, the PW response does reduce. Further studies are required to determine whether the deletion of NOS1 unmasks the ability of GluR1 KOs to undergo depression (see Wright et al., 2008) or if this observation is simply related to the condition of the animal during recording.

Third, there appears to be a gender specific signaling process in the NOS1 KOs, where females essentially have WT levels of plasticity, yet the males are almost fully inhibited. This suggests a more complex role for GluR1 and NOS1 signaling than has previous been proposed. However, although there is substantial inhibition in the male KOs, it is not correct to claim that NOS1 is simply responsible for the lack of plasticity in the GluR1/NOS1 KOs. Single GluR1 KOs are not fully inhibited unlike GluR1/NOS1 KOs and female GluR1/NOS1 KOs are significantly different from female NOS1 KOs which do show strong plasticity. Therefore in GluR1 KOs, NOS1 KOs and NOS3 KOs, plasticity can occur. In the female NOS1 KOs plasticity is likely to be mediated by GluR1 and NOS3. However, in the GluR1/NOS1 KO no plasticity can occur, as NOS3 is unable to fully support potentiation.

Addressing the hypotheses set out at the beginning of the chapter, it is known that potentiation is possible in the GluR1 KO but was reliant on a spike pairing-type protocol (Hardingham and Fox, 2006; Hoffman et al, 2002; Phillips et al, 2008). During later phases of LTP (at one-hour), GluR1 was no longer required; the absence of GluR1 only affects short-term potentiation. Layer II/III GluR1-insertion increases following the D1-spared deprivation protocol (Chem and Barth, 2006), suggesting that GluR1 is required for EDP. Our results certainly support this hypothesis. GluR1 was needed for the full expression of EDP, although like LTP studies (Hardingham and Fox, 2006) there is a GluR1-independent form of EDP. Hardingham and Fox (2006) found that GluR1-

independent potentiation was dependent upon NO, suggesting that in double KO mice there should have been no EDP. My results only partially confirm this hypothesis. GluR1 and NOS1 deletion completely abolished potentiation, while there was no significant difference between EDP in WTs and GluR1/NOS3 KOs. This confirms that in the barrel cortex, GluR1-independent EDP was solely dependent upon NOS1 and not NOS3. Finally, the genetic deletion of NOS isoforms did lower absolute experience-dependent potentiation magnitude. Studies of EDP in the visual cortex did not find these results (Reid et al., 1996; Ruthazer et al., 1996), although NOS antagonism in the barrel cortex has found reductions in LTP (Hardingham and Fox, 2006). The reductions in NOS1 KO EDP were strongly dependent upon gender; male EDP was absent while female EDP was at WT magnitudes. This was not the hypothesized result and is difficult to explain given that NOS1 association with NMDARs requires estrogen (d'Anglemont de Tassigny, 2009). Similarly, potentiation in the absence of GluR1 or in WTs was not dependent upon gender, suggesting that estrogen does not influence overall response magnitude by GluR1 insertion (Srivastava et al., 2008).

The full implications of these results will be considered in the General Discussion.

Chapter 5:

The Dependence upon GluR1 and Nitric Oxide Signalling for Memory

5.1. Introduction

The barrel cortex has established itself as an excellent model system for investigating neocortical experience-dependent plasticity (EDP). However its primary role is to encode tactile information to facilitate navigation and identification of the animal's environment (for example O'Connor et al., 2010). One might therefore reason that molecules / receptors that are found to be required for EDP would also be required for behaviour. Molecular mechanisms that support barrel cortex EDP are also required for barrel cortex LTP (Hardingham et al., 2003), which in turn are also required for hippocampal plasticity (Phillips et al., 2008; Silva et al., 1992). Hence, molecular mechanisms of synaptic plasticity discovered in the barrel cortex are relevant to synaptic potentiation processes in the hippocampus.

The AMPA receptor GluR1 has been shown necessary for the formation of neocortical short-term potentiation but not for LTP at one-hour post induction (Hardingham and Fox, 2006). Barrel cortex potentiation following EDP protocols also requires GluR1 synaptic insertion (Clem and Barth, 2006). Chapter 4 studied the effect of GluR1 receptor removal upon the magnitude of the spared D1 whisker potentiation. GluR1 was necessary for the full expression of EDP, although similar to previous LTP studies a GluR1-independent form of synaptic potentiation was also evident. This potentiation was dependent upon NO signalling, however only removal of the NOS1 isoform abolished it. The NO-dependence of GluR1-independent potentiation has been shown in the neocortex (Hardingham and Fox, 2006) and the hippocampus (Phillips et al., 2008), although potentiation in the hippocampus requires both NOS isoforms. The magnitude of GluR1-independent potentiation was not affected by gender, nor was EDP in WT mice. However, EDP in the absence of only NOS1 was dependent upon gender. NOS1 KO females were unaffected by the loss of NOS1, whereas male KOs did undergo significant potentiation. Therefore while NOS-dependent EDP may vary between males and females, GluR1-independent potentiation that is supported by NOS occurs regardless of gender.

Robust barrel cortex behavioural paradigms have not evolved alongside the electrophysiological developments. Several elegant studies have started to dissect the vibrissae movements during active touch. However in reality, relatively little is known how the barrel cortex processes during awake whisker movements (for review

see Petersen, 2007 and O'Connor, 2010 for recent work). It is known that activity (membrane potential dynamics) within layer II/III depends upon the behavioural state of the animal (either quiet wakefulness or active whisking), although action potential firing is broadly similar between the states (Crochet and Peterson, 2006). The processing of sensory information is also different between quiet wakefulness or active whisking; a whisker deflect during quiet wakefulness (possible most similar to the experimental conditions in Chapter 4) produces a large cortical response whereas it only produces a minimal response during active whisking (Crochet and Petersen, 2006). How cortical responses caused by whisker deflections during active whisking are integrated requires further research (Petersen, 2007).

Technical difficulties in developing sensitive behavioural paradigms for barrel cortex function has been one of the major limitations in examining the relationship between barrel plasticity and behaviour. To link structure to function, mapping of neural activity in relation to whisker movements is required during exploratory behaviour, similar to the place cells of the hippocampus (O'Keefe and Dostrovsky, 1971). This is not methodologically simple given the diffuse nature of layer II/III and the size of the barrel field; it would require a large array of electrodes to be able to sample enough layer II/III cells to understand how sensory integration occurs across multiple barrels. Despite this, there are potential advantages of studying the barrel cortex. Layer II/III of the barrel cortex only descends 270 μm from the pia. This presents the opportunity to use new techniques such as awake *in vivo* intracellular recordings, optogenetic manipulations and 2-photon imaging to study synaptic plasticity occurs and synaptic modifications during behaviour. This is currently difficult to achieve in the hippocampus due to its depth from the skull.

Surprisingly, no one paradigm has yet emerged as a definitive model for studying behaviour in the barrel cortex. Most are modifications of either tactile discrimination or edge detection tasks. The first behavioural experiment using the barrel cortex found that performance dropped dramatically in a gap crossing task (when the opposing platform could only be sensed by whisker touch) when either all whiskers were deprived or the barrel field ablated (Hutson and Masterton, 1986). A single whisker was sufficient to complete that task, with only minor reductions in performance (Harris et al., 1999; Hutson and Masterton, 1986). Rodents readily discriminate

between two opposing stimuli for reward, yet this is not possible is all whisker are deprived (Krupa et al., 2001; O'Connor et al., 2010). Successive removal of the whiskers decreases the discrimination ratio, however two studies have found conflicting results regarding the impact of this deprivation upon performance. Krupa et al. 2001 found that discrimination performance reduced each time following the removal of 4 whiskers (within the same animal), until chance performance occurred with a single whisker. Conversely, O'Connor et al. 2010 found that chance performance was reached only if all whiskers but one were removed straight away; progressive deprivation to only a single whisker did not have a detrimental effect upon discrimination.

Although it is possible to teach rodents these discriminations, it is not known definitively whether modifications similar to those observed with EDP protocols (deprivation) are necessary for barrel behaviour. That is, is one whisker sufficient for tactile representations and subsequent associative learning, or does spared whisker plasticity have to occur before learning? So far only a hypothetical link between EDP and barrel learning has been proposed, and to the best knowledge of the author, no synaptic manipulation has been attempted to disrupt barrel plasticity and thus behaviour. Therefore further work is needed to confirm 1) in examples of single whisker behaviour, is plasticity required or is the tactile representation from the whisker alone sufficient, 2) what synaptic manipulations would affect associative learning without disrupting the 'sense' (for example by inhibiting transmission) and 3) is EDP produced by training a phenomenon expressed in the barrel cortex or other regions (for example somatosensory cortex 2 or motor cortex 1)? An analogous question is during spatial learning tasks, are the primary sensory areas of the cortex that receive the inputs (olfactory, tactile, visual) plastic or does this occur further down the processing order in association cortices? Discriminating between two textures, or 'roughness' has been studied and is known to require the barrel cortex (Guic-Robles et al., 1989). Lesions of the barrel cortex also diminish pre-lesion ceiling discrimination performance to chance, although non-vibrissal (paw touch) discriminations are still possible (Guic-Robles et al., 1992). Although these complex discrimination tasks require the barrel cortex, they do not expressly address the question of whether it is just the tactile sense that is disrupted, preventing further 'downstream' associative pairings from taking place.

Given the lack of detailed knowledge about how the barrel cortex is employed during behavioural paradigms or whether the same molecules that are required for LTP and EDP are required for barrel cortex behaviour, this chapter will consider the role of GluR1 and NOS in learning and memory traditionally supported by the hippocampus. In doing so, it is hoped that the results may inform examination of barrel cortex function and plasticity in the future.

The hippocampus is required for spatial memory (Morris et al., 1986), it undergoes synaptic potentiation (Bliss and Lomo, 1976) and manipulations of synaptic molecules and receptors disrupt both synaptic plasticity and learning and memory (Silva et al., 1992a and b). Many molecular components of synaptic plasticity found in the barrel cortex are also required for LTP in the hippocampus and are necessary for the formation of memory. For example, inhibiting the autophosphorylation of αCaMKII prohibits barrel cortex EDP potentiation (Glazewski et al., 2000), hippocampal LTP and spatial learning in the watermaze (Giese et al., 1998). GluR1 is another such molecule that is required for barrel cortex EDP, hippocampal LTP (Hoffmann et al., 2002) and hippocampal-dependent memory (Schmitt et al., 2003).

5.1.1. The Role of GluR1 in Hippocampal Spatial Memory

The hippocampus is required for spatial learning and some forms of contextual fear conditioning (Richmond et al., 1999). Synaptic plasticity is required for the formation of some hippocampal dependent memories (Morris et al., 1986; but see Bannerman et al., 1995; Bannerman et al., 2006).

As has been discussed previously (Chapter 1, Section 1.4.5), the AMPA receptor subunit GluR1 has been widely implicated in long-term potentiation (LTP) and experience dependent plasticity (EDP). Its genetic deletion has also revealed some curious behavioural phenotypes. GluR1 KO behavioural phenotypes have been discussed in detail elsewhere (Chapter 1, Section 1.6.2). In brief, several studies have found that GluR1 KO mice are able to use spatial reference memory to solve tasks that have varying motivation demands (Reisel et al., 2002; Schmitt et al., 2003, Schmitt et al., 2004, Schmitt et al., 2005). Yet, lesions to the hippocampus in the

GluR1 KO will abolish spatial reference memory (Reisel et al., 2002). Therefore while the hippocampus is required for the formation of spatial reference memory, GluR1 is not. However, GluR1 KO performance on a spatial task working memory task is severely impaired. GluR1 KOs never perform above chance on a rewarded alteration T-maze task (Reisel et al., 2002), the radial-arm maze (Schmitt et al., 2003) and do not exhibit short-term habituation (Sanderson et al., 2009). It has been postulated that the spared spatial reference memory in the GluR1 KO is related to the spared long-term component of LTP (at one hour post induction) in these mice. While the impairments in spatial working memory may reflect the deficits in short term potentiation (Frey et al., 2009).

The hippocampus and the amygdala appear to contribute to required contextual fear conditioning (but see Wiltgen et al., 2006). Deficits in hippocampal synaptic plasticity can result in reduction of the fear conditioned response (for example Abeliovich et al., 1993). 24 hours following conditioning, GFP tagged GluR1-containing AMPA subunits are recruited to mushroom spines in CA1 hippocampal neurons (Matsuo and Mayford, 2008). An extensive study in the GluR1 KO found that not only were tone and contextual conditioning absent, but LTP in the thalamo-LA synapse, the BA and cortico-LA synapse was also either attenuated or abolished (Humeau et al., 2007). Although the amygdala is also required for the formation of contextual fear conditioning, it is likely that deficits in hippocampal plasticity (related to GluR1) affect the ability of the hippocampus to encode the spatial context in which the aversive stimuli are delivered. Humeau et al. (2007) examined fear condition to both a tone and context simultaneously and reported a deficit in both. Nevertheless, the extent to which fear conditioning to the context is sensitive the mutation is unclear. In order to address this issue, the first experiment examined simple contextual fear conditioning. GluR1 KO deficits in contextual fear conditioning therefore might reflect a similar mechanism to that underlying the deficit in spatial learning, i.e., it disrupts the ability to rapidly encode contextual information.

Given that others (Phillips et al., 2008; Romberg et al., 2009) and I have found that GluR1-independent plasticity requires NO signalling, the question remains as to whether GluR1-independent memory formation is also reliant upon NO signalling. Since GluR1 phenotypes have been found using contextual fear conditioning and

spatial learning tasks, GluR1 KO mice will be tested for contextual conditioned freezing and their ability to form spatial working and reference memory. Should a paradigm reveal a GluR1-independent form of memory, its dependency for NOS plasticity mechanisms will be tested.

In Chapter 4 (Section 4.3.5.2, p. 144), I found that NOS1-dependent EDP was gender specific; NOS1 was not required in females but was in males. Other studies have suggested that estrogen is required for the physical association of NOS1 with the NMDA receptor (d'Anglemont et al., 2009). Although gender differences in contextual fear conditioning in the NOS1 KO were not observed (Kelley et al., 2009), other molecules associated with synaptic calcium influx have shown gender specific differences (Mizuno and Giese, 2010). Estrogen could also modulate GluR1 insertion. Acute application of 17β-estradiol (E2) resulted in *de novo* spine formation and subsequent activation of NMDA receptors was found to increase the GluR1 content within the new spines while also increasing AMPA-mediated transmission (Srivastava et al., 2008). This suggests that the concentration of estrogen could serve to modulate spine formation and GluR1-containing AMPA receptor insertion. Hence, GluR1 insertion could vary between males and females. It is therefore possible that the gender specific differences in potentiation could result in a gender-specific behavioural phenotype. As such, gender will be a factor in the experimental design.

LTP and EDP in the neocortex and hippocampus requires GluR1. However, late-phase LTP and experience-dependent potentiation is partially GluR1-independent and supported by NO. GluR1 KOs are impaired in spatial working memory tasks and contextual fear conditioning, but spatial reference memory is unaffected (Schmitt et al., 2003). Given the recruitment of hippocampal GluR1 following fear conditioning (Matsuo and Mayford, 2008), it was predicted that conditioned freezing in the GluR1 KO will be reduced (Experiment 1). In Experiment 2, spatial learning (working and reference memory) was examined in a radial arm water maze (RAWM) task. It was predicted that GluR1 KOs will form a spatial representation of the extramaze cues to navigate to the goal arm. Having established that this was the case, the NO-dependence of spatial reference memory was then subsequently tested (Experiment 3). Since potentiation is abolished when NOS and GluR1 are inhibited (Chapter 4; Phillips et al., 2008), it was predicted that spatial reference memory would not form in

the GluR1 KO. Given there has been no gender difference in potentiation magnitude in either GluR1 KOs or knockouts of GluR1/NOS, it was predicted that gender would not be a factor in behavioural outcomes.

5.2. Experiment 1

5.2.1. Context Fear Conditioning

Previous studies have investigated fear conditioning in the GluR1 KO (Humeau et al., 2007). However, the design of that particular study used auditory cues in the conditioning protocol. It also did not distinguish whether the conditioning deficit was hippocampal or amygdala in origin. Encoding of the conditioning context requires the hippocampus and the amygdala, whereas tone conditioning is generally not affected by lesions to the hippocampus (Phillips and LeDoux, 1992 but see Richmond et al., 1999). Deficits in early phase hippocampal potentiation have been established in the GluR1 KO (Hoffmann et al., 2002), which could correlate to the inability of KOs to rapidly encode spatial memory and undergo short-term habituation of exploratory behaviour (see Sanderson et al., 2010). Based on this theoretical framework, the deficit in fear conditioning could reflect the inability of GluR1 KOs to form a hippocampal-dependent conjunctive representation of the context (Rudy and O'Reilly, 1999); due to the lack of short-term (less that 20 minute) plasticity processes. A recent study has demonstrated that following contextual conditioning, GluR1 is recruited into the synapse (Matsuo and Mayford, 2008). Hence, hippocampal GluR1 could be important for encoding of the context in which the fear event occurs. To test the requirement of GluR1 to form contextual fear memory, GluR1 KOs will be conditioned to the experimental context using unsignalled footshocks and tested for recall 24 hours later. While Humeau et al. (2007) found fear conditioning deficits to both the context and tone, they did not examine the effect of context conditioning in isolation. It is possible that competitive interactions between tone and contextual conditioning could reduce GluR1-independent memory formation. However, the Matsuo (2008) study found that hippocampal GluR1 is required for fear conditioning and predicts that there could be a freezing deficit in the GluR1 KO. Should GluR1-independent plasticity be sufficient to support contextual fear conditioning, it is possible that it could be sensitive to NO manipulation.

5.2.2. Methods

Detailed methods can be found elsewhere (Chapter 2, Section 2.5.1.3, p. 96). 21 WT and 21 GluR1 KO mice were used in the study, of which there were 11 male WTs, 10 female WTs, 9 male GluR1 KOs and 12 female GluR1 KOs. During the conditioning phase, the mouse was transported into the experimental room placed in to the conditioning chamber. The conditioning chamber (Coulbourn Instruments, Whitehall, PA, USA) contained two Perspex walls, two metal walls, house lights and a grid floor, and was housed in a sound attenuation box. A video camera located at the back wall recorded all experiments for offline analysis. Three unsignalled footshocks (US) were delivered under computer control (H13-16, Coulbourn Instruments, Whitehall, PA, USA) through the metal grid floor at 0.4 mA for 2 seconds. After being placed into the chamber, the mouse was allowed a 6 minute acclimatization period (ISI 1) before delivery of the first US (US1). After two minutes (ISI 2) gap, a second US (US2) was delivered, and two minutes after that (ISI 3), a third (US3). 30 seconds (ISI 4) were then allowed to pass before the animal was removed from the chamber and returned to its home cage.

24 hours post-conditioning, the mouse was returned to the same chamber and tested for conditioned freezing to the context for 8 minutes. After 8 minutes had elapsed, the mouse was removed and returned to its home cage. Activity during the context test was recorded to videotape for offline analysis.

Freezing was defined by the lack of all movement except that of respiration as has previously been described (Humeau et al., 2007) and scoring was performed blind to the experimenter. Both the conditioning phase and context test were scored for freezing. Every 5 seconds, a judgment was made as to whether freezing was occurring or not. A percentage score was then calculated by dividing the freezing score by all opportunities to score within the defined block and multiplying by 100. During the conditioning phase, a block was defined as a single inter-shock interaval (ISI) period (either ISI 1, ISI 2, ISI 3 or ISI 4). For the context test, a percentage score was derived from the entire 8 minute period.

5.2.3. Results

5.2.3.1. Impaired Context Conditioning in GluR1 KO Mice

To determine whether the AMPA subunit GluR1 was required for processing the three US presentations, freezing behaviour was assessed both before and after shock delivery during training. Baseline levels of freezing were also measured before the first US to confirm that general activity was not disrupted in the GluR1 KO.

The freezing results of the conditioning trial are shown in Figure 5.1. A repeated-measures three-way ANOVA for genotype, gender and block (ISI number) was carried out. A significant main effect of block ($F_{(3, 114)} = 95.99$, $p < 0.0001$) and genotype ($F_{(1, 38)} = 52.76$, $p < 0.0001$) and an interaction of block by genotype ($F_{(3, 114)} = 16.15$, $p < 0.0001$) was observed. However, there was no significant main effect of gender ($F_{(1, 38)} < 1$, $p > 0.05$) or interactions of block by gender ($F_{(3, 114)} < 1$, $p > 0.05$), block by gender by genotype ($F_{3, 114)} = 1.93$, $p > 0.05$) or genotype by gender ($F_{(1, 38)} < 1$, $p > 0.05$).

Taken together, gender does not affect the conditioning of WT and GluR1 KOs. However, genotypic differences in freezing magnitude can be observed after the delivery of the first, second and third unsignalled stimuli (US1, US2 and US3). To further explore the significant interaction of block and genotype, tests of simple main effects was performed. There were significant differences between WTs and GluR1 KOs at ISI 2 ($F_{(1, 38)} = 12.05$, $p = 0.001$), ISI 3 ($F_{(1, 38)} = 64.11$, $p < 0.0001$) and ISI 4 ($F_{(1, 38)} = 25.05$, $p < 0.0001$). However, there was no significant difference between WTs and GluR1 KOs at ISI 1 ($F_{(1, 38)} = 2.74$, $p > 0.05$), suggesting that prior to the first US footshock delivery, freezing behaviour when first exposed to the conditioning chamber was similar.

Taken together, these results demonstrate that while GluR1 KOs consistently freeze less than their WT counterparts upon successive presentations of the footshock US, they nevertheless do increase their freezing behaviour compared to baseline. Comparing ISI 1 and ISI 4, male GluR1 KOs increased their freezing behaviour 6 fold while females KOs increased 4.3 fold. Gender does not interact with the acquisition of contextual freezing. It therefore seems likely that the KOs do recognise the

presentation of the foot shock, although they cannot reach the freezing magnitudes of WTs. Potential reasons for this will be discussed later.

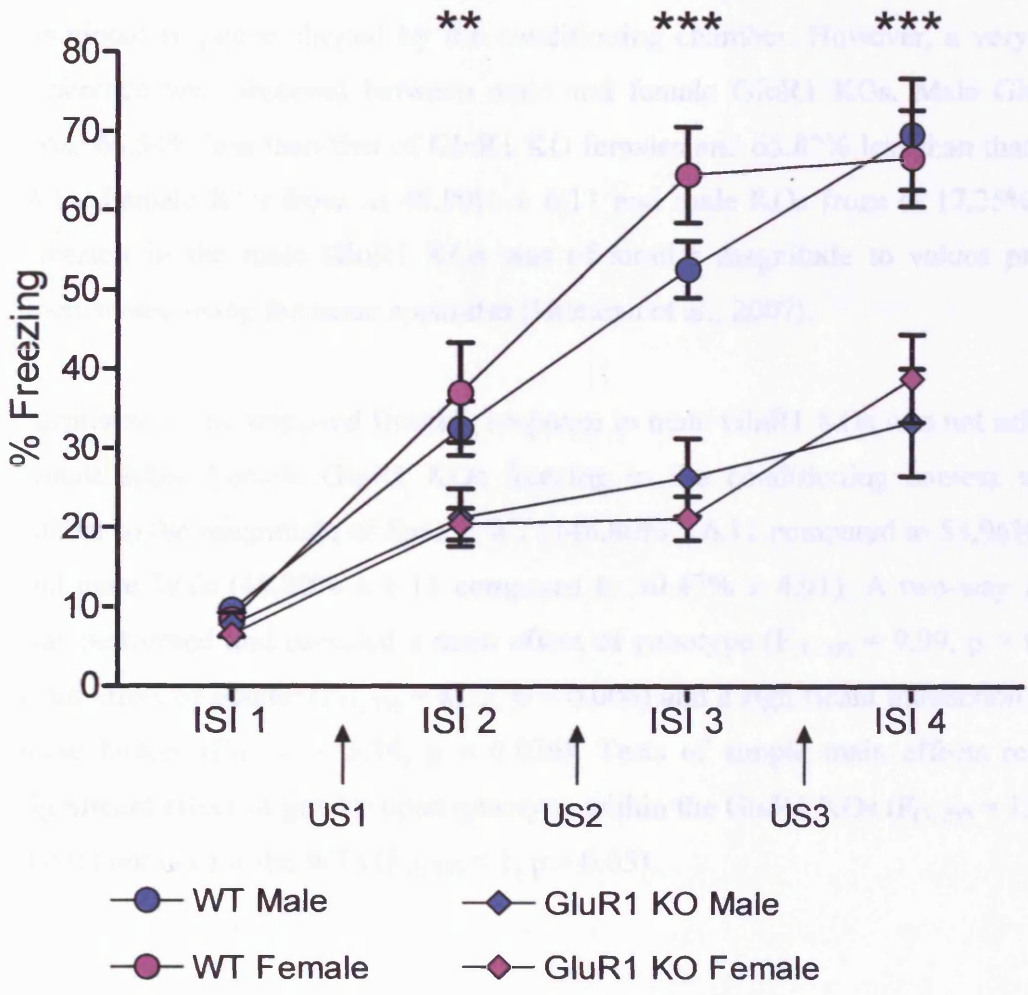


Figure 5.1. *Freezing behaviour of WTs and GluR1 KOs during the conditioning phase. Following 6 minutes exposure to the conditioning chamber (ISI 1), a 2 second foot shock (US1) was delivered, where both WTs and GluR1 KOs increase their freezing behaviour (ISI 2). A test of simple main effects revealed freezing magnitude was significantly different between the genotypes at ISI 2. Freezing behaviour also increased in both genotypes at ISI 3 and ISI 4 following delivery of US2 and US3, respectively. Tests of simple main effects revealed significant differences between the genotypes at both ISI 3 and ISI 4. Although GluR1 KOs froze less than WTs, they did increase freezing upon successive presentations of the US, suggesting that they were aware of the US delivery.*

5.2.3.2. Retention Test: Context Freezing is Dependent upon Gender

Figure 5.2 presents the results of the 24-hour retention test. Inspection of this figure shows that WT male and female mice froze to the context at very similar levels (males 50.47% ± 4.91 and females 53.96% ± 7.37). This confirmed that in the C57/BL6 (Harlan) background, gender was not an important factor in determining the emotional response elicited by the conditioning chamber. However, a very striking difference was observed between male and female GluR1 KOs. Male GluR1 KO froze 64.54% less than that of GluR1 KO females and 65.82% less than that of male WTs. Female KOs froze at 48.80% ± 6.11 and male KOs froze at 17.25% ± 5.01. Freezing in the male GluR1 KOs was of similar magnitude to values previously documented using the same apparatus (Humeau et al., 2007).

Surprisingly, the impaired freezing response in male GluR1 KOs was not reflected in female KOs. Female GluR1 KOs freezing in the conditioning context was very similar to the magnitude of female WTs (48.80% ± 6.11 compared to 53.96% ± 7.37) and male WTs (48.80% ± 6.11 compared to 50.47% ± 4.91). A two-way ANOVA was performed and revealed a main effect of genotype ($F_{(1, 39)} = 9.99$, $p = 0.003$), a main effect of gender ($F_{(1, 39)} = 8.33$, $p = 0.006$) and a significant interaction between these factors ($F_{(1, 39)} = 5.34$, $p = 0.026$). Tests of simple main effects revealed a significant effect of gender upon genotype within the GluR1 KOs ($F_{(1, 39)} = 13.61$, $p = 0.001$) but not for the WTs ($F_{(1, 39)} < 1$, $p > 0.05$).

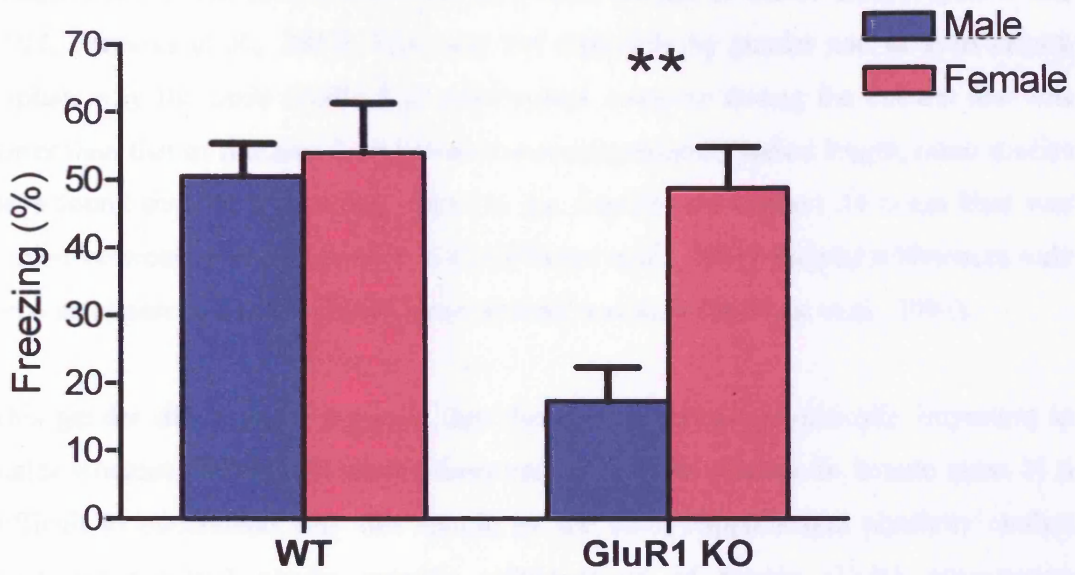


Figure 5.2. *Male GluR1 KOs lack context-induced freezing whereas female GluR1 KOs are unimpaired. There was no effect of gender within the WT group. However, simple main effects analysis following a significant interaction of genotype and gender revealed that male GluR1 KOs were significantly impaired compared to female GluR1 KOs ($p = 0.001$).*

5.2.4. Discussion

Gender is an important factor that dissociates performance in the GluR1 KO during the 24 hour retention test. Freezing behaviour was significantly attenuated in male GluR1 KOs while female performance was comparable to both male and female WTs. Normal levels of freezing during the acclimatisation period suggests that GluR1 KOs did not suffer from extensive neophobia or major locomotor deficits. This has been found in other studies of GluR1 KOs prior to footshock presentation (Humeau et al., 2007), where locomotor activity between WTs and GluR1 KOs was the same. Despite this, GluR1 KO have been found to be hyperactive in an open field (Bannerman et al., 2004). Although this was not performed in this study, the animals were of the same background as the ones used here. It therefore difficult to be definitively sure whether the deficit in freezing was due to a memory impairment or motor deficits. Although GluR1 KOs froze less than WTs following the unsignaled footshock presentation, given that freezing did increase during ISI 2, 3 and 4 it is likely that the KOs were

sensitive to the US. This is consistent with other studies of GluR1 KOs (Feyder et al., 2007; Humeau et al., 2007). This was not separable by gender and as such cannot explain why the male GluR1 KO conditioned response during the context test was lower than that of females. Using a similar acclimatisation period length, other studies have found that freezing when tested in the conditioned context 24 hours later was similar between male and female WTs (Wiltgen et al., 2001). Gender differences only become apparent if the acclimatisation period was short (Wiltgen et al., 2001).

This gender dimorphism suggests that the GluR1 subunit is critically important in males whereas context-US associations can form in its absence in female mice. It is difficult to understand why this should be the case. Hippocampal plasticity studies have not reported gender specific enhancement of female GluR1 potentiation compared to male KOs. That does not necessarily mean that one does not exist. Many of the potentiation studies have either used male mice or not disclosed which gender was used (see Appendix 1 for a summary of genders used in GluR1 studies). A gender specific reduction in hippocampal and amygdaloid LTP could represent the simplest mechanisms behind the results seen here. However, it is clear that some (perhaps compensation) mechanisms exist in the female GluR1 KO that allows fear conditioning to occur and be expressed.

The main aim of this study was to establish whether the conditions were present using the fear paradigm to assess if inhibition of NO would reduce spared learning in GluR1 KO mice (Chapter 4; Hardingham and Fox, 2006; Phillips et al., 2008; Romberg et al, 2009). However, in the case of context fear conditioning, males are at or close to floor performance, which does not allow any further manipulation to explore a subsequent NO contribution to learning. Given that in Chapter 4 (Section 4.3.10.1, p. 165) I demonstrated that potentiation in the GluR1 KO is completely dependent on NO, it is possible that the contextual fear conditioning in the female GluR1 KO is NO-dependent. Although this represents an excellent opportunity to test NO-dependence upon memory, there are a number of caveats that make this difficult. First, hippocampal dependent plasticity requires both NOS1 and NOS3 (Hopper and Garthwaite, 2006; Phillips et al., 2008), meaning that a double knockout animal (GluR1/NOSX) would not be appropriate for testing. Second, delivery of NOS antagonists can provoke non-specific peripheral contraindications (Prendergast et al.,

1997). This is most commonly associated with intraperitoneal (IP) injection of the antagonist. Indeed, IP injection of the non-specific inhibitor L-NAME at 75 mg/kg significantly reduced locomotion compared to sham injected controls (see Appendix 4). Although central delivery of NOS antagonists is possible, the implantation of minipumps is not appropriate for the fear conditioning paradigm. The extreme movements that occur during the footshock delivery would likely cause damage to the incision site and could potentially dislodge the cannula. I therefore sought to determine the NO-dependence of GluR1-independent plasticity using a form of memory that has previously been shown intact in the GluR1 KO; spatial reference memory (Schmitt et al., 2003).

5.3. Experiment 2

5.3.1. Radial Arm Water Maze

Although contextual fear conditioning was GluR1-independent in females, for the reasons stated above it would be difficult to manipulate NOS to test the dependence of freezing upon NO. Previous studies have used the Morris water maze and the radial arm paradigm to investigate memory mechanisms in the GluR1 KOs. Spatial reference memory in the GluR1 KO was comparable to WTs in both the water maze (Zamanillo et al., 1999) and the radial arm maze (Schmitt et al., 2003) although GluR1 KOs did show a spatial working memory impairment in the latter apparatus (Schmitt et al., 2003). The lack of spatial working memory could reflect an inability to rapidly encode contextual cues, explaining the freezing deficit in the male GluR1 KOs. Contrary to this deficit, it is known that the formation of spatial reference memory in the male GluR1 KO is not impaired. This suggests that although male GluR1 KOs were at floor during the context fear test, it is possible to form spatial memory under appropriate experimental conditions. Little is known about female GluR1 spatial memory, although given that females were unaffected in the contextual fear conditioning experiment and no gender differences were observed in barrel cortex potentiation (Chapter 4, Section 4.3.4.2, p. 139), it is probable that female GluR1 KOs will perform similar to males. Curiously, it has been proposed that male mice benefit from a small advantage during radial arm maze tasks over females, whereas females perform slightly better males in the water maze (Jonasson, 2005).

This suggests that motivation demands of the task may influence gender differences in performance.

To test spatial acquisition and retention, male and female WTs and GluR1 KOs were challenged on a 6-arm radial water maze (RAWM). Since the RAWM incorporates elements of both spatial working and reference memory, GluR1 KOs were ran with WTs to determine whether they could learn the platform location and if so, whether the rate of acquisition differed between the genotypes and/or genders?

5.3.2. Methods

During each trial, the mouse was released from a pseudorandom arm and had to navigate to a hidden platform at the end of an arm. This location remained consistent throughout the experiment. 4 training days were conducted with 12 trials per day. For the purpose of analysis, a block consisted of the average of 4 trials (hence 3 block represented one days acquisition). An error was defined as when the hind legs of the mouse had entered an incorrect arm. At that point, the mouse was returned to the start arm and released to continue exploration. Each trial was terminated when the animal found the platform or after 60 sec.

A probe trial was performed on day 5 and then 3 days following this to examine retention of the platform location. The hidden platform was removed from the maze and the mice were released from the arm opposite to the goal. Time spent in the goal arm was measured. Chance was set at 8.6 seconds (equal time spent in 6 quadrants plus the central arena). To assess long-term retention of the goal location, a second probe trial was conducted three days following the first probe. During this time the mice had no contact with the experimenter or the experimental room.

5.3.3. Results

5.3.3.1. GluR1 Deletion Retards Spatial Memory Acquisition

WTs and GluR1 KOs were tested on the RAWM. During the acquisition phase, memory errors (entry to incorrect arms) were counted and analysed. Figure 5.3A show the GluR1 KO made more errors, particularly mid-way during acquisition.

Nevertheless, by the end of training the performance of the GluR1 KO mice was comparable to that of the WT mice. GluR1 KO mice also took longer to find the hidden platform during the middle stages of training but the latencies became similar to WTs at the end of training (Figure 5.3B).

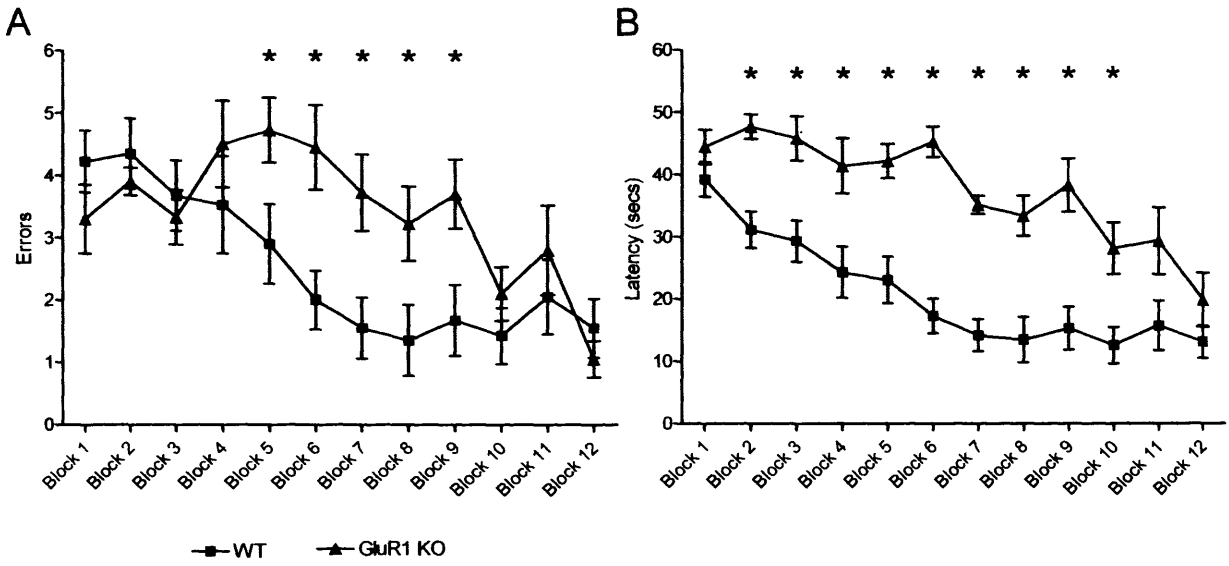


Figure 5.3. *The effect of GluR1 deletion upon RAWM performance. For clarity, WTs and GluR1 KOs in this figure are displayed as a sex balance group. See Figure 5.4 and 5.5 for the results separated by gender.* ***A.*** *WT and GluR1 KO entries into arms that did not contain the platform. Once the hind legs had entered the incorrect arm, the mouse was returned to the start arm, released and allowed free arm choice. Simple main effects of block upon genotype was found at block 6, 7, 8 and 9.* ***B.*** *There was also statistical differences between the latency of GluR1 KOs compared to WTs in finding the hidden platform during acquisition at blocks 2 to 10.*

Figure 5.3 reveals the effect of the removal of GluR1 upon RAWM acquisition as combined, sex-balanced groups. However, to assess the potential role of gender in acquisition, the genotypes were separated and are shown in Figure 5.4. A repeated measures three-way ANOVA was conducted to determine the effect of block number, gender and genotype upon arm entry errors (Figure 5.4A and B). A significant main effect of block ($F_{(11, 176)} = 7.40$, $p < 0.0001$) but not genotype ($F_{(1, 16)} = 3.42$, $p = 0.083$) or gender ($F_{(1, 16)} < 1$, $p > 0.05$) was found. There was a significant interaction

between the block by genotype factors ($F_{(11, 176)} = 3.15$, $p = 0.001$) but not of block by gender ($F_{(11, 176)} < 1$, $p > 0.05$), genotype by gender ($F_{(1, 16)} < 1$, $p > 0.05$) or block by gender by genotype ($F_{(11, 176)} < 1$, $p > 0.05$). Following the significant block by genotype interaction, tests of simple main effects were conducted. Simple effects of genotype upon block were found at block 6 ($F_{(1, 16)} = 7.98$, $p = 0.012$), 7 ($F_{(1, 16)} = 7.23$, $p = 0.016$), 8 ($F_{(1, 16)} = 4.67$, $p = 0.046$) and 9 ($F_{(1, 16)} = 6.93$, $p = 0.018$).

This analysis confirmed that acquisition of the RAWM procedure proceeded more slowly in GluR1 KOs, although by the end of training the KO mice performed as accurately as the WT mice.

Consistent with the memory errors, time taken to find the hidden platform followed a similar pattern. Both WTs and GluR1 KOs start with a similar latency (block 1) but diverge thereafter (Figure 5.4C and D). GluR1 KOs take considerably longer to find the hidden platform from blocks 2 to 10, at which point, the latencies became comparable to WTs.

A repeated measure three-way ANOVA with block number, gender and genotype as factors revealed a significant main effect of block ($F_{(11, 176)} = 16.88$, $p < 0.0001$), genotype ($F_{(1, 16)} = 26.39$, $p < 0.0001$) and a significant interaction between genotype and block ($F_{(11, 176)} = 2.63$, $p = 0.004$). There was no significant main effect of gender ($F_{(11\ 16)} < 1$, $p > 0.05$) and no significant interactions of genotype by gender ($F_{(1, 16)} < 1$, $p > 0.05$), block by gender ($F_{(11, 176)} < 1$, $p > 0.05$) or block by gender by genotype ($F_{(11, 176)} < 1$, $p > 0.05$). Following the significant interaction of block and genotype factors, tests of simple main effects were conducted. Significant differences were observed at block 2 ($F_{(1, 16)} = 19.87$, $p < 0.0001$), 3 ($F_{(1, 16)} = 11.30$, $p = 0.004$), 4 ($F_{(1, 16)} = 7.33$, $p = 0.016$), 5 ($F_{(1, 16)} = 15.35$, $p = 0.001$), 6 ($F_{(1, 16)} = 55.09$, $p < 0.0001$), 7 ($F_{(1, 16)} = 50.28$, $p < 0.0001$), 8 ($F_{(1, 16)} = 14.83$, $p = 0.001$), 9 ($F_{(1, 16)} = 16.74$, $p = 0.001$) and 10 ($F_{(1, 16)} = 10.55$, $p = 0.005$. This analysis confirmed that GluR1 KO mice were slower to find the platform, although by the end of training they performed at a level similar to control mice.

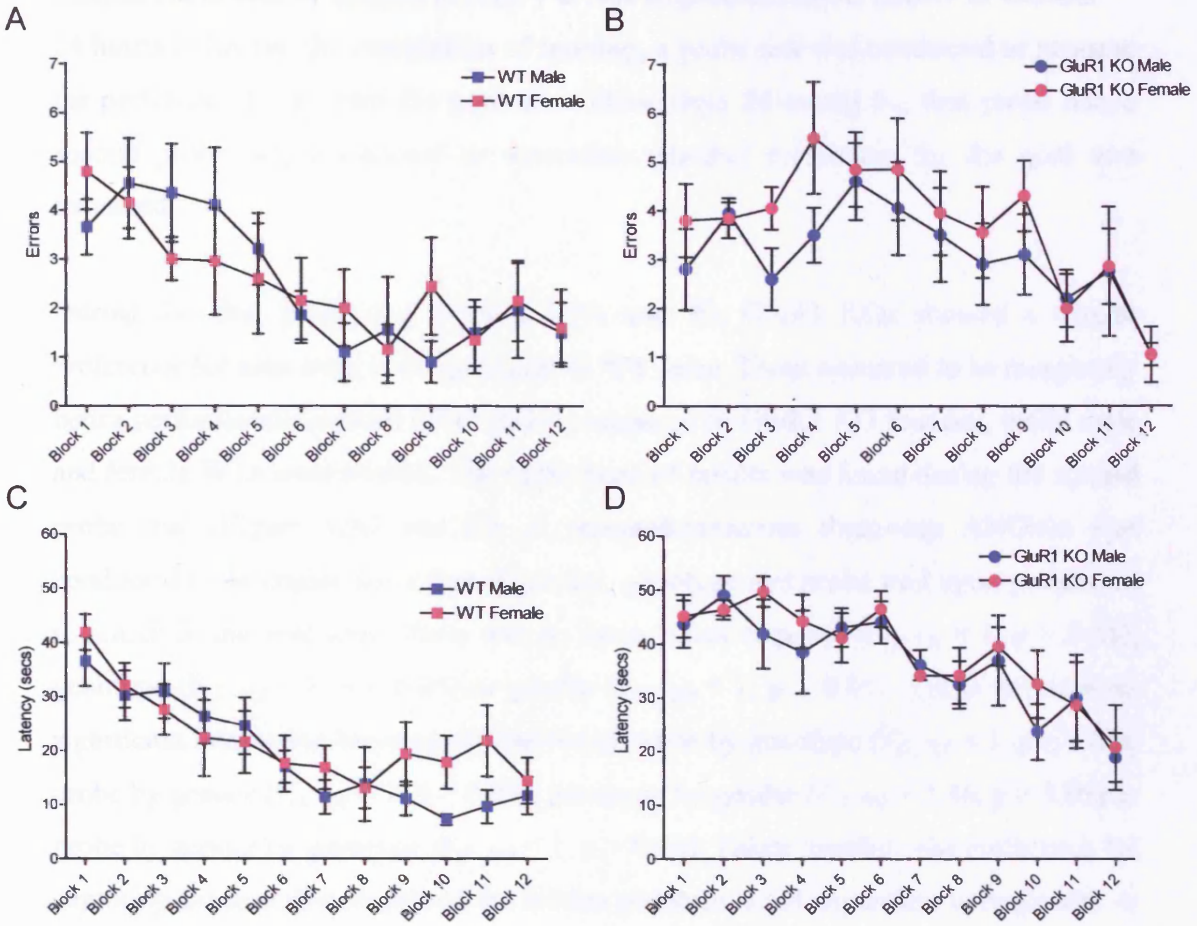


Figure 5.4. *The effect of GluR1 deletion and gender upon RAWM performance. As for Figure 5.3, but separated by gender.* ***A and B.*** *GluR1 KOs make significantly more arm entry errors during training than WTs. However, gender was not a factor in either WT or GluR1 KO performance. Both male and female GluR1 KOs make similar errors to male and female WTs at the end of training.* ***C and D.*** *A similar trend was found for the latency to find the hidden platform. GluR1 KOs took significantly longer to find the hidden platform during the middle of training, although there was no difference compared to WTs at the end of training. Gender was not a significant factor in the latency measure.*

5.3.3.2. Retention of Spatial Memory is Not Dependent upon GluR1 or Gender

24 hours following the completion of training, a probe test was conducted to measure the preference to the visit the goal arm. Three days following the first probe test, a second probe was conducted to determine whether preference for the goal arm remained.

During the first probe trial (Figure 5.5A and B), GluR1 KOs showed a similar preference for searching in the goal arm as WT mice. There appeared to be marginally better performance in GluR1 KO males compared to GluR1 KO females, while male and female WTs were similar. The same trend of results was found during the second probe trial (Figure 5.5C and D). A repeated-measures three-way ANOVA was conducted to determine the effect of gender, genotype and probe trial upon preference to search in the goal arm. There was no main effect of probe ($F_{(1, 16)} < 1$, $p > 0.05$), genotype ($F_{(1, 16)} < 1$, $p > 0.05$) or gender ($F_{(1, 16)} < 1$, $p > 0.05$). There was also no significant interaction between the factors of probe by genotype ($F_{(1, 16)} < 1$, $p > 0.05$), probe by gender ($F_{(1, 16)} < 1$, $p > 0.05$), genotype by gender ($F_{(1, 16)} = 2.36$, $p > 0.05$) or probe by gender by genotype ($F_{(1, 16)} < 1$, $p > 0.05$). Taken together, the preference for exploring the arm that contained the hidden platform is not dependent upon gender or genotype. The spatial memory is also stable for a subsequent three days following the first probe in both genotypes and genders.

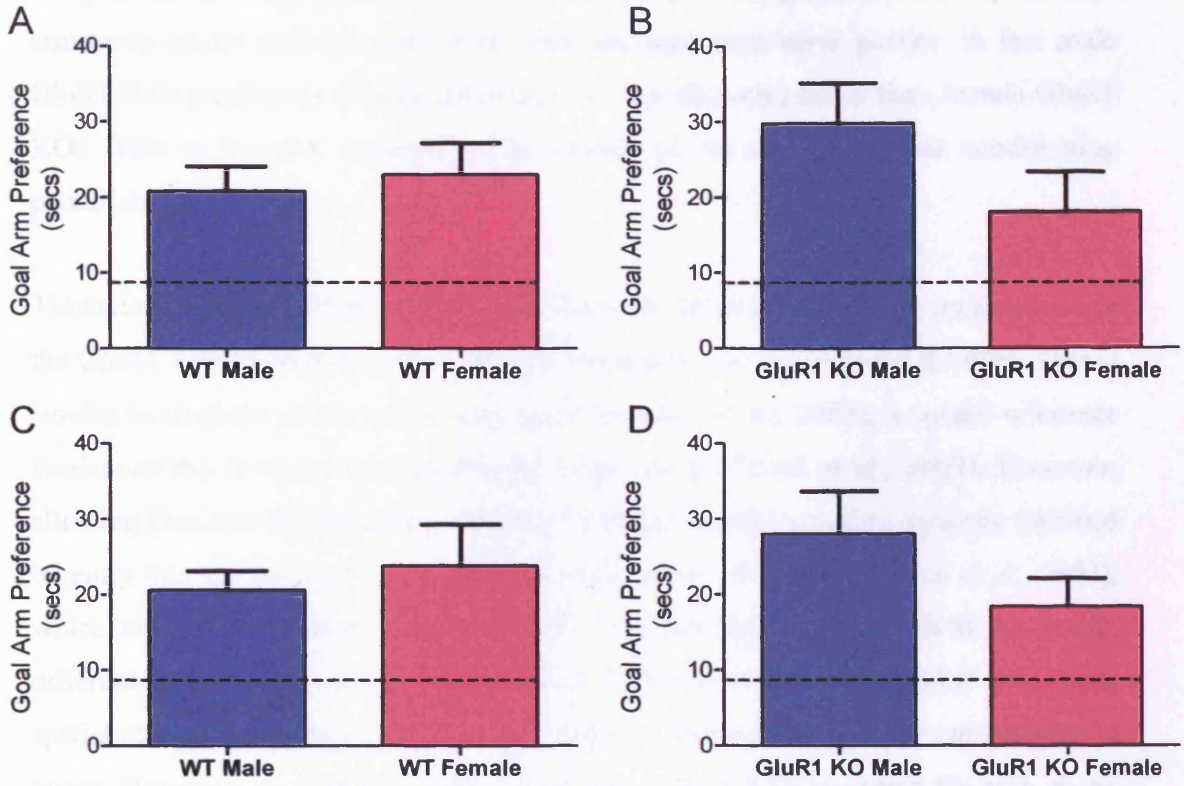


Figure 5.5. *The time spent by male and female GluR1 KOs and WTs in the goal arm during probe tests.* ***A and B.*** *24 hours following training, the hidden platform was removed and the mouse allowed free exploration of the maze for 60 seconds. The time spent in the arm that contained the hidden platform was measured. Chance was set at 8.6 seconds (dashed line).* ***C and D.*** *As for A and B, but the second probe was conducted three days following the first probe to determine whether there had been any reduction in preference for exploring the goal arm. There was no statistical effect of gender, genotype or probe trial number upon goal arm preference. Hence, GluR1 KOs of both genders have similar spatial memory of the goal arm location to WTs.*

5.3.4. Discussion

During training in the RAWM, GluR1 KOs made significantly more errors during the middle stages of training than WTs and were also significantly slower at finding the hidden platform. However, by the end of the training phase, performance was comparable to WTs. Preference for exploring the goal arm during probe tests performed 1 and 4 days following completion of training found no differences

between the genotypes. Also, performance during training as measured by latency, arm entry errors and the probe tests was not dependent upon gender; in fact male GluR1 KOs performed slightly (although not significantly) better than female GluR1 KOs. This is in stark contrast to the results of the contextual fear conditioning paradigm.

These data provide further evidence that there are dissociable memory mechanisms in the GluR1 KO. Spatial reference memory formation can occur in the RAWM. This is similar to findings in the radial arm maze (Schmitt et al., 2003), a spatial reference version of the Y-maze and the Morris water maze (Reisel et al., 2002). However, allowing free arm choice during training increased spatial working memory (defined by entry into the incorrect arms during a trial) errors (similar to Schmitt et al., 2003), which slowed acquisition until block 10. The findings in the RAWM are subtly different to previous studies. Schmitt et al. (2003) found that in a radial arm maze, spatial reference memory could not form when spatial working errors were allowed to occur. However, in a Morris water maze test, GluR1 KOs acquired the task at the same rate as WTs and probe test performance was similar (Reisel et al., 2002). It seems likely that the difference in acquisition compared to the standard water maze is related to free arm choice. Once an arm has been entered, spatial working memory must guide subsequent arm choices to prevent the mouse re entering the incorrect arm. It is known that this system is impaired in the GluR1 KO (Schmitt et al., 2003), and this is likely to be the cause of the slower GluR1 KO acquisition in the RAWM. The ability of the GluR1 mice to acquire the RAWM task to the same level as the WT mice in the present study may reflect the motivational properties of the task (escape form water as opposed to access to food reward used by Schmitt et al., 2003). Very recently, dissociations of spatial working and reference memory have been discussed in terms of short and long term habituation (Sanderson et al., 2010). This new interpretation could be relevant to the memory dissociation seen in the GluR1 KOs and will be explored in detail in the General Discussion section (Chapter 6, Section 6.8, **p**).

5.4. Experiment 3

5.4.1. Nitric Oxide Inhibition in the Radial Arm Water Maze

Previous work in this thesis has found that GluR1-independent potentiation is supported by NOS signalling (Chapter 4, Section 4.3.9.2, p. 160). It is also known that GluR1-indpendent potentiation in the hippocampus requires both isoforms of NOS (NOS1 and NOS3; Phillips et al., 2008). However, the NO-dependent forms of potentiation are most prominent one-hour post-induction (Hardingham and Fox, 2006). It is possible that the late phase of potentiation, which is NO-dependent, is responsible for spatial reference memory in the GluR1 KO. Therefore the dependence of spatial reference memory upon NO mechanisms will be tested. It is predicted that if spatial reference memory is supported by NO, then antagonism of NOS will abolish memory formation in the GluR1 KO.

5.4.2. Methods

For the RAWM minipump experiment, 16 WT and 16 GluR1 KO mice were used. These animals were split into two groups; those receiving ACSF and those receiving L-NAME. Within the ACSF group there were 5 female and 3 male WTs and 4 female and 4 male GluR1 KOs. Within the L-NAME group there were 4 female and 4 male WTs and 4 female and 4 male GluR1 KOs.

The RAWM protocol was the same as for Experiment 2. However, both WT and GluR1 KO of both genders were implanted with minipumps. Alzet osmotic (1002) minipumps were primed 24 hours before implantation in sterile saline at 37°c with either artificial cerebrospinal fluid (ACSF) or the non-specific inhibitor L-NAME at 100 mM. The osmolarity of the ACSF was altered to ~460 mOsm so it was similar to the L-NAME. Both solutions contained 0.1% trypan blue to aid visualisation of the drug delivery. Pumps were fitted with a custom designed cannula (see Materials and Methods, Section 2.5.3.2, p. 101). The pumps delivered their contents at 0.25 µl/hour.

Mice were anesthetised with Avertin and transferred to a stereotaxic frame. An incision was made along the midline for scalp, skin retracted and skull exposed. A hole in the skull was bored with a 30 gauge needle at -0.45 mm posterior and 1 mm

lateral to bregma. A pair of scissors was inserted between the shoulder blades to form a subcutaneous pocket into which the minipump was inserted. The needle of the cannula was inserted through the hole in the skull into the lateral ventricle and glued into place. The incised scalp was sutured and glued together, and the animal transferred to a hot box to recover. 48 hours postoperative recovery was allowed before testing commenced. During this time weight loss was monitored and anti-biotic powder applied to the surgery site. Following the completion of RAWM testing, the minipump was removed and confirmed for content delivery. The animals were sacrificed and brains sectioned to visually confirm drug delivery by trypan blue hippocampal staining. Brain tissue was cut at 50 μm on a microtome and mounted.

5.4.3. Results

5.4.3.1. Histology

Following the completion of RAWM testing, the mice were sacrificed and brains removed to confirm that the contents of the minipump had been delivered. All minipumps contained trypan blue, which stains cells bodies without affecting normal cell processes. The magnitude of the drug penetration into the hippocampus was estimated by visually assessing trypan blue staining through brain slices moving in the posterior axis from the cannula insertion site.

The cannula was inserted into the lateral ventricle of the right hemisphere at -0.46 mm and 1 mm lateral to bregma. The extent of the damage caused by insertion can be visualised in Figure 5.6 (left). The tract through the neocortex (motor cortex 1 and 2) into the lateral ventricle left by the 30 gauge needle is clearly visible. However, it should be noted that this is less than would have been caused if a commercially available Brain Infusion Kit (Alzet) was used, owing to the larger gauge needle. It is also noticeable that there was damage to the fimbria/fornix. This is important as lesions of the fimbria/fornix have previously been found to disrupt spatial learning during water maze tasks (Whishaw et al., 1995). Despite this, as shown below spatial reference memory of the platform location was unaffected by the minipump (Section 5.4.3.3, p. 204). It is therefore unlikely that damage caused to the fimbria/fornix prevented spatial learning. The section in the right of Figure 5.6 was taken from -2.0 mm posterior of bregma. No obvious damage occurred to the any of the hippocampal

subfields in either hemisphere. However, staining of the stratum radiatum and stratum oriens was biased to the hemisphere that contained the cannula. This suggests that drug penetration was weaker in the contralateral hippocampus, and suggests that NOS antagonism might not have been pervasive.

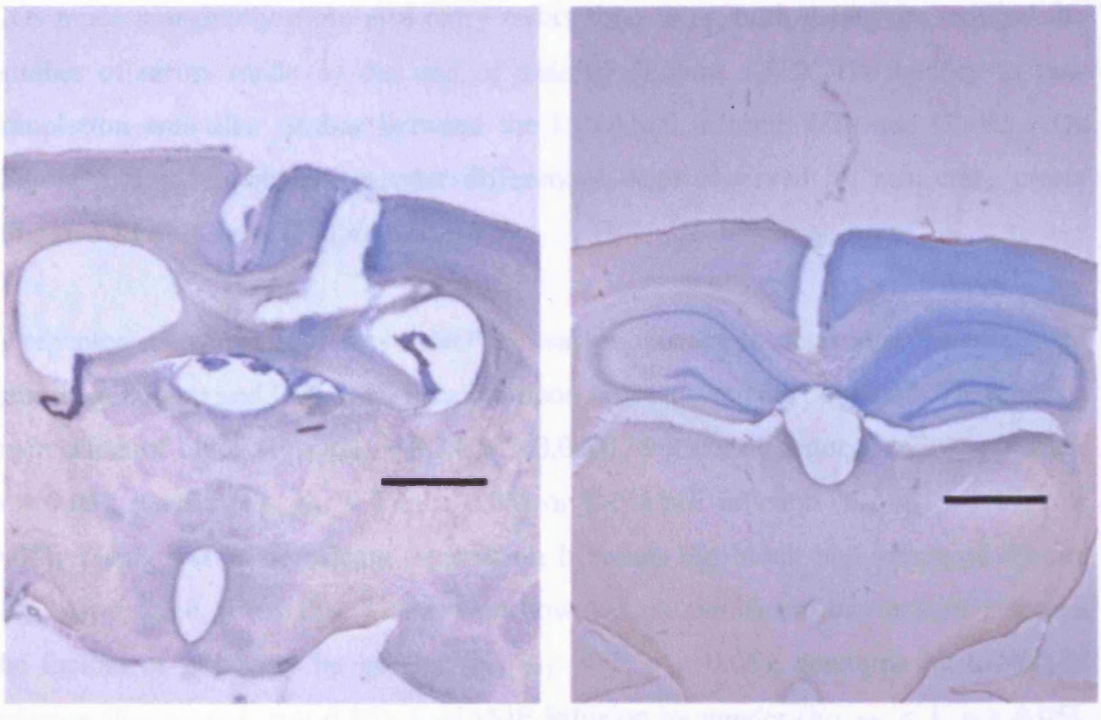


Figure 5.6. *Visualisation of the infusion of ACSF from the minipump post mortem. The ACSF or L-NAME minipumps were loaded with trypan blue to visualise the flow through the lateral ventricle. The cannula was inserted posterior into the right lateral ventricle at bregma -0.46 mm posterior and 1.0 mm lateral. The left panel, sectioned at -0.46 mm from bregma, highlights the damage caused by the cannula and drug flow. The right panel, sectioned at -2.0 mm from bregma, shows hippocampal staining by the trypan blue. The minipump successfully delivers drug to both hippocampi, although the hemisphere that contained the cannula is most strongly stained. Black scale bars represent 1 mm.*

5.4.3.2. Nitric Oxide is Not Required for Spatial Acquisition

Following the postoperative recovery period, male and female WT and GluR1 KO mice were trained in the RAWM using the same protocol as in Experiment 2.

Surprisingly, entries into incorrect arms were similar between WT and GluR1 KO mice that received ACSF (Figure 5.7A). These results are contrary to the statistically higher error rate found at during the middle of training for GluR1 KOs in Experiment 2 (Figure 5.3). The latency to find the hidden platform remained consistently higher in GluR1 KOs compared to WTs (Figure 5.7B). Mice that were infused with L-NAME were not impaired in learning the location of the hidden platform. Although GluR1 KOs made marginally more arm entry errors than WTs, both genotypes reduced the number of errors made by the end of training (Figure 5.7C). The latency to task completion was also similar between the L-NAME infused WTs and GluR1 KOs (Figure 5.7D). No obvious gender differences were observed for arm entry errors (Figure 5.8) or latency (Figure 5.9).

A repeated-measures four-way ANOVA was conducted to determine the effects of gender, genotype and L-NAME infusion upon arm entry errors per block. There was a main effect of block ($F_{(11, 264)} = 8.34$, $p < 0.0001$) but not of genotype ($F_{(1, 24)} = 1.73$, $p > 0.05$), gender ($F_{(1, 24)} < 1$, $p > 0.05$) or L-NAME infusion ($F_{(1, 24)} = 1.13$, $p > 0.05$). There was a significant interaction between the block and genotype factors ($F_{(11, 264)} = 2.70$, $p = 0.03$). There were however no significant interactions between the factors of genotype by gender ($F_{(1, 24)} < 1$, $p > 0.05$), genotype by L-NAME infusion ($F_{(1, 24)} < 1$, $p > 0.05$), L-NAME infusion by gender ($F_{(1, 24)} < 1$, $p > 0.05$), block by L-NAME infusion ($F_{(11, 264)} < 1$, $p > 0.05$), block by gender ($F_{(11, 264)} < 1$, $p > 0.05$), block by L-NAME infusion by genotype ($F_{(11, 264)} = 1.75$, $p > 0.05$), block by gender by genotype ($F_{(11, 264)} < 1$, $p > 0.05$), block by L-NAME infusion by gender ($F_{(11, 264)} = 1.37$, $p > 0.05$), genotype by L-NAME infusion by gender ($F_{(1, 24)} < 1$, $p > 0.05$) or block by genotype by L-NAME infusion by gender ($F_{(11, 264)} = 1.24$, $p > 0.05$).

To test the significant interaction between block and genotype, tests of simple main effects were performed. Significant differences between WTs and GluR1 KOs were observed at block 1 ($F_{(1, 24)} = 4.87$, $p = 0.037$) and block 6 ($F_{(1, 24)} = 11.44$, $p = 0.002$).

A repeated measures four-way ANOVA was also conducted to determine the effects of gender, genotype and L-NAME infusion upon the latency to find the hidden platform during training. There was a main effect of block ($F_{(11, 264)} = 19.65$, $p < 0.0001$) and genotype ($F_{(1, 24)} = 7.71$, $p = 0.01$) but not of gender ($F_{(1, 24)} = 4.04$, $p = 0.056$) or L-NAME infusion ($F_{(1, 24)} = 1.57$, $p > 0.05$). There was no significant interaction between the factors of block by genotype ($F_{(11, 264)} = 1.54$, $p < 0.05$), genotype by gender ($F_{(1, 24)} < 1$, $p > 0.05$), genotype by L-NAME infusion ($F_{(1, 24)} = 1.49$, $p > 0.05$), L-NAME infusion by gender ($F_{(1, 24)} < 1$, $p > 0.05$), block by L-NAME infusion ($F_{(11, 264)} < 1$, $p > 0.05$), block by gender ($F_{(11, 264)} = 1.19$, $p > 0.05$), block by L-NAME infusion by genotype ($F_{(11, 264)} = 1.69$, $p = 0.075$), block by gender by genotype ($F_{(11, 264)} < 1$, $p > 0.05$), block by L-NAME infusion by gender ($F_{(11, 264)} = 1.68$, $p = 0.077$), genotype by L-NAME infusion by gender ($F_{(1, 24)} < 1$, $p > 0.05$) or block by genotype by L-NAME infusion by gender ($F_{(11, 264)} < 1$, $p > 0.05$).

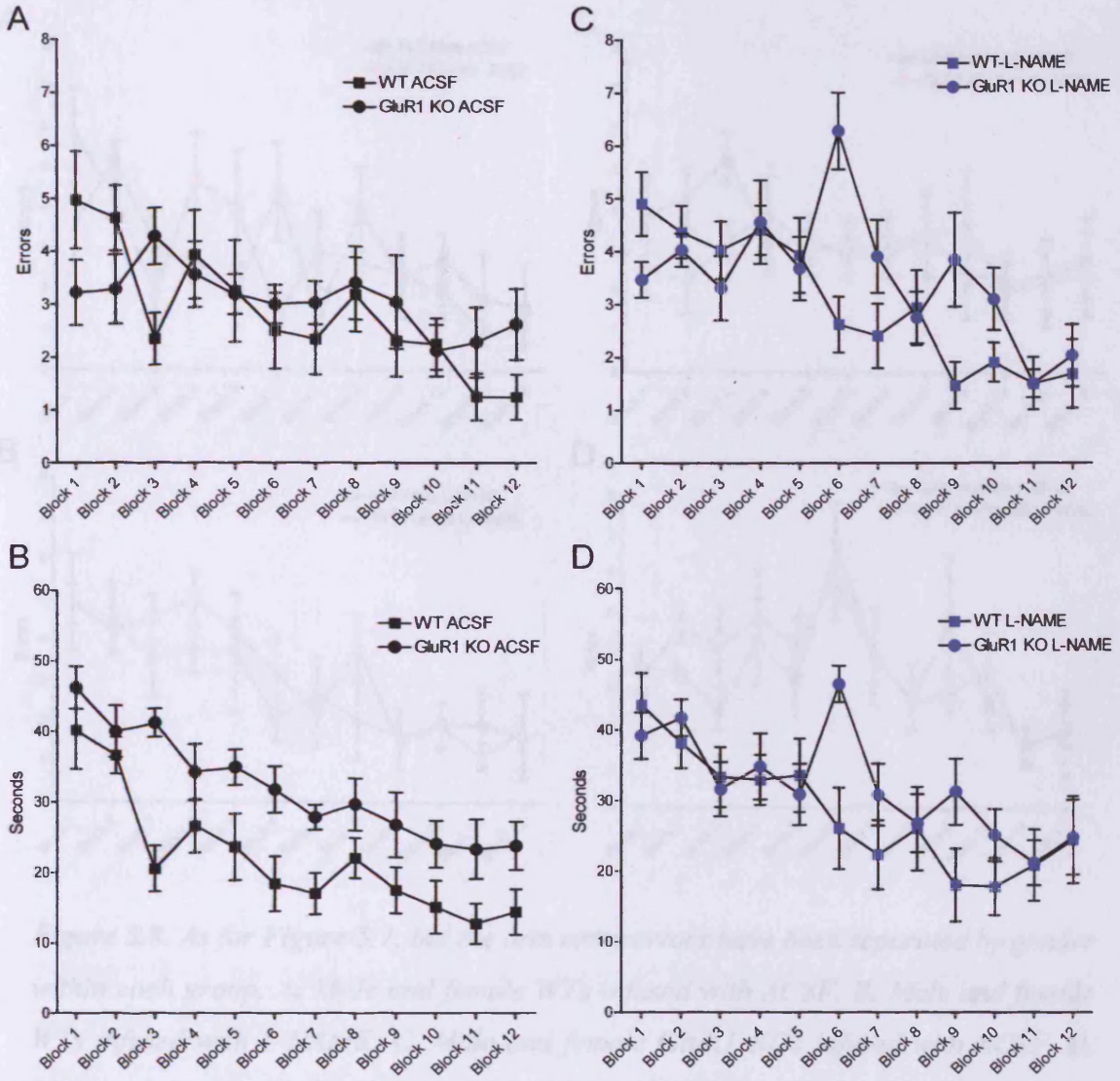


Figure 5.7. *Learning is not impaired by the application of the NOS inhibitor L-NAME.* ***A and B.*** *Errors and latency from both genotypes implanted with ACSF minipumps reduced as training progressed in the RAWM. This pattern was repeated in animals implanted with minipumps infusing the NOS inhibitor L-NAME (**C and D**). A repeated measures four-way ANOVA revealed that neither the drug manipulation or gender were factors in RAWM learning. Genotype was a significant factor for the latency measure but not for the number of errors made. There was however a block by genotype interaction for the number of arm entry errors made.*

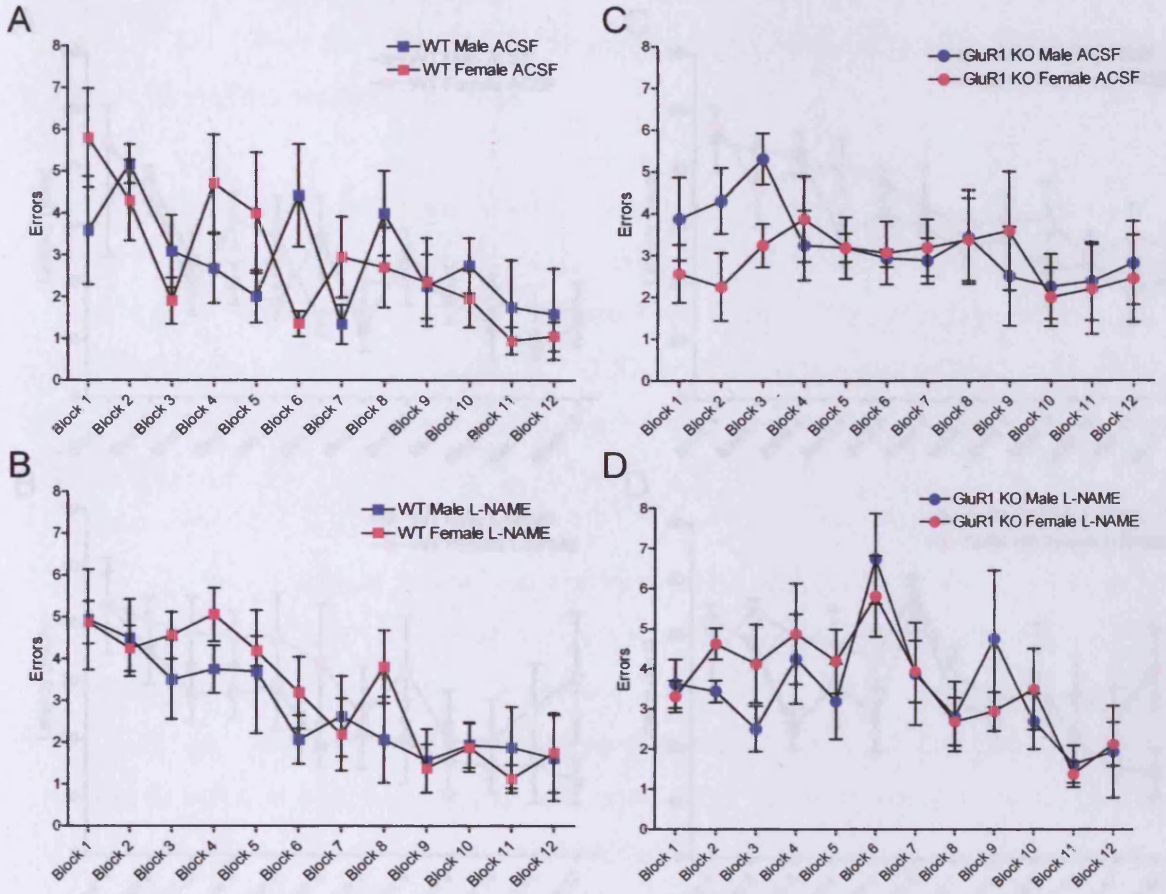


Figure 5.8. *As for Figure 5.7, but the arm entry errors have been separated by gender within each group.* ***A.*** *Male and female WTs infused with ACSF.* ***B.*** *Male and female WTs infused with L-NAME.* ***C.*** *Male and female GluR1 KOs infused with ACSF.* ***D.*** *Male and female GluR1 KOs infused with L-NAME. There was a significant main effect of block and a significant interaction between block and genotype. L-NAME and gender did not influence the number of arm entry errors made.*

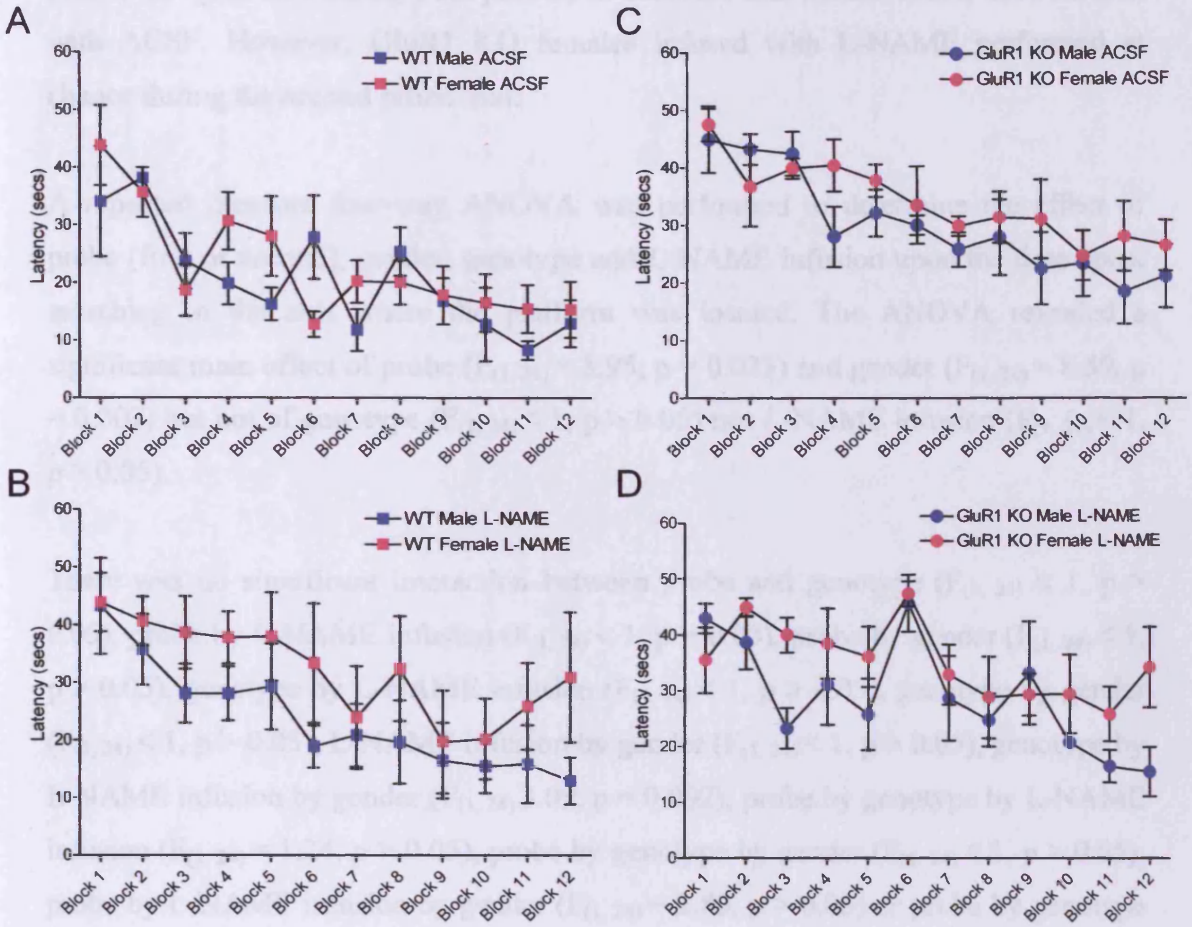


Figure 5.9. *As for Figure 5.7, but the latency to find the hidden platform has been separated by gender.* ***A.*** *Male and female WTs infused with ACSF.* ***B.*** *Male and female WTs infused with L-NAME.* ***C.*** *Male and female GluR1 KOs infused with ACSF.* ***D.*** *Male and female GluR1 KOs infused with L-NAME. A significant main effect of block and genotype was found, although treatment with L-NAME and gender did not affect performance.*

5.4.3.3. Nitric Oxide Antagonism and Retention of Spatial Reference Memory

24 hours after the completion of RAWM training, the mice were subjected to a probe test for 60 seconds. A second probe test was conducted 3 days following the first. Figure 5.10 shows the results of the first and second probe separated by gender. Male WT mice that were infused with ACSF had a slightly higher preference to explore the arm that had contained the hidden platform during the first and second probe compared to female WTs. Male and female WTs infused with L-NAME spent similar

time in the goal arm during both probes, as did male and female GluR1 KOs infused with ACSF. However, GluR1 KO females infused with L-NAME performed at chance during the second probe trial.

A repeated measure four-way ANOVA was performed to determine the effect of probe (first or second), gender, genotype and L-NAME infusion upon the time spent searching in the arm where the platform was located. The ANOVA revealed a significant main effect of probe ($F_{(1, 24)} = 5.95$, $p = 0.023$) and gender ($F_{(1, 24)} = 8.59$, $p = 0.007$) but not of genotype ($F_{(1, 24)} < 1$, $p > 0.05$) nor L-NAME infusion ($F_{(1, 24)} < 1$, $p > 0.05$).

There was no significant interaction between probe and genotype ($F_{(1, 24)} < 1$, $p > 0.05$), probe by L-NAME infusion ($F_{(1, 24)} < 1$, $p > 0.05$), probe by gender ($F_{(1, 24)} < 1$, $p > 0.05$), genotype by L-NAME infusion ($F_{(1, 24)} < 1$, $p > 0.05$), genotype by gender ($F_{(1, 24)} < 1$, $p > 0.05$), L-NAME infusion by gender ($F_{(1, 24)} < 1$, $p > 0.05$), genotype by L-NAME infusion by gender ($F_{(1, 24)} 3.09$, $p = 0.092$), probe by genotype by L-NAME infusion ($F_{(1, 24)} = 1.24$, $p > 0.05$), probe by genotype by gender ($F_{(1, 24)} < 1$, $p > 0.05$), probe by L-NAME infusion by gender ($F_{(1, 24)} = 2.83$, $p > 0.05$) or probe by genotype by L-NAME infusion by gender ($F_{(1, 24)} < 1$, $p > 0.05$).

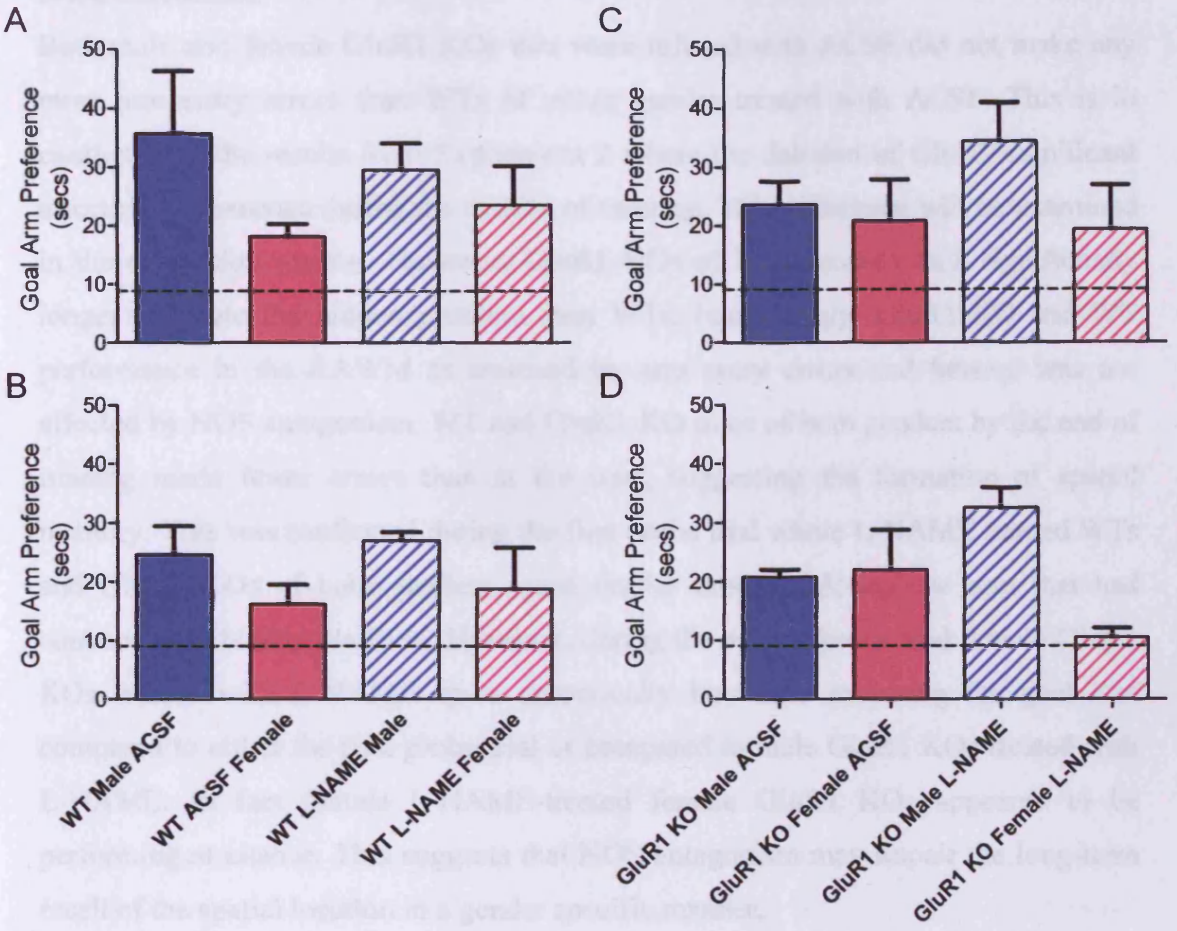


Figure 5.10. *NO inhibition affects spatial reference memory in female GluR1 KOs.* ***A and B.*** *Spatial reference memory was challenged in WT mice by a probe trial 24 and 96 hours following the end of training. Male WT mice infused with ACSF performed marginally better than female WT mice infused with ACSF during the first (**A**) and second (**B**) probe, although the memory of the platform location was not inhibited in either gender. Spatial reference memory formation was similar to controls when NOS was antagonised in male and female WTs.* ***C and D.*** *Time spent in the goal arm was strikingly similar between male and female GluR1 KOs infused with ACSF across both probe trials. Male GluR1 KOs that were infused with L-NAME performed slightly better than controls of both gender in both probe trials. However, although goal arm preference was similar to controls in the first probe, it was at chance during the second probe (**D**).*

5.4.4. Discussion

Both male and female GluR1 KOs that were infused with ACSF did not make any more arm entry errors than WTs of either gender treated with ACSF. This is in conflict with the results from Experiment 2 where the deletion of GluR1 significant affected performance during the middle of training. This difference will be examined in the discussion section. However, GluR1 KOs of both genders took significantly longer to locate the hidden platform than WTs. Interestingly, GluR1 KO and WT performance in the RAWM as assessed by arm entry errors and latency was not affected by NOS antagonism. WT and GluR1 KO mice of both genders by the end of training made fewer errors than at the start, suggesting the formation of spatial memory. This was confirmed during the first probe trial where L-NAME treated WTs and GluR1 KOs of both genders spent similar times exploring the arm that had contained the hidden platform. However, during the second probe trial female GluR1 KOs treated with L-NAME spent numerically less time exploring the goal arm compared to either the first probe trial or compared to male GluR1 KOs treated with L-NAME. In fact female L-NAME-treated female GluR1 KOs appeared to be performing at chance. This suggests that NOS antagonism may impair the long-term recall of the spatial location in a gender specific manner.

This pattern of result is interesting but should be regarded with caution given the low numbers of subjects in each gender in each condition and clearly requires replication. Furthermore, the pattern is somewhat surprising for two reasons. First, potentiation has been shown to be abolished when GluR1 and NOS have both been inhibited, regardless of gender (Chapter 4, Section 4.3.10.1, p. 165). Yet spatial reference memory can form in both female and male GluR1 KOs in the presence of NOS antagonists. Second, although females can form spatial reference memory, its long-term stability seems susceptible to NOS manipulation. In all manipulations so far performed in the RAWM (Experiment 2 and 3), female GluR1 KOs perform marginally worse than GluR1 KO males. It is possible that spatial reference memory is generally less stable in the female KO, although NOS-related plasticity could be required to support the spatial memory beyond 24 hours following completion of training in female KOs (Figure 5.10D). It is also possible that the GluR1-independent spatial memory relies more upon NOS signalling in the female GluR1 KO. This could potentially account for the contextual freezing result in Experiment 1 and the decrease

in goal arm preference in Experiment 3 when NOS was antagonised. Very recently it has been found that NO inhibition does not affect spatial memory recall in a water maze during a probe trial 24 hours following training, yet performance was at chance during a second probe performed 7 days after training (Tanda et al., 2009). Although the results presented in Experiment 3 suggest that GluR1 and NOS inhibition have to occur together for spatial memory to be impaired, it is possible that NOS-dependent plasticity in the GluR1 KO is important for the stability of "remote memories" (Tanda et al., page 11) not simply spatial reference memory assessed one day after training.

5.5. General Discussion

Male GluR1 KO mice were impaired during recall of the fear conditioned context (Experiment 1), which is similar to the findings of others (Humeau et al., 2007). It is possible that the deficit in short-term hippocampal plasticity in the GluR1 KO (Phillips et al., 2008) could prevent the rapid encoding of the spatial context (Sanderson et al., 2010), preventing memory formation. Despite this, female GluR1 KO conditioning was comparable to WTs. The present study showed that using the RAWM procedure, GluR1 KO mice are impaired in acquisition of a reference memory procedure when they have the opportunity to make working memory errors. This is similar to the findings reported by Schmitt et al. (2003). These authors showed that using a food motivated radial arm task, GluR1 KO mice showed greater reference memory errors if the were allowed to commit working memory errors. In contrast to the Schmitt et al. (2003) study, our results differ in that GluR1 KO mice eventually acquired the task to the same level at WT mice. Experiment 2 was conducted in two replications, suggesting that the findings are legitimate. Although GluR1 KOs in Experiment 3 did not show a clear GluR1 phenotype, this is likely due to the minipump implantation causing a further locomotor disruption in addition to that already known of in the GluR1 KO (Bannerman et al., 2004). In addition the results presented in Experiment 2 has shown that gender does not interact with the performance of GluR1 mice in contrast to the context-fear conditioning results reported in Experiment 1. Finally, the results show that inhibition of NO does not exacerbate the acquisition deficit shown by GluR1 KO mice in the RAWM task. However, female GluR1 KO mice appear to performed at chance when tested in a probe trial 4 days after the completion of training, suggesting that NO-related

plasticity may contribute to the long-term stability of spatial memory (Experiment 3). Taken together, these results suggest that although spatial working memory and short-term habituation is GluR1-dependent, GluR1 does not support spatial reference memory. While a large component of late-phase LTP is NO-dependent (Phillips et al., 2008), the contribution of NO signalling to spatial reference memory it is not entirely clear.

The full implications of these results will be considered in the General Discussion.

Chapter 6:

General Discussion

6.1. General Discussion

The first section of this chapter revisits the aims of the thesis followed by a discussion of the important findings and their theoretical implications. The results are then discussed in terms of the mechanisms that could be taking place to drive synaptic plasticity.

6.2. Aims

The general purpose of the study was to investigate the role of the AMPA receptor GluR1 and nitric oxide (NO) in synaptic plasticity. The criticism was raised that the role of the GluR1 subunit has only thus far be studied in juvenile models (~1 month of age) and very few have used electrophysiology to characterise its requirement in naturally occurring plasticity processes *in vivo*. Activity dependent GluR1-insertion during development has been associated with the reduction in silent synapses (Rumpel et al., 2004), and it has been proposed that plasticity is only dependent upon GluR1 until P42 (Jenson et al., 2003).

The first aim was to use *in vivo* electrophysiology to determine whether the barrel cortex could develop normally in the absence of GluR1 and NO. GluR1 and NO are both potentially implicated in activity-dependent synaptic processes, and their removal could result in abnormal development similar to NMDAR antagonism (Fox et al., 1996).

GluR1 is required for neocortical LTP (Hardingham and Fox, 2006) and experience-dependent plasticity (EDP) using the mouse barrel cortex (Clem and Barth, 2006). LTP in the GluR1 KO has been characterised in the barrel cortex and the hippocampus. While GluR1 KO mice show a deficit in the early phase of LTP, the magnitude of LTP was comparable to WT mice at 1 hour post-induction (Hardingham and Fox, 2006; Phillips et al., 2008). However it is not known to what extent the deletion of GluR1 affects the magnitude of adult *in vivo* experience-dependent potentiation of the spared whisker response. Therefore the second major aim was to assess the magnitude of potentiation following whisker deprivation in adult GluR1 KO mice. GluR1-independent LTP was completely sensitive to NOS antagonism in the neocortex (Hardingham and Fox, 2006) and partially in the hippocampus (Phillips

et al., 2008; Romberg et al., 2009). Thus the third major aim was that if experience-dependent potentiation occurred in the barrel cortex of GluR1 KO mice, then similar to the LTP studies, was the GluR1-independent potentiation supported by NOS signalling? And if it was, which NOS isoform, if any, played a more significant role in *in vivo* potentiation? In addition to aims two and three, estrogen has been show to modulate both NOS1 (d'Anglemont et al., 2009) and GluR1 (Srivastava et al., 2008) following activity-dependent processes, suggesting that potentiation could be influenced by gender. As a result experience-dependent potentiation will be examined in both male and female mice of all genotypes.

GluR1 is also required for learning and memory, another form of experience-dependent plasticity. Contextual learning (Matsuo and Mayford, 2008) and spatial learning (Reisel et al., 2002; Schmitt et al., 2003) both require hippocampal GluR1. Despite this, GluR1-independent forms of learning exist (Schmitt et al., 2003). Hippocampal GluR1-independent LTP has been shown to be NO-dependent (Phillips et al., 2008) and aims two and three should reveal whether this plasticity is relevant to *in vivo* mechanisms. It is therefore possible that GluR1-independent learning could depend upon NOS signalling. Thus, aim four will be to establish a form of memory that is GluR1-independent and then test whether it is susceptible to NOS antagonism.

6.3. Main Findings

Barrel cortex development was assessed by anatomical measurements of barrel field size and area, and electrophysiological measurements of principle whisker spike latency, receptive field and the confinement of short latency responses to their topographically related barrel column. Development was not affected by the absence of GluR1 and/or NOS by any of these measures. Thalamocortical afferant (TCA) targeting of layer IV, as assessed by barrel size, area and response latency was comparable in all genotypes. The receptive fields of layer II/III and layer IV were also comparable across genotypes, suggesting that intracortical transmission was unaffected. Hence, GluR1 and NOS are not necessary for activity-dependent barrel cortex formation and development.

Barrel cortex experience-dependent potentiation was assayed by depriving all of the whiskers on the snout except for the D1 whisker for 18 days followed by 6-11 days regrowth to allow whisker for stimulation. Single units were recorded from layer II/III in the barrel columns surrounding the spared D1 barrel. Under control conditions the intracortical connections emanating from the D1 column exert little influence on the receptive field (Fox, 1992). However following deprivation, these responses become strongly biased by the spared whisker input (Fox, 1992). Subjects that underwent single unit recording were aged approximately 5 months of age (range 2-10 months). In WTs, spared whisker responses increased ~4 fold compared to control conditions and is similar to previously reported changes for that age (Glazewski et al., 1996). GluR1 KOs were also able to undergo spared whisker potentiation, but this was 36% lower than the response magnitude reached by WTs. Despite this, shifts in the vibrissae dominance following deprivation were not as convincing (see Chapter 4 Discussion, Section 4.4). Therefore it is possible that GluR1 is required for adult experience-dependent potentiation, although further work is required to confirm this suggestion.

I also established that NO is also required for the full expression of EDP. Mice that had either the neuronal form of NOS (αNOS1) or endothelial NOS (NOS3) knocked out both exhibited an approximate 2.5 fold increase in D1 whisker response following deprivation. This was similar to that achieved by GluR1 KO mice. However, the potentiation in the NOS1 KO mice was related to gender. Female NOS1 KOs were able to reach near WT levels of plasticity (6% lower than females WTs). Male NOS1 KO plasticity was considerably lower than that of either WT plasticity (60% lower than male WTs) or female NOS1 KO EDP (49% lower than female NOS1 KO EDP). Compared to control littermates, no significant potentiation was observed in male NOS1 KOs.

Having established that NO contributed to EDP, I tested the hypothesis that the residual plasticity shown by GluR1 KO mice was NO-dependent. NOS1 and NOS3 mutants were bred with GluR1 mutants to form a double KO mouse (GluR1/NOS1 KO and GluR1/NOS3 KO). Significant plasticity was found in the GluR1/NOS3 double KO (447% increase in the spared whisker response) whereas only a 19%

increase occurred in the GluR1/NOS1 KO. Hence no significant plasticity occurred in the GluR1/NOS1 KO.

This evidence suggests that GluR1 and NOS signalling provide a complementary process of supporting plasticity in the barrel cortex. However NO produced by NOS1 completely abolished potentiation when combined with the GluR1 KO, whereas NOS3 did not. I can conclude therefore that NOS1 is more important for EDP than NOS3 (Chapter 4, Section 4.3.10.1). This finding cannot be easily explained by developmental abnormalities. Despite small variances, no mutant had a depressed or expanded surround receptive field in control conditions that would preclude or exaggerate plasticity (Figure 3.7, p. 121).

Technical difficulties in measuring the critical role played barrel cortex plasticity in behaviour led me to consider whether GluR1 and NO play a similar role in hippocampal plasticity processes. The role of GluR1 in hippocampal dependent tasks was also assessed. In the first instance I examined whether GluR1 KO mice could acquire a simple context-fear conditioning task. Consistent with previous reports (Humeau et al., 2007), male GluR1 KOs showed little freezing response when tested in the context 24 hours following conditioning. In contrast, I observed that GluR1 females froze to a similar magnitude as WTs of both genders (Chapter 5, Section 5.2.3.2, p. 186). Locomotor activity or similar non-specific effects alone cannot explain the difference between the dimorphic conditioning in the GluR1 KO. Male and female GluR1 KOs both had very similar activity profiles during conditioning, with freezing increasing following each successive unsignalled footshock (US) delivery, signifying some perception of the mild shock. Although the freezing was less than WTs during conditioning, female GluR1 KO conditioned response (CR) was similar to that of WTs, suggesting the lack of freezing in males KOs was due to a specific memory impairment.

Mice were tested in a water maze procedure (RAWM) that mirrored the food reinforced radial maze task used by Schmitt et al. (2003). Male and female GluR1 KOs made more memory errors during acquisition training, although after 3 days of trainings they were comparable to WTs. Furthermore, the GluR1 KO mice showed comparable retention of the correct goal arm location 24 hrs and four days following

training (Figure 5.5, p. 195). In contrast to the lack of synaptic plasticity, acquisition of the RAWM task was not further disrupted in the GluR1 KO mice by NOS inhibition (Chapter 5, Section 5.4.3.3, p. 204). Although the level of NOS inhibition was not confirmed in the present study, the lack of effect on the RAWM confirms earlier reports that NO does not play a role in spatial learning (Bannerman et al., 1992). Despite this, female GluR1 KOs that had NOS antagonised appeared to perform at chance in the second probe test, a trend not observed in male GluR1 KOs.

6.4. Barrel Cortex Development Does Not Require GluR1 or Nitric Oxide

Chapter 3 investigated the effect of mutations of the NOS genes and GluR1 on the ability to develop a 'normal' barrel field using morphological and synaptic transmission analysis. NMDA receptors, adenylyl cyclase and the PKA RIIβ subunit are a number of synaptic molecules that have been shown to be required for the full development of the barrel system. The receptors/signalling molecules investigated in this study (GluR1 and NOS) have all been implicated in activity-dependent signalling processes (Garthwaite et al., 1988; Hayashi et al., 2000). It is therefore possible that removal of subunits that mediate the transmission of activity could result in abnormal development. Of further relevance, some subplate neurons have been found to be NADPH-diaphorase positive, implicating NO in refinement (Finney et al., 1998).

Measurement of the individual barrels and the length of the D row revealed no differences between mutants and controls. This therefore suggests that barrel formation is not affected by the absence of NOS or GluR1. Other studies have replicated these results. GluR1 KOs (Watson et al., 2006; Wright et al., 2008), NOS1 KOs and NOS3 KOs (Finney and Shatz, 1998) all had their barrels processed in the same manner and no differences were found compared to WT controls. The reason that GluR1 is not required is likely due to barrel patterning occurring before the GluR1 subunit is expressed. The GluR1 subunit is first measurable at P4, yet at this point in development the barrel field is already topographically formed (Watson et al., 2006). P4 also coincides with the end of the critical period of layer IV (Fox, 1992). Hence, layer IV forms before GluR1 is expressed, which is why GluR1 is unlikely to be required. In the Finney (1998) study, both NOS1 and NOS3 KO also had NOS antagonist applied, making the requirement of NO in development very unlikely.

Therefore, although NADPH-diphorase positive subplate neurons have been identified, either they are too few to affect gross morphology, or they are not required for barrel cortex formation.

Although layer IV barrels can form in the absence of GluR1 and NOS, it is not specifically known whether connections to layer IV occur normally. For example, if NMDAR activity is inhibited shortly (no more than 6 hours) after birth, the layer IV barrels develop normally (by cytochrome oxidase staining; Schlaggar et al., 1993). However, the TCAs targeted multiple barrels across the barrel field as opposed to the normal single whisker to barrel arrangement (Fox et al., 1996). Hence, the absence of GluR1 and NOS could affect the TCA targeting of layer IV despite the visible presence of layer IV barrels.

Measurement of the response latencies from stimulating surrounding whiskers suggests that in both single and double GluR1 and NOS KOs, the TCA targeting of layer IV was normal. Responses to D1 stimulation when the D1 was the PW were generally under 10 ms as expected (Armstrong-James et al., 1992). However, then D1 was stimulated as a surround whisker (that is, the electrode was positioned in a barrel column that surrounded the D1 barrel column) responses were generally above 10 ms. If TCA targeting was disrupted as in the NMDAR inhibitor experiments, short latency responses (<10 ms) to D1 stimulation might have been found in barrel columns that were not the D1 barrel. This was not the case and this trend was not affected by deletion of GluR1 and/or NOS. Transmission to the barrels was also not affected. Latency measurements in layer IV during PW stimulation found no significant differences between the genotypes. Taken together, GluR1 and NOS is not required for layer IV barrel formation by any of these measures. This confirms previous findings that synaptic transmission was normal in young GluR1 KOs (Wright et al., 2008) and double GluR1/NOSX KOs (although synaptic transmission was measured in the hippocampus; Phillips et al., 2008). These results further highlight that although NADPH-diphorase positive subplate neurons exist in the rodent (Finney and Shatz, 1998), their antagonism is unlikely to disrupt neocortical patterning. Similarly, layer IV patterning is complete prior to the GluR1 being detectable (Watson et al., 2006), arguing against a role for GluR1.

6.5. Receptive Fields

Although GluR1 may not be required for barrel cortex patterning, the insertion of AMPARs into the postsynapse is required to form 'active synapses' (that is, non silent synapses). The refinement of connections via LTP and LTD-like processes to form mature, active synapses is likely to be dependent on this process of AMPA insertion (for review see Kerchner and Nicoll, 2008); indeed silent synapses are rare beyond one month of age (Hardingham and Fox, 2006). Considering the topographic map of layer II/III matures at P14 (Stern et al., 2001), GluR1 could be required for connection refinement. A similar role could also exist for NOS. Deficits in activity-dependent layer IV and II/III refinement would most likely be evident within the receptive field of those layers.

The morphology data can only provide a direct insight to the development of layer IV. Layer II/III, with its strong horizontal connections, cannot be revealed by cytochrome oxide staining. Hence electrophsyiological characterization is the most appropriate measure of 'normal' development. In general, the results show very similar patterns across all genotypes and layers. Unlike the morphology and latency analysis, however, subtle visual differences can be observed (Figure 3.7). Although no significant main effects were found, visible expansion of the layer II/III receptive field in the NOS1 and NOS3 KOs can be observed in surround whiskers 2, 3, 4 and 5 (Figure 3.7A). This trend was not evident in layer IV. Conversely, there appears to be depression of the layer IV receptive field of the GluR1/NOS double KOs at surround 1, 2, 3, 4 and 5 (Figure 3.7B). Again, this trend was not observed in layer II/III. Despite there being no significant differences, this variance warrants further discussion. These differences suggest two possible mechanisms. First, horizontal layer II/III connections could have a wider/narrower reach (transmission through the layer) and second, layer IV to II/III connections could be broader/narrower than normal (transmission up and/or across the column). To the best knowledge of the author, there is no mutation that affects layer II/III without disrupting layer IV also, although assessment via the methods used in Chapter 3 is rarely attempted. In this case, changes in layer II/III cannot easily be attributed to layer IV.

The enlargement/reduction of receptive field at only some surrounds in layer II/III but not layer IV (or visa versa) is a very specific effect. A less surprising phenotype

would have been large-scale changes across all surround whiskers. However, as NADPH-diphorase positive subplate neurons have been identified (Finney et al., 1998), albeit in low numbers, and a role for these has so far been elusive, it cannot be ruled out that NOS signalling is associated with fine tuning layer II/III connections. This could be the reason for slightly larger receptive fields in the NOS KOs.

Conversely, there was a trend for receptive fields to decrease in size when GluR1 KO was combined with removal of either NOS isoform. GluR1 KO receptive fields were broadly similar to WTs, except for a slight depression at surround 1 in layer II/III. Considering that in the single NOS KOs the receptive field was slightly larger, it seems curious that the receptive fields were smaller in the double GluR1/NOS KOs. Although simplistic, one could assume that in the double KO mouse, the expanded receptive field of the single NOS KO would have negated the contraction of the GluR1 KO receptive field, which was not the case. It is possible that both GluR1 and NO play a very minor role in cortical refinement, which is only exposed when both molecules are knocked out.

So far, a similar assessment has only been published in the GluR1 KO, where no differences were found by any protocol employed (Wright et al., 2008). However, those animals were considerably younger than the ones used here (P28 vs ~P150). It could be that as age increases the receptive fields become smaller in GluR1 KOs. It has been suggested that hippocampal CA3-CA1 synaptic transmission is attenuated in GluR1 KOs of ~P180 (Romberg et al., 2009), which is broadly similar in age and background to the ones used in the present study. However there is no evidence from this study to suggest that this is the case for the neocortex. There is, however, the possibility that GluR1 and NOS are both required for full receptive field development (layer II/III horizontal transmission), and differences can only be observed when both molecules are absent. If there are reductions in the double GluR1/NOS KO receptive field, then this would disadvantage that genotype from undergoing potentiation. The receptive field represents the intracortical transmission under control conditions; a contracted receptive field would suggest less intracortical transmission. This intra-barrel communication is critical for potentiation, as it is these connections that undergo changes to synaptic efficacy. Hence, mutants with a contracted receptive field could be disadvantaged from potentiating, and conversely mutants with an

expanded receptive field could be advantaged. Despite this, it is shown in Chapter 4 (Section 4.3.8.2) that significant and robust plasticity can occur in a double mutant of GluR1 and NOS3, so this slight difference in receptive field does not prevent potentiation.

Nonetheless, it is unlikely that these minor differences equate to major developmental abnormalities. These results corroborate the Wright et al. (2008) findings that receptive field between WTs and GluR1 KOs were normal, and extends previous studies by demonstrating that NOS KO animals can develop normal receptive fields (see Finney and Shatz, 1998). Major or even minor differences found in layer IV would provide a simple explanation as to deficiencies within layer II/III, yet this was not found in any mutant mouse. To confirm whether the slight differences in layer II/III receptive field size was related to genotypic differences in intracortical connections, a study could be conducted to trace layer IV to II/III connections and layer II/III to II/III horizontal connections. Reduced intrabarrel connectivity would explain the contracted receptive field and theoretically could result in less opportunity for potentiation. Finally, it is possible that recording stability and/or anaesthesia depth could have varied between the genotypes. This is discussed in Section 6.9 (p. 226) and represents the most likely explanation for the genotypic variances. Until further research is performed, it is reasonable to assume that the differences reflect changes in animal state during the recordings and not developmental retardation. Therefore deficiencies in EDP cannot easily be explained by receptive field development.

6.6. The Role of GluR1 in *in vivo* Plasticity

Given that previous studies had highlighted a role for GluR1 in plasticity, I sought to do test experience-dependent plasticity (EDP) in the absence of the GluR1 subunit. Importantly GluR1 KOs exhibited a response magnitude to D1 stimulation that was 36% lower than that of WTs. For the first time this result demonstrates that GluR1 is required for full neocortical EDP in the whole animal at adult ages. Previous studies have proposed a requirement for GluR1 for EDP following single whisker experience (Clem and Barth, 2006). Another study demonstrated that with chessboard deprivation, potentiation could occur in the absence of GluR1 but the response magnitude in the KO was slightly less than that of WTs (Figure 2B, Wright et al.,

2008). The ages used in those studies were considerably lower than this thesis at P14 (Clem and Barth, 2006) and P28 (Wright et al., 2008). Therefore the mechanisms of GluR1 trafficking/potentiation discovered in young animals appears relevant to adult potentiation.

There is one striking difference between my results and previous *in vitro* LTP studies in the GluR1 KO. Virtually every LTP study in both the neocortex and the hippocampus has found that one hour after LTP induction, the magnitude of plasticity between GluR1 KOs and WTs was the same. Given that late-stage LTP was unaffected in the KO, one might presume that EDP could reach a similar level to WTs. This was not the case. Developmental abnormalities cannot explain this decrease in plasticity and studies *in vitro* have found that input/output relationships (a measure of synaptic transmission) are comparable to WTs (Hardingham and Fox, 2006; Phillips et al., 2008). One obvious explanation for this discrepancy is the difference between the plasticity induction protocols. Potentiation induced by whisker deprivation seems at least in part related to structural modification of spines and rewiring of circuits (Cheetham et al., 2007, 2008; Holtmann and Svoboda, 2009; Trachtenberg et al., 2002; Wilbrecht et al., 2010). The stability of spines is also strongly related to postsynaptic density and AMPA content (for review see Holtmann and Svoboda, 2009). Synaptic plasticity requires insertion of GluR1 and GluR1/2 subunits followed by GluR2/3 cycling (Shi et al., 2001). It therefore stands to reason that *de novo* spine formation and stabilisation requires the insertion of GluR1-containing AMPARs, and the absence of which in the GluR1 KO prevents full potentiation from being reached. Similar results have been found in the slice using chemical LTP protocols (Kopec et al., 2007). Although synaptic reorganisation can occur using LTP protocols, the absence of GluR1 during EDP protocols may have a more profound effect. This could be due to the sheer number of neocortical neurons that undergo modification following whisker deprivation and the length for which the deprivation lasts (days opposed to minutes). Despite this, plasticity is still possible in the GluR1 KO (Hoffmann et al., 2002), suggesting that other synaptic structural modifications are possible.

A series of experiments could be performed to confirm the dependence of structural EDP modifications upon GluR1. A simple experiment would be deprive the whiskers

and count spine density compared to deprived WTs and undeprived controls. A more complicated experiment would be to implant a window in the skull and use 2-photon imaging to longitudinally trace the turnover and stability of *de novo* spine formation. Should GluR1 be required for spine formation, it is predicted that in the GluR1 KO spine formation will occur at a slower rate and fewer spines would become mushroom spines.

My results suggest that GluR1 is required for EDP expression. GluR1 is also required for LTP and its synaptic insertion as a functional AMPAR is regulated by phosphorylation events upon GluR1. GluR1 phosphorylation forms a bidirectional type of plasticity; that is, distinct phosphorylation events cause LTP or long-term depression (LTD). The phosphorylation of GluR1 by CaMKII at the S831 site promotes delivery to the synapse, while dephosphorylation promotes internalisation (Hayashi et al., 2000). The phosphorylation of S845 by PKA performs a similar action. LTD dephosphorylates the S845 site while LTP phosphorylates it (Lee et al., 2000). Both are dependent on the initial state of the synapse (potentiated, depressed or naïve) and the mechanism seems relevant to the barrel cortex *in vivo* (Hardingham et al., 2008). In support of this idea, it has been found that mice containing a point mutation at the T286 site preventing autophosphorylation of αCaMKII do not exhibit any barrel cortex EDP (Glazewski et al., 2000). Theoretically the autophosphorylation of CaMKII would enhance its ability to phosphorylate GluR1 at the S831 site, and mutation of αCaMKII prevents GluR1 phosphorylation and insertion.

The total abolition of EDP in the T286 CaMKII mutant cannot explain the partial reduction of EDP in the GluR1 KO (Figure 4.26). The absence of CaMKII would prevent GluR1 insertion via S831 phosphorylation, yet EDP is possible even though the GluR1 subunit is absent. This suggests that CaMKII mediates a role other than GluR1 insertion. In support of this, CaMKII antagonism in the GluR1 KO prevents barrel cortex LTP (Hardingham and Fox, 2006). NOS is known to contain a calmodulin recognition site and CaMK can affect the catalytic activity of NOS (Bredt et al., 1992). It is therefore possible that CaMKII could also modulate NOS activity (either directly or indirectly), affecting NO release depending on synaptic activity. To confirm that CaMKII supports the residual EDP in the GluR1 KO, antagonists could

be applied to GluR1 KOs and subsequent EDP quantified. It is known that GluR1-independent EDP is supported by NOS1 and should CaMKII antagonism also block EDP in the GluR1 KO, it will be likely that CaMKII influences NOS1 activity. Should CaMKII be found to modulate both NOS1 and GluR1 activity/insertion, both processes would be functional in WTs and contribute to EDP.

6.7. The Role of Nitric Oxide in *in vivo* Plasticity

The finding that potentiation occurs in the GluR1 KO raises one obvious question. What supports the plasticity in the GluR1 KO? It is known that LTP in the GluR1 KO is partly NO-dependent in the hippocampus (Phillips et al., 2008) and entirely in the barrel cortex (Hardingham and Fox, 2006). Importantly, individual antagonism of GluR1 or NO cannot fully inhibit plasticity. This result suggests that there are dual processes of plasticity. While some studies have suggested that GluR1 KOs have a presynaptic form of plasticity following LTP (Hardingham and Fox, 2006; Phillips et al., 2008), others have found a mixed pre and postsynaptic loci (Frey et al., 2009; Romberg et al., 2009). Regardless of this debate, NO seems to facilitate potentiation that is in addition to the AMPAR insertion mechanism. Hence, the role of NO can only be fully exposed once GluR1 is deleted. As yet nothing is known about this mechanism *in vivo*. To test the role of NO in plasticity, GluR1 KOs were crossed with animals deficient in either αNOS1 or NOS3 to create double KOs ($GluR1^{-/-}/NOSX^{-/-}$). These animals then underwent the same deprivation protocol to induce layer II/III EDP, which was recorded *in vivo*.

EDP was abolished in the GluR1/NOS1 KOs but not in the GluR1/NOS3 KOs. This suggests that the two NOS isoforms have differing roles in synaptic plasticity. NOS3 produces NO in a calcium concentration-dependent manner (unlike NOS2), but lacks the PDZ domain that allows associations with PSD-95 and NMDARs (Brenman et al., 1996; Cho et al., 1992). αNOS1 is clearly required for D1 potentiation in the absence of GluR1, whereas NOS3 is not. These data support and extend the Hardingham (2006) study. Given that GluR1 and NO is required for *in vivo* experience-dependent potentiation, this highlights that the absence of LTP (Hardingham and Fox, 2006) was not related to the slice preparation process or plasticity induction protocol that has

sometimes confused the field (for discussion see Phillips et al., 2008). Chapter 4 reported that instead of antagonising all NOS to impair plasticity (Hardingham and Fox, 2006), only NOS1 inhibition is required to prevent EDP plasticity. It is important to note that this finding contrasts LTP obtained from the hippocampus. Pyramidal cells in CA1 require both NOS1 and NOS3 antagonism in addition to the GluR1 mutation to reduce potentiation, and even then LTP does not completely fall to baseline levels (Phillips et al., 2008; Romberg et al., 2009).

Although NOS1 seemingly is more important for barrel cortex EDP, its significance is somewhat confusing. αNOS1 seems more important in males as opposed to females. In αNOS1 single KOs, female potentiation was of a magnitude similar to female WTs, whereas compared to controls, male KOs did not show potentiation. Despite this, NOS1 does seem to have a role in EDP in both genders, as potentiation in the GluR1/NOS1 KO is at control levels in both genders. The exact mechanism behind this gender difference remains illusive. The simplest explanation is that NOS1 and GluR1 is required for plasticity in both genders, with NOS1 contributing more in males than GluR1 insertion and visa versa in females. It is known that NOS1 can associate with estrogen to form a NOS1/PSD-95/NMDA complex to promote NO release (d'Anglemont et al., 2009). Indeed, NO release can flux depending on the level of estrogen (d'Anglemont et al., 2009), which would intuitively suggest that NO release via NMDA and NOS1 would be more important for females. Given that female potentiation was unaffected in NOS1 KOs, this mechanism for NO release seems unlikely to affect plasticity *in vivo*. It is important to note that the d'Anglemont et al. (2009) study was conducted in primary cultures of immature tissue. Whether estrogen/NOS1/PSD-95/NMDA associations are important for development remains to be seen, but it appears likely that the gender difference in NOS1-independent potentiation is more complex than can be explained by estrogen. GluR1 insertion following *de novo* spine formation can also be facilitated by estrogen (Srivastava et al., 2008). Again this would suggest that the magnitude of GluR1-dependent plasticity might vary between the genders. Against this, no gender differences in D1 potentiation were found in either WTs or GluR1 KOs (Figures 4.3 and 4.7). The Srivastava et al. (2008) used neuronal cultures and it is possible that the mechanism they proposed reflects a form developmental plasticity but not adult potentiation.

Previous works have also proposed a role for NOS3 in hippocampal LTP (Hopper and Garthwaite, 2006; Phillips et al., 2008; Son et al., 1999). The reduced EDP in the NOS3 KO suggests that NOS3 is also required for full barrel cortex EDP expression (Figure 4.16). Work so far in the neocortex has not distinguished between NOS isoforms (Hardingham and Fox, 2006; Reid et al., 1996; Ruthazer et al., 1996). Studies in visual cortex plasticity have found no role for NO (Reid et al., 1996; Ruthazer et al., 1996), although this conclusion may be challenged for two reasons. First, the result presented in Chapter 4 has found a very definate reduction in potentiation in both knockouts of NOS. Second, the visual cortex studies did not distinguish between potentiation and depression. Since occular dominance plasticity relies heavily on the input from the deprived eye, it is possible that an occular dominance shift could occur independent of any affect of NO on potentiation.

One source of confusion arising from the findings of barrel cortex potentiation in the absence of NOS3 is the very similar response magnitude of NOS3 KOs and GluR1/NOS3 KOs, which are also similar to GluR1 KOs. If independent deletion of NOS3 and GluR1 both reduced potentiation, then when combined one would have predicted that the effect on plasticity would have been additive. That is, the potentiation reduction in the GluR1 KO and the NOS3 KO would summate to reduce potentiation further, which was not the case. Further discussion is provided within the Compensation Mechanism section (6.11, p. 233).

If it is assumed that NO produced by NOS3 does partially contribute to potentiation, then how does it cooperate with NO produced by NOS1 to alter synaptic efficacy? NOS3 is not located in pyramidal cells unlike NOS1 (Blackshaw et al., 2003), so it seems unlikely that NOS3 is able to temporally regulate NO release in response to synaptic activity as NOS1 could. Hopper (2006) proposed an elegant solution to the possible varying roles that the different NOS isoforms could mediate. In NOS3 KOs and inhibitors selective to NOS3, it was found that a low exogenous application of DEA/NO (a NO donor) rescued the ~50% reduction in LTP at 100 minutes. Tonic NO levels were sensitive to NOS3 manipulation as detected by cGMP measurement but not to NOS1 manipulation (Hopper and Garthwaite, 2006). Even though NOS1 antagonism did not reduce tonic NO levels, late-phase LTP (120 minute) was nearly completely abolished. Exogenous application of the same low level of DEA/NO did

not rescue LTP although when the DEA/NO concentration was increased 10 fold, LTP returned to control levels (Hopper and Garthwaite, 2006). By inference this suggests that NO can form a tonic and phasic signal from NOS3 and NOS1, respectively. A tonic NO signal produced by NOS3 in the endothelial tissue that could, in effect, be boosted by NO released from NOS1 by synaptic activity, would be better able to direct a role for NO in plasticity. Single isoform antagonism would not cause a complete abolition of NO-mediated potentiation, instead on causing a partial reduction. This is strikingly similar to my NOS1 and NOS3 KO result. Potentiation was only partially reduced, not abolished. It is therefore possible that 'tonic/phasic' NO signalling could be occurring in the neocortex in response to EDP.

6.8. The Locus of Plasticity

While this thesis has not addressed the controversial argument of whether synaptic plasticity via NOS signalling is pre or postsynaptic, the data from my studies may nevertheless further this debate. Traditionally it has been thought that NO produced a presynaptic form of plasticity (Garthwaite et al., 1988). There is well established evidence that presynaptic forms of plasticity exist (Malinow and Tsien, 1990) but NO was proposed to modify presynaptic release probability (Garthwaite et al., 1988). The interpretation of quantal analysis remains controversial. Nevertheless it has provided support for the idea that NO mediates presynaptic plasticity. $1/CV^{-2}$ analysis has found that inhibition of NOS drives plasticity to a postsynaptic loci (Sjöström et al., 2007), whereas GluR1 KOs have a presynaptic loci of plasticity that is NO-dependent (Hardingham and Fox, 2006; Phillips et al., 2009). However, other investigators have found a mixed loci in the GluR1 KO (Frey et al., 2009; Romberg et al., 2009). NO has recently been shown to be able to promote insertion of GluR1 and GluR2 (Huang et al., 2005; Serulle et al., 2007). This raises the question of whether NO actually supports a mixed loci by modifying presynaptic release and postsynaptic AMPA insertion? This could certainly be the case in WTs mice. However, in the GluR1 KO the presynaptic locus is favoured. Under these conditions, NO could not cause insertion of GluR1 but NO could increase GluR2 insertion (more specifically, GluR2/3 heteromers). However, GluR2/3 heteromers are not driven into the synapse in an activity-dependent manner (Shi et al., 2001; Zhu, 2009), suggesting against GluR2/3-mediated potentiation. This point is expanded further within the

Compensation Mechanisms section (6.11, p. 233). Taken together, although NO can promote postsynaptic AMPAR insertion, it seems probably that during the absence of GluR1, the locus of the EDP observed within this thesis is presynaptic. Further work would have to be conducted *in vitro* to confirm this hypothesis.

6.9. Experimental Limitations

A caveat that could alter the magnitude of recorded potentiation is the quality of recordings across animals and genotypes. For example, one factor that influences the receptive field size is anaesthetic depth (Armstrong-James and Callahan, 1991; Armstrong-James and George, 1988; Freeburg et al., 1999). Unfortunately, there was no way to independently measure anaesthetic depth throughout the recordings, for example by electroencephalography (EEG). Variances in the depth of anaesthesia might provide the simplest explanation for genotypic differences. There is some evidence to suggest that this could be a factor in Chapters 3 and 4. During the neocortical recordings, it was found that mutants of NOS3 (single and double mutant mice) were more susceptible to the urethane/acepromazine anaesthetic (until the anaesthetic protocol was altered (see Material and Methods, Section 2.2.3) mortality was ~80%).

This highlights that anaesthesia can have a profound effect on neocortical responses. Differences in anaesthesia state might also explain why NOS3 and GluR1/NOS3 KO mice had different potentiation magnitudes. If independent measures of depth been taken during the experiment it could have been realised that NOS3 KO mice actually potentiated to WT levels. However without that data it is unclear whether the reduced NOS3 KO potentiation was a confound of anaesthesia depth or a real effect. Yet anaesthesia alone cannot explain the lack of EDP in the GluR1/NOS1 KO. Both double KOs had a similar receptive field profile, yet robust EDP was found in the GluR1/NOS3 KO. Principle whisker depression following deprivation was also present in both double KO mice (Appendix 3). It therefore seems likely that anaesthetic depth was not a major factor that could have precluded any one genotype from under going potentiation of the spared whisker response.

6.10. The Role of GluR1 and Nitric Oxide in Memory

6.10.1. GluR1

Barrel cortex EDP suggests that GluR1 and NO are required for synaptic plasticity induced by sensory deprivation. Assessment of the role of these molecular processes in barrel cortex learning is hampered by the lack of understanding of behavioural paradigms. The deficits in barrel cortex EDP are relevant to other cortical areas; similar plasticity deficits exist in the hippocampus (Phillips et al., 2008). The hippocampus is also necessary for learning and memory, for which synaptic plasticity is required (Morris et al., 1982). To determine the interaction between GluR1 and NO activity in memory, behavioural studies examined their impact on spatial and contextual memory.

WT and GluR1 KO mice underwent contextual fear conditioning that was of the same design as the Humeau (2007) study, with the exception that tone was not used as a conditioned stimuli (CS). 24 hours following conditioning, WTs of both genders displayed an increase in condition responding to the context, while male GluR1 KOs did not freeze to the context. Previously it has been shown that in WTs infected with GluR1-GFP, contextual fear conditioning increased hippocampal GluR1 insertion (Matsuo and Mayford, 2008). This suggests that hippocampal GluR1 is required to encode the context in which emotional learning takes place, and reductions in memory retention in the GluR1 KO reflects this lack of plasticity. One novel and unexpected finding from this experiment was female GluR1 KO mice showed no deficit in freezing elicited by the context during the retention test (Figure 5.2).

GluR1 has been shown to be important for LTP in the thalamo-lateral amygdala, cortico-lateral amygdala and basal amygdala (Humeau et al., 2007). Currently it is not known whether full LTP expression could have been achieved if spike-timing or modified orthodromic protocols were used. This data also only makes assumptions about the plasticity in males, not females. The lack of plasticity in the amygdala complex of GluR1 KOs could account for the lack of conditioning, but this can only explain the male phenotype. Should control levels of LTP be found in female GluR1 KOs, this could represent the reason as to why females display a conditioned response. These experiments would need to be conducted to resolve this issue. Despite this, no gender differences in synaptic plasticity have been observed in the

GluR1 KO within the barrel cortex (Figure 4.7, p. 141). Compensation cannot be ruled out. An increase of GluR2/3 in the basolateral amygdala has been noted in the GluR1 KO (Mead and Stephens, 2003), although the rationale of how this could compensate for plasticity considering this complex is not trafficked in an activity-dependent manner lacks clarity (see Compensation Mechanisms, Section 6.11, p. 233 for detailed explanation). It is also not known whether the increase in GluR2/3 in that study was associated with male or female GluR1 KOs.

The lack of conditioned response in male GluR1 KOs compared to female GluR1 KOs is not due to a non-specific effect such as neophobia, as freezing activity was comparable between the genders for the 6 minutes preceding the first unsignalled footshock. It is also unlikely that female KO conditioning is related to a heightened sensitivity to the footshock. During the conditioning phase, male and female KOs both froze at a similar magnitude directly after each footshock presentation, suggesting the footshock elicits a similar reaction between all GluR1 KOs. It is known that GluR1 KOs nociception is comparable to WTs (Feyder et al., 2007), as is locomotion during the shock delivery (Humeau et al., 2007), highlighting that the mutant mice are aware of the footshock delivery. Taken together, given that male GluR1 KOs froze at the same magnitude to females GluR1 KOs during conditioning but not during the retention test, the lack of conditioned response cannot be explained easily but non-specific conditioning effects in the mutant mice. Hence, the lack of conditioned response likely reflects a memory impairment of the context.

One possibility is that female GluR1 KOs are able to use extra-hippocampal regions to support learning following hippocampal dysfunction. It is known that in the absence of the hippocampus, extra-hippocampal regions can compensate enabling contextual learning to occur, albeit less efficiently (Biedenkapp and Rudy, 2009; Wiltgen et al., 2006). While this is possible, others have found that that spatial learning was no more efficient in female KOs (Sanderson et al., 2009). This does not rule out the possibility that compensation by extra-hippocampal regions is more efficient in the GluR1 KO. Further experiments could be conducted to confirm this hypothesis by selectively antagonising extra-hippocampal areas during conditioning and then measure freezing behaviour during a retention test. It has also been proposed that the ventral hippocampus is required for contextual fear conditioning (Richmond

et al., 1999) and as yet it is not known how GluR1 contributes to ventral hippocampal plasticity. It is therefore possible that the ventral hippocampal GluR1-dependent plasticity is gender dimorphic, explaining the lack of freezing in the male GluR1 KO. However, it is important to note that compartmentalisation of hippocampal involvement in contextual fear conditioning is not widely accepted (Rudy and Matus-Amat, 2005). To challenge this point, female GluR1 KO mice could undergo hippocampal lesioning to determine what effect this would have on the ability to encode the context.

Although the female GluR1 KOs did provide the appropriate conditions to test whether GluR1-independent memory was supported by NOS, there were a number of caveats preventing the experiment from commencing. The reasons for this are outlined in Section 5.2.4. Therefore another form of spatial memory was chosen to be investigated that has previously been found to have a within-task GluR1-dependent and independent phenotype. The GluR1-independent component could then be further challenged by antagonism of NOS. The radial arm water maze (RAWM) combines elements of two standard tests of spatial memory; the 8-arm radial maze (Olton, 1979) and the Morris water maze (Morris et al., 1982), and addresses similar hypotheses to the study of Schmitt et al. (2003).

Although initial performance was comparable to WTs, during blocks 6 to 9 GluR1 KOs made significantly more errors than WTs. However, performance by the end of training (blocks 10-12) was indistinguishable from WTs. No gender difference was evident in this pattern of performance. The finding that more arm entry errors were made by GluR1 KO mice during acquisition is consistent with previous reports (Schmitt et al., 2003) that found allowing working memory errors to occur slows the formation of spatial reference memory. The delayed formation of spatial reference memory in the RAWM could be a result of competitive interaction between working memory errors and reference memory formation (see Smith, 1968). Nevertheless, in contrast to the food motivated radial maze task (Schmitt et al., 2003), the GluR1 KO mice in this study were able to acquire the reference memory component to the same level as WT mice. Others have also found that GluR1 KO spatial reference memory was unaffected in the Morris water maze and hidden platform location was acquired at the same rate as WTs (Reisel et al., 2002). The major difference between the

Morris water maze and the RAWM is the arm choice element, further suggesting that the interaction between spatial working and reference memory was what slowed the acquisition of the platform location in the RAWM.

Gender was not a factor in either RAWM acquisition or memory retention, which was not the case for contextual fear conditioning. Estrogen has previously been shown to increase GluR1 insertion following new spine formation (Srivastava et al., 2008). Spine formation occurs following EDP events (Trachtenburg et al., 2002) and is likely to be important for long-term stability of plasticity. While it was possible the GluR1-insertion could have occurred preferentially in females, memory formation and recall does not seem to be differentially expressed by this mechanism in the RAWM. Although female GluR1 KO mice were not impaired during the context recall test following fear conditioning, deletion of the GluR1 subunit would have prevented estrogen-dependent insertion. Since male and female WTs froze at the same magnitude, estrogen does not seem to promote memory formation as the model of Srivastava et al. (2008) proposes. Taken in corroboration with the lack of gender effects in spared whisker potentiation (Figures 4.3 and 4.7), it seems unlikely that estrogen influences GluR1-dependent or independent memory.

Recently the GluR1 memory dissociation has been described not as working and reference memory but of impairments in short-term habituation (see Sanderson et al., 2010). My findings would certainly support this hypothesis. The increase in memory errors could be because the GluR1 KO does not habituate to the arm during entry. Hence, each arm maintains its novelty value and since mice naturally have preference for entering a novel location (i.e. spontaneous alternation T-maze), re entering an arm during a trial is more likely to occur. In terms of Wagner's Sometimes Opponent Process (SOP), all elements within the context will be primed to the A1 state but cannot rapidly transfer to the A2 state (Wagner, 1981). Despite this, long-term habituation can form via retrieval-generated priming directly to the A2 state. The elements within the context gain associative strength to an outcome (i.e. extramaze cues indicating the submerged platform), but this process is slower in forming, depends upon the strength of the previously formed associative memory and can competitively interact with short-term habituation (Sanderson et al., 2010). The process of retrieval-generated priming and long-term habituation has been shown to

be GluR1-independent (Sanderson et al., 2009). Therefore the deficit in short-term habituation reflects the increase in arm entry errors during acquisition, which is supported by GluR1-dependent plasticity processes.

One potential explanation of the pattern of results summarised above is the GluR1 KO mice have abnormal sensory or motor functions that interacts with acquisition of the fear conditioning and watermaze tasks. This seems unlikely, however, as several studies have shown that sensorimotor deficits were either not present or could not explain the dissociable pattern of memory function shown by GluR1 KO mice (Reisel et al., 2002; Sanderson et al., 2010; Zamanillo et al., 1999). Furthermore, two studies using the watermaze task have found no difference between WTs and GluR1 KOs acquisition (Reisel et al., 2002; Zamanillo et al., 1999), confirming that any sensorimotor deficit arising from the mutation does not disrupt memory formation.

6.10.2. Nitric Oxide

In the hippocampus, antagonism of a single NOS isoform causes a partial reduction of plasticity (Hopper and Garthwaite, 2006; Phillips et al., 2008). Larger plasticity reductions can be achieved if both NOS isoforms are inhibited (Son et al., 1999), but the largest occur when NOS and GluR1 are blocked together (Phillips et al., 2008). Experiments within Chapter 4 demonstrated that this mechanism is relevant to *in vivo* experience-dependent potentiation, although in the neocortex NOS1 seems more significant (Figure 4.27). Since GluR1-independent potentiation is NO-dependent, it is possible that NO could mediated the spatial reference memory observed in the RAWM.

To antagonise hippocampal NOS activity, minipumps were implanted into the GluR1 KOs to constantly infuse the non-specific inhibitor L-NAME into the lateral ventricle. This was undertaken because first, both NOS isoforms need to be inhibited to impair plasticity and second, L-NAME can cause peripheral side-effects that can confound learning (Prendergast et al., 1997; Appendix 4). Surprisingly L-NAME did not affect the goal arm preference of either WTs or GluR1 KOs, although female GluR1 KOs infused with NOS antagonists appeared to perform at chance during the second probe trial. Given the GluR1-independent plasticity is governed in large by NO, it was

hypothesised that this would be deleterious to acquisition of spatial reference memory in the GluR1 KO. These results suggest otherwise. While LTP is reduced by deletion of GluR1 and antagonism of NOS, some plasticity remains (Phillips et al., 2008). This residual potentiation could be sufficient to support spatial reference memory. These results therefore support earlier published reports that NO inhibition does not impair spatial learning (Bannerman et al., 1994; Zou et al., 1998). Despite this, there is considerable evidence for behavioural deficits following NOS antagonism (Chapman et al., 1992; Kelley et al., 2010; Majlessi et al., 2008; Prendergast et al., 1997; Qiang et al., 1997). Although there is an interesting reduction in goal arm preference during the second probe in female GluR1 KOs infused with L-NAME, more experiments are required to confirm this initial finding. If this were to be confirmed, it is possible that NOS acts to maintain the long-term stability of spatial reference memory (beyond 24 hours) following training. This prediction has recently been proposed in another study where NOS1 KO mice performed similar to WTs during a water maze transfer test 24 hours following training but not 7 days following training (Tanda et al., 2009).

One potential explanation for the absence of a consistent deficit in NOS treated mice is that the rate of delivery and/or concentration was too low to inhibit NOS activity in the hippocampus. An immediate improvement to the design would be to measure NOS activity post mortem to better understand the magnitude of NOS antagonism during the trial. Despite this, other experiments have used NOS inhibitors in minipumps, albeit not in the hippocampus. 50 mM of L-NAME delivered at 0.5 µl/hr reduced NOS activity by 57% up to 4 mm away from the cannula tip (Reid et al., 1996). Given that the experiments in Chapter 3 used 100 mM L-NAME delivered 0.25 µl/hr and that the mouse hippocampus does not extend further than 4 mm from where the cannula was inserted, it is probable that there was a similar level of NOS inhibition. For this simple reason, concluding that NO is not required for hippocampal dependent learning is premature and contradictory to the lack of plasticity found in the *in vitro* preparation. It is also possible that locomotor activity was disrupted in GluR1 KO mice following the minipump insertion. 'Floating' (whereby the animal becomes motionless in the water maze) has been noted by others (Reisel et al., 2002) and did occur within this study. While this was generally only temporary and infrequent, it could reflect a tendency towards a GluR1 KO deficit in locomotion. The

implantation of minipumps could have further reduced the swimming ability of the GluR1 KO. This which would account for why there are fewer arm entry errors during acquisition training (Figure 5.8); the GluR1 KO has less opportunity to explore as many arms during the 60 second period. Yet this potential confound does not appear to impair spatial reference memory formation, as no significant genotypic differences were observed for goal arm preference during the probe tests (Figure 5.10). However, this experiment would be improved greatly by finding either a less invasive drug delivery method or using antagonists that do not impair locomotion themselves (see Appendix 4).

6.11. Compensation Mechanisms

The deletion of GluR1 raises the question of whether such a deletion initiates compensatory processes such as the upregulation of other AMPARs subunits. Recent evidence indicates that NO can influence insertion of GluR2 (Huang et al., 2005). Therefore it could be possible that there is also a NO-dependent form of compensation that leads to increased GluR2 insertion. Both of these questions can be challenged by a recent study by Zhu (2009) and relates to the function of other heteromeric AMPARs. First, GluR4 can mostly be excluded from adult studies as it only forms a small percentage of the total AMPA subunit population (Rossner et al., 1993; Wenthold et al., 1996; Zhu et al., 2000; Zhu, 2009), leaving GluR1, R2 and R3. When GluR1 is knocked out, this leaves the heteromeric GluR2/3 complex. It is known that this receptor is not actively trafficked to the synapse. Instead it is continually cycled in and out of the synapse, replacing GluR1 homers and GluR1/2 heteromers in both the hippocampus and the barrel cortex (Shi et al., 2001; Zhu, 2009). It seems unlikely that in the absence of GluR1, cycling of GluR2/3 that would normally replace GluR1 and GluR1/2 could form a compensatory mechanism to now actively deliver receptors to the synapse. While NO can promote GluR2 insertion, it would not be the receptor type that is synaptically delivered by activity, and there is no evidence for GluR2/3 activity-dependent insertion (Zhu, 2009). Indeed it has been hypothesised that the increased somatic GluR2 observed in the GluR1 KO could be related to the absence of a second receptor subunit to form a receptor complex for activity-dependent synaptic delivery (Zamanillo et al., 1999). GluR2, R3 and R4 are also expressed at similar levels in GluR1 KO compared to WTs in the barrel cortex

(Wright et al., 2008) and the hippocampus (Zamanillo et al., 1999), suggesting that no one receptor is upregulated in compensation for the loss of GluR1. In spite of this evidence, some studies do propose that GluR2 could be important in plasticity in the GluR1 KO (Frey et al., 2009; Romberg et al., 2009), although as yet no specific mechanisms are known.

It is interesting to note that in the GluR1/NOS3 KO, plasticity is at a similar magnitude to the GluR1 and NOS3 single KOs. Intuitively one may have predicted that the sum of both KOs would have caused an additive effect, abolishing all plasticity as in the GluR1/NOS1 KO. It could be that in the GluR1/NOS3 KO, compensation occurs to allow some degree of synaptic plasticity. If it is assumed that the other AMPA subunits are unlikely to compensate (see above), then NO via NOS1 could facilitate greater than normal plasticity. It is already known that GluR1-independent potentiation is completely dependent on NOS signalling (Hardingham and Fox, 2006). GluR1/NOS3 KO barrel cortex LTP appears strikingly similar to GluR1 KOs but the potentiation is completely abolished with the application of NOS antagonist (Hardingham et al., 2010 – see Appendix 5). This strongly suggests that NO is required for plasticity in the GluR1/NOS3 KO and is formed via NOS1. It is difficult to determine whether this is due to NOS1 compensation or is a finding that would otherwise occur in WTs. In some respects this debate highlights the importance of NOS1 signalling. Plasticity is not compensated in the GluR1/NOS1 double KO by NOS3, and as previously stated NOS1 and NOS3 are structurally similar but for the PDZ domain in NOS1. It therefore seems more likely that NO is important for plasticity, with NOS1 playing a more significant role than NOS3.

Differences in gene expression between WTs and GluR1 KOs have recently been examined in the hippocampus. Differences in regulation of ~30 genes was found, but most notable was that in the GluR1 KO, the NMDA receptor NR1 had increased expression compared to WTs, whereas αCaMKII expression was reduced (Zhou et al., 2009). This could suggest that NMDARs compensate for the lack of GluR1, although CaMKII has been shown key to synaptic plasticity (Glazewski et al., 2000; Silva et al., 1992), which would seem to negate any benefit from the increase in NR1. This study looked at baseline conditions. A further development would be to look at expression profiles following an experience-dependent plasticity-type protocol, which

could provide an explanation as to what compensation mechanisms are occurring in the KOs during plasticity.

Studies have suggested that neuromodulators, such as noradrenalin (NA) and acetylcholine (ACh), are involved in associative learning and cortical plasticity. Studies of the auditory cortex have found that a cognitive association must occur between auditory stimuli and reinforcement before plasticity can take place (Blake et al., 2006). Indeed, if an animal (termed yoked) is prevented from understanding how stimuli is related to reinforcement, whereas a 'guide' animal is allowed to form this association, plasticity will not occur in the yoked animal (Blake et al., 2006). Recently, pairing sensory stimulation (a specific tone) to stimulation of the nucleus basalis, the major source of cortical ACh, resulted in long-lasting retuning of the receptive field in the auditory cortex to the paired tone (Froemke et al., 2007). It is already known that ACh is required for cortical plasticity to take place (see Shulz et al., 2000, 2003). ACh has diverse roles but is known to modulate the excitability of neurons, and within the barrel cortex will cause excitation of layer II/III and V but inhibition of layer IV (Eggermann and Feldmeyer, 2009). Previous studies have found that ACh receptors, specifically the α7nAChR, can exist alongside GluR1 receptors in the barrel cortex (Levy and Aoki, 2002). Recent evidence has found that in the hippocampus, injection of NA will result in increased GluR1 phosphorylation via the S845 site (Hu et al., 2007). Furthermore, application of NA to hippocampal slices resulted in greater LTP in a time-dependent fashion and S831 and S845 site phosphorylation-dependent delivery of homomeric GluR1 receptors to the synapse (Hu et al., 2007). It is therefore possible that similar facilitation mechanisms could occur in the neocortex. Likewise, ACh and NA can be released in response to NO donors (Satoh et al., 1996; de Vente et al., 2001 but see Morton and Bredt, 1998). It is highly likely that neuromodulators like ACh and NA have some role in the plasticity observed within this study. To the best knowledge of the author, the role of ACh in whisker deprivation paradigms has yet to be established, and although a role in whisker pairing protocols is known, it is likely that frequency dependent pairing and physical whisker removal will invoke different synaptic mechanisms. No specific information is available for possible disruption of the reinforcer system in the knockouts used here, although it does seem that both ACh and NA can interact with NO and GluR1. The most obvious disruption would be to alter the excitability of

neuronal responses, but it is known *in vivo* and *in vitro* that this does not occur in the knockout mice (Chapter 3, **page**; Phillips et al., 2008). This does not rule out more subtle phenotypes, and further study needs to be performed to understand whether this system is disrupted by the removal of GluR1 and NOS.

6.12. Conclusions

Taken together, this study has found that GluR1 is required for adult EDP in the neocortex. Using whisker deprivation, I have found that potentiation was reduced by approximately ~50% in GluR1 KO mice. Given that potentiation of this type requires structural modifications and that spine stability following *de novo* formation is proportional to PSD and AMPA content (Holtmann and Svoboda, 2009), the reduced potentiation could represent a structural plasticity deficit. The potentiation that was possible in the absence of GluR1 was completely sensitive to NOS1 inhibition; NOS3 inhibition did not affect potentiation. This supports and extends the work of Hardingham (2006) by establishing the nature of the NOS isoform required for this residual plasticity. It also is one of the few studies that have used electrophysiological techniques to confirm a role for NO *in vivo*. Furthermore these experiments confirm NOS isoform specific plasticity in the barrel cortex, whereas in the hippocampus both NOS isoforms seem to play an equal role (Phillips et al., 2008). In addition I have found evidence for gender-specific plasticity. Two male specific plasticity and memory impairments were identified (EDP in the absence of NOS1 and contextual fear conditioning in the absence of GluR1). Both EDP and freezing during the extinction trial were unaffected in females despite the deletion of NOS1 and GluR1, respectively. The cellular mechanisms of plasticity are therefore more complicated than can be appreciated by studying a single gender in isolation.

Despite clear plasticity deficits in the barrel cortex and previous reports of LTP in the hippocampus (Phillips et al., 2008), behavioural studies could not find evidence for a NO contribution to spatial learning either in isolation or in combination with GluR1 deletion. GluR1 and NOS antagonism could modulate the stability of long-term spatial reference memory in female mice, although this observation requires further study. Taken together, although GluR1-independent synaptic plasticity is supported

by NO in the barrel cortex, this mechanism is not responsible for GluR1-independent spatial memory formation.

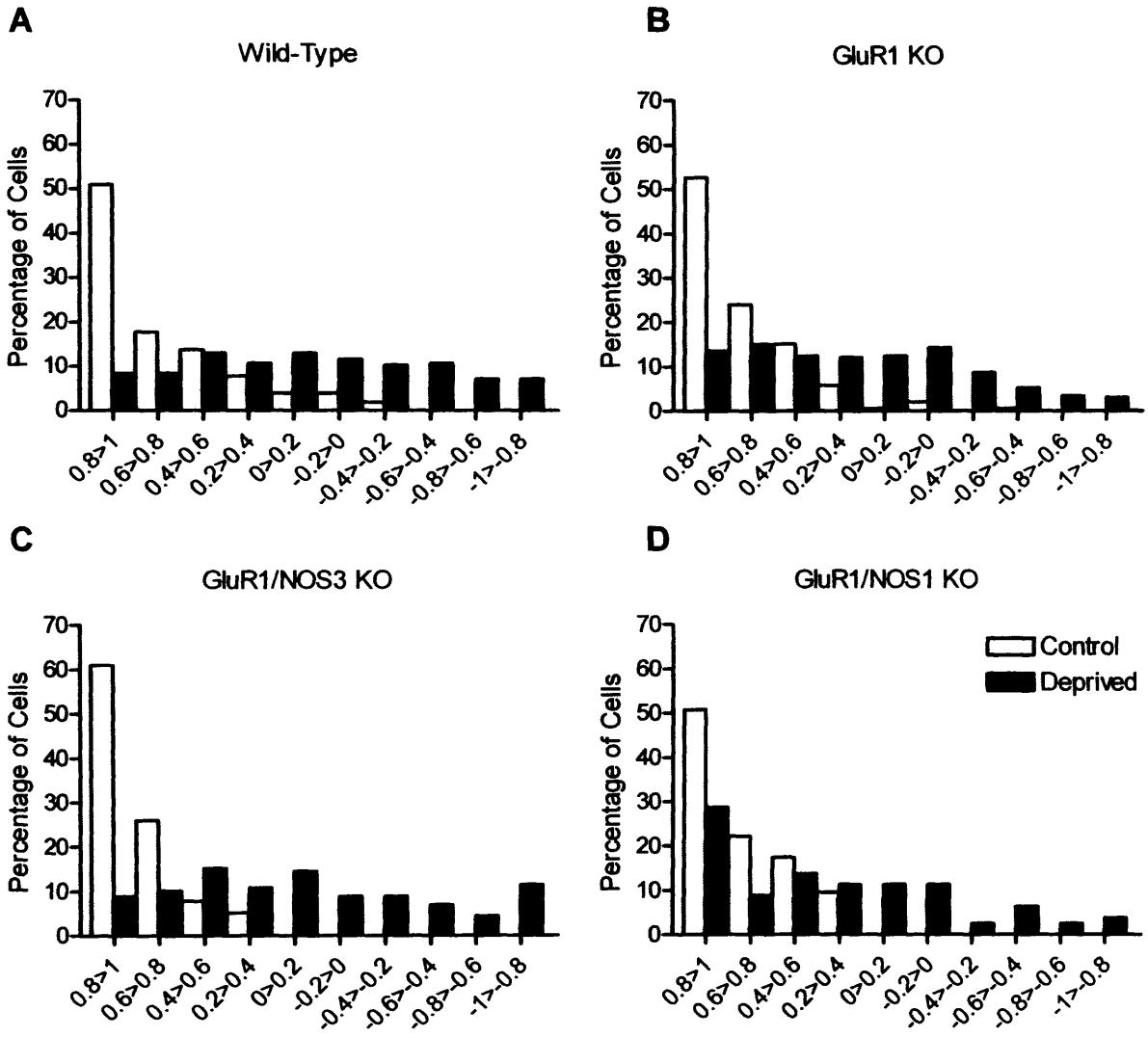
Appendix 1

Reference	Preparation	Age Range	Gender
(Boehm et al., 2006)	DIV	N/A	N/A
(Clem and Barth, 2006)	*Ex vivo*	P13-15	Not Disclosed
(Ehrlich and Malinow, 2004)	DIV	N/A	N/A
(Frey et al., 2009)	*In vitro*	P39-100	Not Disclosed
(Hardingham and Fox, 2006)	*In vitro*	P42-91	Not Disclosed
(Hardingham et al., 2008)	*In vitro*	P28-42	Not Disclosed
(Hayashi et al., 2000)	DIV	N/A	N/A
(Hoffman et al., 2002)	*In vitro*	P41-56	Not Disclosed
(Jensen et al., 2003)			Not Disclosed
(Kopec et al., 2007)	DIV	N/A	N/A
(Lee et al., 2000)	*In vitro*	P21-30	Male
(Lee et al., 2003)	*In vitro*	P21-28	Male
(Makino and Malinow, 2009)	DIV	N/A	N/A
(Phillips et al., 2008)	*In vitro*	P45-64	Not Disclosed
(Romberg et al., 2009)	*In vitro*	>P180	Male
(Serulle et al., 2007)	DIV *In vitro*	N/A ~P84	N/A Not Disclosed
(Shi et al., 2001)	DIV	N/A	N/A
(Takahashi et al., 2003)	*Ex vivo*	P14	Not Disclosed
(Wright et al., 2008)	*In vivo / ex vivo*	~P28	Not Disclosed
(Zamanillo et al., 1999)	*In vitro*	P42-45	Not Disclosed
(Zhu, 2009)	*Ex vivo*	~P28	Not Disclosed

DIV – Days in Vitro. The study has cultured tissue in vitro for a length of time before recording/imaging. Time spent in culture will not be quoted as this table is only to highlight whether cells where cultured, in vitro or in vivo in the study.

Appendix 2

The vibrissae dominance index has previously been described using the classic index of F = D1/(D1 + PW). It is however possible to calculate a dominance index using a contrast measure, similar to that used for visual plasticity. Thus, vibrissae dominance was calculated using F = (D1 – PW)/(D1 + PW). This calculation has the potential to lessen the impact of the PW upon the index. Within the GluR1/NOS1 KOs (D), a rightward shift was evident. A Mann-Whitney U test found this shift to be significant ($U = 1529$, $p < 0.0001$), which was similar to the previous VDI calculations in Chapter 4.

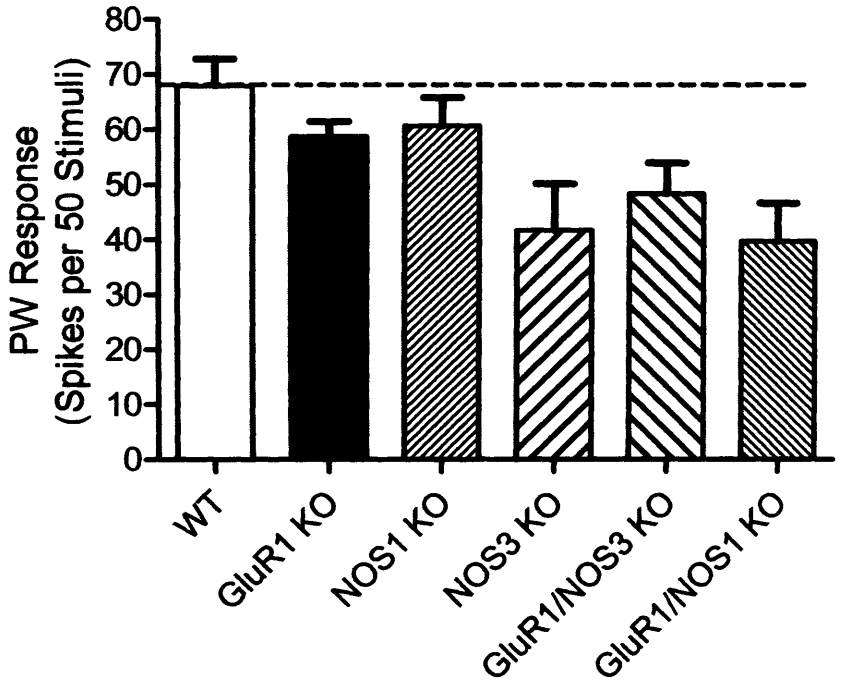


This analysis suffers from the same difficulties as the previous VDI calculation, in that it does not specifically exclude the notion that the PW is undergoing synaptic

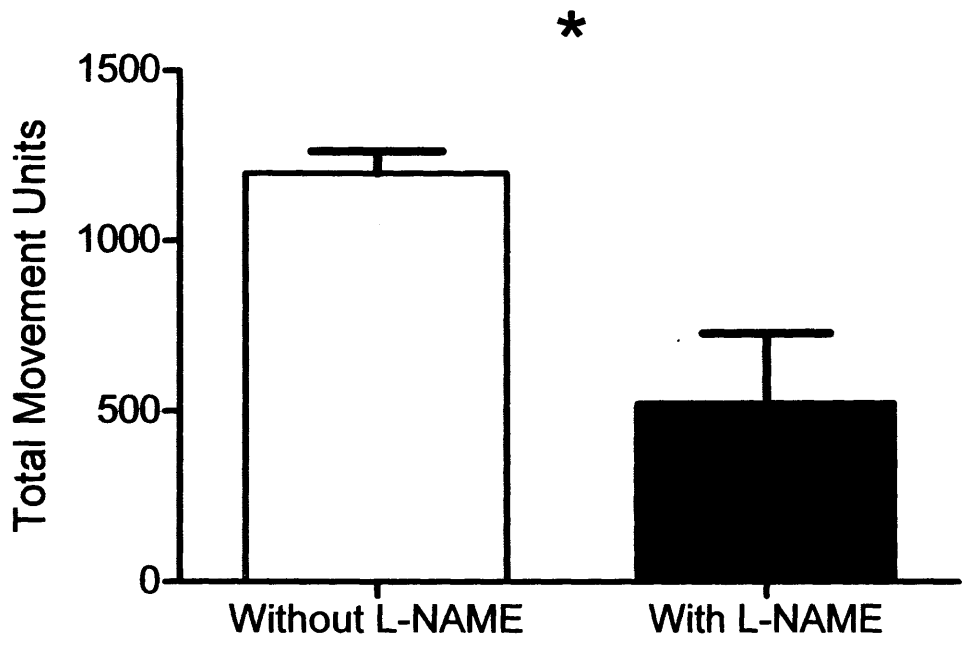
plasticity mechanisms that are independent of the potentiation of the D1 barrel column. Depression of the PW but no potentiation of the D1 barrel column would still result in a dominance index shift, which was the case here. A potential solution that would have the potential to resolve this problem would have been to also record cells from a cortical region that was not the barrel cortex. These cells should not undergo potentiation or depression, but would clearly indicate if depression via anaesthesia had occurred. This would provide a criterion to exclude cells due to anaesthesia but would also allow examination of PW and D1 plasticity independently.

Appendix 3

Recordings were made from the topographically related 'principle' barrel in response to principle whisker stimulation. These recordings were made from barrels surrounding the D1 barrel but not in the D1 barrel. Following deprivation of all but the D1 whisker for 18 days, 7-11 days regrowth was allowed before recording. Compared to control conditions, little difference was observed in the wild-types (control 75.65 ± 7.97 vs deprived 67.96 ± 4.89), GluR1 KOs (71.71 ± 4.59 vs 58.75 ± 2.75) or NOS1 KOs (76.11 ± 6.63 vs 60.69 ± 5.16). However there was a noticeable reduction in PW response in the NOS3 KOs (64.47 ± 8.25 vs 41.70 ± 8.45), GluR1/NOS3 KOs (77.18 ± 7.78 vs 48.36 ± 5.63) and the GluR1/NOS1 KOs (74.70 ± 4.90 vs 39.71 ± 6.89). A two-way ANOVA to compare control and deprived PW responses reveals a main effect of deprivation ($F_{(1, 105)} = 31.63$, $p < 0.0001$), genotype ($F_{(5, 105)} = 2.33$, $p < 0.05$) but not an interaction between the factors ($F_{(5, 105)} = 1.23$, $p > 0.05$). Post hoc analysis revealed that NOS3 KOs differed from WTs ($p < 0.05$). Although this suggests that statistically NOS3 KO PW responses are most strongly affected by deprivation, similar magnitude of depression exists in GluR1/NOS3 and GluR1/NOS1 KOs. It is also important to note that the reduction observed in the GluR1/NOS1 KO is not reflected in the GluR1 or NOS1 single KOs.



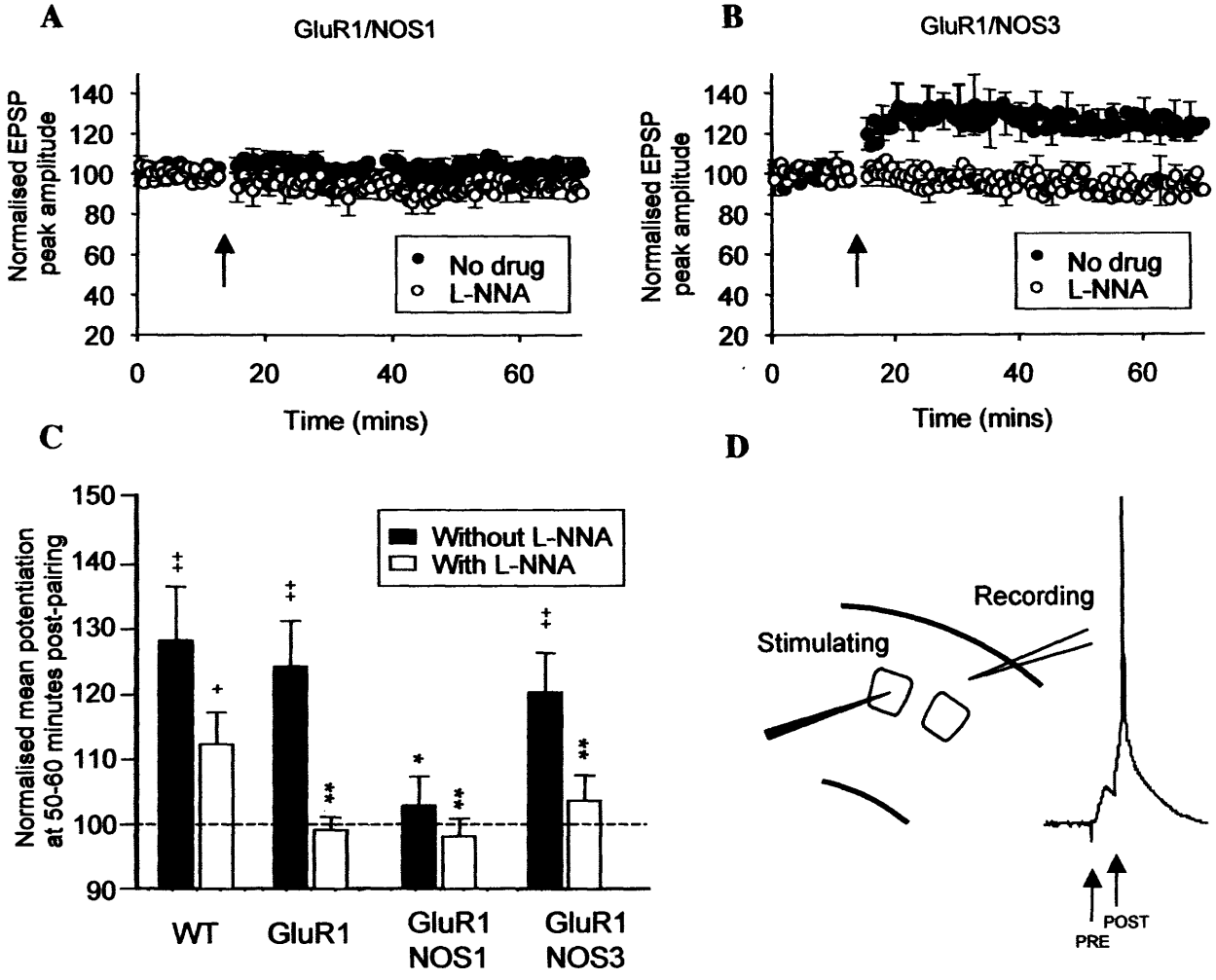
Appendix 4



WT C57/BL6 mice (all littermates) were given either an IP injection of 75 mg/kg L-NAME (n = 4) or saline (n = 4) and were allowed approximately 30 minutes recovery time in their home cages. Each mouse was then transferred to a novel chamber housed within a sound attenuation box. The chamber was well lit by 4 house lights contained within the ceiling, along with an infrared monitor (H24-61MC, Colbourne Instruments, Allentown, PA). The monitor assessed locomotion by calculating 'movement units', with each 'unit' corresponding to whether movement was detected within a 20 ms period. The mouse was placed in the chamber and computer controlled software commenced the movement detection. After 8 minutes, the software ceased detecting locomotion and the mouse was removed.

Mice treated with 75 mg/kg L-NAME were significantly less active than mice treated with only saline (unpaired t-test, $t_{(6)} = 3.2$, $p = 0.02$). This result likely comfirms the findings of others (Prendergast et al., 1997) that L-NAME results in non-specific side effects (for example to locomotion). Any deficit in locomotion potentially risks confounding the acquisition of any spatial learning task, suggesting that L-NAME should be delivered by methods other than IP injection.

Appendix 5



The dependence of LTP upon GluR1 and NO. Following the establishment of a stable baseline period, LTP was induced by x4 of 50 paired spikes at 2 Hz, with a 30 second interval between the trains. The stimulating electrode was placed in layer IV and the recording electrode in the adjacent barrel in layer II/III (see **D**). **A and B.** Pairing LTP could not be induced in GluR1/NOS1 KOs, whereas no deficits were observed in the GluR1/NOS3 KO. The plasticity in the GluR1/NOS3 KO was completely dependent on NO, as inhibition of the residual NOS activity (presumably NOS1) by pharmacological manipulation blocks all LTP. L-NNA represents the NOS antagonist (white circles) whereas no drug represents control ACSF conditions (black circles). **C.** The plasticity in the GluR1/NOS3 KO was of a similar magnitude to the GluR1 single KO, as there was no significant difference in LTP between WTs, GluR1 KOs and GluR1/NOS3 KOs when measured at 50-60 minutes post-pairing. This suggests that

while NO is important for plasticity, only NO produced by NOS1 affects LTP. In the GluR1 KO, LTP is possible in the absence of NOS3 but not NOS1 or pharmacological inhibitors. In WTs this mechanism only represents a proportion of the LTP (~50% reduction in LTP when a NOS inhibitor was applied (**C**), presumably as postsynaptic insertion of GluR1-containing AMPA receptors is possible. Taken from Hardingham et al., 2010.

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