Chapter 1

Introduction

1.1 Molecular mechanisms of brain function

What is the physiological basis of cognition? The full answer to this question requires nothing less than a detailed understanding of the characteristics of every molecule in the brain, how they interact to form operational signalling pathways, how that signalling impacts on neuronal function, how individual neurons are connected into information-processing networks and, finally, how neuronal networks interact with each other and the rest of the body to produce behaviour.

Among higher order cognitive functions, the ability to acquire, store and recall at will vast quantities of information about ourselves and the world around us is one which possibly contributes the most to our humanity. Memory impairment in human cognitive disorders has a severely deterimental impact on quality of life.

Types of memory

Memory can be divided into declarative and reflexive forms. Reflexive memory has an automatic quality and is not dependent on conscious cognitive processes. It includes storage of specific motor skills such as walking. Declarative memory requires conscious thought and involves cognitive processes such as evaluation, comparison and inference. It encodes specific autobiographical events and associations and can be described using specific declarative statements. For example, the memory of the year astronauts first walked on the moon is declarative (Kandel et al., 2000).

Declarative and reflexive memories are encoded by distinct areas of the brain. Lesions to the temporal lobe, and in particular the hippocampus, disrupt the ability to remember declarative events without affecting reflexive memories. This is demonstrated by the well-known case of patient HM, who, having undergone temporal lobe removal to treat epilepsy, forgets new faces so

rapidly and consistently he introduces himself to the same person hundreds of times over many years, each time as if they are a new acquaintance. He can rapidly learn new motor tasks but although in a later test his performance in the task will be retained he insists he has never performed the task before. The hippocampus seems to store declarative memories only for a limited period of time, after which they are consolidated to other areas of brain, since temporal lobe damage causes anterograde amnesia involving inability to remember new information, while pre-damage memories remain intact (Frankland and Bontempi, 2005; Milner et al., 1998).

The hipppocampus encodes declarative memories

Hippocampus-dependent memory formation is a valuable paradigm for understanding complex cognitive function. There is a wealth of clinical data on the consequences of hippocampal damage in humans (Milner et al., 1998) and there exist also a number of rodent memory tasks which rely on hippocampal function (Gerlai, 2001; Morris et al., 1982). Rodent hippocampal anatomy, including its structure, cell types and circuitry, is very well-characterised, as are physiological manipulations allowing modelling of plastic changes in the area. Decades of research using pharmacological and, more recently, genetic manipulations has provided much information on the biochemical processes underlying hippocampal neuronal network activity and hippocampus-dependent behaviour (Kandel et al., 2000).

1.2 Glutamatergic synaptic transmission

The majority of excitatory synapses in the central nervous system use glutamate as their neurotransmitter. Glutamatergic synaptic transmission is mediated by three types of ionotropic glutamate receptors, classified according to their agonist sensitivity: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), N-methyl-D-aspartate (NMDA) and kainate receptors. AMPA receptors (AMPARs) open in rapid response to glutamate binding, allowing sodium ions

into the postsynaptic cell and mediating fast synaptic transmission. NMDA receptors (NMDARs) are unusual in requiring both ligand binding and membrane depolarisation for activation, the latter necessary to release the magnesium ions which otherwise block the channel (Nowak et al., 1984). This property of 'coincidence detection' makes them an attractive potential mediator for storing information about associated stimuli during learning (Blitzer et al., 2005). Once open, NMDAR channels are permeable not just to sodium and potassium but also to calcium, a crucial aspect of their function in activating intracellular signalling pathways to modify synaptic strength (Lynch et al., 1983; Malenka et al., 1988). Kainate receptors can be expressed both presynaptically and postsynaptically. At some synapses they support synaptic transmission along with AMPARs, while at others they act alone. In the hippocampus, activation of presynaptic kainate receptors in Schaffer collateral axons reduces their transmitter release onto CA1 dendrites (Huettner, 2003). Figure 1.1 shows the structure and operation of a glutamatergic synapse.

NMDAR expression patterns and activation properties

A functional NMDAR consists of two NR1 subunits and two NR2 subunits. There are four NR2 subtypes, designated NR2A, NR2B, NR2C and NR2D, each with their own expression patterns and activation characteristics (Dingledine et al., 1999; Ottersen and Landsend, 1997). NR2A and NR2B are predominantly expressed in the forebrain, particularly in the hippocampus, cortex and olfactory bulb. NR2B is more strongly expressed during the early postnatal period while NR2A is dominant during adulthood. NR2C is specific to the cerebellum and thalamus and NR2D is mainly expressed prior to birth (Wenzel et al., 1997; Wenzel et al., 1995). NR2A and NR2B-containing NMDARs have a stronger voltage-gated Mg^{2+} block than those containing NR2C or NR2D, making them more resistant to activation, and specific comparison between NR2A and NR2B subunits show that NR2A-containing receptors have higher open probability when exposed to a brief, synaptic-like pulse of glutamate (Erreger et al., 2005). The time taken for channel closing following activation also varies between subunits: NR2A-containing receptors

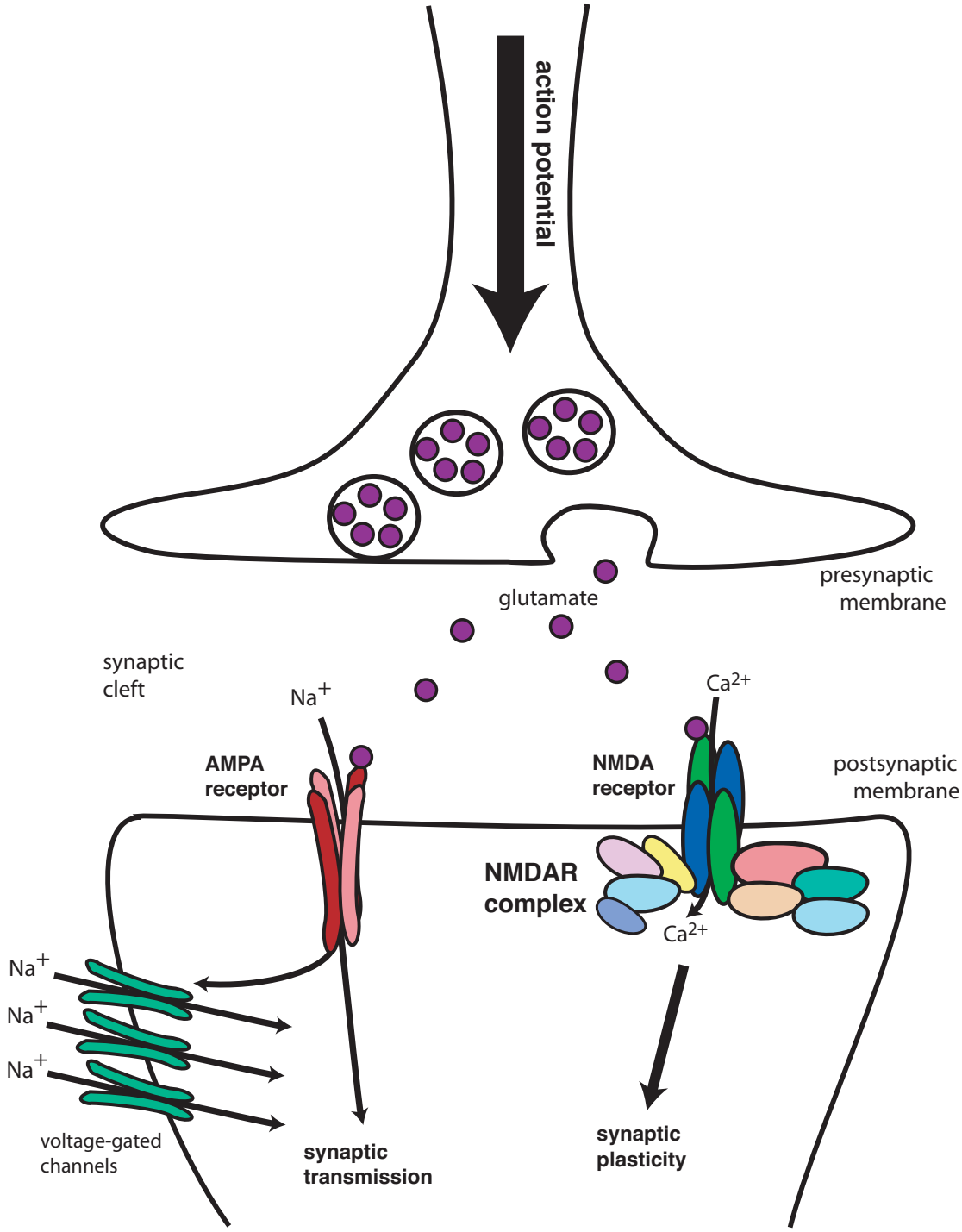

Figure 1.1 The glutamatergic synapse. Arrival of an action potential at the presynaptic terminal stimulates the fusion of synaptic vesicles with the presynaptic membrane and release of glutamate neurotransmitter into the synaptic cleft. Glutamate binding activates AMPA receptor channels, leading to an influx of sodium ions and depolarisation of the postsynaptic membrane. This in turn activates postsynaptic voltage-gated sodium channels further depolarising the membrane and leading to the generation of a new action potential. NMDA receptors are activated preferentially by intense presynaptic activation, since they require both glutamate binding and membrane depolarisation. Activated NMDARs allow an influx of calcium ions which stimulates NMDAR-associated postsynaptic signalling proteins, leading to synaptic plasticity and other modifications of neuronal function.

have the fastest decay time, while those containing NR2B and NR2C have decay time constants around 4-fold greater. NR2D-containing receptors take exceptionally long to close with a decay time constant 40-fold greater than NR2A-containing receptors (Monyer et al., 1994).

1.3 Synaptic plasticity

Information storage in the brain must necessarily be mediated by some persistant physical change in its components. One candidate for such a change is synaptic plasticity, the modification of the strength of a synaptic connection resulting in an increase or decrease in the sensitivity of the postsynapse to presynaptic activation. The origins of modern ideas on the relationship between synaptic plasticity and cognitive function lie in Donald Hebb's 1949 proposal of a physiological mechanism that could potentially translate repeated exposures to associated environmental stimuli into a physical modification of synaptic strength:

When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or 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).

Long-term potentiation is a cellular model of synaptic plasticity

Hebb's theory was bereft of solid physiological basis until more than 20 years later, when Bliss and Lømo discovered that intense synaptic stimulation increases the response of those synapses to subsequent stimuli, a phenomenon called long term potentiation or LTP (Bliss and Lømo, 1973). In the decades since, research effort in this area has focussed on elucidating the physiological characteristics of synaptic plasticity, its molecular mechanisms and its relationship to cognitive function.

LTP is now known to occur at excitatory synapses in virtually every region of the brain but is best characterised in the stratum radiatum of the hippocampus at synapses formed between Schaffer collateral axons projecting from neurons in the CA3 area and the dendrites of CA1 pyramidal cells. In a typical experiment, a stimulating electrode is placed among the Schaffer collateral axons and a recording electrode in stratum radiatum. Baseline synaptic strength is determined by measuring field potentials from the recording electrode in response to repeated pulses low-frequency stimulation. Two pulses of 100 Hz tetanic stimulation are then given followed by a return to baseline stimulation. Potentiation of the slope of excitatory postsynaptic potentials (EPSPs) post-stimulation typically reaches around 150 % of the pre-stimulation level and lasts for several hours. The stimulation frequency threshold for LTP induction is around 5 Hz (Mayford et al., 1995). 1 Hz stimulation produces the opposite effect, a lasting reduction in synaptic response known as long term depression or LTD (Dudek and Bear, 1992).

NMDAR-dependent LTP requires calcium influx

An initial clue to the molecular basis of LTP came with the demonstration that its induction by tetanic stimulation in hippocampal CA1 is prevented by NMDAR antagonists (Collingridge et al., 1983). Pharmacological inhibition of NMDARs also prevents hippocampal-dependent spatial learning in the water maze and a role for NMDARs in these processes fits conceptually well with its coincidence-detecting capabilities (Morris et al., 1986). Low-frequency stimulation-induced LTD in hippocampal CA1 is also NMDAR-dependent (Dudek and Bear, 1992).

It is now known that influx of calcium through activated NMDARs is the critical event for inducing LTP. Most research has focussed on elucidating the mechanisms by which calcium entry leads to changes in synaptic strength. Early emphasis on possible presynaptic mechanisms for LTP induction by increasing transmitter release probability have now mostly given way to a focus on modifications to postsynaptic sensitivity to glutamate. Initial postsynaptic attention focussed

on increasing posttranslational modifications of AMPARs. In particular, LTP induction and/or NMDAR activation increases phosphorylation of GluR1 at ser-831 on its C-terminal tail by CaMKII and PKC, increasing its open channel probability, while LTD dephosphorylates ser-845 and produces the opposite effect (Malenka and Bear, 2004).

More recently it has become clear that the insertion and withdrawal of AMPAR subunits in and out of the postsynaptic membrane is a major mechanism for changing synaptic strength. LTP induction stimulates insertion of AMPARs into the membrane, increasing synaptic response, while LTD has the opposite effect (Bredt and Nicoll, 2003; Malenka and Bear, 2004).

1.4 NMDAR complex and signalling

Targeted mutations in mice have shown that NMDAR subunits and other constituents of the complex are crucial for many aspects of cognitive function including brain development, plasticity, addiction, learning and memory as shown in figure 1.2 (Grant and O'Dell, 2001).

The essentiality of NMDARs for neuronal function is highlighted by the phenotypic effects of their loss in mice (Sprengel and Single, 1999). Germline knockout of NR1 results in perinatal lethality, although the gross anatomy of the central nervous system at death appears undisturbed (Forrest et al., 1994). CA1-restricted NR1 conditional knockout produces viable mice which lack CA1 LTP and show spatial learning deficit in the water maze (Tsien et al., 1996b). Mice with a constitutive knockout of NR2A are viable but also display disrupted CA1 LTP and spatial learning (Sakimura et al., 1995). NR2B knockouts die perinatally from lack of a suckling response (Kutsuwada et al., 1996). Deletion of only the intracellular C-terminal of these subunits produces phenotypes similar to the full knockout, suggesting a default in intracellular signalling is responsible (Sprengel et al., 1998).

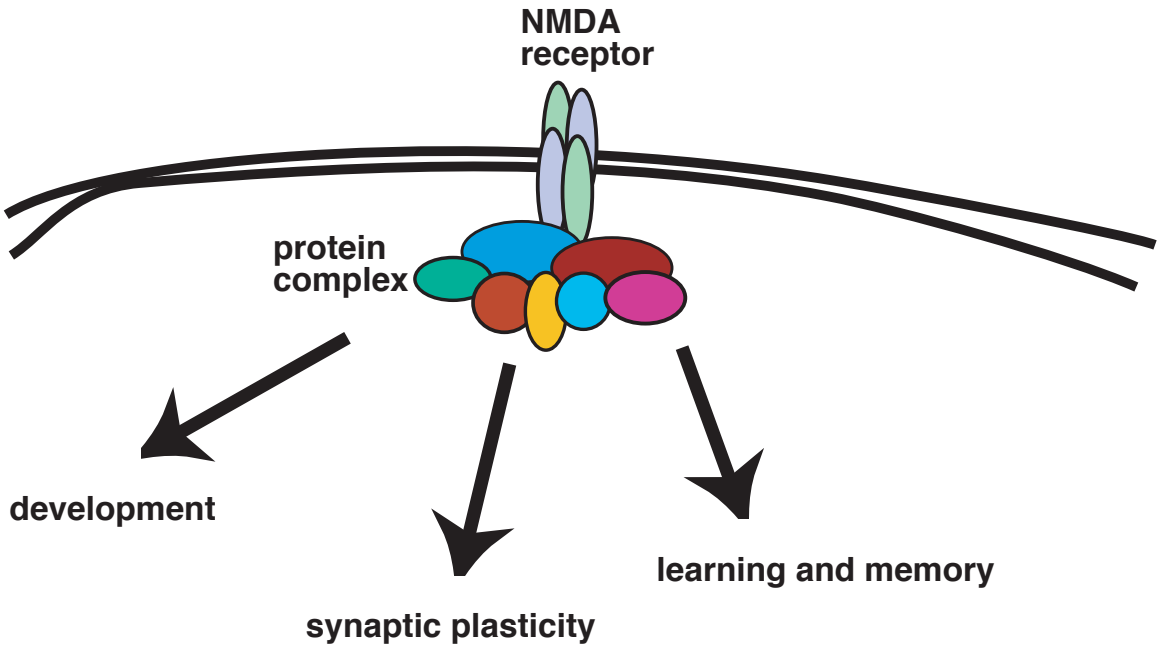

Figure 1.2 NMDA receptors are essential for many aspects of neuronal function. Activation of NMDARs is transduced to a large postsynaptic signalling complex containing adaptors, enzymes and cytoskeletal proteins. Signalling through the receptor and its complex is required for neuronal development, synaptic plasticity and complex cognitive functions.

Purification of NMDARs and their associated proteins show the receptors form part of a large signalling complex of more than 100 adaptors, enzymes and cytoskeletal proteins (Grant and O'Dell, 2001; Husi et al., 2000). The complex connects NMDARs to a multitude of intracellular signalling pathways and, through them, to various mechanisms for changing neuronal properties. The major mechanism by which NMDAR activation impacts on intracellular signalling is by influx of calcium through the open NMDAR channel pore. Calcium activates diverse signalling molecules including cAMP-dependent protein kinase (PKA), calcium/calmodulin-dependent protein kinase II (CaMKII) and the MAP kinase pathway. These changes can modify neuronal properties directly at the synapse, for example by altering postsynaptic sensitivity to glutamate through posttranslational modifications and trafficking of AMPARs, and indirectly through synapse-nucleus signalling to modify the cellular transcriptional programs, for example to alter synaptic structure (Blitzer et al., 2005; Malenka and Bear, 2004).

The interactions and priorities among the plethora of signalling molecules surrounding NMDARs are not well understood. What are the rules governing which pathway responds under what circumstances? The number and complexity of molecules and pathways implies that elucidation of the organising principles surrounding the complex will be a valuable step towards understanding its roles in neuronal function.

Correlations between synaptic plasticity and cognitive function

If the same synaptic plasticity mechanisms that underlie hippocampal LTP were also responsible for hippocampal-dependent spatial learning, manipulations that modified one would also alter the other. This prediction has been extensively explored using mice carrying engineered mutations in postsynaptic proteins. While, it is often the case that such mutations influence performance in both paradigms, no clear correlation has emerged. Null mutations in some genes, such as NR2A

(Tsien et al., 1996b), α-CaMKII (Silva et al., 1992a; Silva et al., 1992b), fyn (Silva et al., 1992a) and synGAP (Komiyama et al., 2002) result in LTP deficits and impaired spatial learning. Overexpression of NR2B produces elevated LTP and enhanced water maze performance (Tang et al., 1999). However, examples also abound in which only one or the other is affected, for example, GluR1 knockout ablates LTP but spares water maze performance (Zamanillo et al., 1999). IP3-kinase(A) knockout mice have enhanced LTP but unaltered spatial learning (Jun et al., 1998). Still other manipulations have apparently opposing effects on the two phenomena, such as mutations in PSD-95 which produce impaired spatial learning but enhanced LTP (Migaud et al., 1998). Clearly the value of LTP is as a model of cellular synaptic plasticity whose underlying mechanisms overlap with, but do not equate with, current spatial learning paradigms.

The MAP kinase pathway in NMDAR-dependent synaptic plasticity and learning

The Mitogen-Activated Protein Kinase (MAPK) pathway was originally described as a cascade inducing cellular division and differentiation in response to extracellular growth signals in non-neuronal cells (Pearson et al., 2001). The discovery of highly-expressed MAPK components in fully differentiated, post-mitotic neurons, however, indicated the pathway must play a somewhat different role in these cells and it is now known to be intimately involved in neuronal signalling in NMDAR-dependent synaptic plasticity, learning and memory (Sweatt, 2004; Thomas and Huganir, 2004).

In classical MAPK signalling, mitogens activate small G proteins like Ras by increasing its GTP-bound state. This is executed by changing the activity of GTPase Activating Proteins (GAPs) which inhibit Ras by enhancing its endogenous GTPase activity and Guanine Nucleotide Exchange Factors (GEFs) which activate Ras by catalysing the production of GTP from GDP. Ras-GTP activates kinases such as the Raf proteins which phosphorylate and activate

MAPK/ERK Kinase (MEK). MEK in turn phosphorylates extracellular signal-related kinase 1 and 2 (ERK1 and ERK2, also called p44 and p42 respectively). The ERKs are themselves serine/threonine kinases whose targets include transcription factors, cytoskeletal proteins, regulatory enzymes and other kinases which mediate cellular growth and differentiation. In neurons, intracellular calcium influx induced by NMDAR activation at glutamatergic synapses activates the MAPK pathway by a currently unknown mechanism (Ghosh and Greenberg, 1995; Thiels et al., 2002; Thomas and Huganir, 2004).

Blum et al. (1999) demonstrated that water maze training increases ERK phosphorylation in hippocampal areas CA1 and CA2 and that administration of the MEK inhibitor P098059 into the hippocampus before or after training impairs retention, but not acquisition, of spatial memories in the maze. Selcher et al. (1999) also found a requirement for hippocampal MAPK activation in water maze learning using the MEK inhibitor S327; in this case the intervention impaired both latency to reach the hidden platform during training and preference for the training quadrant in probe tests, but was only effective when administered prior to training. No impairment was observed during visible platform training. Infusion of P098059 into the entorhinal cortex also impairs spatial learning in the water maze (Hebert and Dash, 2002). SL327 administration one hour prior to training also inhibits hippocampal-dependent contextual fear conditioning (Atkins et al., 1998).

NMDA-dependent synaptic plasticity also requires MAPK activation: P098059 application to hippocampal slices one hour prior to tetanic stimulation greatly reduces both LTP induction and the consequential elevation of phosphorylated ERK levels (Davis et al., 2000; English and Sweatt, 1997). SL327 blocks both NMDAR-dependent LTD in CA1 of the hippocampus and its associated increase in ERK phosphorylation (Thiels et al., 2002).

Studies of the effects of MAPK activation during synaptic plasticity and learning implicate the ERK targets CREB, RSK2 and Elk-1 in activating downstream transcription. LTP induction results in phosphorylation of these targets along with induction of the immediate early genes zif268 and arc. These phosphorylation and transcriptional events are blocked by MEK inhibitor administration (Davis et al., 2000; Impey et al., 1998; Thomas and Huganir, 2004). MEK inhibitors also impair LTP induced by 5 Hz stimulation (Winder et al., 1999).

Opposing roles of NMDAR subunits in synaptic plasticity

Recent evidence suggests the NMDAR subunits NR2A and NR2B may play opposing roles in synaptic plasticity. Treatment of acute hippocampal slices with the NR2B-selective antagonists ifenprodil or Ro25-6981 blocks NMDAR-dependent LTD, but not LTP, in area CA1. In the same region, the NR2A-selective antagonist prevents induction of NMDAR-dependent LTP but has no effect on LTD (Liu et al., 2004; Massey et al., 2004). Biochemically, surface expression of the GluR1 AMPAR subunit is increased by NR2B antagonists and decreased by NR2B overexpression. In contrast, knockdown of NR2A by RNA interference (RNAi) reduces delivery of GluR1 to the cell surface and steady state levels of surface GluR1 (Kim et al., 2005). These results suggest LTP induction preferentially activates NR2A-containing NMDARs, leading to a consequent increase in the number of synaptic AMPAR channels, while LTD-inducing stimuli activate NR2B-containing NMDARs, stimulating AMPAR withdrawal from the synaptic membrane.

A possible mechanism for this phenomenon is provided by the observation that inhibition of NR2B by pharmacological antagonist or RNAi prolongs the NMDA-induced activation of the MAPK pathway constituents Ras and ERK (Kim et al., 2005). SynGAP, which inhibits MAPK signalling through its Ras GTPase activity, interacts with NMDARs through the PSD-95 family of Membrane-Associated Guanylate Kinase (MAGUK) proteins and preferentially associates

with NR2B-containing receptors (Kim et al., 2005; Sans et al., 2000). RNAi knockdown of SynGAP prolongs NMDA-induced ERK activation and reduces GluR1 cell surface expression (Kim et al., 2005). According to these data, then, low-frequency stimulation activates NR2B-containing NMDARs, stimulating synGAP which reduces MAPK activity, leading to synaptic withdrawal of AMPARs. High-frequency stimulation activates NR2A-containing receptors, leading to AMPAR insertion.

Further support for this hypothesis comes from experiments using the known activation characteristics of NMDAR subunits to simulate the responses of NMDARs to different types of synaptic stimulation. Under high-frequency, 100 Hz, tetanic stimulation the greater opening probability and faster rise and decay times of NR1/NR2A receptors means they contribute more to calcium influx than NR1/NR2B receptors, while the reverse is true for low-frequency, 1 Hz stimulation (Erreger et al., 2005).

There remain a number of issues surrounding this model that need explanation, however. Transgenic mice with forebrain-specific overexpression of NR2B display enhanced LTP with unaltered LTD (Tang et al., 1999). Other experiments indicate that robust LTP is possible even under strong pharmacological NR2A inhibition, particularly when induced by multiple tetanic stimuli (Berberich et al., 2005), and LTP can be partially induced in NR2A knockout mice (Sakimura et al., 1995).

1.5 The PSD-95 family of Membrane-Associated Guanylate Kinases

Interacting directly with the intracellular C-terminal tails of NR2 subunits of NMDARs is a family of adaptor proteins whose function may be crucial in organising the response of the plethora of PSD signalling proteins to synaptically-induced calcium influx. This is the PSD-95 family of proteins, which are Membrane-Associated Guanylate Kinases (MAGUKs) localised to

cell-cell junctions. MAGUKs are defined by a common domain structure with one or more PDZ (postsynaptic density-95/discs large/zona occludens-1) domains, a src homology 3 (SH3) domain and a guanylate kinase-like (GK) domain (Funke et al., 2005). The first MAGUK discovered was discs large (dlg) in *Drosophila*, which was initially identified as a recessive oncogenic mutation (Stewart et al., 1972) but not cloned until two decades later (Woods and Bryant, 1991). Its protein, DLG, is the sole *Drosophila* representative of the Postsynaptic Density-95 (PSD-95) family of MAGUKs, which in mammals consists of PSD-95, Postsynaptic Density-93 (PSD-93), Synapse-Associated Protein 102 (SAP102) and Synapse-Associated Protein 97 (SAP97, the mammalian homologue of DLG). PSD-95 family proteins carry three tandem PDZ domains each as shown in figure 1.3.

Domain properties

PDZ domains are modular protein-interaction domains which bind to short peptide motifs at the C-termini of other proteins. They are found in a variety of different proteins and often in tandem in multidomain scaffolding proteins which mediate formation of multiprotein complexes. PSD-95 family proteins carry type 1 PDZ domains which bind the C-terminal motif S/T-X-V (Nourry et al., 2003).

SH3 domains are also protein-interaction domains which occur in proteins of varied function. Unlike canonical SH3 domains, those in MAGUKs rarely bind to polyproline Pro-X-X-Pro motifs. Instead they interact intramolecularly with the GK domain and thus may regulate interactions between MAGUKs and their GK-binding proteins (Masuko et al., 1999).

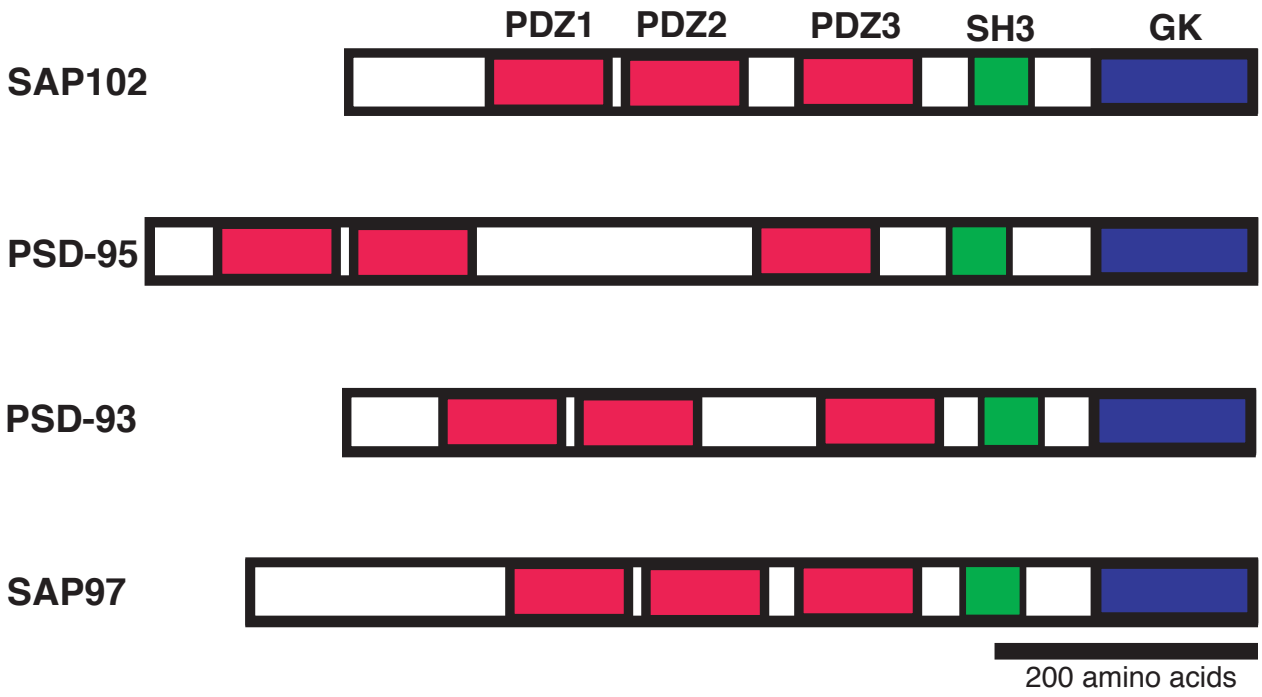

Figure 1.3 Domain structure of the PSD-95 family of Membrane Associated Guanylate Kinase proteins. Each member carries multiple protein-protein interaction domains including three tandem PDZ domains, an SH3 and a GK domain. SAP102, PSD-95 and PSD-93 are associated with NMDA receptors while SAP97 interacts with AMPA receptors. Adapted from Fujita and Kurachi (2000).

The MAGUK GK domain shares only 40 % sequence homology with the true yeast guanylate kinase and has lost its enzymatic capacity to produce guanosine diphosphate. An early study suggested that while the MAGUK GK domain could no longer interact with adenosine triphosphate (the phosphate group donor), it retained some capacity for binding to guanosine monophosphate (the reaction substrate) (Kistner et al., 1995) and there was even some suggestion of a possible role for the nucleotide in regulating GK-dependent PSD-95 protein interactions (Li et al., 2002). Further examination, however, failed to replicate these results and more convincingly showed a lack of GMP binding resulting from the loss of two conserved residues in the GMP-binding pocket of PSD-95 (Olsen and Bredt, 2003).

Regional expression patterns and subcellular localisation

MAGUK proteins are highly expressed in the brain. SAP102, PSD-95 and PSD-93 are most abundant in the hippocampus, cortex and olfactory bulb (Brenman et al., 1996; Fukaya et al., 1999; Fukaya and Watabe, 2000) while SAP97 is more highly expressed in the cerebellum (Muller et al., 1995).

The subcellular localisation of the MAGUKs has historically been the subject of some disagreement. Biochemical fractionation experiments showed a concentration of SAP102, PSD-95 and PSD-93 in the postsynaptic density (Brenman et al., 1998; Cho et al., 1992; Muller et al., 1996) - indeed, PSD-95 was discovered by virtue of its presence in this fraction (Cho et al., 1992) - and electron microscopic (EM) studies showed selective labelling of the postsynaptic junctional membrane (Aoki et al., 2001; Roche et al., 1999; Sans et al., 2000). However, early conventional immunohistochemical experiments presented staining in the cytoplasm of dendritic shafts as well as in neuronal perikayra (Bassand et al., 1999; Hunt et al., 1996; Kim et al., 1996). These data also appeared to contradict regional mRNA expression patterns shown by *in situ* hybridisation (Fukaya et al., 1999). The puzzle was eventually solved by the observation that protease

pretreatment of tissue sections prior to immunohistochemistry resulted in sharp, punctate, synaptic MAGUK staining patterns and empty dendritic shafts, in precise concordance with the EM and mRNA results (Fukaya and Watabe, 2000; Watanabe et al., 1998; Wenzel et al., 1995). The same problem in localising NMDAR subunits had been resolved by this method, suggesting that the inaccessibility of the PSD to antibodies may be to blame (Watanabe et al., 1998; Wenzel et al., 1995). It is now generally agreed that SAP102, PSD-95 and PSD-93 are postsynaptic, while SAP97 occurs both pre- and postsynaptically (Aoki et al., 2001; Funke et al., 2005).

Developmental expression profiles

In a comprehensive examination of the developmental expression patterns of NMDARs and their associated MAGUKs in the mouse hippocampus, Wenthold and his colleagues (Petralia et al., 2005; Sans et al., 2000) found intriguing correlations between NR2A, PSD-95 and PSD-93 on one hand and NR2B and SAP102 on the other. The former group is virtually undetectable by western blot at postnatal day 2 but increase their levels during postnatal development to be robustly expressed at 3 months of age. In contrast, the latter two are both expressed at P2, with NR2B declining during development and SAP102 expression increasing only moderately before levelling off at P35 then declining slightly through adulthood. These patterns are to a greater extent reflected in their respective mRNA levels during development (Fukaya et al., 1999; Monyer et al., 1994; Wenzel et al., 1997). These experiments suggest that SAP102 and PSD-95 may differentially facilitate the function of NR2B- and NR2A-containing NMDARs, respectively, and is consistent with the observations of distinct activation properties, signalling associations and roles in plasticity for the two receptor subunits.

At the synaptic level, immunogold electron microscopy examination reveals the developmental increase in hippocampal PSD-95 is a result of greater numbers of synapses containing the protein, while the number of molecules per synapses remains static. In contrast, both particles per synapse

and number of synapses labelled with SAP102 are at their maximum at P2 and decrease during postnatal development (Sans et al., 2000). This observation is at odds with the total developmental expression levels of SAP102 described above, but could possibly be accounted for by an increase non-synaptic SAP102.

Biochemical associations

Like the NMDAR subunits, SAP102, PSD-95 and PSD-93 are found in the cell membrane fraction of tissue protein extracts (Lau et al., 1996; Sans et al., 2003) and co-immunoprecipitation experiments show that MAGUK proteins associate with their respective glutamate receptors. SAP102, PSD-95 and PSD-93 co-immunoprecipitate with NMDAR but not AMPAR subunits, while the reverse is true for SAP97 (Cai et al., 2002; Garcia et al., 1998; Lau et al., 1996; Leonard et al., 1998; Muller et al., 1996; Sans et al., 2001). The correlated temporal expression patterns of SAP102 and PSD-95 with NR2B and NR2A respectively are recapitulated in their co-immunoprecipitation patterns, that is, more SAP102 co-immunoprecipitates with NR2B and more PSD-95 co-immunoprecipitates with NR2A, lending credence to the idea that each MAGUK supports the function of distinct NMDAR subtypes. The reciprocal experiment, immunoprecipitating each MAGUK and blotting for the receptor subunits, produces a similar result (Sans et al., 2000; Townsend et al., 2003). The C-terminal PDZ binding motif of NR2B also binds SAP102 preferentially over PSD-95 *in vitro* (Lim et al., 2002).

Immunogold double labelling electron microscopy of the NMDAR-associated MAGUKs at CA1 hippocampal synapses suggests it is not the case that distinct populations of synapses carry particular subsets of these proteins. SAP102 and PSD-95 double labelling shows some synapses contain one, some the other, and some both proteins with the proportion of double labelled synapses varies between 10 and 35 % according to their combined abundance at different developmental time points in what appears to be a random distribution of the two proteins.

Similar patterns are seen with other labelling combinations: the maximum proportion of synapses double-labelled for SAP102 and PSD-93 is 16 %, and for PSD-95 and PSD-93 is 33 % (Sans et al., 2000).

Clustering properties

Excitement surrounding the PSD-95 family was first elevated with the observation that PSD-95 possessed a striking ability to cluster the voltage-gated potassium channel Kv1.4 at the cell surface when the two proteins were co-expressed in heterologous cells, suggesting it may function to anchor ionotropic receptors in the synaptic membrane (Kim et al., 1995). This observation was quickly replicated in other laboratories with different cell lines, MAGUKs and membrane-bound receptors and the functional hypothesis was supported by the observation of co-localisation of the receptors and MAGUKs in mammalian neurons (El-Husseini et al., 2000a; El-Husseini et al., 2000b; Imamura et al., 2002).

The general observation is that a MAGUK or ionotropic receptor expressed in isolation in, for example, HEK cells, produces a smooth and uniform staining pattern throughout the cell cytosol. In contrast, co-expression of both the MAGUK and receptor results in co-localisation of the two proteins in clustered staining patterns on the cell surface. The two proteins co-immunoprecipitate with each other and the clustered receptor can be shown to be inserted into the membrane and functional by recording the appropriate ion currents into or out of the cell. Use of mutant receptors lacking the S/TxV binding motif at their C-terminus shows that these phenomena are dependent on interactions between the receptor and the PDZ domains of the MAGUK.

Similar methodologies have been used to show that PSD-95 clusters NR1 (El-Husseini et al., 2000a), the inward rectifying potassium channel Kir4.1 (Horio et al., 1997), the *ShaI* voltage-sensitive potassium channel family member Kv4.2 (Wong et al., 2002), the kainate receptor KA2

(Garcia et al., 1998) and the neuregulin receptor ErbB4 (Huang et al., 2002). PSD-93 clusters NMDA receptors and potassium channels (Kim et al., 1996). SAP102 does not cluster NMDA receptor subunits or potassium channels in heterologous cells, a property attributed to its lack of palmitoylation (El-Husseini et al., 2000b). It has been demonstrated, however, to cluster transfected Kv1.4 at postsynaptic sites in a PDZ interaction-dependent manner in hippocampal neuronal cultures (Firestein et al., 1999).

As will be shown later in this section, however, these results need to be interpreted with caution. A demonstration that two proteins can associate when overexpressed at high levels in a foreign cell by no means proves the interaction occurs endogenously and with functional significance in neurons *in vivo*. Where such a functional interaction does occur it still remains to be proven that the interaction is necessary for the observed phenotype, in this case receptor clustering.

Links with postsynaptic signalling pathways

Links between PSD-95 family proteins and specific postsynaptic signalling pathways currently rely almost exclusively on simple protein interaction data rather than more convincing demonstrations of altered signalling following disruption of MAGUK expression. For example, their association with synGAP implies a role in MAPK signalling. An exception to this is the observation that not only does PSD-95 interact with neuronal nitric oxide synthase (nNOS), but that antisense-mediated PSD-95 knockdown reduces nitric oxide production and NMDA-induced excitotoxicity in culture cortical neurons (Sattler et al., 1999).

Mutations in PSD-95 family proteins *in vivo*

MAGUK expression early in development suggests mice with mutations in these proteins might display developmental phenotypes and there is some evidence of this. SAP97 knockouts are perinatal lethal (Caruana and Bernstein, 2001). PSD-95 homozygous mutants survive but in

reduced numbers and many show delayed postnatal development, catching up in size only upon reaching adulthood at around seven weeks of age (Migaud et al., 1998). In PSD-93 mutant mice viability appears unaffected (McGee et al., 2001).

As a result of their clustering properties in heterologous cells, there has been much interest in whether loss of NMDAR-associated MAGUKs *in vivo* would lead to alterations in NMDAR synaptic localisation. In *Drosophila*, where DLG is the only PSD-95 family protein present, the neuromuscular junction contains two types of glutamate receptors, those containing the GluRIIA subunit and those containing GluRIIB. Dlg colocalises with these glutamate receptors at the postsynapse and flies with a loss-of-function *dlg* mutation exhibit loss of GluRIIB surface expression and concomitant decreases in glutamate receptor currents and spontaneous excitatory junctional currents. They also show an increase in single channel glutamate receptor currents, a phenotype observed in GluRIIB mutant flies (Chen and Featherstone, 2005).

In mammalian cells, however, only PSD-93 has thus far been proven necessary for mammalian ionotropic receptor localisation and then only in specific situations. In the forebrain and spinal cord, loss of PSD-93 results in reduced surface expression of NR2A and NR2B subunits, decreased NMDA component of EPSC amplitudes and lowered sensitivity to NMDAR-dependent neuropathic pain (Tao et al., 2003). In contrast, in cerebellar Purkinje cells, where PSD-93 is the only MAGUK expressed and there is thus no possibility for compensation from other family members, loss of PSD-93 has no effect on EPSCs or short term plasticity measured by paired pulse facilitation or depression, suggesting normal NMDAR localisation and function in these cells (McGee et al., 2001). A truncating mutation in SAP97 in mice which removes the SH3 and GK domains has no apparent effect on AMPAR subunit localisation in cortical neuronal cultures (Klöcker et al., 2002). In the hippocampus of PSD-95 mice no gross disruptions of NMDA receptor subunit distribution are evident at light or electron microscopic levels and NMDA-

induced currents and NMDA component of EPSCs are normal (Migaud et al., 1998). Neither is any disruption of high-density clustering of MAGUK-interacting voltage gated potassium channels Kv1.1, Kv1.2, Kv1.4 or Kvβ2 observed at juxtaparanodes adjacent the nodes of ranvier in the optic nerve where PSD-95 is the only MAGUK expressed (Rasband et al., 2002). These data are not exhaustive, but the emerging picture is that SAP97 and PSD-95 do not function to anchor their respective receptors at the synapse. There is as yet no convincing evidence for a requirement or lack thereof for SAP102 in postsynaptic receptor localisation or function.

MAGUK association with glutamate receptors and other signalling molecules at the postsynapse is also suggestive of involvement in emergent neuronal properties such as synaptic plasticity, learning and memory, however, phenotypic data of this type from mouse mutants is surprisingly patchy. Cerebellar postsynaptic currents and short term synaptic plasticity are normal in PSD-93 knockout mice as described above (McGee et al., 2001). Data on long-term synaptic plasticity is available only for PSD-95, where long-term potentiation (LTP) is strikingly enhanced in stimulation protocols of varied frequency, even with low-frequency (1 Hz) stimulation, such that LTD is effectively abolished (Migaud et al., 1998). The long term synaptic plasticity phenotype of PSD-95 mutant mice is shown in figure 1.4. There is no published data on the involvment of SAP102, PSD-93 or SAP97 in LTP. Data on learning behaviour in MAGUK mutants is available only for PSD-95 mice, which perform normally with a visual cue in the water maze but have impaired spatial learning in the hidden platform version of the task which cannot be improved by overtraining (Migaud et al., 1998).

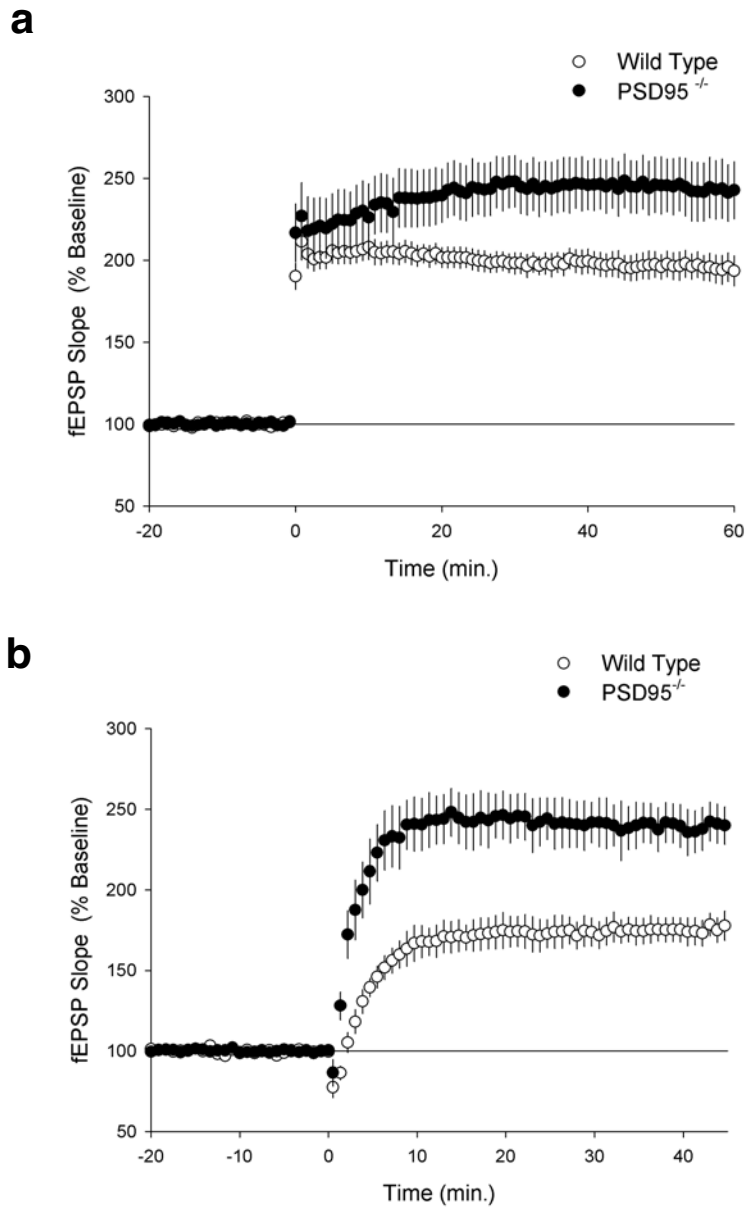

Figure 1.4 Enhanced NMDAR-dependent hippocampal synaptic plasticity in mice with a targeted mutation in PSD-95. Synaptic plasticity is measured here using long term potentiation (LTP), in which the strength of synapses formed between Schaffer collateral axons and CA1 pyramidal cell dendrites is increased following tetanic stimulation delivered at time zero. Potentiation of postsynaptic responses is observed after both **(a)** 100 Hz and **(b)** 5 Hz stimulation. From Migaud et al., 1998.

Hypotheses of the function of PSD-95 family proteins

Current knowledge of the properties of the PSD-95 family of proteins described above has led to two major hypotheses as to their function at the postsynapse (Fujita and Kurachi, 2000; Funke et al., 2005; Montgomery et al., 2004):

1. *Localisation and clustering of postsynaptic transmembrane receptors.*

This hypothesis arises from the observation that the family associates with numerous transmembrane proteins via their PDZ binding motifs and is able to cluster such proteins at the surface of neurons and heterologous cells.

2. *Organisation of intracellular postsynaptic signalling molecules in close proximity to relevant transmembrane receptors to facilitate efficient signal transduction.*

The need for proteins with such a function is suggested by the sheer number of signalling proteins in the postsynaptic density, the array of signalling pathways known to be sensitive to synaptic activation and the rapid response of these pathways to signals such as calcium influx through NMDARs. PSD-95 family proteins, linking multiple signalling molecules with transmembrane receptors, seem ideally placed to fulfill such a role.

1.6 Properties of SAP102

This dissertation focusses on the function of SAP102 (dlg3, neuroendocrine dlg, NE-dlg) as the PSD-95 family protein whose *in vivo* function remains uncharacterised. The following section describes in detail the current knowledge of SAP102 properties.

Expression patterns and localisation

As described above, SAP102 is predominantly expressed in the hippocampus, cortex and olfactory bulb in the brain. Northern and Western blotting of rat tissue fails to detect SAP102

mRNA or protein in liver, heart or muscle (Muller et al., 1996). In humans it has been reported as expressed in trachea, prostate, stomach, spinal cord, cardiac myocytes, islets of Langerhans and cell-cell junctions in the oesophageal epithelium, but absent from lung, liver, skeletal muscle, kidney, placenta, lymph nodes and proliferating cells such as basal oesophageal epithelial cells and cultured cancer cell lines (Makino et al., 1997). SAP102 is also expressed in the retina (Koulen, 1999; Koulen et al., 1998). Developmentally, SAP102 begins to be produced at P2 and increases over the following postnatal week before levelling off into adulthood (Monyer et al., 1994; Petralia et al., 2005; Sans et al., 2000; Wenzel et al., 1997).

Like NMDAR subunits, SAP102 is found in the membrane fraction of tissue protein extracts, being insoluble in Triton X-100, CHAPS and RIPA but soluble in SDS and DOC detergents (Lau et al., 1996; Sans et al., 2003).

Binding partners

SAP102 has numerous and varied interacting protein partners. It is unlikely the following list is complete, however, since many more proteins are known to bind to PSD-95. Most experiments in this area have been performed on only one or two of the three NMDAR-interacting PSD-95 family proteins, thus little is known about the potential differential interaction preferences; such investigations are likely to be valuable in elucidating distinct functions of SAP102, PSD-95 and PSD-93.

Transmembrane receptor binding partners

SAP102 interacts with several postsynaptic transmembrane receptors. These include the NR2A and NR2B subunits of the NMDAR (Garcia et al., 1998; Lau et al., 1996; Lim et al., 2002; Muller et al., 1996; Sans et al., 2003), the 5-HT2c subunit of the serotonin receptor (Becamel et al., 2004), the plasma membrane calcium ATPase 4b which maintains intracellular calcium

homeostasis by pumping calcium out of the cell (DeMarco and Strehler, 2001), the axon guidance molecular semaphorin 4C (de Wit and Verhaagen, 2003; Inagaki et al., 2001), the neuregulin growth factor receptor ErB4 (Garcia et al., 2000; Huang et al., 2002) and the low-density lipoprotein receptor megalin (Larsson et al., 2003). This diverse list is strongly suggestive of a role for SAP102 transmembrane receptor function, for example trafficking them to the cell membrane, anchoring them at particular sites after arrival and/or organising their associated intracellular signalling molecules.

Trafficking and adaptor protein binding partners

SAP102 has been implicated in the trafficking of NMDA receptors via its association with the transport protein Sec8. Sec8 is a member of the exocyst protein complex known to be involved in delivery of vesicles to the cell membrane for exocytosis. It binds to SAP102 via a C-terminal class I PDZ binding motif and co-immunoprecipitates from mouse hippocampal extracts with SAP102 and with NMDAR subunits. This association is formed in the endoplasmic reticulum. Overexpression of sec8 in COS cells, which express endogenous SAP102, results in clustering of NR1 subunits in a manner dependent on the sec8 PDZ binding motif. Sec8 overexpression also amplifies the spontaneous excitoxicity induced by co-expression of NR1 and NR2B; this effect is not observed when the PDZ binding motif of either sec8 or NR2B is mutated. In cultured hippocampal neurons, overexpession of a dominant negative form of sec8 lacking the PDZ binding motif decreases punctate, dendritic NMDAR staining and reduces both whole-cell NMDAR currents and synaptic NMDAR EPSCs. These data suggest that NMDARs are associated with sec8 and the exocyst complex via a mutual interaction with SAP102 and that this complex transports the receptor to the synaptic membrane (Sans et al., 2003).

SAP102 also interacts with stargazin, a protein required for delivery of AMPARs to the cell surface in cerebellar granule cells (Chen et al., 2000). However, since SAP102 is not

biochemically associated with AMPARs (Cai et al., 2002; Lau et al., 1996; Sans et al., 2001), the significance of this interaction is not clear. Cypin, another SAP102 interacting protein, may be involved in trafficking of MAGUKs themselves, since cypin overexpression leads to loss of SAP102 clustering in cultured hippocampal neurons (Firestein et al., 1999; Kuwahara et al., 1999). Cypin also interacts with tubulin and regulates dendritic outgrowth and branching in hippocampal neurons (Akum et al., 2004).

The adaptor proteins guanylate kinase-associated protein (GKAP) and SAP90/PSD-95-associated proteins (SAPAPs) 1-4 interact with SAP102 via its GK domain and may anchor it to the cytoskeleton (Kim et al., 1997; Takeuchi et al., 1997).

Signalling protein binding partners

Direct interactions between SAP102 and a number of postsynaptic signalling proteins suggests the MAGUK may ensure physical proximity between postsynaptic transmembrane receptors and their intracellular signalling networks. This could facilitate rapid signalling responses to receptor activation. Perhaps most intriguingly, SAP102 interacts with the synaptic Ras GTPase activating protein SynGAP, which links NMDARs to the MAP kinase pathway (Kim et al., 1998). Mice with a mutation in SynGAP show a deficit in LTP and spatial learning and enhanced activation of ERK in response to NMDA stimulation (Komiyama et al., 2002), suggesting SAP102 may mediate the physical association between NMDARs and MAPK signalling proteins for synaptic plasticity and cognitive function.

Further suggestion of SAP102 involvement in NMDAR-mediated cognitive function arises from its interaction with the tyrosine kinase Pyk2. Pyk2 activates Src kinase which phosphorylates NR2B. Increases in calcium concentration activate Pyk2 and LTP induction also activates Src and results in increased Src phosphorylation of NR2B. Inhibition of Src can inhibit LTP induction

(Seabold et al., 2003). Thus, SAP102 may hold Pyk2 close to NMDARs, allowing efficient signal transduction for synaptic plasticity.

There is also evidence for interactions between SAP102 and the guanine exchange factor kalirin-7 involved in dendritic morphogenesis (Penzes et al., 2001), the calcium signalling protein calmodulin (Masuko et al., 1999) and the tumour suppressor adenomatous polyposis coli (APC) protein (Makino et al., 1997).

Table 1.1 shows currently known SAP102 binding partners along with the SAP102 domain with which they interact and the experimental paradigm used to demonstrate binding. Only proteins for which there is a evidence for a direct interaction with SAP102 are shown. For example, the kainate receptor subunits GluR6 and KA2 co-immunoprecipitate with SAP102 from rat brain extracts and have been shown to bind the PDZ domains of PSD-95 *in vitro* (Garcia et al., 1998) but have not been shown to bind directly to SAP102.

Mutations in SAP102 cause XLMR

Recently, a large-scale exon-resequencing effort identified four families with NS-XLMR associated with truncating mutations around the second and third PDZ domains of SAP102. Affected males have moderate to severe mental retardation with IQ levels between 31 and 48, developmental delay and learning disabilities. No non-cognitive symptoms are apparent (Tarpey et al., 2004).

Table 1.1 SAP102 interacting proteins. Categories of evidence for interaction with SAP102 are: 1 – yeast 2-hybrid, 2 – co-immunoprecipitation from double-transfected heterologous cells, 3 - in vitro binding assay (3^a - overlay assay, 3^b - GST pulldown, 3^c - ELISA, 3^d - surface plasmon resonance 3^e – affinity chromatography with mass spectrometry or amino acid sequencing), 4 – co-immunoprecipitation of endogenous proteins from brain extracts.

Protein	Description	Function	SAP102 domain	Evidence	References
NR2A	Ionotropic glutamate receptor subunit	Excitatory synaptic transmission, synaptic plasticity	PDZ	3^a, 4	(Lau et al., 1996) (Garcia et al., 1998)
NR2B	Ionotropic glutamate receptor subunit	Excitatory synaptic transmission, synaptic plasticity	PDZ	$3^{a, b, c}$, 4	(Muller et al., 1996) (Sans et al., 2003) (Lim et al., 2002)
5-HT2c	G protein-coupled serotonin receptor subunit	Serotonergic synaptic transmission: mood, sleep, appetite	PDZ	3^e	(Becamel et al., 2004)
Kir2.2	Inward-rectifying potassium channel	Regulation and maintenance of cell excitability	PDZ	3^e	(Leonoudakis et al., 2004)
ErbB4	Neuregulin transmembrane receptor	Growth factor receptor. Regulates expression of voltage- and ligand-gated channels in neurons	PDZ	1	(Garcia et al., 2000) (Huang et al., 2002)
Semaphorin 4C	Transmembrane semaphorin	Axonal guidance	PDZ	1	(Inagaki et al., 2001) (de Wit and Verhaagen, 2003)
PMCA4b	Plasma membrane calcium ATPase	Maintenance of calcium homeostasis	PDZ	1, 2, 3^b	(DeMarco and Strehler, 2001)
Megalin	Low-density lipoprotein membrane receptor	Endocytosis, signalling	PDZ	1, 3^d	(Larsson et al., 2003)
PSD-95	Postsynaptic adaptor protein	Postsynaptic organisation, synaptic plasticity, spatial learning	SH3/GK	1, 3^b	(Masuko et al., 1999)
Sec8	Exocyst complex	Vesicle trafficking and exocytosis	PDZ	1, 3^b, 4	(Sans et al., 2003)
Stargazin	Relative of γ-1 calcium channel	AMPAR trafficking and localisation	PDZ	2	(Chen et al., 2000)
Cypin	guanine deaminase enzyme	Cytoskeleton assembly, regulation of dendritic branching, MAGUK trafficking	PDZ	2, $3^{b, e}$, 4	(Firestein et al., 1999) (Akum et al., 2004) (Kuwahara et al., 1999)
GKAP	Postsynaptic adaptor protein	Assembly of multiprotein complexes, anchoring to cytoskeleton	GK	1	(Kim et al.)
SAPAP	Postsynaptic adaptor protein	Assembly of multiprotein complexes, anchoring to cytoskeleton	GK	1	(Takeuchi et al., 1997)
SynGAP	Synaptic Ras-GTPase activating protein	MAP kinase signalling, synaptic plasticity	PDZ	1, 4	(Kim et al., 1998) (Komiyama et al., 2002)
Kalirin-7	Rac1 guanine nucleotide exchange factor	Dendritic spine morphogenesis	PDZ	1	(Penzes et al., 2001)
Pyk2	Tyrosine kinase	Src activation, synaptic plasticity	SH3	2, 3^b, 4	(Seabold et al., 2003)
Calmodulin	Calcium binding protein	Calcium signalling	SH3/GK	$3^{b, d}$	(Masuko et al., 1999)
APC	Wnt signalling	Tumour suppressor	PDZ	1, 3^b	(Makino et al., 1997)

1.7 Genetic approaches to elucidating gene function

The mouse as a model organism

The best method of analysing the function of a gene is to examine the phenotypic consequences of its loss *in vivo*. The mouse has long been a favoured model organism for such genetic ablation experiments as a result of its small size, short life cycle, prolific breeding capabilities and broad physiological similarity to humans (Capecchi, 2005; van der Weyden et al., 2002). This similarity applies to the brain, where the majority of neuronal structures, cell types and physiological processes found in the human brain are also present in the mouse (Kandel et al., 2000). Equally important for gene function analyses is the genetic similarity between the two organisms, an issue which has recently been become much clearer as a result of genome sequencing projects. The mouse genome contains 2.5 billion base pairs of DNA, slightly smaller than the human at 2.9 billion, but both contain approximately 30,000 genes and the identity of those genes is strikingly similar: 99 % of mouse genes have a human homologue. The broad structure of the two genomes is also similar, with around 90 % of each residing in unambiguously syntenic regions. Coding regions are well conserved at 85 % identity between mouse and human at the DNA level. Untranslated regions have 75 % identity and even introns 69 %. At the protein level, an analysis of nearly 13,000 1:1 orthologues (those with direct equivalents in the other genome which have arisen from the same ancestral gene) have 70.1 % amino acid identity (Consortium, 2001; Consortium, 2002).

Before the advent of mutagenesis technology, geneticists relied on finding naturally-occurring genetic mutations by looking for interesting inherited phenotypes in breeding colonies of mice. In this way the genetic bases of physiological traits such as coat colour, skeletal morphology and behaviour were studied long before the genes involved were cloned and characterised (Morgan et al., 1999; Rakyan et al., 2003; Zeng et al., 1997).

The mid-1980s saw the beginning of a revolution in mouse genetics with the demonstration that a piece of foreign DNA could be inserted into the mouse genome at a predetermined or 'targeted' location by homologous recombination in totipotent embryonic stem (ES) cells (Doetschman et al., 1987; Smithies et al., 1985; Thomas and Capecchi, 1987) and that those targeted ES cells, when injected into an early blastocyst, could contribute to the germline of a mouse (Capecchi, 2005; Schwartzberg et al., 1989; Thompson et al., 1989). With this technology came the ability to ablate any specific murine gene and examine its effect *in vivo*. The most common type of DNA construct now used to target a gene is the replacement vector. This consists of a plasmid containing a positive selectable marker for genomic insertion, flanked by sequences homologous to the site of insertion (homology arms). The plasmid backbone contains a unique restriction site for linearisation and a negative selectable marker such as herpes simplex thymidine kinase (HSVtk) or the diphtheria toxin A fragment (DTA) to discourage integration of the entire construct into a random genomic location. For targeting, the linearised vector is introduced into ES cells and undergoes homologous recombination, replacing the genomic target sequence with the targeting fragment (figure 1.5). Despite their lower targeting efficiency, replacement vectors are now more popular than the alternative insertion vectors for most targeting experiments because they do not involve a duplication of the targeted region, making genotyping and phenotype interpretation more straightforward (Ramirez-Solis et al., 1993).

Current possibilities for manipulating the mouse genome

The past 20 years has seen the efficiency of fundamental targeting technology develop sufficiently that the creation of knockout lines for all 30,000 genes in the mouse genome is now an achievable goal (Adams et al., 2004; Austin, 2004; Valenzuela, 2003). In parallel, expansion of the versatility of genetic manipulations has made available a wide array of strategies for

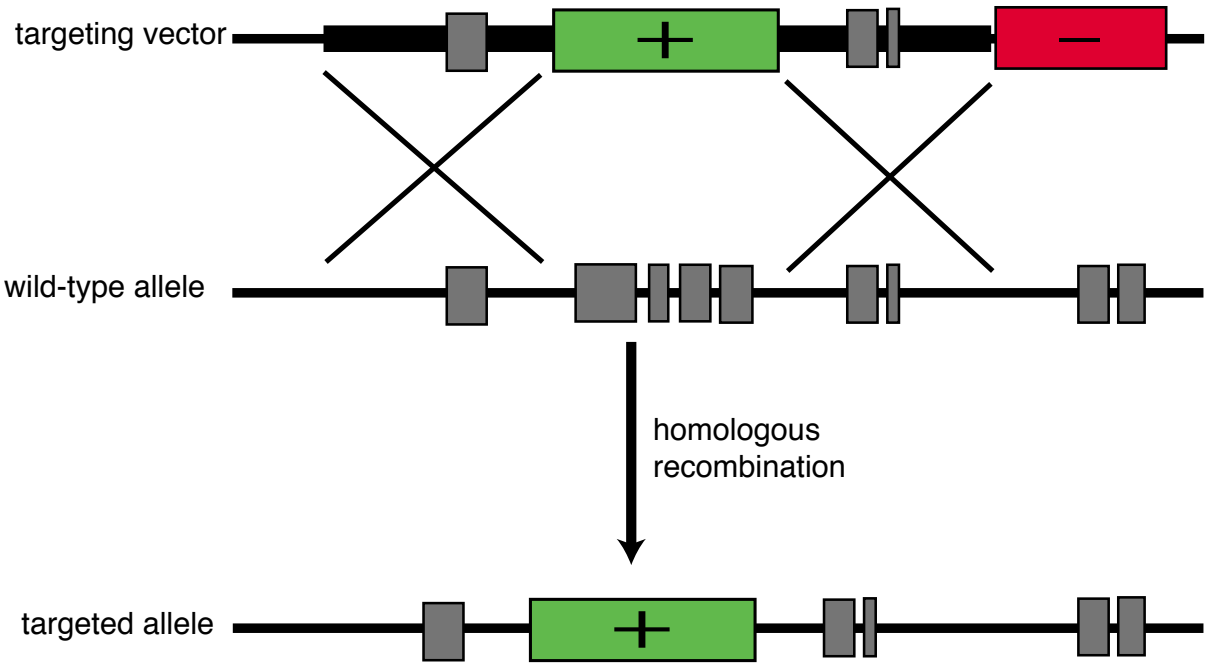

Figure 1.5 Gene targeting with a replacement vector. The replacement vector consists of a positive selectable marker (green box) flanked by regions of homology (thicker black lines) corresponding to the desired site its genomic insertion. Homologous recombination between the homology arms and their endogenous counterparts in the ES cell genome replaces the intervening sequence with the positive marker. Integration of the entire targeting construct at a random location in the genome results in insertion of the negative selectable marker (red box) and the death of the cell.

mutagenisation and it is now possible to make virtually any desired modification to the mouse genome.

Reporter genes such as lacZ can be inserted into a locus and placed under the control of an endogenous promoter to track the transcriptional patterns of a gene (Komiyama et al., 2002; Migaud et al., 1998). BAC transgenesis allows the insertion of an entire structural gene along with its regulatory elements for accurate expression profiles in the new genome (Heintz, 2001). Introduction of a single point mutation or replacement protein domain can be achieved by several different strategies. The double replacement method uses a replacement vector to first introduce positive and negative selectable markers then a second targeting to replace them with the required mutation (Cearley and Detloff, 2001). The hit-and-run method introduces the required mutation in an insertion vector alongside positive and negative selectable markers, then removes the markers and the insertional duplication by intrachromosomal recombination (Dickinson et al., 2000). Finally, a single replacement vector can be used to introduce the required mutation along with an adjacent (intronic) positive selectable marker which is later removed by site-specific recombination (van der Weyden et al., 2002).

The Cre/*loxP* and Flp/*FRT* site-specific recombinase systems allow creation of temporally- and spatially-restricted conditional deletions, described in the following section. Inducible overexpression of a gene can be achieved using the *tet* system, in which a transgenic tetracycline transactivator is driven by a tissue-specific promoter and becomes active upon binding to externally-administered tetracycline. The active transactivator interacts with a tet operator sequence which is located in a second transgene alongside a minimal promoter driving the gene of interest. Thus, the gene is expressed in reversible fashion only upon tetracycline administration and only in the tissue of interest (Gossen and Bujard, 1992; Sakai et al., 2002; Zhu et al., 2001b). Large structural modifications spanning several megabases of DNA can be introduced by

chromosomal engineering to recapitulate rearrangements that cause human cancers and other disorders (Yu and Bradley, 2001). RNA interference reduces, without ablating, the expression of a target gene (Carmell et al., 2002; Elbashir et al., 2001; Paddison et al., 2002; Paddison et al., 2004).

While the reverse genetics approaches above allow functional analysis of a specified gene, forward genetic strategies use random mutagenesis followed by phenotypic screening to identify sets of genes involved in a physiological process of interest. For example, adminstration of the chemical mutagen ethylnitrosurea (ENU) introduces point mutations at random in the genome. Mutagenised mice carrying the phenotype of interest, skeletal deformation for example, are then identified and the causative mutations positionally cloned (van der Weyden et al., 2002). Gene trap strategies involve insertional mutagenesis, where a reporter is introduced into genes at random, disrupting function and placing the reporter under control of the host promoter. Mutagenised cells displaying the phenotype of interest are selected and the disrupted gene identified by rapid amplification of cDNA ends (Stanford et al., 2001).

Engineering conditional mutations in mice using site-specific recombination

The advent of conditional mutations has been an important step in the development of gene function analysis technology. Ablating a gene in the germline and thus removing it from every cell of the animal throughout its lifetime is a valuable method of determining its broad physiological impact and is relevant for creating models of human disease, but it does not allow separation of the gene's function in different tissues or during different periods of development. The most severe example of this problem occurs when loss of a gene causes embyronic or early postnatal lethality, preventing examination of adult phenotypes. A recent example of this is Serum Response Factor (SRF), a neuronally-expressed transcription factor mediating activity-dependent upregulation of many immediate early genes including c-fos and c-jun. Mice with a constitutive knockout of SRF lack mesoderm and die around embryonic day 12.5 (E12.5),

showing the protein is crucial for normal development and survival but precluding an analysis of its role in the adult brain (Arsenian et al., 1998). Even if a constitutive knockout animal is viable, absence of a gene during development will regularly produce morphological or other abnormalities that may complicate examination of the effects of acute absence of the gene during later life. In the brain, a constitutive knockout precludes independent analyses of the function of a protein for the development of normal neuronal networks and synaptic connections and in synaptic transmission and/or plasticity once normal and mature synapses have been formed. The severe disruptions of hippocampal morphology in knockouts of the CREB family of transcription factors are good examples of this phenomenon (Lonze and Ginty, 2002).

Site-specific recombinase systems allow the deletion or rearrangement of DNA between two recombination sites upon the introduction of the appropriate recombinase enzyme. The system most commonly used in mice is Cre-*loxP* from P1 bacteriophage, in which Cre recombinase excises DNA from between two 34 bp *loxP* sequences, leaving only a single *loxP* site behind. A conditional knockout can be created by flanking key exons in the gene of interest with *loxP* sites by standard targeting methods, producing a 'floxed' allele (Kwan, 2002). If *FRT* recombination sites have been included flanking the positive selection cassette used for targeting, crossing the floxed mouse with a line expressing the Flp recombinase from *S. cerevisiae* excises the cassette, preventing its strong promoter from interfering with expression of the target gene and leaving a pseudo-wild-type floxed allele (Farley et al., 2000). Crossing the floxed mouse with a strain expressing Cre results in excision of the flanked exons and ablation of gene function (Dymecki, 2000; Lallemand et al., 1998; Nagy, 2000). Figure 1.6 summarises this strategy, which has recently been used, for example, to circumvent embyronic lethality in SRF knockout mice and show that SRF is required for activity-dependent gene expression and synaptic plasticity but not neuronal survival (Ramanan et al., 2005).

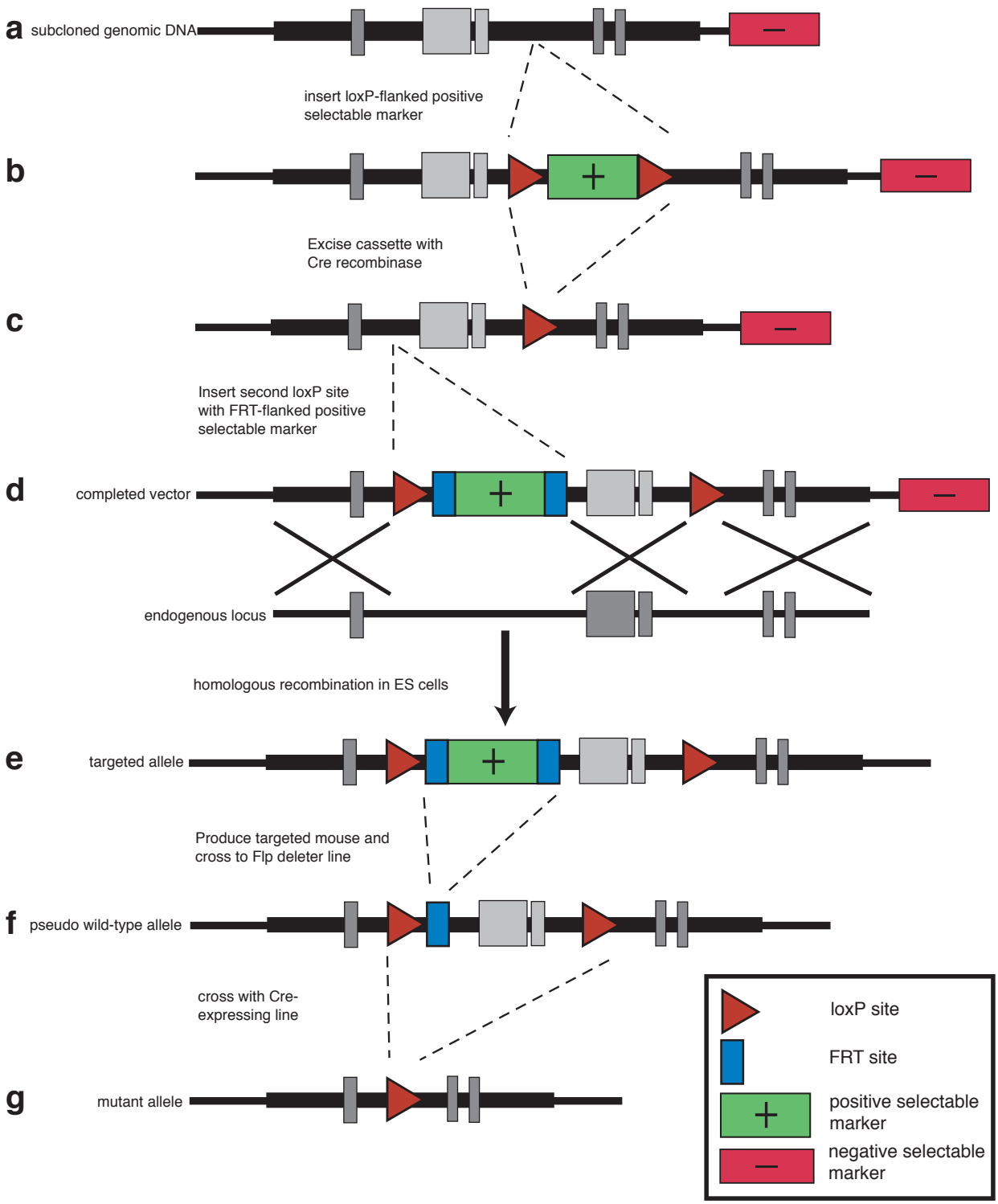

Figure 1.6 Conditional targeted mutagenesis strategy using site-specific recombinases. (a-c) The first loxP site is inserted on one side of the exons of interest in a genomic subclone using a loxP-flanked postive selectable marker followed by excision of the marker in Cre-expressing E.coli. **(d)** The second loxP site is inserted along with an FRT-flanked positive selectable marker. **(e)** The completed targeting construct is introduced into the endogenous locus in ES cells by homologous recombination, the negative selectable marker inhibiting random integration. Targeted ES cells are injected into blastocysts to generate a mouse **(f)** Crossing with a mouse strain ubiquitously expressing Flp recombinase removes the selection cassette along with its strong promoter, leaving a pseudo-wild-type allele containing loxP sites but expressing the gene normally. **(g)** Crossing to a Cre-expressing mouse strain results in recombination between the loxP sites, removing the exons of interest and ablating gene function.

Mouse strains with restricted Cre expression allow conditional gene deletion in a spatially- and/or temporally-controlled fashion. This can be achieved by placing the *cre* gene under the control of a promoter which drives expresion only in the tissue or temporal period of interest. For example, the synapsin-1 promoter drives neuron-specific Cre expression (Zhu et al., 2001a), the D6 promoter/enhancer results in Cre expression only in the neocortex and hippocampus (van den Bout et al., 2002) and the CaMKIIα promoter produces transgenic lines which express Cre postnatally in various regions of the forebrain, the precise specificity varying with each line (Dragatsis and Zeitlin, 2000; Tsien et al., 1996a).

An alternative strategy is to use mice expressing Cre fused with a mutated form of the hormone-binding domain of the oestrogen receptor (Cre-ER^T). The fusion protein can be expressed ubiquitously from a strong promoter but becomes active only with administration of tamoxifen, an oestrogen analog, which can be performed systemically or, for example, stereotaxically injected into a particular brain region (Brocard et al., 1997; Indra et al., 1999; Kellendonk et al., 1999; Vooijs et al., 2001). A third approach is to infect the animal with a virus expressing Cre (Ahmed et al., 2004). Yet another possibility is to produce recombinant Cre fused to a membrane translocation sequence, enabling the protein to be injected directly into the mouse (Chen and Behringer, 2001; Jo et al., 2001).

Caveats in the use of conditional mutagenesis technology

The technologies described above present a wide array of alternative strategies for mouse mutagenesis and other versions and combinations of these methods have also been published. No method is perfect, however, and the limitations of each need to be born in mind when designing a functional analysis strategy. This section focuses on the imperfections of the Cre recombinase system as it is so commonly used and comprises an important part of this dissertation.

The advantages of the Cre/*loxP* system lie in its ability to produce spatially- and temporally-restricted mutations. However, the specificity of expression of Cre transgenes is limited by the availability of an appropriately specific promoter for the purpose in hand. Patterns of Cre expression vary between transgenic lines produced with the same DNA construct (Dragatsis and Zeitlin, 2000) and anecdotal evidence suggests that even a single transgenic line can perform differently in different laboratories and through different generations, possibly as a result of variations in genetic background and/or epigenetic silencing of the transgene. The efficiency of recombinase activity in these lines also varies with the locus being recombined, highlighting the importance of analysing recombination not just with reporter lines but by confirming loss-of-function by immunohistochemistry or similar techniques (Vooijs et al., 2001).

Some of these limitations can be circumvented by delivering Cre virally or by direct injection of the recombinant protein, however these strategies also have disadvantages, notably in relation to achieving efficient recombination in the target population of cells but no others. Finally, high levels of Cre expression in mammalian cells lines causes DNA damage and inhibition of cell proliferation, properties which are dependent upon its endonuclease activity (Loonstra et al., 2001) and may be the result of recombinase activity on cryptic *loxP* sites in the genome (Schmidt et al., 2000; Silver and Livingston, 2001; Thyagarajan et al., 2000).

Targeting vector construction by homologous recombination in bacteria

Targeting vectors have traditionally been constructed using standard manipulation methods, cutting plasmids with restriction enzymes, isolating the required DNA fragments by agarose gel electrophoresis and joining them together using a DNA ligase enzyme. Relying on the presence of unique restriction sites limits the precision with which deletions and insertions can be made in the genome and often requires complicated and time-consuming cloning strategies to build

vectors from large pieces of genomic DNA. The recent development of homologous recombination-based DNA cloning strategies in bacteria ('recombineering'), however, allows insertions and deletions to be made at any position in a target DNA without the need for appropriate restriction sites (Muyrers et al., 2001).

Using recombineering, fragments of DNA can be excised from or inserted into plasmids by a process similar to gene targeting by homologous recombination in ES cells. For insertion, short homologous sequences corresponding to the site of insertion are attached to either end of the DNA fragment. It is then introduced into recombination-competent bacteria harbouring the recipient plasmid and clones carrying the desired recombination event are selected by antibiotic resistance. For an excision, short homology arms defining the ends of the fragment to be excised are attached adjacent to one another in the recipient plasmid. The plasmid is linearised between the homology arms and introduced into recombination-competent bacteria harbouring the donor plasmid and recombinants are selected as before (figure 1.7).

Endogenous homologous recombination activity in *E.coli* relies on the ATP-dependent, dsDNA exonuclease RecBCD, which also degrades linear DNA. To circumvent this problem, recombineering plasmids have been developed which express the λ-bacteriophage *gam* gene, which inhibits RecBCD, along with the Rac prophage genes *recE* and *recT* or the λ-bacteriophage genes *redα* and *redβ* to restore recombination activity. *RecE* and *redα* encode Exo, a 5'-3' exonuclease which acts on linear dsDNA to produce 3' overhangs. Beta, encoded by *recT* or *redβ*, binds to those overhangs and stimulates annealing to a complementary strand. In the plasmid, *gam* and *recT*/*redβ* are constitutively expressed while *RecE*/*redα* is placed under the control of the arabinose-inducible BAD promoter to minimise aberrant recombination (Zhang et al., 1998; Zhang et al., 2000). These plasmids have the advantage of conferring inducible

homologous recombination activity on any host strain, allowing recombineering to be used, for example, in BAC hosts (Copeland et al., 2001; Muyrers et al., 1999).

An alternative system uses a defective λ prophage containing the *redα*, *redβ* and *redγ* genes under the tight control of the temperature-sensitive λ-*cI857* repressor incorporated into the bacterial chromosome of the BAC host strain DH10B, allowing normal culturing at 32ºC and induction of recombination activity at 42ºC (Court et al., 2003; Yu et al., 2000). Cre and Flp recombinase genes have also been introduced, separately and under the control of the BAD promoter, into the chromosome of these cells to produce the new strains EL350 and EL250 respectively (Lee et al., 2001).

Recombineering has been proven an efficient method for inserting and excising DNA fragments into and out of plasmids without the need for restriction enzymes or DNA ligases in situations where positive selectable marker can be used (Zhang et al., 1998; Zhang et al., 2000). Importantly for targeting vector construction, it works efficiently for manipulating large DNA fragments many kilobases in length and has been used to generate deletions of up to 70 kb (Valenzuela, 2003; Zhang et al., 2000). Use of positive selectable marker genes such as neomycin phosphotransferase which confer drug resistance upon both *E.coli* and vertebrate cells, along with dual promoters which drive strong expression in both cell types, means the same selection cassette can be used for homologous recombination in bacteria for targeting vector construction and in ES cells for gene targeting (Angrand et al., 1999). The efficiency, rapidly and flexibility of the technique means it is particularly suited for constructing complex conditional targeting vectors. The EL350 and EL250 strains are especially convenient for vectors utilising the Cre/*loxP* and Flp/*FRT* site-specific recombinase systems (Liu et al., 2003; Muyrers et al., 1999). Recombineering is also useful for inserting reporter genes into BAC clones for producing

transgenic mice (Orford et al., 2000). The efficiency of recombination is such that it can even be used to retrieve a desired DNA fragment or clone from a complex mixture such as a genomic library (Zhang et al., 2001) or fragmented mouse genomic DNA (Zhang et al., 2000).

Recombineering also simplifies the introduction into targeting vectors of fine mutations such as point mutations or very small deletions or replacements spanning only a few base pairs. These modifications can be achieved without positive selection by using single-stranded oligonucleotides consisting of the desired mutation flanked by 35 bp of homology on either side. Because of the low recombination efficiency (1 correct recombinant per several hundred electroporated cells) without a selectable marker a PCR-based screen on pooled DNA is required to select positive clones (Swaminathan et al., 2001). The difficulty of designing efficient PCR primers against such short sequences can be overcome using a double replacement-type strategy (Yang and Sharan, 2003).

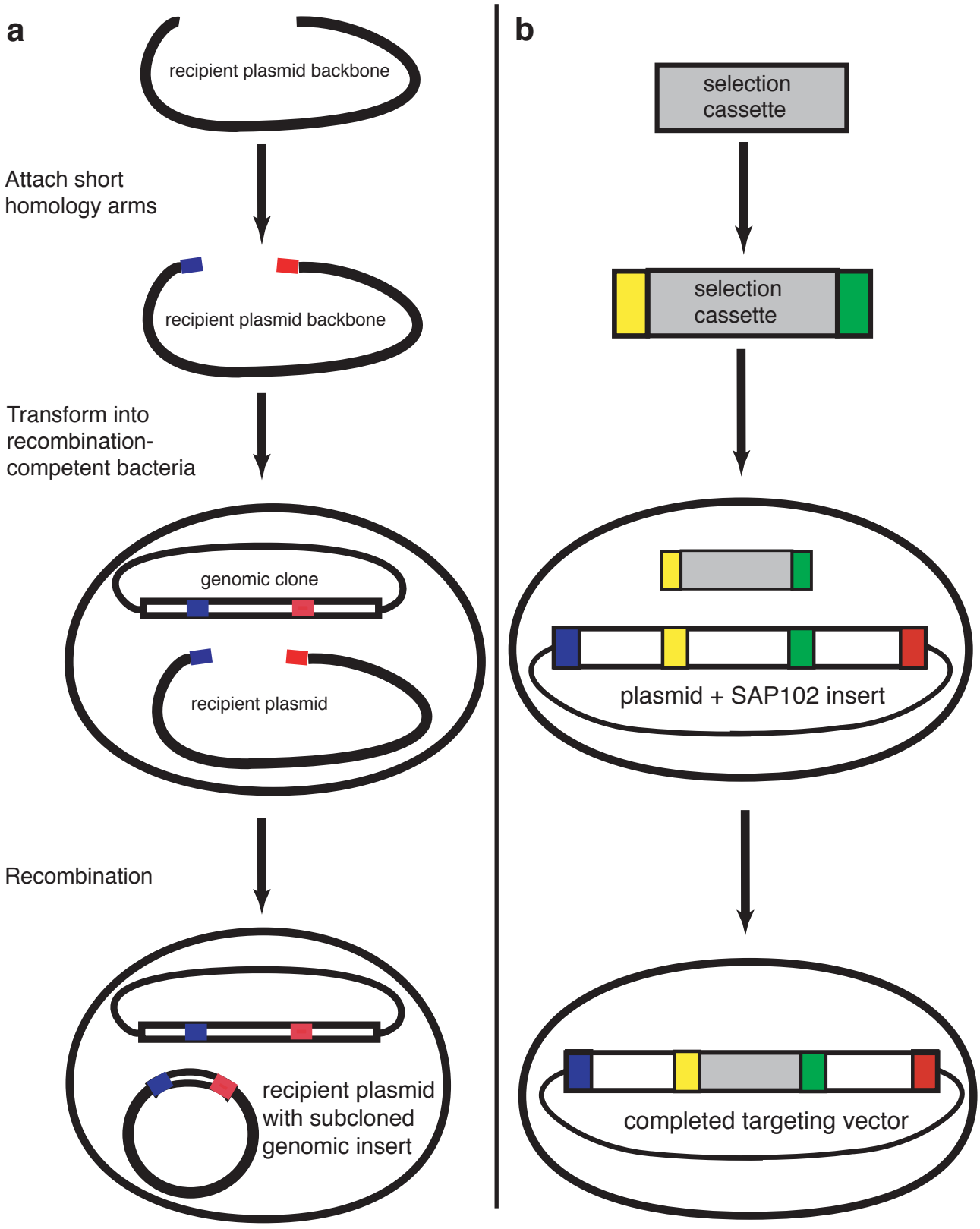

Figure 1.7 Constructing targeting vectors by recombineering. (a) To excise a piece of genomic DNA into the targeting vector backbone, short homology arms (blue and red rectangles) corresponding to the ends of the excision fragment are first attached to the linearised ends of the recipient plasmid. The plasmid is then introduced into recombinaion-competent E.coli harboring the donor vector and resulting antibiotic-resistant clones are screened for correct recombinants. **(b)** To insert a selection cassette into the genomic subclone in the newly-created plasmid, short homology arms (yellow and green rectangles) corresponding to sites of insertion in the subclone are attached to the ends of the cassette. The remainder of the experiment is performed as in (a).

1.8 X-linked mental retardation

The techniques described above allow sophisticated manipulation of the mouse genome. This section examines a genetic, cognitive disorder in humans and how these manipulations are being used to elucidate its causes.

Definition and clinical presentation of X-linked mental retardation

X-linked mental retardation (XLMR) is an inherited cognitive disorder caused by mutations in brain-expressed genes on the X chromosome (Ropers and Hamel, 2005). XLMR primarily affects males as they carry only a single copy of the X chromosome per cell and have thus no backup copy of the majority of their X-linked genes. Females carry two X chromosomes but prevent over-dosage by silencing one, generally at random, in each cell by the process of X inactivation, so that each cell is affected or unaffected depending on whether the wild-type or mutated allele has been silenced.

Mental retardation is clinically defined by three characteristics (American Psychiatric Association, 2000):

1. Sub-average general intellectual functioning, with an intelligence quotient (IQ) of less than 70.
2. Significant limitations in adaptive functioning in at least two of the following skill areas: communication, self-care, ability to live independently, social and interpersonal skills, use of public services, decision making, functional academic skills, work, leisure and health and safety.
3. Onset prior to 18 years of age.

Most investigators distinguish between several levels of severity of XLMR as shown in table 1.2.

Table 1.2 Severity levels of XLMR (Ropers and Hamel, 2005).

Severity of XLMR	IQ level
Mild	50-70
Moderate	35-50
Severe	<35

XLMR is a very heterogeneous disorder. Firstly, it can be divided into two forms: Non-syndromic XLMR (NS-XLMR) has mental retardation as its only symptom, while Syndromic XLMR (S-XLMR) is accompanied by additional abnormalities which often include facial and/or digital dysmorphologies, skeletal abnormalities and macroorchidism, among others (Ropers and Hamel, 2005). Two-thirds of XLMR cases are thought to be non-syndromic, but this is likely to be an underestimate since further symptoms often do not begin until puberty or adulthood. Some forms of the disease originally classed as non-syndromic have since been re-classified following more systematic examination of larger patient cohorts. Even within these two divisions, presentation of the disease can vary widely. IQ can vary from 20 to 70 points (see table 1.2) and syndromic forms can include symptoms as diverse as visual impairment (caused by mutations in *ABCD1* and *NDP*), cleft palate (*PHF8*) and heart defects (*PQBP1*) (Ropers and Hamel, 2005).

Prevalence

Moderate to severe XLMR occurs in around 1 in 2,000 males, although its true frequency is difficult to measure since the genetic basis of many forms of mental retardation remains to be determined and X-linked inheritance patterns are not always clear. Mental retardation in general has a prevalence of 2-3 % in developed countries. The importance of mental handicap as a health issue is highlighted by the fact that it is responsible for 8 % of healthcare expenditure in central

Europe, far greater than that on any other class of disease (Chelly and Mandel 2001, ; Fishburn et al., 1983).

Genes causing XLMR

The heterogeneity in the presentation of XLMR is reflected in its genetic basis. Around 140 syndromic forms of XLMR have been identified so far and causative genetic mutations (including some allelic mutations) have been identified in 66 of these, while partial mapping to distinct regions of the X chromosome has been performed in a further 50. In NS-XLMR mutations in 20 different causative genes have so far been identified, representing approximately 50 % of the total known cases. In total, 59 different genes are currently causatively implicated in XLMR (Ropers and Hamel, 2005).

Current knowledge of the function of XLMR-causing genes indicates heterogeneity but also some common themes emerging. Mutations in Rho GTPase pathways involved in the determination of dendritic spine morphology through actin cytoskeleton remodelling often seem to cause NS-XLMR. Genes in this category are oligophrenin-1 (*OPHN1*), a Rho GTPase activating protein (Billuart et al., 1998); p21-activated kinase (*PAK3*), which links Rho GTPases to the actin cytoskeleton and MAP kinase pathway (Allen et al., 1998); *ARHGEF6* and *FGD1*, both Rho guanine exchange factors (Kutsche et al., 2000; Lebel et al., 2002). The S-XLMR genes *FLNA* and *KIAA1202* are also implicated in this process as a result of their actin-binding properties. Other XLMR genes involved in GTP-based signalling include the Rab3A GTPase effector *SYN1* and the Rab4 and Rab5 GTPase regulator *GDI1* (Chelly and Mandel, 2001). XLMR sufferers often exhibit defects in dendritic spine maturation, resulting in an abundance of long, thin immature spines in the cortex and hippocampus during infancy and low spine density in adulthood (Kaufmann and Moers, 2000). This suggests spine dysgenesis may be a key factor in XLMR cognitive deficits and is consistent with observations that mutations in genes involved in

spine formation cause XLMR. These observations have been linked together in the Rho protein hypothesis of mental retardation (Ramakers, 2002; Renieri et al., 2005).

Transcription and chromatin remodelling forms a second functional theme amongst XLMR genes. *MECP2*, a methyl-binding protein responsible for chromatin condensation, causes the severe mental retardation, including male lethality, observed in Rett's syndrome (Amir et al., 1999). Mutations in *RSK2*, a protein kinase activated by the MAPK pathway which phosphorylates the transcription factor CREB and regulates the histone acetyltransferase CBP, cause a form of S-XLMR called Coffin-Lowry Syndrome whose symptoms include severe mental retardation, dysmorphology of the face and digits and progressive skeletal deformations (Trivier et al., 1996). *ARX* and *FMR2* are other transcriptional regulators which can cause XLMR (Chelly and Mandel, 2001).

Other XLMR genes possess more isolated functions. For example, Neuroligin 4 (*NLGN4*) is localised to the glutamatergic postsynapse and is central in the assembly of presynaptic structures (Yan et al., 2004). *PQBP1* is implicated in RNA splicing (Kalscheuer et al., 2003), *FTSJ1* in translation, *MID1* in protein degradation and *SLC6A8* in energy metabolism (Ropers and Hamel, 2005).

Mouse models of XLMR

Modelling the cognitive deficits of XLMR using genetic engineering in mice is a potentially very valuable method of elucidating the molecular and physiological mechanisms of the disorder. Since the causative genetic mutation is generally loss-of-function, a targeted deletion which knocks out the gene is a relatively straightforward and usually appropriate strategy for generating a mouse model. Cognitive and other behavioural tests can be used to validate the model followed

by morphological, electrophysiological and biochemical analyses to uncover the underlying pathology (Watase and Zoghbi, 2003).

Mouse models generated so far have been generally successful in reproducing some of the phenotypic aspects of the relevant human disorder. By far the most advanced and successful has been the model of Fragile X mental retardation discussed below. Others include a knockout of the NS-XLMR gene *GDI1*, producing mice which display a specific deficit in short-term hippocampal-dependent memory in radial maze and trace fear conditioning tests with normal long-term spatial memory in the water maze. They also exhibit a reduction in aggressive behaviour (D'Adamo et al., 2002). Mice with a deletion in *Fmr2* display impaired contextual fear conditioning and increased pain sensitivity, but none of the syndromic facial dysmorphology observed in the analogous human condition (Gu et al., 2002). Mice with a conditional, neuron-specific mutation in *NF1*, a model for Von Recklinghausen's neurofibromatosis type 1 (NF1), begin to show a growth retardation 3-4 days postnatally which continues into adulthood, where they stabilise at half the weight and size of their wild-type littermates. They also show reduced cortical thickness (Zhu et al., 2001a). *PAK3* mutant mice display normal hippocampal structure and dendritic spine morphology and normal spatial learning in the water maze, but have a deficit in the late phase of hippocampal LTP and more rapid extintion of a learned taste aversion than wild-type controls (Meng et al., 2005).

It is promising that these mutant mice display the types of phenotypes that might be expected in mental retardation. Still lacking, however, is robust demonstration that these phenotypes directly correspond to those observed in the relevant human disorder. This will be an important step in giving confidence that biochemical analyses of the underlying pathologies in these mice may lead to generation of useful therapeutic measures.

Fragile X mental retardation – a case study

The best-characterised XLMR mouse model is the *Fmr1* knockout mouse, a model for Fragile X syndrome. Fragile X is the most common form of mental retardation, accounting for 2-3 % of male retardation (Chelly and Mandel, 2001) and is a syndromic form whose symptoms include mental retardation, developmental delay, facial dysmorphology and macroorchidism (Maes et al., 1994; O'Donnell and Warren, 2002). Fragile X males have abnormally long and thin dendritic spines in cortical neurons without a change in cell density (Hinton et al., 1991; Purpura, 1974).

Fragile X is caused by an expanded CGG trinucleotide repeat in the 5' untranslated region of the *Fmr1* gene on the X chromosome. Presence of the expanded repeat induces methylation of the *Fmr1* promoter region and transcriptional silencing of the gene. Thus, Fragile X is a result of *Fmr1* loss-of-function (Bagni and Greenough, 2005).

The function of the fragile X mental retardation protein (FMRP), the product of *Fmr1*, is also well characterised. FMRP contains several RNA-binding domains including two KH and two RGG box domains. Studies of its RNA-binding properties show that it binds a large number of mRNAs, including its own (Ashley Jr et al., 1993; Brown et al., 2001; Chen et al., 2003; Dolzhanskaya et al., 2003). For example, one study used several approaches including co-immunoprecipitation of RNAs with FMRP followed by identification of the transcripts by microarray hybridisation to show binding of the protein to 432 different transcripts (Brown et al., 2001). The same study used fractionation of translating polyribosomes from human fragile X cells to show that the translational status of 50 % of these transcripts was changed in the absence of FMRP. FMRP is in fact a repressor which binds to mRNAs and prevents their translation, a function mediated via its interaction with microRNAs and the RNA-induced silencing complex (RISC). FMRP's targets include many transcripts localised near synapses and implicated in synaptic plasticity (Bagni and Greenough, 2005).

Fmr1 knockout mice show morphological and behavioural phenotypes mainly consistent with human Fragile X syndrome. 90 % of hemizygous males display macroorchidism, although none have facial dysmorphology. They display mild spatial learning deficits in both the traditional circular water maze and a plus-shaped version, along with a locomotor deficit. No deficit was observed, however, in a contextual fear conditioning test of hippocampus-dependent spatial learning (D'Hooge et al., 1997; Van Dam et al., 2000). Other behavioural abnormalities include increased locomotion in an open field test and reduced anxiety responses in an elevated plus maze. Like fragile X males, *Fmr1* mutant mice have longer and thinner cortical dendritic spines than wild-type controls (Comery et al., 1997).

FMRP is important for normal synaptic function, demonstrated by the observation that synaptic plasticity is disrupted in *Fmr1* knockout mice. Specifically, these mice show enhancement of LTD induced by activation of group 1 metabotropic gluatamate receptors (mGluR) in the CA1 area of the hippocampus. This is observed whether the LTD is induced by low-frequency electrical stimulation or by adminstration of the mGluR agonist DHPG. NMDAR-dependent LTD is unaffected. mGluR-dependent LTD is also dependent on protein synthesis and is associated with increased translation of FMRP protein (Huber et al., 2002).

These observations have led to the mGluR theory of fragile X syndrome, which postulates that mGluR-dependent LTD is a mechanism by which inactive synapses are marked for destruction during brain development, allowing only active, functional synapses to mature. Enhancement of LTD in the absence of FMRP prevents normal synapse maturation leading to developmental delay, aberrant synaptic connections and cognitive deficiency (Bear et al., 2004).

1.9 Aims

The aim of this dissertation was to analyse for the first time the function of the SAP102 protein *in vivo* in the mouse. Several characteristics of the protein suggest it may play crucial roles in fundamental neural processes. It is highly expressed in the hippocampus, a brain region important for memory formation and is localised to the postsynaptic density at glutamatergic synapses and interacts directly with NMDARs that are essential for normal brain development, synaptic plasticity, learning and memory.

SAP102 contains multiple protein-protein interaction domains and is a member of the PSD-95 family of MAGUK proteins which is implicated in the organisation of postsynaptic signalling pathways as well as localisation of postsynaptic transmembrane receptors. Mutations in PSD-95, which has similar domain structure and expression patterns to SAP102, cause enhancement of synaptic plasticity and spatial learning deficits in mice. Targeted mouse mutations have been generated for all PSD-95 family proteins except for SAP102. Mutations in SAP102 cause X-linked mental retardation in humans, providing further evidence for its importance in cognition and adding an urgency to the elucidation of its precise role in neuronal function.

Gene targeting in mice is the most powerful method available for the determination of neuronal gene function since it allows the creation of multiple mutations to assess different functional aspects of the gene in a system sufficiently sophisticated for meaningful analyses of complex cognitive behaviours. Thus, to discover the function of SAP102, a targeted deletion was introduced into the mouse gene with the aim of creating a null allele and assessing its impact on brain development and morphology, postsynaptic signalling, synaptic plasticity and behaviour. In addition to this constitutive knockout mutation, targeting vectors were also constructed for the introduction of a reporter gene knock-in to analyse SAP102 transcriptional patterns and a conditional knockout to facilitate the spatially- and temporally-dependent functions of SAP102.

These targeted mutations will allow a detailed functional analysis of SAP102 in relation to postsynaptic signalling, brain development and morphology, synaptic function and plasticity and behaviour.

A comprehensive understanding of neuronal function requires detailed investigations as to the function of large numbers of brain-expressed genes. To facilitate these types of experiments this dissertation aimed to use the SAP102 gene as a test case for developing a rapid and efficient system for generating mouse targeting constructs utilising recently-developed recombination-based cloning technology in bacteria.