Chapter 8

General discussion

8.1 Summary of results

This dissertation demonstrates for the first time the function of the postsynaptic adaptor protein SAP102 *in vivo* in the mouse. SAP102 allows efficient hippocampal-dependent learning of spatial information. Despite its early postnatal onset of expression and postsynaptic location, SAP102 is not required for the development of normal brain structures, nor for normal operation of basal excitatory synaptic transmission or NMDAR localisation or activation even at synapses where SAP102 is robustly expressed. Instead it controls potentiation amplitude during NMDAR-dependent plasticity induced by specific types of stimulation, a role consistent with its close association with NMDARs at the postsynapse. Biochemically, SAP102 governs basal activation levels of the MAPK pathway.

In addition, a novel vector system has been developed and shown to be broadly useful for constructing targeting vectors by bacterial homologous recombination for the introduction of a variety of mutations into different loci in the mouse. In particular its use to construct additional targeting vectors for SAP102 will facilitate more detailed analysis of the protein's expression patterns and its function in distinct brain regions during specific developmental periods.

8.2 Future directions for recombineering-based targeting vector construction

DNA cloning by homologous recombination in bacteria has been shown here as well as previously in the literature to be a significant step forward in technology for constructing targeting vectors, allowing rapid modification of large DNA fragments with precise control over excision and insertion points and without the need for appropriate restriction sites in those fragments.

This means that simple targeting vectors, such as replacement of one or more key exons of an endogenous gene with a lacZ marker, can be constructed using an almost identical cloning

strategy independent of the locus being targeted. The process can thus be partially automated for the construction of large collections of simple mouse mutations. The process is being further facilitated by improvements in associated areas, such as the availability of end-sequenced BAC clones to alleviate the need for library screening, quantitative PCR for genotyping of targeted ES cell clones and automated procedures for cell culturing and colony picking (Valenzuela, 2003). These technological advances are driving a paradigm shift in gene targeting, away from single genes and individual proteins towards gene families, signalling pathways and entire protein complexes.

As well as the capacity to produce large numbers of mutations, recombineering facilitates construction of sophisticated targeting vectors for detailed *in vivo* analysis of multiple functional aspects of a single gene. *LoxP* and *FRT* sequences for site-specific recombination can be introduced at precisely the required genomic location in a single cloning step for deletion of key exons with minimal disruption to the surrounding genomic sequence. Introduction of point mutations can be performed by recombination using single-stranded oligonucleotides without the use of a selectable marker, although this approach is inefficient and requires several cloning steps in the context of targeting vector construction. A better strategy may be to include the selectable marker in intronic sequence adjacent to the exonic point mutation and insert both elements in a single recombination step mediated by flanking homology arms.

8.3 SAP102 function

Normal hippocampal NMDAR expression levels, distribution patterns and postsynaptic currents suggests that SAP102 is not required for synaptic delivery of these receptors. Instead, changes to postsynaptic signalling and synaptic plasticity in SAP102 mice, together with SAP102's ability to bind numerous postsynaptic proteins support the hypothesis that it mediates the postsynaptic

signalling response to calcium influx through synaptically activated NMDARs to control cellular mechanisms for synaptic plasticity and information encoding in the hippocampus.

What is the causal relationship between the biochemical, synaptic plasticity and behavioural phenotypes in mice lacking SAP102? We have seen that there is no clear correlation between disruption of LTP and disruption of spatial learning by genetic maniupation in mice, but these two processes share many common molecular mechanisms. Although the phosphorylation screen results suggest that basal activity of most postsyaptic signalling pathways is unaffected by the mutation, the screen is not exhaustive and other biochemical effects may be present. Demonstration that disruption of MAPK activity is responsible for the electrophysiological and behavioural phenotypes in SAP102 mice would require rescue of those phenotypes by artificial dampening of the pathway by pharmacological or genetic means.

Elevation of ERK activation in SAP102 knockout mice is consistent with published data suggesting preferential linking of NR2B-containing NMDARs with SAP102 and the MAPK pathway (Kim et al., 2005; Sans et al., 2000). SAP102 interactors and MAPK regulators synGAP or kalirin could mediate this link. Also consistent is the observation that synGAP regional expression patterns correspond more closely with those of SAP102 than PSD-95 (Porter et al., 2005). MAPK activation by NMDARs is necessary for normal hippocampal synaptic plasticity and spatial learning.

Enhancement of LTP in the mutant mice suggests that, as expected, SAP102 has some association with NR2A-containing NMDARs despite its preference for those containing NR2B. It will be important to determine whether MAPK signalling is also disturbed in PSD-95 mutant mice. Preferential association of SAP102 with NR2B predicts that mice lacking SAP102 will show greater disturbance of LTD induced by low-frequency stimulation than of LTP. Changes in

synaptic delivery of GluR1 following NMDAR activation by LTP-inducing stimuli should also be evident.

Developmental versus acute SAP102 function

While germline deletion gives a reliable indication of the effect of SAP102 loss on the entire animal and provides the most genetically accurate model of the equivalent human disorder, it means that the temporal origins of the observed phenotypes cannot always be determined. For a synaptic protein, changes in synaptic plasticity and learning could result from disruption to synapse structure arising from lack of SAP102 during development, or acute alterations in protein trafficking or signalling during each behavioural or electrophysiological experiment. No chronic modifications to gross brain morphology or basal synaptic transmission were observed in SAP102 mutant mice, but this does not preclude ultrastructural changes that are beyond the scope of the current investigation, such as changes in dendritic spine morphology which can be associated with XLMR. Interestingly, both SynGAP and PSD-95 mutant mice display changes in dendritic spine morphology (Vazquez et al., 2004; Cathy Vickers, personal communication). A distinction could be made between developmental and acute roles for SAP102 using mice with floxed SAP102 alleles crossed with Cre-ER^{T} mice followed by stereotaxic tamoxifen administration to ablate the gene solely in the adult hippocampus.

8.4 Distinct roles of PSD-95 family proteins in postsynaptic signalling, plasticity and learning

Elevelated hippocampal expression of SAP102 in PSD-95 mutants and increased association of PSD-95 with NMDARs in the absence of SAP102 suggest that these two proteins may be able to partially compensate for loss of the other. One would expect, then, that loss of both proteins would produce a more severe phenotype, a prediction confirmed by the loss of viability in

SAP102/PSD-95 double mutants. This observation further highlights the importance of these proteins for normal development.

It is unlikely, however, that these two molecules are completely redundant; indeed, several observations here provide the first evidence of distinct functions for SAP102 and PSD-95 at the postsynapse. Without SAP102, mice have difficulty encoding spatial information in the water maze but can acquire the information with additional training. Once acquired, the information is retained and recalled without impairment. In contrast, PSD-95 mutant mice display severe disruption of spatial learning which cannot be improved with overtraining. The observation that spatial learning impairments resulting from deletion of SAP102 and synGAP can be improved with overtraining provides further circumstantial evidence that these two proteins are operate in the same signalling pathway. PSD-95 mice exhibit enhancement of LTP in area CA1 with a variety of induction protocols, while loss of SAP102 elevates potentiation at 5 Hz but has little effect at 100 Hz.

Comprehensive analyses of the protein partners of each of the three NMDAR-associated MAGUKs may link each to distinct postsynaptic signalling pathways and provide further clues as to their individual functions. A bioinformatic search of the human genome sequence in 2002 revealed 54 proteins with a canonical C-terminal class I PDZ binding motif (Lim et al., 2002), suggesting there remain further MAGUK interactions to be characterised.

Further examination of the collective roles of SAP102, PSD-95 and PSD-93 in NMDAR function awaits the generation of mice lacking all three MAGUK proteins as well as double knockout combinations. The SAP102 floxed targeting vector will probably be useful in preventing developmental lethality in some of these combinations.

8.5 Mental retardation

SAP102 mutant mice as a model of XLMR

Several phenotypic observations already made on the SAP102 mice are relevant to the human disorder. Enouragingly, the mice show no apparent gross disruptions to brain morphology, the caveats already discussed notwithstanding. Cognitive deficits in mental retardation sufferers carrying SAP102 mutations may thus be a result of acute alterations in postsynaptic signalling which could be more easily corrected by pharmaceutical intervention. Pharmacological MAPK inhibitors may be a rewarding therapeutic avenue to explore in this regard. Further biochemical characterisation of the mutant mice will inform these investigations. The mice will also provide a useful means for testing promising drug candidates.

Improvement of cognitive performance with additional training in the mutant mice suggests that additional tutoring and assistance for human sufferers may be successful in improving their quality of life. More detailed examination of the cognitive consequences of SAP102 loss using analogous tasks in humans and mice may be valuable.

Future directions in XLMR research

How many different genes have the potential to cause XLMR in humans? The answer to this question is valuable not only because of its clinical implications for the diagnosis and treatment of the disorder but also because its implied insight into the fundamental biology of the brain. Indeed, the question could be rephrased: how many proteins are there in the human brain that are essential for normal cognitive function? Around 350 of the approximately 1,000 genes on the X chromosome are expressed in the brain (Ropers and Hamel, 2005; Ross, 2005) and it seems reasonable to assume the majority of these will impact on cognition. Even if the phenotypic effects of loss-of-function are on occasion masked because of redundancy or environmental

factors, it seems likely that mutations in many of these genes would result in cognitive deficits including, in many cases, mental retardation.

Technology is now sufficiently advanced to make realistic the prospect of analysing the function of every brain-expressed, X-linked gene by targeting in ES cells. Unlike their autosomal counterparts, generation of null ES cell lines for each X chromosome gene requires only a single targeting experiment. Such lines could then be differentiated into neurons for cell-based analysis and only the more interesting of them initially used to generate mutant mice.

Such wide variability in the genetic causes of XLMR presents potential problems for its treatment. If different causative genes impact on cognition through independent molecular mechanisms it will be difficult to design pharmacological interventions to be effective across the different disease forms. On the other hand, if the neuronal function of XLMR genes converges on a limited number of signalling pathways which are crucial for cognitive function, it may be possible to design drugs only against these ‘master’ cognitive pathways.