Chapter 7

Synaptic plasticity and postsynaptic signalling in SAP102 mutant mice

SAP102 is localised to synapses and is greatly enriched in the postsynaptic density. It interacts directly with NMDAR subunits, ionotropic glutamate receptors that are crucial for synapse function. It also interacts with other proteins with play essential roles at the synapse. Among these are the AMPAR and NMDAR synaptic delivery proteins stargazin and sec8 respectively, the ubiquitous calcium-binding protein calmodulin, the MAPK inhibitor and synaptic plasticity regulator synGAP and the controller of synaptic plasicity and complex cognitive function PSD-95 (see sections 1.5 and 1.6). Mutations in SAP102 disrupt cognitive function in humans (Tarpey et al., 2004). It therefore seems likely that SAP102 plays an important role in some aspect of synaptic communication and the first part of this chapter describes experiments performed to test this hypothesis. These electrophysiological experiments were performed by Ann Fink, Patricio Opazo and Tom O'Dell (Department of Physiology, University of California, Los Angeles).

An understanding of the role of SAP102 in postsynaptic signalling is a prerequisite for detailed knowledge of its role in neuronal function. The second part of this chapter begins to examine the biochemical consequences of loss of SAP102 in relation to synaptic function, with emphasis on potentially differing roles of SAP102 and PSD-95.

7.1 SAP102 mutant mice have normal basal synaptic function

Normal synaptic responses in SAP102 mice

To analyse basal synaptic function in the absence of SAP102, the responses of pyramidal cells in CA1 to synaptic stimulation via Schaffer collateral axons projecting from CA3 were recorded in acute, adult, hippocampal slices. Axonal firing, measured by the amplitude of fibre volleys in Schaffer collateral projections, was induced by electrical stimulation eliciting 25 %, 50 %, 75 % and 100 % of maxium fEPSP amplitude. Synaptic responses were measured by the slope of field excitatory postsynaptic potentials (fEPSPs) in the stratum radiatum layer of CA1. Figure 7.1a shows that no differences were observed in the postsynaptic responses of SAP102 mutant slices

compared to wild-type controls at any of the stimulation intensities used, suggesting that basal hippocampal function is undisturbed by the mutation.

Normal AMPAR and NMDAR conductance in SAP102 mutant mice

The conductance of AMPARs and NMDARs in SAP102 mutant mice was next examined. CA1 pyramidal cells were subjected to whole-cell voltage clamp while induced excitatory postsynaptic currents (EPSCs) were recorded. Since AMPAR channels open rapidly in response to glutamate but also close quickly afterwards, while NMDAR channels take longer to respond but remain open open for longer, the relative contributions of the two receptor subtypes can be determined using the ratio of the EPSC amplitude 5 ms after onset, when mainly AMPARs are active, and 50 ms after onset, when mainly NMDARs are open. Furthermore, when the experiment is performed with membrane potential held at –80 mV, EPSC is mediated almost exclusively by AMPARs since NMDAR channels are subject to the magnesium block, while at +40 mV both AMPARs and NMDARs can be activated (Nowak et al., 1984). Figure 7.1b shows that absence of SAP102 had no effect on relative contribution of AMPARs and NMDARs to CA1 EPSCs at either membrane potential. Figure 7.1c shows representative EPSC traces from wild-type (top) and SAP102 mutant (bottom) slices. To analyse the subunit composition of NMDARs at these synapes, the EPSC decay constant was calculated by fitting a single exponential curve to the decaying phase of the synaptic currents measured at +40 mV. Different NMDAR subunits have differing decay characteristics, so a change in NMDAR composition in SAP102 mutants would be reflected in a change in the decay constant. However, figure 7.1d shows that this was not the case.

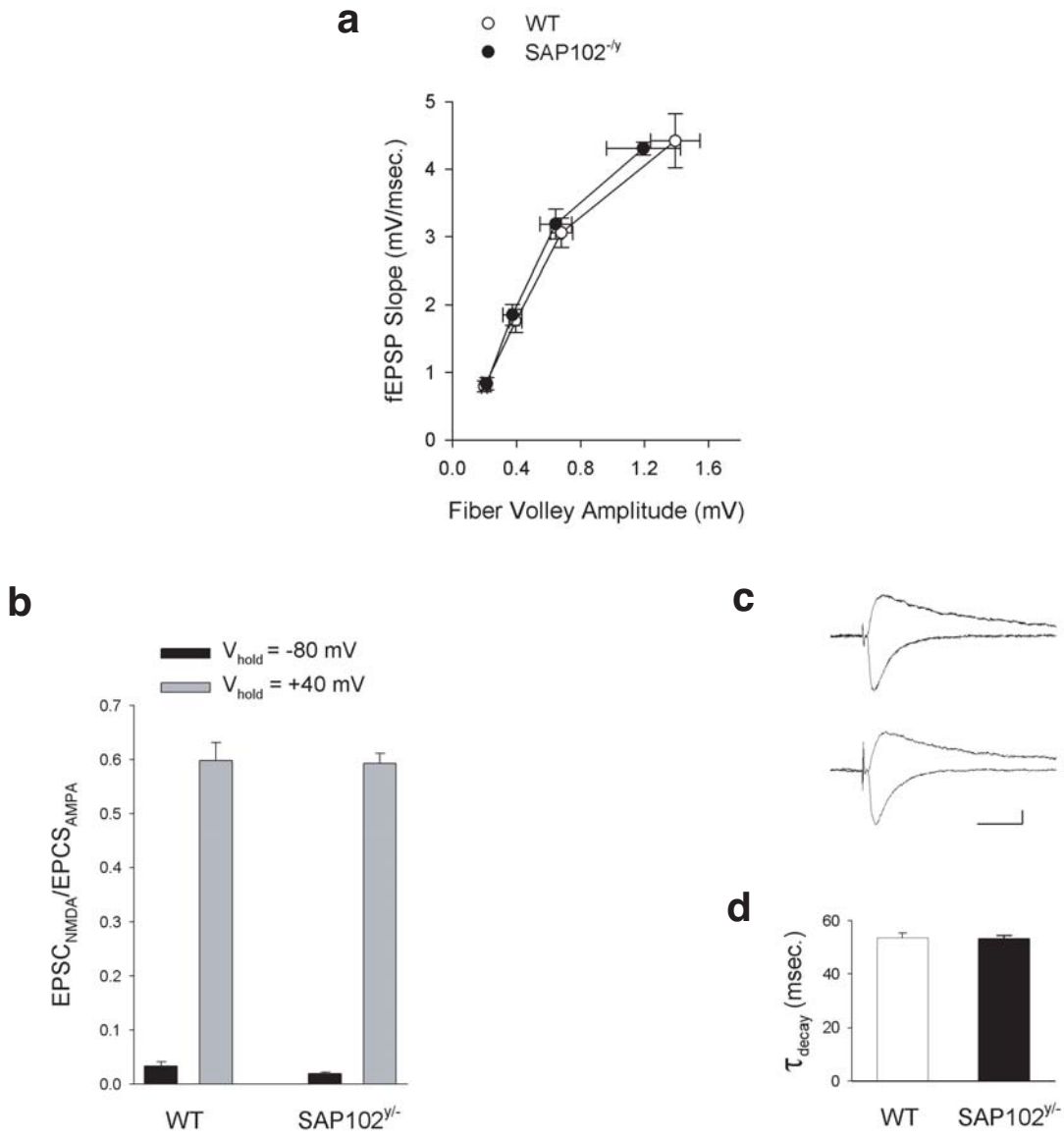

Figure 7.1 Basal hippocampal synaptic function is undisturbed in SAP102 mutant mice. (a) Basal synaptic responses were measured by recording field excitatory postsynaptic potentials (fEPSPs) from CA1 stratum radiatum in following Shaffer collateral axonal fibre volleys induced by stimulation at levels which invoked 25 %, 50 %, 75 % and 100 % of the maximum fEPSP amplitude. There were no differences between wild-type and SAP102 -/Y fEPSP responses at any of the four stimulation intensities. **(b)** Synaptic glutamate receptor function in SAP102 mutant mice. AMPAR and NMDAR conductance was measured using excitatory postsynaptic currents (EPSCs) at membrane potentials of -80 and +40 mV under whole-cell voltage clamp conditions in CA1. The AMPAR ($EPSC_{AMPA}$) and NMDAR ($EPSC_{NMDA}$) contributions to EPSCs were determined by the EPSC amplitude 5 and 50 ms after onset respectively. No differences between wild-type and mutant responses were observed at either membrane potential. **(c)** Representative EPSC traces recorded at -80 and +40 mV in wild-type (top) and SAP102 -/Y (bottom) slices. Horizontal and vertical calibration bars are 20 ms and 50 pA respectively. **(d)** NMDAR subunit composition was examined by fitting a single exponential curve to the decaying phase of the synaptic currents recorded at +40 mV. A change in the time course of the delay would be indicative of a change in NMDAR subunit composition. Again, no difference between wild-type and SAP102 -/Y cells was observed.

7.2 SAP102 loss enhances synaptic plasticity induced by low-frequency stimulation

Synaptic plasticity is often hypothesised to be a mechanism underlying information storage in the brain. Some types of synaptic plasticity, including tetanus-induced long term potentiation (LTP) in hippocampal CA1, require functional NMDARs, as does hippocampal-dependent spatial learning (Collingridge et al., 1983; Morris et al., 1986). SAP102 interacts directly with NMDAR subunits and is strongly expressed the hippocampus. Loss of SAP102 or PSD-95 causes spatial learning deficits in the water maze and PSD-95 mice have enhanced hippocampal long term potentiation.

To analyse the consequences of SAP102 loss on synaptic plasticity, NMDAR-dependent hippocampal long term potentiation was examined in the CA1 area of SAP102 mutant mice using a stimulating electrode in the Schaffer collateral projections and a field recording electrode in stratum radiatum. Figure 7.2 shows the results of these experiments. Tetanic stimulation at a frequency of 100 Hz resulted in potentiation of CA1 fEPSP slope to approximately 200 % of baseline in both wild-type and SAP102 mutant slices (figure 7.2a). There was no significant difference in fEPSP slope between the genotypes at 60 min post-tetanus [$t(5) = 0.72$, $p = 0.51$].

When LTP was induced with 3 min of continuous stimulation at 5 Hz, a striking enhancement of potentiation was observed in mutant slices compared to wild-type controls (figure 7.2b), the statistical significance of which was confirmed by comparing fEPSP slopes 45 min post-tetanus [$t(8) = 4.68$, $p = 0.005$].

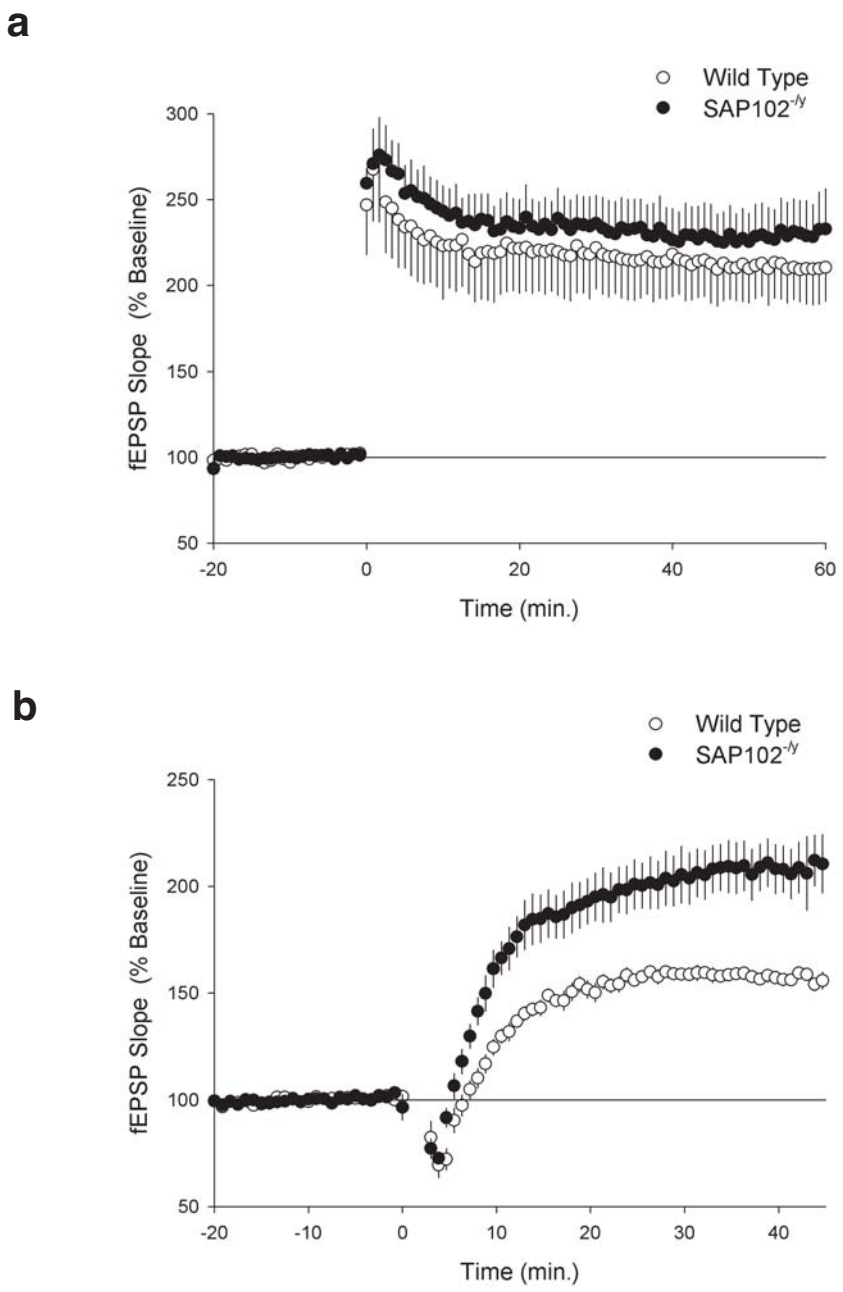

Figure 7.2 Enhanced hippocampal long-term potentiation induced by low-frequency stimulation in SAP102 mutant mice. (a) Loss of SAP102 does not affect potentiation of fEPSP slope in CA1 pyramidal cells induced by 100 Hz tetanic stimulation of Schaffer collateral axons. At 60 min post-tetanus: wild-type = 209 +/- 20% of baseline, n = 3 mice (5 slices); SAP102 -/Y fEPSPs = 230 +/- 20% of baseline, n = 4 mice (9 slices). t (5) = 0.72, p = 0.51. **(b)** Potentiation is enhanced in SAP102 mutant mice compared to wild-type controls when induced by 3 min of 5 Hz stimulation. At 45 min post-tetanus: wild-type = 156 +/- 3% of baseline, n = 6 mice (12 slices); SAP102 -/Y = 207 +/- 12% of baseline, n = 4 mice (10 slices). t(8)=4.68, p = 0.005.

7.3 Upregulation of MAP kinase activity in SAP102 mutant mice

The experiments in this section were performed by Marcelo Coba (Wellcome Trust Sanger Institute). To identify potential signalling changes that may underlie the SAP102 mutant phenotype, a differential proteomics strategy was adopted. Since phosphorylation is a common post-translational modification in postsynaptic signalling molecules (Collins et al., 2005), is an important modulator of activity in numerous signalling NMDAR-related signalling pathways and is associated with changes in signalling and LTP induction (Atkins et al., 1998; Blitzer et al., 2005; Collins et al., 2005; Davis et al., 2000; English and Sweatt, 1997), an analysis of protein kinase function was used. Hippocampal protein extracts from two wild-type and two SAP102 -/Y mice are subjected to a Kinexus screen of postsynaptic phosphorylation sites. This commercial screen uses phospho-specific antibodies to quantify phosphorylation states of the following proteins (phospho-sites shown in parentheses): ADD1 (S724), ADD3 (S693), B23 (S4), CDK1/2 (T14/Y15), CREB1 (S133), ERK1 (T202/Y204), ERK2 (T185/Y187), GSK3α (S21, 279), GSK3β (S9, Y216), JNK (T183/Y185), Jun (S73), MEK1 (S217/S221), MEK3 (S218), MEK6 (S207), MSK1 (S375), NR1 (S896), p38α MAPK (T180/Y182), PKBα (T308, S473), PKCα (S657), PKCα/β2 (T638/T641) PKCδ (T505) PKCε (S729), PKR1 (T414), Raf1 (S259), Rb (S773, S800/S804), RSK1/2 (T359/T365), S6Kα (T412), Smad1/5/9 (S463/S465/S428/S430), Src (Y423, Y543) STAT1 (Y701), STAT3 (S727) and STAT5A (Y694).

Of the 32 proteins only extracellular signal-related kinase 2 (ERK2), a component of the MAP kinase signalling pathway, showed a change in phosphorylation state in both pairs of samples, being more phosphorylated in SAP102 -/Y than in wild-type extracts. To confirm this result, ERK phosphorylation levels were determined in additional hippocampal extracts from wild-type and mutant animals using western blotting and ELISA assays. Western blots of hippocampal extracts

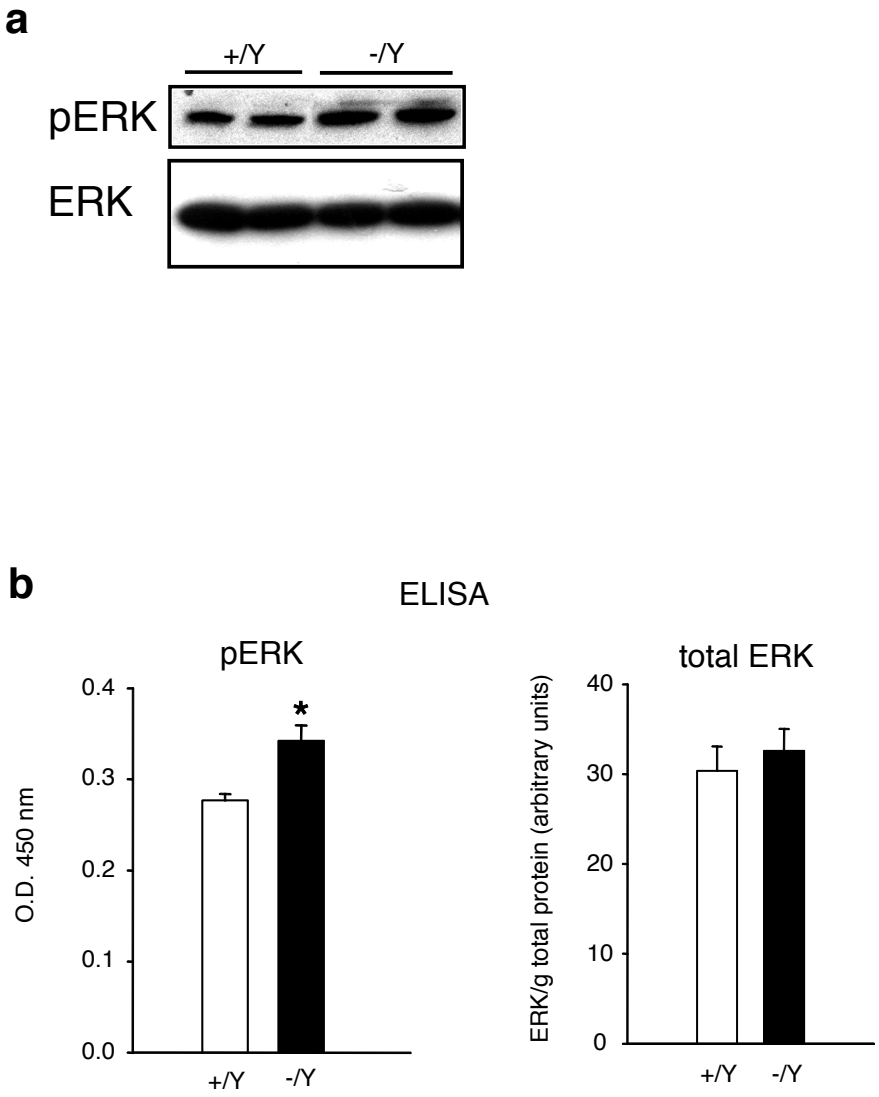

Figure 7.3 Elevated ERK phosphorylation in SAP102 mutant mice. (a) Western blotting of hippocampal protein extracts with an antibody against phospho-ERK (pERK) shows a consistent elevation of the phosphorylated form with no change in levels of total ERK. Representative samples from 15 wild-type and 15 SAP102 -/Y animals are shown. **(b)** A sandwich ELISA assay on 9 wild-type and 9 SAP102 -/Y animals confirms the increase in ERK phosphorylation [$t(16) = 3.38$, $p = 0.004$] without a change in total ERK [$t(28) = 0.06$, $p = 0.95$].

showed a consistently reproducible increase in phospho-ERK (pERK) in 15 mutant animals compared to 15 wild-type controls as shown in figure 7.3a. A sandwich ELISA experiment on 9 wild-type and 9 SAP102 -/Y animals further confirmed the increase, showing an approximately 25 % elevation of pERK in mutant extracts over controls but no significant change in total ERK levels (figure 7.3b).

7.4 SAP102 and PSD-95 perform distinct yet overlapping functions

SAP102 and PSD-95 have similar protein domains, binding partners, subcellular localisation and regional expression patterns (Fukaya and Watabe, 2000; Cho et al., 1992; Fujita and Kurachi, 2000; Muller et al., 1996), yet their functional relationship *in vivo* remains undefined. This issue was examined using biochemical and genetic strategies in the SAP102 mice along with previously generated PSD-95 mutant mice (Migaud et al., 1998).

First, it was asked whether SAP102 expression is altered in PSD-95 mutant mice. Western blots of hippocampal extracts from 10 wild-type and 10 mutant animals showed a robust and reproducible elevation of SAP102 (figure 7.4a), suggesting a partial compensation for loss of PSD-95 in these mice. We had already found no change in PSD-95 protein levels in SAP102 mice (see figure 5.5b), however, this did not preclude a change in localisation of PSD-95 as a compensatory mechanism. Co-immunoprecipitation was used to determine the amount of PSD-95 associated with NMDARs. More PSD-95 co-immunoprecipitated with NR1 in forebrain extracts from 10 SAP102 mutant mice than from 10 of their wild-type littermates as shown in figure 7.4b.

These results suggest that SAP102 and PSD-95 have partial functional overlap and that the phenotypes observed in the two mutant mouse strains may be tempered by compensation from the other MAGUK. If this was the case, mice with mutations in both proteins should display a more

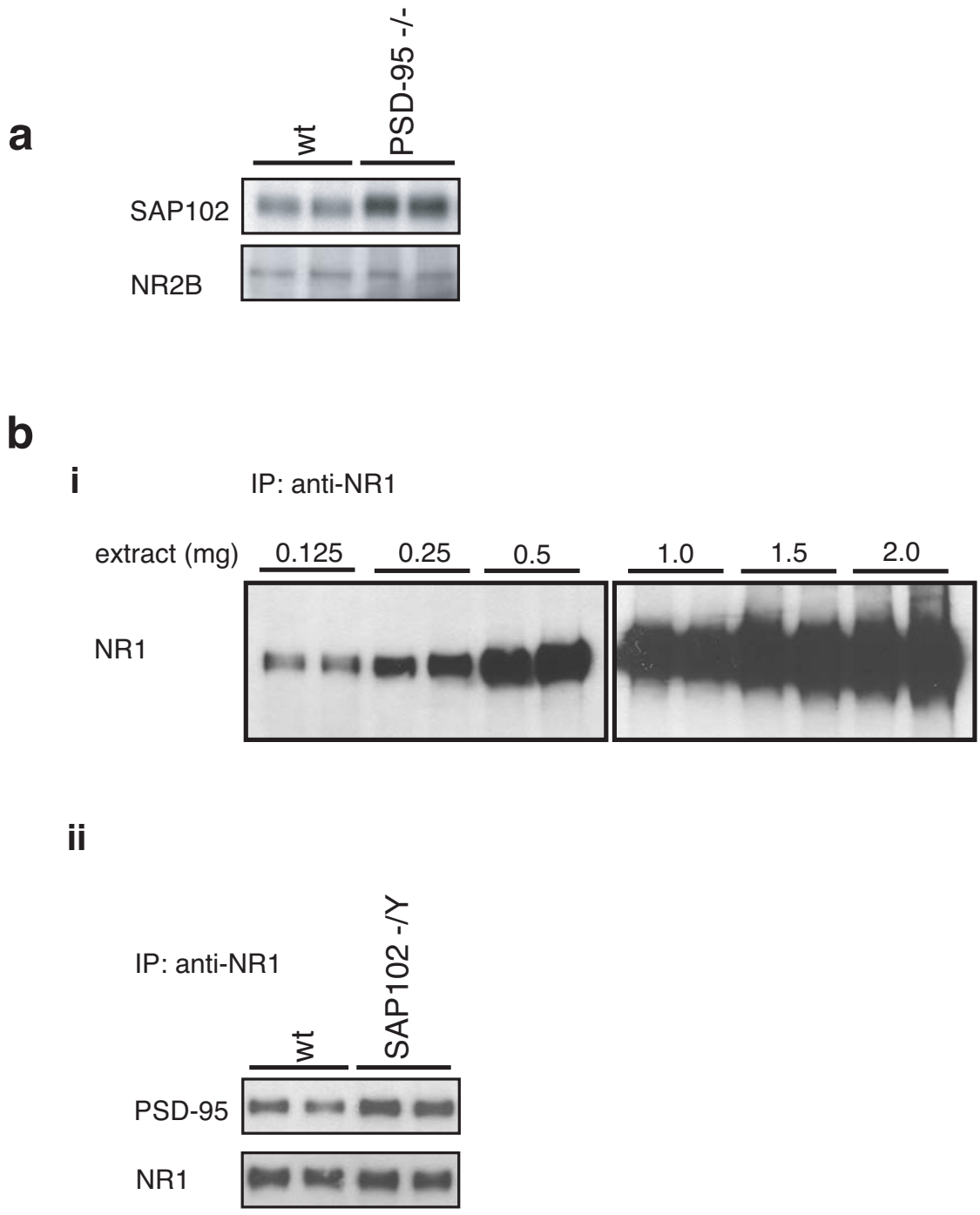

Figure 7.4 Changes in MAGUK expression and localisation in SAP102 and PSD-95 mutants **(a)** Western blot shows SAP102 protein levels are elevated in the hippocampus of PSD-95 mutant mice. **(b)** Co-immunoprecipitation of NR1 and PSD-95 from forebrain extracts of SAP102 mutant mice. **(i)** NR1 immunoprecipitations are quantitative over a range of wild-type input amounts. **(ii)** Increased PSD-95 co-immunoprecipitating with NR1 in SAP102 mutants (0.5 mg extract per IP).

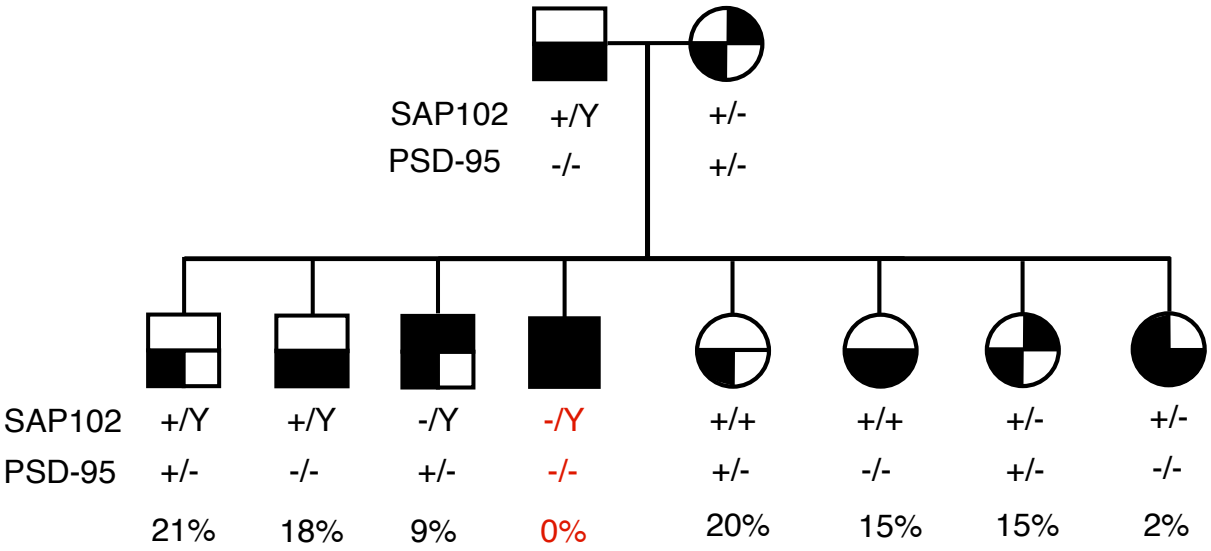

Figure 7.5 SAP102/PSD-95 double mutation is lethal. Pups from crosses between SAP102 +/Y, PSD-95 -/- males and SAP102 +/-, PSD-95 +/- females were weaned and genotyped at 4 weeks of age. The distribution of genotypes among weaned pups differed significantly from that expected under mendelian inheritance (χ^2 = 34.37, n = 94, p = 0.000014). No viable double knockout (SAP102 -/Y, PSD-95 -/-) pups and only a small proportion (2 %) of SAP102 +/-, PSD-95 -/- pups were produced.

severe phenotype than that of the individual mutations. To examine this prediction we crossed the SAP102 and PSD-95 strains. Of 94 weaned pups from these crosses none were double null and only two were heterozygous for SAP102 and homozygous for PSD-95, reflecting a severely skewed distribution of genotypes among the offspring (figure 7.5, $\chi^2 = 34.37$, $p = 0.000014$). These data show that SAP102 and PSD-95 have partial functional overlap and that the presence of at least one is necessary for viability.

7.5 Discussion

Despite localisation of SAP102 at the glutamatergic postsynapse and its interaction with numerous proteins implicated in synaptic communication and postsynaptic signalling, SAP102 does not appear to be necessary for basal synaptic transmission, since evoked synaptic responses in hipppocampal CA1 are normal in its absence.

The amplitude and time course of ion influx through AMPARs and NMDARs at these synapses in response to presynaptic stimulation is also unaffected in the mutant mice, suggesting lack of SAP102 does not alter the number of these receptors in the postsynaptic membrane, their activation properties or their conductance characteristics upon activation. SAP102 does not therefore appear to be required for trafficking of AMPARs or NMDARs to the synaptic membrane under these circumstances. Absence of a deficit in NMDAR-dependent LTP in the mice further implies normal localisation of the receptor. This result is surprising because SAP102 is often presented as an adaptor responsible for trafficking postsynaptic proteins (Fujita and Kurachi, 2000) and in one study has been directly implicated in trafficking of NMDARs in association with sec8, in experiments overexpressing a dominant negative form of sec8 lacking its C-terminal PDZ interaction motif in heterologous cells and cultured hippocampal neurons (Sans et al., 2003). Synaptic delivery of AMPARs requires an indirect interaction with a PDZ protein,

possibly SAP102, mediated by stargazin, a protein which contains a PDZ interaction motif and interacts directly with the AMPAR GluR1 subunit (Chen et al., 2000). It is possible that PDZ proteins other than SAP102 perform these functions or that they are at least able to functionally compensate when SAP102 is absent. Growing evidence from postsynaptic receptor localisation studies using targeted mutations in situations where other MAGUKs are not present, however, suggests that in fact, with occasional exceptions (Tao et al., 2003), the family does not function in this capacity at all (Klöcker et al., 2002; Migaud et al., 1998; Rasband et al., 2002).

Enhancement of 5 Hz LTP in the mutant mice, however, demonstrates that SAP102 has an essential role in regulating hippocampal synaptic plasticity. The presence of this phenotype in the absence of a disruption of basal synaptic transmission or NMDAR activation or conductance implies that SAP102 controls the intracellular signalling response to NMDAR stimulation, an observation in keeping with SAP102's intracellular location and interactions with multiple postsynaptic signalling proteins.

As with spatial learning, the effect of SAP102 loss on hippocampal synaptic plasticity is distinct from that of PSD-95 (compare figures 1.5 and 7.2). The comparison can be made with confidence since LTP experiments on the two targeted strains have been performed by the same research group, using the same protocol and experimental conditions. Enhancement of LTP in both mutants is consistent with the similarity of function suggested by the proteins' common localisations, domain structures and interaction partners. While the enhancement in PSD-95 mice is seen across a number of stimulation protocols, for SAP102 it is present mainly when potentiation is induced by low-frequency stimulation. Intriguingly, this may indicate that each of the MAGUKs mediates postsynaptic response to different patterns synaptic activation. Indeed, ERK activation is required for 5 Hz but not 100 Hz LTP (Winder et al., 1999).

An alternative explanation is that the differential involvement of the two proteins in different forms of LTP is amplitude- rather than frequency-dependent. That is, loss of PSD-95 may have a greater effect on LTP amplitude, while the smaller effect of SAP102 can be seen only following stimulation protocols, like 5 Hz, which induce a greater amount of potentiation. This would be consistent with the slight but non-significant enhancement of LTP under 100 Hz induction (see figure 7.2a) and could be further investigated by using saturating stimulation protocols to determine the maximum possible potentiation in each strain (Migaud et al., 1998). The effect of SAP102 mutation on LTD also remains to be investigated.

Initial biochemical investigations presented here on the consequences of SAP102 loss have focussed on two aspects: the identification of disruptions to postsynaptic signalling pathways which may underlie the observed synaptic plasticity and spatial learning deficits in mice and cognitive impairments in humans, and potential functional overlap between SAP102 and PSD-95. In relation to postsynaptic signalling, several different experimental protocols demonstrate that lack of SAP102 leads to a steady state elevation of phosphorylation of ERK, a member of the MAP kinase signalling pathway required for hippocampal LTP and spatial learning. This change is likely a result of modified NMDAR-dependent intracellular signalling – this could be confirmed using NMDAR agonists in acute hippocampal slices or neurons in primary culture. It will be important to determine the proteins mediating the link between SAP102 and ERK as well as the consequences for downstream MAPK signalling and how these may underlie the observed effects on plasticity and cognitive function.

The observation that mutations in either SAP102 or PSD-95 result in compensatory-like changes in the expression or localisation of the other supports the notion that partial functional overlap between the two proteins may limit the extent of phenotypic impairment in the individually targeted mouse strains. Indeed, absence of both MAGUKs resulted in loss of viability, a far more

severe phenotype, demonstrating a requirement for at least one of these proteins for postnatal survival.