# Chapter 2

# Materials and methods

## 2.1 General procedures and materials

Molecular biological procedures were performed as described (Sambrook and Russell, 2001) except as detailed below. All chemicals were analytical grade and purchased from Sigma except where specified.

General cloning was performed in DH10B or XL1Blue *E.coli*. The *dam*$^-$ strain SCS110 was used to prepare plasmids for digestion with *dam*-sensitive restriction endonucleases. Recombineering was performed in JC9604, HS996 or EL350 strains as specified. Cre and Flp recombinase-mediated site-specific recombination was performed in EL350 and EL250 strains respectively.

Mice were treated in accordance with the UK Animals (Scientific Procedures) Act, 1986 and all procedures were approved by the British Home Office Inspectorate.

## 2.2 Restriction digestion and DNA fragment purification

DNAs were digested with the appropriate type II restriction endonucleases from New England Biolabs. For digestion of DNA vectors for use in ligations with other DNA fragments 1 U of Shrimp Alkaline Phosphatase (USB) was added to the reaction to prevent subsequent self-ligation of the vector. Restriction fragments were size separated by agarose gel electrophoresis on an i-mupid electrophoresis system (Eurogentec) and/or purified from the gel using Spin-X columns (Corning). Synthetic linkers were annealed prior to ligation by combining equimolar amounts of sense and antisense strands in annealing buffer (10mM Tris, 50mM NaCl, 1mM EDTA pH 8.0), heating to 95°C and cooling gradually.

### 2.3 Ligation and transformation

DNAs were ligated using 1 U of T4 DNA ligase (Roche) at 20ºC for 4 hours or at 16ºC overnight. For transformation of bacteria, 1 µl of ligation reaction was mixed with 40µL electrocompetent *E.coli* which were produced as described by Sharma and Schimke (1996). The mixture was transferred to an ice-cold, 1mm-gap electroporation cuvette (Bio-Rad) and subjected to an exponentially decaying pulse of 1.8kV and 200µF in a Gene Pulser Xcell electroporation unit (Bio-Rad). The cells were then mixed in 1ml room temperature LB medium and incubated at 37ºC for 1hr with shaking at 250 rpm before being spread onto LB agar plates containing the appropriate antibiotic(s) and incubated at the same temperature overnight. Antibiotics were used at the following concentrations:

| | |
|---|---|
| ampicillin | 100µg/ml |
| kanamycin | 30µg/ml |
| tetracycline | 14µg/ml |
| chloramphenicol | 12.5µg/ml |

### 2.4 Plasmid DNA preparation and sequencing

Single *E.coli* colonies from were picked from agar plates into 3ml LB containing the appropriate antibiotic(s) at the concentrations above and incubated at 37ºC overnight with shaking. For general use, DNA was extracted from the liquid cultures using a boiling lysis procedure as follows: 1.5ml of culture was centrifuged at 10,000 x g for 1.5 minutes and the supernatant discarded. The cell pellet was resuspended in 180µl STET (10mM Tris.Cl,100mM NaCl, 1mM EDTA, 0.5% Triton X-100, pH 8.0), lysozyme was added to a final concentration of 1.4mg/ml and the mixture was left at room temperature for 1-5 min before boiling at 100ºC for 1 min. The solution was then centrifuged at maximum speed for 10 min and the plasmid DNA precipitated

from the resulting supernatant by the addition of 0.15 volumes of 4M ammonium acetate and 1 volume isopropanol. Precipitated DNA was washed with 70% ethanol, dried and dissolved in water or 10mM Tris, pH 8.0. RNA was degraded by the addition of 0.6 μg/ml RNase A (Sigma).

For high-quality preparations, DNA was extracted using a Wizard Plus Miniprep system (Promega) or a Qiagen Plasmid Midiprep kit. Routine plasmid sequencing from unique primers was performed by the Wellcome Trust Sanger Institute Plasmid Sequencing Facility using standard dideoxy methods. Full sequencing of *TARGETER* plasmids was performed by David Willey, Wellcome Trust Sanger Institute, using transposon-mediated shotgun sequencing.

### 2.5 BAC library screen

An adult 129Sv mouse genomic BAC library (ResGen, Release 11, 96021RG) was screened by PCR according to the manufacturer's instructions using the primer pair SAP3'PDZ, slightly 3' of the PDZ-encoding exons of the SAP102 gene. The identity of the positive clone was confirmed using two separate primer pairs, SAP5'PDZ and SAP5'PDZ2 both 5' of the PDZ-encoding exons. PCR reactions are described in section 2.7. The genomic clone was then end-sequenced to further confirm its identity and to establish the extent of the clone's coverage of the SAP102 locus. Multiple restriction digests were used to confirm an absence of structural alterations in the clone for each experiment in which it was used.

### 2.6 Protein extraction

Mouse tissue was thoroughly homogenised in DOC buffer [1% (w/v) DOC, 50mM Tris, 50mM sodium fluoride, 1mM sodium vanadate, 20μM zinc chloride, 1x Roche Complete protease inhibitor], centrifuged at maximum speed for 15 min at 4ºC and the protein-containing

supernatant retained. Extract concentrations were quantified by BCA assay (Pierce) according to the manufacturer's instructions.

### 2.7 Polymerase chain reaction

Oligonucleotides for use as PCR primers were designed using the web-based program Primer3 (Rozen and Skaletsky, 2000). Oligonucleotide sequences are listed in appendix 2.

General amplification

PCR for BAC library screening, amplification of DNA probes for Southern blotting and cDNA amplification following RT-PCR was performed using approximately 1ng (plasmid) or 1 μg (genomic) template DNA in a reaction containing 1.5 U *Taq* DNA polymerase (Promega), 1 x amplification buffer (Promega), 200 μM each dNTP, 0.5 μM each primer and 25 mM $MgCl_2$. The cycling protocol is shown in table 2.1. For BAC screening 30-40 amplification cycles were used; for DNA probes 25 cycles were used.

**Table 2.1 Cycling protocol for general PCR amplification**

| Temperature (°C) | Time (s) | Cycle number |
|---|---|---|
| 94 | 60 | 1 |
| 94 | 30 | see text |
| 55 | 30 | |
| 72 | 60/kb | |
| 72 | 120 | 1 |

Mouse genotyping

For SAP102 PCR genotyping, approximately 1 μg of genomic DNA was placed in a reaction containing 1x PCR master mix (Promega), 0.5 μM each primer and an additional 0.25 mM $MgCl_2$ (total $MgCl_2$ concentration 1.25 mM). Amplification was as described in table 2.1 using 33 cycles with an extension time of 60 s. Two separate reactions were performed for each tail, one using primers P4 and P5 to amplify the wildtype allele and the other using primers P4 and P3 to amplify the mutant allele.

The PSD-95 PCR genotyping assay was developed by Karen Porter. Approximately 1 μg of genomic DNA was placed in a reaction containing 1.25 U HotStar *Taq* DNA polymerase (Qiagen), 1x amplification buffer (Qiagen), 0.8 μM each primer, 300 μM each dNTP and 10 % (v/v) DMSO. Each tail was genotyped with a single, multiplex PCR reaction containing primers P6 and P7 to amplify the wildtype allele and P8 and P9 to amplify the mutant allele. Table 2.2 shows the cycling conditions for this assay.

Sex determination PCRs were performed as described (Lambert et al., 2000).

**Table 2.2 Cycling protocol for PSD-95 PCR genotyping assay**

| Temperature (°C) | Time (s) | Cycle number |
|---|---|---|
| 95 | 900 | 1 |
| 94 | 30 | 35 |
| 58 | 60 | |
| 72 | 60 | |

ES cell genotyping

Amplification was performed using the Expand Long Template PCR System (Roche), utilising a mixture of thermostable *Taq* DNA polymerase and thermostable *Tgo* proofreading DNA polymerase. Approximately 1 μg of genomic DNA was placed in a reaction containing 1.75 U polymerase mix, 1x Expand amplification buffer 3, 0.2 μM each primer and 200 μM each dNTP. Two separate PCR reactions were performed for each clone, one with primers P1 and P2 to amplify the wildtype allele and the other with primers P1 and P3 to amplify the mutant allele. Table 2.3 shows the cycling conditions for this assay.

**Table 2.3 Cycling protocol for SAP102 targeted ES cell genotyping assay**

| Temperature (°C) | Time (s) | Cycle number |
|---|---|---|
| 97 | 120 | 1 |
| 96 | 10 | 40 |
| 60 | 30 | |
| 68 | 240 | |
| 68 | 10 | 1 |

High fidelity amplification

For amplification of short homology arms and cassettes for recombineering, and of the SV40polyA signal fragment, where accurate replication of the DNA template is essential, high-fidelity Platinum *Pfx* DNA polymerase (Invitrogen) was used. Approximately 50 ng of plasmid DNA was placed in a reaction containing 3 U *Pfx* polymerase, 1x *Pfx* amplification buffer, 0.3 μM each primer, 300 μM each dNTP and 1mM $MgSO_4$. Table 2.4 shows the cycling conditions for this assay.

**Table 2.4 Cycling protocol for high-fidelity PCR with Platinum *Pfx* polymerase**

| Temperature (°C) | Time (s) | Cycle number |
|---|---|---|
| 94 | 300 | 1 |
| 94 | 15 | 26 |
| 53 | 30 | |
| 68 | 60/kb | |
| 68 | 120 | 1 |

## 2.8 Reverse Transcription-PCR

Reverse transcription reactions were performed using 1 µg total RNA, 200 U SuperscriptII reverse transcriptase (Invitrogen), 1 x SuperscriptII first-strand buffer, 2 µM oligo dT primer, 500 µM each dNTP, 10 mM DTT and 40 U RNaseOUT RNase inhibitor (Invitrogen). The RNA, oligo dT and dNTPS were first combined and incubated at 65°C for 5 min, then placed on ice while the buffer, DTT and RNase inhibitor was added. The reaction was incubated at 42°C for 2 min, then the reverse transcriptase was added. The incubation then continued at the same temperature for 50 min then the enzyme was inactivated by incubation at 70°C for 15 min. 1-2 µl was used in the PCR reaction (see 'general amplification', section 2.7).

## 2.9 Recombineering

Short homology arms of up to 70 bp for recombineering were attached to DNA fragments by PCR amplification of the entire fragment using composite synthetic oligonucleotides containing

the homology arm sequence adjacent to the PCR primer sequence (see section 2.7 for PCR conditions and oligonucleotides). PCR products were purified with a Geneclean spin kit (Bio 101), treated with *dam*-sensitive restriction enzyme *Dpn*I to remove residual (*dam*-methylated) template plasmid, purified by organic extraction followed by ethanol precipitation then redissolved in water for electroporation.

Short homology arms of length greater were PCR-amplified individually using primers carrying restriction sites on their ends, then digested and ligated either side of the selection cassette for recombineering. Fragments containing the selection cassette flanked by homology arms were released by restriction digestion, isolated by agarose gel electrophoresis, purified through a Spin-X column (Corning), ethanol precipitated and redissolved in water for electroporation.

JC9604 and HS996 *E.coli*

Recombineering experiments using JC9604 cells, which carry endogenous, constitutive recombination activity, were performed as described (Zhang et al., 1998). Electrocompetent cells carrying the appropriate recipient or donor plasmid for recombination were prepared by incubating a 70ml culture in LB medium at 37ºC with shaking to $A_{600}$ = 0.4 and harvesting by centrifugation for 10 min at 4,000 rpm, -5ºC. Cells were then washed twice in ice-cold water and once in 10 % glycerol, each time resuspending the cells in the wash solution then recovering them with the centrifugation step. After the final wash the cells were resuspended in approximately 100µl 10 % glycerol and immediately transformed with the previously prepared linear DNA fragment.

Recombineering in HS996 cells was performed by first transforming the cells with the p*R6K116/BAD/αβγ* plasmid which confers inducible recombination competence. When preparing

the cells for electroporation, expression of *redα, redβ and redγ* was induced by the addition of arabinose to a final concentration of 0.1 % to the culture at $A_{600}$ = 0.15. The remainder of the protocol was identical to that used for JC9604 cells.

EL350 and EL250 *E.coli*

EL350 and EL250 cells were grown at 32ºC except during induction for recombineering. Recombineering was performed as described (Liu et al., 2003). Electrocompetent cells carrying the appropriate recipient or donor plasmid were prepared by incubating a 20ml culture in LB medium at 32º with shaking to $A_{600}$ = 0.5. At this point *Redα, redβ and redγ* expression was induced by transferring half the culture to 42ºC while the remainder was left at 32ºC. 15 min after the transfer both cultures were chilled in wet ice for 20 min then harvested by centrifugation at 5,000 rpm for 6 min at 2ºC. Cells were washed three times in 1ml water per wash, being recovered after each wash by centrifugation at 10,000 x g for 1 min at 2ºC. After the final wash both induced and uninduced cells were resuspended in an approximate total volume of 50 µl ice-cold water and immediately transformed with the previously prepared linear DNA fragment.

To check for differences in transformation efficiency between the induced and uninduced cells, each recombineering experiment was performed in parallel with an additional experiment in which induced and uninduced cells were transformed with 10 pg supercoiled plasmid. Induced cells were generally around 3-fold more efficient in transformation, however this had little bearing on the interpretation of the results since under transformation with the linear DNA fragment for recombination the induced cells produced thousands of antibiotic-resistant colonies (see table 3.1) while the uninduced cells never produced more than 5 colonies.

To perform Cre or Flp recombinase-mediated site-specific recombination between loxP or FRT sites in plasmids, EL350 or EL250 cells respectively were prepared for electroporation as above, except that when the culture reached $A_{600}$ = 0.3, arabinose was added to a final concentration of 0.1 % to induce recombinase expression.

**2.10 DNA cloning strategies**

Sequences of oligonucleotide linkers used in DNA cloning are listed in appendix 2. Full sequences of p*TARGETER*, p*IRESlacZneoflox*, p*neoflox* and *ploxPneoflrt* are listed in appendix 3.

p*TARGETER*

A *Bcl*I-*Pvu*II fragment from pACYC184 (New England Biolabs), containing p15A replication origin and tetracycline antibiotic resistance gene, was ligated with a synthetic oligonucleotide containing multiple, unique restriction enzyme sites to form the multiple cloning site (MCS). A *Hind*III-*Xho*I fragment containing MC1-DTA-PGKpA from pMC1DTApA (a gift from Noboru Komiyama) was then ligated into the 3' end of the MCS to complete the plasmid.

IRES-lacZ-polyA plasmids

A synthetic oligonucleotide linker (L1) containing an MCS was ligated between the *Aat*II and *Hpa*I sites of pSP72 (Promega). A *BamH*I-loxP-PGK-EM7-neo-PGKpolyA-loxP-*BamH*I cassette (a gift from Karen Porter) was ligated into the *BamH*I site in the new MCS. A 324 bp *Sca*I-*Ssp*I fragment containing the ampicillin resistance gene (*bla*) was then removed from the plasmid and the remaining fragment self-ligated. Absence of *E.coli* colonies containing the plasmid after plating on ampicillin confirmed the success of this strategy. This plasmid was named p*SP75neoflox*.

A *BamH*I-T3-IRES-lacZ-*BamH*I fragment (a gift from Douglas Strathdee) was ligated into the *Bcl*I site of p*SP75neoflox* to produce p*SP76*. p*SP76* was used for initial recombineering attempts to insert the T3-IRES-lacZ-polyA-neoflox cassette into pBSSK.SAP102 by PCR-amplifying the cassette using PCR primers carrying short homology arms on their ends, and also by cloning longer homology arms either side of the cassette and then releasing the entire construct from the plasmid by restriction digestion.

For further recombineering experiments with only the neo cassette, the T3-IRES-lacZ-polyA section was removed from p*SP76* by digesting the plasmid with *EcoR*I, isolating each fragment other than that containing the T3-IRES-lacZ-polyA cassette and back together.

To construct p*IRESlacZneoflox*, an *EcoR*V-*Hpa*I fragment containing the cassette PGK polyA signal was removed from p*SP75neoflox* and, by blunt-end ligation, was replaced with an SV40 polyA fragment which had been PCR-amplified from p*CAGGS* (a gift from Noboru Komiyama) using the primer pair AAC+SV40pA (see appendix 1) to create p*SP75SV40pA*. A *BamH*I-T3-IRES-lacZ-polyA-*BamH*I fragment was then ligated into the *Bcl*I site 5' of the neo cassette to produce p*IRESlacZneoflox*.

<u>p*neoflox* and p*loxPneoflrt*</u>

p*neoflox* was constructed by ligating the *BamH*I-loxP-PGK-EM7-neo-SV40pA-loxP-*BamH*I cassette from p*SP75SV40pA* into the *BamH*I site of p*SP72*.

p*loxPneoflrt* was constructed using a *BamH*I-loxP-FRT-PGK-EM7-neo-PGKpolyA-FRT-*BamH*I cassette in a p*Bluescript* backbone (a gift from Noboru Komiyama). The PGK polyA signal in this cassette was replaced with and SV40 polyA using the same strategy as in p*SP75SV40pA*, then the new cassette was ligated into the *Bgl*II site of p*SP72L*.

SAP102 constitutive targeting vector

A 13 kb section of the SAP102 locus extending from 1 kb upstream of exon 1 to 3.2 kb downstream of exon 10 was excised from the SAP102 BAC clone into p*BSSK.DTA* (a gift from Noboru Komiyama) by recombineering. First the entire p*BSSK.DTA* plasmid was amplified by high-fidelity PCR using primers (SAP1A, SAP1B) containing a short sequence hybridising to the plasmid to prime the reaction adjacent to 70 bp of sequence corresponding to the ends of the SAP102 genomic section to be cloned. The purified PCR product was transformed into recombination-competent bacteria containing the BAC clone and a clone carrying the correctly recombined plasmid (p*BSSK.DTA.SAP102*) isolated as described in section 2.9.

Attempts to insert a selection cassette into the SAP102 fragment in *pBSSK.DTA.SAP102* by recombineering were first performed by PCR-amplifying the cassette from p*SP76* using primers, SAP2A (911 bp product) and SAP2B (863 bp product), containing short homology arms matching the sequence flanking the site of insertion in exon 4. After that, 5' and 3' homology arms were amplified by PCR using primers SAP2Afwd/rev and SAP2Bfwd/rev and ligated into the *Not*I-*Asc*I and *Pme*I-*Pac*I sites either side of the p*SP76* cassette respectively. The construct was then released with *Not*I and *Pac*I for recombination.

When the recombineering strategy failed, the cassette was instead inserted by tradition cloning methods. The *BamH*I-loxP-PGK-EM7-neo-PGKpA-loxP-*BamH*I fragment from p*SP75neoflox* (see above) was isolated and ligated into a *Bam*HI site flanked by *Ssp*I sites in a synthetic linker (L2) previously inserted between the *Pst*I and *EcoR*I sites of p*SP72*. The new plasmid was digested with *Ssp*I and the blunt-ended fragment containing the selection cassette isolated for insertion into the SAP102 targeting vector. p*BSSK.DTA.SAP102* was digested with *Afl*II, removing a fragment of SAP102 sequence from exon 2 to 8 inclusive. The remaining, larger fragment was isolated and treated with mung bean nuclease (New England Biolabs) to cleave the

single strand overhangs left by the restriction enzyme, then blunt-end ligated with the cassette fragment. For targeting, the completed vector was linearised with a unique *Not*I restriction site in the plasmid backbone.

SAP102 reporter knock-in vector

To build this vector a 10.9 kb SAP102 genomic fragment extending from 350 bp downstream of exon 1 to 3.2 kb downstream of exon 10 was excised from the SAP102 BAC clone into p*TARGETER* by recombineering. To do this, 2 short homology arms, aa1 and aa2, each 643 bp in length and matching the 5' and 3' ends respectively of the genomic fragment to be excised, were PCR-amplified using primers aa1fwd/rev and aa2fwd/rev and cloned adjacent to each other, separated only by an *Asc*I site, between the *Kpn*I and *Pme*I restriction sites in p*TARGETER*. The plasmid was linearised with *Asc*I and transformed into recombination-competent *EL350 E.coli* carrying the SAP102 BAC clone. Clones from antibiotic-resistant colonies were purified and analysed for the correct recombination event by restriction digestion and DNA sequencing. The recombined plasmid was named p*TARGETER.SAP102*.

To insert the T3-IRES-lacZ-pA-neoflox cassette into the vector, the same SAP2A and SAP2B short homology arms as were used in the first attempt to produce this vector (see 'SAP102 constitutive targeting vector', section 2.10) were re-amplified and cloned into the *Not*I-*Asc*I and *Pme*I-*Sal*I sites either side of the cassette in p*IRESlacZneoflox*. The cassette was released by digestion with *Not*I and *Sal*I, isolated and recombined into p*TARGETER.SAP102* to complete the targeting vector.

SAP102 conditional targeting vector

This vector used the p*TARGETER.SAP102* plasmid generated above as its base. To insert the first loxP site, short homology arms matching the sequence flanking the required site of insertion in

intron 5 were PCR-amplified using primers SAPneoflox5'fwd/rev (402 bp product) and SAPneoflox3'fwd/rev (420 bp product) and cloned into the *Xba*I-*Xho*I and *Kpn*I-*Bgl*II sites either side of the loxP-PGK-EM7-neo-pA-loxP cassette in p*neoflox*. The construct was released by *Xba*I and *Bgl*II digestion and recombined into p*TARGETER.SAP102*. Cre recombinase expression was induced by arabinose exposure in *EL350* cells carrying the new plasmid to remove the neo cassette, leaving only a single loxP site in intron 5. This plasmid was named p*TARGETER.SAP102.loxP*.

To insert the second loxP and selection cassette for targeting, short homology arms matching the sequence flanking the required site of insertion in intron 1 were PCR-amplified using primers SAPloxPneoflrt5'fwd/rev (601 bp product) and SAPloxPneoflrt3'fwd/rev (485 bp product) and cloned into the *Not*I-*Asc*I and *Pme*I-*Sal*I sites either side of the loxP-FRT-PGK-EM7-neo-pA-FRT cassette in p*loxPneoflrt*. The construct was released by *Not*I and *Sal*I digestion and recombined into p*TARGETER.SAP102.loxP* to complete the targeting vector.

### 2.11 Genomic DNA extraction

Extraction from ES cells

For extraction of DNA from ES cells in a 24-well plate, the culture medium was removed and replaced with 300µl DNA lysis solution (50mM Tris pH 8.0, 100mM EDTA, 100mM NaCl, 1% SDS) containing 0.7mg/ml proteinase K (Sigma) and thoroughly triturated. The solution was incubated at 55ºC for 2 hours with occasional mixing by inversion. 3µg RNase A (Sigma) was then added and the solution was incubated at 37ºC for 30 min.

For use in PCR, 20µl was removed and the genomic DNA precipitated by the addition of 0.8 volumes of isopropanol. The solution was centrifuged at maximum speed for 10 min and the

DNA pellet retained, washed in 70% ethanol, dried and dissolved in 10µl TE (10mM Tris, 1mM EDTA, pH 8.0). 1 µl was used per PCR reaction.

For use in restriction digestions, the remaining proteinase K- and RNase-treated DNA was extracted once with an equal volume of phenol, once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated with isopropanol as above and dissolved in 50µl TE

Extraction from mouse tissue

Mouse tissue was digested overnight at 55ºC in 500µl DNA lysis solution containing 1.6mg/ml proteinase K and then treated with 3µg RNase A for 30 min at 37ºC. For PCR the solution was incubated in dry ice for 1 min then centrifuged at maximum speed for 15 min at 4ºC. A small amount of supernatant was removed from the surface and diluted between 5- and 15-fold with water. 1ul of the diluted solution was used per PCR reaction. For restriction digestion the proteinase K- and RNase-treated DNA solution was thrice organically extracted, precipitated and dissolved in TE as described for the ES cell DNA above.

**2.12 Southern blot**

DNA probes were amplified by PCR and cloned into pGEM-T Easy vectors (Promega). The 5' probe (784 bp) was amplified using primer pair SAP5'probe and the 3' probe (886 bp) with SAP3'probe. The internal probe (969 bp) used for verification of the Ensembl SAP102 genomic sequence was amplified with primer pair SAP5'PDZ3. For hybridisation, probes were released from their vectors by restriction digestion, purified by agarose gel electrophoresis and radiolabelled with $^{32}$P-deoxycytosine using a Rediprime II random priming kit (Amersham Biosciences).

Genomic DNA for hybridisation were digested overnight with the appropriate restriction endonuclease and resolved by electrophoresis through a 0.6% agarose gel. Separated DNAs were UV-nicked by subjecting the gel with 0.08 J/cm$^2$ ultraviolet radiation in a UV crosslinker (Stratagene) and transferred to Hybond-N$^+$ nylon membrane (Amersham Biosciences) by capillary transfer using alkaline transfer buffer (1.5M NaCl, 0.5M NaOH). Membranes were hybridised with radiolabelled probe overnight in 0.1ml phosphate-SDS hybridisation buffer [0.5M sodium phosphate, 1mM EDTA, 7% (w/v) SDS, 1% (w/v) fraction-V BSA, pH 7.2]. Excess probe was removed by washing at 65ºC with phosphate-SDS wash solution I [40mM sodium phosphate, 1mM EDTA, 5% (w/v) SDS, 0.5% (w/v) fraction-V BSA, pH 7.2] followed by phosphate-SDS wash solution II [40mM sodium phosphate, 1mM EDTA, 1% (w/v) SDS, pH 7.2]. The membrane was then exposed to a phosphor screen and developed in a Typhoon imager (Amersham Biosciences).

### 2.13 Protein extraction

Mice were killed by cervical dislocation. They were then decapitated, the skin and top of the skull removed and the brain extracted from the resulting opening. For forebrain protein extracts, the hindbrain, cerebellum, midbrain and olfactory bulbs were dissected away. For hippocampal extracts, bilateral hippocampii were further dissected from the forebrain.

Adult forebrain was homogenised in 4 ml DOC buffer [1 % (w/v) DOC, 50 mM Tris pH 9.0, 50 mM NaF, 20 μM ZnCl, 1 mM sodium orthovanadate, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin] in a 5 ml manual homogeniser. Adult hippocampus was homogenised in 1.5 ml DOC buffer in a 2 ml homogeniser and P6 forebrain was homogenised in 2 ml DOC buffer in a 5 ml homogeniser. The homogenised tissue was centrifuged at 32,000 rpm for 20 min at 4ºC and the supernatant retained. Extracts were quantified using a bicinchoninic acid assay (Pierce).

### 2.14 Western blot

15 μg protein extract was diluted in Laemmli sample buffer (Bio-Rad) and subjected to SDS-PAGE using Tris-HCl Polyacrylamide ReadyGels (Bio-Rad) in Tris/Glycine/SDS buffer [25 mM Tris, 192 mM Glycine, 20% (v/v) SDS, pH 8.3] in a mini-Protean cell (Bio-rad). Proteins were transferred onto Hybond-P membrane (Amersham Biosciences) in a mini trans-blot cell electroblotter (Bio-Rad) at 400mV for 1 hour in Tris/Glycine buffer (25mM Tris, 192mM Glycine, pH 8.3). Membranes were stained in Ponceau S (Sigma) then incubated overnight at 4ºC in PBS/Tween (PBS with 0.1% (v/v) Tween 20) containing 5% (w/v) skimmed milk powder (Marvel). Membranes were washed 5 times in PBS/Tween and then incubated with primary antibody in PBS/Tween for 2 hours at room temperature. Membranes were washed again 5 times then incubated with secondary antibody in PBS/Tween for 1 hour at RT. Secondary antibodies were anti-mouse or anti-rabbit IgG HRP-linked whole antibody (Amersham Biosciences). Binding of the secondary antibody was detected with an ECL Plus kit (Amersham Biosciences) followed by exposure to Hyperfilm (Amersham Biosciences). Primary antibodies are listed in appendix 2.

For repeated probing, the primary antibodies were stripped from membranes by incubation in stripping buffer [1.5% (w/v) glycine, 0.05% (w/v) Tween 20, pH 2.5] at 80ºC for 1.5 hrs with one change of solution. They were then washed 3 times in PBS/Tween at room temperature, 5 min per wash, and then blocked as normal with 5% milk before incubation with the new primary antibody.

### 2.15 Co-immunoprecipitation

Protein extracts in DOC buffer were incubated with 5 μg of the precipitating antibody with rotation at 4ºC for 1 hour in a total volume of 400 μl. 15 μl Protein G Sepharose beads (Amersham Biosciences) were equilibrated in DOC buffer and added to the mixture before incubating for further hour under the same conditions. Precipitated complexes were washed 3 times in DOC buffer, resuspended in Laemmli sample buffer, boiled at 85ºC for 5 minutes, centrifuged and the supernatant subjected to Western blotting as described above.

### 2.16 Phosphorylation screen and sandwich ELISAs

These experiments were performed by Marcelo Coba (Wellcome Trust Sanger Institute). Hippocampal protein extracts were subjected to a KPSS-1.3 phosphorylation screen (Kinexus Bioinformatics Corporation). Extracts were prepared by homogenising dissected hippocampi in 20 mM MOPS pH 7.4, 2 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 30 mM NaF, 40 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1mM PMSF, 3mM benzamidine, 5 μM pepstatin and 10 μM leupeptin and quantifying the protein concentrations using a BCA assay (Pierce).

Sandwich ELISAs were performed according to the manufacturer's instructions to determine endogenous levels of ERK1/2 (Biosurce total ERK1/2) and phospho-ERK1/2 T202/Y204 (Pathscan Cell Signaling).

### 2.17 ES cell culture and targeting

Culture and targeting was largely performed as described (Nagy et al., 2003). Reagents were purchased from Gibco-BRL except where indicated. HM-1 mouse ES cells were cultured on

0.1% gelatin in ES cell medium consisting of BHK-21 (Life Technologies) supplemented with 10% FBS (Stem Cell Technologies), 2mM L-glutamine, 100U pencillin, 100µg/ml streptomycin, 1x non-essential amino acids (Gibco-BRL 11140-035), 1mM sodium pyruvate, 100µM 2-mercaptoethanol and 700U/ml ESGRO leukemia inhibitory factor (Chemicon International).

For targeting, 1 x $10^7$ cells were electroporated with 100 µg linearised, purified targeting vector in a 0.4mm gap electroporation cuvette (Bio-Rad) at 0.8kV, 3µF using a Gene Pulser Xcell electroporation unit (Bio-Rad). Cells were plated into 10cm petri dishes and selection with 300µg/ml G418 sulphate was begun 24 h after plating. Single G418-resistant colonies were picked 5-7 days later for expansion, analysis and freezing.

Colonies were expanded gradually into 2 wells of a 24-well plate, of which one was used for DNA extraction and analysis and the other stored at –80ºC in ES cell medium containing 10% DMSO and 20% FBS. For blastocyst injection, frozen positive clones were thawed and further expanded, then passaged a final time into medium without G418 before trypsinising, washing and resuspending into fresh G418-free medium for injection. Blastocyst injection was performed as described (Ramirez-Solis et al., 1993).

To test the functionality of the SV40 polyA signal for targeting, approximately 1 x $10^6$ cells were electroporated with 5 µg linearised plasmid and subjected to G418 selection as above. After 5 days of selection, drug-resistant colonies were fixed and permeabilised in methanol then stained with Giemsa for analysis.

### 2.18 Histology

Histological experiments were performed essentially as described (Bancroft et al., 1996). Mice were deeply anaesthetized with sodium pentobarbitone and perfused intracardially with 4 % paraformaldehyde. The whole brain was then dissected and placed in 4 % paraformaldehyde overnight, then impregnated with wax using a Shandon Exelsior tissue processor (Thermo Electron Coorporation). This consisted of dehydration in increasing ethanol concentrations over 6 hrs following by 3 hrs of xylene incubation with fresh solution each hour, then 3 hrs of incubation in molten paraffin wax at 60ºC with fresh solution each hour. The brains were then embedded in paraffin wax blocks and cut into 5 μm sections.

For Nissl staining, slides were incubated for 10 min in xylene then 10 min in ethanol, each with one change of solution, then rehydrated sequentially in 90 %, 70 %, 50 % and 30 % ethanol, 30 s per incubation. The slides were washed briefly in PBS then placed in Nissl stain [0.4 % (w/v) cresyl violet, 80 mM sodium acetate, 120 mM acetic acid, 29 % (v/v) methanol] for 15 min. They were then sequentially dehydrated to 100 % ethanol, cleared in xylene and immediately coverslipped.

Immunohistochemistry was performed on a Ventana Discovery machine according to the manufacturer's instructions. For NR1, NR2A, NR2B, SAP102, PSD-95 and PSD-93 staining, slides were pretreated with Ventana Cell Conditioning 1 followed by 10 min of Protease 1. For MAP2B staining, slides were pretreated with Ventana Mild Cell Conditioning 1 only. Primary antibodies are listed in appendix 1. Secondary antibodies were biotin-conjugated rabbit anti-mouse (Dako Cytomation) or biotin-conjugated donkey anti-rabbit (Jackson). Antibody binding was detected by chromogenic oxidation of diaminobenzidine (DAB) by streptavidin-conjugated

HRP enzyme. Images were captured using a Zeiss Axioplan 2 microscope with Axiovision 4.2 software.

### 2.19 Electrophysiology

Electrophysiological experiments were performed by Anne Fink, Patricio Opazo and Tom O'Dell (Department of Physiology, University of California, Los Angeles) as described (Mayford et al., 1995). Experimenters were blind to the genotypes of the mice. 400 μm-thick slices of mouse hippocampus were maintained at 30ºC in an interface-type recording chamber perfused with a murine artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 4.4 mM KCl, 25 mM $Na_2HCO_3$, 1 mM $NaH_2PO_4$, 1.2 $MgSO_4$, 2 mM $CaCl_2$ and 10 mM glucose. EPSPs were elicited once every 50 s (0.02 Hz, 0.01 – 0.02 ms duration pulses) using tungsten wire bipolar stimulation electrodes in stratum radiatum of the CA1 region. The resulting potentials were measured using low resistance glass microelectrodes (5-10MΩ, filled with ACSF), also in CA1 stratum radiatum.

For basal synapse function experiments, fibre volleys in Schaffer collateral axons and field excitatory postsynaptic potential (fEPSP) slopes were measured at stimulation intensities inducing 25 %, 50 %, 75 % and 100 % maximum fEPSP amplitude. Postsynaptic currents were measured using whole-cell voltage-clamp recordings at postsynaptic membrane potentials of –80 and +40 mV. The AMPAR-mediated component of the excitatory postsynaptic current (EPSC) was determined by the peak amplitude of the EPSC at 5 ms after EPSC onset. The NMDAR-mediated component was determined by the amplitude of the EPSCs 50 ms after EPSC onset. For long term potentiation, the 100 Hz stimulation protocol consisted of two trains of 100 Hz stimulation, each 1 s in duration and separated by 10 s. The 5 Hz protocol consisted of continuous 5 Hz stimulation for 3 min (900 pulses delivered in total).

## 2.20 Behaviour

Two cohorts of mice were used for behavioural testing. The first cohort was tested only in the water maze at eight weeks of age. A second cohort of mice was tested at seven weeks of age in the T-maze and olfactory habituation tasks. Four weeks later they were tested in the elevated plus maze and another four weeks later they were tested in the open field, grip strength and elevated plus maze tasks. All behavioural experiments were performed blind with respect to the genotypes of the mice.

Water maze

This experiment was performed and analysed by Jamie Ainge (Division of Neuroscience, University of Edinburgh) and Lianne Stanford. The water maze was 2 m in diameter with opaque water of temperature 25 ± 1 ºC. The escape platform was 30 cm in diameter and was made visible when required by lowering the water level in the pool and adding a flag to the centre of the platform. A black curtain was drawn around the maze when required to eliminate the visible cues from within the room. Data analysis was performed with automated swim path analysis software (Actimetrics).

The testing protocol was adapted from Migaud et al. (1998) and Komiyama et al. (2002). Each training trial began when the mouse was placed in the water and ended when it climbed onto the platform or after 90 s had elapsed, whichever was shorter. If the mouse had not reached the platform after 90 s it was led to the platform and allowed to climb onto it. Average swimming speed, distance travelled and latency to reach the platform were calculated for each trial. Mice were first trained with a visible platform for three days, then with a hidden platform for five days and finally to the opposite platform location for five days. Four training trials were given per day. In probe tests, each mouse was placed in the pool without the platform present and its swim path recorded for 60 s. Time spent in each quadrant of the pool and number of crossings of the

platform site were recorded for each test. Probe tests were performed 10 min (H1) and 24 hrs (H2) after the final hidden platform training trial, 10 min after the final trial on each day of reversal training (R1-5) and 1, 7, 14 and 56 days after the final reversal training trial (M1, M7, M14 and M56 respectively).

Two-way (genotype x day) mixed ANOVAs were completed for latency, distance and swim speed. For the probe trials, 2-way (genotype x quadrant) mixed ANOVAs were completed. Single mean *t*-tests with the null mean set to 15 s were also performed to assess whether the mean 'training quadrant' times for each genotype were above chance (15 s).

T-maze

This experiment was performed in collaboration with Lianne Stanford. T-maze design and testing protocols were adapted from Gerlai (Gerlai, 1998). The maze was constructed by the maintenance department of the Wellcome Trust Sanger Institute. The floor was a single piece of black acrylic sheet mounted onto plywood. The T was created by a 90º bisection of a 102 cm x 12 cm alley with a second alley of 45 cm x 12 cm. Walls of clear acrylic sheet of height 20 cm were attached to the sides with All Purpose clear adhesive (Bostick Findley Ltd, Stafford, UK) and screws. 10 cm from the end of the bottom arm of the 'T', vertical aluminium guides of length 15 cm were attached to the walls. These held a clear acrylic door which separated the end of the arm from the remainder of the maze, creating a start box. Similar guides were placed close to the T-intersection in the other two arms so that access to either or both arms could be prevented. Thin white lines, 12 cm into each of the right and left arms, were added to the floor of the maze. A second set of lines was drawn 25 cm from the entrance of the two arms. The maze was placed on the floor in a room containing a number of spatial landmarks including a bin, table, chair and door. The room was dark except for some dim white light entering though the slightly opened door.

The T-maze test was run in continuous, non-rewarded fashion (Gerlai, 1998). Each mouse completed one forced-choice followed by 10 choice trials. In the forced-choice trial, entrance to one of the two goal arms (chosen at random) was blocked and the mouse was placed in the start box with the starting gate closed. The starting gate was then raised to begin the trial and closed once the animal had left the start box. Once both rear paws of the mouse had crossed the first white line in the unblocked goal arm, the starting gate was again raised. When the mouse re-entered the main arm of the maze the forced-choice arm was closed behind it. The trial ended when the mouse re-entered the start box and the starting gate was closed. The remaining 10 choice trials were performed the same way except both goal arms were unblocked and available. Arm choice in each trial and total time taken to complete the 11 trials (session duration) was recorded.

Olfactory habituation-dishabituation task

This experiment was performed in collaboration with Lianne Stanford. The habituation-dishabituation protocol was adapted from those previously published (Brown et al., 1987; Wrenn et al., 2003). Mice were tested individually in translucent, empty, housing cages, of dimensions 13 x 30 x 15 cm, with metal lids. The test was performed under dim lighting with only one animal in the room at any one time. Urine samples for use as olfactory stimuli were collected separately from male C57BL/6J and CD-1 strain mice. The experimenter changed gloves between trials and used separate pipettes for each urine type. Mice were placed in an empty cage for 30 minutes prior to the test for acclimatisation. A cotton bud (Sainsbury's Safety Buds, London, UK) containing 9.5 μl urine on its lower end was then suspended from the cage lid so that the urine-soaked tip hung 65 mm below the lid. The cotton bud was removed after 120 s. Each mouse was presented with seven stimuli in this fashion, with an inter-trial interval of 60 s. The first stimulus was distilled water only (vehicle), followed by three presentations of urine from one donor strain,

then three presentations of urine from the other strain. Half the mice received the C57BL/6J urine first and half received the CD-1 urine first in a randomized manner. Time spent sniffing each stimulus was recorded.

Grip strength

This experiment was performed in collaboration with Lianne Stanford. The protocol was adapated from published experiments (Lalonde et al., 2004). The apparatus consisted of a trapeze bar made by bending a 5 cm, 21 g syringe needle, attached to a force displacement transducer (FT03C, Grass Instruments, Mass, USA) transmitting through a Mac Lab Bridge amplifier to a Powerlab/4SP and then into a computer running Powerlab ADInstruments Chart v 4.12. Each mouse was held by its tail and its front paws placed on the trapeze bar. Once the mouse had grasped the bar with both paws, it was pulled horizontally backwards in a continuous motion until the mouse lost its grip. This was performed seven times and the trials with the greatest and least peak force discarded. The score for each mouse consisted of the mean of the peak generated force in the remaining five trials.

Rotorod

This experiment was performed in collaboration with Lianne Stanford. The rotorod was a fixed-speed, Series 8, Model 755 (IITC Life Science, Harvard Apparatus UK) apparatus with a rod diameter of 32 mm. The test was performed during the light period of the light-dark cycle in a brightly-lit testing room adjacent to the vivarium. Each trial consisted of the mouse being placed on the stationery rod which then began to rotate at a constant speed. The trial continued until the mouse fell off the rod or until 60 s had elapsed. Latency to fall was recorded for each trial. Mice were given three trials at each of 4, 16 and 32 rpm on each of three days. Trials were given in order of increasing speed on each day. An additional set of three trials at 2 rpm were given prior

to the 4 rpm trial on the first day only, to habituate the animals to the apparatus. The results were analysed using a three-way (genotype x trial x day) mixed ANOVA.

Open field

This experiment was performed in collaboration with Lianne Stanford. The open field test was performed during the light period of the light-dark cycle. Each mouse was transported alone from its home cage in a small container and placed directly into a clean, opaque, plastic box of dimensions 45 cm x 28 cm x 13 cm with no lid in a brightly-lit room for 15 min. Behaviour was recorded by camcorder for later analysis. Analysis was performed using Hindsight 1.5 behavioural scoring software. The field was divided into thirds along its length and width and the resulting inner rectangle denoted the inner zone (see figure 6.5). The behaviour of the mice was scored for the following measures: Latency to enter inner zone, number of times entering inner zone, duration in inner zone, incidence and duration of immobility, line crosses, supported (against the wall) and unsupported rearing (Crusio, 2001).

Elevated plus maze

This experiment was performed in collaboration with Lianne Stanford. It was designed in accordance with published protocol (Lister, 1987). The elevated plus maze consisted of a black perspex cross with four arms and a central, square platform. The arms of the cross were 5 cm wide and 30 cm long. Two opposite arms had clear perspex walls of height 15 cm and the remaining two arms were open with only a 3 mm lip around the edge. The entire apparatus was supported 45 cm above the floor. The test was performed during the light period of the light-dark cycle in a room lit with dim red light. The behaviour of the mice was recorded using a Sony video camera fitted with an infra-red filter and the data was analysed using Ethovision 3.0 tracking software.

Each mouse was given a single trial in which it was released on the centre square facing an open arm and allowed to explore for 5 minutes. A trained observer recorded the number of times the mouse dipped its head over the edge of the open arms of the maze (head dips) and the number of times it assumed a stretch-attend posture. The maze was cleaned with alcohol-free disinfectant wipes (Trigene Antiseptic Wipes, Medichem UK) between trials.