2 Materials and Methods

2.1 Materials

2.1.1 Chemicals, reagents and consumable materials

Chemicals were used from the companies Sigma (München), Roth (Karlsruhe), Merck (Darmstadt), Fluka (Neu-Ulm), Serva (Heidelberg), Gibco BRL (Karlsruhe) and Pharmacia (Freiburg) if not noted otherwise. Restriction enzymes were from New England Biolabs (Bad Schwallbach) or Roche (Mannheim), other enzymes, DNA polymerases, kits, equipment and consumables were used as stated in the corresponding sections. Radioactive reagents were purchased from Amersham (Braunschweig).

2.1.2 Plasmid vectors

- pBKS, Bluescript KS (Stratagene, Heidelberg)
- p1029 is based on pGEM-7Zf(+) (Promega, Mannheim) and modified by Dr. M. Koenen.
- pCRII-TOPO, pCR-BluntII-TOPO (Invitrogen, Karlsruhe)
- pRK5 expression vector (Schall, 1995) contains an early cytomegalovirus (CMV) promoter/enhancer 5´ of the multiple cloning site (MCS) that drives the expression of downstream coding DNA fragments. 3´ of the MCS are SV40 termination and polyadenylation signals.
- p958 contains a 5,8 kb XhoI-XbaI promoter fragment of the ChAT gene in pBKS, provided by Dr. M. Koenen.
- p974/2 contains a 3 kb XbaI fragment comprising exon 2 of the ChAT gene in pBKS (overlaps with p958 in the promoter region), provided by Dr. M. Koenen.
- pTR-UF2 contains the humanized, red-shifted GFP sequence under the control of the CMV promoter (Zolotukhin *et al*., 1996).

- ploxneo3.repair was provided by Dr. R. Sprengel, MPI für Med. Forschung (Heidelberg), Abt. Molekulare Neurobiologie. The vector contains a CMV promoter and a loxP flanked PGK-neo sequence.
- pEGFP-C1 and pDsRed2-C1 (BD Biosciences Clontech, Heidelberg)
- pRK5-GluRA-GFP and pRK5-GluRB-GFP were also provided by Dr. R. Sprengel.
- In pEGFP-N3/synaptophysin the rat cDNA for synaptophysin is cloned in the way that GFP is fused to the N-terminus and is directed to the cytosol. The plasmid was provided by Verena Wimmer and Dr. T. Kuner.

2.1.3 Bacteria strains, cell lines and animals

- *E. coli* strain HB101 for general cloning (Promega, Mannheim)
- *E. coli* strain BS591 expressing cre recombinase (Sauer and Henderson, 1988a)
- COS-1 cells (Gluzman, 1981) were purchased from the American Type Culture Collection (Manassas, USA)
- Embryonic stem cells E14-1were from the laboratory of Prof. K. Rajewski (Institut für Genetik, Köln) and were provided by Dr. R. Kühn (Kuhn *et al*., 1991).
- To produce ChAT$^{(GFP/+)}$ animals, the targeted ES cell clone ChAT-GFP-6F11 was injected into blastocysts of C57Bl/6 mice by the Transgene Lab, ZMBH, Universität Heidelberg.
- Seven day-old neonatal Wistar rats were used for preparation of organotypic hippocampal slice cultures.

2.1.4 Primers and Oligonucleotides

All primers were purchased and synthesized by MWG-Biotech (Ebersberg).

- Primers for sequencing

JR25seq.GFP2for 5´-gat ggc gat gtg aat ggg-3´

JR25seq.GFP3for 5´-gcg ctg aag tca agt tcg-3´

JR25seq.GFP7rev 5´-ctt gta gtt ccc gtc atc-3´

JRChATseq.6for	5´-gcc agt gcc ccc act gca g-3´
JRChATseq.7for	5´-cag cta agg ttt gca gcc-3´
JRChATseq.9for	5´-ggc acc tgc ggt gtg gtg-3´
JRChATseq.10for	5´-ggc ttt acc aga ggc tgg-3´
JRChATseq.11for	5´-cct gtg caa aga gcc acc-3´

· Primers for PCR amplification

JRChATfor	5´-gct act ctg gat taa gaa tc-3´
JRChATamp9	5´-tca aga ttg ctt ggc ttg-3´
hGFPHIIIfor	5´-ctc tca agc ttc taa ggc cg-3´
hGFPHIIIrev	5´-gag aga agc ttg ctt gta c-3´
JRPCRscreen3	5´-gga aaa gat gat gtt tgg-3´
pgk 3´(F)	5´-ctg aag aac gag atc agc ag-3´
pgk-Prom (R)	5´-gag gcc act tgt gta gcg-3´
JR18.seqBsaBI	5´-gga ttc aga gag tac act g-3´
JR38BstBIfor	5´-gtc aag ttc gaa ggt gac acc ctg g-3´
JR38SalIrev	5´-cat tgg gtc gac tca aga ttg ctt ggc ttg gc-3´
neoSalIloxPfor	5´-ttt tgt cga ccc tgc agg tca att cta cc-3´
neoSalIloxPrev	5´-ttt tgt cga cgg acc taa taa ctt cgt ata g-3´

· Primers for site-directed mutagenesis

Mut-SalI.for	5´-gtt tag ccc att ggg tcg act gga acc aga gtt gg-3´
Mut-SalI.rev	5´-cca act ctg gtt cca gtc gac cca atg ggc taa ac-3´
Mut-NotI.for	5´-cat tcc aga agc tcc agc ggc cgc cat gct tca gtc cag-3´

Mut-NotI.rev	5´-ctg gac tga agc atg gcg gcc gct gga gct tct gga atg-3´
Mut-JR14.for	5´-cct gta caa gct agc aag ggc gag g-3´
Mut-JR14.rev	5´-cct cgc cct tgc tag ctt gta cag g-3´
Mut-ChATGFP.for	5´-ggc atg gac gag ctg tac aag caa gct tct agc tgt gag gag gtg ctg-3´
Mut-ChATGFP.rev	5´-cca gca cct cct cac agc tag aag ctt gct tgt aca gct cgt cca tgc-3´
Mut-JR18BglIIfor	5´-cca ggt cgg cag cca gat cta ttc tgc tac tc-3´
Mut-JR18BglIIrev	5´-gag tag cag aat aga tct ggc tgc cga cct gg-3´

· 5´-Fluoresceine-labeled oligonucleotide as probe for PCR screen of ES cell clones

JRscreenChATPCR	5´-ggt tta gga gtg ttc agt tga tct gga act aag cac tgg aga atc tgt gaa agg-3´

· loxP encoding oligonucleotides

JRloxPfor	5´-gat ctc cct taa tat aac ttc gta taa tgt atg cta tac gaa gtt att agg tcc a-3´
JRloxPrev	5´-gat ctg gac cta ata act tcg tat agc ata cat tat acg aag tta tat taa ggg a-3´

2.1.5 Buffers and solutions

TBE buffer (20x)	1.78 M Tris; 1.78 M borate; 40 mM EDTA (pH 8.0)
DNA loading buffer (10x)	12.5 g Ficoll; 2.5 ml TBE (20x); 0.25 % xylene xyanol; 0.25 % bromphenol blue made up to 50 ml using dH_2O
Ethidiumbromide stock solution	10 mg/ml ethidiumbromide in dH_2O
GITC buffer	4 M GITC; 25 mM Na-citrate (pH 7.0); 0.1 M β-mercaptoethanol

DEPC-H_2O	1 ml diethylpyrocarbonate made up to 1 l using dH_2O, incubate 12 h at RT then autoclave
SSC buffer (20x)	3 M NaCl; 0.3 M Na-citrate
ECL hybridization buffer	5x SSC; 0.1 % SDS; 10 µg/µl salmonsperm DNA; 5 % dextran sulfate; 5 % Liquidblock (Amersham, Freiburg)
Buffer A (ECL)	100 mM Tris-HCl (pH 7.5); 600 mM NaCl
Denhards solution (100x)	20 g Ficoll; 20 g PVP; 20 g BSA made up to 1 l using dH_2O
NTX hybridization buffer	50 % formamide; 5 x SSC; 100 mM phosphate buffer (pH 7); 5x Denhards solution; 0.2 % SDS; 100 µg/ml; salmonsperm DNA
Extraction buffer (COS-1 cells)	0.2 M NaCl; 0.2 % Triton X-100
ChAT reaction buffer	100 mM Na-phosphate (pH 7.5); 300 mM NaCl; 40 mM EDTA; 6 mM cholin chloride; 200 µM eserine; 35 µM ^{3}H-acetyl-CoA (SA 200 mCi/mmol)
"Kalignost" reagent	85 % toluene; 15 % acetonitrile; 0.5 % tetraphenylboron
Toluene scintillation solution	0.05 % diphenyloxazole; 0.02 % 1,4-bis(4-methyl-5-phenyloxazole-2yl)benzene in toluene
Protein sample buffer (2x)	125 mM Tris-HCl (pH 6.8); 4 % SDS; 4 % β-mercaptoethanol; 10 % glycerine; 0.02 % bromphenol blue
Stacking gel buffer	0.5 M Tris-HCl (pH 6.8); 0.4 % SDS
Separating gel buffer	1.5 M Tris-HCl (pH 8.8); 0.4 % SDS
Acrylamide solution	29.99 % acrylamide; 0.78 % bisacrylamide
APS solution	10 % in dH_2O
Electrophoresis buffer	12 g Tris; 57.6 g glycine; 40 ml 10 % SDS adjusted to pH 8.5 and made up to 5 l with dH_2O using dH_2O
Coomassie stain solution	0.05 % Coomassie Blue G250; 25 % isopropanol; 10 % acetic acid
Destain solution	7.5 % acetic acid; 2 % methanol
Western blotting buffers	Anode: 300 mM Tris-HCl (pH 10.4); 20 % methanol Kathode: 25 mM Tris-HCl (pH 9.4); 40 mM amino-n-

	caprone acid; 20 % methanol
TBS-T buffer	20 mM Tris-HCl (pH 7.5); 150 mM NaCl; 0.1 % Tween 20
Blocking buffer	1 % BSA in TBS-T buffer
PBS (10x)	43 mM $Na_2HPO_4 \cdot 7H_2O$; 14.7 mM KH_2HPO_4; 27 mM KCl; 1.36 M NaCl
GBS	560 µl 1 M $MgSO_4 \cdot 7H_2O$; 40 ml 1 M HEPES (pH 7.2); made up to 2 l using "Ringer" component1 with component2 (Biometra, Göttingen)
4 % PFA solution	4 % paraformaldehyde in 1x PBS
Mowiol anti-fade agent	2.4 g Mowiol (Calbiochem, Braunschweig) + 6 g glycerol + 6 ml dH_2O, stirring 2 h at RT; add 12 ml 0.2 M Tris (pH 8.5) and incubate 10 min at 50 °C with frequent stirring, add DABCO to 0.1 % final and stir, centrifuge to clarify, aliquot and store at –20 °C
Electroporation buffer (ES cells)	20 mM HEPES (pH 7.2); 137 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4; 6 mM glucose; 0.1 mM β-mercaptoethanol
Normal Rat Ringer (NRR)	1.35 mM NaCl; 5.4 mM KCl; 1 mM $MgCl_2$; 1.8 mM $CaCl_2$; 5 mM HEPES
Physiological salt solution	"Ringer" component1 and 2 (Biometra, Göttingen)

2.1.6 Media and solutions for bacteria, cell and tissue culture.

LB medium	20 g tryptone; 5 g yeast extrakt; 0.5 g NaCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 0.2 % Glucose made up to 1 l using dH_2O
TFB I buffer	100 mM RbCl; 50 mM $MnCl_2$; 30 mM K-acetate; 10 mM CaCl; 0.5 mM LiCl; 15 % glycerin adjusted to pH 5.8 using H-acetate
TFB II buffer	10 mM MOPS; 10 mM RbCl; 75 mM CaCl; 15 % glycerin adjusted to pH 7 using NaOH
COS-1 cell medium	DMEM; 100 U/ml penicillin; 100 µg/ml streptomycin; 2 mM glutamine; 15 % fetal calf serum

E14-1 medium	DMEM (4.5 g/l glucose); 100 U/ml penicillin; 100 µg/ml streptomycin; 2 mM L-glycine; 1 mM MEM-Na-pyruvate; 1x MEM non-essential amino acids; 0.1 µM β-mercaptoethanol; 15 % fetal calf serum; 100U/ml LIF factor
Trypsin/EDTA solution	2.5 g/l trypsin; 0.4 g/l EDTA; 1g/l glucose; 3g/l Tris made up to 1 l using PBS (1 x)
G418 (Gibco BRL, Karlsruhe) stock solution	100 mg/ml in 10 mM HEPES (pH 7.4)
Mitomycin stock solution	2 mg Mitomycin C; 200 µl DMSO made up to 4 ml using PBS (1x)
Emptage medium	200 ml MEM; 100 ml EBSS; 100 ml horse serum; 8 ml B27 supplement (Gibco BRL, Karlsruhe); 8 ml glucose (32.5 % in MEM); 100 U/ml penicillin; 100 µg/ml streptomycin

2.2 Methods

1.1.1 Molecular Biology

2.2.1.1 Preparation of plasmid DNA

The preparation of small amounts of plasmid DNA (miniprep) was carried out by using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden) according to the supplier's protocol. The preparation of plasmid DNA in the µg range (maxiprep) was carried out by using QIAfilter Maxi Cartridges and QIAtip 500 columns (QIAGEN, Hilden) according to the supplier's protocol. For centrifugation steps a Beckman J2-21M/E centrifuge with a JA-14 and a JA-13.1 rotor (Beckman, Palo Alto, USA) was used.

2.2.1.2 Determination of nucleic acid concentration

The concentration of nucleic acids was determined by measurement of the absorption at 260 nm using the Helios β spectral photometer (Unicam Instruments, Cambridge, UK). The optic density at 260 nm (OD_{260} = 1) is equivalent to 50 µg/µl of double strand DNA, 33 µg/µl of single strand DNA and 25 µg/µl of single strand RNA.

2.2.1.3 Sequence analysis

Sequence analysis was performed according to the chain termination method with ddNTPs.

Sample mix	500 ng template DNA 0.5 µl sequencing primer (10 µM) 4 µl ABI Prism BigDye Terminator Cycle Sequencing Mix (Perkin-Elmer, Weiterstadt)

made up to 10 µl using dH_2O

The following profile was run in the TGradient cycler (Biometra, Göttingen): After an initial denaturation step of 1 min at 96 °C 25 cycles followed:

1. Denaturation at 96 °C for 15 sec
2. Annealing at 55 °C for 15 sec
3. Extension at 60 °C for 4 min

10 µl H_2O, 2 µl 3 M Na-acetate and 50 µl of 87 % Ethanol was added to the sample. After incubation for 10 min on ice the sample was centrifuged for 10 min at 13000 rpm using the Biofuge 13 (Heraeus, Hanau) and washed with 200 µl of 75 % Ethanol. The air-dried pellet was diluted in 4 µl formamide buffer, denatured for 2 min at 95 °C, immediately chilled on ice and applied to the ABI Prism 377 DNA Sequencer (Perkin-Elmer, Weiterstadt).

2.2.1.4 Digestion with restriction enzymes

The sample volume and the amount of enzyme were varied according to the amount of DNA. For the analysis of minipreps approx. 500 ng plasmid DNA was digested with approx. 2.5 U restriction enzyme in a final volume of 30 µl. For cloning of DNA fragments approx. 4 µg of plasmid DNA was digested with approx. 10 U restriction enzyme in a volume of 60 µl. For genomic Southern blot hybridization 10 µg DNA was digested with approximately 20 U restriction enzyme in a volume of 100 µl. The incubation at 37 °C varied between 2-16 h.

2.2.1.5 Agarose gel electrophoresis

DNA fragments were separated electrophoretically on horizontal agarose gels (Ultrapure Agarose, Gibco BRL, Karlsruhe) for purification or restriction analysis.

The concentration of agarose gels was 1 % in 1x TBE buffer including 10 µl of a 10 mg/ml ethidiumbromide stock solution per 100 ml. The DNA samples were mixed with 10x DNA loading buffer and applied to the gel that was subsequently run at 7.5

V/cm^2 in 1x TBE buffer. The fragments were visualized and documented upon UV illumination using Epi Chemi II Darkroom (UVP Laboratory Products, Cambridge, UK). EcoRI/HindIII digested λ−DNA served as a marker.

2.2.1.6 DNA fragment isolation

The isolation of DNA fragments from agarose gels or the purification of DNA restriction samples was performed using the QIAquick Gel Extraction Kit (QIAGEN, Hilden) according the supplier's protocol.

2.2.1.7 5´-Dephosphorylation

Vector DNA was dephosphorylated to reduce re-ligation of the empty plasmid. After the digestion of the vector DNA with restriction enzymes (see 2.2.1.4) and subsequent purification (see 2.2.1.6) the linearized vector was 5´ dephosphorylated using 5 U CIAP (Roche, Mannheim), gently mixed and incubated 1h or overnight at 37 °C. The sample was purified by using QIAquick Gel Extraction Kit (QIAGEN, Hilden).

2.2.1.8 Ligation

Vector and insert DNA were set into the reaction in a relation of 1:4. The amount of vector DNA was approx. 100 ng. The ligation was performed in 20 µl with 400 U T4 DNA Ligase (New England Biolabs, Bad Schwallbach) for 1h at RT. A control ligation of the vector without the insert was done to estimate the efficiency of insert incorporation and the quality of vector DNA.

2.2.1.9 Transformation

Approximately 100 ng DNA was mixed with freshly thawed competent bacteria (HB101 strain) in an "Eppendorff" tube and incubated for 15 min on ice. Then a heat

shock of 2 min at 37 °C was applied followed by incubation on ice for 2 min. 800 µl LB medium was added to the bacteria and 30 min incubation at 37 °C followed upon shaking. 200 µl of the bacterial suspension was plated on LB agar plates containing the appropriate antibiotic. The plates were incubated overnight at 37 °C.

2.2.1.10 Preparation of competent cells

One clone of *E. coli* strain HB101 was grown in 5 ml LB medium at 37 °C overnight. 2 ml of this culture were added to 200 ml LB medium and incubated at 37 °C until OD_{600} reached 0.4-0.5. The culture was incubated 10 min on ice and centrifuged at 4000 rpm (Beckman JA-14 rotor). The pellet was suspended in 200 ml TFB I buffer and incubated on ice for 2 h. After centrifugation at 4000 rpm the pellet was suspended in 8 ml TFB II, split on ice into 100 µl aliquots and frozen in liquid N_2. The cells were stored at -80 °C.

2.2.1.11 RNA preparation from mouse (C57Bl/6) spinal cord (Chomczynski and Sacchi, 1987)

1g tissue was homogenized in 15 ml GITC buffer with a Polytron (Chinematica, Littau, CH) and mixed with 1.5 ml 2 M Na-acetate (pH 4.0). 15 ml phenol was added and mixed. Then 6 ml chloroform were added, mixed and incubated on ice for 15 min. After centrifugation at 9000 rpm in a Beckman J2-21M/E centrifuge with JA-13.1 rotor (Beckman, Palo Alto, USA) the upper phase was transferred in a "Corex" glass tube. The RNA was precipitated by the addition of an equal volume of isopropanol and incubated for 1 h at -20 °C. After the centrifugation at 9000 rpm the pellet was dissolved in 1 ml GITC and transferred to an "Eppendorff" tube. Again the RNA was precipitated by the addition of isopropanol and incubated at –20 °C overnight. After centrifugation at 13000 rpm with a Biofuge 13 table top centrifuge (Heraeus, Hanau) the pellet was air dried and dissolved in 300 µl DEPC-H_20 and the concentration was determined by measurement of the absorption at 260 nm. For storage at –20 °C 30 µl 3 M Na-acetate (pH 6.0) and 900 µl Ethanol were added.

2.2.1.12 Reverse transcription

The RNA concentration was adjusted to 1-5 µg/10 µl and incubated for 5 min at 68 °C, immediately put on ice and briefly centrifuged. Then the RT reaction sample was added to the following Master Mix:

Master Mix	4 µl 5x first strand buffer 1 µl RNA guard (Amersham, Freiburg) 1 µl dNTP mix (4 mM each) 1 µl pd(N_6)-Primer 2 µl 0.1 M DTT 1 µl M-MLV Reverse Transcriptase (Gibco BRL, Karlsruhe)

The sample was mixed and incubated for 1 h at 37 °C. The resulting cDNA was denatured for 5 min at 95 °C and immediately put on ice. 1 µl was used for the PCR reaction.

2.2.1.13 Site-directed mutagenesis

The mutagenesis was performed by using the QuickChange-Site-Directed Mutagenesis Kit (Stratagene, Heidelberg) according to the supplier's protocol.

Sample Mix	5-50 ng template DNA 5 µl 10x buffer 125 ng of any forward and reverse primer 5 µl dNTP mix (2 mM each) 2.5 U *Pfu Turbo* DNA Polymerase made up to 50 µl using dH_2O

The following profile was run in the TGradient cycler (Biometra, Göttingen):
After an initial denaturation step of 30 sec at 95 °C 12-18 cycles followed:

1. Denaturation at 95 °C for 15 sec
2. Annealing at 55 °C for 1 min

3. Extension at 68 °C for 2 min/kb (plasmid size)

10 U DpnI were directly added and incubated for 1 h at 37 °C. 1 µl was transformed (see Section 2.2.1.9).

2.2.1.14 PCR amplification of DNA fragments (Saiki *et al*., 1988)

"Hotstar" DNA Polymerase (QIAGEN, Hilden) was used for standard amplification protocols. "ProofStart" DNA Polymerase (QIAGEN, Hilden) contains 3´-5´exonuclease activity ("proofreading") which recognizes and removes incorrectly incorporated deoxynucleotide. This polymerase was used for the amplification of fragments larger than 2 kb which were generated for subcloning. "ProofSprinter" DNA Polymerase is a mixture of Pwo and Taq Polymerase with improved fidelity (Hybaid, Heidelberg) and was used for the subcloning of fragments less than 2 kb.

Standard PCR sample	ca. 100 ng Template DNA 5 µl 10 x PCR-Puffer 5 µl dNTP mix (2 mM each) 3 µl of any forward and reverse primer at 10 µM 0.5-2 U DNA Polymerase made up to 50 µl using dH_2O

The individual profile for each PCR was determined by gradient or touchdown runs in the TGradient cycler (Biometra, Göttingen).

After an initial denaturation step at 95 °C (Hotstar 15 min; Proofstar 5 min; Proofsprinter 2 min) 25-45 cycles followed:

1. Denaturation at 94 °C for 30 sec
2. Annealing at 50-65 °C for 30 sec
3. Extension at 72 °C for 1 min/kb (Hotstar) and 2 min/kb (Proofstar), respectively

At the end of the run a 10 min incubation at 72 °C followed.

The PCR sample was purified by gel electrophoresis (see Section 2.2.1.5).

2.2.1.15 PCR screen of ES cell clones in the 96 well format

25 µl of PCR Master Mix were applied to each tube of a 96 well PCR plate using a multi pipette.

Master Mix	850 µl dH_2O
	500 µl 10x PCR-Puffer
	500 µl dNTP-Mix (2 mM of any dNTP)
	300 µl of pgk 3´(F) and JRscreen3 primer at 10 µM
	50 µl "Hotstar" Taq DNA Polymerase (250 U)

Using an eight channel pipette, 25 µl of genomic DNA (see Section 2.2.1.16) were applied to the 96 well plate.

After an initial denaturation step of 15 min at 95 °C, 45 cycles followed:

1. Denaturation at 94 °C for 30 sec
2. Annealing at 60 °C for 30 sec
3. Extension at 72 °C for 3 min

At the end of the run a 10 min incubation step at 72 °C followed.

After the run 25 µl DNA loading buffer was applied to each well and 60 µl were separated by agarose gel electrophoresis for further analysis by Southern blot hybridization using the ECL system (see Section 2.2.1.18).

2.2.1.16 Preparation of genomic ES cell DNA from 96 well culture plates (Schwarz *et al.*, 1998)

The preparation was performed using the DNeasy 96 Tissue Kit (QIAGEN, Hilden).

For two 96 well blocks	=> 36 ml ATL buffer + 4 ml Proteinase K
	=> 40 ml AL buffer + 42 ml EtOH

With an eight-channel pipette using sterile filter tips 200 µl ATL-Proteinase K mixture was added to each well and incubated for 2 h at 56 °C. Then 20 µl RNase A (10mg/ml) was added and incubated for 30 min at 37 °C. The lysate was mixed with 410 µl AL-EtOH mixture and applied to the 96 well column block. The block was

centrifuged at 6000 rpm in a Sigma 6-10 centrifuge, equipped with a Sigma/QIAGEN rotor (QIAGEN, Hilden) for 5 min. Each column was first washed with 500 µl AW1 buffer and then with 500 µl AW2 buffer. After drying the block at 70 °C for 15 min 100 µl dH_2O was applied to each column, incubated for 5 min at 70 °C and the DNA was eluted by centrifugation. Then the DNA was stored at 4 °C and analyzed by PCR screen (see Section 2.2.1.14).

2.2.1.17 Southern transfer of DNA fragments onto membranes (Southern, 1975)

DNA, separated by agarose gel electrophoresis (see Section 2.2.1.5), was transferred onto Biodyne A membrane (Pall, Ann Arbour, USA) for hybridization with radiolabeled probes or Hybond-N^+ membrane (Amersham, Freiburg) for ECL according standard protocols (Maniatis, 1982) overnight using 10x SSC buffer. The membrane was washed briefly in 6x SSC and the DNA was UV cross-linked to the membrane with 2x $1.2x10^5$ µJ using the UV Stratalinker 1800 (Stratagene, Heidelberg).

2.2.1.18 Detection of PCR products of using the ECL system (Amersham, Freiburg)

The membrane was preincubated in hybridization buffer at 60 °C for 30 min. 1 µg/25 ml fluorescein-labeled oligonucleotide probe JRscreenPCR was added and further incubated overnight. The membrane was washed in 1x SSC/0.1 % SDS at 60 °C for 15 min and incubated in 25 ml buffer A containing Liquidblock (Amersham, Freiburg) in 1:20 dilution at RT for 30 min. The buffer was changed to 25 ml buffer A containing 125 mg BSA and anti-fluorescein-HRP conjugate (Amersham, Freiburg), diluted 1:1000, and the incubation was extended for a further 30 min. The membrane was washed three times in 25 ml buffer A containing 0.1 % Tween 20 for 10 min each and developed using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, USA). An Hyperfilm ECL film (Amersham, Freiburg) was exposed 1 h to the film and developed.

2.2.1.19 Detection of genomic digestion fragments with radiolabeled probes

For all Southern blot hybridizations, a NotI fragment (see Figure 3.16A) served as probe. 20 ng DNA probe was labeled with 50 µCi α−^{32}P-dCTP using the Random Primed DNA Labeling Kit (Roche, Mannheim) according to the suppliers protocol and purified using Bio-Spin 6 chromatography columns (BioRad, Minden). The yield was determined by measuring the radioactivity of 1 µl using an LS3801 counter (Beckman, Palo Alto, USA). The membrane was preincubated in 25 ml NTX hybridization buffer at 42 °C for 1 h. The buffer was exchanged with 15 ml NTX hybridization buffer containing radioactively labeled probe in an amount of at least 10^6 cpm and incubated overnight. Then the membrane was washed two times with 0.1 % SDS/1x SSC at 42 °C for 10 min and two times with 0.1 % SDS/0.5x SSC at 60 °C for 10 min. The membrane was air-dried and a Kodak BioMax MS-1 Film (Sigma, München) was exposed to the membrane at –80 °C overnight and developed.

2.2.2 Biochemistry

2.2.2.1 Choline acetyltransferase activity assay (Fonnum, 1975)

Transfected COS-1 cells of one 10 cm culture dish were lysed in 1 ml extraction buffer at 4 °C and 20 µl of a 1:4000 dilution were incubated with 20 µl of ChAT reaction buffer at 37 °C for 10 min. The reaction was stopped with 200 µl 10 mM phosphate buffer (pH 7.5) on ice and 1 ml "Kalignost" reagent was added. After intense shaking and centrifugation at 13000 rpm with the Biofuge 13 table top centrifuge (Heraeus, Hanau) for 2 min, 650 µl of the organic phase was added to 10 ml of toluene scintillation solution and radioactivity was determined using an LS3801 counter (Beckman, Palo Alto, USA).

The synthesized ACh is proportional to the measured radioactivity and thus the enzyme activity.

2.2.2.2 Determination of protein concentration (Bradford, 1976)

Protein concentration was determined using the Bradford Reagent (Sigma, München) by measuring the absorbance at 595 nm. A standard curve was prepared in a volume of 1 ml with sample buffer using BSA dilutions (2.5 µg/ml, 5 µg/ml, 10 µg/ml and 15 µg/ml BSA). 1 ml Bradford reagent was added and mixed gently. The absorption at 595 nm was determined with the Helios β spectral photometer (Unicam Instruments, Cambridge, UK) after 5 min incubation at RT. Dilutions of the unknown protein solution were equally treated. Sample protein concentrations were calculated from the standard curve.

2.2.2.3 Protein gel electrophoresis (Laemmli, 1970)

5 µl COS-1 cell extract (see Section 2.2.2.1) were mixed with 5 µl 2x protein sample buffer and incubated at 95 °C for 5 min. The samples were applied to the slots of the stacking gel (3.9 %) and separated on a 10 % separating gel at 15 mA on a denaturing SDS-polyacrylamide gel system under discontinuous conditions.

Stacking gel mixture (3.9 %)	5 ml dH_2O; 1 ml acrylamide/bis-acrylamide solution; 2 ml stacking gel buffer; 40 µl APS solution; 8 µl TEMED
Separation gel mixture (10 %)	7 ml dH_2O; 5.6 ml acrylamide/bis-acrylamide solution; 4.2 ml separation gel buffer; 66 µl APS solution; 10 µl TEMED

Prestained broad range protein marker (New England Biolabs, Bad Schwallbach) ranging from 16.5 kDa to 175 kDa was added to the gel run.

2.2.2.4 Staining of proteins in polyacrylamide gels

The gel was incubated in Coomassie stain solution at RT for 1 h and then incubated in destain solution until the bands of the proteins were clearly visible. Then the gel was dried between cellophane sheets for storage.

2.2.2.5 Western blotting and immunodetection

Proteins, separated by SDS-PAGE (see Section 2.2.2.3), were transferred onto PolyScreen PVDF membrane (NEN Life Science Products, Boston, USA) for antibody staining in a semi-dry transfer chamber using blotting buffers (see Section 2.1.5) at 2.5 mA/cm^2 membrane for 25 min.

Following blotting the membrane was incubated with blocking buffer at RT for 1 h and immunostained at 4 °C overnight with polyclonal affinity purified goat anti-mouse ChAT antibody (AB144P, Chemicon, Temecula, USA), diluted 1:5000 in TBS-T buffer, or monoclonal mouse anti-GFP antibody (IL-8, BD Bioscience Clontech, Heidelberg), diluted 1:10000. After washing 3x 5 min in TBS-T the membrane was incubated with horseradish-peroxidase-conjugated secondary mouse anti-goat or anti-mouse IgG (Sigma, München) respectively, diluted 1:250000 in TBS-T for 1 h at RT. After intensive washes in TBS-T the bound antibodies were detected with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, USA) and exposed to Hyperfilm ECL (Amersham, Freiburg).

2.2.2.6 Fixation of cells and tissues

COS-1 cells were rinsed in PBS, plunged into methanol chilled to -80 °C and slowly heated to -30 °C in a self assembled apparatus (Neuhaus *et al*., 1998). After plunging in PBS, the fixed cells or tissue were embedded into Mowiol or immunostained (see Section 2.2.2.7).

Slice cultures were fixed by incubation of the whole Millicell insert (see Section 2.2.3.3) in 4 % paraformaldehyde in 1x PBS for 15 min at RT. Then the cultures were washed three times with PBS and mounted with Vectashield (Vector Laboratories, Burlingame, USA)

2.2.2.7 ChAT immuncytochemistry of transfected COS-1 cells

Cells grown on coverslips were incubated in 1 mg/ml BSA in PBS at RT for 30 min and then at 4 °C overnight with polyclonal affinity purified goat anti-mouse ChAT antibody (AB144P, Chemicon), diluted 1:150 in PBS. After three washes in PBS (10 min each) cells were incubated with ZyMax FITC-conjugated rabbit anti-goat IgG (Zymed, Berlin), diluted 1:150 in PBS, for 1 h at RT, extensively washed and mounted. Fluorescence of GFP and antibody-stained cells were visualized by confocal microscopy on a Leica TCS NT confocal laser scanning unit (Leica Microsystems, Heidelberg) equipped with an argon/krypton ion laser and by using dedicated CLS software (Leica Microsystems, Heidelberg).

2.2.3 Cell culture

2.2.3.1 Transfection of COS-1 cells

Cells were plated one day prior transfection. Change of medium was performed 2-3 h prior transfection. For transfection of 10 cm dishes $6x10^5$ cells were plated and the following solution was mixed with 9.5 ml medium:

(10 µg DNA + 62.5 µl 2 M $CaCl_2$ ad 500 µl) + 500 µl 2 x BBS buffer; 10 min at RT

Cells were incubated for 24 h at 3 % CO_2 and 37 °C then the medium was changed and incubated for further 24 h at 10 % CO_2 in the incubator.

The same procedure was performed for the transfection of cover slips. $4x10^4$ cells were plated and the following solution was mixed with 1.9 ml medium:

(2.4 µg DNA + 12.5 µl 2 M $CaCl_2$ ad 100 µl) + 100 µl 2 x BBS buffer; 10 min at RT

2.2.3.2 ES cell culture

Cells were maintained at 10 % CO_2 and 37 °C.

2.2.3.2.1 Preparation of feeder cells

10^7 primary fibroblasts were thawed and plated on a 15 cm dish pretreated with 0.1 % gelatin solution. After 5-7 days, depending on confluence of the cells, cells were split and distributed on four new dishes. After 5-7 days the cells were split again. Of each subculture 1-2 dishes were not split but treated with mytomycine C (prevents cells from DNA synthesis and arrests cells in S-phase of the cell cycle). Starting from the 6^{th} subculture, all dishes were treated with mytomycin C. Mytomycin C-treated fibroblasts are not able to proliferate any more but their metabolism is not affected. Mytomycin C treatment lasts 8-10 days after the last split. The medium was exchanged to 20 mg/ml mytomycin C containing fresh medium and incubated 3 h. The cells were washed three times with PBS, trypsinized and the number of cells was determined. The cells were either frozen or plated. One day after the plating of 0.7-1x10^5 feeder cells/cm^2 gelatin-treated petri dish, ES cells could be plated.

2.2.3.2.2 Electroporation of ES cells

3-5x10^6 ES cells (E14-1) were thawed and plated on 10 cm dishes with a monolayer of feeder cells. The medium was changed each day. After 5 days the cells were trypsinized and washed three times in PBS. Cells were counted and 1x10^7 cells were diluted in 100 µl electroporation buffer containing 25 µg targeting vector DNA. Similarly 1x10^7 cells were diluted in electroporation buffer without DNA as control. Both samples were electroporated using the pulse generator EPI 2500 (Intergen, Heidelberg) with 300 V/2.5 msec and incubated for 10 min at RT. The short-duration electric field pulse leads to the formation of mini pores through which DNA can be taken into the cell and integrated into the genome. The DNA containing sample was plated on three 10 cm dishes, the sample without DNA on one 10 cm dish with feeder

cells. The next day the medium was changed and on the second day the selection with G418 was started. The medium was changed every day to remove dead cells.

2.2.3.2.3 Picking of G418 resistant clones

Only cells expressing the neomycin marker gene can survive the selection with G418. Resistant clones were picked 10 days after electroporation. Therefore clones were overlaid with PBS and single clones were aspirated and separated with an Eppendorff pipette with crystal tip and transferred into 25 µl trypsin/EDTA. The trypsinization was stopped after 5-10 min with ES cell medium. The cells of one clone were separated by pipetting up and down and the cell suspension was divided and plated on three 96-well culture plates with feeders. Grown to confluency two of the plates were frozen and stored at –80 °C while the third plate was used for DNA preparation, followed by PCR analysis. Clones identified by PCR were further analyzed by genomic Southern blot hybridization (see Section 2.2.1.19).

2.2.3.3 Organotypic tissue culture (Stoppini *et al*., 1991)

Seven day old neonatal Wistar rats were decapitated and the heads were sprayed with 70 % ethanol. The heads were chilled in ice cold GBS solution. The skin was removed in order to expose the skull. With fine scissors the skull was cut rostrally from the foramen magnum along the midline and two small cuts at the level of the frontal suture. The skull was then removed and the brain was placed into a petri dish with GBS solution. Under the stereomicroscope (WILD, Heerbrugg, CH) the corpus callosum was cut and both hemispheres were separated. Then the hippocampus was separated from the fimbria and the temporal cortex. With a longitudinal cut along the hippocampal fissure the hippocampus was isolated from the entorhinal cortex. In the next step the hippocampus was placed on the teflon stage of a McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co. Ltd, Guildford, UK) and cut into slices of 350 µm thickness. The slices were separated with two spatulas. The best slices were selected and three (one for SCE experiments) of them plated on a Millicell CM insert (Millipore, Molsheim) which was preincubated with 1 ml Emptage medium in the incubator at 5 %

CO_2 and 36 °C. The medium was changed 24 h after plating and then every 48 h. The slices were maintained on the membrane at the interface between air and medium.

2.2.4 Electroporation of single neurons in brain slice cultures (SCE) (Haas *et al.*, 2001)

The transfection of single neurons was performed using DNA- filled micropipettes. Micropipettes with a tip diameter of about 1-2 µm that were pulled from GB150F-8P capillary glass with filament (Science Products, Hofheim) on a P-97 Micropipette Puller (Sutter Instrument Company, Novato, USA). Plasmids were used at an initial concentration of 1 µg/µl in dH_2O. The concentration of tetramethylrhodamine dextran, MW 3000, (Molecular Probes, Eugene, USA) was 2 mM in dH_2O. The micropipettes were mounted on a three-axis micromanipulator (Luigs & Neumann, Ratingen) and connected to a SD9 stimulator (Grass-Telefactor, West Warwick, USA). The Millicell culture well was placed in a 35 mm petri dish containing culture medium on the stage of an Axioplan microscope (Zeiss, Jena) with a 20x lens and infrared differential interference (IR-DIC) optics. The micropipette was inserted into the stratum pyramidale of the hippocampus area CA3. Voltage pulses were delivered between an electrode placed inside the micropipette in contact with the solution, and a ground electrode placed into the medium. For negatively charged DNA the micropipette was connected to the cathode and the ground electrode to the anode. For positively charged dextran the polarization was reversed. The dextran was electroporated with one to two 40 msec long pulses with an amplitude of 15 V for each neuron. The current passing through the micropipette was determined by measuring the voltage drop across a 100 kΩ resistor with an oscilloscope applying Ohm's law. This also provided a control to determine whether the pipette was clogged during electroporation.

To improve the efficiency of SCE the following modifications were performed. The culture well insert containing only one slice was placed in a customized perfusion chamber and was perfused with oxygenated physiological salt solution during SCE. The neurons were visualized by a 40x water immersion objective and further 2x magnification using IR video microscopy with "Dodt" gradient contrast illumination. Individual cell somata and apical dendrites could be identified. Under optical control it

was possible to precisely place and push the pipette close to the soma, so that the tip touched the cell membrane. The SD9 stimulator was exchanged for an isolated voltage stimulator (WPI, Berlin) that was controlled by a tetanizer (Sigmann Elektronik GmbH, Hüffenbart). The DNA was diluted to 33 ng/µl in NRR salt solution. To prevent the tip from clogging a back-pressure of 3-4 mbar was applied to the pipette. Finally the following electroporation parameters were used: one 1 sec long train of 1 msec square pulses with an amplitude of 10 V delivered at 200 Hz. Electroporated cells were either imaged with a Zeiss AxioplanII fluorescence microscope equipped with the Seescan 3CCD video camera system LC100C (Intas, Göttingen) or by confocal microscopy on an inversed Leica TCS NT confocal laser scanning unit equipped with an argon/krypton ion laser and by using dedicated CLS software (Leica Microsystems, Mannheim).