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Tetracycline-dependent gene expression in cholinergic neurons of transgenic mice— a tool to study Neuregulin-1 function *in vivo*

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Zusammenfassung

Das Ziel dieser Arbeit bestand in der Herstellung eines Mausmodels, mit dessen Hilfe die Funktion von Neuregulin-1 (NRG1) im adulten Nervensystem untersucht werden kann. NRG1 ist ein Wachstumsfaktor mit einer 'epidermal growth factor like' Domäne, der als Ligand für Tyrosinkinase-Rezeptoren der ErbB-Familie wirkt. Durch alternative RNA-Prozessierung enstehen mindestens 15 NRG1-Isoformen, welche in die Typen I, II und III unterteilt werden. Bisher wurde NRG1 primär als Wachstumsfaktor betrachtet, der eine wichtige Rolle bei der Entwicklung des Nervensystems spielt. Neuere Beobachtungen weisen jedoch darauf hin, dass auch im erwachsenen Tier ein kontinuierlicher Bedarf an NRG1 besteht. Der frühe Tod der NRG1-Mausmutanten hat allerdings Untersuchungen zur Funktion von NRG1 im postnatalen und adulten Nervensystem erschwert.

Im Rahmen dieser Arbeit wurde das System zur tetrazyklinabhängigen Genregulation (Tet-System) eingesetzt, um eine regulierbare adulte Überexpression von NRG1-Isoformen zu erreichen. NRG1 wird von nahezu allen cholinergen Neuronen exprimiert. Um die Expression des tetrazyklinabhängigen Transaktivators $tTA2^S$ in allen cholinergen Zellen zu gewährleisten, wurden daher regulatorische Elemente des Cholin-Azetyltransferase Gens gewählt. Für eine möglichst präzise Expressionskontrolle wurde ein etwa 200 kb großes ‚bacterial artificial chromosome' (BAC) verwendet, welches das gesamte ChAT-Gen und flankierende Bereiche umfasst. Durch homologe Rekombination in Bakterien wurde die $tTA2^S$-cDNA in das ChAT-Gen eingebracht und das resultierende ChAT-$tTA2^S$ BAC-Konstrukt zur Herstellung transgener Mäuse verwendet. Die Charakterisierung ChAT-$tTA2^S$ transgener Mäuse erfolgte durch *in situ*-Hybridisierung und Verpaarung mit Reporterlinien, die ein tTA-induzierbares β-Galaktosidase-Gen tragen. Es wurden zwei transgene ChAT-$tTA2^S$ Linien identifiziert, die eine cholinerg-spezifische tTA-Aktivität aufweisen. Weiterhin wurden Mauslinien hergestellt, die eine tTA-abhängige Expression von NRG1-Isoformen ermöglichen sollten. Diese Mauslinien wurden durch Verpaarung mit der α-CamMKII-tTA Mauslinie charakterisiert, die tTA in Prinzipalneuronen des Vorderhirns exprimiert. Es konnte eine Mauslinie identifiziert werden, die sich durch eine stringente, doxyzyklinabhängige Expression von NRG1 Typ I-mRNA auszeichnet.

Mit Hilfe ChAT-tTA:NRG1 Typ I doppelt-transgener Mäusen wird es erstmals möglich sein, die Auswirkungen einer erhöhten NRG1 Typ I-Expression auf die Funktion von Synapsen und die Myelinisierung im adulten Nervensystem zu untersuchen. Darüber hinaus stellen ChAT-tTA transgene Mäuse ein wertvolles Werkzeug dar, mit dessen Hilfe die Funktion von Genen in cholinergen Neuronen untersucht werden kann.

I. Summary

The goal of this thesis was to create a mouse model permitting the analysis of neuregulin-1 (NRG1) function in the adult nervous system. NRG1 is a polypeptide growth factor, which contains an 'epidermal growth factor like' (EGFL) domain and serves as a ligand for the erbB receptor tyrosine kinase family. Alternative splicing of the NRG1 gene gives rise to at least 15 NRG1 isoforms, which can be grouped into type I, II and III variants. NRG1 has been traditionally viewed as a growth factor important for development. However, recent studies have suggested a continuous requirement for NRG1 signaling in the mature animal. Thus far, the study of NRG1 function *in vivo* has been hampered by the early embryonic or perinatal death of complete and isoform-specific null mutants.

To overcome the limitations of the available mouse mutants, the tetracycline regulated gene expression system (Tet-System) was utilized in this thesis. NRG1 is expressed by virtually all cholinergic neurons, therefore the choline acetyltransferase (ChAT) gene was selected to direct the expression of the tetracycline-dependent transactivator ($tTA2^S$) to cholinergic neurons. In order to increase the likelihood that all required regulatory elements were present, a bacterial artificial chromosome (BAC), roughly 200kb in size, containing the ChAT gene and flanking sequences was used. Homologous recombination in bacteria was used to insert the $tTA2^S$ cDNA into the ChAT gene. The resulting ChAT-$tTA2^S$ BAC construct was used for the generation of transgenic animals by oocyte injection. The ChAT-$tTA2^S$ transgenic mice were subsequently characterized by *in situ* hybridization and by mating them to reporter mice carrying a tTA-inducible β-galactosidase gene. In two independent transgenic founder lines, a faithful cholinergic expression was observed. In addition, mouse lines for the tTA-inducible expression of NRG1 isoforms were generated. Their functionality was assessed by mating them to the α-CaMKII-tTA mice, in which tTA expression is directed to principal neurons of the forebrain. One mouse line was identified that showed a tight doxycyline-dependent regulation of transgene-derived NRG1 type I mRNA expression.

Through the use of ChATtTA:NRG1 type I doubly transgenic animals, it is now possible to examine the effects of increased NRG1 type I expression on synaptic function and myelination in the mature nervous system. Moreover, the ChATtTA transgenic mouse lines that have been generated are universal tools to regulate the expression of any transgene of interest in cholinergic cells.

II. Introduction

Cell-cell interactions regulate fundamental processes during embryogenesis, organ morphogenesis and nervous system formation. In the developing nervous system, neuron-glia interactions regulate many processes that are required for proper nervous system function. Glial cells provide attractive or repulsive guidance cues for axons en route to their target area, which are needed for the formation of a proper neuronal circuitry in the central nervous system. In addition, both, neurons and glial cells, are dependent on reciprocal trophic support from each other: While, for example, electrical activity in neurons evokes the activity-dependent release of adenosine, which is a potent neuron-glia transmitter, glial cells provide trophic support to neurons through the production of factors that induce sodium channel clustering, through myelination and through the sectretion of neurotrophins (Wilkins et al., 2001; Dai et al., 2003). Synaptogenesis in the central nervous system is another example that is, at least in part, dependent on neuron-glia interaction. Here, glial cells serve two functions, on the one hand they are required for homeostasis and on the other hand they have a profound influence on activity-dependent remodeling processes of the synapses and synapse number (Ullian et al., 2004).

Neuregulin-1 (NRG1), a polypeptide growth factor expressed by neurons, is one example of a signaling molecule that provides trophic support to glial cells. NRG1 signaling through erbB receptor tyrosine kinases has been shown to be important in regulating several aspects of glial cell biology. Schwann cells and oligodendrocytes, the myelinating glia of the peripheral and central nervous system, are depending on NRG1 signaling for proliferation, survival and myelination (Falls, 2003; Michailov et al., 2004).

1. The Neuregulin-1-erbB signaling network

NRG1 is a polypeptide growth factor of the epidermal growth factor family and belongs to a family of 4 members of which it is the best characterized (Busfield et al., 1997; Carraway et al., 1997; Higashiyama et al., 1997; Zhang et al., 1997; Harari et al., 1999). NRG1 is known to play critical roles in the developing heart, mammary gland and nervous system. NRG1 was identified as the biological activities known as heregulin/neu differentiation factor (NDF) for its apparent ability to bind to and activate the HER2/neu oncogene and as glial growth factor (GGF) for its ability to promote Schwann cell proliferation (Peles et al., 1992) (Raff et al.,

1978; Brockes et al., 1980). It was also identified as the acetylcholine receptor-inducing activity (ARIA), as it increased the expression of acetylcholine receptors when added to muscle cells grown *in vitro* (Jessell et al., 1979; Corfas et al., 1993; Falls et al., 1993). It was later discovered that heregulin/NDF, GGF and ARIA are all splice variants of the same gene, which was subsequently called "neuregulin" combining the "neu" and "heregulin" nomenclature (Marchionni et al., 1993). Although the NRG1 isoforms have been traditionally viewed as molecules important for neural development, recent studies suggested that there is a continuing requirement for NRG1 signaling in mature animals (Sandrock et al., 1997).

1.1. Structure of Neuregulin-1

Common to all NRGs is an epidermal growth factor-like (EGF-like) domain of 50-60 amino acids in length, which is characterized by two sets of anti-parallel β-pleated sheets held together by 3 pairs of disulfide-bonded cysteine residues (see Fig.1).

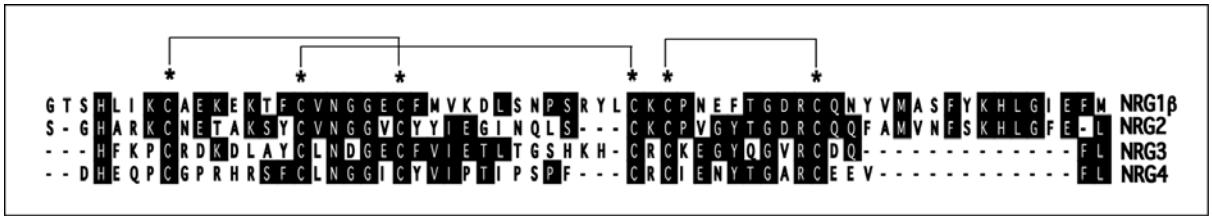
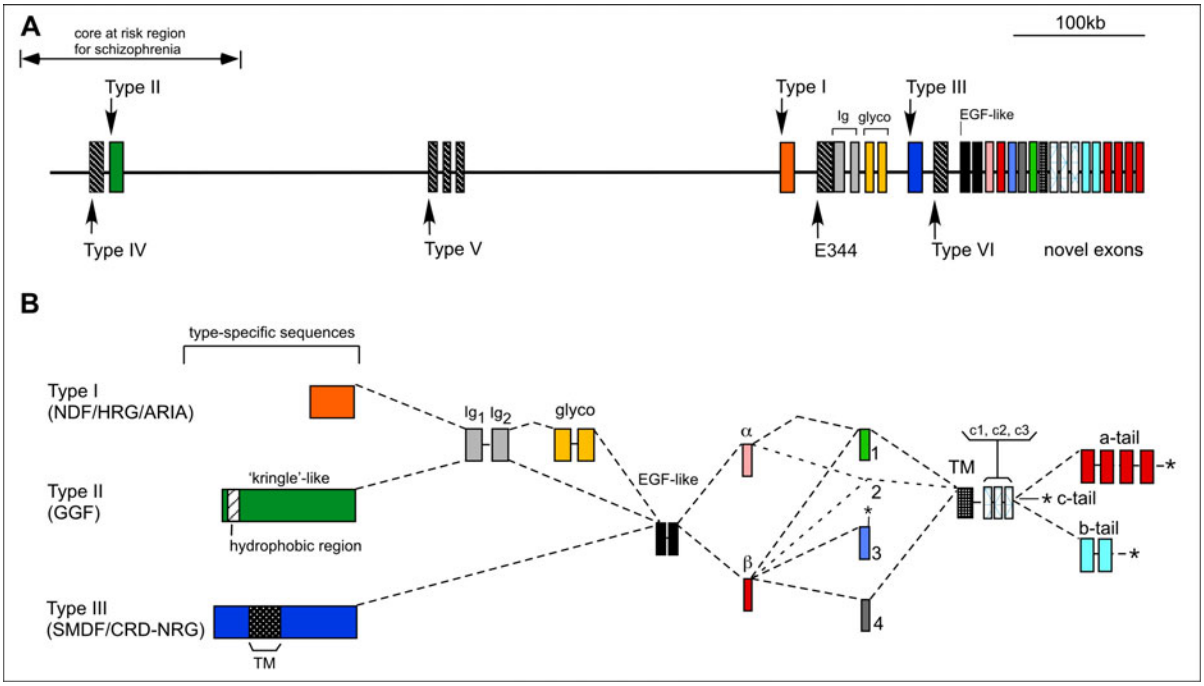


Figure 1: Sequence alignment of the EGF-like domains of NRG1–4.
Shaded in black are conserved amino acid residues in the EGF-like domains of NRG1 – 4. Asterisks denote conserved cysteine residues that form disulfide bonds within the EGF-like domain as indicated by brackets. Pairwise sequence alignment of the NRG1 EGF-like domain with the EGF-like domains of NRG2, NRG3 and NRG4 reveals amino acid homologies of 47%, 31% and 33% respectively.

The study of NRG1 has been complicated by the identification of more than 15 cDNA variants that arise by alternative splicing and the use of 3 alternative promoters (reviewed in (Lemke, 1996; Rosen et al., 1996; Falls, 2003). On the genomic level this complex splicing pattern is reflected by the number of exons in the NRG1 gene. NRG1 spans ~800kb on chromosome 8qA3 in the mouse genome and has 23 identified exons (see Fig.2) (Stefansson et al., 2002). The recent identification of 6 novel exons within the human NRG1 gene, for which there are syntenic regions in the murine NRG1 gene, suggests that the regulation of NRG1 might be even more complex (Steinthorsdottir et al., 2004). However, it is unclear whether the novel exons are expressed in the mouse, or whether their expression is restricted to humans.



(adapted from Falls, 2003 and Steinthorsdottir et al., 2004)

Figure 2: Genomic organization and splicing of the murine NRG1 gene.
(A) Genomic organization of the murine NRG1 gene. Exons are represented by rectangles and are not drawn to scale. The colors used in (A) match the exons in the splicing scheme in (B). The 6 recently identified novel exons are indicated below the cartoon. The core at risk region for schizophrenia, which was identified in a genetic linkage study in an Icelandic population surrounds the genomic region of exons encoding types II and IV (Stefansson et al., 2002).
(B) Dashed lines indicate possible splicing events. Type-specific exons are shown on the left with type I referring to splice variants NDF, HRG and ARIA, type II to glial growth factor (GGF) and type III to SMDF/CRD-NRG1. The type III exon has a second transmembrane (TM) domain, which is indicated by the box within the type III-specific exon. The hydrophobic region in the type II-specific exon does not function as a TM domain, but is thought to be an uncleaved signal sequence. Type II and type III isoforms lack the 'glyco' exons; in NRG1 α2 and β2 isoforms the EGFα and EGFβ domain is directly spliced into the TM-encoding exon following the α or β exon. NRG1β3 splice variants contain an in-frame stop codon (indicated by '*') 5' to the TM domain so that these isoforms are secreted. All TM-domain containing NRG1 isoforms contain the exons c1, c2 and c3; TM domain-containing NRG1β isoforms are always spliced to the a-tail, whereas NRG1α isoforms are not confined to the a-tail (Ben-Baruch and Yarden, 1994). The novel NRG1 isoforms, as described by Steinthorsdottir (Steinthorsdottir et al., 2004), were omitted from the scheme, since little is known about their expression and underlying splicing in mouse. It should be noted that not all splice variants depicted in this scheme have been observed *in vivo*.

In mice, alternative splicing gives rise to three major isoforms, types I, II and III that differ in their N-termini and contain either an immunglobulin-like (Ig-like) domain (types I, II) or a cysteine-rich domain ('CRD', type III), which is also referred to as "sensory and motor neuron-derived factor" (SMDF) (see Fig.2) (Ho et al., 1995).

The Ig-containing isoforms can be further subdivided into type I and type II, which differ not only by the type II-specific exon, but also by the presence of a glycosylation-rich segment (type I, “glyco”, see Fig. 2). The type III splice variants are the predominantly expressed isoforms in the nervous system and they are characterized by an apolar region within the CRD domain, which serves as an uncleaved, internal membrane insertion signal (Ho et al., 1995; Meyer et al., 1997). Splicing in the C-terminal part of the EGF-like domain yields "α" and "β" isoforms, which display different affinities for the ErbB receptors (Pinkas-Kramarski et al., 1998; Jones et al., 1999). The "α" splice variants exert their biological function outside the nervous system where they are required for lobuloalveolar development of the mouse mammary gland (Li et al., 2002), whereas the "β" isoforms are predominantly expressed in the nervous system.

The NRG1 gene encodes isoforms which are secreted (β3 isoforms) or that contain a transmembrane domain (α2, β 1, β2 and β4 isoforms). Isoforms with a transmembrane domain can be proteolytically processed to yield soluble forms (Burgess et al., 1995; Loeb et al., 1998; Shirakabe et al., 2001). The proteolysis appears to be mediated by different proteases and involves distinct regulatory mechanisms. Proteolytic processing of NRG1 can be induced by neuronal electric activity and interaction of NRG1 with its receptors. A PKC-controlled mechanism seems to be underlying the proteolytic cleavage upon electrical stimulation of NRG1 expressing neurons, whereas NRG1 receptor interaction induces γ-secretase-mediated intramembrane cleavage (Bao et al., 2003; Ozaki et al., 2004). Extracellular proteolytic processing of β-isoforms is mediated by β-meltrin and does not appear to be regulated by electric activity or NRG1-receptor interaction (Shirakabe et al., 2001).

Upon proteolytic processing of type III-β1 isoforms at their stalk region the new C-terminus becomes membrane associated through a poorly understood mechanism (Wang et al., 2000; Cabedo et al., 2002). Type III isoforms were shown to dimerize in the plasma membrane following proteolytic processing accompanied by a redistribution into lipid rafts. The redistribution of type III fragments into lipid rafts is inhibited by O-glycosylation of the protein, which also interferes with the dimerization process (Cabedo et al., 2004). In addition, type I and type II isoforms can remain associated with the cell surface or the extracellular matrix (ECM) as the Ig-like domains bind heparin through interactions with N-sulfates, 2-O-sulfates and 6-O-sulfates of the heparan-sulfate proteoglycans of the ECM (Loeb et al., 1999; Pankonin et al., 2005).

The cytoplasmic tail of NRG1-β1a ('a-tail' or intracellular domain, 'ICD') that is released by γ-secretase, was shown to translocate into the nucleus of spiral ganglion neurons *in vitro*, where it serves as a transcription factor increasing the expression of anti-apoptotic genes (Bao et al., 2003). Recent studies found that increased synaptic activity in the mouse cochlea leads to increased nuclear localization of the NRG1 ICD (Bao et al., 2004). In the nucleus the NRG1 ICD interacts with the zinc-finger transcription factor Eos. This NRG1-ICD-Eos complex binds to the promoter of PSD-95 and stimulates its transcription (Bao et al., 2004). These findings provide evidence for an activity-dependent modulation of synaptic plasticity by NRG1. The C-terminus of the NRG1 cyoplasmic a-tail is furthermore characterized by a class II PDZ consensus binding motif, but thus far no PDZ domain-containing protein has been reported to interact with the a-tail.

1.2. Neuregulin-1 expression in the nervous system

NRG1 expression has been studied by *in situ* hybridization as well as by knock-in approaches of β-galactosidase into the NRG1 gene in mice (Chen et al., 1994; Corfas et al., 1995; Meyer et al., 1997). NRG1 expression is first detected in the mesoderm of mouse embryos at E7.5/E8 in the ectoplacental cone and rhombomeres 2, 4 as based on lacZ expression in *nrg1*$^{\Delta EGF\text{-}lacZ}$ mutant embryos, in which the EGF-like domain was replaced by the lacZ gene (Meyer et al., 1997). By E8.5 NRG1 expression can be detected in the brachial arch cartilage, the developing heart, the dorsal neural tube and rhombomere 6 next to rhombomeres 2 and 4. Isoform-specific expression analysis by ISH has revealed that NRG1 type I and type III are both expressed during the formation of cranial ganglia. NRG1 type I can be detected around E10 in trigeminal, glossopharyngeal and vagal ganglia; at E18 some DRG neurons are found to express NRG1 type I (Meyer et al., 1997).

NRG1 type III is the predominantly expressed isoform in the CNS and type III transcripts can be detected in cranial ganglia, DRGs and developing spinal cord between E10-E18. NRG1 type III expression persists in the adult animal, where it is expressed by motor neurons, in the cortex, the medial septal area and at high level in the facial nuclei and the habenulae (Chen et al., 1994; Meyer et al., 1997). NRG1 type II transcripts are found in the notochord at E10 as well as in spinal cord and DRGs by E12 – E18. NRG1 type II also appears to be expressed at lower levels in muscle between E12 – E18 (Meyer et al., 1997). Taken together, the

experimental data suggest that NRG1 is expressed by virtually all cholinergic neurons in the CNS including the spinal cord.

1.3. Cholinergic neurons in the CNS and structural organization of the ChAT gene

Immunohistochemical and ISH studies on rodent brains have revealed that cholinergic neurons in the CNS are aggregated in 8 major groups: the medial septal nucleus, vertical nucleus of the diagonal band of Broca, the horizontal limb of the diagonal band of Broca, the nucleus basalis of the substantia innominata (Meynert), the pedunculopontine nucleus, the laterodorsal tegmental nucleus, the medial habenulae and the parabigeminal nucleus. In addition to the 8 major groups, cholinergic neurons are also found in the striatum and as motor neurons in the anterior horn of the spinal cord (Oda, 1999). The earliest time point at which cholinergic neurons have been described in the mouse forebrain is E13.5. At this time mitotic ChAT positive cells can be found near the ventricular lumen of the germinal zone of the olfactory, lateral and third ventricles (Schambra et al., 1989). The generation of cholinergic neurons peaks between E14 and E16 and ChAT positive neurons can be found in various areas of the developing brain, from which they migrate to their target areas.

ChAT is the key enzyme for the synthesis of acetylcholine from choline and acetyl-CoA and the most specific marker for cholinergic neurons in both CNS and PNS. The murine ChAT gene is located on the long arm of chromosome 14 and possesses 15 exons (Ishii et al., 1990). Analysis of the resulting mRNAs revealed that exons R, N and M, which are located on the 5' end of the gene, are non-coding, alternatively spliced exons (see: results, Fig. 1). Alternative splicing results in the generation of 7 different mRNAs in the mouse.

An important and unusual structural feature of the ChAT gene is the presence of the intron-less vesicular acetylcholine transporter (VAChT) gene within the first intron of the ChAT gene, located between exons R and N. Both genes appear to be co-regulated, which would provide a reason for this unusual arrangement (Berrard et al., 1995; Berse and Blusztajn, 1995; Misawa et al., 1995).

1.4. Neuregulin receptors

The neuregulins bind to and activate members of the ErbB receptor-tyrosine kinase family. Four ErbB receptors have been identified: EGFR (ErbB1/HER1), ErbB2 (neu/HER2), ErbB3 (HER3) and ErbB4 (HER4), with the "HER" nomenclature referring to "human epidermal growth factor receptor". The ErbBs are large integral membrane glycoproteins with a molecular weight ranging from 170-185kDa. They consist of four extracellular domains (I-IV), a single transmembrane domain and an intracellular tyrosine kinase domain. The extracellular region of erbBs is heavily glycosylated, which can contribute to up to 20% of the molecular weight (Lax et al., 1990). Domains I (L1) and III (L2) mediate ligand binding whereas the cysteine-rich domains II (S1) and IV (S2) are involved in receptor dimerization (Burgess et al., 2003). The cytoplasmic tail of erbBs is also characterized by the presence of 8-18 tyrosine residues, which are sites of autophosphorylation (Carraway and Cantley, 1994).
Most of the structural analyses for ligand-receptor interaction studies for the erbB RTK family were performed on EGFR – EGF or TGF-α complexes. It was shown that the EGFR undergoes a conformational change upon ligand binding by the L1 and L2 domains. The conformational change leads to the binding of a second ligand to the EGFR dimer. The primary dimerization mediator between the two EGFR molecules is a β-hairpin loop (dimerization loop), which protrudes from the S1 domain (Burgess et al., 2003).
ErbB2 is an unusual receptor in that it has no known ligand, but it increases ligand-binding affinity when recruited into a receptor heterodimer. Its crystal structure revealed that domains L1 and L2 show a strong interaction, mimicking a ligand-bound state. The consequence of this interaction is a constitutively extended conformation of the dimerization loop, making erbB2 a promiscuous dimerization partner. It also explains the inherent inability of erbB2 to bind EGF-like ligands (Citri et al., 2003). ErbB3 is also an atypical receptor in that it possesses little or no tyrosine kinase catalytic activity (Guy et al., 1994). This deficit appears to be the result of the lack of conservation of critical amino acids residues, which are believed to be essential in the kinase active site. Despite this lack of catalytic activity, ErbB3 can be phosphorylated *in trans* by a catalytically active ErbB dimerization partner. ErbB4 is similar in its overall structure to EGFR and is capable of forming functional homodimers as well as heterodimers with ErbB2 and ErbB3. There are 2 regions within ErbB4 at which alternative splicing occurs, one located in the stalk region, the other one in the C-terminal domain, which potentially can result in 4 alternative splice variants. The C-terminal splice variants are

characterized by the presence (CYT-1) or absence (CYT-2) of a PI3K binding site (Carpenter, 2003). The C-terminus ending in –TVV further harbors a PDZ binding motif mediating the interaction of ErbB4 with PSD-95 at neuronal synapses (Garcia et al., 2000; Huang et al., 2000).

ErbB4 is also proteolytically processed by TNFα-converting enzyme (TACE), which sheds its ectodomain and subsequently by γ-secretase, which releases the cytoplasmic tail from the membrane. The cytoplasmic tail was shown to translocate into the nucleus of the erbB4 expressing cell. Although the cytoplasmic tail was shown to act as a transcriptional activator in cultured oligodendrocytes increasing the expression of myelin basic protein (MBP), its biological function is poorly understood (Lai and Feng, 2004).

Upon ligand binding the tyrosine residues on the cytoplasmic tails of the ErbB receptor dimers are phosphorylated through phosphorylation in *trans*. Different EGF-like domain-containing ligands have been shown to exhibited distinct patterns of receptor autophosphorylation. The specific subset of tyrosines that are phosphorylated is thought to determine the signaling pathways that are induced, as the 5-8 amino acids surrounding the phosphotyrosine residue confer the specificity of SH2 and phosphotyrosine binding (PTB) domain binding (Marmor et al., 2004). The 3 best characterized signaling pathways induced by ErbB signaling are the Ras-MAPK, PI3K-Akt and the phospholipase-Cγ (PLCγ)-PKC pathways (reviewed in (Yarden and Sliwkowski, 2001; Marmor et al., 2004). All activated ErbBs feed into the Ras-MAPK pathway, either directly through SH-2 domain-mediated recruitment of Grb-2, or indirectly through PTB domain-mediated binding of Shc (Marmor et al., 2004).

1.5. Neuregulin-1 function

NRG1 has been studied for over 25 years as the biological activities known as the acetylcholine receptor (AChR) inducing activity (or "ARIA"; reviewed in Fischbach and Rosen, 1997; Buonanno and Fischbach, 2001). Next to its *in vitro* function as ARIA, several studies suggest that NRG1β can serve an ARIA-like role at neuronal synapses. The addition of recombinant NRG1β to cultured cerebellar granule cells stimulates the expression of NMDA-receptor subunit NR2C as well as the expression of $GABA_A$β2 subunits (Ozaki et al., 1997; Rieff et al., 1999). In a related finding recombinant NRG1β was able to induce the expression of α7-nAChR in cultured hippocampal neurons (Liu et al., 2001). NRG1 type III

appears to possess similar inductive properties: Its application *in vitro* to cultured chick sympathetic neurons increases the expression of α3, α5, α7 and β4 nAChR subunits (Yang et al., 1998). *In vivo* NRG1 type III is expressed by preganglionic visceral motor neurons that innervate these sympathetic neurons.

The presence of ARIA *in vivo* however, is less clear. NRG1-Ig heterozygous mice, lacking one functional copy of the Ig domain encoding exon, display roughly a 50% reduction in AChR number at the NMJ and are myasthenic, suggestive of an ARIA function of the Ig-containing isoforms (Sandrock et al., 1997). However, neither NRG1 type I nor type II are required for the expression of AChRs embryonically (Yang et al., 2001). Therefore, based upon current evidence, the early appearance of AChRs at the developing NMJ is a NRG1-independent event, but AChR number in the mature Ig-NRG1 heterozygous animal is suggestive of an ARIA-like function. The Ig-domain containing forms have recently been identified as being essential for muscle spindle development, revealing a role for NRG1 signaling in the differentiation of proprioceptors (Hippenmeyer et al., 2002). These findings are supported by recent *in vitro* data demonstrating that the addition of NRG1 to cultured primary human myotubes induces the expression of not only AChRs, but also of the muscle spindle-specific transcription factors Egr1-3 and myosin heavy chains (Jacobson et al., 2004).

In vivo function of NRG1 has also been addressed genetically: Homozygous NRG1 mouse mutants die during embryogenesis at E10.5 due to a heart defect (Meyer and Birchmeier, 1995). Histological data reveal that structures within the developing heart, known as trabeculae fail to form. This phenotype is shared by mice that are deficient in either ErbB2 or ErbB4 indicating that both receptors are required to transduce NRG1 signals (Gassmann et al., 1995; Lee et al., 1995). In the developing heart, NRG1 is expressed in endocardial cells and the receptors, ErbB2 and ErbB4 are expressed by the adjacent myocardial cells. Genetic rescue of the cardiac phenotype of ErbB2 null mutants provided further insight into ErbB2 function in the peripheral nervous system (Morris et al., 1999). Rescued animals survive until birth, but show a severe loss of motor and sensory neurons. Schwann cells along peripheral nerves are missing, although Schwann cell precursors are present in DRGs indicating that their ability to migrate may be impaired.

Mice lacking the type I and type II isoforms (NRG1 Ig-specific knockout) die of the same cardiac defect as NRG1 complete null mutant animals as well as ErbB2 and ErbB4 null mutant animals (Kramer et al., 1996). This indicates that the Ig-domain containing NRG1 isoforms are required for proper heart development and that the ErbB2/4 heterodimer transduces the signal in the opposing myocardium. The importance of Ig-NRG1 signaling for

cardiovascular function throughout life is underscored by recent findings that NRG1-Ig$^{+/-}$ animals display an increased sensitivity towards the chemotherapeutic doxorubicin in comparison to wild type animals. NRG1 heterozygous animals develop a dilated cardiomyopathy and have an increased mortality rate upon doxorubicin treatment (Liu et al., 2005).

In contrast to NRG1 type I and type III null mutants, homozygous NRG1 type II-specific knockouts are viable and fertile and show a developmental phenotype in the cerebellum of incomplete penetrance. (Alistair N. Garratt, MDC Berlin, personal communication; unpublished).

Multiple linkage studies have identified specific haplotypes within the genomic region surrounding the NRG1 type II exon as risk factors for schizophrenia in Icelandic and Scottish populations (Stefansson et al., 2002; Stefansson et al., 2003). Interestingly, the polymorphisms found, were non-coding and the mechanism by which they contribute to schizophrenia are not understood. It has been suggested that the polymorphisms alter regulatory elements required for normal expression levels of NRG1 type II. Behavioral analysis of NRG1 Ig$^{+/-}$ mice revealed that mutant animals exhibit behavioral aspects that are related to schizophrenia-like behaviors: Administration of the atypical antipsychotic drug clozapine suppresses open field and running wheel activity and it impairs latent inhibition (Stefansson et al., 2002; Rimer et al., 2005).

It is thought that aberrant NMDA receptor (NMDAR) function is one of the causes in the pathophysiology of schizophrenia (Tsai and Coyle, 2002). A potential link between NRG1 signaling and NMDAR function has been provided through a recent finding that demonstrates a reduction of whole-cell NMDAR currents in acutely dissociated and cultured prefrontal pyramidal neurons (Gu et al., 2005). The findings suggest that a reduction in NRG1 signaling may reduce NMDAR-mediated activity. The use of pharmacological inhibitors has identified PLC/IP_3 and Ras/MEK/ERK signaling pathways as potential mediators of the NRG1-induced down-regulation of NMDAR currents. It is thought that activation of both signaling pathways by NRG1 leads to an actin-dependent internalization of the NMDAR NR1 subunit.

It has also been reported that hippocampi of schizophrenics display increased levels of $GABA_A$ receptors (Benes and Berretta, 2001). A potential involvement of NRG1 signaling in the reported pathological changes is supported by the finding that the application of NRG1 to hippocampal slices decreases the expression of $GABA_A$ receptor subunits. Therefore, a dysregulation of NRG1 signaling could contribute to the observed increase of $GABA_A$ receptors (Okada and Corfas, 2004). Another link between NRG1 and schizophrenia is

provided by its ability to induce the expression of α7-nAChRs subunits and by the finding that in schizophrenics the frontal cortex appears to express lower levels of α7-nAChRs (Yang et al., 1998; Guan et al., 1999; Liu et al., 2001). This suggests that a deficit in NRG1 signaling could account for the observed downregulation of α7-nAChRs in schizophrenics.

Alterations in interneuronal function through aberrant cell placement and migration during development has been proposed to be another likely cause underlying the pathophysiology of schizophrenia (Benes and Berretta, 2001). Recent findings implicate NRG1 type I and type III in interneuronal migration during development: NRG1 type I was identified as a long-range attractant for embryonal GABAergic interneurons migrating from the medial ganglionic eminence (MGE) to the developing cortex (Flames et al., 2004). In the same study NRG1 type III was identified as a 'permissive substrate' for the migrating cells, suggesting that it promoted their migration out of the MGE. In related studies by Anton et al. (2004), which examined the consequences of the loss of ErbB4 in the adult brain, the NRG1 type III isoform was also identified as a permissive substrate for migrating interneuron precursors from the subventricular zone (SVZ) to the olfactory bulb. Phenotypically, the ErbB4 deficient mice were characterized by defects in the final placement of olfactory interneurons (Anton et al., 2004). Despite the fact that the existence of a rostral migratory stream in humans is less clear, it is interesting to note that schizophrenics also suffer from olfactory deficits (Moberg et al., 1999). These findings provide causal links as to how alterations in NRG1 signaling can contribute to changes in neural connectivity and neurotransmission, strengthening a role of NRG1 in the pathophysiology of schizophrenia. It has also been proposed that alterations in NRG1-erbB signaling are the underlying cause for the myelin defects that are found in schizophrenics (Corfas et al., 2004).

NRG1 type III is the predominantly expressed NRG1 isoform expressed at the time of synaptogenesis of somatic and visceral motor neurons (Meyer et al., 1997; Yang et al., 1998). It continues to be expressed postnatally in spinal motor neurons and in the brain. NRG1 type III deficient animals survive until birth and die as a result of neuromuscular defects resulting in an inability to breathe. Schwann cells are initially present along embryonic peripheral nerves, but die during later stages of embryogenesis, which leads to nerve degeneration (Wolpowitz et al., 2000).

The DRGs of null mutant animals are characterized by a marked loss of sensory neurons, as indicated by a 60% reduction in Islet1^{+} cells. Sensory neurons are initially present, but degenerate between E14.5 and E18.5. Nerve-muscle synapses begin to form in type III mutant

animals, but the nerve-muscle interactions are not sustained as peripheral axons fragment, retract and degenerate (Wolpowitz et al., 2000).

1.6. NRG1 as a regulator of myelin sheath thickness

At the time this thesis was started, the signals that determine myelin sheath thickness were poorly understood. It was well established, however, that axon diameter and myelin sheath thickness are strongly correlated and that small caliber axons in the PNS (<0.8μm) are usually not myelinated (Friede, 1972; Friede and Bischhausen, 1982; Smith et al., 1982). Recent studies have provided evidence that Schwann cells read signals on axonal surfaces that can be uncoupled from axonal diameter (Elder et al., 2001). Evidence for a direct involvement of NRG1-erbB signaling in myelination was obtained through the analysis of conditional erbB2 mutants in which erbB2 function was ablated at a time point where Schwann cells express the transcription factor Krox-20 (Garratt et al., 2000). Krox-20 is expressed in Schwann cells as they make the transition from a Schwann cell precursor to an immature Schwann cell. The resulting erbB2 mutant animals exhibited considerably thinner myelin sheaths, a finding that was interpreted to suggest that erbB2 function was required for the formation of myelin sheaths of normal thickness.

Analysis of NRG1 heterozygous mice revealed that these animals are characterized by thinner myelin sheaths, suggestive of NRG1 as being the axonal signal regulating myelin sheath thickness (Michailov et al., 2004). The production of transgenic mice, which overexpress NRG1 type III under transcriptional control of the Thy1.2 promoter revealed that the myelin sheath thickness of neurons expressing the transgene was significantly increased. This is an intriguing finding as it suggests that NRG1 type III is one of the factors that determine myelin sheath thickness. Subsequent analyses also revealed the presence of ectopically myelinated small caliber fibers (<0.8μm), indicating that NRG1 expression that exceeds a certain threshold level induces ectopic myelination (Dr. M.H. Schwab, unpublished observations).

In addition to NRG1, other factors are known to influence myelination. Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and the modulation of NRG1 signaling by integrins were all shown to affect myelination (Colognato et al., 2002; Cosgaya et al., 2002; Tolwani et al., 2004).

1.7. Neuregulin-2, -3 and -4

NRG2 is similar in its overall structure to the Ig-like domain containing forms of NRG1 types I, II and shares an overall 35% amino acid sequence identity when compared to NRG1 type I β1a. Within the EGF-like domain NRG1 and NRG2 share a 50% sequence identity (Carraway et al., 1997). The murine NRG2 gene is smaller than the NRG1 gene, covering ~178kb on chromosome 18qB2 and contains 12 exons (Yamada et al., 2000). There are at least 9 different splice variants for NRG2 with "α" and "β" splice variants C-terminal to the EGF-like domain, in analogy to NRG1. Unlike for NRG1, the "α" and "β" domains are not mutually exclusive and can be spliced into the same transcript (Yamada et al., 2000). Despite the sequence similarity to NRG1 type I, NRG2 does not promote Schwann cell proliferation and does not possess a significant ARIA-like function (Rimer et al., 2004 and C. Lai, unpublished results).

An additional region of high sequence homology surrounds the transmembrane-domains of NRG1 and NRG2 (see Fig. 3), suggesting that NRG2 may be proteolytically processed in a fashion similar to that of NRG1. The cytoplasmic tail of NRG2 also harbors a nuclear localization signal, but it is unknown whether the cytoplasmic tail acts as a transcription factor, as has been suggested for the NRG1 cytoplasmic a-tail.

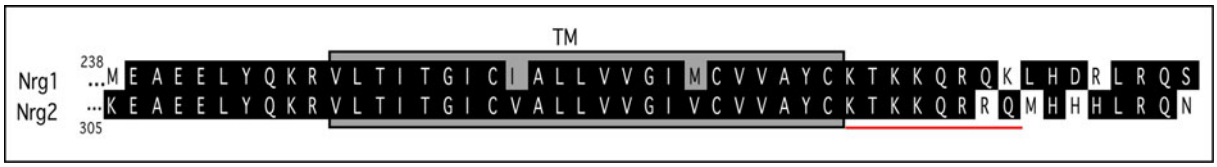


Figure 3: Sequence alignment of the transmembrane domains of NRG1 and NRG2.
Displayed are the amino acid sequences of the highly conserved transmembrane domains of NRG1 and NRG2 including parts of their stalk region and juxtamembrane region. Underlined in red is the nuclear localization signal (NLS) sequence, which was shown to target the cytoplasmic tail of NRG1 to the nucleus of spiral ganglion neurons in culture (Bao et al., 2003). The putative NLS of NRG2 was shown to target EGFP–NRG2 cytoplasmic tail fusion proteins to the nuclei of transfected COS1 and primary hippocampal neurons in culture (personal unpublished observations). The numbering refers to amino acid positions within the published sequences for NRG1 type I and NRG2 (NCBI accession#: AAT68241, ENSEMBL accession#: ENSMUSP00000044380). Boxed in grey are the predicted transmembrane (TM) domains of both proteins.

NRG3 shares little sequence similarity outside the EGF-like domain with NRG1 and NRG2, lacking an obvious Ig-like domain. Instead NRG3 contains a unique alanine/glycine rich segment at the N-terminus (Zhang et al., 1997). *In situ* and Northern blot analysis have shown that NRG3 is predominantly expressed in the nervous system and in the testis.

NRG4, the most recently identfied member of the neuregulin family is distinct from the other NRGs. Its extracellular region consists of only the EGF-like domain and the cytoplasmic tail is mere 33 amino acids in length, making it the shortest NRG known. It also differs from the other NRGs in that it is detected at very low levels in the nervous system, but has been observed in human pancreas and brown adipose tissue in mouse (Harari et al., 1999) (M.H. Schwab, personal communication).

1.8. Transgenic approaches to study gene function *in vivo*

Several methodologies have been developed to study gene function *in vivo*: Generation of transgenic animals through pronuclear injection (Gordon et al., 1980; Palmiter et al., 1982; McKnight et al., 1983) and targeted gene ablation in embryonic stem (ES) cells either by a loss-of-function mutation ('knock-out') or by the insertion of a reporter gene into the genetic locus of interest ('knock-in') or by conditional ablation of gene function in a specific cell type (Doetschman et al., 1987; Thomas and Capecchi, 1987; Gu et al., 1994; Wang et al., 1996).
In contrast to knock-in approaches, the promoters used in transgene constructs often do not provide the same pattern of expression seen for the endogenous promoter, but instead give rise to varying expression profiles. This variation in expression may be attributed to *cis*-acting regulatory elements that surround the site of transgene integration into the genome (Caroni, 1997). Thus, multiple founders have to be analyzed to identify an animal with the desired transgene expression profile.

1.8.1. Modification of BACs by homologous recombination in bacteria

The use of larger genomic DNA fragments for the generation of transgenes increases the likelihood that the regulatory elements required for proper expression are contained within the transgene construct. Large genomic fragments have become available through the generation of bacterial artificial chromosomes (BACs) (Shizuya et al., 1992) and subsequently BAC-based libraries (Shizuya et al., 1992; Kim et al., 1996; Osoegawa and de Jong, 2004; Osoegawa et al., 2004). As a result of their large size, BACs cannot be readily manipulated by restriction-ligation based methodologies and have thus required the development of different techniques for their modification. The precise insertion of DNA fragments into BACs is now

possible through the use of homologous recombination systems devised in bacteria (Yang et al., 1997; Zhang et al., 1998; Yu et al., 2000)

The first recombination that was successfully used for the modification of BACs by homologous recombination in bacteria and the subsequent generation of transgenic mice, was introduced by Dr. N. Heintz in 1997 (Yang et al., 1997). The system utilizes recA-mediated homologous recombination in a recombination deficient bacterial strain. The recE/recT-based homologous recombination system as described by Stewart and co-workers uses transient expression of recombinogenic proteins in a recombination-deficient bacterial strain (Zhang et al., 1998). The recombination functions are contained on a plasmid, which encodes a truncated single strand binding protein, a 5'-3' exonuclease and an inhibitor of the bacterial *recBCD* helicase/DNAse complex.

Stewart and co-workers were able to demonstrate that the systems worked very efficiently with homology arms as short as 50-70bp, which permitted the addition of homology arms to targeting constructs by PCR without the need for multiple cloning steps. Furthermore, the recombinogenic factors are encoded on one plasmid and can thus be easily transferred into most BAC hosts. However, the system suffers from the fact that constitutive expression of *recBCD* inhibitor is toxic to *E.coli*, thereby reducing the efficacy of the homologous recombination event. Furthermore, transient transfection of plasmids generally leads to a 'leaky' expression of plasmid-encoded proteins, which results in a higher background of unwanted recombination events.

Yu and colleagues improved the recombination system by using a defective λ-prophage to stringently control the expression of *Red* recombinogentic factors *exo*, *beta* and *gam* (Yu et al., 2000). The proteins are functionally analogous to the Rac-encoded recET system: *exo* is a 5'-3' exonuclease that generates 3' ssDNA overhangs, which are then bound by the ssDNA-binding protein *beta* forming proteonucleic filaments that initiate strand invasion. Both proteins are functionally assisted by *gam*, which inhibits the bacterial recBCD helicase/DNAse complex. The recombination functions were placed under transcriptional control of the temperature-sensitive λ-repressor cI857, which is tightly bound to its operator sequenc es at 32°C thereby prohibiting transcription. Shifting the cells to 42°C for 15-18 minutes inactivates the repressor and leads to the coordinate expression of the *exo*, *beta* and *gam* genes at very high levels from the λ-pL promoter.

In addition to *exo*, *beta* and *gam* the defective prophage was engineered to encode either *Cre* or *eFlp* recombinases, whose transcription was placed under the control of the pBAD

promoter (Lee et al., 2001). These prophages were then used to generate 2 bacterial strains, EL250 and EL350 harboring the inducible *eFlp* and *Cre* recombinases, respectively. In this manner, 'floxed' or 'flped' selectable markers can be removed from modified BACs through the addition of L-(+)-arabinose to the growth medium (Fig.4). This system works efficiently with short homology arms of 50-70bp in length making it a very reliable and convenient method to modify BACs. A major disadvantage of the λ-prophage-based strategy is, however, the fact that the BACs have to be transferred to the corresponding bacterial strains by electroporation making it less flexible in use.

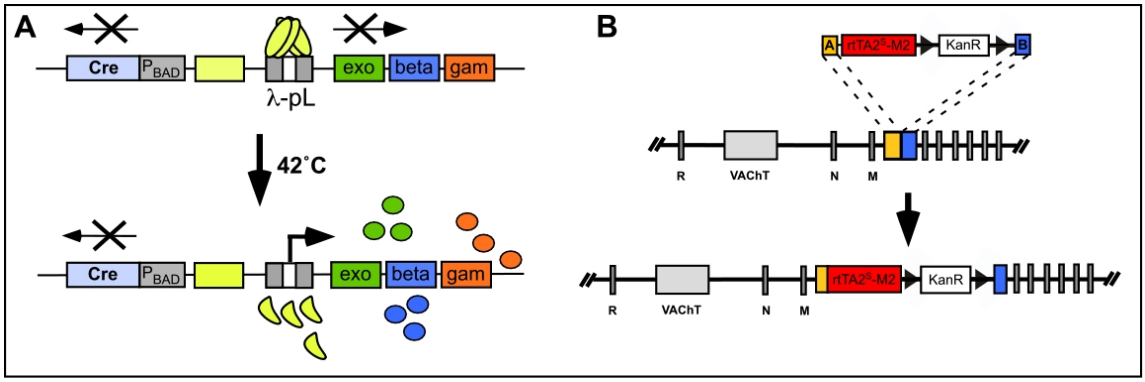


Figure 4: Schematical representation of the λ-prophage-based *Red* recombination system.
(A) At 32°C the temperature-sensitive λ-repressor cI857 is tightly bound to its repressor thereby prohibiting transcription of *exo*, *beta* and *gam* from the λ-pL promoter. At 42°C the repressor becomes unstable and allows the coordinate expression of *exo*, *beta* and *gam*. The prophage also harbors a site specific recombinase (shown here is *Cre* recombinase), which is under transcriptional control of the L-(+)-arabinose-inducible P_{BAD} promoter. (B) Shown is a recombination event of rtTA2^S-M2 into exon 4 of the choline acetyltransferase gene. A, B denote homology arms, which were added to the targeting construct by PCR. Addition of L-(+)-arabinose to the growth medium will induce *Cre* expression form the P_{BAD} promoter, which will result in the excision of the floxed kanamycin resistance, leaving one loxP site behind (not shown).

1.8.1.1. Inducible transgenes

A number of systems have been devised to achieve the inducible and reversible expression of transgenes *in vitro* an *in vivo*. One of the first examples of a system permitting inducible gene regulation was the metallothionein-1 promoter, which was responsive to heavy-metals (Chalifour et al., 1990). The use of this system was very limited, however, since the administration of heavy-metals has severe side effects, particularly at higher doses, which are needed for the maximal induction of transgene expression. Another inducible system was engineered by Evans and colleagues utilizing a modified ecdysone – ecdysone-receptor based switch (No et al., 1996). Here, the ecdysone receptor was N-terminally fused to the VP16 trans-activation domain of the herpes simplex virus (VpEcR) and when co-expressed with the retinoic X receptor (RXR), ecdysone/muristerone induced the heterodimerization of VpEcR

and RXR. This heterodimer is then able to bind to ecdysone response elements in a target promoter with high affinity to stimulate transcription.

Recently, Dr. Heidi Scrable and colleagues have adapted the *E.coli* lac operon for controlled gene expression in the mouse (Cronin et al., 2001). The system is a binary system requiring 2 lines of mice: One that ubiquitously expresses a synthetic lac repressor, *syn*lacI, and a second line that harbors a tissue- or cell type-specific promoter into which operator sequences were inserted surrounding the transcriptional start of the gene to be expressed. In this case a 4.1kb promoter fragment of the tyrosinase gene was used. The bacterial origin of lacI required a codon optimization of its cDNA for the use in eukaryotic cells or transgenic mice. Almost all CpG islands had to be mutagenized to avoid transcriptional silencing of lacI by methylation through mammalian DNA methyltransferases. Additionally, a cryptic splice donor-acceptor site was removed that gave rise to aberrantly spliced lacI mRNA (Scrable and Stambrook, 1997).Transgene induction is achieved through IPTG, which can simply be added to the drinking water of the mice. IPTG does not have any toxic effects at concentrations required for transgene induction (0.1 – 1mM), which makes the system suitable for transgenic work. However, the system has not been widely used due to several factors. First, the system was published recently and additional mouse lines need to be generated to better validate the technology in other tissues and through the use of promoters other than the tyrosinase promoter. Second, the need to insert operator sequences surrounding the transcriptional start site of the transgene can have adverse effects on transgene expression by altering the secondary structure of the resulting mRNA.

1.8.2. Tetracycline-regulated gene expression

The most widely used system for inducible gene expression in the mouse is the tetracycline-regulated gene expression system (tet system). The system was invented by Gossen and Bujard and is based on the tetracycline resistance of *E.coli* encoded on transposon Tn10 (Gossen and Bujard, 1992). Tetracycline (Tc) resistance in *E.coli* is conferred by the export of tetracycline-magnesium complexes $[Tc{\cdot}Mg]^+$ by the membrane-embedded proton-$[Tc{\cdot}Mg]^+$ antiporter tetA. The expression of tetA is under tight transcriptional control of the tetracycline repressor tetR. Both genes, tetA and tetR, are transcribed in opposite directions from two palindromic operator sites tetO1,2 (Hillen and Berens, 1994). In the absence of $[Tc{\cdot}Mg]^+$ complexes, tetR is tightly bound to the operator sites inhibiting the expression of tetA and its own. The association constant for the binding of tetR to its operator sequences is very high

($K_a \sim 10^{11}\ M^{-1}$). This tight regulation of tetA is vital for bacteria, since its uncontrolled expression results in an unspecific cation influx into the cell, reducing its membrane potential, which can be lethal. $[Tc{\cdot}Mg]^+$ binds to the 30S subunit of ribosomes with $K_a \sim 10^6 M^{-1}$ thereby inhibiting protein synthesis. The affinity of tetR to $[Tc{\cdot}Mg]^+$ is $\sim 10^9 M^{-1}$ and binding of $[Tc{\cdot}Mg]^+$ reduces its binding constant for tetO by a factor of $\sim 10^9$ making it a sensitive switch controlling the expression of tetA at tetracycyline concentrations that are 1000-fold lower than the affinity of tetracycline to the 30S ribosomal subunit.

1.8.2.1. Components of the tet-system

In order to provide tight transcriptional control in eukaryotic cells Gossen and Bujard converted the prokaryotic tetR into a eukaryotic transcriptional activator by fusing it to the negatively charged C-terminal domain of HSV-VP16 (Gossen and Bujard, 1992). The resulting tetracycline-controlled transactivator, tTA, binds to its operator sequences within the tetracycline-inducible promoter P_{tet} with high affinity and thereby strongly stimulates transcription.The tetracycline-inducible promoter itself is comprised of the human cytomegalovirus minimal promoter, spanning the region of –53 to +75 relative to its transcriptional start and heptamerized tetO sequences inserted 5' to it.

Transcriptional regulation of P_{tet}-controlled genes by tTA is achieved with tetracycline or one of its derivatives, doxycycline, anhydrodoxycycliene, minocycline or 4-epidoxycycline. Binding of one of the effectors reduces the affinity of tTA to its operator sequences by a factor of $\sim 10^9$, which represses transcription from P_{tet} to undetectable levels. In luciferase assays tTA was shown to lead to a 10^5-fold reporter gene activation when bound to P_{tet}, which was reduced to background levels at tetracycline concentrations of 100ng/ml (Gossen and Bujard, 1992).

The continuous requirement for tetracycline in order to repress the transcription of P_{tet}-controlled genes by tTA has potential disadvantages. Using tTA, the kinetics by which a target gene can be switched from "OFF" to "ON" is slowed by the clearance of residual tetracycline that is stored in lipid-rich tissues or membranes. This is particularly important in transgenic animals where larger amounts of tetracycline or its more lipophilic analog doxycycline can be stored in adipose tissue or bone, which can lead to a significant delay in the activation or re-activation of tTA-mediated transcription. Bujard and colleagues therefore screened tetR mutants to identify variants that possessed the opposite DNA-binding properties that would enable the tetracycline/doxycycline-mediated activation of transcription. One

mutant was identified that was characterized by 4 amino acid substitutions (E71K, D95N, L101S and G102D) and that exhibited the desired DNA binding properties. When this variant was fused to the C-terminal transactivation domain of VP16, the reverse tetracycline-controlled transactivator, or "rtTA" was produced (Gossen et al., 1995).

The initial version of rtTA was characterized by a residual affinity to P_{tet} in the absence of doxycycline and it also displayed a lower sensitivity to doxycycline than tTA. These properties still made tTA the transactivator of choice, despite the favorable pharmacokinetics of rtTA. Urlinger and co-workers therefore subjected rtTA to repeated cycles of mutagenesis in *S. cerevisae* to obtain rtTA mutants with a lower residual affinity to P_{tet} as well as an increased sensitivity to doxycycline (Urlinger et al., 2000). The mutagenesis screen yielded two new rtTA mutants, rtTA2^S-S2 and rtTA2^S-M2, both of which have no residual affinity for P_{tet} increasing the induction range from 10^3 to 10^5 orders of magnitude. The sensitivity to doxycycline was also increased about 10-fold in the case of rtTA2^S-M2.

The use of the viral transactivation domain of VP16 can lead to a 'squelching' of cellular factors (Gill and Ptashne, 1988). It was later also discovered that high levels of VP16 protein can induces changes in chromatin structure (Tumbar et al., 1999). Furthermore, the viral VP16 moiety is potentially able to provoke an immune response, which would have deleterious consequences for the transgenic animal. Therefore, the viral VP16 transactivation domain of tTA and rtTA was replaced by 3 minimal transactivation domains of the "F" type, which have the same transactivation potential as VP16, but lack the ability to 'squelch' cellular factors (Baron et al., 1997).

1.9. Aims of this thesis

The analysis of NRG1 gene function in the mouse is limited by the early embryonic death of NRG1 null mutant animals. Isoform-specific ablation of NRG1 gene function led to an improved understanding, as NRG1 type I/II mutant animals die, like the complete null mutant, *in utero* at E10.5, indicating that either or both of these forms is required for cardiac development. NRG1 type III mutant animals, however, proved to be more informative, as at least some animals lacking this isoform survive until birth. The loss of this isoform is correlated with a near total loss of Schwann cells and while neuromuscular synapses initially formed, they could not be maintained. The analysis of NRG1 heterozygous animals provided additional insight into NRG1 gene function in the mature nervous system, where NRG1 continues to be expressed. Findings by Sandrock and co-workers suggested that the Ig-NRG1 isoforms help to regulate acetylcholine receptor (AChR) number at the neuromuscular junction (NMJ), as heterozygous animals show a ~40% reduction in receptor number (Sandrock et al., 1997). Together, these genetic studies have revealed that different NRG1 isoforms serve distinct biological roles with NRG1 type I/II isoforms appearing to serve in the role of "ARIA" and NRG1 type III forms serving as glial growth factor.

The observations of Sandrock et al. lead to the hypothesis that the level of expression of NRG1 in mature animals may help to regulate AChR at the NMJ throughout the life of the animal. In order to test this hypothesis, it was reasoned that an inducible overexpression of the type I/II isoforms of NRG1, should lead to detectable increases in AChR number at the NMJ. Furthermore, the reversibility of these changes could be assessed by halting the increased expression of NRG1 and returning the system to the normal level of expression. In order to test this hypothesis, an animal model had to be generated that would permit the inducible expression of distinct NRG1 isoforms. The binary tet system was selected, requiring the production of 2 distinct types of mice. The first set of mice, described in Aim 1, permits the expression of the tetracycline-dependent transactivator (rtTA or tTA) allowing for an inducible gene expression in cells that normally express NRG1. The second type of mice, described in Aim 2, would permit the expression of distinct NRG1 isoforms under transcriptional control of tTA or rtTA in a doxycycline-dependent fashion.

Although the initial motivation was based on studying the "ARIA"-like role of the type I/II isoforms, the analysis of $NRG1^{+/-}$ mice also revealed a reduction in the thickness of the peripheral myelin sheath, suggesting that the NRG1 type III isoform may serve as a regulator

of sheath thickness. Indeed, recently published findings by Michailov and co-workers, which were initiated after this thesis was started, have demonstrated that the constitutive overexpression of NRG1 type III does lead to increases in sheath thickness (Michailov et al., 2004). In view of these findings, the proposed inducible NRG1 mouse model should permit determining if NRG1 type III can regulate myelin sheath thickness throughout the life of the animal or if its actions are confined to specific developmental periods.

Thus, the availability of an animal model permitting the inducible expression of NRG1 should permit testing the hypothesis that distinct isoforms of NRG1 can exert their effects either on muscle cells at the NMJ to regulate AChR number or on myelinating glia to influence myelin sheath thickness throughout the life of the animal.

1.9.1. Aim 1: Generation of a transgenic mouse model permitting regulated gene expression in cholinergic neurons

Although the ideal promoter to regulate NRG1 expression is the NRG1 promoter itself, the available evidence at the inception of this project suggested the existence of 3 distinct promoters (one for each of types I, II and III), complicating the selection of the most suitable promoter. Based on the observation that NRG1 is known to be expressed in virtually all cholinergic neurons as well as in a subset of non-cholinergic cells, a mouse model permitting expression of NRG1 in cholinergic cells was elected (Chen et al., 1994; Corfas et al., 1995; Meyer et al., 1997). This would limit expression to cells that, in general, already expressed this factor. This approach seemed reasonable as it had been reported that a promoter fragment from the choline acetyltransferase gene had been used to direct the expression of a lacZ reporter gene to cholinergic neurons in the mouse (Naciff et al., 1999). Unfortunately, the promoter fragment was lacking functionally important regulatory sequences, which resulted in low expression levels of the transgene as well as in incomplete and ectopic expression. Despite these problems, their findings suggested that the approach was feasible, however there was a need to identify a promoter containing the regulatory elements conferring faithful expression in all cholinergic cells. The use of a bacterial artificial chromosome harboring the ChAT gene was therefore thought to provide a means to achieve cholinergic expression of $rtTA2^{S}$-M2.

1.9.2. Aim 2: Generation of transgenic animals permitting the doxycycline-regulated expression of distinct NRG1 isoforms.

In order to generate a mouse model permitting regulated NRG1 expression in cholinergic cells, transgenic mouse lines have to be generated that express distinct NRG1 isoforms under transcriptional control of the tTA/rtTA-responsive P_{tet} promoter.

For the generation of rtTA2^{S}-M2-inducible NRG1 expression constructs, full-length murine NRG1 type I and type III clones have to be assembled and placed under control of P_{tet}. The expression constructs will then be used to generate transgenic animals through pronuclear injection and subsequently be analyzed for their inducibility and expression pattern of the transgene by mating them to the well characterized α–CaMKIItTA mouse line (Mayford et al., 1996).

P_{tet}NRG1 transgenics will then be used to assess the consequences of NRG1 overexpression during various developmental time points: NRG1 type I overexpressing animals may be evaluated for structural alterations of neuromuscular synapses while NRG1 type III overexpressing animals should be suitable for the analysis of alterations of myelin ultrastructure and changes in Schwann cell number.

III. Results

The role of NRG1 in the mature nervous system is very limited as most experimental approaches to its function are based on loss-of-function experiments, which are hampered by the early embryonic or perinatal death of NRG1 null mutant animals (Meyer and Birchmeier, 1995; Wolpowitz et al., 2000). The goal of this thesis was therefore to generate a mouse model that would allow for the regulated overexpression of NRG1 during various time points in development as well as in the mature animal. A BAC-based approach was chosen to target rtTA2S-M2 and subsequently $tTA2^S$ expression to cholinergic neurons, as NRG1 appears to be expressed in virtually all cholinergic neurons (Chen et al., 1994; Corfas et al., 1995; Meyer et al., 1997). In addition, full-length NRG1 cDNA clones encoding NRG1 type I and type III were assembled and used for the generation of $rtTA2^S$-M2/$tTA2^S$-responsive transgenic mouse lines.

1.1. Characterization of pBelo11 ChAT-BAC

As proposed in aim 1 of the thesis, a BAC that would potentially confer cholinergic expression of $rtTA2^S$-M2 was identified through a custom pBeloBAC11 library screen at Incyte Genomics. For the screen ChAT promoter-specific primers were used as indicated in Fig. 1. The identified BAC clone contained a 135kb 129/SvJ-dervied genomic DNA fragment of the ChAT gene. Restriction mapping and subsequent BAC end sequencing revealed that the BAC contained ~96kb of sequence 5' to the ATG of the ChAT gene and ended 2kb 3' to coding exon 12, missing the last 3 coding exons of the gene (Fig.1). As shown in Fig.1 the first intron of the CHAT gene contains the intron-less vesicular acetylcholine transporter (VAChT) gene.

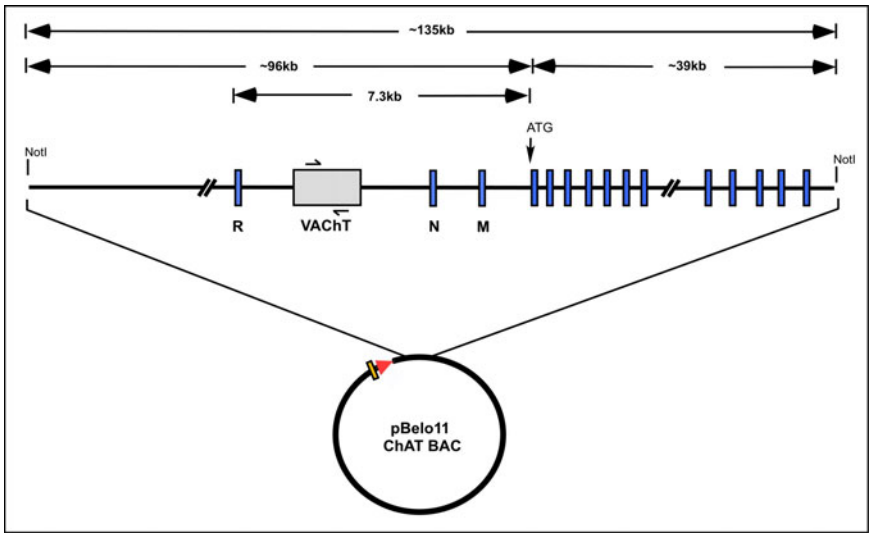


Figure 1: Organization of pBelo11 ChAT BAC.
Structural organization of pBelo11 ChAT-BAC as determined by restriction mapping, Southern blotting and BAC end sequencing. Exons are indicated by blue boxes and are not drawn to scale. Exons R, N and M represent untranslated, alternatively spliced exons of the ChAT gene. The intron-less VAChT gene is located within the first intron of the ChAT gene. Arrows represent the primers, which were used to screen the BAC library. The genomic fragment contained in pBelo11 is flanked by 2 NotI restriction sites, which were used to release the insert from the pBelo11 vector. Indicated by a red triangle is a loxP site within the pBelo11 backbone, the yellow rectangle represents a cosN site.

1.2. Generation of pBelo11 ChAT rtTA2^{S}-M2

For the modification of pBelo11 ChAT BAC using homologous recombination in bacteria, the first step was to inactivate the loxP site in the pBelo11 vector backbone, as its presence would interfere with subsequent manipulation. The loxP site was replaced by a β-lactamase resistance cassette through homologous recombination. Subsequently, the VAChT gene was inactivated by targeting it with a construct that introduced two in-frame stop codons 3' to the 4th codon. This construct also contained a FRT-flanked kanamycin cassette, which served as a selectable marker for the recombination event.

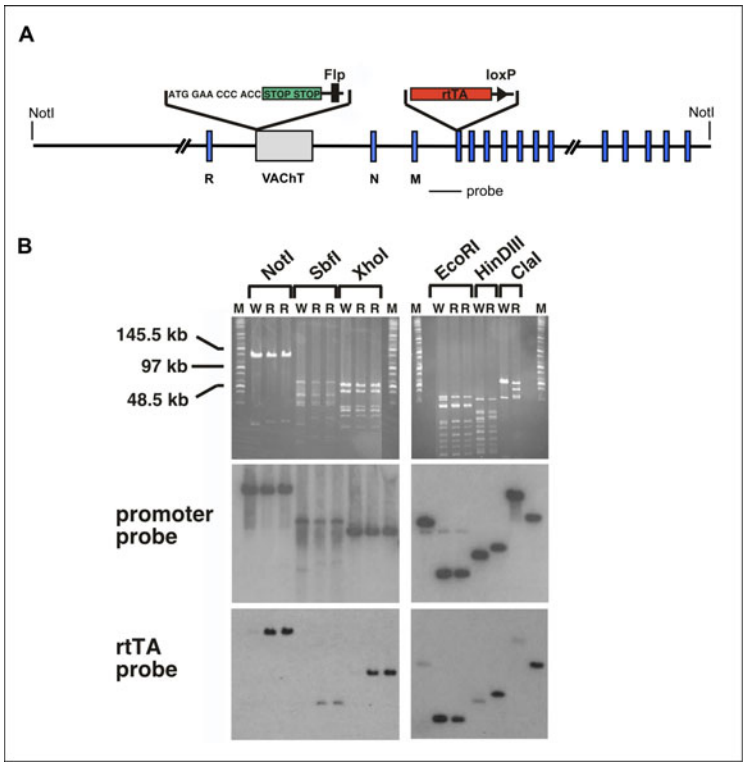


Figure 2: (A) Final pBelo11 ChAT rtTA2^S-M2 BAC after three rounds of homologous recombination. Shown is the genomic insert without the modified pBelo11 backbone vector. Shown are the sites of integration of the targeting constructs to inactivate the VAChT gene as well as for the insertion of rtTA2^S-M2 into the start codon of the ChAT gene. Indicated below the scheme is the location of the 800bp ChAt promoter probe, which was used for Southern blotting.
(B) Restriction digest and Southern blot of unmodified and final pBelo ChAT rtTA2^S-M2 BAC. "W" denotes the wild type and "R" the final, recombined ChAT rtTA2^S-M2 BAC. The upper panel shows the restriction digest of one unmodified BAC and two clones after the homologous recombination events. The restriction digests were resolved by PFGE, blotted and probed with a ChAT promoter-specific and rtTA2^S-M2-specific probes. The enzymes that were used for the restriction digests are indicated.

The resistance cassette was removed by L-arabinose-induced *eFlpe* recombinase expression in the bacterial strain EL250. In a third homologous recombination step rtTA2^S-M2 was inserted into the start codon of the ChAT gene. The targeting construct used contained a floxed kanamycin cassette 3' to the rtTA2^S-M2 ORF, which was excised upon PCR verification of the homologous recombination event. The integrity of the final pBelo11 ChAT-rtTA2^S-M2 BAC was verified by Southern blotting with a ChAT promoter-specific and a rtTA2^S-M2-specific probe (Fig.2). The rtTA2^S-M2 ORF was PCR amplified in independent reactions and PCR products were sequenced following the homologous recombination event prior to pronuclear injection.

1.3. Generation of pBelo11 ChAT rtTA2^{S}-M2 BAC transgenics

For the generation of ChAT rtTA2^{S}-M2 BAC transgenics, the genomic insert was released from the pBelo backbone by a NotI restriction digest and resolved by PFGE. The 135kb fragment was cut from the gel and electro-eluted, followed by repeated steps of dialysis against injection buffer.

Pronuclear injection of the 135kb fragment into C57Bl6 oocytes resulted in very few pups, none of which harbored the transgene. Linearization of the BAC with λ-terminase at the cosN site within the vector backbone to circumvent electro-elution did not result in sufficient amounts of linearized, full length BAC DNA for pronuclear injection. Therefore, circular, maxi-prep grade BAC DNA was used for pronuclear injections at a concentration of 0.6ng/µl. Additionally, the injection buffer was modified and the polyamines spermine and spermidine were omitted from the buffer, as they appeared to be toxic to the oocytes in control experiments.

Multiple rounds of injection resulted in 106 pups, 13 of which had integrated the transgene into their genome. 11 animals were able to transmit the transgene to the F1 generation. This corresponded to an efficiency of transgenesis of 10.4%, compared to ~20% for smaller, conventionally-sized transgene constructs.

1.4. Analysis of ChAT rtTA2^{S}-M2 BAC transgenics

The resulting founder animals were mated to GFPG3 reporter mice to assess the functionality and the expression pattern of the integrated ChAT rtTA2^{S}-M2 transgene (Fig. 3) (Krestel et al., 2001). The use of circular BAC DNA for the generation of transgenic animals was expected to give rise to founder animals with varying expression patterns for rtTA caused by circular permutation, increasing the number of founder animals to be screened.

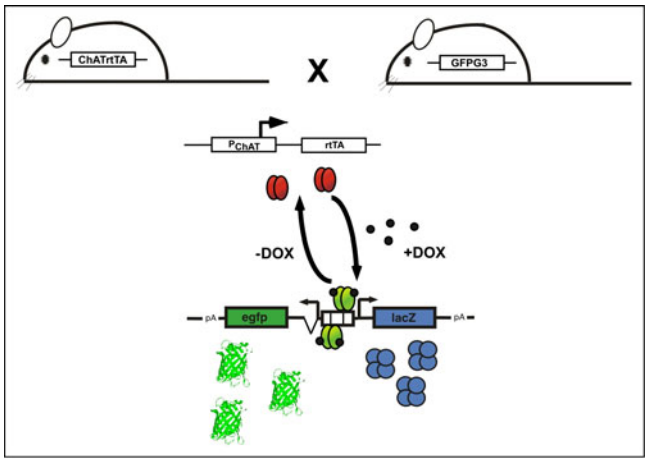


Figure 3: Characterization of ChAT rtTA2^S-M2 BAC transgenic animals.
ChAT rtTA2^S-M2 BAC transgenic animals were mated to GFPG3 reporter mice. GFPG3 reporter mice harbor a bi-directional tTA/rtTA responsive transegene construct that allows the simultaneous expression of eGFP and β-galactosidase from the bidirectional promoter P_{tet}-bi (Krestel et al., 2001). ChAT rtTA2^S-M2:GFPG3 doubly transgenic offspring was fed DOX food (6mg dox/g food) to induce the expression of the GFPG3 reporter transgene.

ChAT rtTA2^S-M2:GFPG3 doubly transgenic offspring were fed DOX food for 10 days to induce the rtTA-dependent expression of the GFPG3 transgene, after which they were sacrificed and β-galactosidase expression was assessed by X-Gal staining.

None of the lines examined showed significant levels of lacZ expression, even after doxycycline administration in both food and drinking water for 2 weeks. In order to analyze whether the absence of detectable lacZ activity was caused by the lack of rtTA2^S-M2 expression, a subset of the ChAT rtTA2^S-M2 mouse lines was further analyzed for rtTA expression by RT-PCR (Fig. 4, Table 1).

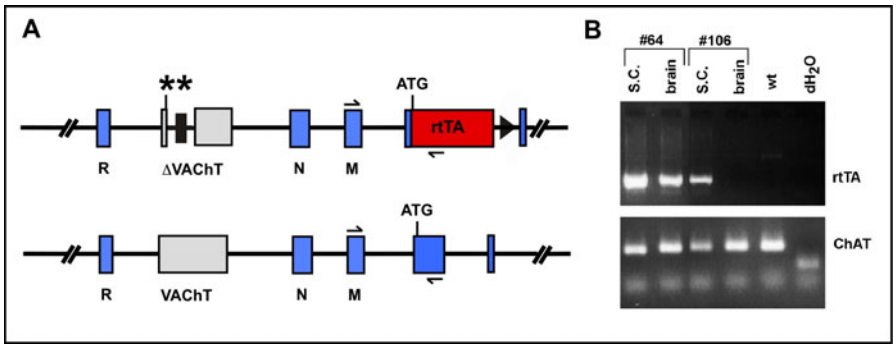


Figure 4: RT-PCR analysis of two ChAT rtTA2^S-M2 BAC transgenic lines.
(A) Displayed are the organization and primer locations for the endogenous ChAT locus and the BAC-derived ChAT rtTA2^S-M2 locus. The asterisks denote the two in-frame stop codons that were introduced into the VAChT ORF. The filled triangles represent a residual FRT site (for VAChT) and a loxP site (for ChAT). Exons are not drawn to scale. **(B)** RT-PCR analysis of cDNA prepared from spinal cord (s.c.) and brain of transgenic mouse lines #64, #106 and wild type (wt) control using primers that amplify endogenous ChAT and a fusion product of the ChAT M-exon and rtTA2^S-M2. Both lines were generated using circular BAC DNA and random breakage during chromosomal integration (circular permutation) might account for the absence of rtTA expression in the brain of line#106.

MOUSE LINE	BRAIN	SPINAL CORD	LIVER
ChATrtTA#18	+	+	N.D.
ChATrtTA#27	+	N.D.	-
ChATrtTA#51	+	-	-
ChATrtTA#64	+	+	-
ChATrtTA#77	-	-	-
ChATrtTA#106	-	+	-

Table 1: RT-PCR analysis of rtTA expression in 6 ChAT rtTA2^{S}-M2 mouse lines.
Based on the detectable rtTA expression in both brain and spinal cord, lines#18 and #64 were further examined by *in situ* hybridization. N.D.:rtTA expression was not determined. Liver cDNA was used as a negative control.

Two mouse lines ChATrtTA#18 and #64 were further examined by *in situ* hybridization, as both lines showed rtTA expression in brain and spinal cord.

A comparison of the rtTA and endogenous ChAT mRNA expression are shown in Fig. 5. It appears that neither line exhibited the pattern observed for ChAT, as evidenced by the ectopic expression of rtTA detected in the cortex of line#18 and the absence of rtTA expression in the septum and striatum in line#64. The RT-PCR product from the brain cDNA of line#64 appears to be due to a weak and ectopic expression of rtTA in the hippocampus of that line (not shown).

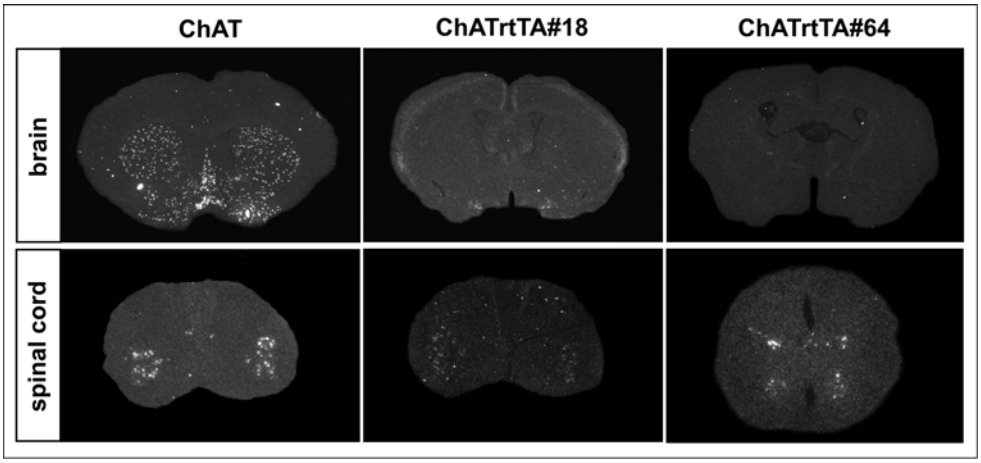


Figure 5: Analysis of ChAT and rtTA2^{S}-M2 expression by *in situ* hybridization.
Shown are *in situ* hybridizations of cross sections in the medial septal area of the brain and the spinal cord for ChAT and for rtTA for 2 mouse lines, #18 and #64. In comparison to the endogenous ChAT mRNA, ectopic rtTA transcripts can be detected at low levels in the cortex of line#18. Low levels of rtTA are also found in motor neurons of the spinal cord. rtTA expression is absent from the brain section of line#64, the expression of rtTA in the spinal cord does not appear to be restricted to motor neurons.

The failure to observe β-galactosidase activity, despite the expression of rtTA led to concerns that the blood brain barrier (BBB) may have prevented doxycycline from entering the brain in sufficient amounts to activate transcription from P_{tet}-bi by rtTA2^S-M2. In support of this concept, measurements conducted by Dr. S. Berger in the laboratory of Prof. H. Bujard indicated that the oral administration of doxycycline at a dose of 2mg DOX/g food resulted in an effective concentration in the brain of 0.5ng/ml. Based on dose-response measurements from that laboratory, the transcriptional activation achieved by rtTA2^S-M2 at this concentration of doxycycline was <1% of its maximal transactivation potential (Berger, 2003).

In light of these findings, a slice culture experiment was set up to circumvent the BBB and to determine whether the rtTA2^S-M2 expressed in line#18 was able to induce reporter gene transcription. For this purpose P5 ChATrtTA2^S-M2 BAC:GFPG3 and GFPG3 transgenic animals from line#18 were sacrificed and brain slices were kept in culture for 3 days in the presence or absence of doxycycline as indicated in Fig. 6, followed by fixation and X-Gal staining. None of the slices derived from the doubly transgenic animal in the presence of DOX at a concentration of 1μg/ml showed detectable β-galactosidase activity, suggesting the absence of functional rtTA protein. Sequencing of PCR amplified rtTA2^S-M2 transcripts from this mouse line did not reveal any mutations.

Surprisingly, sequencing of independent RT-PCR reactions that amplified the splicing product of the ChAT N-exon and rtTA revealed no mutation in the transcript.

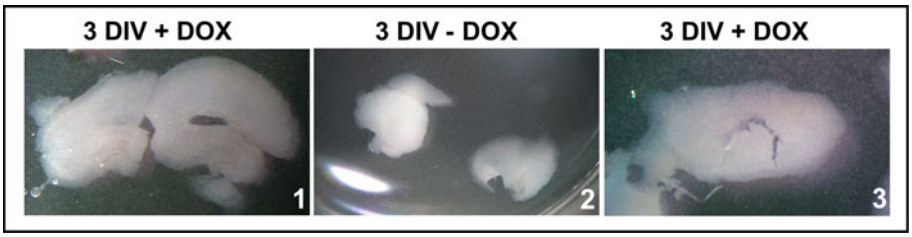


Figure 6: LacZ-stained slice cultures of doubly and singly transgenic animals from a ChAT rtTA2^S-M2#18 x GFPG3 litter.
Brain slices from P5 animals were kept in culture for 3 DIV with or without doxycycline as indicated, fixed with 4%PFA and lacZ stained ON. Slices shown in (1,2) are derived from a doubly transgenic animal, the slice in (3) was taken from a GFPG3 singly transgenic animal. "+DOX" indicates the addition of 1μg/ml doxycycline to the culture medium.

1.5. Alternative approach to cholinergic expression of tTA

The analysis of the ChATrtTA2^{S}-M2 BAC transgenic mouse lines indicated that despite the presence of ~89kb of putative promoter sequence 5' to the R-exon of the ChAT gene, important regulatory elements important for proper cholinergic expression of rtTA2^{S}-M2 were not contained on the pBelo11ChAT BAC. Based on the genomic organization of the BAC, it appeared likely that the missing regulatory elements were located 3' of the gene. A similar problem was also subsequently observed by Dr. N Heintz during the generation of a ChAT-EGFP BAC transgenic mouse line for the GENSAT database (Gong et al., 2003).
Meanwhile, through advances in the assembly and annotation of the public murine genomic databases (such as ENSEMBL), the location of BAC clones in the genome became readily available. A new BAC that contained the complete ChAT gene and roughly equal amounts of genomic 5' and 3' flanking sequence was identified through the "cytoview" application implemented in the ENSEMBL database. The structural organization of the new BAC, RP24-70D4, is depicted in Fig. 7.

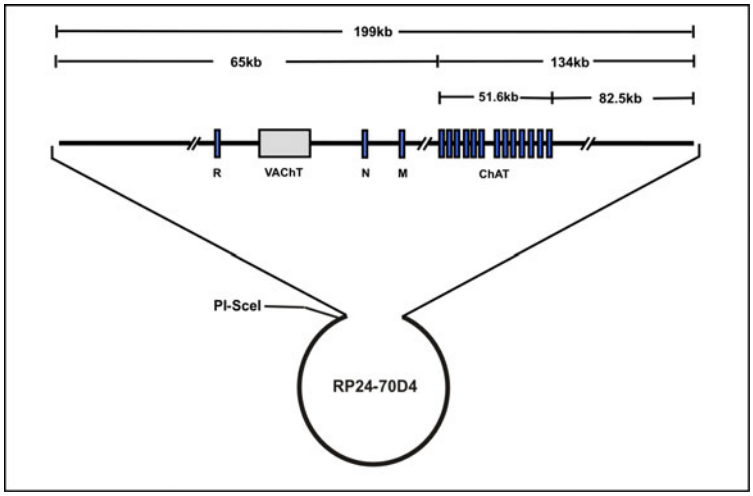


Figure 7: Structural organization of the RP24-70D4 BAC.
The 199kb RP24-70D4 BAC contains all coding and non-coding exons of the ChAT gene, which span 58.9kb in the genome. It is furthermore characterized by ~58kb of promoter 5' to the R-exon and ~82kb 3' flanking sequence to the last exon of the ChAT gene. An important feature of the pTARBAC1 BAC backbone is the recognition site for the homing endonuclease PI-SceI, which was used for linearization of the final BAC. Exons are indicated by rectangles, which are not drawn to scale.

In addition, as the BBB prevented the levels of doxycycline required for the transcriptional activation of P_{tet}-bi by rtTA2^{S}-M2 from entering the brain, a humanized, second generation tTA, htTA, was chosen to achieve doxycycline-dependent gene expression in cholinergic neurons. htTA does not require doxycycline for transcriptional activation of P_{tet}, thus full transcriptional activation can be achieved in the absence of doxycycline. Due to a roughly 10-fold greater sensitivity of htTA to doxycycline, repression of transgene expression to low levels was thought to be possible, despite the presence of the BBB.

1.6. Generation of RP24-70D4 ChATtTApA ('ChATtTApA')

For the generation of ChATtTApA, the same recombination scheme was used as for the generation of pBelo11 ChAT rtTA2S-M2 (see Fig. 8). However, a different selection marker were used for the generation of the homologous recombination fragements: For the insertion of tTA into the start codon of the ChAT gene, the tTA cDNA was first cloned into pFRTzeo5'MCSpA (see: appendix, plasmid maps), which also contained a polyadenylation signal, as this was not contained in the htTA cDNA.

The resulting construct, pFRTzeo5'MCSpA-htTA was used as a PCR-template to add the 55bp homology arms to the targeting construct. The PCR product was subcloned into pGemT, sequenced and used for homologous recombination in bacterial strain EL250 harboring the RP24-70D4ΔVAChATΔneo BAC.

The recombination event, the subsequent excision of FRT-flanked zeocin resistance and the presence of both 5' and 3' BAC ends were PCR verified as shown in Fig. 8. The final BAC was linearized with PI-SceI and column purified over Sepharose ClL-4B. Based on the results of the PFG-analysis of the collected fractions after column purification of the linearized BAC, fraction#10 was chosen for pronuclear injection.

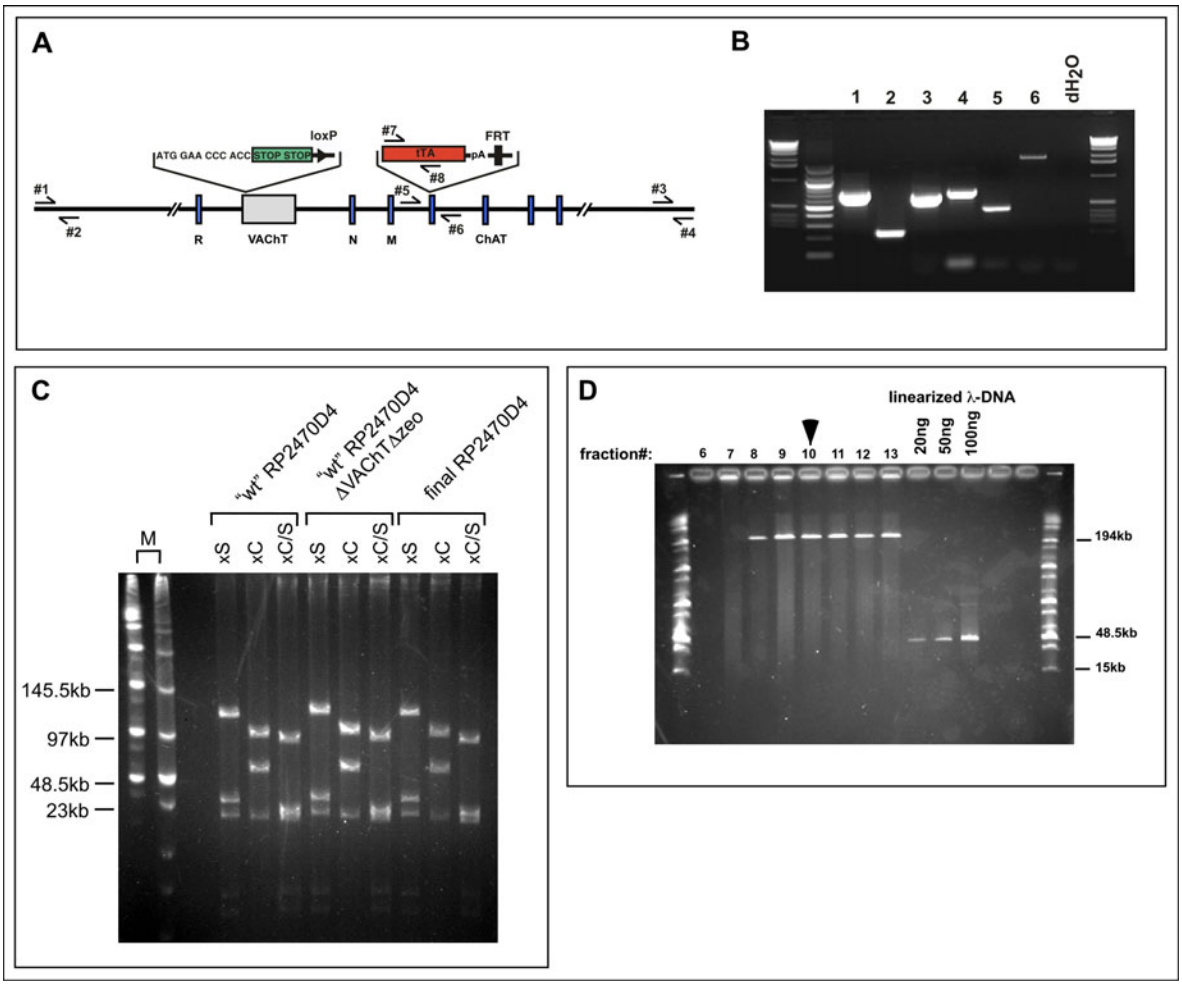


Figure 8: Analysis and linearization of the final ChATtTApA BAC.
(A) Structure of the final ChATtTApA BAC. Primers that were used to verify the integrity of the BAC are indicated. (B) Results of the PCR verification of the BAC integrity. 1: primers#5/#8 (5' ChAT/tTA), 2: primers#7/#8 (tTA), 3: primers#6/#7 (3' ChAT/tTA as, post Flp expression), 4: primers#1/#2 (5' BAC end), 5: primers#3/#4 (3' BAC end), 6: primers #6/#7 (tTA FRTzeo clone, prior to Flp expression). **(C)** PFGE analysis of the wt RP24-70D4, ΔVAChTΔneo and the final, RP24-70D4:ΔVAChTΔneo:tTApA:Δzeo, BAC. The enzymes used for restriction analysis are indicated above the corresponding lanes, "S": SalI, "C":ClaI, "C/S": ClaI/SalI. **(C)** Shown is a PFG of different fractions of the PI-SceI-linearized final RP24-70D4 BAC after column purification for pronuclear injection. Based on OD_{260} 300ng of DNA were loaded for each lane. As a standard the indicated amounts of linearized λ-DNA were loaded. Fraction #10 was used for pronuclear injection.

This fraction appeared to have the best ratio of full length BAC to sheared BAC DNA, which arises as a contamination during linearization and subsequent handling.

1.7. Generation of RP24-70D4 ChATtTApA BAC transgenics

Injection of the linearized BAC DNA from fraction#10 into CByB6F1 oocytes resulted in the birth of 66 pups 9 of which had stably integrated the transgene into their genome. One founder did not transmit the BAC transgene to the F1 generation and was therefore sacrificed.

The remaining 8 animals were mated to GFPG3 mice in the absence of doxycycline to permit reporter gene expression.

During the analysis of the founders generated using linearized BAC DNA, circular BAC DNAs were also used for the production of a second set of transgenic mice. Three rounds of injections yielded a total of 40 pups. Of these, 6 animals that were found to be tTA positive using PCR were mated to GFPG3 mice to assess their tTA expression pattern.

1.8. Analysis of ChATtTApA BAC transgenics generated with linear BAC DNA

ChATtTApA:GFPG3 doubly transgenic animals from each of the 8 founder lines were perfused with 4% PFA, brains and spinal cords were removed and sectioned on a vibratome. Coronal brain sections containing the medial septal area and the habenulae, brain regions known to contain a large number of cholinergic neurons, as well as coronal sections of the spinal cord were subjected to X-Gal histochemistry. Unexpectedly, 7 of the 8 lines examined did not show any detectable lacZ activity. Of the 7 lines two were analyzed further by RT-PCR. Both lines showed tTA expression in brain and spinal cord. One of the lines was then chosen for a more detailed analysis by *in situ* hybridization to determine the spatial distribution of tTA transcripts (Fig. 9).

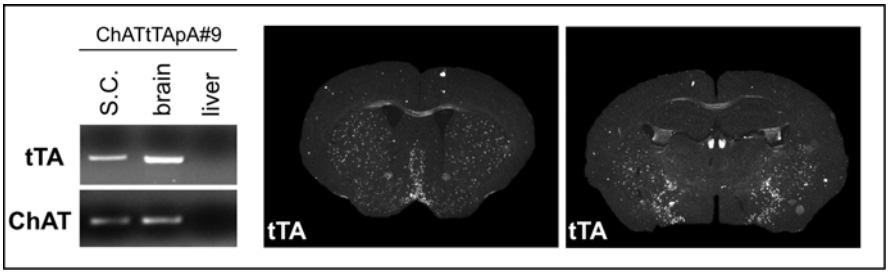


Figure 9: tTA-specific RT-PCR and in situ hybridization for line ChATtTApA#9.
Shown on the left are tTA-specific and ChAT-specific RT-PCRs demonstrating that tTA is expressed in both brain and spinal cord. Liver cDNA was used as a negative control. Shown on the right are in situ hybridizations with a tTA-specific probe. Strong hybridization signals for tTA were detectable in the medial septal nucleus, the striatum (middle panel), as well as in the lateral habenular nuclei and amygdaloid structures (right panel), areas known to contain cholinergic neurons. tTA expression was not seen in the cortex or hippocampus.

It was determined that despite the absence of detectable lacZ expression, tTA was being faithfully expressed in a cholinergic cells. The reasons for an apparently cholinergic, but non-functional tTA expression were not additionally pursued.

1.9. Analysis of the ChATtTApA#44 BAC transgenic line

A preliminary X-Gal histochemical analysis of line ChATtTApA#44 suggested cholinergic expression of tTA as judged by the location of lacZ-positive cells in the brain as well as by lacZ positive cells in the spinal cord and sciatic nerve (Fig. 10A). Administration of doxycycline for 10 days in both food and drinking water was used to achieve maximal repression of the transcriptional transactivation by tTA (Fig. 10B). Following this initial characterization, *in situ* hybridization was used to compare the expression pattern of ChAT and tTA, revealing prominent expression of tTA in apparent cholinergic structures in the brain and spinal cord (Fig. 11).

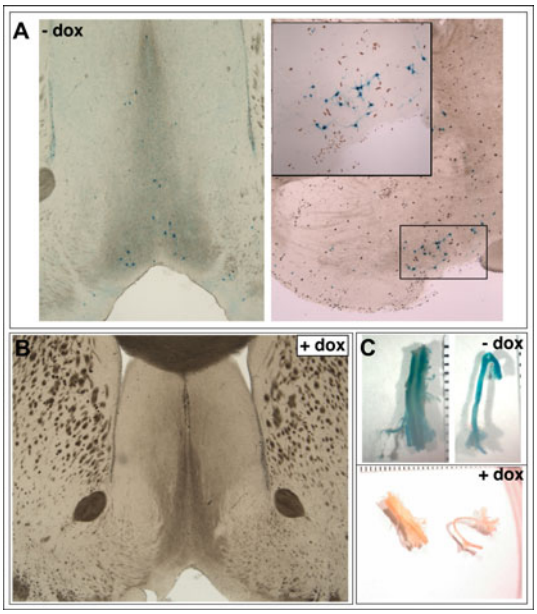


Figure 10: Doxycycline-dependent histo-chemical detection of lacZ-activity in ChATtTApA#44:GFPG3 doubly transgenic animals. (A) A coronal cross section through the medial septal area of a doubly transgenic animal is shown in the left panel. Shown on the right is the medial septal area in a sagittal plane of section of a doubly transgenic littermate. Inset: Higher magnification of the boxed area. **(B)** The medial septal area of a doubly transgenic animal after 10 days of doxycycline administration in both food and drinking water. **(C)** LacZ-stained spinal cords (left) and sciatic nerves (right) of doubly transgenic animals that were fed doxycycline for 10 days in both drinking water and food (lower panel, "+dox") or were fed regular food ("-dox").

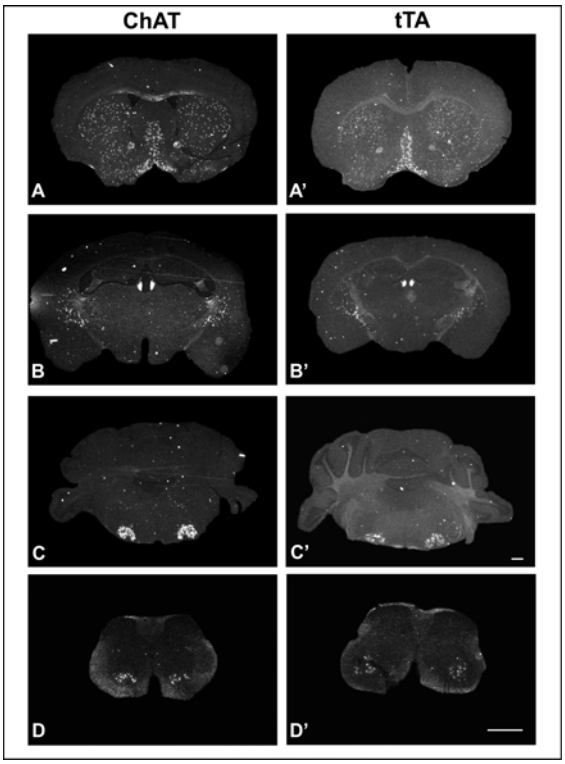


Figure 11: Comparative *in situ* hybridization for a ChATtTApA#44 transgenic animal using ChAT- and tTA-specific probes. Panels (**A-C'**) show cross sections through brain areas known to contain characteristic cholinergic structures: (**A, A'**) medial septal area and striatum, (B, B') Habenular nuclei, caudal striatum, (**C, C'**) facial nuclei. (**D, D'**) Panels show motor nuclei in the spinal cord. Scale bars: 500μm.

In the next step ChAT and transgene-derived EGFP co-localization were assessed by immuno-histochemistry to address faithful cholinergic expression (Fig. 12).

Based on the co-localization of ChAT and transgene-derived EGFP founder line ChATtTApA#44 appeared to exhibit a cholinergic expression pattern of tTA in the forebrain and the spinal cord.

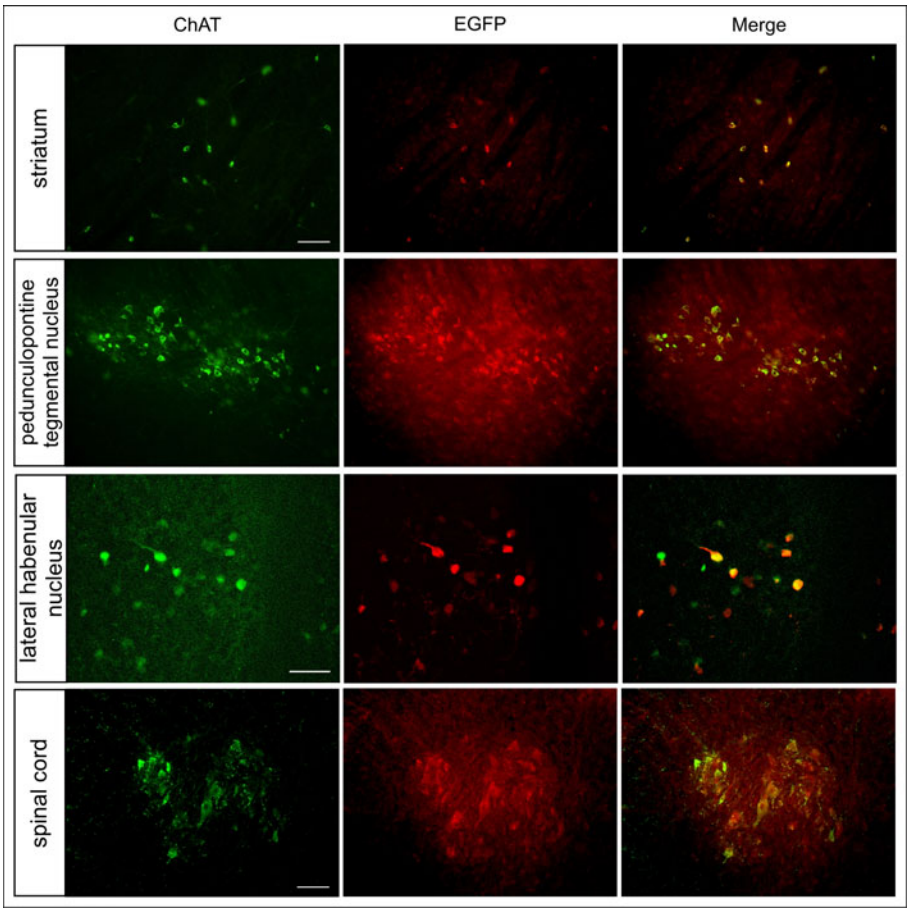


Figure 12: Immuno-histochemical co-localization of ChAT and EGFP in line ChATtTApA#44.
Shown are 45µm vibratome sections of cholinergic neurons in different brain areas and motor neurons in the spinal cord stained with α-ChAT (in green) and α-EGFP antibodies (in red). The merged images show a high degree of co-localization of both signals. Scale bars: striatum, pedunculopontine tegmental nucleus, lateral habenular nucleus: 50µm, spinal cord: 20µm. It should be noted that the images taken for the habenular nucleus were acquired with a confocal microscope and have thus a different spatial resolution than the other images in the composite. Differences in the localization of the EGFP and ChAT signals are due to different fluorescent intensities of tyramide and AlexaFluor568, which are not apparent by conventional microscopy.

1.10. Detection of β-galactosidase-positive Bergmann glia in ChATtTApA#44 transgenics

Histo-chemical processing of sagittal brain sections revealed the presence of lacZ-positive cells in the cerebellum appear to be Bergmann glia based on location and morphology (Dr. F. Kirchhoff, MPI f. Exp. Med., personal communication) (Fig. 13). The transgene expression in Bergmann glia was initially thought to be ectopic, however α-ChAT immunohistochemical analyses have revealed that a subset of these cells are ChAT positive. In addition, the expression of lacZ in those cells was repressed upon doxycycline administration (Fig. 13C).

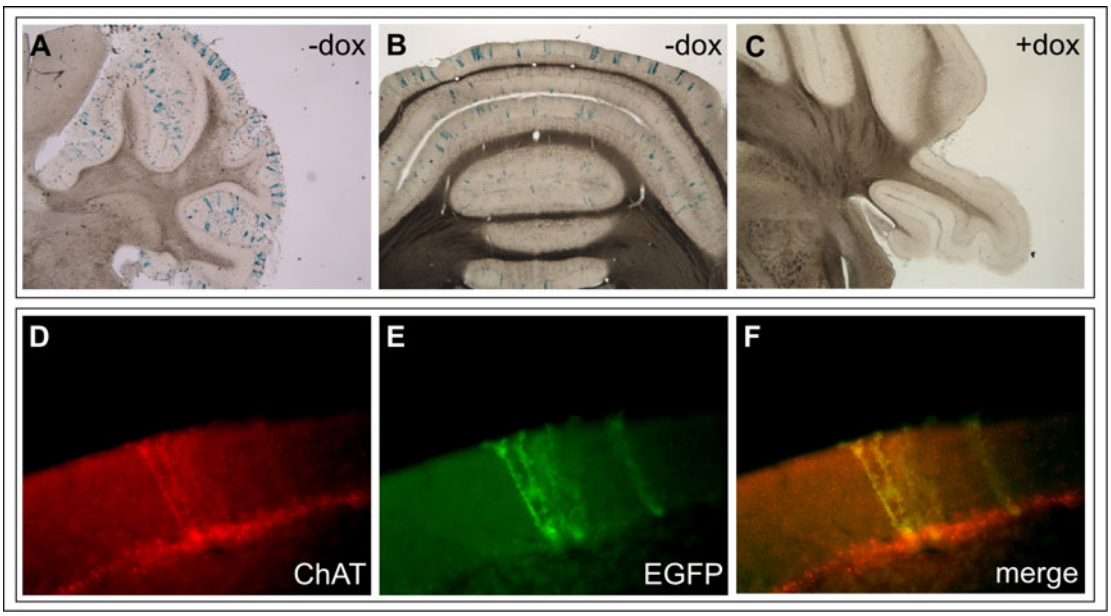


Figure 13: Detection of lacZ-positive Bergmann glia in ChATtTApA#44:GFPG3 doubly transgenic animals. (**A**) X-Gal-stained sagittal section of a cerebellum from a doubly transgenic animal. (**B**) LacZ-positive cells can also be seen in a coronal plane of section in a doubly transgenic littermate. (**C**) Doubly transgenic animal after 10 days of doxycycline administration in the food. (**D, E, F**) Immuno-histochemical detection of ChAT and EGFP in Bergamann glia in the cerebellum of a doubly transgenic mouse.

1.11. Analysis of ChATtTApA BAC transgenics generated with circular BAC DNA

The use of circular BAC DNA for the generation of transgenic animals was believed to result in a higher percentage of animals displaying either a failure in expression or ectopic expression of tTA as a result of the random breakage of the circular DNA prior to integration. This would have led to a permutation of genomic sequences relative to the linear map depicted in Fig. 7. Of the 6 founders analyzed 2 displayed ectopic or partial cholinergic expression of tTA. One animal however, ChATtTApA#73, appeared to exhibit faithful expression in cholinergic cells.

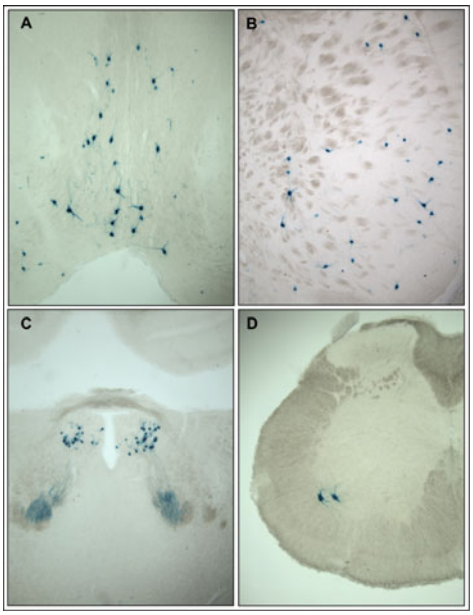


Figure 14: Coronal sections of the brain and spinal cord of a ChATtTApA#73:GFPG3 doubly transgenic animal after histo-chemical lacZ detection. Depicted are the medial septal area (**A**), the striatum (**B**), the lateral habenular nuclei and their projections into the dorsal thalamus (**C**) and the spinal cord (**D**).

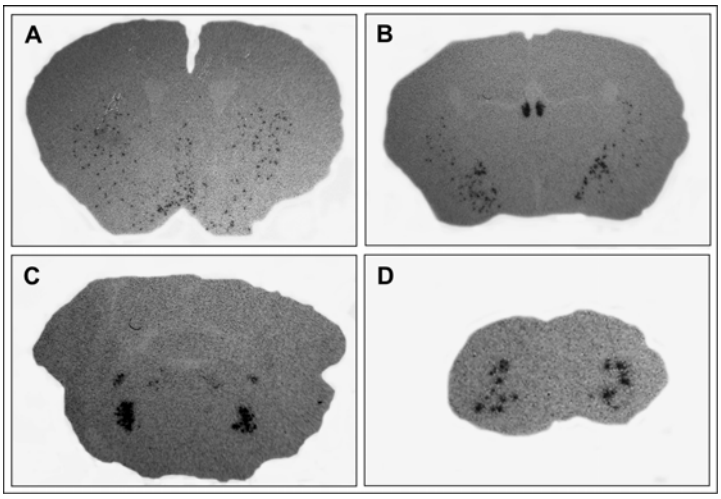


Figure 15: Autoradiographic *in situ* hybridization images of a ChATtTApA#73 transgenic animal. Shown are the hybridization signals of a ^{33}P-labeled tTA-specific probe after a 72h exposure. (**A**) Hybridization signals can be seen in the medial septum and the striatum, (**B**) the habenular nuclei and the basal nucleus of Meynert and (**C**) the facial nuclei. Shown in panel (**D**) are tTA-positive motor neurons in the spinal cord.

This mouse line was then further evaluated by in situ hybridization as shown in Fig. 15. Based on the number and location of tTA-positive neurons, which resembles the pattern observed for line#44, these results suggest that line#73 also expresses tTA in a cholinergic fashion.

Upon comparison of the number of tTA-positive versus lacZ-positive neurons, significant disparities in cell number were observed (compare Fig.10A, 11A'). This apparent difference was found in both mouse lines and it was initially assumed that technical reasons could account for the observed differences, as it was possible that the radioactive signals were more readily detected than the X-Gal stain due to a better tissue penetrance. Subsequent analysis (see Fig. 19 and discussion), indicated that the problem is most likely caused by the lacZ reporter gene.

1.12. Determination of BAC copy numbers in mouse lines ChATtTApA#44 and #73

Both linear and circular BAC DNA forms were successfully used for the generation of mouse lines with cholinergic expression of tTA. In order to determine whether tTA expression levels correlated with transgene number, BAC vopy number for each line was determined by Southern blotting (Fig. 16).

The Southern blots shown in Fig. 16 demonstrate that wild type and BAC-derived ChATtTApA locus can be distinguished by restriction digests, due to the presence of tTA in the BAC, which results in a size shift of roughly 1kb. A MacBas5000 phosphoimager was used to quantify the signal ratios between wild type and transgene-derived bands.

The signal ratios revealed that mouse line ChATtTApA#44 harbors 3 copies of the BAC in its genome, while line #73 carries 5 BAC transgene copies. It should be noted that in one restriction digest for ChATtTApA#73 using the restriction enzymes SacII, EcoRV only half of the DNA was cut to a fragment of the expected size.

A potential reason for this incomplete cleavage was a single nucleotide polymorphism (SNP) leading to a loss of the SacII site in half of the DNA sample. In order to clarify the situation, a 500bp genomic DNA fragment surrounding the SacII restriction site in question was amplified by PCR and digested with SacII. In the event of an underlying SNP, only half of the DNA should be cut and thus result in 3 fragments: uncut, and 2 smaller fragments. However, no difference was found in the restriction digest pattern for wt and ChATtTApA#73-derived fragments, indicating that the underlying cause was likely not a SNP, but may instead be attributed to differences in the methylation of one of the two CpG dinucleotides within the SacII restriction site 5'-CCGCGG.

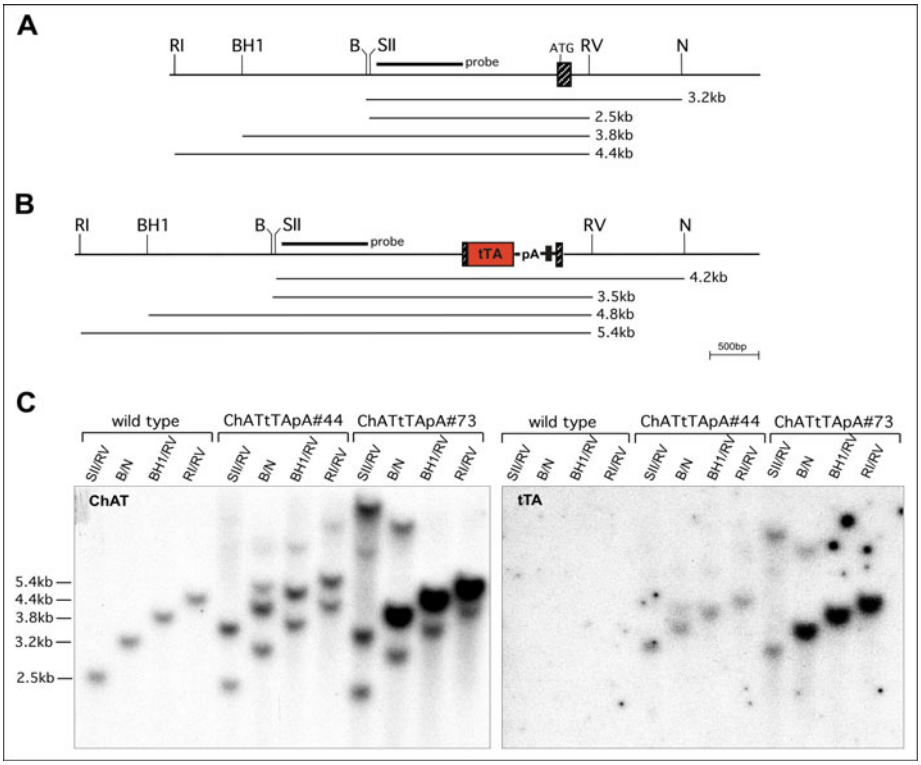


Figure 16: Southern blot scheme and blots of mouse lines ChATtTApA#44 and #73. Shown in **(A)** is organization of the wt ChAT locus surrounding the ChAT start codon. The resulting restriction fragment sizes are indicated below the cartoon. **(B)** Structural organization of the recombined ChAT-tTApA locus, along with the resulting restriction fragment sizes below the cartoon. The location of the ChAT promoter probe is indicated in both cartoons. **(C)** ChAT and tTA-specific Southern blots of wt, ChATtTApA#44 and ChATtTApA#73 genomic DNA, which was cut as indicated above each lane. The lower band in the ChAT-specific Southern blot corresponds to the wt ChAT locus, while the upper bands, which are shifted by 1kb, correspond to the BAC-derived ChATtTApA locus. Restriction sites that were used for the analysis are: RI: EcoRI, BH1: BamH1, B: BspE1, RV: EcoRV, N: NdeI, SII: SacII.

1.13. Whole-mount histo-chemical detection of lacZ in ChATtTApA:GFPG3 doubly transgenic embryos

The embryonic expression of tTA in each of the ChATtTApA lines was assessed. Previous studies have indicated that during development cholinergic neurons can be first detected using α-ChAT antibodies at E12.5 in the upper thoracic spinal cord (Phelps et al., 1991). Studies by Schambra et al. have demonstrated that the majority of cholinergic neurons emerge between E14-E16 in the mouse forebrain (Schambra et al., 1989). In view of these observations, tTA expression was assessed between E14 – E16 in ChATtTApA:GFPG3 doubly transgenic embryos. Later time points were not chosen for evaluation, as the clearing process of the embryos for whole mount lacZ histo-chemistry is inefficient and prohibits a detailed analysis. Timed-pregnant females from matings that would result in doubly transgenic offspring were

sacrificed at the desired gestation day. For genotyping purposes a small piece of tail was removed and the remainder of each embryos was subjected to whole mount lacZ histochemistry, the results of which are shown in Fig. 17.

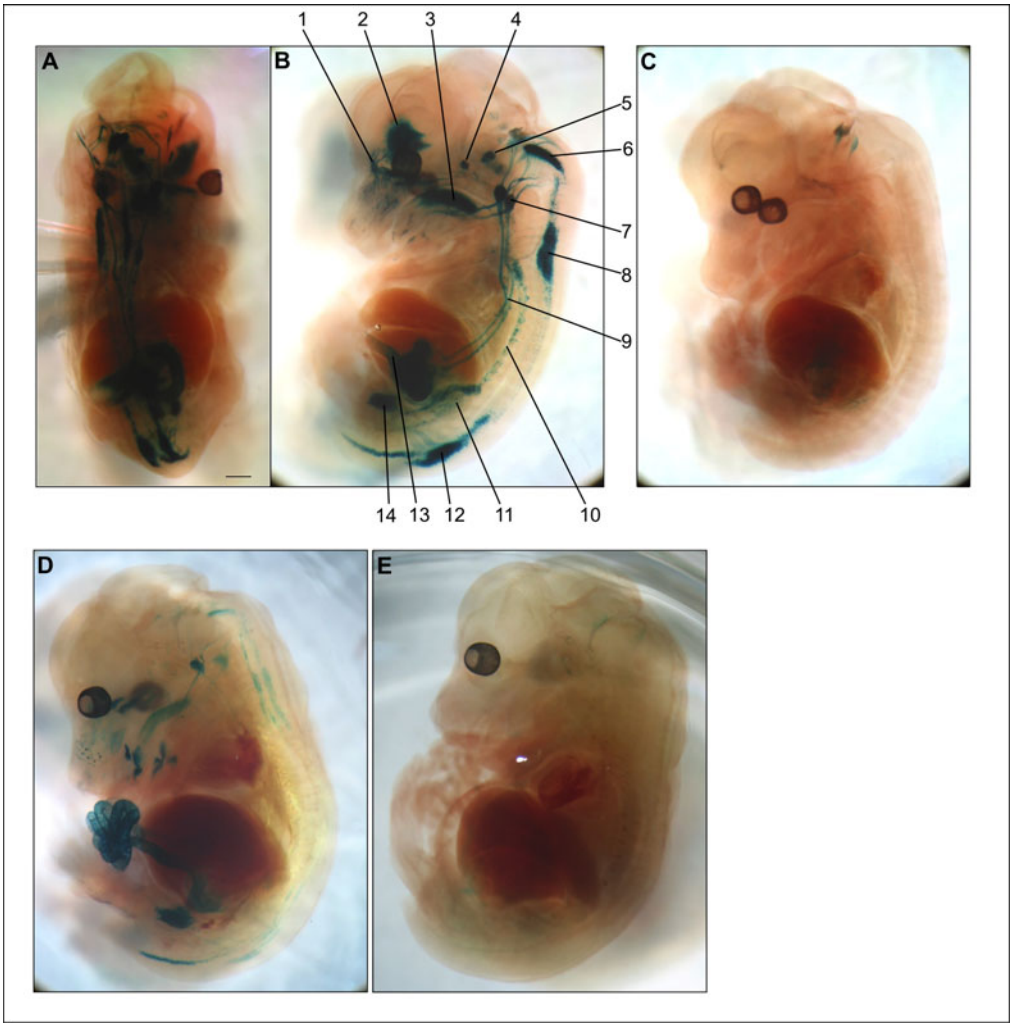


Figure 17: Whole mount lacZ-stained ChATtTApA#73:GFPG3 embryos.
Embryos depicted in panels A-C were fixed at E14, embryos in D, E at E15. The embryos shown in (C) and (E) are GFPG3 singly transgenic control embryos.
(A) Dorsal, (B) lateral view of a tTA:GFPG3 doubly transgenic embryo at gestation day 14 after lacZ detection and clearing of the embryo. The structures visible are (1) olfactory nerves converging on olfactory lobe of brain, (2) germinal zone of lateral ventricle, (3) extrinsic tongue muscle [m. genioglossus], (4) Ggl. oticum (pterygopalatinum), (5) Ggl. trigeminale, (6) Ggl. cervicothoracicum, (7) superior cervical ganglion, (8) Intumescencia cervicalis, (9) Nervus vagus, (10) Truncus sympathicus, (11) Plexus hypogastricus, (12) Plexus sacralis, (13) parts of the umbilical cord, (14) developing enteric nervous system. Shown in (D) is a lacZ-stained E15 embryo. Some of the lacZ-positive structures as seen in (A), (B) are still visible, although the staining is much weaker and appears incomplete. Scale bar: 0.5mm

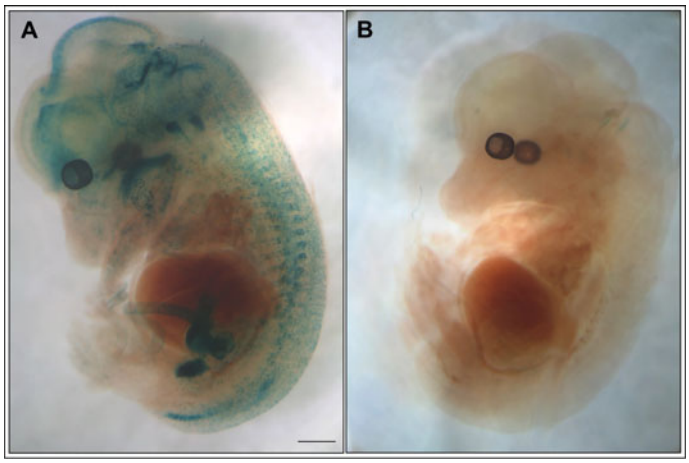


Figure 18: Whole mount lacZ-stained and cleared ChATtTApA#44 E15.5 embryos.
(A) A lacZ-stained and cleared ChATtTApA#44:GFPG3 doubly transgenic E15.5 embryo. Despite the less efficient clearing of the embryo at E15.5, an increased number of cholinergic neurons is visible in comparison to the E14 embryo shown in fig.17. Most notably are the migrating cholinergic neurons in the cortex and lateral ventricle as well as the intercostals motor neurons. (B) GFPG3 singly transgenic control embryo. Scale bar: 1mm.

The later developmental time point of the embryos shown in Fig. 18 does not allow for a complete clearing of the embryonic tissue, but cholinergic neurons are still visible.
The incomplete lacZ stain as seen in Fig. 17D may indicate potential functional limitations of the lacZ reporter transgene that was used to generate the GFPG3 mouse line. This finding does not appear to be restricted to mouse line ChATtTApA#73, but it is also apparent in line ChATtTApA#44. In this line, lacZ expression appears to be incomplete in the adult animal and prominent cholinergic structures are characterized by a small number of lacZ-expressing cells, although tTA is expressed by virtually all cholinergic neurons (see Fig. 10 and 11A').

1.14. Analysis of β-galactosidase expression in ChATtTApA#73:GFPG3 transgenic animals

The difference in the number between tTA-positive neurons seen by *in situ* hybridization and by lacZ histo-chemistry prompted the question whether this lack of β-galactosidase activity was due to improperly expressed tTA or caused by the lacZ gene in the GFPG3 reporter.
In situ hybridization with tTA- and lacZ- specific probes was used to assess the expression of each of the two transgenes in a ChATtTApA#73:GFPG3 doubly transgenic animal (Fig. 19).

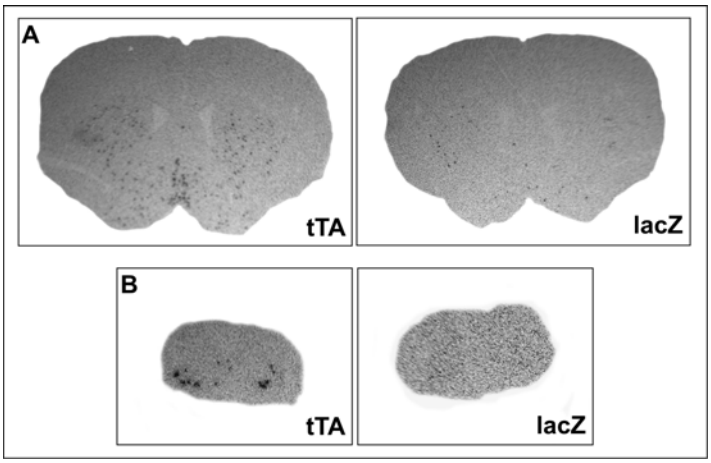


Figure 19: Autoradiographic *in situ* hybridization images for tTA- and lacZ specific probes on coronal brain and spinal cord sections of a ChATtTApA#73:GFPG3 doubly transgenic animal. (A) Adjacent coronal brain sections are shown that were hybridized with a tTA- and lacZ-specific probe. Cholinergic expression can be seen in interneurons of the striatum and the cholinergic neurons of the beginning medial septum. LacZ-expressing cells are sparsely distributed and are absent from the medial septal area. (B) Displayed are adjacent sections of the spinal cord. Whereas tTA is expressed by motor neurons, no lacZ transcripts can be detected.

The results of the *in situ* hybridization suggest that the lack of lacZ-positive cholinergic neurons is not due to an improperly expressed tTA, since tTA transcripts can be readily detected and they appear to be expressed in a cholinergic fashion. The possibility that the tTA gene was not functional as a result of mutation did not appear to be the case, as lacZ-positive cells were detected, although their number appeared to be decreasing with each succeeding mouse generation.

The finding that lacZ was subject to a 'silencing' effect in cholinergic neurons was unexpected and of great importance for the generated tTA-inducible NRG1 transgenic lines. The parental plasmid for the construction of the NRG1 expressing lines was the same construct used to generate the GFPG3 reporter line and thus lacZ silencing could potentially influence or repress the expression of the NRG1 transgene in those lines.

2. Generation of P_{tet}-bi NRG1 transgenic animals

For the tTA-regulated overexpression of NRG1 isoforms in cholinergic neurons NRG1 cDNAs encoding the β1a variants of the type I, type II and type III isoforms were generated by standard molecular cloning techniques. The NRG1 expression constructs were subsequently tested in tissue culture prior to cloning the transgene constructs.

2.1. Molecular cloning of NRG1 isoforms

A full-length murine NRG1 type III β1a cDNA clone ('GGF#9') was used as the source plasmid for the transmembrane domain and cytoplasmic tail regions to produce full-length clones for NRG1 types I, II and III. The full-length NRG1 type III clone was amplified by PCR to add SalI restriction sites to its 5' and 3' end and sequenced. A unique SacI restriction site that was located 5' to the transmembrane domain in conjunction with the 3' SalI restriction site of the modified NRG1 type III cDNA were subsequently used to assemble NRG1 type I and type II β1a cDNAs. NRG1 type I and type II cDNA fragments encoding the extracellular parts of the corresponding proteins up to the SacI restriction site, were PCR amplified from brain cDNA libraries and EST clones. The resulting clones were sequenced and subcloned into CMV promoter containing expression plasmids.
The nucleotide sequences of NRG1 type I and type III, along with their conceptual translations were also deposited in the NCBI database (accession numbers: AY648976, AY648975).

2.2. Expression of NRG1 cDNAs in cultured cell lines.

The generated NRG1 types I, II and III β1a expression constructs were transfected into cos7 fibroblasts and PC12 cells to test whether the assembled cDNAs could be expressed and detected in cell lysates with a NRG1β1a-specific antibody (Fig. 20).

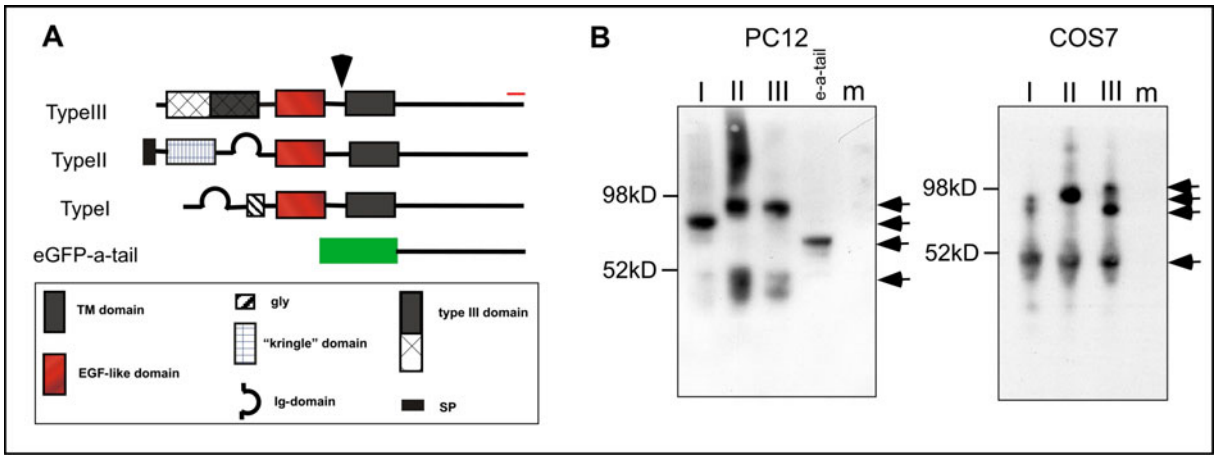


Figure 20: Examination of NRG1 cDNA expression in heterologous cells.
(A) Depicted are the domains of the NRG1β proteins encoded by the assembled cDNAs. 'EGFP-a-tail' denotes an expression construct that was used as a positive control for the sc-348 antibody that was used to detected recombinant NRG1 protein in cell lysates. The red bar indicates the epitope recognized by the sc-348 antibody, the arrow head indicates the MMP cleavage site within the stalk region of NRG1. (B) Western blots from PC12- and cos7-derived cell lysates probed with the NRG1β-a-tail-specific sc-348 antibody. Roman numerals indicate NRG1 type I-III, m: mock transfected sample, e-a-tail: eGFP-a-tail fusion. Protein sizes are indicated. Arrows mark the bands of interest. The expected (calculated) protein sizes are: NRG1 type I: 71kD, type II: 90kD, type III: 77kD, EGFP-a-tail: 69.5kD and a-tail (post MMP cleavage) ~44kD.

Western blotting indicated that all isoforms were expressed in non-neuronal and neuronal-like cell lines. Interestingly, processing differs between cos7 and PC12 cells; cos7 cells appear to favor a cleavage of the full-length NRG1 isoforms, indicated by the presence of the 44kD a-tail specific band. In PC12 cells, however, most of the NRG1 protein appears to be full length and uncleaved, since only little a-tail fragment can be detected.

2.3. Generation of P_{tet}-bi NRG1 transgenics.

NRG1 type I and type III cDNAs were subcloned into pGFPlacZ, which carries a bi-directional tTA/rtTA-responsive promoter, P_{tet}-bi, that enables the expression of lacZ and EGFP from the same promoter in opposite directions (Baron et al., 1995). The cDNA fragment encoding EGFP was released form pGFPlacZ through a NotI digest and the NRG1 type I or type III cDNA were inserted into this site. The resulting transgene constructs, pNRG1typeI-lacZ ('IgBetaBi') and pNRG1typeIII-lacZ ('SMDFBi'), were linearized with AseI, gel purified and used for pronuclear injection at a concentration of 6ng/µl.
The pronuclear injections of the pNRG1typeI-lacZ ('IgbetaBi') construct resulted in the birth of 46 pups, 9 of which had stably integrated the transgene into their genome and were able to transmit it to the F1 generation. The pronuclear injection of pNRG1typeIII-lacZ resulted in the birth of 64 pups of which 8 were founders.

2.4. Analysis of pNRG1typeI-lacZ ('IgBetaBi') transgenic animals

Conventionally-sized transgenes are often subject to positional effects that are caused by neighboring regulatory elements in the genome. These elements may influence the expression pattern of the transgene and can lead to ectopic expression or to the silencing of the transgene. Inducible transgenes are prone to be influenced by positional integration effects through transactivation by a nearby enhancer. It is therefore important to establish that the expression of pNRG1-lacZ transgenes is not affected by positional effects, which can lead to abberant transgene expression. The α-CaMKII-tTA line is the best characterized and most widely used tTA-expressing mouse line and it was therefore chosen for the characterization of pNRG1typeI-lacZ founder animals. Doubly transgenic mice were used to assess the ability of doxycycline to induce or repress lacZ reporter gene expression. Animals that displayed a α-CaMKII-tTA-like expression of lacZ were further analyzed by RT-PCR for a doxycycline-dependent expression of transgene-derived NRG1 type I mRNA.

Out of the 9 founder animals analyzed, 1 line was characterized by a α-CaMKII-tTA-like expression pattern of β-galactosidase as well as by a reversible doxycycline-dependent expression of the pNRG1typeI-lacZ transgene (Fig. 21).

As evidenced by RT-PCR a weak transgene expression can be detected in IgbetaBi#24:α-CaMKIItTA doubly transgenic animals after a week of doxycycline administration. A likely reason is the BBB, which prevents sufficient amounts of doxycycline form entering the brain that are needed for a complete repression of transgene expression. Based on RT-PCR and X-Gal stains it is apparent that after a week of doxycycline withdrawal transgene expression is not fully re-induced.

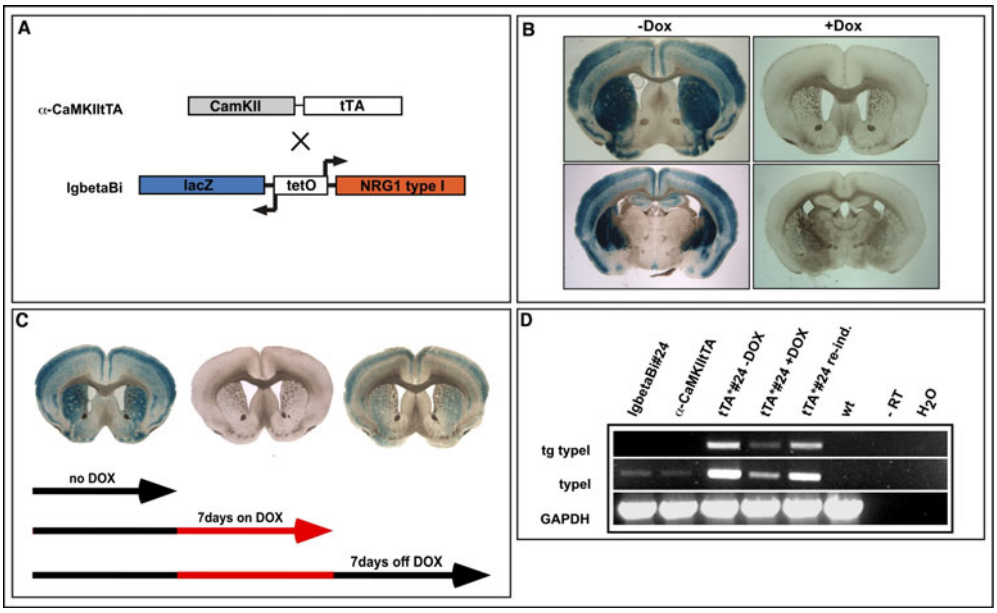


Figure 21: Characterization of IgbetaBi#24:α-CaMKII-tTA transgenics. (**A**) Mating scheme for the generation of α-CaMKII-tTA:IgbetaBi#24 doubly transgenic animals. (**B**) Depicted are coronal brain sections from 2 mice on (+Dox) and off (-Dox) doxycycline for an initial assessment of transcriptional repression of transgene expression through doxycycline administration (**C**) Shown is a time course for the re-induction of transgene expression after doxycycline administration for 7 days and doxycycline withdrawal for 7 days. The mice were sacrificed subsequently sacrificed and lacZ expression was assessed by X-Gal staining. (**D**) RT-PCR analysis of transgene expression in singly and doubly transgenic animals, which were fed DOX food as indicated. "tTA*#24": α-CaMKII-tTA:IgbetaBi#24 doubly transgenic animal. "wt": wild type, "-RT": no reverse transcriptase added to RT-PCR. The wild type control cDNA used was generated independently with less total RNA and overall PCR product yields are lower. "tg typeI": transgene-derived NRG1 type I transcripts. "type I": total NRG1 type I transcripts. All lacZ-stains were allowed to proceed for 90 minutes at 37°C, after which the reaction was stopped.

2.5. Analysis of pNRG1typeIII-lacZ ('SMDFBi') transgenic animals

The SMDFBi founder mice were mated to GFPG3 animals and lacZ expression was assessed as done for IgbetaBi founders. Of the 8 founder animals analyzed one animal, SMDFBi#26, displayed a α-CaMKII-tTA-like expression of lacZ (Fig. 22A), however the administration of 6mg dox/g food for 9 days was not able to substantially repress transgene expression (Fig. 22A). To address the question whether this line is functional in cholinergic neurons despite the poor repression of transgene expression, SMDFBi#26 animals were mated to ChATtTApA#44 mice. Doubly transgenic SMDFBi#26:ChATtTApA#44 animals were sacrificed and transgene expression was assessed by X-Gal staining. LacZ-positive cells were found in cholinergic structures in the brain (Fig. 22B, C), but they were absent from the spinal cord. Silencing effects of lacZ expression, which were also seen in the GFPG3 line, were also observed in SMDFBi#26:ChATtTApA#44 doubly transgenic animals as shown in Fig. 22D.

In comparison to Fig. 22B, only few, randomly distributed lacZ-positive cells can be found in the medial septal area.

It should be noted that continuous dox administration throughout embryogenesis lead to a full repression of transgene expression in SMDFBi#26:ChATtTApA doubly transgenic animals (not shown).

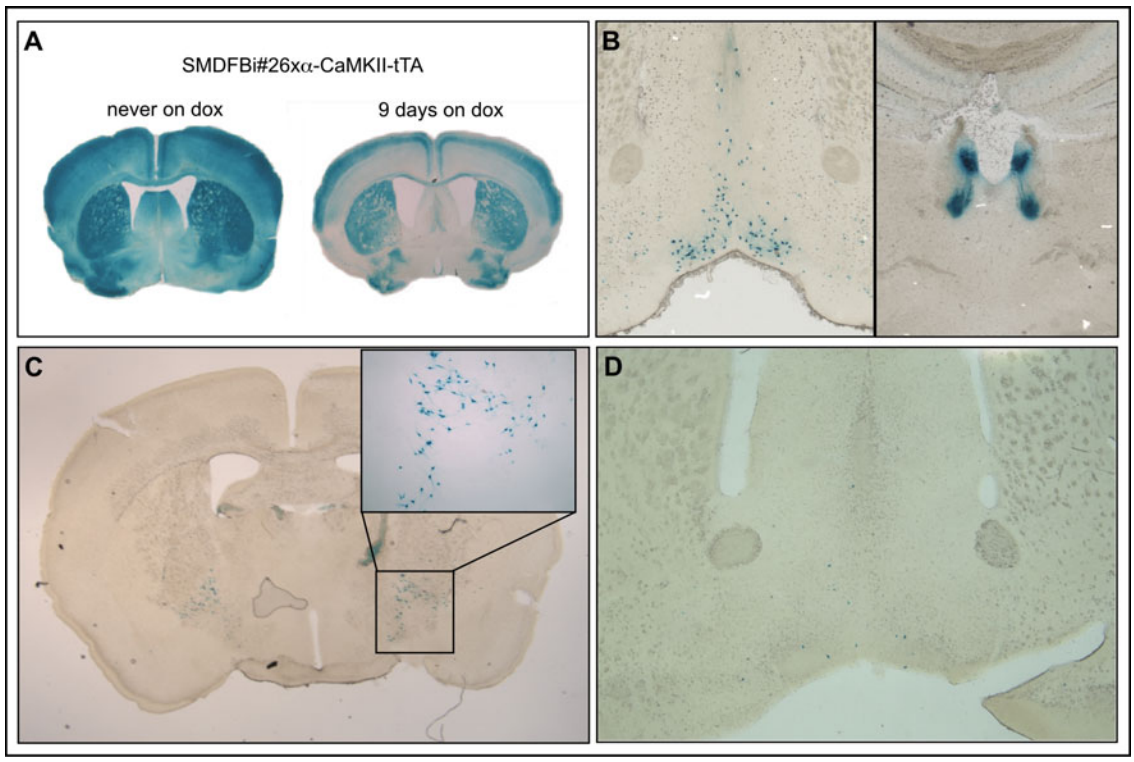


Figure 22: Histo-chemical analysis of lacZ expression in mouse line SMDFBi#26. (A) LacZ expression in coronal brain sections of SMDFBi#26:α-CaMKII-tTA doubly transgenic animals. The brain sections are derived from animals that were fed dox (6mg dox/g food) as indicated. Dox administration for 9 days did not result in a complete repression of transgene expression as evidenced by the residual lacZ activity. (B) Shown is the lacZ expression in a ChATtTApA#44:SMDFBi#26 doubly transgenic animal in the medial septum (left) and the lateral habenular nuclei along with their projections into the dorsal thalamus. (C) LacZ expression in a ChATtTApA#44:SMDFBi#26 doubly transgenic animal in the posterior limb of the interstitial nucleus and substantia innominata. Shown in the inset is a higher magnification of the boxed area. (D) Medial septal area of a doubly transgenic animal in which lacZ expression was silenced. Only few cells are lacZ-positive, which are randomly distributed within the medial septal area.

RT-PCRs for the detection of transgene-specific NRG1 type III transcripts in ChATtTApA#44:SMDFBi#26 doubly transgenic animals failed to detect transgene-derived transcripts. This could be attributed to a very low effective mRNA copy number in the sample, which may be in part due to the limited number of cholinergic neurons in the sagittal brain section utilized.

IV. Discussion

The primary goal of this thesis was to test the hypothesis that the polypeptide factor, Neuregulin-1 (NRG1), is required throughout the life of the animal in the peripheral nervous system in order to perform at least 2 functions, first, to regulate acetylcholine receptor (AChR) expression at the neuromuscular junction and second, to maintain the myelin sheath. In order to test this hypothesis, it was reasoned that if one were able to increase the expression of distinct NRG1 isoforms in adult animals, one should be able to determine if there were detectable changes in either AChR number or the thickness of the myelin sheath. Therefore, we elected to produce a mouse model that would permit the regulated expression of distinct NRG1 isoforms in cells known to normally express this factor. As the NRG1 gene was believed to have at least 3 distinct promoter elements, none of which had been characterized, a different promoter was utilized that would restrict expression to cholinergic cells. This selection was based on the observation that virtually all cholinergic neurons appeared to express NRG1 (Chen et al., 1994; Corfas et al., 1995; Meyer et al., 1997). In order to obtain regulated expression, we elected to use the tetracycline-regulated transcriptional transactivator system (Gossen and Bujard, 1992). Thus the overall strategy required the production of 2 types of transgenic mice, one type expressing the transactivator in cholinergic cells and the other type expressing distinct NRG1 isoforms under the control of a tetracycyline transactivator-regulated promoter. When these two types of transgenic mice are mated together, the doubly transgenic offspring were anticipated to inducibly express NRG1 isoforms in a doxycycline-dependent fashion in cholinergic neurons.

Although these studies are still ongoing, significant progress has been made toward accomplishing these goals. In the sections that follow, the progress that has been made on the construction and characterization will be summarized and the efforts on the construction of the NRG1-expressing lines will be evaluated. In general, the cholinergic lines of mice appear to be functioning at least by the criterion that the transactivators are being expressed in cholinergic cells and not in other cell types. However, when mated to the NRG1-expressing lines, or other reporter lines, reporter gene expression is not detected in all cholinergic neurons. The potential reasons for this incomplete pattern of gene expression are discussed in detail and possible approaches to surmount these obstacles are explored.

1. The generation of BAC transgenic mice for rtTA/tTA-dependent gene regulation in cholinergic neurons

1.1. The use of pBelo11 ChAT BAC to achieve cholinergic-specific gene expression

At the time these studies were initiated, two attempts had been made to target transgene expression to cholinergic neurons in the mouse (Lonnerberg et al., 1995; Naciff et al., 1999). In both cases relatively small genomic fragments of 2.3kb and 6.4kb located in the 5' region of the ChAT gene were used. Based on the patterns of transgene expression observed in their lines, it appeared that important regulatory elements were not provided by these small putative promoter regions. In order to overcome the problems associated with promoters of insufficient size, it was thought that the large BAC-based transgenes could provide cholinergic transgene expression in the mouse, as this methodology had been used successfully to mimic the expression pattern of the zinc finger transcription factor RU49 (Zipro1) (Yang et al., 1997).

The first ChAT BAC that was utilized was obtained using a PCR-based screen of a custom BAC library at Incyte Genomics. At the time, there was relatively little murine genomic sequence available and the associated map of overlapping BAC clones was not generally available. Using primers that were located within the VAChT gene, the screen was intentionally biased towards the 5' end of the gene, in order to increase the likelihood that the resulting BAC contained sufficient amounts of promoter sequence to provide cholinergic-specific regulation. The identified BAC, pBelo11ChAT BAC, contained about 96 kb of promoter sequence as well as most, but not all of the ChAT gene. It was hoped that this large amount of 5' sequence would be capable of directing rtTA2^{S}-M2 expression to cholinergic neurons, as most regulatory elements that are required for proper expression of a gene are located in its 5' region along with intronic enhancers, which are usually located within the first intron (Caroni, 1997; Chan et al., 1999; Uveges et al., 2002).

Homologous recombination in bacteria was used to perform 3 separate manipulations on this BAC. First, it was used to insert the rtTA2^{S}-M2 cDNA into the start codon of the ChAT gene, next is was used to inactivate the VAChT gene and in the final step it was used to remove a loxP site from the pBelo11 vector backbone. Pronuclear injection of circular BAC DNA resulted in 13 founder animals, of which 11 were able to transmit the BAC transgene to the F1 generation. The use of circular BAC DNA for pronuclear injection was expected to give

rise to multiple lines with varying expression patterns, due to random breakage of the BAC during integration into the mouse genome. However, none of the lines analyzed by *in situ* hybridization displayed a proper cholinergic expression pattern of rtTA2^S-M2.

It was reasoned that 2 factors may have contributed to the apparent lack of cholinergic expression of rtTA2^S-M2. First, it was possible that for stochastic reasons the analyzed ChAT-BAC transgenics did not display a proper cholinergic expression of rtTA2^S-M2 due to an insufficient number of founder animals analyzed. Secondly, it was conceivable that regulatory elements that are required for faithful cholinergic transgene expression were not contained on the BAC. It was speculated that the missing regulatory elements were located 3' to the ChAT gene, as no 3' flanking sequence was contained on pBelo11 ChAT BAC. Thus, it appeared likely that the use of an alternative BAC, that contained 3' flanking sequence to the ChAT gene would be better suited to direct transgene expression to cholinergic neurons. In addition to the decision to use a new BAC for the generation of transgenic animals, a switch from rtTA to the use of tTA2^S was selected for reasons that will be discussed in detail below.

1.2. Cholinergic expression of tTA2^S through the use of the RP24-70D4 BAC

Advances in BAC end nucleotide sequence annotation in the public ENSEMBL database allowed for the selection of a new BAC, RP24-70D4, that harbored the complete ChAT gene as well as roughly equal amounts of 5' and 3' flanking sequence to the ChAT gene. This BAC was engineered by homologous recombination in bacteria using manipulations analogous to those used with the pBelo11 ChAT BAC. For the subsequent generation of BAC transgenic animals both, circular and linear BAC DNA was used.

Two ChATtTApA mouse lines, ChATtTApA #44 and #73, which were generated with linear and circular BAC DNA, displayed apparent cholinergic expression of tTA based on X-Gal staining of GFPG:ChATtTApA doubly transgenic animals. The cholinergic expression of tTA2^S in these lines was confirmed by *in situ* hybridization and immuno-histological co-localization of ChAT and transgene-derived EGFP. Next, BAC copy number was determined for both lines by Southern blotting, which revealed that line #44 had 3 copies of the BAC integrated into its genome, while line #73 harbored 5 copies. It remains to be shown whether the higher BAC copy number in line #73 is correlated with a stronger transgene expression from the tTA-responsive P_{tet} promoter in the GFPG3 or P_{tet}-bi NRG1 lines. Both lines differ,

however, in their expression of tTA in cells that appear to be Bergmann glia in the cerebellum: While line ChATtTApA#44 tTA expression can be found in this cell type, line #73 lacks tTA expression in these cells. The fact that a subset of Bergmann glia appears to be cholinergic is a novel finding. Immuno-histochemical co-localization of ChAT and transgene-derived EGFP was used to verify the cholinergic nature of these cells to exclude ectopic expression of the ChAT-BAC transgene in line ChATtTApA#44. The number of cholinergic Bergmann glia as well as the extent of tTA expression in these cells will be determined in future studies. The functional consequences of this difference between the 2 mouse lines for the study of NRG1 function remains to be evaluated.

2. Generation of inducible Ptet-bi NRG1 transgenic mice

The generation of inducible NRG1-expressing mice was the second set of transgenes that was needed to study the potential ARIA- and GGF-like properties of NRG1 type I and type III, respectively. For the generation of the tTA/rtTA-responsive expression constructs that would allow for the inducible overexpression of these NRG1 isoforms, the parental pGFPlacZ plasmid, which had been successfully used for the generation of the GFPG3 mouse line, was chosen (Krestel et al., 2001). As conventionally-sized transgene constructs are often influenced by positional effects upon integration into the mouse genome, the resulting P_{tet}-bi NRG1 founder animals had to be analyzed with regard to these issues. Positional effects can have a profound influence on the activation of the P_{tet} promoter by tTA as well as on the expression pattern of the transgene. Therefore, the well characterized α-CaMKII-tTA mouse line was used to assess tTA-mediated induction of transgene expression in the resulting founder animals and to exclude that positional effects spatially restrict the expression of the transgene in brain. Although this assessment is biased towards the functionality of the Ptet-Bi transgene in predominantly principal neurons of the forebrain, it appeared to be a reasonable approach to address transgene functionality.

The screen of 9 founder animals identified one P_{tet}-bi NRG1 type I line, IgbetaBi#24, which displayed a stringent tTA-dependent expression of the transgene based on RT-PCR analysis and whose expression pattern was not influenced by positional effects. This line was subsequently mated to mice of the ChATtTApA#44 line to address its functionality in cholinergic neurons of the CNS. It was determined that with succeeding backcrosses of the IgbetaBi#24 line to C57Bl6 mice, fewer cholinergic cells were found to express transgene-

derived lacZ. In some cases, no lacZ expression was detected in the brains and spinal cords of doubly-transgenic animals, indicative of a functional limitations of either the ChATtTApA mouse line or the IgbetaBi#24 mouse line. This phenomenon was also observed in ChATtTApA:GFPG3 doubly transgenic animals and will be discussed below.

The generation and identification of a P_{tet}-bi NRG1 type III line that was characterized by a stringent control of transgene expression through dox administration, proved to be less efficient. Out of 8 lines analyzed, one line, SMDFBi#26, displayed a α-CaMKII-tTA-like expression pattern. However, doxycycline-dependent repression of transgene expression was incomplete in this mouse line, despite the high concentrations of doxycycline (6mg dox/g food) used. Interestingly, the continuous administration of dox throughout embryogenesis into adulthood lead to a complete repression of transgene expression in this line. Considering the fact that the lacZ expression pattern appears to be α-CaMKII-tTA-like, transactivation of P_{tet}-bi by nearby enhancers does not seem to be the underlying cause for the observed incomplete repression. When line SMDFBi#26 was crossed to ChATtTApA#44 animals, the lacZ expression pattern in doubly-transgenic animals (Fig. 22D), suggested that this line was also, like IgbetaBi#24, characterized by a lack of lacZ-positive cells.

3. Expression analysis of ChATtTApA animals using the GFPG3 mouse line

The use of the GFPG3 reporter line to analyze $tTA2^S$ or $rtTA2^S$-M2 expression in ChAT-BAC transgenic animals was thought to be an efficient way to assess transgene expression. This reporter mouse line expresses both EGFP and lacZ upon transcriptional activation by $tTA2^S$ or $rtTA2^S$-M2 and can thus be used for an initial characterization of transgene expression by X-Gal visualization and also for co-localization studies with EGFP and ChAT-specific antibodies.

However, the characterization of ChATtTApA:GFPG3 doubly transgenic animals from both ChATtTApA lines revealed a discrepancy between the number of tTA-positive cholinergic cells seen by *in situ* hybridization and the number of cells seen by X-Gal-based detection. This discrepancy appeared to increase with each backcross of the ChATtTApA animals to the C57Bl6 background resulting in an almost complete loss of lacZ-positive cells in some animals. There are a number of mechanisms that could account for the apparent lack of lacZ-positive cells in ChATtTApA:GFPG3 doubly transgenic animals: First, it was conceivable

that the GFP3 reporter mouse line was not competent to monitor tTA expression in cholinergic neurons and as a result functional β-galactosidase was not being made by that specific neuronal subset. This hypothesis is based on the fact that the GFPG3 reporter mouse line was characterized through the use of the α-CaMKII-tTA mouse. The α-CaMKII-tTA transgene, however, is not expressed in cholinergic neurons so that the functionality of the GFPG3 line in cholinergic neurons could not be assessed. Another possibility is that the ChAT promoter could not direct the production of a sufficient number of tTA transcripts and molecules of tTA protein to obtain reliable reporter expression. Thus, the ChAT promoter could be viewed as being "weaker" than promoters such as that for the α-CaMKII gene used for the generation of the α-CaMKII-tTA mouse line. However, the analysis of ChATtTApA#73:GFPG3 doubly transgenic animals by tTA- and lacZ-specific ISH revealed that tTA transcripts could be readily detected, but lacZ transcripts were only found in few cells (Fig. 19). In light of this finding, a third possibility is that lacZ expression may be absent or reduced due to events leading to the epigenetic silencing of its expression. A number of studies have suggested that methylation of CpG dinucleotides within the lacZ reporter gene might be responsible for the observed 'silencing' effect (Chevalier-Mariette et al., 2003). Methylation of a CpG dinucleotide occurs at position C5 of the cytosine (5meCpG) and is the only known epigenetic modification in mammals. The DNA methylation machinery in mammals is comprised of two components, the DNA methyltransferases (DNMTs) and methyl-CpG binding proteins (MDBs). DNA methylation is catalyzed by three DNMTs: DNMT1 and DNMT3b, which are essential for embryonic development, and DNMT3a. Mice lacking DNMT3a die within a few weeks of birth (Robertson, 2005). *De novo* methylation can occur at foci from which the methylation spreads to neighboring regions. 5meCpGs are bound by MDBs, which are associated with histone deacetylases (HDACs). Histone deacetylation in turn leads to chromatin remodeling processes, which result in transcriptional silencing of the affected region.

Experiments by Chevalier-Mariette et al. elegantly demonstrate that the expression of a YAC-lacZ transgene is strongly correlated with the CpG content of the lacZ gene (Chevalier-Mariette et al., 2003). Bacterial derived lacZ, which was also used for the generation of the GFPG3 mouse line, contains 291 CpGs per 3076bp corresponding to a relative frequency of 9.24%. The use of synthetic lacZ genes, in which the occurrence of CpG dinucleotides was decreased to 1.6% and 0.06% inversely correlated with the expression levels of the gene. It is

certainly possible that these epigenetic modifications are contributing to the observed silencing of lacZ expression in ChATtTApA:GFPG3 doubly transgenic animals.

In a related finding by Sutherland et al. varying lacZ expression was oberserved within the same litter of globin-lacZ transgenic animals ranging from strong (high-expressors) to no detectable lacZ activity ('non-expressors') (Sutherland et al., 2000). Interestingly, lacZ expression could be reestablished in non-expressors by crossing the mice into a different mouse strain. The finding by Sutherland et al. could potentially explain the functional lacZ expression in ChATtTApA#44:GFPG3 and ChATtTApA#73:GFPG3 doubly transgenic animals during the initial characterization: Both founder animals had not been backcrossed onto the C57Bl/6J background and were still CByB6F1 hybrids. Backcrossing the founder animals to C57Bl/6J animals for 5 generations and thereby reducing the CByB6F1 background to ~3% could explain a reversion to a silenced lacZ gene, which was observed during later stages of the characterization of both lines. It has been suggested that the *ssm1* locus (strain-specific modifier 1), could be the underlying cause leading to a transgene methylation and subsequent silencing in C57Bl6 mice (Padjen et al., 2005). *Ssm1* has been mapped to the distal arm of chromosome 4 in the mouse genome and its activity has been linked to enhanced transgene methylation. The gene or genes encoded by the *ssm1* locus have not been identified yet, but it is believed that *ssm1* does not encode DNMTs, but rather 'monitors' evoked chromatin changes that are induced by the presences of repetitive elements or bacterial DNA (Padjen et al., 2005). In the case examined *ssm1* activity lead to a strong methylation of a transgene, which resulted, through the recruitment of MDBs and HDACs, in its silencing. However, if the same transgene was crossed into a different strain of mice, transgene methylation was minimal, resulting in functional expression. It is therefore conceivable that *ssm1* activity influences the expression of the P_{tet}-bi NRG1 lines as well as the expression of the GFPG3 transgene in cholinergic neurons. As mentioned earlier, promoter strength might be an important determinant for transgene function and it is possible that the use of a strong promoter, like the α-CaMKII promoter, prevents *ssm1* from directing methylation to the transgene. The use of alternative mouse strains, such as the CBA/Ca strain, for the maintenance of the GFPG3 reporter line, will help to provide further insight into the strain-specific influence of C57Bl6 mice on reporter gene expression. CBA/Ca mice have been reported to result in lower transgene methylation levels and may thus be better suited for the characterization of transgenic lines with weaker promoters, such as, presumably, the ChAT promoter (Sutherland et al., 2000).

Immuno-histological detection of transgene-derived EGFP in ChATtTApA:GFPG3 doubly transgenic animals suggested that the expression of EGFP was not affected by this potential silencing effect so that despite the lack of lacZ expression, doubly transgenic animals could still be used for co-localization of ChAT and EGFP. It cannot be excluded, however, that in succeeding generations of backcrosses to C57Bl6 mice EGFP expression will also be affected.

It is very well possible that due to the potential reasons mentioned above, the use of the GFPG3 reporter line in C57Bl6 mice is limited to the use of strong promoters driving the expression of tTA and that for the characterization of transgenic mouse lines in which the expression of tTA is under transcriptional control of a weaker promoter, GFPG3 mice have to be maintained in a mouse strain other than C57Bl6.

It is also possible that the ChATrtTA2^{S}-M2:GFPG3 doubly transgenic animals were also affected by these silencing effects, which could explain the absence of lacZ-positive cells in the slice culture experiment (Fig. 6).

Despite the factors that influence lacZ expression in ChATtTApA:GFPG3 doubly transgenic animals, a subset of the doubly transgenic embryos allowed for the visualization cholinergic neurons in whole mount X-Gal stainings. According to database searches this is a novel approach to visualize cholinergic neurons in the developing mouse embryo, without the need for subsequent immuno-histochemical procedures or microscopy to visualize cholinergic cells.

3.1. The use of circular versus linear BAC DNA for transgenesis

For these studies both circular and linear BAC DNA was used for the generation of transgenic animals. The use of linear or circular BAC DNA is associated with a distinct set of technical problems that arise during purification and subsequent handling of the DNA as well as during the integration process into the genome.

Circular BAC DNA is easily purified through standard DNA purification procedures and due to the fact that intact BAC DNA is supercoiled, subsequent handling of the DNA by pipetting does not lead to significant shearing of the DNA. The major problem associated with the use of circular BAC DNA arises during its integration into the genome. Random breakage of the BAC DNA during integration into the genome can result in linear BAC DNA fragments in which regulatory elements from the 3' end of the gene are placed 5' to the gene and vice

versa. The resulting circular permutation can have profound influence on transgene expression. During the characterization of the ChATtTApA-BAC transgenics that were generated with circular BAC DNA, circular permutation, that was cuased by random breakage of the BAC during the integration into the genome, lead to varying expression patterns of tTA. In one animal tTA expression was detected in basket cells of the cingulate cortex, while in others incomplete cholinergic expression of tTA was observed.

The use of linear BAC DNA does not lead to circular permutation, but the purification and handling of linearized DNA results in DNA damage through shearing. This is evident on the PFG presented in Fig.8: Based on DNA concentration as determined by OD_{260}, 300ng of BAC DNA were loaded per gel lane, but relative to the linearized λ-DNA, which was used as a standard, it is evident that only ~75ng of full length linear BAC DNA are present. Most of the DNA appears to be sheared and is visible as a 'smear' of lower molecular weight.

The presence of sheared BAC DNA can impose problems on the production of BAC transgenic animals, if its concentration is considerably high. The sheared DNA will contribute to the production of transgenic animals in the same way as full length DNA and thus can increase the number of founder animals that need to be screened to achieve faithful transgene expression. Based on the results obtained in this thesis, the use of both circular and linear BAC DNA appears to be equally well suited for the generation of transgenic animals through pronuclear injection. It is not possible to favor one of the two topological isomers as in both cases the frequency with which ChAT $tTA2^S$ BAC transgenic animals were generated was about the same (1:8 for linearized BAC DNA and 1:6 for circular BAC DNA).

3.2. Functional consequences of the blood brain barrier for doxycycline-dependent regulation of transgene expression

Despite the ectopic expression of rtTA in ChATrtTA BAC transgenics, transcriptional activation of the bi-directional tTA/rtTA-responsive promoter P_{tet}-bi should be possible in the presence of dox. However, none of the lines examined displayed lacZ-positive cells in the brain or spinal cord, even though high doses of dox were simultaneously administered to ChATrtTA2^S-M2:GFPG3 doubly transgenic animals in both food and drinking water (6mg dox/g food and 2mg dox/ml drinking water). This was an unexpected finding as previous work had demonstrated that through the use of rtTA inducible transgene expression in the brain could be achieved in dox-dependent fashion (Mansuy et al., 1998). The published

mouse line was claimed to be able to induce transgene expression in doubly transgenic animals upon administration of 6mg dox/g food for 6 days. The rtTA used for the generation of this mouse line was the first generation rtTA, which requires about 10-fold higher dox concentrations than rtTA2^{S}-M2 and it is thus even more surprising that no lacZ-positive cells could be detected in ChATrtTA2^{S}-M2:GFPG3 doubly-transgenic animals, even after administration of dox in food and drinking water for 2 weeks.

Work form Prof. Bujard's laboratory (ZMBH, Heidelberg) indicated that the underlying cause for of rtTA's inability to activate transgene expression in the CNS was most likely not due to an insufficient number of founder animals analyzed, but rather attributable to insufficient amounts of dox within the CNS caused by the blood brain barrier (Dr. S. Berger, personal communication and Berger, 2003). Measurements conducted with luciferase reporter:α-CaMKII-rtTA2^{S}-M2 doubly-transgenic animals, which were fed 2mg dox/g food, revealed that the effective dox concentration in the brain reaches ~0.5ng/ml, a concentration at which the transcriptional transactivation potential of rtTA2^{S}-M2 is less than 0.1% of its maximum. The measurements also indicated that a theoretical 10-fold increase of the dox concentration in the brain would only result in induction levels of <1% for rtTA2^{S}-M2.

In light of these findings, tTA appears to be the transactivator of choice to achieve doxycycline-dependent transgene expression in the CNS. Both tTA and tTA2^{S} are characterized by a roughly 10-fold greater sensitivity towards dox than rtTA and rtTA2^{S}-M2. Therefore, dox-dependent transcriptional repression is much more effective for tTA2^{S} than transcriptional induction through rtTA2^{S}-M2. Whereas 0.5ng/ml dox only induce a transcriptional activation of <0.1% by rtTA2^{S}-M2, transcription is repressed to roughly 16% at this concentration by tTA2^{S}. It appears likely that the transcriptional repression can be further enhanced through the use of higher dox concentrations, as an increase in the effective dox concentration in the brain to 1ng/ml would reduce tTA2^{S}-mediated transcription to roughly 5%. However, higher dox concentrations can have adverse effects on embryonic development and re-induction of transgene expression. Recent work has shown that dox administration in the food at concentration >2.5mg dox/g food can result in placental abnormalities and that these abnormalities increase with increasing dox concentrations in the food (Moutier et al., 2003). The placental defects can become very severe at dox concentrations of 10mg/g food leading to early embryonic death. It has been suggested that the toxic effects of dox are most likely to an inhibition of proinflammatory cytokines and matrix metalloproteinases (Moutier et al., 2003). The adverse effects were not observed, when dox was administered in the drinking water in a range of 1-5mg/ml.

In addition to the effects on placental development, prolonged administration of dox, that is needed to study the effects of regulated transgene expression in adult mice, can lead to accumulation of dox in white matter. The stored dox is released upon dox withdrawal, which results in a slower re-induction of the transgene in brain regions of high myelin content. This can potentially lead to a non-uniform re-induction of the transgene.

3.3. Outlook

3.4. Future experiments

3.5. Generation of additional P_{tet}-bi NRG1 lines

Future experiments will be focused on the generation of additional P_{tet}-bi NRG1 type III transgenic mouse lines as well as on the further characterization of mouse line IgbetaBi#24. NRG1 type I expression in this mouse line will be assessed by immuno-histochemical analyses and Western blotting with NRG1-specific antisera. P_{tet}-bi NRG1 type II mouse lines will be generated to further to help evaluate the potential involvement of this isoform in the pathogenesis of Schizophrenia. Linkage studies in an Icelandic and Scottish population mapped certain haplotypes surrounding the genomic region of the type II-specific exon to an increased risk of contracting Schizophrenia. Regulated overexpression of NRG1 type II and other NRG1 isoforms during various time points in development or in the mature animal may help to generate a mouse model that mimics certain aspects of Schizophrenia. In addition, this line will also be useful to establish, whether it is the NRG1 type I or type II isoform that serves an "ARIA"-like function *in vivo*.

Furthermore, by generating homozygous ChATtTApA transgenic animals, the effects of ChATtTApA BAC copy number on transgene expression will be addressed. An increase of tTA expression could potentially overcome the incomplete lacZ expression observed in GFPG3 mice as well as in P_{tet}-bi NRG1 lines. In parallel, new transgene constructs for the inducible overexpression of NRG1 type I and type III will be generated, that no longer harbor the bacterial lacZ gene, but a synthetic CpG-free lacZ reporter gene. This will increase the likelihood for the P_{tet}-bi NRG1 lines not to be influenced by epigenetic phenomena, such as CpG methylation. Furthermore, alternative mouse strains, such as CBA/Ca, will be used for

the maintenance of the GFPG3 reporter mouse line to address the influence of *ssm1* on transgene methylation. If *ssm1* activity in C57Bl6 mice is indeed the underlying cause for the observed discrepancy in the number of tTA-expressing cells versus lacZ-positive cells observed, then the use of CBA/Ca mice should result in a re-activation of the GFPG3 transgene in GFPG3:ChATtTApA doubly transgenic animals.

In an attempt to minimize the number of P_{tet}-bi NRG1 founder animals that are needed to be screened to identify transgenics that are characterized by a stringent tTA-dependent transgene expression, the recently identified "LC1" locus will be utilized (Schonig et al., 2002). This locus was identified during the generation of a P_{tet}-Cre mouse line that displayed a tight transcriptional control of *Cre* recombinase expression by tTA (Schonig et al., 2002). A corresponding BAC that harbors the genomic LC1 locus was identified by Dr. Bujard's laboratory. The BAC as well as a targeting construct for homologous recombination in bacteria for the LC1 locus were obtained from Dr. Bujard and may facilitate the generation of inducible NRG1 transgenic mouse lines that are characterized by a stringent doxycycline-dependent induction of NRG1 expression.

4. Alternative uses of ChATtTApA mice

Next to the use of the ChATtTApA mouse lines in helping to understand NRG1 function in the adult animal, the mice will be a useful tool in neuroscience research. Mouse line ChATtTApA#44 is used in a collaborative effort by Dr. D. Isbrandt (ZMNH, Hamburg) to study the role of KCNQ2/KCNQ3 (M-channel) potassium channels in the pathogenesis of a disease known as benign familial neonatal convulsions (BFNC). BFNC is characterized by seizures that start in the first postnatal week, but spontaneously disappear after several weeks. It has been reported though that the disease progression resulted in a poor prognosis for 10-16% of the patients where it persisted into adulthood with reoccurring seizures.

Thus far, M-channel function has been studied through the regulated overexpression of dominant-negative M-channel pore mutants for which a prion protein promoter-tTA transgenic mous line was used (Prnp-tTA) (Tremblay et al., 1998; Peters et al., 2005). However, KCNQ2/KCNQ3 appear to be predominantly expressed in cholingeric neurons of the CNS, so that a cholinergic-specific overexpression of the M-channel mutants is thought to provide a better understanding of their involvement in BFNC (Cooper et al., 2001).

The ChATtTApA mouse lines might also be a valuable tool for studying the neuropathology of Alzheimer's disease, as cholinergic neurons of the basal forebrain are severely affected in Alzheimer's patients. Current mouse models, that mimick neuropathologic hallmarks of the disease were generated through the expression of mutant human amyloid precursor (hAPP) driven by the platelet-derived growth factor-β promoter or through CMV promoter driven expression of α-NGF antibodies (Games et al., 1995; Capsoni et al., 2000). The use of ChATtTApA mouse lines for a regulated and cholinergic overexpression of mutant hAPP or α-NGF antibodies might facilitate the understanding of the pathology in Alzheimer's disease.

V. Materials

1. Chemicals and laboratory supplies

All chemicals and laboratory equipment and supplies used were purchased from Fisher Scientific, VWR or Sigma-Aldrich, unless specified otherwise:

1.1. Laboratory equipment

CCD B/W camera "Orca ER"	Hamamatsu, Bridgewater, NY
Coffee maker	Krupps, Medford, MA
Computer software:	
Adobe Photoshop 7.0	Adobe, San Jose, CA
Acrobat Reader 6.0	Adobe, San Jose, CA
Canvas 8	Deneba Systems, Inc., Miami, FL
DNAStrider 1.3	CEA, France
DNAStar 5.5	DNAStar, Inc., Madison, WI
Endnote 6.0	Thomson ResearchSoft, Carlsbad, CA
Firefox web browser	Mozilla Foundation, Mountain View, CA
iTunes 4	Apple, Cupertino, CA
Keynote	Apple, Cupertino, CA
Microsoft Office X	Microsoft, Redmond, WA
Openlab 3.1.7	Improvision, Lexington, MA
RasMol v2.7	www.openrasmol.org
Safari web browser 1.2.4	Apple, Cupertino, CA
X11	Apple, Cupertino, CA
Computer hardware:	
Apple iPOD 20GB	Apple Cupertino, CA
Apple PowerBook G4	Apple, Cupertino, CA
Apple G4, 800Mhz	Apple, Cupertino, CA
Apple G4, dual 1Ghz	Apple, Cupertino, CA
HP Color LaserJet 4600	Hewlett-Packard, Palo Alto, CA
Digital camera, Nikon CoolPix 4200	Nikon Corp.
Electrophoresis chambers	JM Specialty Parts, San Diego, CA
Eppendorf Mastercycler Gradient PCR machine	Eppendorf, Westbury, NY
Eppendorf Thermomixer compact	Eppendorf, Westbury, NY
GenePulserII	BioRad, Hercules, CA
Gilson Pipetman P10, P20, P200, P1000	Gilson Inc., Middleton, WI
Microscope Olympus BC-50	Olympus, Melville, NY
Microwave oven	Samsung
Multichannel pippettor 1-10µl, 10-100µl	Eppendorf, Westbury, NY
Photospectrometer Beckman DU 7400	Beckman Coulter Inc., Fullerton, CA
Polytron	GlennMills, Clifton, NJ
Scintillation counter Beckman LS6500	Beckman Coulter Inc., Fullerton, CA

Video imaging System “EagleEye”	Stratagene, La Jolla, CA
X-ray film developer KODAK XOMAT	Kodak, Rochester, NY

1.2. Laboratory supplies

Cryo-vials	Nalgene, Rochester, NY
DNeasy Tissue kit	Qiagen, Inc., Valencia, CA
Filter units 0.22µm, 500ml	Nalgene, Rochester, NY
MinElute Gel Extraction kit	Qiagen, Inc., Valencia, CA
Nucleobond AX-500 colums	BD Biosciences, San Jose, CA
Nucleobond buffer set	BD Biosciences, San Jose, CA
Nylon membrane “HyBond”	Amersham-Pharmacia, Piscataway, NJ
PVDF membrane “Immobilon P”	Millipore, Billerica, MA
Qiaquick Gel Extraction kit	Qiagen, Inc., Valencia, CA
Qiagen Plasmid Purification kits	Qiagen, Inc., Valencia, CA
Rapid DNA Ligation Kit	Roche, Indianapolis, IN
RNALater, RNA stabilization reagent	Ambion, Austin, TX
RNeasy Mini, Midi kits	Qiagen Inc.,Valencia, CA
rNTPs	Promega Corp., Madison, WI
Sequencing gel mix “Long Ranger”	BioWhittaker, Walkersville, MD
Syringe filters, 0.22µm	Nalgene, Rochester, NY
VECTASTAIN ABC Kit	VectorLabs, Burlingame, CA
VECTASHIELD HardSet Mounting Medium	VectorLabs, Burlingame, CA
Water, molecular biology grade	Eppendorf, Westbury, NY
Zeocin	Invivogen, San Diego, CA

1.3. Enzymes

Alkaline phosphatase, CIP	New England Biolabs, Beverly, MA
HotStartTaq MasterMix	Qiagen, Inc., Valencia, CA
ProofStart Taq polymerase	Qiagen, Inc., Valencia, CA
Restriction enzymes	New England Biolabs, Beverly, MA Roche, Indianapolis, IN
RNAseH	New England Biolabs, Beverly, MA
RNAsin, RNAse inhibitor	Promega Corp., Madison, WI
RQ1 DNAse, RNAse-free	Promega Corp., Madison, WI
SuperScriptII, III	Invitrogen, Carlsbad, CA
Sp6, T3, T7 RNA polymerases	Promega Corp., Madison, WI
T4 DNA ligase	Promega Corp., Madison, WI

1.4. Antibodies

Goat α-ChAT IgG (polyclonal)	Chemicon International, Temecula, CA
Rabbit α-EGFP IgG (polyclonal)	BD Biosciences, San Jose, CA
Donkey-α-goatIgG biotin-SP-conjugated, AffiniPure	JacksonImmuno, West Grove, PA
Alexa Fluor® 594 donkey anti-rabbit IgG	Molecular Probes, Eugene, OR
α-HA, rat monoclonal (3F10) IgG1	Roche Diagnostics, Penzberg Germany
α-Nrg1, rabbit polyclonal IgG, sc-348	Santa Cruz Biotechnolgy, Santa Cruz, CA

1.5. Mouse lines

GFPG3 (Krestel et al., 2001)	Dr. R. Sprengel, MPI for Medical Research, Heidelberg
α-CaMKII-tTA (Mayford et al., 1996)	Dr. M. Mayford, The Scripps Research Institute, La Jolla, CA

1.6. Bacterial strains

E.coli XL-1 blue	Stratagene, La Jolla, CA
E.coli DH10B	Invitrogen, Carlsbad, CA
EL250, EL350	Dr. N. Copeland, NCI Frederick, MD

1.7. Oligonucleotides

Oligonucleotides used for genotyping:

Standard primers:	2285: 5'-ATGAAGCTTCCCAGTCCAAATGAC
	2286: 5'-CCTATTGGAGTCAATTCTTGTTCTGGAT
rtTA:	3019: 5'-AGGCGAGTCATGGCAAGACTTTCTG
	2259: 5'-GGCAGGTTCGGCTCCCTGCCGGTC
htTA:	2321: 5'-CCGGGATGGTGCCAAAGTTCAC
	2322: 5'-CAGCAGTGGTGGCATGGAGTCAGT
tTA:	2413: 5'-CATCAAGTCGCTAAAGAAGAAAGGGAAACA
	2414: 5'-CCGCGGGGAGAAAGGACAGG
GFPG3:	2008: 5'-GCGGAGAGGGTGAAGGTGATGC
	2009: 5'-CAGGGCCGTCGCCGATTGG
lacZ	2384: 5'-TGGCAGGCGTTTCGTCAGTATCC
	2385: 5'-GCGGTAGTTCAGGCAGTTCAATCAA

Oligonucleotides used for homologous recombination:

BAC library screening primers (Incyte Genomics, Inc.):

1723: 5'-GGCGTCATGTTCGCCTCCACAGTCATG (s)
1724: 5'-ACGCCTAACACGTGTGGCACGAAAGCC (as)

rtTA homologous recombination, inner primer (s):

3069:5'-CCAGGTCGGCAGCTCTGCTACTCTGGATTAAGAATCGCTAGGATGTCT
AGACTGGACAA

rtTA homologous recombination, outer primer (s):

3070:5'-GCTGAGTCTCCTCTGTTCCCAGGTCGGCAGCTCTG

rtTA homologous recombination inner primer (as):

3071:5'-CAGCTAGAAGCTTGTACAGGCATCTTTGGGGGGACCTTTTCCCCA
AGCTACTCGCGACC

rtTA homologous recombination outer primer (as):

3072:5'-AAGTCCAGCACCTCCTCACAGCTAGAAGCTTGTAC

ΔVAChT inner primer (s):

3076:5'-GGAAGAGCAGCGGGTAGGGGCATGGAACCCACCTAGTGACTACT
TCACTAACAACCGG

ΔVAChT outer primer (s):

3075:5'-CCGGTGGAGGCATCTTAGGAAGAGCAGCGGGTAGG

ΔVAChT inner primer (as):

3078:5'-GGCCGCCGCCCGGGCCTGACCGGTTGGCGCGGTAGTGCCAAGCTA
CTCGCGAC

ΔVAChT outer primer (as):

3077:5'-GCTTCCGACAGCTTGGTGGCCGCCGCCCGGGCCTG

ΔVAChT screening primer:

3079:5'-CTGGAGCATCTAAGAGCAGCGGCACC

htTA homologous recombination inner primer (s)

2302:5’-CCAGGTCGGCAGCTCTGCTACTCTGGATTAAGAATCGCTAGGATG
TCCAGACTGGACAAG

ChATtTA homologous recombination screening primers:

3066:5’-CTCACGTGCCCTCTACTCTATACCTGA (s)

3068:5’-AGGTGTTGCATGCACTGAAGGTAGGT (as)

Neo/Kan anti-sense screening primer:

3045:5’-CGCGGATCCGGCGTCGCTTGGTCGGTCA (as)

Oligonucleotides used for molecular cloning of Neuregulin-1 typeI and typeIII:

2001: 5’-CTGGTAGAGCTCCTCCGCTTCCAT (as)
2002: 5’-CCAATAGCCGGCGGGCCAAAAG (s)
2003: 5’-ATAGGTACCGTCGACTAGGTTTTATACAGCAATAGG (as)
2010: 5'-GAAGCGGAGGAGCTCTACCAG (s)
2011: 5'-ATATCTCGAGGTCGACGCCACCATGTCTGAGCGCAAAGAAG (s)
2012: 5'-CTTCTGGTAGAGCTCCTCCGC (as)
2014: 5'-ATACTCGAGGTCGACGCCACCATGGAGATTTATCCCCCAG (s)
2064: 5'-ATATATGTCGACTTATACAGCAATAGGGTCTTGG (as)

Oligonucleotides used for RT-PCR:

2420: 5'-GGCTGATCAGCGAGCTCTAGCATTTC (as)
2347: 5'-GGATCTGCCTTTTCTCAGTCATGAAAGTTG (s)
2348: 5'-GCCTCCAGCCCTGCTTGGTGTGG (s)
2419: 5'-ACCCGCGGCCGCGCCACC (s)
2059: 5'-CCAGAGTCAGCCAGGGACGC (as)
2421: 5'-CTACATGGTCTACATGTTCCAGTA(s)
2422: 5'-TGATGGCATGGACTGTGGTCAT (as)
2450: 5'-GCGAGCACAGCTTCTTTGCAGC (s)
2451: 5'-CCACACGCAGCTCATTGTAGAAGG (as)

Oligonucleotides used for sequencing:

2058: 5'-GAGCCTTAGAGACAGGTGAAG (as)
2059: 5'-CCAGAGTCAGCCAGGGACGC (as)
2061: 5'-GTCCTCGTGGGCCCCCAGCTGG (as)
2104: 5'-GACGCAAATGGGCGGTAGGCG (s)
2105: 5'-TTCAGGGGGAGGTGTGGGAGG (as)
2378 5’-GTGAGGGCCATTCGCTATGTTCAC (as)

Additionally, standard sequencing primers Sp6, T3 and T7 were used.

2. Buffers and solutions

Acetylation buffer:

625µl acetic anhydride
250ml 0.1M tetraethyl ammonium

BAC-TE:

10mM Tris pH7.5
0.1mM EDTA pH8

β-gal staining buffer:

X-Gal (20 mg/ml in N,N-DMF)	600µl (f.c. 1.2 mg/ml)
K-Ferricyanid (500 mM in PBS)	100 µl (f.c. 5 mM)
K-Ferrocyanid (500 mM in PBS)	100 µl (f.c. 5 mM)
$MgCl_2$ (2 M)	10 µl (f.c. 2mM)

Add 1xPBS to 10ml.

Blocking buffer for Western blotting:

5g non-fat dry milk

add TBS-T to 100ml.

Blocking buffer for immunohistochemistry:

5%	donkey serum (heat inactivated)
0.3%	tween-20

in TBS. Filter blocking buffer prior to use.

Denaturing buffer (Southern):

1.5M NaCl
0.5M NaOH

50xDenhardt's:

1% (w/v)	Ficoll 400
1% (w/v)	polyvinylpyrrolidone
1% (w/v)	bovine serum albumin (fraction V)

DNA loading dye II:

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Ficoll (type 400)	15%

dH_20 to 10ml, store in 1ml aliquots at –20°C.

High SDS hybridization buffer:

2.1.1. 1mM EDTA, pH8

0.25M NaH_2PO_4, pH7.2
7% SDS

2.1.2. Pre-heat to 65°C prior to use.

Hybridization buffer (ISH):

25ml formamide
10ml 50% dextran sulfate
3ml 5M NaCl
1ml 50X Denhardts'
2.4 ml 1.2M phosphate buffer
0.1 ml 0.5 M EDTA
2.5 ml 10 mg/ml tRNA
1.0ml depc dH_2O

Injection buffer, conventional transgene constructs:

10mM	Tris pH 7.5
0.1mM	EDTA pH 8.0

Use molecular biology-grade water to make the buffer, sterile filter buffer through a 0.2μm filter.

Injection buffer, BAC constructs:

10mM	Tris pH 7.5
0.1mM	EDTA pH 8.0
100mM	NaCl

Use molecular biology-grade water to make the buffer, sterile filter buffer through a 0.2μm filter.

ISH perfusion mix, 500ml:

20g PFA
38.14g $NaBH_4$
2g NaOH

dH_2O to 500ml, pH 9.5

Hydrolysis buffer:

80mM $NaHCO_3$
120mM Na_2CO_3
10mM DTT

2x KCM buffer:

1.0M	KCl
0.30M	$CaCl_2$
0.50M	$MgCl_2$

sterile filter, store at RT.

0.02M KPBS:

2.91g K_2HPO_4
0.45g KH_2PO_4
18.0g NaCl
dH_2O to l000ml

LB-Medium:

Bacto Trypton	10g
Bacto Hefeextrakt	5g
NaCl	10g

Dissolve in 1000ml dH_2O, adjust pH to 7.5 with 10M NaOH, autoclave.

For selective LB media the following antibiotics were used:

Ampicillin, f.c. 100µg/ml
Chloramphenicol, f.c. 20µg/ml
Kanamycin, f.c. 50µg/ml
Tetracycline, f.c. 12.5µg/ml
Zeocin, f.c. 25µg/ml

LB plates:

LB-medium
1.2% Bacto-agar

Autoclave, cool to ~55°C in a waterbath, add desired antibiotic. Store plates at 4°C.

LDS sample buffer:

106mM	Tris HCl
141mM	Tris base
2%	lithium dodecyl sufate (LDS)
10%	glycerol
0.51mM	EDTA, pH8.0
0.22mM	Serva Blue G250
0.175mM	phenol red

10x modified Gitschier buffer (MGB):

6.7ml	1M Tris pH 8.8
1.66ml	1M $(NH_4)_2SO_4$
650µl	1M $MgCl_2$

add PCR-grade dH_20 to 10 ml

For 10ml of 1xMGB combine:

1ml	10xMGB
100µl	β-mercaptoethanol
500µl	10% Triton X-100
8.4ml	PCR-grade dH_2O

0.9% NaCl solution:

9g	NaCl

add dH_2O to 1000ml, filter solution.

Neutralization Solution (ISH):

200mM sodium acetate
170mM glacial acetic acid
10mM DTT

Neutralization Solution (Southern):

1.5M	NaCl
1M	Tris, pH7.5

20x NuPAGE MES SDS running buffer:

97.6g	MES
60.6g	Tris base
10g	SDS
3.0g	EDTA

Dissolve in 500ml dH_2O, store at 4°C.

20x NuPAGE transfer buffer:

81.6g	Bicine
104.6g	Tris base
6g	EDTA

Dissolve in 1000ml dH_2O, store at 4°C.

1x NUPAGE transfer buffer:

50ml	20xNUPAGE transfer buffer
100ml	100% methanol

Add dH_2O to 1000ml.

Mowiol 4-88:

2.4g	Mowiol 4-88
6.0g	glycerol
6.0ml	dH_2O
12.0ml	0.2M Tris, pH8.5

Mix glycerol and Mowiol 4-88 thoroughly. Add dH_2O and incubate for 2 hours at RT, add Tris and incubate at 55°C until the Mowiol 4-88 has dissolved. Clear solution by centrifugation (20 minutes, 5000g). Aliquot into 1ml samples, store at –20°C.

Buffer P1:

50mM Tris-HCl, pH8.0
10mM EDTA
100µg/ml RNAseA

Buffer P2:

200mM NaOH
1% SDS

Buffer P3:

3.0M potassium acetate, pH5.5

10x PBS:

NaCl	100g
KCl	2.5g
Na_2HPO_4 x $2H_2O$	7.2g
KH_2PO_4	2.5g

dH_2O to 1000ml, dilute 1:10 for 1xPBS

1.2M Phosphate buffer:

0.6M NaH_2PO_4
0.6M Na_2HPO_4

Mix in a 1:1 ratio, sterile filter.

Phosphate buffer, according to Soerensen:

Solution A:
27.6g $NaH_2PO_4*H_2O$, dH_2O to 1l

Solution B:
57.2g Na_2HPO_4, dH_2O to 1l

Combine 115ml solution A, 385ml solution B for 500ml of a 0.2M buffer solution.

4%PFA, phosphate buffered, pH7.4:

Disolve 20g PFA in 230ml dH_2O and add 1g NaOH pellets. Heat carefully in microwave to 65˚C. Add dH_2O to 250ml. Add 57.5ml solution A and 192.5ml solution B.

Proteinase K buffer:

25ml 1M Tris, pH 8.0
250µl proteinase K (10mg/ml, f.c. 10µg/ml)
25ml 0.5M EDTA, pH 8.0
to 250ml dH_2O

SOC-Medium:

NaCl	10mM
KCl	2.5mM
MgSO4	10mM
Bacto Pepton	2%
Bacto yeast extract	0.5%
Glucose	20mM

Autoclave and store at –20°C in 50ml aliquots.

Slice culture medium:

For 100ml:

10ml	10x Gibco#79-01012
25ml	horse serum
25ml	Hank's
3.5ml	glucose (@ 200mg/ml, f.c. 6.5mg/ml)
1ml	glutamax
1ml	PenStrep
34.75ml	dH_2O (sterile)

sterile filter and store at 4°C.

20x SSC:

0.3M	sodium citrate
3M	NaCl

dissolve in 800ml dH_2O, adjust pH to 7.0, bring to 1000ml. Autoclave.

50x TAE:

Tris-Base	242g
Gliacil acetic acid	57.1ml
EDTA (0.5 M, pH 8)	100ml
dH_2O to 1000ml	

10x TBE:

Tris-Base	108g
Boric acid	55g
EDTA (0.5 M, pH 8)	40ml
dH_2O to 1000ml, filter solution	

20x TBS:

Tris-Base	1M
NaCl	3M

Dissolve in 700ml dH_2O, adjust pH to 7.4 with HCl, add dH_2O to 1000ml.

TBS-T:

1xTBS supplemented with Tween20, f.c. 0.02%

TE:

10mM	Tris pH7.5
1mM	EDTA pH8

TENS buffer:

50mM Tris HCl pH8.0
0.1M EDTA
0.1M NaCl
1%SDS

TSB:

LB	73.5ml
50% PEG (MW 3350)	20ml, f.c. 10%
100% DMSO	5ml, f.c. 5%
2M $MgCl_2$	500µl, f.c. 10mM
1M $MgSO_4$	1ml, f.c. 10mM

sterile filter, store at 4°C.

WB stripping buffer:

100mM	2-mercaptoethanol
50mM	Tris-HCl, pH 6.8
2%	SDS

VI. Methods:

1. DNA transfer into *E.coli*

1.1. Generation of competent *E.coli*

1.1.1. Generation of electro-competent cells

An overnight culture of the desired bacterial strain (DH10B, XL-1 blue, EL250, EL350) was inoculated from a single colony or directly from a glycerol stock into 4ml of LB or LB-tet (10µg/ml) medium (for Xl-1 blue). Typically, 50ml of LB medium were inoculated with 500µl of the ON culture. Cells were then grown at 37ºC (or 32ºC for EL250, EL350) to an OD_{600}~0.5. The cells were then spun down at 5000g for 10min at 2-4ºC. The supernatant was then carefully removed and the cells were then re-suspended on ice in 50ml of ice-cold 10% glycerol/dH_2O. The wash was repeated 2 more times and after the final wash the cells were re-suspended in 100-150µl of 10% glycerol/dH_2O and snap-frozen in liquid nitrogen. The electro-competent cells were stored at -70ºC. The competence of the cells was tested by transformation of super-coiled pUC18 plasmid DNA and usually resulted in $5x10^7$ – $8x10^8$ transformants/µg pUC18 DNA.

1.1.2. Generation of electro-competent and recombination-competent EL250, EL350

To obtain recombination-competent EL250 or EL350, 50ml cultures were grown in LB medium (see 1.1.1) to an OD_{600}~0.5. At this point the bacteria were heat-shocked in a water bath at 42C for 15min under slight agitation to obtain a uniform heat-shock. The cells were then chilled on ice for 15min, washed and frozen as described (see 1.1.1). The competence of EL250, EL350 cells was usually comparable to electro-competent DH10B. As a control, a second 50ml culture of EL250/350 was grown and processed in parallel, but was not heat-shocked.

1.1.3. Generation of chemically-competent *E.coli* Xl-1 blue

A 4ml ON culture of E.coli XL-1 blue was grown in LB-tet medium (10µg/ml) and a 200ml culture was inoculated with the ON culture (1:100). The cells were then grown to OD_{600}~0.4 and spun down in a GSA rotor at 5000g. The supernatant was discarded and the cells were re-suspended in $1/15^{th}$ of the volume in TSB and incubated on ice for 10 minutes. The cells were then snap frozen in 300µl aliquots and stored at –70ºC. For the transformation of the cells, the bacteria were incubated with the DNA in 1xKCM buffer on ice for 20 minutes and then shifted to room temperature for 10 minutes. 800µl of LB were added and the cells were incubated at 37°C for 30-45 minutes in a shaker. 100-150µl of the bacteria were then plated on the appropriate selective LB agar plates and incubated at 37°C for 16-18 hours.
The competence of these cells was usually lower than for the electro-competent cells ($4x10^7$ – $1x10^8$ /µg pUC18 DNA), but sufficient for standard cloning procedures.

1.1.4. Electroporation of *E.coli*

Electrocompetent *E.coli* were thawed on ice and 40µl were given into a pre-cooled electroporation cuvette (gap size: 1mm) along with the DNA to be transformed. The bacteria and DNA were mixed in the cuvette, followed by a 15 minute incubation on ice. The cells were then placed into the shocking chamber of a "GenePulserII" (BioRad) and electroporated with the following settings: Voltage: 1.75kV, resistance: 200Ω, capacitance:25µF. The cells were then resuspended in 800µl – 1ml LB or SOC medium, transferred into a Falcon 2059 tube and incubated for 30-45 minutes at 37°C in an orbital shaker. The cells were then plated on LB plates containing the appropriate antibiotic. EL250/350 were incubated for 90 minutes at 32°C before being plated.
High salt concentration and PEG interfere with electroporations and have to be removed by ethanol precipitation of the DNA and several washes with 80% ethanol.

1.2. DNA isolation and purification

1.2.1. Standard DNA isolation

1.2.1.1. Small scale DNA purification, "DNA mini preps"

Small scale DNA preparations were carried out using Qiagen's "Plasmid Mini" kits following the manufacturer's instructions. The basic principle is a modified alkaline lysis protocol (Birnboim and Doly, 1979) followed by binding of the DNA to an anion-exchange resin under appropriate pH and salt conditions and subsequent wash and elution steps. In brief, 4ml ON cultures of a clone were grown in LB medium with the appropriate antibiotic. 2 x 1.5ml of each culture were then spun down into the same tube and the supernatant was discarded. The bacterial pellet was re-suspended in 250µl P1, lysed by adding 250µl P2 and neutralized by adding 350µl of P3. The preps were then spun at maximum speed in an Eppendorf table-top centrifuge for 10min to pellet cellular debris and chromosomal DNA. The supernatant was then transferred onto a Qiagen mini column and centrifuged at ~8000g for 1min. The flow-through was discarded and 750µl of buffer PE were added followed by another centrifugation step. The flow-through was discarded and the column was dried by a spin at maximum speed for 2min. The purified plasmid DNA was eluted into 50µl of dH_2O.

1.2.1.2. Large scale DNA preparations, "maxi preps"

Preparative purification of plasmid DNA was carried out using Qiagen's "Plasmid maxi kit", which is based on an alkaline lysis procedure (Birnboim and Doly, 1979) coupled to anion-exchange resin purification under appropriate low-salt and pH conditions. In this procedure contaminating RNAs and low-molecular weight impurities are removed by medium salt washes. The plasmid DNA is then eluted from the resin by a high-salt buffer and precipitated with isopropanol followed by several washes to remove residual salt from the elution buffer.

1.2.1.3. BAC DNA preparation

Small scale preparation of bacterial artificial chromosomal DNA, "BAC minis"

For the purification of small amounts of BAC DNA for BAC fingerprinting a modified alkaline lysis procedure was used (Birnboim and Doly, 1979). In brief, 4ml of LB medium with the appropriate antibiotic were inoculated from a single BAC clone or glycerol stock and grown ON at 37ºC or 32ºC (for bacterial strains EL250, EL350). 2x1.5ml of ON culture were then spun down into the same tube, the supernatant was discarded and the pellet was re-suspended in 400µl of buffer P1. The cells were lysed by adding 400µl of buffer P2 and proteins and bacterial chromosomal DNA were precipitated by adding 400µl of chilled buffer P3. The cellular debris was removed by centrifugation and the supernatant was transferred into a new tube. BAC DNA was then precipitated by adding 450µl ice-cold isopropanol followed by an incubation at –20ºC for 1h. The BAC DNA was pelleted by centrifugation (13.000g, 10 minutes) in a table-top centrifuge. The supernatant was discarded and the DNA was washed once with 1ml of 80% ethanol, air dried and re-suspended in 40µl of BAC-TE.

1.2.1.4. Large scale purification of bacterial artificial chromosomal DNA, "BAC maxis"

Larger amounts of BAC DNA were obtained by an alkaline lysis procedure (see 2.2) followed by anion-exchange resin chromatography (NucleoBond kit, Clontech). 250ml of selective LB medium were inoculated with 2.5ml of an BAC ON culture and grown for 18 hours at the appropriate temperature. Cells were spun down and either frozen away or directly processed.
Cells were re-suspended in 12ml of chilled buffer S1, lysed by adding 12ml of buffer S2. Due to the large amount of cells, the lysis time was extended to 5 minutes. 12ml of chilled buffer S3 were added and the lysate was cleared by filtration through a pre-wetted filter. The lysate was then passed onto a AX-500 tip, which was pre-equilibrated with 5ml of buffer N2 followed by 2 x 12ml washes with buffer N3. The BAC DNA was eluted from the column by two 3ml elution steps with buffer N5 heated to 55ºC. The DNA was precipitated by adding 8.4ml of isopropanol and pelleted by centrifugation at 12.000g for 25 minutes. The BAC-DNA pellet was then washed with 2x4ml of 80% ethanol, air dried and re-suspended in 200-300µl of BAC-TE.

1.2.2. Preparation of genomic DNA for Southern blotting

For the preparation of mouse genomic DNA for Southern blotting, ~200mg of tissue (liver or tail) were digested in 700µl TENS buffer supplemented with proteinase K (f.c. of 0.5µg/µl) ON at 55°C. The debris was pelleted by centrifugation (5min, 10.000g) and the supernatant was transferred to a new Eppendorf tube. An equal volume of phenol:chloroform:isoamyl alcohol (49:49:2), pH8, was added and incubated under constant agitation for 20-30 minutes. The aqueous phase was separated by centrifugation (10min, 10.000g) and transferred into a new tube. An equal volume of isopropanol was carefully pipetted onto the sample and the genomic DNA was spooled onto glas rods by stirring and twisting. The DNA was washed twice by stirring it in 70% ethanol, followed by a wash in absolute ethanol to facilitate drying. The tip of the glas rod was broken into a new tube and the DNA resuspended in 200µl TE. This method usually yielded 150-200µg of genomic DNA.

1.2.3. Extraction of DNA from agarose gels

DNA fragments between 200bp-12kb were extracted from agarose gels using Qiagen's "Qiaquick Gel Extraction kit" following the manufacturer's instructions. The Qiaquick kit is based on the ability of DNA to bind to silica-membranes under high-salt conditions at a pH ≤7.5. In brief, the desired DNA fragment was cut out of the agarose gel on a UV screen and placed into an Eppendorf tube. For each 100mg of agarose, 100µl of buffer QG were added and the sample was placed in a heat block at 50ºC until the agarose was dissolved. For DNA fragments larger than 4kb the appropriate amount of isopropanol was added to the mix prior to loading it onto the spin column. The sample was then centrifuged at 3000g in a table-top centrifuge and washed with 750µl of buffer PE. The flow-through was discarded and the column was spun dry for 1 minute at 10,000g. The DNA was then eluted by adding either dH_2O or buffer EB onto the column followed by a 1 minute incubation prior to centrifugation. Depending on the amount of DNA present in the agarose the elution volume was typically between 25-40µl. To facilitate the elution process for DNA fragments ≥4kb the dH_2O was pre-heated to 50ºC.
For the extraction of small amounts of DNA from agarose gels Qiagen's MinElute Gel Extraction Kit was used, which allows for the elution of the adsorbed DNA into a volume of 10µl dH_2O.

1.2.4. Purification of DNA fragments and oligonucleotides by spin column chromatography (gel filtration chromatography)

For de-salting or simple clean-up procedures after restriction digests with endonucleases or kinasing reactions, Amersham-Pharmacia S-200, S-300 or S-400 spin columns were used. These columns contain sepharose beads with a defined pore size and allow for the purification of DNA fragments of different sizes. Prior to use, the buffer was removed from the columns by centrifugation at 1,500g for 1min in a table-top centrifuge.
For the purification the sample volume was adjusted to at least 50µl and loaded onto the column bed followed by a centrifugation at 1,500g for 1min.

1.2.5. Purification of nucleic acids by phenol-chloroform extraction

To quantitatively remove proteins form nucleic acid solutions, an equal sample volume of phenol (buffered to pH8):chloroform:isoamyl alcohol (49:49:2) was added to the sample. For smaller DNA fragments or RNA the sample was vortexed for 30 seconds – 1 minute and then centrifuged at 15,000g in a table-top centrifuge. For the purification of genomic DNA, the sample was carefully mixed by inversion for several minutes using a rotating tube holder and subsequently centrifuged at 15,000g for 10 minutes in a table-top centrifuge. The aqueous phase was then carefully transferred into a new tube, precipitated and re-suspended in dH_2O or TE.

1.2.6. Preparation of mouse genomic DNA for genotyping

For the preparation of genomic mouse DNA for genotyping either two ear punches or 2-3mm of mouse tail were placed in a 96 well plate and 90µl of 1xMGB were added to each well and the wells were sealed with cap strips. The 96 well plate was then heated to 95ºC for 5 minutes in a PCR machine. The plate was removed from the PCR machine and allowed to cool for

several minutes prior to the addition of 10µl of proteinase K per well. The 96 well plate was then placed back into the PCR machine and incubated at 55ºC for 2 hours followed by a 5 minute heat kill at 95ºC. The plate was placed on a vortex for several seconds and then centrifuged at 2500g for 10 minutes. For PCR 1-2µl of this prep were used.

1.2.7. Concentration of nucleic acids by precipitation

A standard procedure for concentrating nucleic acids is ethanol precipitation. In this method, 1/10 of the sample volume of 3M sodium acetate (pH 5) is added and 2 to 2.5 sample volumes of absolute ethanol are added. The sample is mixed by pipetting and the nucleic acids are precipitated by an incubation at –20ºC for 1 hour. The nucleic acids are then pelleted by centrifugation in a table-top centrifuge (10 minutes, 15,000g), the supernatant is discarded. The pellet is washed twice with 80% ethanol, air dried and re-suspended in dH_2O or TE. In the case that the concentration of the nucleic acids was <100ng/ml, carrier substances such as tRNA or glycogen were added to the sample to enhance precipitation and to help visualize the resulting pellet.

1.2.8. Purification of DNA for pronuclear injection

1.2.8.1. Purification of DNA for pronuclear injection of conventional transgenes

For the generation of DNA suited for pronuclear injection, the desired construct was linearized with the appropriate restriction endonucleases and gel purified using Qiagen's "Qiaquick Gel Exctraction kit". The bound DNA was washed twice with 750µl of wash buffer PE. The column was dried by centrifugation (2 minutes at 15,000g in a table-top centrifuge). The DNA was then eluted with injection buffer and the integrity was checked by running a fraction of the eluate on a gel. The DNA concentration was determined photometrically and subsequently diluted to a concentration of 8ng/µl.
This purification protocol was used for transgene constructs for up to 12kb in size.

1.2.8.1. Linearization and purification of RPCI23, -24 BAC DNA for pronuclear injection

For the generation of linearized, full-length RPCI-23- or RPCI-24-derived BAC DNA for pronuclear injection, roughly 40µg of BAC DNA ('maxi prep' grade) were digested with the homing endonuclease PI-SceI in a 100µl reaction volume at 37°C for 12-16 hours. The enzyme was then heat-killed by an incubation at 70°C for 15 minutes. The sample volume was increased to 195µl and 5µl of a 0.25% bromphenol blue solution were added.
Using a Pasteur pipet the sample was carefully loaded onto a 4CL-B sepharose column (bed volume ≈ 5ml), which was pre-equilibrated with 4 column volumes of BAC injection buffer. The sample was allowed to enter the column bed and 1 column volume of injection buffer was loaded onto the column. One column volume in 200µl fractions was collected until the brome-phenol blue had migrated out of the sepharose matrix. The DNA concentration in each fraction was determined photometrically and the fractions with the highest DNA concentration were run on a PFGE gel next to λ-DNA standards. The fraction with the highest DNA concentration and the highest ratio of full-length BAC DNA to sheared DNA was chosen for transgenesis and diluted to 1ng/µl in BAC injection buffer.

1.3. Preparation of RNA

1.3.1. Small scale RNA purification, Qiagen "RNeasy mini prep"

Small scale RNeasy mini preps were used to purify up to 100µg of total RNA from tissues or tissue samples. The kit is based on a selective binding of RNAs >200 bases to a silica-gel-based membrane under high-salt conditions, which excludes 5S, 5.8S and tRNAs from the prep. RNAs were purified following the manufacturer's instructions. In brief, cultured cells were washed on ice with chilled PBS to remove residual culture medium. The PBS was removed and the cells were lysed by the addition of buffer RLT and scraped off the culture dish with a cell scraper and the lysate was transferred to an Eppendorf tube. The appropriate amount of RNA-grade 70% ethanol was added and mixed by pipetting. The sample was then transferred onto the RNeasy column and centrifuged at 8,500g for 1 minute. The flow-through was discarded, the column was washed with 700µl RW1, followed by two washes with 500µl of buffer RPE. The column was dried by centrifugation. The RNA was eluted from the column by adding 30-50µl of RNase-free dH_2O. RNA quality was assessed by gel electrophoresis or by analysis on an Agilent 2100 Bioanalyzer. For the isolation of total RNA form mouse tissues, the tissue was homogenized with an Ultra-Turrax in buffer RLT and purified as described above.

1.3.1. Lare Scale RNA purification, Qiagen "RNeasy midi prep"

Larger amounts of total RNA (up to 1mg) were purified using Qiagen's "RNeasy midi prep" kit. The kit is based on the same principle as the "RNeasy mini kit" and was primarily used to obtain RNAs from mouse tissues according to Qiagen's instructions.
Mouse tissues that were not processed immediately were stored in the RNA stabilization agent "RNAlater" at 4ºC for up to 10 days prior to RNA isolation.

1.4. Determination of nucleic acid concentration

1.4.1. Photometric determination of nucleic acid concentration

According to the Lambert-Beer law the absorption of an aqueous solution of a substance is directly proportional to its concentration,

$$A=\varepsilon c l ,$$

where ε is the molar extinction coefficient (unit: $M^{-1}cm^{-1}$), c the concentration (unit: M) and l is the path length of the light through the sample (unit: cm). The extinction coefficients for nucleic acids are (at λ=260nm):

guanine : ε=12010 $M^{-1}cm^{-1}$
cytosine : ε=7050 $M^{-1}cm^{-1}$
adenine : ε=15200 $M^{-1}cm^{-1}$
thymine/uracil : ε=8400 $M^{-1}cm^{-1}$ (8111 $M^{-1}cm^{-1}$ for uracil)

The absorption was measured at 260nm and 280nm, which represent the absorption maxima for nucleic acids and proteins, respectively. The measured absorption is the sum of the

absorptions of all the bases in the solution. For working purposes the following OD_{260} – concentration relations were used:

1 OD_{260} for dsDNA	: 50mg/ml
1 OD_{260} for RNA	: 40mg/ml
1 OD_{260} for ss-oligonucleotides	: 33mg/ml

The purity of a given sample was assessed by the ratio OD_{260}:OD_{280}. For clean preparations of nucleic acids, the ration should be between 1.7-2.0, lower ratios usually indicate a protein contamination.
Depending on the expected concentration of a sample it was diluted 1:50 to 1:1000 prior to measuring.

1.4.1. Estimation of BAC DNA concentration on PFGE gels

To estimate the concentration of linearized full-length BAC DNA, several dilutions of the BAC DNA were run on a PFGE gel next to defined amounts of linearized λ-DNA as a standard. The gel was then stained with ethidium bromide and placed on a UV screen to estimate the concentration and subsequently photographed.

1.5. Analysis of nucleic acids

1.5.1. Gel electrophoretic analysis of nucleic acids

1.5.1.1. Separation of DNA and its topological isomers in agarose gels

Due to the constant mass-to-charge ratio of nucleic acids, their migration velocity in a constant electrical field depends on the properties of the matrix (gel), the molecular weight and the topology of the nucleic acid. Since the side chains of the nucleic acids have the same same pK_S value, it is not their charge that determines the migration velocity in the gel (unlike for proteins), but their diffusion coefficient. The migration velocity is inversely proportional to the decimal logarithm of the molecular weight.
Two different gel systems can be used depending on size and nature of the nucleic acid: The para-crystalline meshwork of agarose gels allows for the separation of DNA ranging from 100bp to several Mb. The polymerization-based and covalently coupled matrix of polyacrylamide gels allow for the separation of smaller nucleic acids (up to only a few kb). The separation properties of the polyacrylamide gels not only depends on the gel concentration, but also on the degree to which they are cross-linked.

A detailed description of the different gel systems, their handling and generation is described in Sambrook “Molecular Cloning – A Laboratory Manual”, CSHL Press, 2001.

1.5.1.2. Separation of DNA fragments by “conventional” gel electrophoresis

For the separation of DNA fragments between 100bp and 14kb agarose gels ranging from 0.8% to 2.5% were used.
For the generation of agarose gels, the appropriate amount of agarose was dissolved in 1xTAE buffer by heating the suspension in a microwave until the agarose had completely

dissolved. After the agarose had cooled to approx. 60ºC, ethidium bromide was added (f.c. 1µg/ml) and the agarose was poured into a sealed horizontal gel tray, combs were placed in the setup and the agarose was allowed to solidify. The gel was then placed in a gel chamber filled with 1xTAE buffer so that the gel was submerged by 2-3mm in the buffer. One 10^{th} of the sample volume was added as "loading dye II" to each sample prior to loading it on the gel. The gels were run at 4-6Vcm^{-1} until the desired separation was achieved. DNA fragments were visualized by placing the gel on an UV screen (wavelength 260nm) and photographed.

1.5.1.3. Separation of DNA fragments by pulsed-field gel electrophoresis (PFGE)

PFGE allows for the separation of linear DNA fragments up to 10Mb and was first described by Schwartz and Cantor (Schwartz and Cantor, 1984). Unlike conventional electrophoresis, PFGE requires DNA molecules to periodically change their direction of migration.
Several types of PFGE have been described, all of which utilize the basic principle of applying an electric field alternating in two orientations. The difference lies in the manner in which the alternating electrical fields are generated. The method described by Schwartz and Cantor and orthogonal field-alternation gel electrophoresis (OFAGE) utilizes arrays of point electrodes that yield non-homogenous electrical fields and non-linear lanes in the final migration pattern. In transverse alternating field electrophoresis (TAFE) the distortion of DNA migration, as seen in OFAGE, is eliminated by running the gel vertically and applying the electrical field at an angle to the face of the gel plates (see: (Gardiner et al., 1986; Gardiner et al., 1988)). In another version of PFGE, the field inversion gel electrophoresis (FIGE; (Carle et al., 1986)) involves a periodic inversion of the electrical field. Forward movement of the DNA molecules is obtained by either a shorter pulse time or a lower field strength for the reverse direction. This results in a linear migration of the molecules, although DNA migration is not always monotonic with size for molecules >2Mb.
For this thesis a fourth variant of PFGE was used, contour-clamped homogenous electric field electrophoresis (CHEF). This method was first described by (Chu et al., 1986) and uses multiple electrodes arranged along a closed contour, which are clamped to predetermined electrical potentials equal to those calculated to be generated by two parallel, infinitely long electrodes. With this arrangement of electrodes, the distortion of DNA migration is overcome, which is observed in non-homogenous electrical fields. The system used here (BioRad CHEF DRII system) generates a homogenous electrical field that alternates between two orientations that are 120º apart. The CHEF system was further advanced by using a hexagonal array of 24 electrodes which could be independently controlled (programmable autonomously controlled electrode gel electrophoresis (PACE)).
As for conventional gel electrophoresis, a number of factors are known to influence the resolution of linear DNA fragments: the topology of DNA, the time interval between reorientation of the electrical field (the pulse time), the angle at which the alternating electrical field is applied, field strength, the gel concentration and the temperature at which the gel is run. Linear and circular DNA molecules of the same size migrate differently in PFGE. Open circular DNA molecules do not enter the gel matrix, thus BACs have to be linearized by restriction endonucleases prior to analysis by PFGE.
The pulse time has a major effect on the migration of the DNA and it therefore depends on the size of the DNA molecules that need to be resolved. Generally speaking, the larger the DNA molecule the longer is the pulse time required for resolution. At any given pulse time, DNA molecules greater than an upper limit do not have enough time to re-orient in the alternating electrical field. Consequently, they migrate along the direction of the average electrical field and cannot be resolved. Conversely, DNA molecules smaller than the lower

limit become completely re-oriented and thus move as in conventional gel electrophoresis. These molecules usually migrate off the gel.
The mobility of DNA increases with increasing temperature of the electrophoresis, thus permitting shorter run times. However, the resolution of the fragments is decreased at higher temperatures. Since these effects are more pronounced in PFGE than in conventional gel electrophoresis, PFGE gel buffers are kept at a constant temperature, usually 14ºC.
Increasing the gel concentration decreases the mobility of all sizes of DNA molecules, thus improving resolution. Although smaller sized fragments may appear sharper, the resolution of larger DNA fragments will decrease.
For this thesis 1% agarose gels made with 0.5XTBE were used which allowed for the resolution of DNA fragments between 3kb – 280kb. The gels were run in a CHEF DRII system with 0.5XTBE buffer chilled to 14ºC and programmed to the following settings:

Run time : 16 hours
1st switch : 5 seconds
final switch : 15 seconds
field strength : 6 Vcm^{-1}

After the gel run was finished, the gels were placed in a tray filled with 0.5XTBE that contained 1µg/ml ethidium bromide for 20 minutes and then photographed.
As a standard linear, multimerized λ-DNA that was contained in agarose was used (MidRange PFGE marker I or II, NEB). For electrophoresis a thin slice of the marker was cut and placed in a well, which was then sealed with agarose.

1.5.1.4. Separation of RNA by denaturing polyacrylamid gel electrophoresis

For the electrophoretic separation of RNAs, it is necessary to include denaturing agents into the gel system to resolve intra-molecular secondary structures of the RNAs, which would influence their migration behavior in the gel matrix. Usually urea or formamide are used for this purpose. As a consequence of the low ionic strength of the gel system, the electric resistance is high leading to a heating of the gel system. This thermal energy in turn additionally improves the resolution of the gel by 'melting' secondary structures that might still exist.
For the analysis of *in vitro* transcribed RNAs and the analysis of hydrolyzed ISH probes PAA gels were prepared as follows:

35ml 10M urea (f.c. 8M)
6ml 50% PAA (BioWhitttaker Long Ranger sequencing gel mix)
5ml 10XTBE
4ml dH_2O

The polymerization was started by adding 250µl of 10%APS and 25µl of TEMED. A 3µl sample + 22µl loading dye were loaded per well, and the samples were run at 260V, 25mA in 1XTBE running buffer.
The electrophoretic separation was allowed to proceed until the bromophenol blue dye, which corresponding to a fragment size of 29 nts, migrated out of the gel. The gel was then exposed to a Kodak Biomax MR film for 6- 24h.

1.6. Fragmentation of DNA with endonucleases

1.6.1. Fragmentation of DNA with restriction endonucleases ("DNA digests")

Restriction endonucleases are enzymes that recognize specific sequences within dsDNA ~~and~~ cut both DNA strands. There are 3 groups of restriction endonucleases:
Type I and type III restriction enzymes each carry both the endonuclease and methylase activity on a single protein. Type I enzymes cleave the DNA at a possibly random site located at least 1000bp from the recognition sequence, whereas type III enzymes do so 24-26bp distant from the recognition sequence. Type II restriction enzymes, which were discovered and characterized by Smith and Nathans (Nathans and Smith, 1975), are separate entities from their corresponding modification methylases. They cleave at specific sites within their recognition sequence. The recognition sequence of type II restriction enzymes usually ranges from 4-8bp in length and is in most cases palindromic. The hydrolysis of both dsDNA strands can generate 5'-protruding, 3'-protruding or blunt ends. The 5' ends are always phosphorylated and the 3' ends always carry a hydroxyl group. These characteristics make them a useful tool in molecular biology for sequence specific fragmentation of DNA.
Each restriction enzyme has its own reaction conditions, which are specified by the supplier. Important parameters are reaction temperature and the components of the restriction buffer, which specify the pH of the reaction mix and ionic strength of a restriction digest. If two enzymes require the same reaction conditions, both can be used within the same reaction. In the case that the reaction conditions are incompatible, the DNA has to be cut with one of the enzymes first, then precipitated and subsequently cut with the second enzyme. Alternatively, the DNA can be cut with the enzyme that requires the lower salt concentrations first, then the reaction conditions are adjusted for the second enzyme in a volume of 60-80µl.
For analytical DNA digests, usually 500ng-1µg of DNA were used and digested with 1-20 units of the corresponding enzyme. A unit is defined as the amount of enzyme required to cut 1µg of λ-DNA in 1 hour at 37°C. Commercially available enzymes are usually supplied in 50% glycerol, and glycerol concentrations of >10% in the restriction digest can lead to "star activity" of the enzyme (George et al., 1980). This limits the amount of enzyme that can be used in a given reaction volume to avoid unspecific cleavage of the DNA. Usually, a restriction digest was allowed to proceed for 45 minutes to 2 hours at 37°C and was setup as follows:

x µl DNA (500ng - 2µg)
4 µl 10X reaction buffer
1 µl enzyme 1
1 µl enzyme 2
dH_2O to 40µl.

Restriction enzymes can be inactivated by a heat-kill at 65°C for 20 minutes (for heat sensitive enzymes), or the enzymes can be removed either by phenol-cholorform extraction or by purifying the DNA fragment after electrophoretic separation in an agarose gel using a Qiagen "QiaQuick gel extraction" kit.

1.6.1. DNA fragmentation with homing endonucleases

Homing endonucleases are dsDNAses that have large, asymmetric recognition sites of 12-40bp in size. Their coding sequences are usually embedded in either introns or inteins. Homing endonucleases are named using conventions similar to those used for restriction endonucleases: Intron-encoded endonucleases begin with "I-" and intein-encoded endonucleases begin with "PI-".
The recognition sequences for homing endonucleases are extremely rare; for example, an 18bp recognition sequence will occur only every $7x10^{10}$ bases. However, homing endonucleases tolerate some sequence degeneracy within their recognition sequence resulting in an effective recognition sequences of 10-12bp.
The extremely rare cutting frequency makes homing endonucleases a valuable tool in BAC-based genome analysis, eg. in linearizing BACs for the production of transgenic animals. For the linearization of BACs digests were setup as follows:

x µl BAC-DNA (~40 µg, maxi prep grade)
10 µl 10x PI-SceI buffer
8 µl PI-SceI (5U/µl)
dH_2O to 100µl

The reaction was allowed to proceed ON at 37°C followed by a heat-inactivation of the enzyme (10minutes, 70°C).

1.7. DNA sequencing

Custom DNA sequencing was carried out by Retrogen, Inc. San Diego, CA using a modified enzymatic DNA sequencing procedure, the dideoxy-chain termination method, that was first described by Sanger (Sanger et al., 1977).This method is based upon an *in vitro* replication of DNA with a modified *taq* polymerase ('Sequenase'). The sequencing reactions were analyzed using an "Applied Biosystems 3730 DNA-Analyzer" and the proprietary "BigDye Terminator v3.1" chemistry.

1.8. Sequence analysis

Data obtained through DNA sequencing was analyzed using DNAStar's "Lasergene" software package version 5.5 as well as applications available at the "National Center for Biotechnology Information" (NCBI, http://www.ncbi.nlm.nih.gov) and ENSEMBL (http://www.ensembl.org).
For the analysis of protein and amino acid sequences, the "Lasergene" software package as well as the resources at NCBI, ExPASy (http://www.expasy.org/) and the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/) were used.

2. Modification of DNA

2.1. *In vitro* modification of DNA

2.1.1. Synthesis of oligonucleotides

Custom oligonucleotides were obtained through the following companies: Proligo, Inc., Operon, Inc and Eurogentec, Inc. The oligonucleotides were synthesized using a phosphoramidite-based chemistry (Caruthers et al., 1983). Oligonucleotides >50bp were also HPLC purified to eliminate aberrant synthesis products.

2.1.2. Covalent ligation of DNA fragments with T4 DNA ligase

The bacteriophage T4-encoded enzyme DNA ligase catalyses the formation of phosphodiester bonds between neighboring 3'-hydroyl- and 5'-phosphoryl-termini. The enzyme requires Mg^{2+}-ions and ATP as co-factors. The enzymatic activity is measured in Weiss units. 1 Weiss unit is the amount of enzyme required to catalyze the exchange of 1nmol of ^{32}P from pyrophosphate to ATP in 20 minutes at 37°C. Another commonly used unit definition is the 'cohesive end ligation unit', and it is defined as the amount of enzyme required to give a 50% ligation of HindIII digested λ-DNA in 30 minutes at 16°C in 16µl at a 5' termini concentration of 0.12µM. One Weiss unit corresponds to 67 cohesive-end-ligation units.
The efficacy of a ligation reaction is influenced by several factors, e.g. incubation temperature, reaction volume and the concentration of DNA termini. Commercially available "rapid ligation kits" also use inert polyethylene glycol in their ligation buffer to enhance the ligation efficacy, which in turn reduces the incubation time.
For a typical ligation reaction 0.08 to 0.15 pmol of vector DNA and the 3-5 molar excess of insert was used. When a commercial kit was used for the ligation of 'sticky' ends, the ligation reaction was allowed to proceed at room temperature for several hours. "Blunt" end ligations were carried out at 16°C for >12 hours.

2.1.3. Phosphorylation of 5' hydroxyl ends using polynucleotide kinase (PNK)

Polynucleotid kinase catalyzes the transfer and exchange of a γ-P_i from ATP to the 5'-OH end of dsDNA, RNA and single-stranded oligonucleotides. For the quantitative phosphorylation of double-stranded oligonucleotides, ATP was added to the reaction at a final concentration of 1mM to strongly favor the transfer of γ-P_i onto the 5'-OH ends of the double-stranded oligonucleotides. Typically, a kinasing reaction was setup as follows:

xµl ds-oligonucleotide (~50pmol)
4µl 10xPNK buffer
4µl dATP (f.c. 1mM)
1µl PNK (10 units)
dH_2O to 40µl.

The reaction was allowed to proceed for 30 minutes at 37°C and it was stopped by a heat kill of PNK at 70°C for 15min. The oligonucleotides were allowed to re-anneal and were purified using a S-400 sepharose column.

2.1.4. Dephosphorylation of 5'-ends of DNA fragments

Terminal 5'-phosphoryl groups can be removed enzymatically by treating the dsDNA with the enzyme calf intestinal phosphatase (CIP). The enzymatic treatment of vector DNA with CIP prevents unwanted re-ligation. The CIP used was compatible with the NEB (NEB) and SURE cut (Roche) restriction buffer systems and for the removal of 5'phosphoryl groups, 1-2 U of CIP were added to a completed restriction digest and incubated for 30 minutes at 37°C. Since CIP cannot be heat inactivated, the treated DNA was subsequently gel purified using the "Qiaquick gel extraction kit". One unit of CIP is defined as the amount of enzyme required to hydrolyze 1µM p-nitrophenolphosphate to nitrophenol in 1 minute at 37°C.

2.1.5. Amplification of DNA fragments *in vitro* using polymerase chain reaction (PCR)

The polymerase chain reaction allows the *in vitro* amplification of a specific DNA sequence (Mullis et al., 1986). The DNA synthesis is started at two primers that are flanking the sequence to be amplified. One of the primers anneals to the sense and the other one to the anti-sense strand of the amplicon. The synthesis reaction is catalyzed by the DNA polymerase of the thermophilic bacterium *Thermus aquaticus* (taq polymerase). The enzyme is stable at 95°C and catalyzes the synthesis of DNA at a temperature of 72°C. DNA synthesis therefore can be achieved in a thermocycler shifting temperatures between 95°C, the specific annealing temperature of the primers and 72°C for DNA synthesis in the presence of a DNA template, primers and dNTPs and corresponding salt and pH conditions for taq polymerase.
Usually, 24 – 40 cycles are necessary to obtain sufficient quantities of the PCR product for subsequent steps. For this thesis Qiagen's "HotStar taq PCR Master Mix" was used, which contains a taq polymerase that is inactive at ambient temperatures to avoid misprimed PCR products. The "Master Mix" also contains dNTPs and a proprietary buffer. The enzyme can be activated by an initial denaturing step at 95°C for 15 minutes.
Standard PCR reactions were setup as follows:

10µl HotStar PCR MasterMix
1µl primer1 (@ 15pmol/µl)
1µl primers (@ 15pmol/µl)
1µl DNA template (100pg – 25ng)
7µl PCR-grade dH_2O
20µl

For so called "multiplex" PCRs in which several PCR products are amplified in the same PCR reaction using multiple primers, up to 3 primer pairs were used in a total reaction volume of 20µl. Multiplex PCRs were exclusively used for genotyping transgenic mice.
PCR was also used to introduce new restriction sites into the amplified product. The new restrictions sites to be added were contained within the PCR primers used.
Taq polymerase does not possess a 3'-5' exonuclease activity ("proofreading"), which on average leads to the introduction of a mutation every $1.3x10^5$ nucleotides. For high-fidelity amplifications, the DNA polymerase of *pyrococcus furiosus* (pfu polymerase) was used, which has a proofreading activity that results in a >10-fold reduced mutation rate.
PCR products were ran on an agarose gel for either visualization or purification followed by gel extraction. Alternatively, DNA fragments were purified using a S-200 spin column.

2.2. Homologous recombination in bacteria

2.2.1. Bacterial artificial chromosomes

Bacterial artificial chromosomes (BACs) are F-factor based plasmids, which are capable of harboring large pieces of DNA of up to 1Mb (Shizuya et al., 1992).They are maintained at a low copy number of 1-2 copies per bacterial cell, which reduces the likelihood of unwanted recombination between BACs.
The first BAC backbone vector described was based on the mini F-factor pMBO131 and contained, next to the required regulatory sequences oriS, repE, parA and parB, a multiple cloning site which was flanked by Sp6 and T7 promoters respectively (Shizuya et al., 1992). Furthermore, the vector contained a cosN and a loxP site 5' to the T7 promoter, which can be used for linearization.
Large pieces of genomic DNA of up to 300kb can be generated through partial digests with restriction endonucleases. The genomic DNA fragments can then be used to create BAC libraries by ligating them into the BAC backbone vectors.
Improvements have been made to the first generation BAC backbone vectors, and they now also contain a recognition sequence for the homing endonuclease PI-SceI, which can be used to linearize a given BAC.
For this thesis pBelo11- and RPCI-24-based BAC clones were used. The pBelo11 BAC clones were obtained from Incyte Genomics, Inc. (now part of Research Genetics, Inc). The RPCI-24 based clones were obtained through "BAC/PAC Resources", Oakland, CA (http://www.chori.org/bacpac). The DNA used to construct the RPCI-24 library was generated by a partial MboI digest of spleen and brain genomic DNA of a male C57Bl/6J mouse, which was then cloned into pTARBAC1 (Zeng, C et al., 2001). The DNA used for the construction of the pBelo11-based library was derived from SV129 ES cells.
The "pBelo11-ChAT-BAC" was identified through a BAC library screen at Incyte Genomics using a ChAT promoter-specific probe. With the advances of the ENSEMBL database (http://www.ensembl.org), a suitable "cholinergic" BAC (RPCI-24-70D4) was identified through the use of the implemented "Cytoview" option.

2.2.2. Homologous recombination in bacteria

The use of restriction endonucleases is a standard procedure to modify a given piece of DNA. It is limited by the frequency with which an enzyme cuts the target DNA and the presence of restriction sites in a given piece of DNA. In recent years recombination systems have been developed that utilize phage-derived factors that enable a site-specific recombination without the restriction-ligation-based methodology (Zhang et al., 1998; Lee et al., 2001). This new technique makes it possible to modify virtually any target DNA independent of its size to generate complex targeting vectors for 'knock-out' or transgene constructs. This methodology is also referred to as 'recombineering'.
Several publications have described this and related systems and their properties in great detail (Zhang et al., 1998; Muyrers et al., 2000; Yu et al., 2000; Liu et al., 2003), therefore, only a brief overview of the technique shall be given here:
Efficient homologous recombination in *E. coli* is made possible by the use of λ-phage encoded Red genes. Copeland and colleagues (Lee et al., 2001) constructed several bacterial strains that carry a defective λ-prophage encoding the Red proteins *exo*, *bet* and *gam*, which have been placed under the transcriptional control of a temperature-sensitive λ-repressor. *Exo* encodes a 5'-3' exonuclease that acts on 5'ends of linear dsDNA fragments to produce 3' ssDNA overhangs; *bet* encodes a pairing protein that binds to 3' overhangs produced by *exo*

and promotes their annealing to the complimentary strands on the target DNA. The *gam* protein inhibits the RecBCD exonuclease activity of *E. coli* thereby protecting the linear dsDNA targeting DNA from degradation. By shifting the growth temperature from 32°C to 42°C for 10 – 15 minutes the temperature-sensitive λ-repressor is no longer able to bind to the P_L promoter allowing the transcription of *exo*, *bet* and *gam* at high levels. This system works very efficiently so that homology arms of 50-70bp are already sufficient for homologous recombination. For this thesis homology arms were routinely added to targeting constructs by PCR followed by sequence verification.
The bacterial strains EL250 and EL350 also carry inducible *cre* and *flpe* site-specific recombinases under the transcriptional control of the P_{BAD} promoter. This way their expression can be controlled by adding L-arabinose (0.5% w/v) to the LB medium. The recombinases were used to excise selectable markers, such as the zeocin resistance or kanamycin resistance, which were either flanked by loxP or FRT sites.
For the excision of a selectable marker an ON culture of the desired clone was grown at 32°C. A 2ml LB-only culture was then inoculated with 20µl of the ON culture and grown to an OD_{600} of ~0.5. At this point 50µl of a 20% w/v L-arabinose solution (f.c. 0.5%) were added and the cells were allowed to grow for an additional hour. From this culture a new 2ml culture was inoculated (1:50) and the cells were grown for another hour. 5-10µl of this culture were then plated on LB-Cm plates and grown ON at 32°C.
The next day clones were picked into 3ml of LB-Cm and grown ON. 1µl of each culture was used in a PCR screen to verify the excision of the selectable marker.

3. *In situ* hybridization

3.1. Synthesis of RNA by *in vitro* transcription

RNA polymerases of the bacteriophages T3, T7 and Sp6 have a high affinity to their own promoters and can thus be used for the in vitro synthesis of RNA. The polymerases only require a DNA-template, Mg^{2+}-ions and rNTPs as co-factors. A polymerase unit is defined as the amount of enzyme required to catalyze the incorporation of 5nmol CTP into acid-insoluble product in 60 minutes at 37°C in a total volume of 100µl.
For the generation of radioactive *in situ* hybridization probes ^{33}P-labelled rUTP (111TBq/mmol) can be used. The probes were generated according to the following protocol:

- 2µg of the plasmid harboring the in situ probe fragment was linearized with the appropriate enzyme, gel purified and eluted into RNase-free Eppendorf tubes with 40µl dep dH_2O.

The in vitro transcription was setup as follows for each probe:

3.0	µl 5X transcription buffer
1.5	µl 100mM DTT
0.25	µl Rnasin (40u/µl)
0.75	µl rATP (10mM)
0.75	µl rCTP (10mM)
0.75	µl rGTP (10mM)
0.75	µl ^{33}P-rUTP (10mM)
5.25	µl dep dH2O
1.0	µl linearized DNA template
1.0	µl DNA polymerase
15	µl

The reaction was incubated at 37°C for 1 hour, then 0.8µl of RNase-free DNAse were added followed by a 15 minute incubation at 37°C.
3µl of tRNA (10mg/ml) were added and a 1µl aliquot was analyzed on a 6% PAA gel, the rest of the probe was stored at –70°C.

3.2. *In situ* hybridization of ^{33}P-labelled RNAs on murine tissues

In situ hybridization (ISH) is a very sensitive method to detect RNAs in morphologically preserved animal tissues or cells. The technique was first described by Gall and Pardue (Gall and Pardue, 1969) and by John (John et al., 1969). ISH procedures have been improved to not only work with radioactively-labeled probes but also with digoxigenin and fluorescein-labeled RNA probes allowing a chromogenic detection of RNAs (Schaeren-Wiemers and Gerfin-Moser, 1993).
For this thesis ISH was carried out on tissue section of 20-30µm thickness mounted on Fisherbrand Superfrosted microscope slides, or poly-L-lysine/gelatine coated microscope slides which were dried ON in a dessicator at RT.
The radioactively labeled RNA probes were hydrolyzed to achieve a greater tissue penetration:
25µl of hydrolysis solution were added to each probe and incubated for 15 mintes at 58°-60°C. The samples were then cooled on ice and 25µl of neutralization solution were added. The probes were then purified using a S-200 spin column pre-equilibrated with 50µ of a SDS/tRNA/EDTA solution (40µ 10% SDS, 16µ 10mg/ml tRNA, 40µl 0.5M EDTA, 280µl dep-H_2O).
3µl (+23µl formamide-based loading dye) of the probe were run on a denaturing PAA gel next to 1µl of the non-hydrolyzed probe to assess the quality of the generated probes. A BioMAX-MR x-ray film was then placed on the gel and exposed for 8-16 hours. In addition, 1µl of the final probes was diluted 1:10 and 1µl of the dilutions was added to 14ml of Scintiverse fluid in scintillation vails, and counts were measured in a Beckman LS6500 scintillation counter. Probes were stored for up to a week at –70°C.

3.2.1. Pre-hybridization treatment of mounted tissue sections

The sections were removed from the dessicator and placed in 10% phosphate-buffered formalin for 30 minutes followed by 4x 5 minute washes in 0.02M KPBS. The samples were then treated in 0.001% proteinase K for 10 minutes in a waterbath at 37°C and gently rinsed with depc dH_2O. The slides were then equilibrated in 0.1M triethanolamine (TEA), pH 8.0 for 2-3 minutes, followed by a 10 minute acetylation step in acetylation buffer.
The samples were then rinsed 2x in 2xSSC (RNA grade) for 2 minutes followed by dehydration in ascending concentrations of fresh ethanol:

50% EtOH, 3 minutes
70% EtOH, 3 minutes
95% EtOH, 3 minutes
100% EtOH, 3 minutes
100% EtOH, 3 minutes

The slides were then drained on a paper towel and air-dried and placed in the dessicator for at least 1 hour prior to applying the ^{33}P-labeled probe.

3.2.2. Hybridization

The probes were thawed on ice and spun briefly to collect all liquid. For hybridizations ~$2x10^7$ cpm/ml in hybridization buffer were used:

900 µl hybridization buffer
µl probe
10 µl tRNA (10mg/ml)
add depc dH_2O to 1ml

The probe hybridization mix was heated to 70°C for 5 minutes, placed on ice and centrifuged briefly. 100µl of the solution were applied to a cover glass and inverted onto the slide. The hybridization was allowed to proceed ON at 55°-60°C in a humidified "slidemoat" chamber.

3.2.3. Post hybridization treatment of samples

The slides were removed from the "slidemoat" chamber and placed in a clean staining rack filled with 4xSSC and were incubated for 30 minutes allowing the cover glass to slide off followed by 4 additional 5 minute rinses with 4xSSC. The samples were then RNAseA treated for 35 minutes at 37°C in RNAse buffer and subsequently washed and desalted as follows:

2X SSC for 5 minutes
2X SSC for 5 minutes
1X SSC for 5 minutes
0.5X SSC for 5 minutes
0.1X SSC for 30 minutes at 65°C.
0.1X SSC for 2 minutes at RT

The tissue was dehydrated in ethanol again:

50% EtOH + 1 ml 20X SSC for 3 minutes
70% EtOH + 1 ml 20X SSC for 3 minutes
95% EtOH for 3 minutes
100% EtOH, 3 changes of 3 minutes each

The microscope slides were then drained on paper towels and placed in a dessicator until they were completely dried.
For preliminary ISH results, the slides were mounted on cardboard with scotch tape, placed in a film cassette and exposed to BioMAX-MR film for 24 hours and in a second exposure for 3 days.
Prior to autoradiography the tissue was treated with xylenes, 2 changes of 10 minutes and 1 hour, to remove all residual fats from the slides.

3.2.4. Autoradiography

The slides were dipped in Kodak NBT-2 emulsion mixed 1:1 with dH2O in a darkroom and allowed to dry in a humidified box at RT. They were then placed in a box with dessicant and kept in a refrigerator for 5 days to 4 weeks depending on the signal intensity expected.
Developing of the slides:

The slides were allowed to warm to RT and developed as follows:

1. 4 minutes in Kodak D-19 at 15°C
2. 30 second wash in dH_2O at 15°C
3. 6 minutes in Kodak Polymax-T fix at 15°C (diluted 1:4 with dH_2O)
4. Slides were washed in running tap water for 30 minutes.

The slides were then dehydrated again in ethanol and coversliped with DPX.

4. Generation, handling and analysis of transgenic mice

All animals used for this thesis were kept and treated in the mouse facilities of The Scripps Research Institute in accordance with the guidelines for animal welfare established by the National Institutes of Health (NIH) and TSRI.

4.1. Generation of transgenic mice

The generation of transgenic mice by pronuclear injection was carried out at the transgenic core facility of TSRI. A detailed description of this complex technique can be found in "Manipulating the mouse embryo: A laboratory manual" A. Nagy (ed.), CSHL Press, 2002.
In brief, super-ovulated mice are mated to a male and sacrificed 24 hours later and the fertilized oocytes are removed from the ovaries. At this stage the male and female pronuclei have not fused and DNA can be injected into the male pronucleus, which is larger in size than the female pronucleus. Hybrid strains such as B6D2F1 or FVB/N are usually used for this

purpose, since their pronuclei are exceptionally large and they tolerate the manipulations better than oocytes derived from C57Bl/6J mice. The injected oocytes are then implanted into the ovaries of a pseudo-pregnant foster mouse. After the resulting pups have been weaned, a piece of tail is cut and the genomic DNA is extracted. Potential founders are identified by PCR using transgene-specific primers. Depending on the transgene construct, founders were either mated to reporter mice or to wild-type C57Bl/6J mice.

4.2. Analysis of transgenic mice

4.2.1. Transgene-specific genotyping PCRs

For the identification of transgenic animals, either at founder level or for regular breeding, genomic tail DNA was isolated and transgene-specific primers were used for PCR (see: Materials). The PCRs were setup as multiplex PCRs and contained next to the transgene-specific primers a primer pair (2285, 2286) that amplified a 157bp fragment from exon1 the erbB4 gene. PCRs were setup as follows:

10µl HotStar PCR MasterMix
1µl primer 1(sense)
1µl primer 1(as)
1µl primer 2(sense)
1µl primer 2(as)
1µl primer 2285
1µl primer 2286
1µl genomic DNA
3µl PCR-grade dH_2O
20µl

The PCR parameters for the amplification were:

(1) 15min @ 95°C
(2) 45s @ 95°C
(3) 30s @ 60°C
(4) 30s @ 72°C
(5) go to (2) 37x
(6) 5min @ 72°C

The PCR products were resolved on a 2.5% agarose gel.

4.2.2. Generation of cDNA from total RNA extracts from cells or tissues

The conversion of RNA into DNA is catalyzed by the enzyme reverse transcriptase, a RNA-directed DNA polymerase. The enzyme synthesizes DNA in the 5' to 3' direction and requires short RNA:DNA hybrids as primers. Commercially available enzymes have been improved through point mutations yielding enzymes with a higher processivity due to the removal of the endogenous RNAseH activity and an improved thermal stability enabling 1st strand cDNA synthesis at temperatures of up to 55°C.

Different priming methods can be used for cDNA synthesis with the 2 most commonly used being oligo-dT priming and random-priming. Oligo-dT priming initiates cDNA at the polyA tail of eukaryotic mRNAs and thereby introduces a 3' bias into the generated cDNAs. For this thesis cDNAs were generated with random-priming, which initiates the reverse transcription at multiple, unspecified positions on the target RNA.
The protocol used was adapted from the ATLAS-array cDNA-synthesis protocol from Clontech, Inc. and carried out in 500µl Eppendorf PCR tubes in a thermocycler:

µl total RNA (~500ng)
up to 11.5µl RNA-grade dH_2O
1µl random nonamers @ 20µM

- incubate RNA-primer mix for 2 minutes at 70°C
- quickly place tube on ice, then the following components were added:

4	µl 5x reaction buffer
1	µl 0.1M DTT
1	µl dNTPs (10mM each)
0.5	µl RNAsin (40U/µl)
1	µl Superscript II or Superscript III (200U/µl)
20	µl

- mix the reaction by pipetting
- incubate the reverse transcription reaction at 50 – 55°C for 1 hour
- heat kill the reverse transcriptase by a 5 minute incubation at 94°C

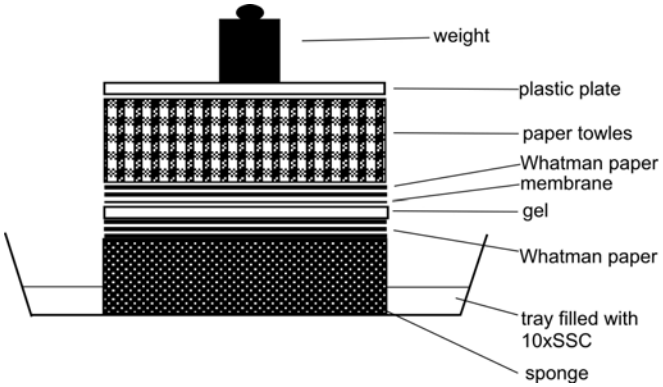
For the removal of the RNAs from the 1st strand cDNAs, the reaction was RNAseH treated, which specifically degrades RNA in RNA:DNA hybrids:

20µl cDNA synthesis reaction
+4µl 10x RNAseH buffer
+16µl dH_2O
40µl

The reaction was incubated for 30 minutes at 37°C, followed by a 5 minute incubation at 94°C to inactivate the enzyme. 40µl dH_2O were added to a final volume of 80µl. 1-2 µl of the cDNA were then used for PCR.

4.2.3. Southern blotting

Southern blotting was introduced by Ed Southern in 1975 (Southern, 1975) and is used for the detection of specific DNA fragments in complex mixtures of DNA fragments separated by gel electrophoresis. The DNA fragments are transferred onto a nylon membrane by capillary transfer and cross-linked by baking the membrane for 1h at 80°C or by exposing it to UV light.
For this thesis mouse genomic DNA or BAC DNA was digested ON with the desired restriction enzymes and separated by gel electrophoresis on a 1% agarose gel. As a standard for subsequent analysis, a ruler was placed next to the gel and the gel was photographed. For a more efficient transfer of larger DNA fragments, the DNA was partially depurinated by incubating the gel for 15 minutes in 0.25M HCl. The gel was then briefly rinsed with dH_2O and incubated for 30 minutes in denaturing buffer. The gel was rinsed in dH_2O and transferred into neutralizing buffer for 15 minutes after which the buffer was replaced by fresh buffer and the gel was incubated for an additional 15 minutes. The gel was equilibrated in 10xSSC for 10 minutes prior to placing it in the blotting setup. A piece of nylon membrane was cut to the appropriate size and equilibrated in 10xSSC for several minutes. The capillary transfer was then assembled as follows:



The transfer was allowed to proceed for 12 hours, after which the blot was disassembled and the membrane was rinsed in 10xSSC to remove unwanted pieces of agarose from the membrane. The membrane was allowed to air-dry for several minutes after which it was placed into a StrataLinker (Stratagene) to cross-link the DNA to the membrane. The membrane was stored at –20°C until use.

4.2.3.1. Preparation of ^{32}P-labeled probes for Southern blotting

For the generation of a ^{32}P-labeled probe, ~20ng of DNA were labeled with the "random prime-it kit II" (Stratagene). The kit was used according to the instructions provided by the manufacturer. In brief, the reaction was setup as follows:

25ng of DNA
10µl random oligonucleotide primers
dH_2O to 34µl

The mix was then boiled for 5 minutes in a water bath, briefly centrifuged and placed on ice. The following components were then added:

10µl 5xdCTP buffer
5µl [α-^{32}P]-dCTP (3000 Ci/mmol)
1µl exo(-) Klenow

The labeling reaction was incubated for 10 minutes at 37°C, after which the reaction was stopped by adding 2µl "stop mix". Unincorporated radionucleotides were removed by column purification using a S-300 column (Amersham).

4.2.3.2. Hybridization of ^{32}P-labeled probes

Southern membranes were pre-hybridized with 20ml of high-SDS hybridization buffer for 30 minutes at 65°C prior to adding the ^{32}P-labeled probe. For hybridization, the ^{32}P-labeled probe was denatured by boiling it for 5 minutes in a water bath after which it was placed on ice. The pre-hybridization buffer was removed, 10ml of pre-heated high-SDS hybridization buffer were added to the hybridization bottle and the denatured probe was pipetted into the hybridization bottle. The hybridization was allowed to proceed for 18 hours at 65°C under constant agitation.
The hybridization solution was then discarded and the membrane was subjected to 2 –3 low stringency washes at 68°C in 2xSSC/0.1%SDS in a water bath under constant agitation. A final high stringency wash was carried out in 0.2xSSC/0.1%SDS for 5-10 minutes. Membrane background was monitored with a hand-held β-counter between washes.
Following the washes, the membrane was wrapped in saran wrap and exposed to a FUJIFILM BAS-5000 screen for 2 hours at RT or to a KODAK BioMAX MS film with enhancer screen for 12-48 hours at –70°C.

4.2.4. Doxycycline administration

Doxycycline was administered to mice either via the food or through the drinking water.
Doxycycline-containing food (DOX-food) (6mg/g food) was purchased from BioServ, Frenchtown, NJ. The food contained green food coloring to more readily identify animals on DOX-food. DOX-food was used for up to 180 days after its mill date, after which it was autoclaved and discarded.
Several factors have to be taken into account when administering DOX in the drinking water. Ca^{2+} and Mg^{2+} ions efficiently chelate doxycycline and therefore destilled water should be used to avoid unwanted precipitation of doxycycline. The drinking water should also be supplemented with 5% sucrose, since doxycycline is bitter tasting. Furthermore, doxycycline is light sensitive, and the drinking bottles should therefore be wrapped with aluminum foil. For this thesis the drinking water was supplemented with 2mg/ml doxycycline and 5% sucrose and was replaced every 2 days.

4.2.5. Transcardial perfusion of mice

Animals were kept and treated in accordance to the guidelines for animal welfare in the mouse facilities of The Scripps Research Institute.
Mice were deeply anesthetized by injection with sodium-pentobarbitol (0.25mg/g body weight). The thorax was opened and a 25G needle was inserted into the left ventricle, followed by a cut into the right atrium. The mouse was then perfused with 0.9% NaCl

solution, pre-warmed to 37°C. The flow rate of the perfusion pump was set to 1-3ml/minute and the perfusion was allowed to proceed until all blood was replaced by NaCl solution. Depending on the intended use of the mouse, it was then perfused with either cold phosphate-buffered 4%-PFA, pH7.4 for 10-15 minutes for subsequent immuno-histochemical analysis or with 4%PFA-borate for ISH analysis.
Organs were removed and post fixed for 2 – 24 hours in the same solution used for perfusion.

4.2.6. Histochemical and Immuno-histochemical procedures

4.2.6.1. Detection of β-galactosidase in tissue sections, 'X-Gal' staining

The bacterial enzyme β-galactosidase catalyzes the cleavage of the O1 bond of the sugar β-D-galactose to a substituent. Due to a broad substrate specificity the enzyme can also be used to cleave organic compounds such as 5-bromo4-chloro-3-indolyl-β-D-galactoside (X-Gal) giving rise to a colorful indigo-colored precipitate (5-bromo-4-chloro-3-hydroxyindole) under oxidizing buffer conditions. Furthermore, the enzyme can be expressed in mammalian cells, when placed under the appropriate regulatory elements and is usually well tolerated. This property of the enzyme allows the use of β-galactosidase in transgenic mice as a reporter for the detection of transgene activity or for the detection of promoter activity of an endogenous gene in 'knock-in' approaches (Goring et al., 1987; Sanes, 1994).
For the detection of β-galactosidase activity in transgenic mice, PFA-fixed tissues were cut on a Leica VT-1000S vibratome (50-75μm sections). The tissue was then placed in 'β-gal staining buffer' for 3-24 hours at 37°C in the dark. The reaction was stopped by washing the samples 3x15 minutes in PBS. Sections were then mounted on FisherBrand 'superfrosted' slides, air-dried and cover-slipped with Mowiol-488. The mounting medium was allowed to solidify and the cover slips were then sealed with clear nail polish. Tissue sections were stored for up to 6 months at 4°C in the dark without any detectable loss of tissue integrity or diffusion of the indigo-colored precipitates.

4.2.6.2. Whole mount β-glactosidase staining of mouse embryos

For the detection of β-galactosidase activity in transgenic embryos (E13-E16), time-pregnant mice were sacrificed and the embryos were removed. A piece of tail was cut for genotyping and the embryos were fixed in cold phosphate-buffered 4%PFA for 4 hours. Embryos of the desired genotype were incubated ON in 'β-gal staining buffer' at 37°C in the dark followed by 3x20 minute washes in PBS. The embryos were then dehydrated in ascending concentrations of methanol (50%, 70%, 90%, 100%) at 4°C. The embryos were cleared in a benzyl benzoate-benzyl alcohol (2:1) mix for 20 minutes to 1 hour and photographed.

4.2.7. Western blotting

Western blotting allows the immunological detection of proteins in biological samples and was first introduced by Towbin and colleagues (Towbin et al., 1979). In this thesis the technique was used to detect endogenous and transgene-derived protein in tissue lysates from transgenic mice.
Proteins from tissue lysates were subjected to denaturing gel electrophoresis (SDS-PAGE) to detect HA-tagged neuregulin-1 a-tail protein by immunoblotting. For the detection of the HA-tagged protein, a rat monoclonal IgG α-HA antibody was used.

4.2.7.1. Generation of protein lysates for Western blotting

Mouse tissues were removed from sacrificed animals, frozen on dry ice and stored at –70°C until processing. For tissue lysis 1ml of 'WB lysis buffer' was used per 100mg of tissue, which was disrupted in an Ultrathurrax homogenizer. The cellular debris was removed by centrifugation and the supernatant was transferred into a new tube and snap-frozen in liquid nitrogen and stored at –70°C.

4.2.7.2. Protein concentration determination according to Bradford

This assay was first introduced by Bradford and is a quick and reliable means of determining the protein concentration of a given solution (Bradford, 1976).
Basic amino acid side chains of proteins form complexes with Coomassie Brilliant Blue G-250 in solution that appear blue with an absorption maximum at λ=595nm. The absorption is proportional to the amount of protein in solution. Through comparison with a calibration curve the protein concentration in a sample can be determined photometrically by measuring its OD_{595}. Calibration curves have to be generated for each assay with defined protein concentrations. The interactions of Coomassie Brilliant Blue G-250 and the amino acid side chains are detergent sensitive; thus detergent concentrations of >1% in the assay should be avoided.
Usually, a Bradford assay was setup as follows:

1-10µl protein lysate
dH_2O to 40µl
+160µl Bradford reagent

The mix was incubated for 20 minutes at RT prior to measuring OD_{595}. If the OD_{595} was >1, the sample was diluted further and the assay was repeated.

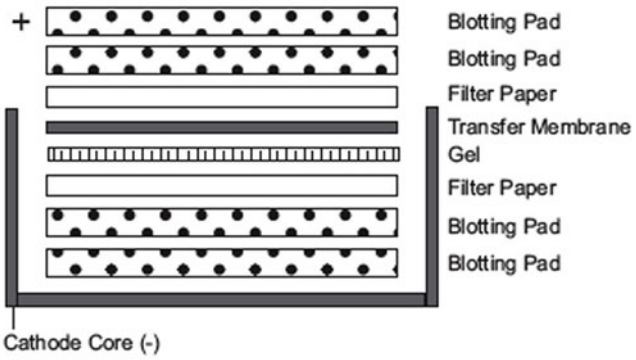
4.2.7.3. Separation of proteins through discontinous denaturing PAA gel electrophoresis (SDS-PAGE)

Unlike DNA, proteins do not have a constant mass-charge ratio due to their differing amino acid sequence. Through heat-denaturing of the protein in the presence of the detergent sodium dodecylsulfate (SDS), the detergent is able to form a 'micelle' with the unfolded protein as its core. The length of this structure is proportional to the polypeptide chain length and therefore to its molecular weight. In addition to SDS, a reducing agent like β-mercaptoethanol or DTT is added to reduce any disulfide bonds.
The gel electrophoresis is carried out in a 2 step gel system consisting of a stacking gel and a running gel (Laemlli, 1970). The stacking gel consists of a low-percentage PAA gel (usually 3%, pH6.7) and the running gel of a higher-percentage PAA gel at a basic pH (8-12% PAA, pH8.9). The focusing of proteins in the stacking gel relies on the fact that the mobility of glycine is made to be less than the mobility of the chloride ion. The amino proton of glycine is only feebly dissociated below pH 9 so that most of the glycine in the stacking buffer exists in an electrically neutral zwitterion form resulting in a low mobility. When voltage is applied, the chloride ions begin to move faster than the glycine. Any tendency for a "gap" to appear in the ion concentration will lead to a drop in conductivity in the boundary region. Since the current has to be the same at every point in the gel, the voltage gradient will increase across this boundary and slower ions will be accelerated so as to keep up. When the boundary passes from the stacking gel into the running gel, 2 important changes occur. First, the higher pH in

the running gel favors the formation of glycine$^-$ ions resulting in a greater mobility of the glycine. Second, the higher gel concentration in the running gel impedes on the proteins. As a consequence, glycine overpasses the proteins and a glycine-chloride boundary moves ahead. The proteins are now electrophoresing in a uniform glycine buffer and are being separated by the molecular sieving effect of the running gel. During the course of this work, pre-cast gels were used (Novex and Invitrogen) as well as NuPAGE pre-cast gels. NuPAGE gels differ from the the SDS-PAGE system described by Laemmli in that their running gel has a pH of 7, reducing running artifacts like band distortion and loss of resolution caused by the basic pH:
12µl sample were mixed with 4µl of 4x LDS sample buffer, heated to 75°C for 10 minutes followed by a brief centrifugation prior to loading them onto the gel. The gels were run at 6Vcm^{-1} until the desired separation was achieved.

4.2.7.4. Immunoblot for the identification of recombinant proteins

The transfer of proteins resolved with SDS-PAGE onto a PVDF membrane was carried out in a "XCellSureLock" wet blotting chamber (Invitrogen, Carlsbad, CA). The blot was setup as follows:



Before setting up the blot, the PVDF membrane was wetted in methanol for 30s and then washed with transfer buffer.
When an electric current is applied to the XCell blotting module, the negatively charged proteins migrate from the cathode to the anode and are retained on the PVDF membrane by electrostatic and hydrophobic interactions. For the transfer the blotting chamber was placed in a bucket with ice to avoid heating of the setup, which was carried out at 150mA and a maximum of 25V for 2-3 hours. The blot was then disassembled and the membrane was placed in blocking buffer for 1 hour at RT or ON at 4°C. The primary antibody was diluted into blocking buffer and incubated with the membrane for 2-3 hours at RT followed by 4x15m minute washes in TBS-T. The secondary HRP-coupled antibody, diluted into blocking buffer, was then applied to the membrane and incubated for 1-2 hours at room temperature followed by 4x15 minute washes in TBS-T.
ECL solution was given onto the membrane for 1 minute (Amersham-Pharmacia) according to the manufacturer's recommendations. The membrane was then covered with Saran wrap and an ECL hyperfilm (Amersham) was exposed to the membrane. The exposition times varied depending on the signal intensity. The films were then developed in a KODAK X-O-MAT developer.

For re-probing of the membrane with a second antibody, the membrane was placed into 'WB stripping buffer' at 60°C for 1-2 hours in a water bath followed by 3x15 minute washes with TBS-T.

4.2.8. Immunohistochemistry

This standard procedure allows for the detection of proteins with mono- or polyclonal antibodies. The technique has been described in great detail in (Harlow and Lane, 1988) and thus will only be outlined briefly.
For the detection of transgene-derived EGFP, a polyclonal peptide antibody (rabbit-α-EGFP, BD Biosciences) was used 1:100. As a secondary antibody, α-rabbit-IgG-AlexaFluor568 was used, 1:5000 – 1:8000. For the detection of ChAT protein, a tyramide-based amplification protocol was used (Winsky-Sommerer et al., 2000). This protocol was also used to detect EGFP and ChAT on the same specimen. "A" and "B" refer to components of the "VectaStain ABC kit" (Vectorlabs):

- Block sections for ~ 2h @ 4°C in blocking buffer
- Incubate tissue ON @ 4°C with primary antibodies (anti-CHAT: 1:1000, anti-EGFP: 1:100) in incubation buffer
- Wash 3x 15min @ RT in TBS-T
- Incubate with 2° antibody (Biotin SP-donkey α-goatIgG, 1:300, 45min –1h @ RT) in incubation buffer

  in the meantime: mix 5ml PBS
  + 1 drop "A"
  + 1 drop "B"
  -> vortex
  ->incubate 30-45min @ RT (ABC/PBS solution)
  Tubes "A" and "B" were vortexed prior to use.

- Wash 3x with TBS-T
- Incubate 45min in ABC/PBS solution
- Wash 3x 5min in TBS
- Incubate 10min in TSA amplification buffer (dilute TSA in dilution buffer [TSA kit] 1:100)
- Wash 3x 5min in TBS
- Incubate with 2nd 2° antibody (α-rabbitIgG-Alexa568, 1:5000 – 1:8000) for 45min @ RT in blocking buffer (use goat serum for blocking)
- Wash 3x 10min with TBS-T
- Mount sections with VectaShield HardSet Mounting medium (VectorLabs, Burlingame, CA)

4.3. Tissue culture

4.3.1. Culturing of mammalian cells

For this thesis the following cell lines were cultured according to well established protocols: cos7 (Gluzman, 1981) and PC12 (Greene and Tischler, 1976). In brief, cos7 cells were maintained in DMEM supplemented with 10%FBS and 1xPenStrep in a humidified incubator in a 5%CO_2 atmosphere. Cells were grown on 15cm dishes and split 1:10 upon reaching 90% confluence.
PC12 cells were grown on PLL-coated 15cm dishes in DMEM supplemented with 10%FBS, 5%HS and 1xPenStrep. The cells were split upon reaching 80% confluence, the number of passages was marked on the culture dish and cells were discarded after 35 passages. The PC12 cells were triturated prior to plating to obtain a single cell suspension. PC12 cells were usually split 1:10.
For passaging, both cell lines were detached from their culture dishes by mild treatment with trypsin/PBS for 5 minutes after which the cells were washed off the culture dish with culture media, transferred into 15ml canonical tubes and centrifuged for 3 minutes at 900g. The supernatant was discarded and the cells were re-suspended in the desired volume of culture medium and plated.

4.3.2. Slice cultures of mouse brains

This method was adapted from (Schwab et al., 2000). In brief, P5 animals were sacrificed by decapitation and their brains were removed. The brains were then transferred into cold slice culture medium and the meninges were removed. The prepared brains were placed in a slicer and 400μm sections were cut and collected into cold slice culture medium. Brain slices were then transferred onto TC inserts (0.4mm pore size, Greiner, cat#657641), which had been placed into 6 well plates containing slice culture medium. In case of GFPG3:rtTAS^2-M^2 doubly transgenic brain slices, doxycycline was added toa f.c.of 1μg/ml.
The slices were cultured for 3 days in a humidified 5% CO2 atmosphere, after which they were fixed with 4%PFA and assessed by lacZ histo-chemistry.

VII. Abbreviations

aa	amino acid
α-CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II-α
AChR	acetylcholine receptor
Amp	ampicillin
APS	ammoniumpersulfate
ARIA	acetylcholine receptor-inducing activity
BAC	bacterial artificial chromosome
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BGHpA	bovine growth hormone polyadenylation site
bla	beta-lactamase
bp	base pair
ChAT	choline acetyltransferase
CIP	calf intestinal phosphatase
CMV	cytomegalovirus
CNS	central nervous system
CpG	deoxy(cytidylyl-guanosine) dimer
depc	diethyl-pyrocarbonate
DIV	days *in vitro*
DNAse	deoxyribonuclease
dNTPs	deoxyribonucleic acid tri-phosphate
DMSO	dimethyl sulfoxide
dox	doxycycline
DRG	dorsal root ganglion
dsDNA	double stranded DNA
E	embryonic day (mouse development)
EDTA	Ethylene Diamine Tetra Acetic Acid
EGF	epidermal growth factor
EST	expressed sequence tag
EtOH	ethanol
f.c.	final concentration
floxed	flanked by loxP sites
flped	flanked by FRT sites
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Ggl.	ganglion
GGF	glial growth factor
HRG	heregulin
kb	kilo base pairs
l	liter
lacZ	β-galactosidase
LB	Luria broth
KanR	kanamycin resistance
m.	musculus
Mb	mega bases
ml	milliliter
MMP	matrix metallo-proteinase

NMDA	N-methyl-D-aspartate
NRG1	neuregulin-1
OD	optical density
ON	over night
ORF	open reading frame
ori	origin of replication
P	postnatal day (mouse development)
P_{tet}-bi	bi-directional tTA/rtTA responsive promoter (Baron et al., 1995)
pA	polyadenylation signal
PAC	P1 artificial chromosomes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	para-formaldehyde
PFG	pulsed-field gel
PFGE	pulsed-field gel electrophoresis
PH	pleckstrin homology
pH	$pH=-lg[H^+]$
PNS	peripheral nervous system
PVDF	polyvinylidene fluoride
RNAse	ribonuclease
RNAsIn	recombinant RNAse inhibitor
RT	room temperature
SDS	sodium dodecyl sulfate
ssDNA	single stranded DNA
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA solution
TEA	Triethanolamine
TEMED	N,N,N',N'-Tetramethyl-1-,2-diaminomethane
Tris	Tris(hydroxymethyl)aminomethane
UV	ultra violet
VAChT	vesicular acetylcholine transporter
WB	Western blot
wt	wild type
@	at
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
zeoR	zeocin resistance

VIII. References

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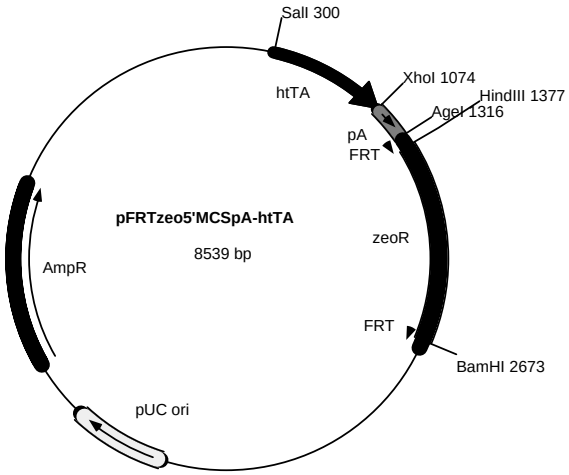
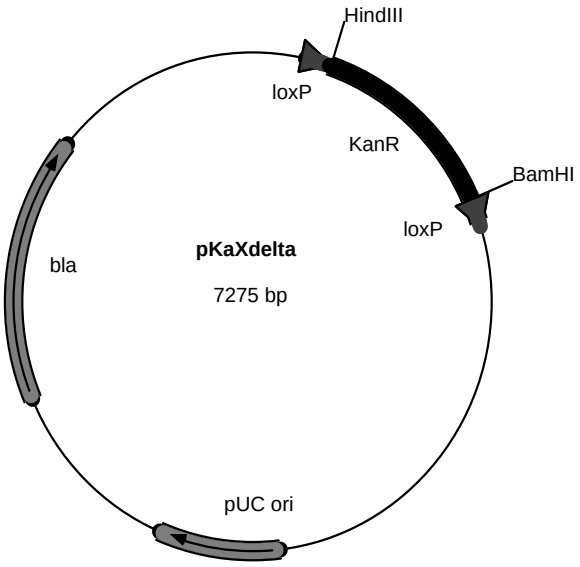
IX. Appendix

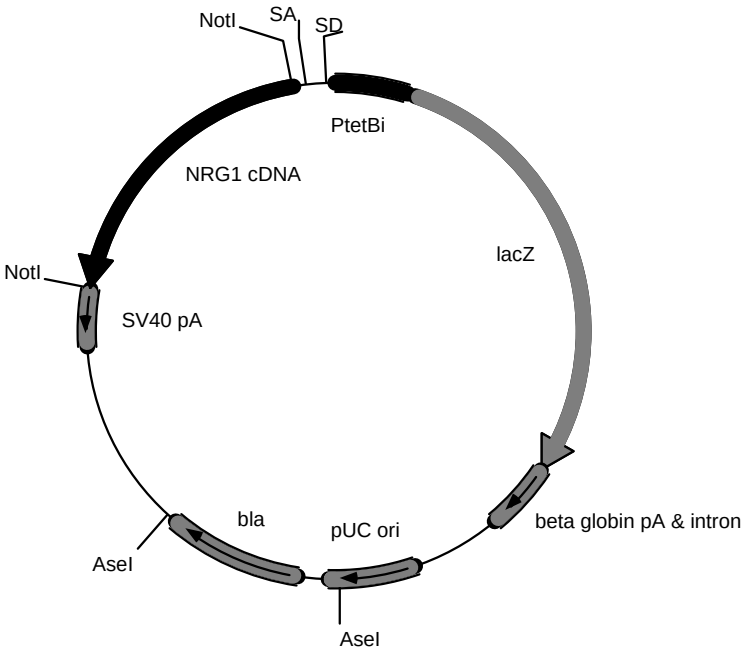
1. Plasmid maps



pFRTzeo5'MCSpA-hTTA: This plasmid was used as a PCR template for the generation of the homologous recombination fragment to insert tTA2S into the start codon of the ChAT gene. Functional elements are: htTA, tTA2S; pA, polyadenylation signal, FRT, FRT site for Flp-mediated excision of the zeocin resistance; zeoR, zeocin resistance; pUC ori, pUC18 origin of replication; AmpR, ampicillin resistance.



pKaXdelta: Plasmid used as a PCR template for the generation of targeting constructs for homologous recombination in bacteria. The functional elements are: loxP, loxP site for a Cre-mediated excision of the kanamycin resistance; KanR, kanamycin resistance; pUC ori, pUC 18 origin of replication; bla, β-lactamse.



P_{tet}-bi NRG1 vector: Expression construct for the tTA-dependent expression of NRG1 isoforms and β-galactosidase in transgenic mice from the bi-directional P_{tet}-bi promoter.The construct was linearized for pronuclear injection by an AseI digest. Functional elements are: NRG1 cDNA. NRG1 cDNA cloned into the vector as a NotI insert; lacZ, β-galactosidase gene; bla, β-lactamase gene; SA, splice acceptor site; SD, splice donor site.