1 Introduction

The enzyme choline acetyltransferase (ChAT) catalyzes the synthesis of the neurotransmitter acetylcholine (ACh) in presynaptic nerve terminals. ACh was the first neurotransmitter described by Loewi in 1921. It plays a pivotal role in fundamental brain functions like learning and memory formation, awareness, the sleep and awaking cycle as well as motor behavior. The ChAT protein was described 1943 by Nachmansohn und Machado who initiated the research of the physiology of the cholinergic nervous system.

The generation of specific ChAT antibodies (Eckenstein and Thoenen, 1982) facilitated the purification of the enzyme and allowed a more detailed analysis of the localization of ChAT in the brain and studies of changes during pathological states. The analysis was further refined by *in situ* hybridization using the ChAT cDNA as probe (Oh *et al*., 1992).

Organotypic brain slice cultures were used to study the development of the cholinergic system *in situ*. Co-culturing slices of different brain areas made it possible to investigate cholinergic fiber growth and targeting (Baratta *et al*., 2001; Gahwiler and Hefti, 1984) and to study the impact of neurotrophic factors on the survival of cholinergic neurons (Gahwiler *et al*., 1987; Gahwiler *et al*., 1990).

Very recently a method for the introduction of foreign genes into individual pyramidal cells of organotypic hippocampal brain slice cultures has been developed, namely single-cell electroporation (SCE) (Haas *et al*., 2001; Rae and Levis, 2002).

1.1 Organization of the cholinergic gene locus, mRNA transcription and translation

The cholinergic gene locus comprises two functionally related genes, the ChAT gene and the vesicular acetylcholine transporter (VAChT) gene which are co-regulated in their expression.

The VAChT transmembrane protein transports ACh synthesized by ChAT into synaptic vesicles. Since the expression of ChAT and VAChT in the central and

peripheral nervous system is restricted to cholinergic neurons, they both serve as presynaptic markers (Eiden, 1998).

The rat ChAT gene consists of one first noncoding exon and of 14 coding exons which are distributed over at least 64 kb. The translation starts in exon 2 (Hahn *et al*., 1992). The first intron of the ChAT gene encodes the single exon of VAChT (Bejanin *et al*., 1994). The genomic structure and the resulting mRNAs are shown in Figure 1.1.

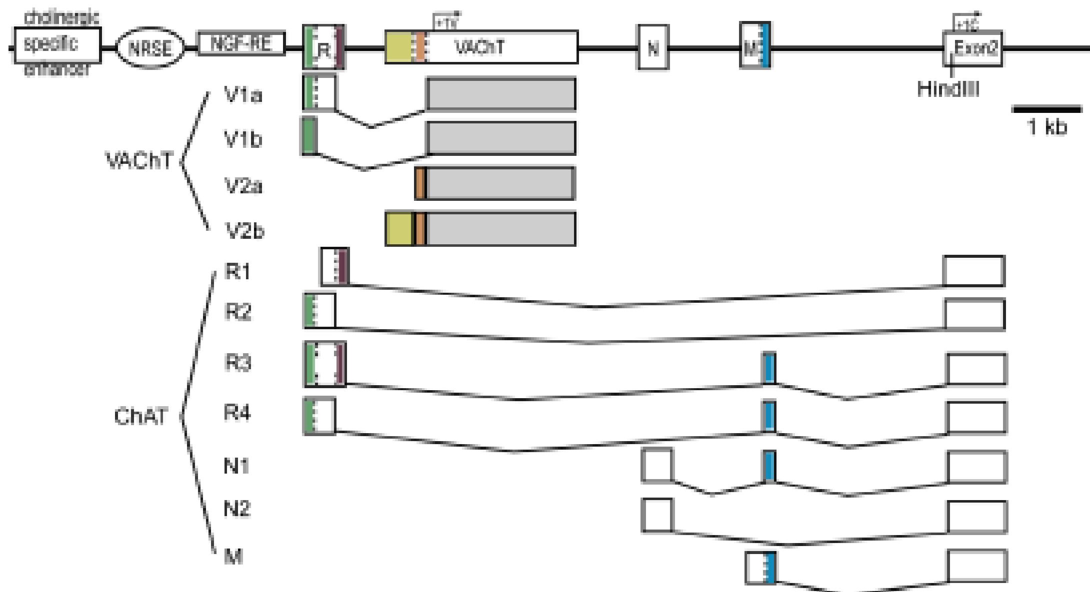

Fig. 1.1 Genomic organization of the cholinergic gene locus. R, N and M are the first noncoding exons of the ChAT gene. The translation of ChAT starts in exon 2. The single coding exon for VAChT is located within the first intron of the ChAT gene. Cis-acting regulatory promoter elements are a cholinergic specific enhancer, a neuron-restrictive silencer element (NRSE) and a nerve growth factor responsive element (NGF-RE). Four splice variants are found for VAChT and seven splice variants for ChAT. Taken from Eiden (1998).

In rodent tissues ChAT is encoded by seven mRNAs with different 5´ends resulting from alternative usage of three noncoding first exons (R1, R2, N and M) (Misawa, 1992). Mechanisms regulating production of the different transcripts and their physiological roles have not been elucidated so far. In the human mRNAs the translation is initiated at an ATG 30 bp downstream of the corresponding ATG found in rodents and produces a 69 kDa ChAT protein (Chireux *et al*., 1995; Misawa *et al*., 1997). The human M-type transcript has two translation-initiation sites and yields, in addition to the 69 kDa form, a 82 kDa protein which is localized in nuclei (Oda *et al*., 1996; Resendes *et al*., 1999).

A 6.4 kb promoter fragment upstream of the ChAT start ATG contains regulatory elements that directs the expression of a transgenic lacZ reporter gene to cholinergic neurons (Naciff *et al*., 1999). In this region cis-acting sequences are present which are responsible for neurotrophic factor actions. A nerve growth factor responsive element is located about 2 kb upstream the R-exon (Bejanin *et al*., 1992). A 21 bp long consensus sequence for a neuron-restrictive silencer element (NRSE) exists in rat and human about 3kb upstream the VAChT start ATG (De Gois *et al*., 2000; Lonnerberg *et al*., 1996; Schoenherr and Anderson, 1995).

1.2 ChAT protein purification and characterization

The abundance of the ChAT protein in mammalian brain is very low (~0.0001 % of brain protein (Eckenstein and Thoenen, 1982)). Therefore purification was very difficult (McGeer *et al*., 1984). This problem was resolved by the availability of antibodies against ChAT using immunoaffinity chromatography (Bruce *et al*., 1985). Protein sequence information led to the cloning of the ChAT cDNA from pig spinal cord (Berrard *et al*., 1987). Subsequently the ChAT cDNAs of rat, mouse (Ishii *et al*., 1990) and human (Oda *et al*., 1992) were cloned.

Bruce and Hersh (1989) studied the phosphorylation of human ChAT as a possible regulatory mechanism. Recently it has been shown that phosphorylation of the recombinant human 69 kDa form by protein kinase C and -calcium/calmodulin-dependent kinase II significantly increased the specific activity while dephosphorylation with alkaline phosphatase reduced the specific activity (Dobransky *et al*., 2000). Phorbol ester treatment of HEK293 cells, transiently transfected with recombinant human ChAT, mediated phosphorylation of ser440 and led to increased ChAT enzyme activity. This was abolished in a ser440ala mutant and resulted in a decreased membrane association indicating a major role of ser440 in regulation of enzyme activity and subcellular targeting (Dobransky *et al*., 2001). Functional analysis of Drosophila ChAT revealed a conserved his426 at the active site (Carbini and Hersh, 1993). In addition an active site arg452 was identified in rat ChAT which was assumed to interact with acetyl-CoA and a arg452ala mutation resulted in a decreased affinity for coenzyme A (Wu and Hersh, 1995). Recombinant ChAT activity can be increased by limited

proteolysis (Wu *et al.*, 1995) but there is no evidence that this activation mechanism occurs *in vivo*. Biochemical studies have shown that ChAT exists as a non-ionically membrane-bound form in rat brain synaptosomes and is at least in part associated with cholinergic synaptic vesicles (Carroll, 1994). This membrane-bound form amounts to 10-20 % of the total enzyme activity present in cholinergic nerve terminals. However, it is neither clear how membrane-bound ChAT is anchored to the membrane nor to which membrane.

1.3 The cholinergic synapse

Acetylcholine (ACh) is synthesized by the transfer of the acetyl group from acetyl-CoA to choline which is catalyzed by ChAT. VAChT actively transports ACh into synaptic vesicles by a proton-motive force. When an action potential reaches the nerve terminal the vesicles fuse with the presynaptic membrane and ACh is released into the synaptic cleft. ACh binds to ACh receptors (AChR) which are highly enriched in the postsynaptic membrane. There are two types of AChRs:

- Nicotinic AChRs (nAChRs) are ionotropic receptors and are activated by the ACh agonist nicotine. They are ion channels selective for Na^+ and K^+ that open upon ACh binding.
- Muscarinic AChRs (mAChRs) are metabotropic receptors and are activated by the ACh agonist muscarine. They are G-protein coupled, putative seven transmembrane receptors and activate downstream signal transduction pathways by activation of phospholipase C (PLC) upon ACh binding. mAChRs are widely distributed in the CNS.

After binding of ACh to AChRs it is cleaved by acetylcholinesterase (AChE) and free choline is transported back into the nerve terminal by the high-affinity choline transporter (Okuda *et al.*, 2000).

The most frequently studied cholinergic synapse is the neuromuscular junction, which is composed of three types of cells: the motoneuron, the muscle fiber and the Schwann cell isolating the nerve terminal. The presynaptic side of the motoneuron and the postsynaptic side of the muscle fiber are separated by the synaptic cleft. The arriving action potential of the motoneuron activates voltage-gated Ca^{2+}-channels located in the

presynaptic membrane. Due to the influx of Ca^{2+}, ACh-filled vesicles fuse immediately with the presynaptic membrane and release ACh into the synaptic cleft (Calakos and Scheller, 1996). ACh binds to nAChRs that are located at the tops of the postsynaptic folds at a density of 10,000/μm^2 (Raftery *et al*., 1974; Salpeter and Elderfrawi, 1973). nAChRs are pentamers of four different subunits (reviewed in Hucho *et al*., 1996) composed of $_2$, , and in the empryonic stage. The -subunit is postnatally replaced by the subunit (Mishina *et al*., 1986). Activation of the receptor leads to an influx of Na^+ ions into the postsynaptic specialization and the depolarization of the membrane, followed by the activation of voltage-gated Na^+ channels in the vicinity of the AChRs. This evokes an action potential which finally results in muscle contraction (Cooper and Jan, 1999).

1.4 The central and peripheral cholinergic nervous system

1.4.1 Anatomy

The distribution, organization and projection pattern of cholinergic neurons was studied by using *in situ* hybridization and immunohistochemistry for the ChAT- or VAChT-mRNA or -protein. At first cholinergic neurons were identified by AChE staining (Friedenwald, 1949). However, this cannot clearly evaluate the cholinergic nature because AChE can be present in both acetycholine-transmitting and cholinoceptive neurons. One of the most specific markers to date are antibodies against ChAT (Eckenstein and Thoenen, 1982). The ACh producing enzyme should be present only in acetylcholine transmitting and not in cholinoceptive neurons. A continuing problem with immunocytochemical methods is the possible cross-reactivity of the primary antibody with structurally distinct proteins sharing similar epitopes. A complementing and highly specific approach is *in situ* hybridization with ChAT cDNA fragments. Although the specificity of this label is very high detailed morphologic assessments of neuronal arbors is difficult. Another recent study describes the cholinergic system by *in situ* hybridization and immunohistochemistry for the other cholinergic marker enzyme VAChT (Schafer *et al*., 1998).

The first overview of the central cholinergic pathways in the rat was provided in 1983 by Mesulam *et al*. In this immunohistochemical study with a monoclonal antibody against ChAT, cholinergic neurons are aggregated in six major groups. These main cholinergic cell groups and their site of innervation are summarized in Table 1.1.

Tab. 1.1 Cholinergic cell groups and their site of innervation.

Cholinergic cell groups	Nuclei that contain cholinergic neurons	Principal target regions of cholinergic projections
Ch1	Medial septal nucleus	Hippocampus
Ch2	Vertical limb nucleus of the diagonal band	Hippocampus
Ch3	Horizontal limb nucleus of the diagonal band	Olfactory bulb
Ch4	Nucleus basalis magnocellularis, globus pallidus, Substantia innominata, Nucleus of the ansa lenticularis, Preoptic magnocellular nucleus	Neocortex and amygdala
Ch5	Nucleus pedunculopontinus	Thalamus
Ch6	Laterodorsal tegmental nucleus	Thalamus

The cholinergic innervation of the hippocampus and the olfactory bulb is provided by neurons in the medial septal nucleus and the vertical and horizontal limb nuclei of the diagonal band of Broca (Ch1-Ch3). Innervation of the neocortex and amygdala occurs via neurons in the nucleus basalis and the substantia innominata (Ch4). Neurons in the nucleus pedunculopontine and the laterodorsal tegmental nucleus of the rostral brainstem project to the thalamus (Ch5-Ch6). Most of these immunohistochemical results were supported by *in situ* hybridization studies with ChAT mRNA riboprobes (Oh *et al*., 1992).

There are three types of cholinergic neurons that originate in the CNS and send their terminals to the periphery. These include motor neurons, preganglionic sympathetic neurons and preganglionic parasympathetic neurons. Cholinergic neurons are found in the ventral, but not in the dorsal horns of the spinal cord at all levels and in the intermediolateral cell column at thoracic, lumbar and sacral levels. Large ventral horn cholinergic cells are probably -motoneurons, whereas these with comparatively small perikarya are arguably -motoneurons (Butcher, 1995; Oh *et al*., 1992). The -motoneurons innervate skeletal muscles of the arms and hands, trunk, legs and feet.

1.4.2 Development

Regulation of the cholinergic phenotype occurs progressively during development. In rat brain and spinal cord, the levels of ChAT mRNA increase slowly during embryonic life and more rapidly after birth, reaching maximal levels in the adult. Neurons may also become cholinergic in response to injury (Ibanez *et al*., 1991).

Nerve growth factor (NGF) plays a fundamental role in the survival and targeting of the basal forebrain cholinergic neurons (Gahwiler *et al*., 1987; Gahwiler *et al*., 1990). Receptors for NGF are the tyrosine kinase A (TrkA) receptor and the low-affinity p75 neurotrophic ($p75^{NTR}$) receptor. Basal forebrain cholinergic neurons are enriched with both receptors which are internalized and retrogradely transported from basal forebrain cholinergic nerve terminals innervating the target regions upon NGF binding (Johnson *et al*., 1987). Regions of cholinergic basal forebrain innervation (e.g. hippocampus and cortex) express high levels of NGF (Korsching *et al*., 1985). This signal pathway may be changed during neurodegenerative diseases and could lead to the specific loss of cholinergic neurons in the basal forebrain (see Section 1.4.4).

The important role of NGF is substantiated by studies of several genetically modified mouse lines. For instance NGF "knock-out" mice display severe cholinergic cell loss in sensory and sympathetic ganglia and a reduced viability (Crowley *et al*., 1994). TrkA "knock-out" mice have a decrease in the cholinergic basal forebrain projections to the cortex and hippocampus (Smeyne *et al*., 1994). $p75^{NTR}$ null mutant mice display an increase in cholinergic neuron number and size and ChAT activity (Yeo *et al*., 1997). The direct effect of NGF has been shown by Auld *et al*. (2001) by the treatment of basal forebrain neurons in primary cultures.

Many other factors were described to induce and maintain the cholinergic phenotype. The NGF-related growth factor neurotrophin-3 (NT3) which binds to the tyrosine kinase C (TrkC) receptor was shown to promote cholinergic differentiation (Auld *et al*., 2001; Brodski *et al*., 2000). Brain-derived neurotrophic factor (BDNF) acts on ChAT and Trk receptor levels (Klein *et al*., 1999). Bone morphogenic protein-9 (BMP-9) was found to be highly expressed in the embryonic mouse septum and spinal cord and was shown to induce and maintain the cholinergic phenotype (Lopez-Coviella *et al*., 2000). The neuropeptide galanin co-localizes with ChAT and regulates the postnatal survival of a subset of basal forebrain cholinergic neurons (O'Meara *et al*.,

2000). Fibroblast growth factor-5 (FGF-5) promotes the differentiation of cultured septal neurons (Lindholm *et al*., 1994).

Survival and development of motoneurons is assumed to be regulated by neurotrophic factors from various sources (reviewed in Sendtner *et al*., 2000). Table 1.2 summarizes these factors so far identified.

Tab. 1.2 Neurotrophic factors that are assumed to function in regulating the survival and development of motoneurons.

	Receptor on motoneuron
1. Neurotrophins	
Brain derived neurotrophic factor (BDNF)	$p75^{NTR}$, TrkB
Neurotrophin-3 (NT-3)	$p75^{NTR}$, TrkC
Neurotrophin-4/5 (NT-4/5)	$p75^{NTR}$, TrkB
2. CNTF/LIF family	
Ciliary neurotrophic factor (CNTF)	CNTFR , LIFR , gp130
Leukemia inhibitory factor (LIF)	LIFR , gp130
Cardiotrophin-1 (CT-1)	?, LIFR , gp130
Cardiotrophin-1-like cytokine (CLC)	?, LIFR , gp130
3. Hepatocyte growth factor/scatter factor (HGF/SF)	c-met
4. Insulin like growth factors	
IGF-I	IGFR-1
IGF-II	IGFR-1, mannose-6P-receptor
5. Glial-derived neurotrophic factor and related factors	
Glial-derived neurotrophic factor (GDNF)	GFR 1, c-ret
Neurturin (NTR)	GFR 2, c-ret
Persephin	GFR 3, c-ret
Artemin	GFR 4, c-ret

1.4.3 Physiology

Cholinergic neurons originating from basal forebrain or brain stem loci project to cortical and hippocampal areas and to the peripheral nervous system as described in section 1.4.1. The physiological properties underlying central cholinergic pathways are very complex and occur either via nAChRs or mAChRs (see Section 1.3). The physiological characteristics are quite different from those produced by glutamate or other excitatory amino acids. One is a slow onset (up to seconds after ACh application until the neuron begins responding) and a similar prolonged action after removal of ACh. These characteristics could be blocked by atropine, indicating that they are due to mAChR. ACh can also activate some cortical neurons via nAChR. The responses to nicotine are usually of a rapid onset and offset (Stone, 1972).

The cortical innervation is diffuse and only roughly topographic which led to the assumption that ACh plays a neuromodulatory rather than a direct synaptic role. Due to the low incidence of cholinergic synapses in the rat cortex it has been suggested that a non-synaptic ACh transmission could be the principal manner through which cholinergic terminals could influence cortical activity (Umbriaco *et al*., 1994). However, direct evidence that the cholinergic innervation of the cerebral cortex follows a precise synaptic pattern has recently been provided (Turrini *et al*., 2001).

ACh, which is released from septohippocampal terminals, is assumed to play a major role in cognitive processes of hippocampal lerning and memory formation by modulation of the most extensively studied model of plasticity, long-term potentiation (LTP). For instance, in the CA3 region of the hippocampus activation of mAChRs depressed LTP (Williams and Johnston, 1988) whereas in the dentate gyrus and CA1 region cholinergic agonists facilitated LTP (Blitzer *et al*., 1990; Burgard and Sarvey, 1990).

1.4.4 Disease

The impairment of central cholinergic function is an early and constant finding in a number of mental disorders associated with amnesia and dementia like Alzheimer’s disease (AD). Apart from central impairments, cholinergic motor neuron degeneration occurs in Amyotrophic Lateral Sclerosis (ALS).

In AD the concentrations of acetylcholine and ChAT are markedly reduced in the cerebral neocortex and hippocampus (Bartus *et al*., 1982) and also the remaining cholinergic neurons insufficiently transcribe ChAT mRNA (Strada *et al*., 1992). The number of cholinergic neurons in the cholinergic basal forbrain, including the nucleus basalis of Meynert (Ch4) and the medial septum (Ch1) is markedly decreased which significantly correlates with the severity of dementia (Wilcock *et al*., 1982). At the same time -amyloid protein plaques, which are a result of the abnormal processing of amyloid precurser protein, are deposited (Geula *et al*., 1998; Selkoe, 1991). How or if -amyloid plaques lead to cholinergic neuron loss has not been elucidated yet. Possibly the -amyloid plaques disturb the metabolism of neurons by inflammation, followed by the formation of intracellular neurofibrillary tangles, which consists of

hyperphosphorylated, highly polymerized cytoskeletal tau protein. This process probably induces damage to neurons, particularly to cholinergic axons in the cortex and cholinergic neurons in the basal forebrain, or causes retrograde alteration in basal forebrain neurons with axons projecting to the cerebral cortex.

In ALS a loss of motoneurons is observed in the cerebral motor cortex, the motor nuclei in the brain stem and the anterior horn of the spinal cord which results in the progressive weakness and wasting of muscles. Biochemical and morphological studies have shown that ChAT activity and mRNA in tissue samples from the anterior horn of ALS spinal cord is markedly reduced (Kato, 1989; Virgo *et al*., 1992). ALS seems to be linked genetically to mutations in the superoxide dismutase gene (Morrison and Morrison, 1999).

1.5 Organotypic cultures of neural tissue

Slices derived from different brain regions are very important tools for studying the physiological and developmental properties of neuronal systems. Organotypic slice cultures were developed with explants of various anatomical origins that can be used for long-term studies. Several alternative techniques are currently available:

- In roller-tube cultures, the tissue is embedded in a plasma clot on glass coverslips and is continuously rotated which ensures oxygenation (Gahwiler, 1981).
- In membrane cultures, the slices are placed at the air medium interface on Millicell semiporous membranes. They obtain oxygen from above and medium from below (Stoppini *et al*., 1991).
- In slices grown in culture dishes, the cultures are completely covered by medium and due to the limited oxygenation the tissue is either derived from fetal animals and is cultured only a few days or very thin sections are prepared (Tucker *et al*., 1996).

Cultures grown on membranes by the interface method are prepared easily and can be observed during all stages of culturing as the tissue is never covered by an embedding material. Early postnatal periods (P0 to P7) of rodents are ideally suited for culturing because the essentials of the cytoarchitecture are already established in most brain areas, the brain is larger and easier to dissect, and the nerve cells survive explantation more readily. Many areas of the nervous system were successfully cultured including the

hippocampus, the cerebellum, the thalamus, the hypothalamus, the cortex, brainstem structures, the spinal cord, the substantia nigra, the striatum, the medial basal forebrain and the retina. These cultures are termed "organotypic" since the phenotypic anatomy of neuronal types and the tissue organization are similar to those *in situ*. Organotypic slice cultures enable many applications, ranging from physiology, pharmacology, anatomy and development to endocrinology. They are very suitable for experiments that require long-term survival of the preparation, such as studies that involve chronic application of drugs and factors (Gahwiler *et al*., 1990), video-microscopy, analysis of fiber growth and synaptic transmission or axon targeting in co-cultures derived from different brain areas (Gahwiler *et al*., 1990) and transfection of neurons with DNA (Haas *et al*., 2001).

1.6 Transfection of neurons by electroporation

Electroporation is a recently developed method to insert DNA, RNA, dyes, peptides and proteins into cells (Neumann *et al*., 1999). Particularly for neurons electroporation has advantages since methods like viral gene transfer, "gene gun" biolistics, lipofection and microinjection are either time consuming or have potentially toxic and damaging effects. In addition selective lebeling of a single cell with dye or transfection is nearly impossible.

Electroporation occurs when the voltage applied across the membrane exceeds the dielectric breakdown voltage of the membrane (~200 mV-1.5 V). The large external electric field induces high transmembrane potentials, that lead to the formation of minute pores (20-120 nm diameter) with higher voltages producing larger pores. During the electric pulse charged molecules are actively transported by electrophoresis across the membrane. Non charged molecules could also enter through these pores by passive diffusion. The opening of the pores occurs very quickly (in the µs range) whereas they reseal over tens to hundreds of msec. Traditionally short-duration dielectric field pulses are applied between two relatively large plate electrodes (Teruel *et al*., 1999). However, this approach does not allow the transfection of single cells. Very recently methods have been developed to electroporate individual cells. Here, a micropipette is filled with DNA solution and connected to the anode of a pulse voltage generator through a thin silver wire, which is in contact with the DNA-solution. The ground electrode (medium

or buffer reservoir) is connected to the cathode. Under visual guidance using a dissection microscope and with the help of a micromanipulator the pipette can be positioned into neural tissue. This method can be applied to transfect not only single cultured cells but also individual neurons in organotypic slice cultures and even in living animals in areas with very high cell density (Haas *et al.*, 2001). However, the transfection rate for neurons was low. Higher transfection rates (up to ~84 %) could be achieved by optimizing the electroporation conditions using the lens epithelial cell line -NT4 (Rae and Levis, 2002).

1.7 *In vivo* imaging of the mammalian nervous system using fluorescent proteins

The complicated cellular morphology of neurons makes it difficult to study the nervous system *in vivo*. This was changed with the introduction of the green fluorescent protein (GFP) (Chalfie *et al.*, 1994). Using specific promoters or vector-encoded protein fusions, GFP can be targeted to specific cell types or subcellular compartments. The introduction of the transgene can be achieved temporally and transiently using viral vectors, "gene gun" biolistics or electroporation (see Section 1.6). An alternative approach to circumvent the short period of gene expression, toxicity and vector delivery problems is the generation of transgenic mice to monitor expression patterns, protein transport and distribution.

The GFP chromophore consists of a cyclic tripeptide comprising ser65-tyr66-gly67 which undergoes cyclization and oxidation and is only fluorescent when embedded within the complete GFP protein. GFP combines the features of gene-based reporter usage with non-invasive detection. It therefore can be used not only to tag gene expression or promoter activity, or be engineered as a fusion protein thereby providing subcellular localization, but also reporter activity can be monitored in real-time *in vitro* or *in vivo*. GFP is small (27 kDa) and functions primarily as a monomer. These features make GFP suitable for "knock-in" gene targeting and transgenics in mice.

Several GFP mutants with altered excitation and emission spectra, in addition to improved thermostability and fluorescence, were described and are available for use in mice. The most widely used GFP is the red-shifted variant, enhanced GFP with humanized codon usage (EGFP) for increased expression efficiency in mammalian

systems (Cormack *et al*., 1996; Zolotukhin *et al*., 1996). Red-shifted variants contain mutations (Phe64Leu and Ser65Thr) that shift the maximal excitation peak toward the red end of the spectrum at approximately 489 nm and emission peak at 508 nm and fluoresces approximately 30-40 fold brighter than wildtype GFP. The wavelengths comprise the band width of commonly used filter sets and argon ion lasers in confocal microscopes. Another red-shifted variant is the enhanced yellow fluorescent protein (EYFP) containing four amino acid substitutions that shift the emission from green to yellow at 527 nm with an excitation maximum at 514 nm (Ormo *et al*., 1996). Apart from GFP a red fluorescent protein (DsRed) is available with excitation at 558 nm and emission at 583 nm. At the other end of the spectrum are the blue/cyan emission variants (ECFP) with four amino acid substitutions that shift the excitation and emmission to a shorter wavelengths of 434 nm and 477 nm, respectively (Heim and Tsien, 1996).

1.8 Cre/loxP mediated gene deletion or conditional gene "knock-out"

Deletion or modification of a functionally important gene may cause severe developmental impairments or even leads to the death of the embryo. To circumvent these effects a method of tissue and developmental specific gene deletion in mice has been introduced (Gu *et al*., 1994). This cell-specific "gene targeting" is based on the Cre/loxP recombination system from the bacteriophage P1 (Sauer and Henderson, 1988b). The 38 kDa Cre recombinase protein (Cre) is able to remove DNA fragments flanked by two 34 bp long loxP sequences (Sternberg and Hamilton, 1981) leading to a deletion of the so-called "floxed" fragment. Targeting a "floxed" gene fragment into mice and breeding them to the homozygous state generates a conditional "knock-out". Subsequent breeding with mice that express Cre in a tissue and/or development specific manner will evoke the loss of gene function only in this tissue or at this developmental phase.

1.9 Creation of mutant mouse lines by homologous recombination

Genetically modified mice are used increasingly as tools to define or clarify the *in vivo* function of molecules that have been studied in vitro. Mutant mice which survive the manipulation allow the analysis of a single gene alteration on a complex behavior. In addition to being a tool for understanding the physiological function of particular proteins, mutant mice can also be used to model human diseases. Foreign genes can either be modified ("knock-in") or inactivated ("knock-out") by homologous recombination (reviewed in Soriano, 1995).

Homologous recombination mediates the *in vivo* exchange of genomic sequences within a selected gene. At first, the genomic fragment encoding all or parts of the gene of interest must be isolated from a genomic mouse library. Then a targeting vector harboring the modification will be cloned. The genomic fragment, which is identical to the gene that should be modified, is separated by a selection marker (neomycin gene, neo^r, driven by the murine phosphoglycerate kinase 1 (PGK-1) promoter) in a long and a short arm of the targeting vector. At the same time the screening strategy to identify homologous recombination events must be developed. The targeting vector is linearized and electroporated into embryonic stem (ES) cells derived from the 129 mouse strain. After antibiotic selection colonies are picked for DNA isolation and are screened by PCR and confirmed by genomic Southern blot hybridization to detect ES cell clones in which the genomic locus is correctly recombined. Recombined ES cell clones are then microinjected into 3,5-day-old blastocysts derived from C57BL/6 mice. Injected blastocysts are reimplanted into the uterus of pseudopregnant females. Usually a fraction of the resulting offspring is chimeric, meaning that they have partly developed from the targeted ES cells and in part from cells of the donor blastocyst. Chimerism can be assessed visually with different coat color. Male chimerae (with more than 50 % chimerism) are then crossed to female wildtype mice and germline transmission of the resulting offspring is assessed by DNA analysis. Mice containing one wildtype and one targeted allele (heterozygotes) can then be intercrossed to generate mice homozygous for the targeted allele.

1.10 Aims of the project

All methods for visualizing cholinergic neurons such as immunohistochemistry, *in situ* hybridization and AChE-staining require fixation of the tissue. Thus it is not possible so far to study the cholinergic nervous system in terms of physiology, development and degeneration *in vivo*. Moreover, these methods do not gain the highest specificity to identify cholinergic neurons.

Therefore, in one part of the project, the widely used *in vivo* marker GFP will be fused to the marker of cholinergic neurons (ChAT). The enzymatic properties as well as the subcellular distribution of a ChAT-GFP fusion protein will be assessed and compared to that of the wildtype enzyme. GFP will then be targeted to the cholinergic gene locus by homologous recombination in mouse embryonic stem cells when it has been shown that GFP introduction has no influence on the physiological properties of ChAT. This approach will provide the highest sensitivity and specificity. Neurons expressing a ChAT-GFP fusion protein can be visualized in cell and tissue culture and even in living animals.

In addition, exon 2 of the ChAT gene will be flanked by loxP sites. This will allow deleting the ChAT gene in later developmental stages and circumvents the lethality of a ChAT "knock-out" during embryogenesis. Furthermore one will be able to delete ChAT specifically in subregions of the brain.

In another part of the project both organotypic brain slice culture and single-cell electroporation (SCE) will be established and optimized. SCE is a very promising method for the transfection of individual neurons in organotypic brain slice cultures. Organotypic brain slice cultures, which will later be prepared from ChAT-GFP or ChAT-loxP targeted mice, enables one to study the development of the cholinergic system in real-time. By using this method it will be possible to manipulate cholinergic neurons individually by introducing foreign genes and to study developmental changes in culture.