4 Discussion

The understanding of the cholinergic nervous system is of vital interest since cholinergic neurons have been implicated to control motor behavior and learning and memory processes. Loss of cholinergic neurons or alterations in cholinergic innervation may cause amyotrophic lateral sclerosis (ALS) or Alzheimer's (AD) and other neurodegenerative diseases.

The commonly used methods to identify cholinergic cells are immuno-histochemistry and *in situ* hybridization for the cholinergic marker enzyme ChAT. However, still unsolved problems are the cross-reactivity of antibodies and a rather poor resolution of the cellular morphology with radiolabeled riboprobes (Butcher, 1995; Oh *et al*., 1992). Both methods require in addition the fixation of the cells and thus preclude studies in living cells or animals. Here, a ChAT-GFP fusion protein was generated, which can be visualized directly by optical methods and thus allows *in vivo* studies. Moreover, the sensitivity of fluorescent detection is at least as high as for histochemical methods and problems due to cross-reactivity do not longer obscure the results. The functionality of the recombinant ChAT-GFP fusion protein was tested in COS-1 cell culture and neuronal expression systems. Having shown that introduction of GFP into the ChAT sequence does not change the enzyme's properties, GFP was targeted to the ChAT gene in mouse embryonic stem cells. In parallel an ES cell clone was generated in which exon 2 of the ChAT gene is flanked by loxP sites that will serve to produce conditional ChAT "knock-out" mice.

Once the ChAT-GFP targeted mice will be available the introduction of foreign genes that modulate cholinergic neurons will be necessary. To this end an organotypic brain slice culture method in combination with single-cell electroporation (SCE) was established and it is now possible to manipulate the physiological properties of neurons *in situ*. Single pyramidal neurons of hippocampus slices were transfected with expression vectors encoding fluorescent cytosolic, presynaptic and postsynaptic localized proteins resulting in strong and efficient expression. The widely used electroporation method for transfection of cultured cells has been adopted and optimized to transfect neurons in an "organotypic" environment. The efficiency of SCE has been significantly increased compared to previous studies.

4.1 A recombinant ChAT-GFP fusion protein has been generated which is efficiently expressed in COS-1 cells

The mouse ChAT cDNA was amplified by RT-PCR from total RNA prepared from spinal cord. The selected primer sequences were based on the published mouse ChAT cDNA sequence (Ishii *et al*., 1990). The ChAT cDNA was cloned into an expression vector under the control of the strong CMV promoter. The open reading frame of the ChAT cDNA was 1923 bp coding for 641 amino acids identical to the sequence of Ishii et al. (1990). The cloned cDNA comprises only the coding sequence without the 5´ends of any of the different ChAT splice variants. The enhanced, red-shifted form of green fluorescent protein with humanized codon usage was cloned into a unique HindIII site 48 bp downstream of the start codon of the ChAT cDNA corresponding to the position in exon 2 of the ChAT gene. This cloning step evoked frameshift mutations in both HindIII sites, which subsequently were corrected by site-directed mutagenesis.

Transfection of COS-1 cells with the expression vectors led to an efficient and functional expression of both the wildtype ChAT and the ChAT-GFP fusion protein. Western blot analysis confirmed that wildtype ChAT with a molecular weight of 71.7 kDa (Figure 3.5B) for the mouse protein was of similar size as the recombinant rat protein as shown here and previously (Wu *et al*., 1995; Wu and Hersh, 1995). When GFP with a molecular weight of 27 kDa is inserted into the N-terminal part of ChAT the molecular weight of the fusion protein is shifted to 98.6 kDa. This was also confirmed by Western blot analysis (Figure 3.5B).

The polyclonal affinity purified anti-ChAT antibody used for Western blot analysis showed a strong cross-reactivity (Figure 3.5B). Several monoclonal antibodies against ChAT have been described but they are unfortunately not commercially available or they are generated against proteins of other species (Eckenstein and Thoenen, 1982; Mechawar *et al*., 2000). In contrast to that, the monoclonal antibody against GFP is very specific and revealed no cross-reactivity (Figure 3.5C). Considering the cross-reactivity of the anti-ChAT antibodies the fluorescence of ChAT-GFP expressing mice represents a better tool with higher specificity to visualize cholinergic cells and to analyze the subcellular targeting of ChAT. The high specificity of the monoclonal anti-GFP antibody will, in addition, be useful for immunohistochemistry at

an ultrastructural level to further resolve the subneuronal distribution and localization of ChAT in the presynapse. Furthermore immunoprecipitation experiments can be performed with high specificity to biochemically characterize the interaction of ChAT-GFP with other proteins.

4.2 Influence of GFP insertion in ChAT enzyme activity

Since GFP should be targeted to the gene locus of ChAT by homologous recombination it was essential that the resulting ChAT-GFP fusion protein is enzymatically active. Otherwise a ChAT "knock-out" would be generated. To test the enzyme activity of recombinant wildtype ChAT and ChAT-GFP both proteins were expressed in COS-1 cells which have no endogenous ChAT protein (as shown by Western blot analysis, Figure 3.5) and were hence capable for this assay. The enzyme activities were measured in crude cell lysates according to the method of Fonnum (1975) without further protein purification (Table 3.1). The activity of the wildtype mouse enzyme was 14 nmol/h/mg. This value is lower than previously measured for the recombinant human wildtype enzyme (48.6 nmol/h/mg) (Robert and Quirin-Stricker, 1998). The deviation might be due to shorter expression times after transfection (36 versus 72 hours), different culture conditions of the COS-1 cells and/or different enzymatic properties between the human and mouse isoform.

The insertion of GFP into the N-terminal part of ChAT causes a decrease of enzyme activity of about 27 % indicating that the relatively large insert (239 amino acids) affects the catalytic properties of ChAT. Nevertheless, the function of the enzyme is largely retained and will be high enough to produce sufficient amounts of ACh in the ChAT-GFP "knock-in" animals. Thus mice homozygous for ChAT-GFP would have retained about 73 % of the ChAT activity and the development and viability of these animals should remain unaffected by this modification. This prediction is substantiated by the finding that heterozygous ChAT "knock-out" mice display 40-50 % ChAT activity and seem to develop normal (Brandon, 2000). However, homozygous null mice lacking ChAT are stillborn apparently because no cholinergic neurotransmission can occur at all.

4.3 Distribution of wildtype ChAT and ChAT-GFP in COS-1 cells

COS-1 cells expressing ChAT-GFP and wildtype ChAT were compared for the subcellular distribution of the recombinant proteins to assure that the inserted GFP introduction does not change the subcellular localization. The fluorescent ChAT-GFP fusion protein appears to have the same distribution as the wildtype ChAT which has been visualized by immunocytochemistry using a polyclonal anti-ChAT antibody and a FITC-conjugated secondary antibody. The imaging of several different cells did not reveal any apparent differences between ChAT-GFP and the wildtype protein (Figure 3.6). Both proteins were absent from the nucleus as previously shown for a human ChAT isoform tagged with GFP at the C-terminus and expressed in HEK 293 cells (Resendes *et al*., 1999). Another human isoform of 82 kDa with nuclear localization signal exists that translocates to the nucleus shown in HEK 293 cells (Resendes *et al*., 1999). The role that a neurotransmitter-synthesizing enzyme could play in the nucleus is unclear and a mouse isoform that was targeted to the nucleus has not been detected. GFP is a cytosolic protein but is also present in the nucleus (Ogawa *et al*., 1995). The comparison of the distribution of ChAT-GFP and the wildtype ChAT with that of GFP leads to the assumption that ChAT is located in the cytosol.

It was shown by Western blot analysis that ChAT-GFP is not rapidly degraded by proteolysis meaning that the fluorescence of the cells does not result from free GFP or fragments of ChAT-GFP but rather reflects the real subcellular distribution of ChAT-GFP.

4.4 ChAT-GFP is expressed in pyramidal neurons and appears to be associated to synaptic vesicles

Biochemical results indicate that ChAT is primarily a cytosolic protein. Minor amounts, however, seem to be bound to membranes or are associated with synaptic vesicles (Carroll, 1994; Pahud *et al*., 1998). Signals necessary for subcellular targeting have not been identified so far. Nevertheless, it was important not to destroy any putative signal sequences in the N-terminus by introducing mutations. Therefore GFP was introduced 16 amino acids behind the N-terminus.

ChAT must translocate along the axon to nerve terminals over long distances and it is likely that there are mechanims responsible for axonal transport. For *Drosophila* it has been shown that axonal transport of ChAT requires Kinesin-II (Ray *et al*., 1999).

To see whether ChAT-GFP diffuses uniformly throughout neurons, expression and subcellular distribution of ChAT-GFP was analyzed in hippocampal primary cultures (Figure 3.7) and in pyramidal neurons of organotypic hippocampal brain slice cultures (Figure 3.8 and 3.9). ChAT-GFP became expressed in both neuron culture systems upon transfection and the transgenic protein could be visualized directly under the fluorescence microscope. In primary neurons ChAT-GFP is distributed in the soma and translocates to neuritic processes. It was difficult to discriminate between axonal and dendritic processes in these neuronal cell cultures since the polarity and original morphology was not preserved after the preparation and the culturing of the cells. In contrast, hippocampal neurons of organotypic brain slice cultures retain their morphology very well and their dendritic tree and the axonal processes were clearly distinguishable. The dendrites displayed spines at a very high density contrary to the axon, which had a smooth surface without spines but with swellings that could represent presynaptic boutons. ChAT-GFP expressed in hippocampal neurons of slice cultures displayed a similar distribution as cytosolic GFP which means that it is also present in the cytosol of the soma, dendrites and the axon.

In both primary cultures and organotypic slice cultures ChAT-GFP appeared to be concentrated in spines. This localization was reminiscent of the subcellular distribution of the postsynaptic marker proteins such as the AMPA receptor subunits. Both GluRA-GFP and GluRB-GFP were present in spines (Figure 3.14b and c) as previously reported (Shi *et al*., 2001). So far it is not known why ChAT, that acts in the presynaptic compartment, is translocated to the postsynaptic compartment. One has to consider that this translocation could result from expression of ChAT-GFP by the CMV promoter. Since this promoter is probably more efficient than the native ChAT promoter ChAT may be expressed to levels which cause an unnatural subcellular distribution and the postsynaptic localization observed here may not reflect the distribution of the native protein. This will be clarified as soon as the ChAT-GFP targeted mice are available. Another reason might be that ChAT is a cytoplasmic protein that is probably targeted in part actively to presynaptic terminals (by binding to synaptic vesicles), however, it is not retained from other compartments except from the nucleus. GFP is obviously

present in the nucleus of neurons but ChAT is not (Figure 3.8 and 3.9). This is consistent with the findings in COS-1 cells.

The imaging of ChAT-GFP expressing hippocampal neurons of slice cultures prior to the fixation by fluorescence microscopy as well as after fixation by confocal microscopy revealed an axonal distribution similar to the distribution of synaptophysin-GFP. Synaptophysin is the major transmembrane protein of synaptic vesicles and has been implicated in neurotransmitter release (Wiedenmann and Franke, 1985). It is expressed almost exclusively on small synaptic vesicles and is therefore a common marker for nerve terminals. Synaptophysin-GFP expression analyzed by confocal microscopy displayed bright fluorescent puncta along axonal processes. It has been previously shown that these puncta were actively transported over long distances (Nakata *et al*., 1998). One synaptophysin-GFP expressing neuron located in the CA3 area of the hippocampus projects its axon with the puncta over large areas of CA1 (Figure 3.14).

ChAT-GFP displayed a dense network of axonal processes with a similar punctate structure (Figure 3.8a and 3.9a´´) and higher magnification of the axonal terminals revealed varicosities, i.e. boutons (Figure 3.8a´´´), in a similar manner like synaptophysin-GFP (Figure 3.13a and 3.14a´). However, the fluorescence-intensities along the axonal shafts between the puncta were higher for ChAT-GFP than for synaptophysin-GFP. The fluorescence in these axonal regions, however, resulted from the cytosolic fraction of ChAT-GFP. Synaptophysin-GFP for comparison is exclusively targeted to vesicle membranes and hence appeares only in puncta. The distribution of ChAT-GFP indicates that it is at least partly associated with synaptic vesicles. This would be in agreement with the biochemical results of Carroll et al. (1994) who showed that 10-20 % of ChAT is associated with synaptic vesicles. In this study synaptosomal fractions prepared from rat brain were incubated with anti-ChAT antibody-coated immunobeads. These immunobeads co-immunoprecipitated synaptophysin as shown by Western blot analysis. Thus it might be possible that fractions of ChAT-GFP bind to synaptic vesicles that are associated with transport packets and, thus, are transported to the presynaptic terminal (see Section 4.7). It will be interesting to co-express ChAT-GFP together with synaptophysin, fused to a fluorescent protein with different spectral properties than GFP, and to compare the distribution within the same cell. In a very

recent study analyzing the cholinergic innervation of the hippocampus with high resolution a similar axonal localization could be observed (Aznavour *et al*., 2002).

In contrast to ChAT-GFP, GFP translocates into dendrites as well as into the axon. However, only the main axon fluoresces (Figure 3.8b and 3.9b) probably since GFP was not actively transported but rather distributed by diffusion into neuritic processes. It is important to note that differences between ChAT-GFP and GFP in the axonal localization could only be seen when the expression level and expression time was in the same order of magnitude. This was accomplished by imaging the neurons at identical time points after transfection and comparison of neurons with similar expression levels of the fluorescent proteins. Otherwise GFP was also present in axonal processes far away from the soma with a similar distribution like ChAT-GFP. Obviously ChAT-GFP translocates faster along axonal processes than GFP. This could be directly observed and quantified by time-lapse imaging with a laser-scanning microscope.

Generally the *in vivo* imaging is of immense value since the observations made here showed that the fixation procedures reduce fluorescence and the quality of imaging and thus impair the assessment of neuronal structures in slices. Confocal or multi-photon microscopy of living slices will circumvent the "fixation problem" and yield images of as yet unreached quality and precision.

4.5 Establishing organotypic brain slice cultures

Organotypic brain slice cultures have many advantages compared to dissociated primary cultures. The "organotypic" morphology of the explanted brain area is very well retained as well as the cytoarchitecture of the tissue and the neurons. Furthermore, the cultures are easy to prepare and their maintenance is less time- and material-consuming than primary cultures.

Hippocampal slice cultures of P7 Wistar rats were used for the single-cell electroporation experiments for the following reasons: The hippocampus is easy to dissect out of the brain due to its characteristic anatomy and these preparations are the most frequently studied and well characterized slice cultures. The slices cultured on Millicell membranes can be simply transferred into a customized chamber and are then

accessible for the micropipette. Pyramidal cells of the stratum pyramidale of the hippocampal area CA3 and CA1 grow very dense and are good targets for the electroporation. In addition, the slices grown on membranes can be observed directly under the fluorescence microscope. Brain slices from other areas, especially from the basal cholinergic forebrain and the cortex can be probably cultured in a similar way after the preparation of corresponding brain slices has been adopted. The culture medium and the culture conditions are identical for brain tissue obtained from mice and rats and it should be possible to apply the slice culture technique to brain tissue from transgenic mice.

The culture of three slices per membrane was considered to be good at the beginning but later only one slice was cultured per membrane because the SCE process is time consuming and the survival of the slices seemed to be dependent on the time they were out of the incubator and under perfusion. Although the slices were manipulated very often under septic conditions (electroporation, visualization) they were never contaminated. This means that the antibiotics in the medium were sufficient to prevent contamination.

The hippocampus was cut into 350 µm thick sections that flattened considerably during the first week to about 100-150 µm. This corresponds to one to four cell layers of stratum pyramidale of CA3 to CA1 that also broadened significantly (Figure 3.10A). The neurons within the slices were viable and healthy. This was shown by labeling with tetramethylrhodamine-dextrane or by GFP expression (see Section 4.6 and Figure 3.11). The characteristic morphology of pyramidal neurons has been shown to be identical as previously reported (Stoppini *et al*., 1991). The neurons visualized by Stoppini et al. were labeled by intracellular filling with HRP, fixation and incubation with diaminobenzidine hydrochloride. They assessed the state of neuronal health by determining electrophysiological parameters. The neurons here were electroporated *in vivo* with fluorescent dyes or by the expression of GFP confirming that the cells were healthy and that electroporated neurons could be observed by the intrinsic fluorescence up to 14 days, the longest time tested.

This culturing technique applied on brain tissue of ChAT-GFP expressing (or "floxed" ChAT) mice opens new experimental approaches. For instance co-cultures of septal, hippocampal and cortical slices as previously done by Baratta *et al*. (2001) can provide further insights into the growth and targeting or even degeneration of

cholinergic projections. The possibilities will be expanded if SCE (see Section 4.6) is applied to these co-cultures. The introduction of foreign genes into septal cholinergic neurons, which are identified by their intrinsic fluorescence, or into the target neurons might help to understand the processes leading to the survival of the cholinergic phenotype.

4.6 Improved single-cell electroporation (SCE) is a powerful transfection method for single neurons

The electroporation of cells is a widely used method to introduce DNA into cells. Teruel *et al*. (1999) have developed an effective method to introduce RNA, DNA and dextrane molecules into hippocampal neurons of primary cultures by electroporation. However, this method can not be used for the transfection of individual neurons and can not be applied to organotypic slice cultures. As part of the project electroporation, as previously described by Haas *et al*. (2001), has been established and significantly optimized to transfect individual neurons of organotypic hippocampal brain slice cultures.

This approach has several advantages over commonly used methods as intra-nuclear injection, viral infection or biolistic bombardment with a "gene gun". The injection is only possible for dissociated primary cultures, grown on smooth supports as coverslips and seems not be suitable for slices that are a few cell layers thick. Viral infection or "gene gun" biollistics generally transfects more than one cell within a slice. Further cellular damage and limited viability result from these methods. All these disadvantages can be avoided by SCE.

In the beginning, a similar setup as described by Haas *et al*. (2001) (Figure 3.10B) was used. The electroporation of tetramethylrhodamine-dextrane into pyramidal neurons of CA3 was very efficient (nearly 100 %). Since the dye molecules are very small only one to two 40 msec square pulses with an amplitude of 15 V were required for the transfer of the dye. This ensured that cells were hardly damaged and survived at good health. Virtually every cell in the vicinity of the pipette tip became electroporated. Electroporation tests with tetramethylrhodamine-dextran were done to optimize the shape of the pipettes and to control that the neurons in the slice cultures were healthy.

However, the labeling efficiency of the cells was greatly reduced when plasmid DNA was filled into the electroporation pipette. Due to the larger size of the DNA molecules stronger pules were necessary to transfer sufficient DNA into the cell. Consequently the pulse parameters and experimental procedure had to be optimized to reach an efficient DNA transfer together with a high cell survival rate. At first the parameters reported by Haas et al. (2001) were used. The pipette tip was randomly inserted into stratum pyramidale. Five 1 sec long trains of 1 msec square pulses with an amplitude of 20 V were delivered at 200 Hz. With these parameters an efficiency (i.e. fluorescent cells of electroporated cells) of about 5 % could be reached for the electroporation of the plasmids encoding GFP or DsRed. The co-electroporation of *pEGFP-C1* and *pDsRed2-C1* was also realized. Sometimes more than one cell was transfected by the same pulse. This happened more often when using pipette tips greater than 1 µm in diameter. The efficiency of about 20 % of attempts for hippocampus slices reported by Haas et al. is valid only for the *in vivo* electroporation of tadpole optic tectum neurons but not for hippocampal slices (personal communication). The efficiency of 5 % for *pEGFP-C1* and *pDsRed2-C1* was further reduced to about 1-2 % when larger GFP fusion constructs were electroporated (which means that 2-3 slices were necessary to obtain one transfected cell). This may indicate a dependence of the transfection efficiency on the plasmid size and would also explain why it is that easy to electroporate small dye molecules. However, other studies did not observe a difference in the efficiency for plasmids up to 14 kb (Rae and Levis, 2002).

Because the transfection efficiencies were so low it was essential to analyze the transfer process and to improve SCE. This required the visualization of the electroporation process. To this end a fluorescein-conjugated oligonucleotide was electroporated. The transfer to the identified cell could be followed in real-time by using a setup equipped with a two-photon laser-scanning microscope (Figure 3.12, measurements were performed by Thomas Nevian, Zellphysiologie, MPImF). This revealed that the applied 5 trains of pulses were definitely too strong and lethal for almost every cell explaining the low transfection rate. It was shown that when the pipette tip was in touch with the membrane of the identified cell only one or two trains were sufficient to fill the cell with the fluorescein-conjugated oligonucleotide. However, pushing the pipette tip gently against the membrane of the cell could only be accomplished under microscopic control at sufficiently high magnification.

In order to reach the high precision of the pipette guidance an electrophysiology setup was modified. The Millicell membrane with the slice culture was placed in a customized perfusion chamber and was perfused with oxygenated physiological salt solution during the electroporation. The slice was illuminated by “Dodt” gradient contrast and the neurons were visualized by IR video microscopy with a 40x water immersion objective and further 2x magnification. The limitation of the perfusion time to 20 minutes, which is sufficient to set the pipette to stratum pyramidale and to electroporate 6-10 cells, probably improved the survival of the cultures. The pulses were applied by an isolated voltage stimulator controlled by a tetanizer - devices widely used for the stimulation of neurons. A back-pressure of 3-4 mbar was applied to the pipette to reduce the clogging of the pipette tip and to avoid that bath solution enters the tip by the hydrostatic pressure which would further dilute the DNA solution. The diameter of the pipette tip was about 1-2 µm to limit the electroporated area of the cell membrane resulting in low co-transfection of neighboring cells. The DNA was diluted to 33 ng/µl in NRR salt solution which presumably resulted in an increased solubility and hence mobility of the DNA. The largest plasmid successfully electroporated was about 12 kb. The pulse parameters finally used were one 1 sec long train of 1 msec square pulses with an amplitude of 10 V delivered at 200 Hz.

These modifications finally resulted in an overall efficiency of about 25-50 % similar to the efficiency reported by Rae and Levis (2002) for cultured cells and represented a significant improvement compared to the initial 1-5 % (Table 3.2). The efficiency after optimization did not depend on the plasmid size. The deviation between the efficiencies from experiment to experiment may result from an accumulation of material that adheres to the pipette tip and creates a diffusion barrier. In spite of these improvements it is not possible to control how much DNA actually has entered the cell. Although identical pulses were applied to each cell the later fluorescence varied significantly. This may derive from different conditions for each cell like the distance between the tip and the membrane or when debris clogs the tip which will reduce DNA transfer. One should also keep in mind that each cell may behave different after pulse application or DNA transfer.

The consideration that the pulses required for electroporation might affect or interfere with the physiological properties of the cell seems unlikely since it could be

demonstrated that neurons electroporated with 1 msec pulses maintain normal synaptic transmission (Teruel *et al*., 1999).

4.7 Gene transfer of fluorescent proteins via SCE

Individual neurons can be intrinsically labeled by the expression of fluorescent proteins like GFP or DsRed. This is useful to study the morphology or morphological changes of cells, e.g. as it happens during the differentiation and development. Here, the expression of GFP or GFP in combination with DsRed over several days indicates that the cells of the brain slices were healthy and alive (Figure 3.11).

SCE represents an expression system, complementary to primary neuron cultures, however, with the advantages discussed earlier (see Section 4.5), to study the subcellular targeting of recombinant proteins. In this context the expression and distribution of ChAT-GFP has been discussed in Section 4.4. In addition the postsynaptic AMPA receptor subunits GluRA and GluRB as well as the presynaptic vesicle protein synaptophysin were expressed as GFP fusion proteins (Figure 3.13 and 3.14).

As expected both GluR subunits were selectively targeted to postsynaptic sites at dendritic shafts and spines. GluRB (Figure 3.13C and 3.14C) is more abundant in the heads of the spines compared to GluRA (Figure 3.13B and 3.14B) which is in agreement with previous results (Shi *et al*., 2001; Shi *et al*., 1999).

Synaptophysin-GFP, expressed by a single neuron located in CA3, was efficiently transported into axonal processes along the "Schaffer collateral" pathway to terminals in CA1. The dense network of axonal processes in CA1 is visualized by the intense punctate fluorecence (Figure 3.13A and 3.14A). One part of this punctate fluorescence may derive from cytoplasmic transport packets as previosly described by Ahmari *et al*. (2000) which move along the axon of cultured hippocampal neurons to presynaptic active zones. The other part probably represents axonal presynaptic varicosities. Ahmari et al. found out that typical aggregates, labeled with VAMP-GFP, another presynaptic vesicle protein, are about 1 µm in diameter. This is in agreement with the puncta size found here (Figure 3.13a´´). Another group has recently shown that synaptophysin is inserted into the presynaptic plasma membrane, juxtaposed to the presynaptic active

zone, in the same way as VAMP after action potential application. Based on this result they assumed that both proteins were transported in the same set of vesicles (Li and Murthy, 2001). Thus, the puncta containing synaptophysin-GFP observed here could indeed be part of these transport packets and/or presynaptic varicosities.

A further advantage of SCE becomes obvious: only one single CA3 neuron was transfected with synaptophysin-GFP. Hence it is quite clear that all the fluorescent axonal varicosities in the whole slice derived from this single cell. If another cell also expressed synaptophysin-GFP it would be impossible to discriminate which axon in CA1 came from which cell in CA3.

4.8 Modification of the ChAT gene in ES cells by homologous recombination

Transgenic expression of GFP directed by the cholinergic promoter can lead to a mosaic distribution if the integration of the transgene occurs at multiple sites or different genomic loci. Therefore the targeting of GFP to the cholinergic gene locus by homologous recombination is a highly specific approach to label cholinergic neurons selectively. Moreover, since these neurons will fluoresce intrinsically it will be possible to study the cholinergic nervous system *in vivo*. As a result this will provide further insights into the physiology of the central cholinergic pathways in the brain. Consequently cholinergic neurons can then be studied by means of electrophysiology. It has not been possible so far to label cholinergic neurons in brain slices without fixation of the tissue. In addition to this it will be possible to study complex processes as the development, maintenance and the degeneration of the cholinergic phenotype. Fluorescent cells can simply be observed in real-time and their treatment with neurotrophic factors or drugs and the resulting effects can be directly visualized.

Transgenic mouse lines which express fluorescent proteins in subsets of neurons under the control of the Thy-1 promoter are already available (Feng *et al*., 2000). Some of these lines were selected for YFP expression in motor neurons, however, this expression is not specifically cholinergic. Another transgenic line has been established that expresses the lacZ gene under a 6.4 kb promoter fragment of the ChAT gene (Naciff *et al*., 1999). The expression pattern of lacZ was similar to the distribution of cholinergic neurons which were previously defined by immunohistochemistry and *in*

situ hybridization but it has not been possible so far to observe this *in vivo*. Furthermore Naciff *et al*. (1999) observed lacZ staining also in areas in which the existence of cholinergic neurons is controversial.

In general, gene targeting experiments are based on modification of DNA sequences of the gene of interest with recombinant cloning strategies. At first it was necessary to identify the genomic sequence of the first coding exon of ChAT and the adjacent regions. This was done by Dr. M. Koenen who provided the overlapping plasmids containing the 5.8 kb ChAT promoter fragment (*p958*) and the ChAT exon 2 with downstream sequences (*p974/2*). Starting from these plasmids the ChAT-GFP targeting vector was assembled.

After analyzing the DNA sequence downstream of exon 2 of plasmid *p974/2* which was unknown until then, site-directed mutagenesis was performed to introduce a SalI restriction site shortly downstream of the exon 2 splice site. In a later step this SalI site enabled the cloning of the neo^r cassette for selection of targeted ES cells. In addition, a NotI site was introduced about 1500 bp downstream of the SalI site. The section between the SalI and the NotI site represents the short arm for homologous recombination of the targeting vector. The GFP sequence was cloned as PpuMI-HindIII fragment from the ChAT-GFP expression construct (*pJR38*), followed by the subcloning of the 5.8 kb XhoI-XbaI promoter fragment from *p958* which represents the long arm for homologous recombination of the targeting vector. Finally the neo^r cassette was introduced into the SalI site resulting in the final targeting vector *pJR48* (Figure 3.2).

At the same time the ChAT-loxP targeting vector was assembled. Mice with a "floxed" exon 2 of ChAT will serve as a conditional "knock-out" avoiding the lethality of a real ChAT "knock-out" (Brandon, 2000). Breeding of mice homozygous for the "floxed" exon 2 with mice that express the Cre recombinase in specific brain regions during later stages of development will lead to the excision of exon 2. Subsequently cholinergic neurons would be supposed to degenerate. Such animals will serve as models to study diseases like Alzheimer's disease (AD) or amyotrophic lateral sclerosis (ALS) in which cholinergic neuron loss is specifically involved. To date the deletion of cholinergic neurons in the living animal is achieved by intracerebroventricular injection of an antibody against the $p75^{NTR}$ receptor, which is specific for cholinergic neurons of the basal forebrain. This antibody is coupled to the toxin saporin, a ribosome-

inactivating protein. The binding of this antibody consequently leads to lesions of cholinergic basal forebrain neurons (Berger-Sweeney *et al*., 2001). However, this also provokes the damage of the surrounding brain tissue and may have side effects such as the toxicity for other than cholinergic neurons.

Into the same precursor construct (*pJR18*), which was used for the ChAT-GFP targeting vector, a BglII site was introduced between the 5´-splice site of exon 2 and the start ATG in which the 34 bp loxP site was integrated. The downstream loxP site flankes the 3´-end of the neo^r cassette (Figure 3.3). Cre recombinase mediated recombination of the ChAT-loxP targeting vector *in vitro* led to the excision of a 2.2 kb fragment comprising exon 2 with the sart ATG and the neo^r cassette which confirmed that both loxP sites were functional (Figure 3.4).

Both targeting vectors were linearized at the unique XhoI restriction site and electroporated into ES cells. For both the ChAT-GFP and the ChAT-loxP construct 800 G418 resistant ES cell clones were isolated and analyzed for homologous recombination of the ChAT gene. In the case of ChAT-GFP two positive clones were found by the PCR screen which is, however, not as specific as genomic Southern blot hybridization. Only one clone could be confirmed by the genomic Southern blot hybridization that was correctly targeted. Similarly to the GFP targeting the PCR screen for ChAT-loxP targeted cells provided one positive clone, which was subsequently confirmed to be correct by genomic Southern blot hybridization. Compared to earlier targeting experiments done for the AChRε gene (Güth, 1999) the frequency of homologous recombination was lower. For the ChAT targeting experiment the overall efficiency was 0.125 %. The targeting experiment of the AChRε gene provided three targeted clones from 576 that were analyzed. This corresponds to an efficiency of 0.52 %. Obviously the efficiency of the homologous recombination is dependent on the identity of the genomic locus that should be modified.

4.9 Outlook

The generation of embyonic stem cells has been described as one part of the project that will serve to establish ChAT$^{(GFP/+)}$ and ChAT$^{(GFP/GFP)}$ "knock-in" mice. ChAT$^{(GFP/+)}$ mice should be available soon since ChAT$^{(GFP/+)}$ ES cells have been already transferred into blastocysts resulting in 14 chimeric animals of 20-70 % chimerism. From these male animals will be bred with wildtype females and the offspring will be analyzed for germline transmission of the targeted ChAT gene. Heterozygous animals will be used for both the breeding and fluorescence analysis. If one supposes that both alleles will be transcribed with similar efficiency ChAT-GFP fluorescence should be already detectable in ChAT$^{(GFP/+)}$ mice. However, the fluorescence intensity will be increased in the homozygous state. In order to obtain optimal conditions ChAT$^{(GFP/GFP)}$ mice are essential for further experiments.

Using these animals it will be possible to analyze the electrophysiological properties of cholinergic neurons in acute brain slices as well as in slice cultures. A neuron will then be selected by its intrinsic fluorescence. Developmental studies will be performed and observed *in vivo*. ChAT$^{(GFP/GFP)}$ mice will be bred with mutant mice that have modifications in AChR subunits of the neuromuscular junction. Here it will be interesting to see how postsynaptic modifications influence the behaviour of presynaptic nerve terminals of motoneurons. ChAT$^{(GFP/GFP)}$ mice bred with Alzheimer's disease (AD) model mice (e.g. APP transgenic mice) or amyotrophic lateral sclerosis model (ALS) mice (e.g. SOD1 transgenic mice) will give insights how cholinergic neurons selectively degenerate. Fluorescence-Activated Cell Sorting (FACS) will enable the preparation of pure cholinergic primary cultures from ChAT$^{(GFP/GFP)}$ brain tissue. These primary cultures will then be studied by genomic and proteomic approaches.

During the other part of the project organotypic brain slice cultures and single-cell electroporation have been established and optimized. These methods will be applied for studying ChAT$^{(GFP/GFP)}$ mice. SCE will allow introducing genes or agents selectively into cholinergic neurons. For example antisense cRNAs for genes of interest, that must have prior been identified by cDNA array analysis, will be used to delete gene function in cholinergic neurons. In addition to this cholinergic neurons will be observed in real-time in organotypic brain slice cultures and the neurotrophic effect of drugs and factors will be studied.

The existing ChAT-loxP targeted embryonic stem cells will soon be transferred into blastocysts. ChAT$^{(loxP/loxP)}$ "knock-in" mice will serve as a new model for neurodegenerative diseases which affect the cholinergic system as for AD or ALS. Breeding of these ChAT$^{(loxP/loxP)}$ mice with brain specific deleter mice will result in the "knock-out" of ChAT depending on the spatiotemporal Cre recombinase expression. There are several neuron specific deleter lines available. Interesting for the cholinergic system will be Cre expression in cortex and hippocampus (Jin *et al*., 2000), forebrain and septum (Cinato *et al*., 2001) and spinal cord (Hirasawa *et al*., 2001) in later developmental stages.