

**Gene targeting and single-cell electroporation to
analyze cholinergic neurons using choline
acetyltransferase as marker**

Inaugural-Dissertation zur Erlangung des Doktorgrades
im Fachbereich Biologie, Chemie, Pharmazie
der Freien Universität Berlin

vorgelegt von
Jan Rathenberg
aus Heidelberg

Tag der Disputation: 13.9.02

Heidelberg 2002

Gutachter: Priv.-Doz. Dr. Veit Witzemann
Prof. Dr. Ferdinand Hucho

The work leading to the submission of this thesis was carried out under the supervision of Priv.-Doz. Dr. Veit Witzemann from August 1999 to July 2002 at the Max-Planck-Institut for Medical Research, Department of Cell Physiology, in Heidelberg. Parts of this thesis have been published in the following scientific paper:

Rathenberg, J., Gartner, A., Koenen, M. and Witzemann, V. (2002) Modification of choline acetyltransferase by integration of green fluorescent protein does not affect enzyme activity and subcellular distribution. *Cell Tissue Res* 308: 1-6.

Further, I presented parts of this work on the following posters:

Rathenberg, J., Koenen M., Witzemann V. Generation and characterization of a ChAT-GFP fusion protein. 8th Gentner Symposium on Cellular and Molecular Processes in Biological Signaling (2001) in Berlin.

Rathenberg, J., Koenen M., Witzemann V. Functional characterization of a ChAT-GFP fusion protein and targeting of GFP to the mouse cholinergic gene locus. European Forum of Neurosciences (2002) in Paris

Danksagung

Ganz besonders danke ich Herrn Priv.-Doz. Dr. Veit Witzemann für die engagierte wissenschaftliche Betreuung und das intensive Interesse am Erfolg meiner Dissertation.

Für die Übernahme des Zweitgutachtens und das Interesse an meiner Arbeit danke ich Herrn Prof. Dr. Ferdinand Hucho.

Herrn Prof. Dr. Bert Sakmann danke ich für die Überlassung des Arbeitsplatzes.

Herrn Dr. Michael Koenen danke ich für die Diskussionsbereitschaft und Unterstützung während meiner Arbeitszeit.

Mein besonderer Dank gilt weiterhin Thomas Nevian für den intensiven Wissensaustausch und die Zwei-Photonenmessungen und Dr. Jack Waters für das kritische Gegenlesen des englischen Manuskripts.

Weiterhin möchte ich mich bei allen Mitarbeitern der Abteilung, besonders bei Ulrike Mersdorf, Ursel Warnke, Michaela Bauer und Karina Barenhoff für die stetige Hilfsbereitschaft und die angenehme Arbeitsatmosphäre bedanken.

Anke, meiner Schwester Verena und meinen Eltern danke ich für die vielseitige Unterstützung von ganzem Herzen.

Table of Contents

1	Introduction	1
1.1	Organization of the cholinergic gene locus, mRNA transcription and translation	1
1.2	ChAT protein purification and characterization	3
1.3	The cholinergic synapse	4
1.4	The central and peripheral cholinergic nervous system	5
1.4.1	Anatomy	5
1.4.2	Development	7
1.4.3	Physiology	8
1.4.4	Disease	9
1.5	Organotypic cultures of neural tissue	10
1.6	Transfection of neurons by electroporation	11
1.7	<i>In vivo</i> imaging of the mammalian nervous system using fluorescent proteins	12
1.8	Cre/loxP mediated gene deletion or conditional gene "knock-out"	13
1.9	Creation of mutant mouse lines by homologous recombination	14
1.10	Aims of the project	15
2	Materials and Methods	16
2.1	Materials	16
2.1.1	Chemicals, reagents and consumable materials	16
2.1.2	Plasmid vectors	16
2.1.3	Bacteria strains, cell lines and animals	17
2.1.4	Primers and Oligonucleotides	17
2.1.5	Buffers and solutions	19
2.1.6	Media and solutions for bacteria, cell and tissue culture	21
2.2	Methods	23
2.2.1	Molecular Biology	23
2.2.1.1	Preparation of plasmid DNA	23

Table of Contents

2.2.1.2	Determination of nucleic acid concentration	23
2.2.1.3	Sequence analysis	23
2.2.1.4	Digestion with restriction enzymes	24
2.2.1.5	Agarose gel electrophoresis	24
2.2.1.6	DNA fragment isolation	25
2.2.1.7	5'-Dephosphorylation	25
2.2.1.8	Ligation	25
2.2.1.9	Transformation	25
2.2.1.10	Preparation of competent cells	26
2.2.1.11	RNA preparation from mouse (C57Bl/6) spinal cord	26
2.2.1.12	Reverse transcription	27
2.2.1.13	Site-directed mutagenesis	27
2.2.1.14	PCR amplification of DNA fragments	28
2.2.1.15	PCR screen of ES cell clones in the 96 well format	29
2.2.1.16	Preparation of genomic ES cell DNA from 96 well culture plates	29
2.2.1.17	Southern transfer of DNA fragments onto membranes	30
2.2.1.18	Detection of PCR products of using the ECL system	30
2.2.1.19	Detection of genomic digestion fragments with radiolabeled probes	31
2.2.2	Biochemistry	31
2.2.2.1	Choline acetyltransferase activity assay	31
2.2.2.2	Determination of protein concentration	32
2.2.2.3	Protein gel electrophoresis	32
2.2.2.4	Staining of proteins in polyacrylamide gels	32
2.2.2.5	Western blotting and immunodetection	33
2.2.2.6	Fixation of cells and tissues	33
2.2.2.7	ChAT immunocytochemistry of transfected COS-1 cells	34
2.2.3	Cell culture	34
2.2.3.1	Transfection of COS-1 cells	34
2.2.3.2	ES cell culture	35
2.2.3.2.1	Preparation of feeder cells	35
2.2.3.2.2	Electroporation of ES cells	35
2.2.3.2.3	Picking of G418 resistant clones	36
2.2.3.3	Organotypic tissue culture	36

Table of Contents

	Electroporation of single neurons in brain slice cultures (SCE)	37
3	Results	39
3.1	Generation of expression and targeting vectors	39
3.1.1	ChAT-GFP and wildtype ChAT expression vectors	39
	ChAT-GFP targeting vector	40
3.1.3	ChAT-loxP targeting vector	42
3.2	Functional characterization of the ChAT-GFP fusion protein	45
	Expression of ChAT-GFP and wildtype ChAT	45
	Enzyme activity of ChAT-GFP compared to the wildtype enzyme	46
3.2.3	Subcellular distribution of ChAT-GFP and wildtype ChAT in COS-1 cells	47
	Subcellular distribution of ChAT-GFP in hippocampal neurons	49
3.3	Transfection of individual neurons in organotypic hippocampal slice cultures by single-cell electroporation (SCE)	53
3.4	Expression of presynaptic and postsynaptic marker proteins	58
3.5	Generation of ChAT-GFP and ChAT-loxP targeted mouse E14 stem cell clones	61
4	Discussion	65
4.1	A recombinant ChAT-GFP fusion protein has been generated which is efficiently expressed in COS-1 cells	66
4.2	Influence of GFP insertion in ChAT enzyme activity	67
4.3	Distribution of wildtype ChAT and ChAT-GFP in COS-1 cells	68
4.4	ChAT-GFP is expressed in pyramidal neurons and appears to be associated to synaptic vesicles	68
4.5	Establishing organotypic brain slice cultures	71
4.6	Improved single-cell electroporation (SCE) is a powerful transfection method for single neurons	73
4.7	Gene transfer of fluorescent proteins via SCE	76

Table of Contents

4.8	Modification of the ChAT gene in ES cells by homologous recombination	77
4.9	Outlook	80
5	<i>Summary</i>	82
6	<i>Zusammenfassung</i>	84
7	<i>Abbreviations</i>	86
8	<i>References</i>	87