ADAPTIVE RESPONSES OF CENTRAL CHOLINERGIC SYSTEMS IN TRANSGENIC MICE

by

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A DISSERTATION

IN

PHARMACEUTICAL SCIENCES

Submitted to the Graduate Faculty of
Texas Tech University Health Sciences Center
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

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August 2006

“Auguste, glaube ich.” –“Auguste, I think.”

Answer of Auguste D. when asked about her husband’s name.
She was the first person to be diagnosed by Alois Alzheimer with the
disease which later came to bear his name.

ACKNOWLEDGEMENTS

There are many people that supported me during my work on this thesis. Without them, this thesis would not have been possible.

I would like to acknowledge Dr. Jochen Klein, my thesis advisor, for taking me up in his lab, for providing the thesis topic and the necessary resources. Also, I acknowledge him for giving insights into the day to day business of a principal investigator, and for discussions on the direction of the project.

I acknowledge the members of my graduate advisory committee: Drs. Oksana Lockridge, Konrad Löffelholz, Reza Mehvar and James Stoll for their continuous support of my work.

I want to thank the graduate students in Dr. Klein/Dr. Löffelholz' lab at the Department of Pharmacology, University of Mainz, Germany: Dipl.-Biol. Michael W. Hilgert for teaching me the general handling of rats and mice, microdialysis and operation of the HPLC equipment. Dr. Marie-Luise Buchholzer is acknowledged for her demonstration of microdialysis in mice and for some initial help with literature references. I thank Claudia Dvorak, MTA, for help with the high-affinity choline uptake assay and for general lab assistance. All of the above, as well as Dr. Beate Schatter and the medical students are acknowledged for the general friendly atmosphere in the group and for their great collegiality. The assistance of the departmental workshop, especially Reinhold Stutz, was invaluable and is deeply missed in Amarillo.

At the no longer existing CNS research department at Bayer Healthcare, Inc. in Wuppertal, Germany, I thank the collaborators on the first part of this thesis: Drs. Karl Heinz Baumann, Ulrich Ebert, and in particular, Dr. Christina Erb. Dr. Erb made sure

that the animals became available to me and coordinated the collaborative effort that resulted in my first first-author publication.

At the University of Nebraska in Omaha, I would like to thank Dr. Oksana Lockridge, member of my graduate advisory committee, for making available the AChE-deficient mice. Ellen G. Duysen, B.Sc. receives my wholehearted gratitude. Ellen is in charge of breeding and keeping AChE-deficient mice and made all the arrangements to get the mice to Amarillo safely.

I thank Dr. Nigel H. Greig, National Institute of Aging, Baltimore, MD for supplying the selective cholinesterase inhibitors, tolserine and bisnorcymserine.

At Texas Tech, I acknowledge Dr. Vikas Kumar, postdoctoral research associate, Markus Hillert, intern, and Runa Naik, fellow graduate student at our lab, for their collegiality.

I want to especially thank Cornelia Kiewert, fellow graduate student and my Significant Other. Cornelia and I set up the laboratories at Amarillo, and Cornelia has helped me with the experiments for the AChE-project. I am deeply grateful for her help and for the discussions about our research, for constantly refocusing me and for simply being there.

Finally, I extend the greatest appreciation to my parents, Helma and Willi Hartmann. There are no words to appropriately express my gratitude for all they have done for me, so I just leave it like this: Thank you for all of it.

CONTENTS

ABSTRACT

In the current thesis, the function of the septohippocampal cholinergic nervous system was investigated in transgenic mouse models pertinent to Alzheimer's disease. First, a transgenic model of increased amyloid formation and deposition was investigated to see whether a cholinergic deficit, as observed in the human disease, is present in those animals. These mice express both a mutated human amyloid precursor protein and human presenilin-1 and generate amyloid peptide and neuritic plaques in an age-dependent manner. High-affinity choline uptake (HACU) into corticohippocampal synaptosomes showed no difference between double mutant transgenic mice and controls, indicating unchanged turnover of the neurotransmitter, acetylcholine (ACh). Extracellular levels of ACh, measured in the dorsal hippocampus using microdialysis, were not significantly different between groups. The response of these levels to stimulation with either scopolamine or by exposure of animals to a novel environment was also unchanged between mutant mice and controls, indicating retained capability of the central cholinergic system to respond to different challenges. In conclusion, this study demonstrated that amyloid pathology can occur without compromising hippocampal cholinergic neurotransmission.

For the second part of this thesis, mice deficient for the enzyme acetylcholinesterase (AChE) were obtained; they serve as a model of the predominant treatment used in Alzheimer's disease, inhibition of AChE. Microdialysis in dorsal hippocampus revealed vastly elevated baseline levels of ACh, whereas baseline levels of Ch were reduced. Selective inhibition of butyrylcholinesterase (BChE) further increased these levels of

ACh in AChE-deficient, but not in control mice. This observation, for the first time, provides clear evidence that BChE can hydrolyze ACh in the brain of a living organism. Elevated levels of ACh were sensitive to the absence of calcium and to tetrodotoxin, confirming their neuronal origin. A compensatory increase in HACU was found, indicating increased transmitter turnover. Furthermore, it was demonstrated that extracellular levels of Ch become rate-limiting for ACh release in the absence of AChE. Finally, a relative failure of presynaptic negative autofeedback receptors was observed. The conclusion is reached that, in the absence of AChE, ACh hydrolysis is maintained to a considerable degree by BChE. Moreover, compensatory changes in the absence of AChE include upregulation of HACU and functional loss of inhibitory autofeedback receptors.

In summary, both mouse models demonstrate that central cholinergic systems can respond to a wide range of challenges with a remarkable degree of adaptation.

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase (EC 3.1.1.7)
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
APP	Amyloid precursor protein
AU	Arbitrary units
AUC	Area under curve
Aβ	Amyloid beta
BChE	Butyrylcholinesterase (EC 3.1.1.8)
BNC	Bisnorcymserine
BW284c51	1,5-Bis(4-allyldimethylammonium-phenyl)-pentan-3-one dibromide
Ch	Choline
ChAT	Choline acetyltransferase (EC 2.3.1.6)
ChOx	Choline oxidase (EC 1.1.3.17)
CHT-1	Choline transporter 1
CNS	Central nervous system
DLB	Dementia with Lewy bodies
DNA	Deoxyribonucleic acid
dpm	Degradations per minute
GABA	Gamma-amino butyric acid
HACU	High-affinity choline uptake
HPLC	High-performance liquid chromatography
IMER	Immobilized enzyme reactor
KHB	Krebs-Henseleit buffer
KO	Knockout

MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
PCR	Polymerase chain reaction
PD	Parkinson's disease
PE	Polyethylene
PS-1	Presenilin-1 (protein)
PSEN1	Presenilin-1 (gene, human)
sAPP	Soluble fragment of amyloid precursor protein
TTX	Tetrodotoxin
VAChT	Vesicular acetylcholine transporter
wt	Wild-type
τ	Tau

CHEMICAL STRUCTURES

This section gives structural formulas, systematic names, and some additional information on the pharmacologically active compounds utilized in the experiments described in this thesis. First, the cholinesterase inhibitors are presented. After that, other compounds are presented, including a short explanation of their activity.

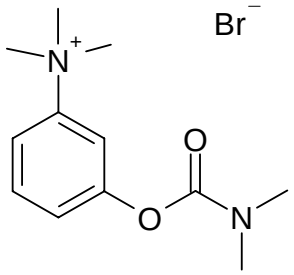
Cholinesterase inhibitors

Neostigmine

3-(N,N-Dimethylcarbamoyloxy)-N,N,N,-trimethylanilinium bromide

Barely selective inhibitor of both acetylcholinesterase and butyrylcholinesterase

IC_{50} (guinea pig): 36nM (AChE), 190nM (BChE) → Selectivity: 5-fold AChE (Kishibayashi et al., 1994)

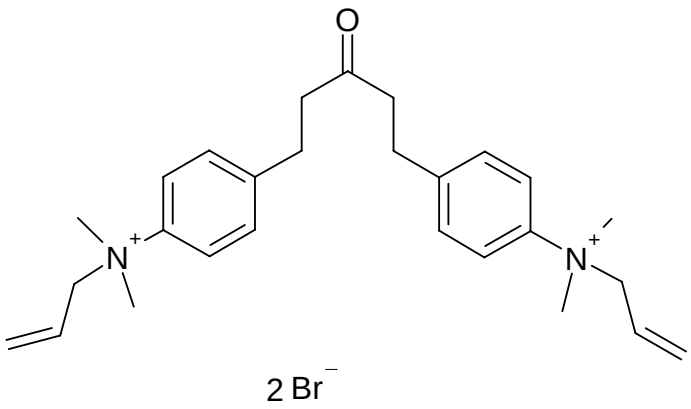


BW284c51

1,5-Bis(4-allyldimethylammonium-phenyl)-pentan-3-one dibromide

Selective inhibitor of acetylcholinesterase

IC_{50} (rat): 18.8nM (AChE), 48μM (BChE) →Selectivity: 2550-fold AChE (Giacobini, 2003)

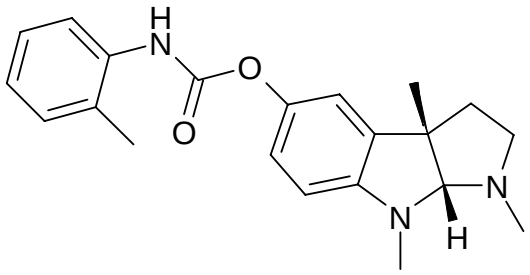


Tolserine

N-2-Methylphenylphysostigmine,
(-)-(3a*S*)-1,3a,8-Trimethyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indol-5-yl-*N*-2'methylphenylcarbamate

Selective inhibitor of acetylcholinesterase

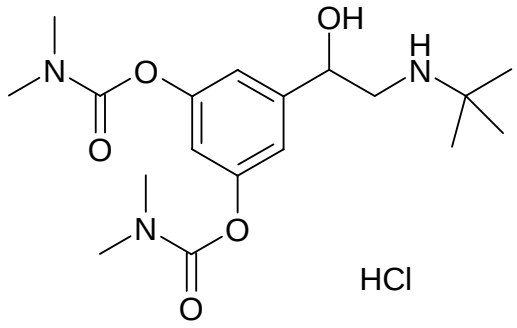
IC_{50} (human): 10.3nM (AChE), 2μM (BChE) →Selectivity: 195-fold AChE (Yu et al., 1999)



Bambuterol

5-[2-(*tert*-butylamino)-1-hydroxyethyl]-m-phenylene-bis(dimethylcarbamate) hydrochloride
Selective inhibitor of butyrylcholinesterase

IC_{50} (human): 30μM (AChE), 3nM (BChE) →Selectivity: 10000-fold BChE (Tunek and Svensson, 1988)

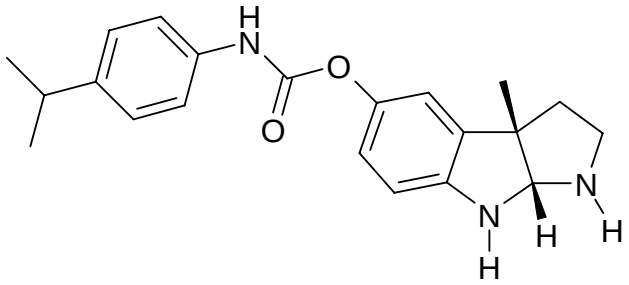


N[1],*N*[8]-Bisnorcymserine

(-)-(3a*S*)- 3a-Methyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indol-5-yl-*N*-4'isopropylphenylcarbamate

Selective inhibitor of butyrylcholinesterase

IC_{50} (human): 110nM (AChE), 1nM (BChE) →Selectivity: 110-fold BChE (Yu et al., 1999)
IC_{50} (rat): 150nM (AChE), 1nM (BChE) →Selectivity: 150-fold BChE (Greig et al., 2005)



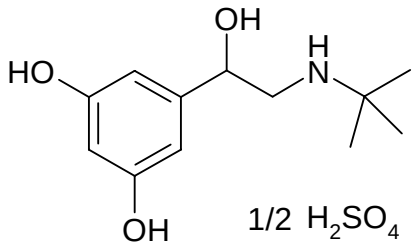
Compounds other than cholinesterase inhibitors

Terbutaline

1-(3,5,-dihydroxyphenyl)-2-*tert*-butylaminoethanol hemisulfate

Hydrolysis product of bambuterol
β_2-adrenoceptor agonist, weak inhibitor of BChE

IC_{50} (human BChE): 0.18-3.3mM; Kovarik and Simeon-Rudolf, 2004

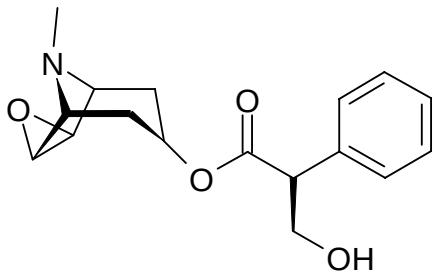


Scopolamine

α-hydroxymethylphenyl-acetic acid-9-methyl-3-oxa-9-azatricyclo[3.3.1.0.^{2,4}]-non-7-yl-ester

Unselective muscarinic acetylcholine receptor antagonist

IC_{50} (porcine brain): 0.95nM (Haga and Haga, 1985)

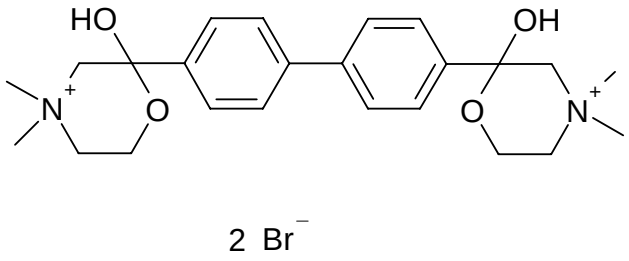


Hemicholinium-3 (HC-3)

2,2'-(4,4'-Biphenylene)-bis-(2-hydroxy-4,4'-dimethylmorpholinium) bromide

Inhibitor of High-affinity choline uptake

IC_{50} (rat): 50-60nM (Guyenet et al., 1973; Simon et al., 1975)

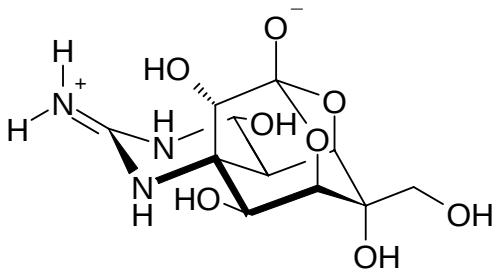


Tetrodotoxin (TTX)

Octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol

Blocker of voltage operated sodium channels

IC_{50} (rat): 1-8nM at sensitive subtypes (Catterall et al., 2005)



CHAPTER I
OVERVIEW

Acetylcholine (ACh) is the earliest known neurotransmitter. Dale published in 1914 on the muscarine-like activities of several ethers and esters of choline, and Loewi demonstrated in 1921, using the vagal nervous input to the frog heart, that neurotransmission is chemical in nature and subsequently identified ACh as the responsible chemical entity. Both scientists were awarded the 1936 Nobel Prize in Physiology or Medicine "for […] discoveries in respect of the chemical transmission of nerve action" (Liljestrand, 1936).

Neurons utilizing ACh as their signaling molecule are found in virtually all animal species, from nematodes and flies to human. In the mammalian body, cholinergic neurons are found in various locations. They are important in motor control, the autonomic, the enteric, and in the central nervous system (CNS). The most completely characterized central locations of cholinergic neuronal cell bodies are in the brain stem, the striatum, and the basal forebrain. Brain stem cholinergic neurons are part of the reticular formation, which is important for processes of arousal and respiratory control. Short cholinergic interneurons are found in the striatum, where they are involved in motor coordination. Finally, cholinergic neurons with cell bodies in the basal forebrain, namely septum and nucleus basalis, send projections to the hippocampus, cortex, amygdala and other brain regions and perform an important role in attention, learning and memory processes.

The basal forebrain cholinergic system has received extensive attention during the last three decades due to the demonstration that these neurons deteriorate selectively during Alzheimer's disease (AD) and that the decline of these neurons correlates well with dementia, the major symptom of the disease. Moreover, even in healthy subjects, interference with the basal forebrain cholinergic system produces effects on attention and memory. Besides AD, cholinergic dysfunction has also been demonstrated in a number of other diseases, including, but not limited to: Myasthenia gravis, Parkinson's disease (PD), dementia with Lewy bodies (DLB) and Schizophrenia.

The overall physiology of cholinergic neurons is very similar to that of neurons using one of the classic neurotransmitters from the class of amines (dopamine, norepinephrine, serotonin) or amino acids (glutamate, γ-aminobutyric acid [GABA]). The major difference exists in the mode of inactivation of transmitter and thus termination of neurotransmission: amines and amino acids are recaptured into nerve cells or glia by reuptake transporters; ACh, however, is hydrolyzed in the synaptic cleft by the enzyme acetylcholinesterase (AChE; EC 3.1.1.7), yielding acetate and choline. Choline in turn is taken up by the cholinergic nerve terminal and utilized for re-synthesis of ACh.

Many of the secondary metabolites found as bioactive compounds in plants have been demonstrated to act on the cholinergic system; two of them, muscarine and nicotine, became the name sakes of the two classes of cholinergic receptors due to their utilization in early studies to distinguish cholinergic responses. Examples of cholinergic drugs from plants include the cholinesterase inhibitor physostigmine, the muscarinic receptor agonists muscarine and pilocarpine, the muscarinic receptor antagonists atropine and scopolamine, and the nicotinic receptor agonist nicotine. Often derived from these plant constituents, a number of drugs used today for different diseases interfere with several aspects of cholinergic neurotransmission. Additionally, synthetic compounds used as insecticides or nerve agents interfere with the enzymatic degradation of ACh causing cholinergic hyperstimulation.

Considering the widespread existence of cholinergic systems in human, their involvement in disease processes and the use of compounds for therapeutic or combative purposes that interfere with these, it is very clear that a deep understanding of cholinergic neuropharmacology is warranted. The development of gene targeted animals has become an important tool to elucidate the function of single components of the body. Genes deleted or selectively mutated in recent mouse models pertinent to cholinergic function include those encoding muscarinic receptor subtypes, nicotinic receptor subunits, the high affinity choline transporter, CHT-1 (knockout not viable), the ACh synthesizing enzyme, Choline acetyltransferase (ChAT; EC 2.3.1.6; knockout not viable), AChE, and butyrylcholinesterase (BChE; EC 3.1.1.8).

Furthermore, gene targeted mouse models of diseases involving cholinergic dysfunction are available, including mouse models for AD, PD and DLB. Most often in these models, genes considered to be important for the respective disease are expressed either in increased amounts, or mutations have been inserted into the genes that are associated with familial forms of the respective disease. These include amyloid precursor protein (APP) and presenilin-1 (PS1) for AD, α-synuclein for PD and DLB, and tau (τ) for several neurodegenerative diseases. Additionally, mouse models of lacking trophic support of septohippocampal cholinergic fibers have been described.

The research presented in this thesis deals with the function of the septohippocampal cholinergic system in two of these mouse models:

1) An AD model, in which a mutated human *APP* gene is expressed alone, replacing the murine gene, or co-expressed with either a wild-type or a mutated human PS1 gene.
2) The mouse model in which the gene for AChE has been deleted.

These models were chosen due to their importance for central cholinergic function and their applicability to understanding both basic pathophysiology and long-term effects of current treatments for AD: The first model was chosen to test the hypothesis that mouse models which mimic AD by expression of mutated disease-related genes should display cholinergic dysfunction, as seen in the human disease. The second model was chosen, because inhibition of AChE is still first line treatment for AD. However, long term effects of such treatment on cholinergic function have not been investigated thoroughly yet. The research hypothesis was that the long-term absence of AChE will trigger modifications in the cholinergic system, including ACh hydrolysis, transmitter turnover and receptor function, which might be either beneficial or detrimental to the therapeutic aim of increasing synaptic concentrations of ACh in an attempt to restore compromised cholinergic function.

CHAPTER II
SPECIFIC BACKGROUND

2.1. The cholinergic nervous system

2.1.1. Synaptic organization

The nerve endings of cholinergic neurons contain all the basic elements necessary for synthesis, storage and controlled release of neurotransmitter, termination of neurotransmission, reuptake of substrate, and reception of the own signal for fine-tuning. The subsynaptic cholinoceptive cells contain the necessary receptors to receive cholinergic signals and the machinery to transduce these signals into cellular responses, and are also involved in termination of the neurochemical signal (Cooper et al., 2003).

What defines a neuron as cholinergic is the unique and exclusive presence of three proteins: The transmitter synthesizing enzyme, choline acetyltransferase (ChAT; EC 2.3.1.6), the vesicular acetylcholine transporter (VAChT), and the high-affinity choline uptake transporter (CHT-1).

Synthesis of the transmitter acetylcholine (ACh) is catalyzed by ChAT. Substrates for the synthesis are acetyl-Co-enzyme A, a central substrate of energy metabolism, and free choline. Approximately half of the bodies' choline requirement is met by dietary intake, the remainder is synthesized by the liver; negligible synthesis also occurs in the brain (Zeisel, 1981). Upon uptake into the brain (Allen and Smith, 2001) and cellular uptake, choline is either utilized directly for ACh synthesis (see below) or stored in choline-containing phospholipids, such as phosphatidylcholine and sphingomyelin, from which it can be liberated by activation of phospholipase D (Klein, 2005). ChAT is predominantly found in the cytosol, and it appears to be present in abundance, so that it is not considered to be rate-limiting for ACh synthesis (Jope, 1979; Cooper, 1994).

Once ACh is synthesized, it is pumped into and concentrated approximately 100-fold in exocytotic vesicles by the action of VAChT. This transporter employs a proton gradient between vesicle lumen and cytosol generated by a vesicular proton ATPase to

facilitate the translocation of ACh into these vesicles in exchange for protons. Concentrations of ACh in the cytosol of cholinergic nerve terminals are estimated to be in the low millimolar concentration range, whereas vesicular concentration is estimated at 500mM (Parsons, 2000; Eiden et al., 2004).

Upon depolarization of the cholinergic neuron, the common electrochemical processes occur: Activation of voltage-dependent sodium channels, leading to opening of voltage-dependent calcium channels. The influx of calcium activates the exocytotic machinery to attach ACh containing vesicles to the presynaptic membrane, to fuse the vesicular and the plasma membrane and thus to connect the intravesicular lumen with the extracellular space, allowing for diffusion of the stored ACh across the synaptic cleft (Südhof, 2004).

In cholinergic neurotransmission, the transmitted signal is terminated by cleavage of the transmitter, ACh, yielding acetate and choline. This cleavage is mediated by acetylcholinesterase (AChE, EC 3.1.1.7), an enzyme of the α/β-fold family of proteins present in all target organs of cholinergic neurons (Grisaru et al., 1999; Soreq and Seidman, 2001; Scholl and Scheifele, 2003). It is located predominantly in association with pre-, but also postsynaptic neurons, as well as on the extracellular matrix and surrounding glial cells. AChE comes in different splice variants and in different assemblies, either mono-, di- or tetrameric and usually tethered to the cell membrane by a collagen-like anchor. AChE-mediated cleavage of ACh is extremely fast. The high turnover number of 5000 molecules of ACh per molecule AChE per second indicates that ACh hydrolysis is merely limited by diffusion (Cooper et al., 2003).

A second cholinesterase is found in mammals, termed butyrylcholinesterase (BChE, EC 3.1.1.8). It shares a high degree of homology with AChE (Chatonnet and Lockridge, 1989; Sussman et al., 1991; Nicolet et al., 2003) and is known to be capable of ACh hydrolysis *in vitro* (Darvesh et al., 2003). However, its distribution is widespread in the entire body and not confined to cholinergic synapses (Li et al., 2000). Therefore, it seems likely that ACh hydrolysis is not the only, maybe just a secondary function of this enzyme *in vivo* (Clitherow et al., 1963; Darvesh et al., 2003).

Once ACh is cleaved, about half of the generated choline is taken back up into cholinergic cells by a high-affinity mechanism and is directly utilized for re-synthesis of ACh. This uptake of choline is mediated by CHT-1, which is distinct from the ubiquitous low-affinity transporter which supplies choline for biosynthesis of phospholipids of membranes (Kuhar and Murrin, 1978; Jope, 1979; Apparsundaram et al., 2000, 2001; Koepsell et al., 2003).

Acetylcholine acts on two sets of receptors, named after natural compounds which selectively activate either kind. The muscarinic receptors are G-protein coupled seven transmembrane domain proteins. So far, five subtypes have been identified (Caulfield and Birdsall, 1998) which are classified as those coupling to G_q/G_{11}-type G-proteins ($M_{1,3,5}$) and those coupling to G_i/G_0-type G-proteins ($M_{2,4}$). Second messenger systems include activation of phosphatidylinositol-specific phospholipase C (G_q/G_{11}), inhibition of adenylyl cyclase (G_i/G_0), activation of the I_{KACh} current (M_2, in the atrium of the heart), inhibition of the M-current (M_1 in hippocampal and cortical neurons, sympathetic ganglia) and other potassium channels, activation of phospholipase D and stimulation of the Ras-Raf-MAPK-pathway (Dutar et al., 1995; Löffelholz, 1996). The nicotinic receptors are ligand-gated cation channels, which are permeable to sodium, potassium and calcium. The individual subunits assemble either as homo- or heteropentamers. A distinction between the receptors at the muscle fibers and the neuronal nicotinic receptors is made: Muscle type receptors are made up of two α_1- and one each of β_1-, δ-, and ε- (γ- in the embryo) subunits (Kopta and Steinbach, 1994). Neuronal nicotinic receptors contain only $\alpha_{(2-10)}$- and $\beta_{(2-4)}$- subunits which demonstrate distinct, partly overlapping expression patterns in different neuronal tissues, with $\alpha_4\beta_2$ and α_7 being the most prevalent ones in brain (Wonnacott, 1997; Drago et al., 2003; Jensen et al., 2005).

2.1.2. Anatomy

Cholinergic nervous systems are demonstrated to innervate virtually all tissues of the mammalian body. In the periphery, the major cholinergic system is the parasympathetic branch of the autonomic nervous system. Its first order neurons leave the central nervous

system either in the brain stem in one of the cranial nerves (III, VII, IX, X) or in the sacral regions of the spinal cord. These neurons project to ganglia near the target organs of parasympathetic innervation and form synapses on second order neurons which in turn execute the effects of parasympathetic stimulation. Both the first order central neuron and the second order neuron utilize acetylcholine as neurotransmitter, which acts on nicotinic receptors in the ganglia and on muscarinic receptors at the target organs. Target organs of the parasympathetic nervous system include the heart (predominantly the atria), lungs, secretory glands, eye, gastrointestinal and urinary tract (Kirstein and Insel, 2004).

The other part of the autonomic nervous system, the sympathetic branch, also utilizes acetylcholine as a neurotransmitter, albeit only at the synapse from the first order to the second order neuron, furthermore in those second order neurons that innervate sweat glands, and in the adrenal medulla. Virtually all cholinergic neurotransmission in the sympathetic nervous system is mediated by nicotinic receptors with the exception of sweat glands, which are stimulated by muscarinic receptors (Kirstein and Insel, 2004).

Cholinergic innervation is prominent in the gastrointestinal tract, both as parasympathetic fibers as well as neurons in the enteric nervous system. Parasympathetic input to the intestine terminates in both the enteric and submucosal plexus, causing a general stimulation of the enteric nervous system. However, the enteric system is capable of independent operation without parasympathetic or sympathetic input. Enteric cholinergic neurons are of paramount importance for propulsive intestinal contractions (Goyal and Hirano, 1996).

The motor neurons whose purpose it is to enable movement by transmitting nerve impulses to the skeletal musculature are cholinergic as well. Their cell bodies are localized in the anterior horn of the spinal cord.

In the central nervous system, cholinergic innervation is widespread (Mesulam et al., 1983; Dutar et al., 1995; Mesulam, 1996; Semba, 2000). Major systems include the reticular formation in the brain stem, short interneurons in the striatum and neurons with cell bodies in the basal forebrain. Further locations of cholinergic neurons in the central

nervous system include those in the Pre-Bötzinger complex in the brain stem, hypothalamic neurons and cortico-cortical fibers (present in rodents, but not in primates).

Cholinergic fibers originating in the basal forebrain shall be discussed in more detail, since the studies described here were performed in a major target area of these fibers, the hippocampus.

Cholinergic neurons with cell bodies in the basal forebrain can be characterized as projection neurons with a large cell body. Many of them are sending axons either to most cortical areas (those with their cell body predominantly in the nucleus basalis: group Ch4 according to the nomenclature of Mesulam et al., 1983) or, via the fiber tracts of fimbria, dorsal fornix and supracallosal striae, to the hippocampal formation (cell bodies predominantly in the medial septum: group Ch1, and the vertical limb of the diagonal band: group Ch2). In addition, amygdala, olfactory bulb, some thalamic nuclei and other brain structures are also innervated by cholinergic fibers originating in the basal forebrain (Dutar et al., 1995; Mesulam, 1996; Everitt and Robbins, 1997; Semba, 2000).

Input to the basal forebrain arises from different afferent fiber tracts, including cholinergic fibers originating in the pedunculopontine and in the laterodorsal tegmental nucleus (Ch5 and 6, according to Mesulam). In addition, there is noradrenergic input from the locus coeruleus. Glutamatergic fibers both directly and indirectly, by multisynaptic circuits, innervate the basal forebrain. Major regions of origin of these fibers include the cortex, amygdala, and the pedunculopontine tegmental nucleus. GABAergic input to the basal forebrain originates in the ventral tegmental area. In addition, several septohippocampal fibers extend collaterals back to their own or neighboring cell bodies in the septum, and hippocampo-septal fibers have also been described. The exact mechanisms by which all these systems modulate cholinergic and non-cholinergic basal forebrain transmission is not clearly described so far (Sarter and Bruno, 2000).

2.1.3. Physiology

The physiologic function of an activation of the parasympathetic system has been described as to enable the body to "rest and digest" or to perform functions that are not desirable during times of high activity and stress. The effect of parasympathetic activation on the heart is mainly a reduction of the contraction rate (negative chronotropic effect), which is mediated by M_2 receptors on the atria. On the gastrointestinal tract, parasympathetic stimulation supports all aspects of digestion, starting from secretion of gastric acid by stimulation of M_1 receptors on parietal cells. Stimulation of the enteric and submucosal plexus of the intestine increases general activity of the intestine, including propulsion of chyme and secretion of digestive enzymes; in addition, the defecation reflex is also stimulated by parasympathetic activation. Urogenital smooth muscle relaxes upon parasympathetic stimulation (M_3), facilitating urination. Furthermore, parasympathetic stimulation of the eye causes miosis and near accommodation, enabling the organism to focus on nearby objects (Caulfield and Birdsall, 1998; Rang et al., 2001).

Activation of the fibers innervating skeletal musculature causes opening of nicotinic receptors located on muscle fibers at the neuromuscular junction. The subsequent influx of sodium in turn depolarizes the membrane. The action potential travels along the muscle fiber and activates voltage-gated sodium channels, causing full depolarization. Subsequent calcium release from the sarcoplasmic reticulum allows for stimulation of the contractile machinery within the muscle fibers, causing contraction of the muscle (Van der Kloot and Molgo, 1994; Niggli, 1999).

Central nervous cholinergic tracts are involved in a plethora of brain functions, including arousal (reticular formation), respiratory control (brain stem), coordination of voluntary movement (striatum), attention and cognitive function, including dreaming (fibers originating in the basal forebrain), and control of cerebral blood flow (Everitt and Robbins, 1997; Sarter and Bruno, 2000; Gold, 2003; Sato et al., 2004). Furthermore, both spinal and supraspinal involvement of muscarinic and nicotinic receptors in nociception has been demonstrated (Bannon et al., 1998; Dussor et al., 2004).

A characteristic feature of the physiology of cholinergic neurons with cell bodies in the basal forebrain is their dependence on trophic signals from their target areas for development and survival – with the exception of those neurons projecting to the amygdala (Heckers et al., 1994). This requirement is met by nerve growth factor (NGF). Axon endings of basal forebrain cholinergic neurons express both the low-affinity (p75) and high affinity (trkA) receptors for NGF (Mesulam, 1996). Upon binding of NGF to its receptors, the receptors dimerize. Then, the receptor-ligand complexes are internalized and transported retrogradely to the cell bodies (Salehi et al., 2003), where the PI3K-Akt-, the Ras-MAPK- and other intracellular signaling pathways are stimulated, causing the expression of distinct genes which ultimately leads to increased probability of survival of these neurons and increased neurite outgrowth (Lad et al., 2003)

Regarding the septohippocampal pathway, it is estimated that more than 60% of those fibers are cholinergic and that 80% or more of the cholinergic input to the hippocampus is mediated by these fibers (Dutar et al., 1995; Hilgert et al., 2003). In addition, GABAergic neurons are present, accounting for 10-20% of septohippocampal fibers (Pepeu and Blandina, 1998; Semba, 2000; Pang et al., 2001). Furthermore, some neuropeptides, like galanin, and other mediators, like nitric oxide, were found to be involved in septohippocampal transmission in some, but not in all species investigated (Semba et al., 2000). Many of these neuromodulators are co-released with one of the classical neurotransmitters.

Whereas cholinergic fibers impinge on many cell types in the hippocampus, including pyramidal neurons in the hippocampus proper and granule cells in the dentate gyrus, GABAergic fibers selectively synapse on hippocampal interneurons, most of which are themselves GABAergic. Additionally, collaterals of the septohippocampal cholinergic fibers also stimulate the cell bodies of septohippocampal GABAergic fibers via M_3 receptors (Dutar et al., 1995; Wu et al., 2003). As a result, stimulation of septohippocampal fibers leads to a stimulation of major hippocampal neurons, both directly, mediated by muscarinic inhibition of potassium currents, and indirectly, by disinhibition of the impact of GABAergic interneurons on principal neurons through

septohippocampal GABAergic neurons. One effect of an activation of septohippocampal neurons is the generation of theta oscillation in the hippocampus, an electroencephalographic pattern characterized by synchronous neuronal discharge with a frequency of 4-15Hz. It is indicative of higher states of arousal and mnemonic processing (Dutar et al., 1995). A feedback inhibition of hippocampal activation might be mediated by inhibitory hippocampo-septal GABAergic neurons, which are also stimulated by septohippocampal neurons, thus further enabling generation of rhythmic activity (Semba, 2000).

Physiologic correlates of an activation of the basal forebrain cholinergic system have been described as increased vigilance, attention, and memory processing (Dutar et al., 1995; Everitt and Robbins, 1997; Semba, 2000). The mechanism by which these cognitive functions are mediated has been conceptualized as a filtering of cortical input, adding an emphasis on relevant incoming signals relative to the constant background activity and thus increasing the signal-to-noise ratio (Sarter et al., 2005).

2.2. Diseases associated with cholinergic dysfunction

2.2.1. Alzheimer's disease

Alzheimer's disease (AD) is a disorder of deteriorating cognitive function. It is the most common neurodegenerative disease with a prevalence of <1% at age 65-69 and roughly doubling for every five years of age, reaching 10-40% at age 85-89 (Manca et al., 2002). Today, approximately 5 million people in the United States are affected by this disease. Besides age-related sporadic forms, there are also familial forms, where first signs of memory dysfunction occur in mid life. However, these inherited cases account for no more than 5% of all cases of AD (Cruts and Van Broeckhoven, 1998). AD is characterized by the progressive loss of cognitive function and psychological disturbances, resulting from widespread loss of function and subsequent degeneration of entire populations of neurons in cortical and limbic areas, with cholinergic neurons

originating in the basal forebrain being damaged early on in the disease process and to a large extent (Mesulam, 2004).

The disease was first described in 1907 by Alois Alzheimer, a German neurologist, in a middle aged woman, Auguste D, whom he attested a state of "allgemeine Verblödung" (general imbecility). At that time, it was disputed whether the presenting pathology represented a distinct disease entity on its own. It anyway occurred rather rarely, so it remained as a mere curiosity in neurology textbooks. With the increase in life expectancy over the course of the 20th century, more and more age related cases of the disease occurred, triggering widespread interest in the pathology. Starting from the observation that ingestion of muscarinic receptor antagonists, such as scopolamine, induces cognitive impairment both in animals and in healthy human volunteers, it was recognized that central nervous cholinergic fibers play an important role in processes of memory and cognition (Deutsch, 1971). Subsequently, evidence was found for a loss of basal forebrain cholinergic neurons in autopsy samples of deceased AD patients (Davies and Maloney, 1976; Whitehouse et al., 1982) which in turn triggered further research culminating in the "Cholinergic Hypothesis of Geriatric Memory Dysfunction" (Bartus et al., 1982). Although it is clear today that a cholinergic deficit does exist in AD and that the cognitive demise correlates relatively well with cholinergic dysfunction (Bierer et al., 1995), it is evident that cholinergic degeneration alone can not account for the entirety of cognitive and non-cognitive symptoms present (Everitt and Robbins, 1997; Mesulam, 2004). However, it can not be envisioned to successfully treat AD without correcting the existing cholinergic deficit.

The etiology of AD is still unknown. Current research on AD gravitates around the discovery of abnormal processing of the Amyloid Precursor Protein (APP) in the disease. Mutations have been identified as causative for approximately half of familiar early-onset forms of the disease; some of them are located in the *APP* gene, but most are found in *PSEN1*, the gene encoding presenilin-1, a component of one of the proteases acting on APP (Suh and Checler, 2002; Kar et al., 2004). APP is a ubiquitous class 1 transmembrane protein with a large, glycosylated extracellular N-terminus and a short

intracellular C-terminus (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987). The physiologic function of APP is still unknown, but it has been speculated that it is involved in different processes, including axonal transport, metal-ion homeostasis in brain and signaling functions. However, the current evidence for these postulates is still weak (Mattson, 1997; Barnham et al., 2004; Gunawardena and Goldstein, 2004; von Bartheld, 2004). APP is cleaved by three enzymes called secretases. Shedding of the large extracellular portion of APP by α-secretase or β-secretase catalyzed cleavage occurs first, followed by intramembrane cleavage by an aspartyl protease activity termed γ-secretase (Hardy and Selkoe, 2002; Selkoe, 2002). Beta- and subsequent γ-cleavage forms the 39-42 amino acid amyloid- β peptide (Aβ), a compound with demonstrated neuro- and synaptotoxic properties (Selkoe, 2001; Kar et al., 2004), whereas α-secretase cleavage occurs within the Aβ sequence, precluding amyloid formation. Instead, the soluble neurotrophic fragment, sAPP, is generated. Amyloid occurs in several aggregational states, including soluble monomers and oligomers, protofibrils and large deposits, termed amyloid plaques, which are commonly found in cortex, hippocampus and amygdala of AD patients, but also occur in the non-diseased aging brain and after traumatic brain injury, albeit far less numerous than in AD (Jellinger, 2004). Interestingly, amyloid deposits were already described by Alzheimer (1907) in his initial presentation of an AD patient, which included histochemical observations after autopsy.

An intricate interplay between cholinergic activity and amyloid metabolism has been established: Nitsch and colleagues (1992) were the first to demonstrate that activation of M_1 and M_3 receptors in stably transfected HEK 293 cells shifted APP metabolism away from the amyloidogenic pathway. The effect was mediated by protein kinase C, and subsequent work has demonstrated that this activation is not specific for cholinergic agonists, since other agonists, including bradykinin, and direct activators of protein kinase C, i.e. phorbolester, are capable of shifting APP-processing to the non-amyloidogenic pathway (Slack et al., 1993; Nitsch and Growdon, 1994). Additionally, it has been demonstrated that both AChE and BChE immunoreactivity can be detected in amyloid plaques in AD, although the functional significance of this finding is unclear

(Soreq and Seidman, 2001). In contrast, amyloid has been shown to interfere with many steps of cholinergic neuronal biochemistry, including high affinity choline uptake and nicotinic receptor signalling (Auld et al., 1998; Kar et al., 2004), although several of these effects were observed at amyloid concentrations that are very unlikely to occur *in vivo*.

Another early histopathologic observation by Alzheimer is the occurrence of neurofibrillary tangles. Molecular studies identified these as filaments consisting predominantly of hyperphosphorylated protein tau (τ), a small microtubule associated protein. Tau comes in different lengths, predominantly as versions with 3 or 4 repeats of the microtubule binding domain. The causes of tau-hyperphosphorylation are under investigation, with an increase in kinase activity, including glycogen synthase kinase 3β, and compromised phosphatase activity being the direct mediators of the hyperphosphorylated state of tau (Buée et al., 2000). The conformational changes imposed on tau by excessive phosphorylation cause decreased interactions of tau with microtubules and subsequently interruption of axonal transport. Mutations in τ were so far not reported in cases of AD, but they are identified in other neurodegenerative diseases, such as "Frontotemporal Dementia with Parkinsonism associated with chromosome 17" (Goedert, 2004). In fact, profound tau pathology is present in several neurodegenerative diseases, commonly referred to as the tauopathies (Lee et al., 2001).

An additional finding which might hold mechanistic clues for the causes of AD is the observation that the genotype of apolipoprotein E, a protein involved in cholesterol homeostasis, increases or decreases the likelihood of humans to develop AD. Carriers of the E4 allele have a severalfold increased likelihood to develop AD, whereas E2 carriers seem to be less likely to develop the disease, both with respect to carriers of the most common E3 allele (Beffert et al., 1998; Schappert et al., 2002).

Additional mechanisms that might be important for the development of AD are disturbances in glucose utilization, inflammatory responses, increased oxidative stress, probably due to an imbalance in metal homeostasis, excitotoxicity and disturbed calcium homeostasis (Mattson, 2004).

2.2.2. Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease, and the major disease of motor dysfunction. It is named for James Parkinson, a British naturalist of the $18^{th}/19^{th}$ century (Bryson, 2003). The vast majority of cases are of idiopathic etiology; however, mutations in the genes encoding α-synuclein and parkin (see below) were recently identified in familial cases of PD (Goedert, 2001; Moore et al., 2005). In PD, dopaminergic neurons originating in the basal ganglia, predominantly the substantia nigra, and projecting to the striatum undergo selective and massive (>70%) degeneration. As a consequence, the interaction between neuronal circuits involved in motor program control is disturbed, including the subthalamic nucleus, substantia nigra, globus pallidus, thalamus and motor cortex (Gibb, 1997; Obeso et al., 2000): The loss of dopaminergic inhibition of cholinergic activity in the striatum causes an imbalance between dopaminergic and cholinergic modulation of the striatal output to the motor program, leading to the characteristic extrapyramidal symptoms of tremor, rigidity and akinesia. Additionally, a subset of patients (10-50%) also develops dementia (Gibb, 1989). Histologic investigations revealed the presence of numerous inclusion bodies primarily in the basal ganglia, called Lewy bodies, named for Fritz H. Lewy, a disciple of Alois Alzheimer's. They consist predominantly of the protein α-synuclein and also stain positive for ubiquitin (Goedert, 2001). It has been hypothesized that a disturbance occurs in the endosomal degradation of α-synuclein, a notion supported by the observation that mutations in parkin, which acts as an E3 ubiquitin modifying protein on α-synuclein, cause autosomal recessive juvenile Parkinsonism (Bennett, 2005). Failure in the ubiquitin-tagging processes or in the endosomal machinery to process this tagged protein might cause its deposition. Alternatively, the mutations might cause a toxic gain of function of α-synuclein, disrupting its interaction with other cellular proteins (Bennett, 2005). Other diseases with formation of Lewy bodies in different parts of the nervous system, the so called synucleinopathies, include Dementia with Lewy bodies (see below), Amyotrophic lateral sclerosis and multiple system atrophy (Goedert, 2001; Bennett, 2005).

In the subset of PD patients suffering from dementia, prefrontal deficits and compromised function of basal forebrain cholinergic neurons were observed (Whitehouse et al., 1983; Perry et al., 1993). The incidence of dementia in PD is twice that of age matched controls (Gibb, 1989). Additionally, PD sufferers have been reported to be more susceptible to the memory impairing effects of scopolamine than age-matched control subjects, indicating that a profound cholinergic dysfunction, encompassing fibers involved in cognitive functions, is likely to occur during this disease, even in the absence of blatant cognitive symptoms (Dubois et al., 1987). In fact, Tiraboschi and colleagues (2000) reported that activity of ChAT in PD and Dementia with Lewy bodies in midfrontal cortex is even more compromised than in AD, whereas hippocampal ChAT activity is similar in AD and PD.

2.2.3. Dementia with Lewy bodies

In Dementia with Lewy bodies (DLB), α-synuclein- and ubiquitin-positive Lewy bodies are present not predominantly in the basal ganglia, as in PD, but in a more diffuse pattern, encompassing mainly cortical, but also subcortical brain regions. Neuropathologic similarities to AD exist, since amyloid deposits are also present, although they are by far less numerous than in AD. Furthermore, tangle-pathology is scarce or absent, and neuronal degeneration is less prominent than in AD (McKeith et al., 2002). Intriguingly, the cholinergic deficit in DLB is more pronounced than that in AD, and an association between activity of BChE in cortical grey matter and decline in cognitive function was described in a preliminary study with a small number of patients: The higher the BChE-activity, the worse the symptoms of the disease (Tiraboschi et al., 2000; Perry et al., 2003). It has also been discussed that those patients responding to cholinesterase inhibitor treatment for suspected AD showing the greatest improvement were in fact misdiagnosed and suffered from DLB (Perry et al., 1990). Clinically, the predominant features of DLB are cognitive symptoms with a more rapid onset compared to AD, and they often present in a fluctuating fashion. Visual hallucinations, which are rarely observed in AD, are common in DLB (McKeith et al., 2002).

2.2.4. Schizophrenia

Schizophrenia is a psychiatric disorder affecting approximately 1% of the population in all societies. It is considered to be a disease with a strong hereditary component and multifaceted etiology, probably including neurodevelopmental disturbances, traumatic life events, exposure to ill-defined toxins or certain viral infections (Andreasen, 2000). In schizophrenia, mesolimbic dopaminergic fibers were shown to be hyperreactive (Lewis and Levitt, 2002). One consequence of this state is a dopaminergic inhibition of GABAergic inhibitory fibers from the Nucleus accumbens to the basal forebrain (Sarter et al., 2005). In addition, the occurance of hypertrophy and hyperactivity of pedunculopontine cholinergic neurons has been suggested (Perry and Perry, 1995). Together, this might lead to altered activation patterns of basalocortical cholinergic fibers, which is considered to be responsible for attentional and cognitive deficits observed in schizophrenia (Sarter et al., 2005).

Experimental pharmacotherapeutic approaches to treat these cognitive deficits include cholinesterase inhibitors, M_1-selective muscarinic agonists and allosteric potentiators of nicotinic receptors (Friedman, 2004). In addition, atypical neuroleptic drugs already in use for treatment of schizophrenia show M_2-antagonistic properties and can elevate hippocampal ACh levels. It has been speculated that this activity contributes to their overall clinical efficacy in this disease (Johnson et al., 2005).

2.2.5. Myasthenia Gravis

Translated as severe muscle weakness, this disease is characterized by progressive loss of motor function. In the majority of cases, this is caused by autoantibodies directed against the nicotinic acetylcholine receptors on muscle fibers, causing an impairment of impulse transmission from nerve to muscle, resulting in muscle weakness and paralysis (Hughes et al., 2004).

2.2.6. Nerve gas/ insecticide poisoning

Most of the nerve gas agents developed for chemical warfare are irreversible inhibitors of AChE. Compounds include tabun, soman, sarin, and VX. In addition, many insecticides target cholinesterases and other serin-hydrolases in insects, the inhibition of which is lethal to many agricultural pests. Inhibition of AChE allows for the accumulation of ACh in the synaptic cleft, causing overstimulation of its receptors. In humans, the symptoms of poisoning with these agents are those of cholinergic hyperactivity, resulting in diarrhea, diaphoresis, vision impairment, muscle weakness and seizures, ultimately leading to death by respiratory failure, if severe enough (Karczmar, 1998).

2.3. Animal models with relevance to septohippocampal cholinergic dysfunction

2.3.1. Non-transgenic models

Primary models of septohippocampal dysfunction were pharmacological models, where either muscarinic or nicotinic agents or cholinesterase inhibitors were administered systemically or locally. Furthermore, lesion approaches were undertaken. This included transsection of the fimbria and fornix (the major nerve bundles from septum to hippocampus), aspirative lesion of septum or hippocampus, or excitotoxic damage to the septum. Septal damage differed by the agent used, with infusion of N-Methyl-D-aspartate (NMDA) or the NMDA agonist, ibotenate, causing unselective, widespread septal cell loss. More selective cholinergic damage was obtained by injection of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) (Everitt and Robbins, 1997). An early mechanistically highly selective cholinergic toxin to be developed was AF64A, the aziridinium analog of ethylcholine, which irreversibly blocks choline transport (Curti and Marchbanks, 1984; Hanin, 1996). Another, more recent approach was the development of ^{192}IgG-saporin, an immunotoxin consisting of an antibody targeting the p75 NGF-receptor coupled to the ribosome inactivating protein saporin

(Santanché et al., 1997; Savino et al., 2000). The antibody bestows selectivity towards p75 expressing basal forebrain cholinergic neurons, when administered intraseptally, while saporin causes disruption of protein synthesis and subsequent cell death (Heckers et al., 1994; Walsh et al., 1995).

2.3.2. Gene targeted models

Advances in the understanding of the reproductive biology of mice allows for the controlled manipulation of fertilized eggs by molecular biological methods, thus either deleting (knock-out) or modifying (transgenesis; knock-in) specific genetic information, which subsequently can be introduced into the blastocyte of pseudopregnant mice (Tecott and Johnson, 2002). The advantages of genetic targeting over conventional approaches are clear: Virtually no other manipulation can produce effects as selective as interruption of a protein at the genetic level. On the other side, since the genetic modification occurs at the stage of totipotent cells, it is present for the entire developmental period and in all tissues. Therefore, resulting phenotypes (or the lack thereof) might not necessarily be those caused by the pure absence or mutation of the targeted gene, but they might as well be caused by compensatory mechanisms occurring during development, by alternate interaction between the transgene and other endogenous genes or by insertional mutagenesis, an insertion of the new genetic information in a way that disrupts an existing gene (Crawley et al., 1997). Attempts to overcome some of these problems are made by developing conditional transgenic mice, i.e. mice in which the expression of a transgene can be activated (or suppressed) by controlled environmental conditions, or by the generation of mice with a more targeted expression of the transgene under control of tissue- or cell type-specific promoters (Jankowsky et al., 2005; SantaCruz et al., 2005).

2.3.2.1. General cholinergic function

Mice deficient for distinct muscarinic receptor subtypes

Some of the first mouse models with gene targeted alterations in the cholinergic system were mice deficient for the genes for individual muscarinic receptors. With time,

all five muscarinic receptor genes have been deleted in mouse models, allowing for detailed studies of the functions of individual receptors (Wess, 2004). All mice generated so far that are deficient for one or two of the muscarinic receptors are viable, fertile and devoid of any major phenotypical disturbances. Since the predominant subtypes in the basal forebrain are M_1, M_2 and M_4 (Levey, 1996), only mice deficient for these receptor subtypes shall be discussed.

Mice deficient for M_1-receptors show no major cognitive deficits. Furthermore, the amnestic effects of the non-selective muscarinic antagonist, scopolamine, are unchanged in these mice, indicating that other muscarinic receptors are likely involved in cognitive functions. Along that line, the ACh stimulated inhibition of the M-current in hippocampus, once thought to be predominantly caused by M_1-stimulation, is fully intact in these animals. These results might be due to functional redundancy occurring in wild-type animals or due to developmental processes that result in functional backup by other muscarinic receptors in the absence of M_1-receptors. The exact reason is unknown.

Pilocarpine induced convulsions are completely absent in M_1-deficient mice, giving clear cut evidence for the almost exclusive involvement of this subtype in convulsions. Strong decreases are also seen in the hydrolysis of inositol phosphates and stimulation of the MAPK pathway. Both these behavioral and biochemical results were not seen in mice deficient for M_2-M_4.

Deficiency for M_2 abolishes the tremor observed after oxotremorin administration. Hypothermia induced by the same agonist was reduced, but not abolished. Interestingly, discrete cognitive dysfunction was observed in the absence of M_2 (Wess, 2004). In addition, baseline extracellular levels of ACh in hippocampus are increased and scopolamine-induced increase of hippocampal ACh release is attenuated, but not abolished in these mice. Conversely, novelty-induced increases are accentuated. Similar observations were made in M_4-deficient mice, and scopolamine-induced ACh release was completely abolished in M_2/M_4-deficient mice, as expected (Tzavara et al., 2003).

Mice deficient for nicotinic receptor subunits

Barely any information on specific effects of deletion of any of the major nicotinic receptor subunits on basal forebrain cholinergic function or neurodegenerative processes has been reported. A few reports indicate modest effects on cognition in β_2-deficient mice and a lowered seizure threshold in α_4-deficient mice (Drago et al., 2003).

ChAT deficient and haplodeficient mice

Genetic deficiency for choline acetyltransferase, the ACh synthesizing enzyme, is lethal immediately after birth, most likely due to lack of muscular activity, as evidenced by signs of flaccid paralysis and lack of movement. Muscular development in utero is also severely disturbed (Misgeld et al., 2002).

Haplodeficiency for ChAT results in a very mild phenotype, lacking overt cognitive or other behavioral symptoms (Brandon et al., 2004). Although ChAT activity in brain is halved, ACh-content and depolarization induced release of ACh from hippocampal slices were unchanged. An almost two-fold upregulation of the high-affinity choline transporter, CHT-1, both at the mRNA- and protein level, was observed as well as an increase in the amount of choline transported by this carrier and utilized for ACh synthesis. The up-regulation of high-affinity choline transport was interpreted as a compensation to offset deficiencies in ACh synthesis due to ChAT haplodeficiency.

CHT-1 deficient and haplodeficient mice

Mice deficient for CHT-1, the protein responsible for the high-affinity choline uptake into cholinergic neurons, die within one hour after birth (Ferguson et al, 2004). However, hemizygote mice are viable and live without major anatomic or physiologic problems to adulthood, due to normal CHT-1 activity despite reduced protein levels.

hAChE transgenic mice

Mice overexpressing human AChE show an age-dependent, progressive impairment of cognitive function (Beeri et al., 1995, 1997). The only difference in their

septohippocampal cholinergic system is an upregulation of high affinity choline transport and [^{3}H]-hemicholinium-3 binding, indicative of increased turnover of ACh, and an altered response of hippocampal ACh levels to halothane anesthesia (Beeri et al., 1997; Erb et al., 2001). The changes in the high-affinity choline uptake are fully reversible upon low-dose application of the cholinesterase inhibitor, physostigmine, indicating that these changes are the direct result of increased cholinesterase activity (Erb et al., 2001).

AChE deficient mice

Mice deficient for AChE are viable (Xie et al., 1999, 2000; Li et al, 2000), an unexpected finding in view of the lethality of poisoning with inhibitors of AChE and the lethal phenotype of AChE deletion in both the fruit fly, *Drosophila melanogaster*, and the zebrafish, *Danio rerio* (Greenspan et al., 1980; Behra et al., 2002). However, only 20% of pups from heterozygote breeders are nullizygote, as opposed to an expected 25% assuming Mendelian genetics. Their development is retarded and their final body size and weight is considerably smaller than that of wild-type litter mates. However, overall sensory and neurologic performance is not severely compromised (Xie et al., 2000; Duysen et al., 2002). Gross anatomy and histology do not show any abnormalities in knockout mice; however, incomplete formation and age-dependent degeneration of the retina as well as ultrastructural changes at the neuromuscular junction were observed (Adler et al., 2004; Bytyqi et al., 2004). Heterozygote mice are phenotypically indistinguishable from wild-type littermates. Behavioral deficits of nullizygotes include untypical vocalizations, lack of exploratory activity, housekeeping and mating behavior. Their survival until adulthood is only possible after early weaning and change to high-caloric liquid diet, which is maintained throughout their life. Fifty percent of nullizygotes die within 100 days after birth, with seizures being the predominant cause of death (Duysen et al., 2002).

Biochemical studies confirmed the absence of AChE. At the same time, these studies demonstrated an activity of BChE which is largely indistinguishable between wild-type, heterozygote and nullizygote mice in any of the tissues investigated (Li et al., 2000).

Studies on central cholinergic aspects revealed no overall disturbances in cholinergic innervation and neuroanatomy (Mesulam et al., 2002). Internalization of muscarinic ($M_{1,2,4}$) receptors in striatum and hippocampus (M_2 was also investigated in cortex) without changes in mRNA levels, but unchanged plasma membrane levels of the β_2 subunit of nicotinic receptors in striatum are reported. In addition, plasma membrane levels of the high-affinity choline carrier, CHT-1, were increased by 60%, whereas no changes were observed in levels of ChAT or VAChT (Li et al., 2003; Volpicelli-Daley et al., 2003a,b). Muscarinic receptor induced activation of the ERK-pathway in hippocampus and cortex was compromised (Volpicelli-Daley et al., 2003b), and in pharmacological studies, a lack of sensitivity of nullizygotes to the muscarinic agonists, oxotremorin and pilocarpine, was observed (Li et al., 2003).

In addition to mice entirely lacking AChE, transgenic mice lacking discrete exons of the *Ache* gene were generated, which showed prominent effects on the expression of AChE in distinct tissues without affecting catalytic activity of AChE (Camp et al., 2005).

BChE deficient mice

A first study utilizing mice deficient for butyrylcholinesterase (BChE) has been published recently (Li et al., 2005). However, a thorough description of the characteristics of these mice is pending (Lockridge et al., 2005).

2.3.2.2. Models for Alzheimer's disease and related neurodegenerative disorders

APP and PS-1 transgenic mice

Several transgenic strains of mice carrying either the normal human gene for the amyloid precursor protein or genes with mutations associated with familial early onset forms of AD have been generated. The same holds true for presenilin-1. Additionally, APP and PS-1 transgenic mice have been cross-bred to generate double transgenic mice (Hock and Lamb, 2001; Köhler et al, 2005). Although most of these transgenic mice show an up to 10-fold overexpression of the *APP* gene, pathology is generally limited to amyloid plaques and mild to moderate cognitive deficits. Even plaques are not very

prominent or absent in some models, unless mice are expressing very high levels of APP or are double mutants for both mutated human *APP* and *PSEN1*. The mice under investigation here express a mutated human APP in place of the murine APP and under control of the endogenous promoter, thus at physiologic levels, in physiologic places and at physiologic times (see also 3.1.1., Materials and Methods). Invariably, none of the APP or PS-1 transgenics generated so far shows neurofibrillary tangles or considerable nerve cell loss. Parameters of the basal forebrain cholinergic system were investigated in some transgenic models, but results were inconclusive and showed only mild cholinergic deficits, if any at all (Wong et al., 1999; Bronfman et al., 2000a; Jaffar et al., 2001; Apelt et al., 2002; Gau et al., 2002; Ikarashi et al., 2004). With the exception of the work by Apelt and colleagues, all the studies published before inception of the work presented here investigated structural markers, like abundance of ChAT activity, number of ChAT positive neurons, radioligand binding, tissue content of ACh, etc. The study presented as first part of this thesis was the first one to conduct a thorough functional investigation of the cholinergic system in APP/PS-1 transgenic mice. Since then, two more studies with a similar design have been presented (Watanabe et al., 2005; Bales et al., 2006).

Tau-transgenic mice

The first tau-transgenic mice were generated before the identification of disease-causing mutations. Both mice overexpressing human 3-repeat and 4-repeat tau show hyperphosphorylation of the protein and signs of muscular weakness, but fail to develop neurofibrillary tangles (Spires and Hyman, 2005).

Overexpression of the mutation P301L, which in humans causes “Frontotemporal Dementia with Parkinsonism associated with chromosome 17”, produces a robust pathology of tau-hyperphosphorylation and NFT’s, accompanied by motor deficits and behavioral disturbances, including learning deficits (Spires and Hyman, 2005).

Additionally, conditional transgenic P301L mice have been developed with forebrain-specific transgene expression under the control of a tetracycline responsive element,

allowing for controlled on- and off-switching of the transgene (SantaCruz et al., 2005). These mice, when expressing the transgene, show dramatic and gross forebrain atrophy.

Mice transgenic for a combination of APP ± PS-1 and tau

To study the interaction between disturbed amyloid metabolism and altered tau processing, mice were generated that express a combination of the dominant AD related transgenes. The first model, mice overexpressing mutated human APP (Tg2576) and the P301L mutation of tau, demonstrated both amyloid and tangle-pathology. Tangles were worse, compared to single tau transgenic controls, but amyloid deposition was unchanged by the presence of mutated tau (Lewis et al., 2001). Another model also included mutated PS-1, leading to triple transgenic mice (Oddo et al., 2003a,b). Amyloid was shown to precede tangle formation. Unfortunately, no comparison to the double APP/tau transgenic mouse was made. Interestingly, the triple transgenic mouse has been used to test the effectiveness of potential treatments for AD and it has been shown that application of an M_1 muscarinic agonist ameliorated both plaque and tangle pathology (Caccamo et al., 2006).

Apolipoprotein E-transgenic mice

Mice have been generated that are deficient for apolipoprotein E. Besides dramatic effects on general cholesterol homeostasis, distinct effects on hippocampal and cortical neurons have been observed. In these mice, those neurons display significantly reduced activity of the ACh synthesizing enzyme, ChAT, which is reflected by impaired memory performance in these mice. Furthermore, a reduced resilience to toxic insults and retarded recovery after such insults has been shown, as well as increased τ phosphorylation (Genis et al., 1995; Gordon et al., 1995; Oitzl et al., 1997). However, another study failed to observe any differences in cholinergic neuroanatomy, AChE- and ChAT-activity and [^{3}H]-hemicholinium-3-binding (Bronfman et al., 2000b).

Knock-in mice expressing the human Apo E4 variant have also been generated. They displayed an aberrant glial response to brain lesions (Sullivan et al., 2004; Blain et al.,

2006). Just as the Apo E deficient mice, the Apo E4 transgenic mice did not demonstrate cholinergic deficits in the study by Bronfman and colleagues (2000b).

Crossing of ApoE null-mice with amyloid depositing transgenic mice attenuates amyloid deposition, suggestive of a physiological role of Apo E in the processing of APP and/or amyloid (Spires and Hyman, 2005).

Nerve growth factor-deficient mice and anti nerve growth factor mice

In order to develop a mouse model to study the biology of nerve growth factor (NGF), an attempt was made to delete the gene encoding NGF. Most of the genetically engineered mice lacking NGF developed poorly and died within a few days after birth, and only few lived for one to four weeks, so no thorough characterization of the phenotype was possible (Crowley et al., 1994). However, despite dramatic deficits in sensory and sympathetic function, the basal forebrain cholinergic system seemed to be adequately developed, with reduced size of cholinergic neurons as the only obvious change. Heterozygote ($ngf^{+/-}$) mice are viable and were also studied. They were phenotypically indistinguishable from wild-type litter mates and showed only moderate deficits in the basal forebrain cholinergic system, as evidenced by moderate memory impairment, and virtually no deficits in other targets of NGF such as sensory and sympathetic neurons (Chen et al., 1997).

Another model was developed that would target the NGF system not during development, but only postnatally: Mice expressing genetic information for a neutralizing monoclonal antibody against NGF (Capsoni et al., 2000; Ruberti et al., 2000). These mice are overall healthy and develop normally up to one month of age. Coinciding with a massive increase in antibody expression, a decrease in forebrain NGF content, as well as clear signs of deficits in the basal forebrain cholinergic system (>50% loss of ChAT positive neurons), accompanied by learning deficits are observed starting at 2 months of age, and further increasing until 6 months. Concomitantly, other hallmarks of AD pathology, both amyloid deposits and phospho-tau positive neurofibrillary tangles,

were observed. This is intriguing, as neither tau-transgenic nor amyloid-transgenic mice develop any one of the other hallmarks of AD, whereas this model shows all of them.

Mouse models for trisomy 21 (Down syndrome)

Segments of mouse chromosome 16 are homolog to parts of human chromosome 21. Initial attempts to create mice triploid for chromosome 16 resulted in lethality in utero, precluding studies in adult triploid mice. More recently, mice were generated which are triploid only for segments of chromosome 16. Both lines, Ts65Dn and Ts1Cje, live to adulthood, and all three mouse lines display characteristic features of human trisomy 21. Ts65Dn and Ts1Cje were studied in behavioural paradigms, and deficits in cognitive function, in particular spatial navigation, were found for both, although they differed slightly. Moreover, cell culture studies with Ts65Dn neurons and glia showed decreased size and number of ChAT positive neurons in Ts. Furthermore, co-culture of Ts glia with diploid neurons compromised cholinergic markers in these neurons. However, diploid glia could rescue the phenotype of Ts neurons (Galdzicki et al., 2001).

α-synuclein transgenics

Mice overexpressing human forms of α-synuclein have been generated. The transgenes represented either wild-type or PD-related mutant genes (A53T, A30P) and were under the control of strong promoters, either Thy-1, hPDGF-β or PrP. In either case, Lewy-body like inclusion bodies that stained positive for α-synuclein and ubiquitin were detected both in brain stem/substantia nigra and hippocampus/cortex as well as motor neurons, with nigral and motor neurons being the more susceptible populations and the A53T mutation causing the most severe damage (Masliah et al., 2000; van der Putten et al., 2000; Lee et al., 2002; Martin et al., 2006). In contrast, nigrostriatal neurons of mice deficient for α-synuclein have been shown to be protected from mitochondrial toxins, such as MPTP, malonate and 3-nitropropionic acid, pointing to a role of α-synuclein in processes that ultimately damage mitochondria (Klivenyi et al., 2006).

CHAPTER III
MATERIALS AND METHODS

3.1. Animal Models

3.1.1. Amyloid precursor protein and presenilin-1 transgenic mice

Three groups of mice were compared: The first one consisted of mice transgenic for a mutated human form of the amyloid precursor protein (APP). The two other lines were cross-breds of this line with mice transgenic for either a normal or a mutated human presenilin-1 (PS1). As a result, the following lines were under investigation: APP_{SL}, APP_{SL}/PS-1wt, and APP_{SL}/PS-1mut.

Mice were generated at Bayer Healthcare Inc., (Wuppertal, Germany). As described in detail (Köhler et al., 2005), a gene targeting vector encoding a humanized amyloid precursor protein gene carrying the so called Swedish (K670N/M671L) and London (V717L) mutations has been introduced into an embryonal stem cell line by electroporation, replacing the murine *App* gene. Embryonal stem cells identified to contain the transgene were injected into blastocytes of pseudopregnant mice, and chimeric male mice were crossed with female 129/SvEv and Black Swiss mice to achieve germ-line transmission of the transgene and homozygote transgenic mice.

Homozygote APP mutant mice were additionally cross bred with mice carrying the gene for wild type or mutated (M146L) human presenilin-1 (Duff et al., 1996), generating double-transgenic mice. Genotypes were verified by PCR of DNA from tail-clips.

3.1.2. Mice deficient for acetylcholinesterase

As described in detail (Xie et al., 1999; Li et al., 1999, 2000), heterozygote AChE deficient mice were generated in a 129/Sv background by deletion of exons 2-5 of one allele of the *ache* gene. Mice nullizygote for AChE were obtained by breeding haplozygote AChE mice. Genotyping of the offspring was performed by DNA testing from tail clips to positively identify the three genotypes in the offspring (+/+, +/-, -/-). In the knockout allele, 93% of the coding sequence, including the signal peptide and the

catalytic triad, is deleted. Neither AChE activity nor immunoreactivity has been detected in any of the tissues investigated.

For the studies described here, adult male and female AChE-deficient mice (Figure 3.1.) were transported in batches of 5 to 10 animals from Omaha, NE to Oklahoma City, OK overnight by commercial transportation. To reduce transit time, mice were picked up in Oklahoma City by car and delivered to Amarillo, TX, cutting the overall transit time to 24-28 hours. Once in Amarillo, mice underwent probe implantation and experiments within a week, but not on the day following transport. Wild type control mice were delivered by standard animal transport and were housed in the animal facility for extended periods of time.

Both wild-type and AChE-deficient mice were kept in the animal facility under controlled temperature and humidity conditions, with a 12/12h light/dark cycle. Controls stayed in standard cages with standard bedding. Knockouts, however, were kept in the same type of cage, but with the wire top removed. Instead, a domed filter top closed the cage. Moreover, to provide these frail mice with a dark compartment where they could hide and cuddle together, an inverted 200µl pipette tip box with a hole cut into one side was placed in each cage and its interior walls were covered with paper towels to provide extra warmth. Additionally, instead of standard bedding, paper towels were used to cover the cage floor to prevent bedding to be kicked into their food (see below).

While control mice were provided standard rodent chow (Purina RMH) and water ad libitum, knockouts, as reported (Duysen et al., 2002) had to be kept on a special diet (Ensure® Fiber with FOS, vanilla flavor, Abbott Laboratories, Columbus, OH). This liquid diet was provided in 35mm plastic Petri dishes placed on the cage floor, and was exchanged daily.

Animal studies were approved by the local Animal Care and Use Committee (protocol 02033-12).

Figure 3.1: Comparison of an adult AChE-deficient and a wild-type mouse. The knockout mouse (left) is considerably smaller than the wild-type litter mate (right). The mice displayed here had a body weight of 15 and 30g, respectively.

3.2. Microdialysis

3.2.1. General description

Microdialysis is a minimally invasive procedure that allows for sampling of compounds from extracellular body fluids (Westerink, 1995; de Lange et al., 1997). Probes are implanted into the tissue of interest (here: into the dorsal mouse hippocampus) during anesthesia and under stereotaxic control. After recovery of the animal from surgery overnight, probes are perfused with a physiologic fluid. A semipermeable membrane at the tip of the probe allows for exchange between the perfusion fluid and the tissue compartments in contact with the membrane. Compounds of interest are sampled in the outflow of the probe and subsequently undergo analysis.

3.2.2. Probes

The probes used in these studies were self-built (Figure 3.2.). As opposed to commercially available probes, the self-made design allows for adjustment of probe dimensions to experimental needs. Additionally, the cost of the materials for a self-made probe of the design used here runs at approx. US$ 5, compared to costs of >$50 for commercially available probes. The design and geometry of the probes was according to that used in previous studies from our lab (Erb et al., 2001; Kopf et al., 2001).

For building of the probes, a 1.5cm length of polyethylene tubing (0.38mm inner diameter, 1.09mm outer diameter; Portex Ltd., Hythe, Kent, UK) is used. A hypodermic needle (26G) is inserted into one end of the tubing for approx. 5mm. The tubing is then bent so that the needle perforates the tubing. A 1.5cm piece of fused silica (type TSP, 75µm inner diameter, 150 µm outer diameter; Polymicro Technologies LLC., Phoenix, AZ) is inserted through the opening punched by the needle and is allowed to protrude approx. 6mm from the end of the tubing.

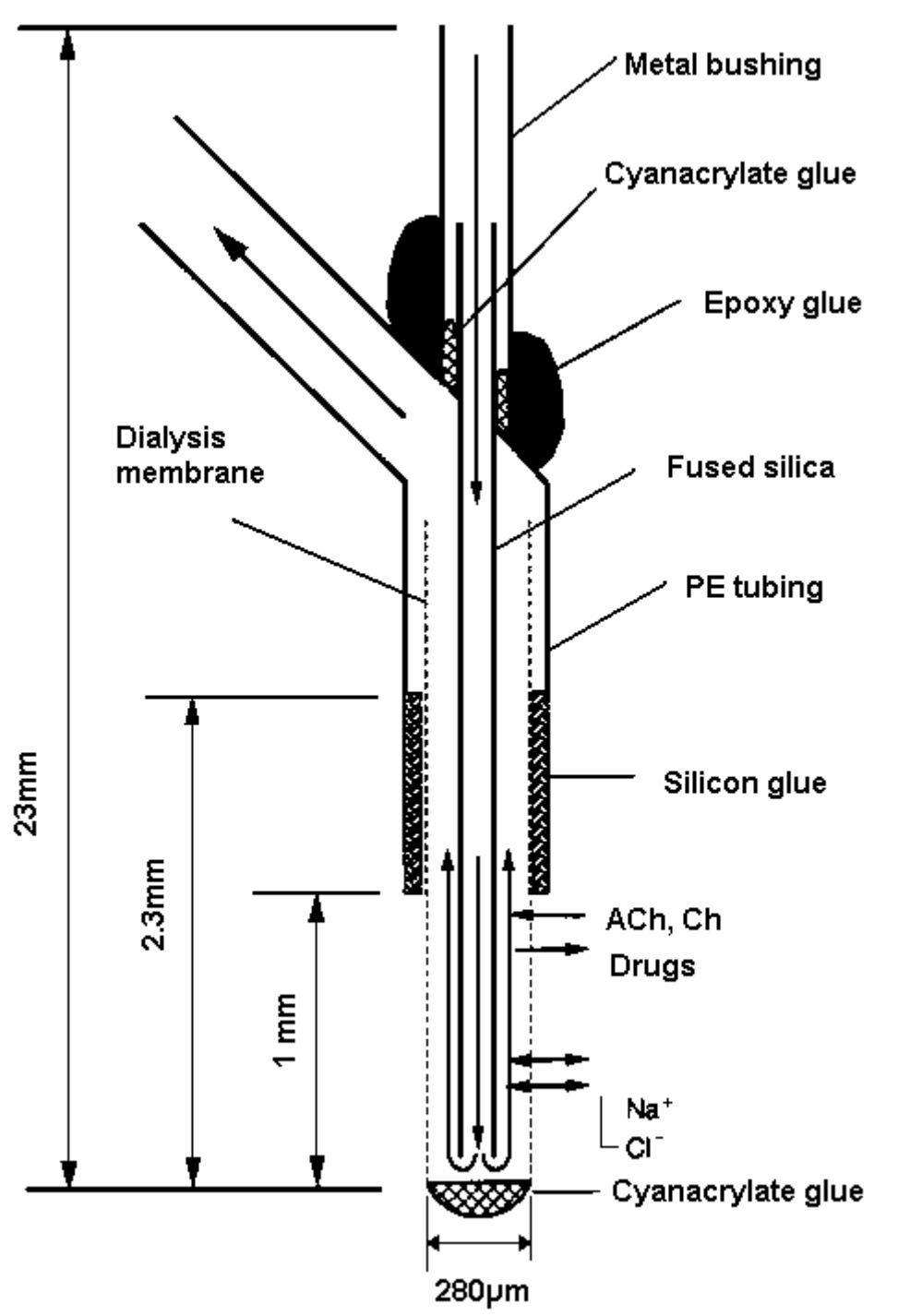


Figure 3.2: Schematic representation of a microdialysis probe (not to scale).

A metal bushing (Reinhold Stutz, workshop, Dept. of Pharmacology, University of Mainz, Germany; length: 8mm, 25G) is put around the fused silica where it protrudes from the needle punched hole in the tubing. It is attached to the tubing and the fused silica with cyanacrylate glue gel (Pattex Blitz Gel, Henkel KGaA, Düsseldorf, Germany).

After drying for at least one hour, the microdialysis membrane is carefully pulled over the free ending of fused silica and inserted into the PE tubing as deeply as possible. (Membranes are derived from hemodialysis cartridges: Filtral 12, AN69 HF, Hospal Industrie, Meyzieu, France. They are made of an acrylonitrile/methallylsulfone-sodium copolymer with a molecular weight cut-off of 10kDa.) Fused silica and membrane together are then cut to the size required for the microdialysis study. In this study, they were required to protrude from the tubing for approx. 2.5mm. After that, the membrane is pulled back out from the fused silica for 1 to 2mm. A small dab of cyanacrylate glue gel is used to seal the front opening of the membrane.

After at least one hour of drying time, the membrane is pushed back onto the fused silica as deeply as possible and fixed to the end of the PE tubing with cyanacrylate glue gel. After another one hour drying period, the exchange length desired for the experiments (here: 1mm) is measured from the tip of the probe, not counting the dab of cyanacrylate glue. A felt pen mark is made, and the remaining length of membrane sealed with silicon glue (704 RTV; IBF Chemotechnik Elektronik GmbH, Munich, Germany).

After drying overnight, the junction of PE tubing and the metal bushing is reinforced with epoxy glue (Epoxy-Rapid; Bison International, Goes, The Netherlands). After drying of this glue, the probes are ready to use. They are tested for permeability with sterile saline prior to implantation. Useful probes will show minimal resistance towards pushing saline through them with a syringe. Additionally, the exchange area at the tip of the probe will be covered with tiny droplets of saline, which might or might not coalesce.

3.2.3. Surgical procedure

Commonly, four mice underwent surgery on the same day between 9am and 4pm. Mice are anesthetized with 4% isoflurane (USP grade; Phoenix Pharmaceuticals, St. Joseph, MO) in a mixture of oxygen and nitrous oxide (25/75%, respectively; medical grade, Southwest Airgas, Corpus Christi, TX), delivered by a vaporizer (Dräger, Lübeck, Germany) at a flow rate of approx. 1.5liter/minute. After mice are unconscious, isoflurane is reduced to 2.5% and later to 1-1.5%, as needed. (For the amyloid transgenic mice, halothane was used instead of isoflurane. A change in anesthetic was made after this project due to the lower toxicity of isoflurane in humans). Mice are transferred to a stereotaxic frame (Stoelting Co., Wood Dale, IL), where they are fixed in a custom-made adapter for mice (Reinhold Stutz, workshop, Dept. of Pharmacology, University of Mainz, Germany): Mice are placed with their teeth into an incisor bar, and a metal clamp is used to fix the nose from above. Two lightweight metal bars are inserted into the ear canals, until a firm support of the skull is achieved. Delivery of the anesthetic gas to the mouse in the stereotaxic frame occurs via standard polyethylene tubing, ending in a mask (finger of a nitrile glove, severed at the tip) that covers the animals' snout.

After fixation, the fur on top of the head is clipped with a curved scissor. Subsequently, a longitudinal midline incision is made with a scalpel, after antiseptic treatment of the skin with povidone-iodide. The incision is extended with fine scissors, so it extends rostrally between the eyes and caudally as far as possible without severing the musculature attached to the base of the skull. The skull is treated with 3% hydrogen peroxide, applied with a cotton swab. The peroxide denatures the fine membrane below the skin and increases contrast of the sutures between skull bones. Surplus peroxide is absorbed with another dry cotton swab. Afterwards, the skin is captured with bulldog clamps and flapped to either side, allowing for a clear surgical field.

Now the stereotaxic (anterior-posterior and lateral) coordinates of bregma, the point where the frontal sutures between skull bones meet, are taken. The point targeted for insertion of the probe was determined according to the stereotaxic atlas of the mouse brain by Franklin and Paxinos (1997) and marked with a felt pen. It was 2mm posterior

and 1.8mm lateral (here: to the right side of the animal) to bregma. Then, with the use of a dental drill (ISO 310 104 001 001 018; Meisinger, Düsseldorf, Germany), the surface of the skull is roughed up to allow for increased grip of dental cement (see below). Following this, the same drill is used to drill a hole through the skull bone at the mark. Once the skull is penetrated, integrity of the dura mater is tested. If it is still intact, it is severed with a hypodermic needle; cessation of the resulting bleeding is accelerated by pressure compression with soft tissues. In the meantime, a microdialysis probe is placed into the manipulator arm of the stereotaxic frame and adjusted so its tip is pointing down perpendicularly. The probe is positioned so that the tip barely touches the surface of the brain at the hole. Now, the last stereotaxic coordinate, dorsoventral, is taken. The probe is then lowered slowly and carefully into the brain, for a total depth of 2.3mm. Once the probe is in place, a plastic shell, made from a pipettor tip and cut open at one side, is placed around the probe. Fast setting dental cement (Harvard schnellhärtend; Richter & Hoffmann Harvard Dental GmbH, Berlin, Germany) is put into the plastic shell around the probe. After hardening of the cement, which takes approximately 5-10 minutes, the probe is carefully released from the manipulator arm of the stereotaxic frame. More dental cement is applied to cover the exposed skull entirely, and the skin wound is closed by putting its edges into the cement plate. Cement is furthermore built up to cover the branching of the dialysis probe, stabilizing it and facilitating the attachment of tubing during the consecutive experiments. Once the entire cement is dried, local anesthetics (lidocaine and bupivacaine) are applied to the skin close to the incision. The animal is released from the stereotaxic frame and anesthesia is terminated. The animal stays in a big, rounded plastic bowl for recovery and for the subsequent experiments. Mice usually gain consciousness within 10 minutes after end of surgery. The total time of anesthesia is between 45 and 75 minutes. Figure 3.3 shows an AChE-deficient mouse at the end of the probe implantation surgery.

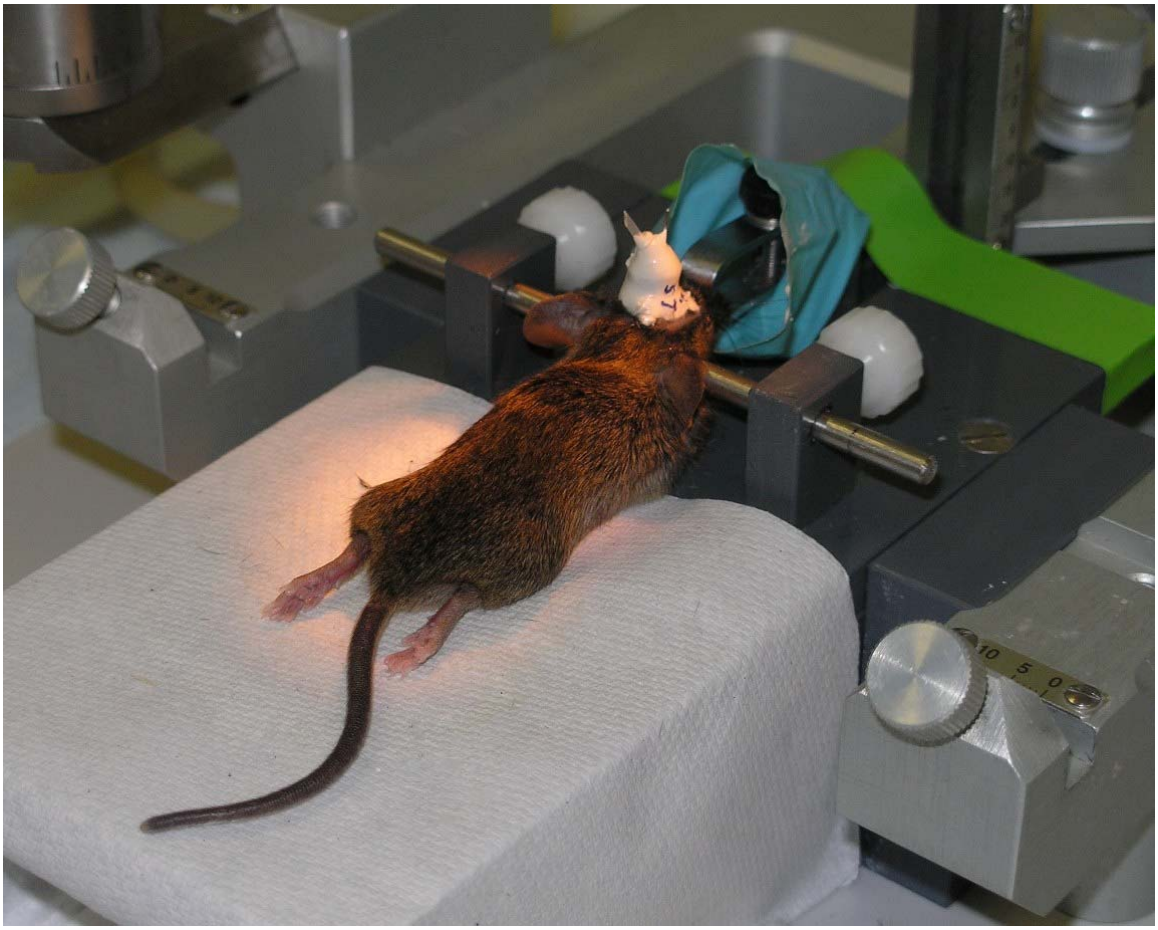


Figure 3.3: AChE-deficient mouse at the end of probe implantation surgery. The head is still fixed in the stereotaxic frame with both a nose clamp and two ear bars. The volatile anesthetic agent, isoflurane, is delivered from the vaporizer to the mouse with the help of standard lab tubing, ending in the blue face mask. The microdialysis probe is fixed to the skull with dental cement. Visible are the inlet (metal) and outlet (PE tubing) of the probe. Animal identification is applied to the dried dental cement by a felt pen.

3.2.4. Experimental procedure

Microdialysis experiments are carried out on up to two consecutive days following probe implantation, allowing for at least 18h of recovery time after surgery. This ensures that tissue trauma, including disruption of the blood-brain barrier, could heal to a large extent. In addition, levels of stress hormones and alterations in other neurochemical processes can subside considerably. Experiments more than two days after probe implantation carry the risk of altered probe permeability, due to reactive gliosis that becomes prominent after 48-72 hours and scarifies the tissue (Westerink, 1995; de Lange et al., 1997).

During the experiments, mice are kept in high, rounded plastic cages with free access to water. AChE knockout mice, due to their scare of open places and general frailty, were kept in standard mouse cages without wire top, but with a filter top, which was as close a match to their home cages as possible. Additionally, they were provided with a cardboard box (self made from Kimwipe boxes, design: C.K.) open on one side and on top, serving as shelter and compartment with reduced light intensity. Since it was perceived that fasting knockouts for the extended periods of experiments (≥8hrs/day) would be detrimental to their general well being, these mice had free access to their liquid diet throughout the experiments. In turn, to increase comparability, wild-type control mice in this group also had free access to standard pelleted food and water throughout the experiment.

Probes were perfused with artificial cerebrospinal fluid (aCSF), containing: NaCl, 147mM, KCl, 4mM, $CaCl_2$, 1.2mM, $MgCl_2$, 1.2mM. All drugs applied directly through the microdialysis probe were diluted to the appropriate concentration in aCSF. All compounds used were readily soluble in this fluid in the concentrations used.

Tubing for experiments was prepared from standard polythene tubing (Portex) with an inner diameter of 0.28mm and a length of 95cm. Suitable adaptors were constructed from other polyethylene and silicone tubing to allow connection of tubing to the syringe containing aCSF, and to both probe inlet and outlet.

Experiments typically started at 9am. Gastight syringes (Type 1001, Hamilton Corp. Reno, NV) with a capacity of 1ml were filled with aCSF. Tubing was flushed with this solution and subsequently attached to the microdialysis probes. While most control mice held still and allowed for attachment of tubing to the probe in their head while they were in their cage, some mice required restraint. All AChE knockouts had to be held gently, but firmly in hands to steady their head, which was otherwise constantly in motion from their uncontrolled jerky moves and tics. Perfusion fluid was pumped through the tubing at a constant flow rate of 2µl/min (APP transgenic mice) or 1µl/min (AChE-project) with the aid of a syringe pump (type 22, Harvard Apparatus, Holliston, MA and type 540200, TSE-systems, Bad Homburg, Germany). Fractions were collected in standard 1.5ml microcentrifuge vials with the aid of autosamplers (type 2110, BioRad, Hercules, CA) in intervals of 15 minutes each. Every 30-45 minutes, tubes were removed and placed in a freezer (-20°C), where they remained until analysis. A typical experimental setting is depicted in Figures 3.4 and 3.5.

Typically, after allowing for equilibration between perfusate and tissue for 60-90 minutes, during which time no samples were collected, four to six samples were collected, which were used to determine baseline levels of the analytes of interest. After that period, syringes filled with other perfusion fluid were set into the pump or animals received systemic treatment by intraperitoneal injections or were exposed to behavioral stimulation. In the case of local infusion of drugs of interest, this perfusion was maintained for one to two hours. Perfusion with regular aCSF was maintained after either kind of stimulation for three to four hours. Owing to the fact that at these low flow rates it takes 30 (at 2µl/min) or even 60 minutes at 1µl/min until fluid originating at the dialysis membrane reaches the sampling vial, time corrections in the analysis of effects had to be made accordingly.

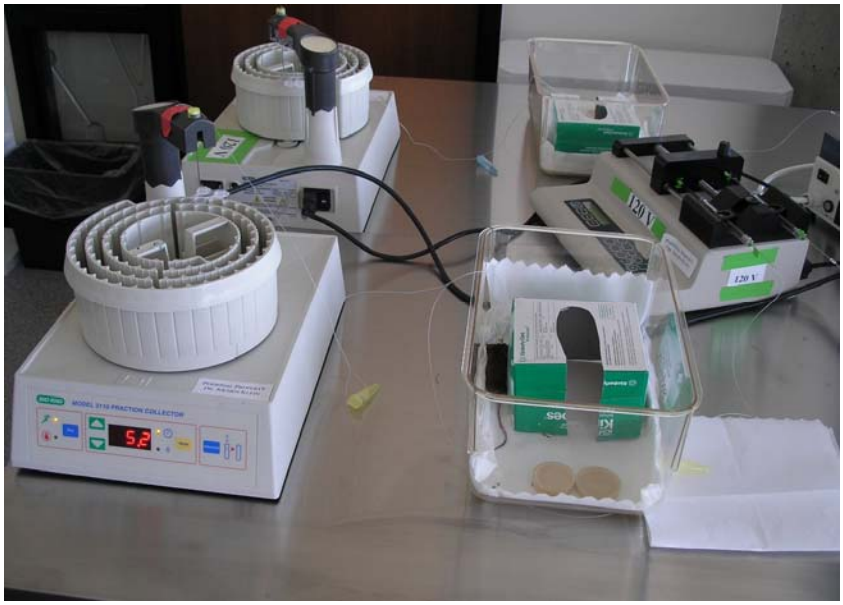


Figure 3.4: Microdialysis setup. Perfusion fluid is pumped from a syringe (top right) through PE tubing to the probe implanted in the mouse hippocampus (center). Outflow is transferred by another piece of PE tubing to the fraction collector (left). Up to four mice in parallel underwent perfusion (background).

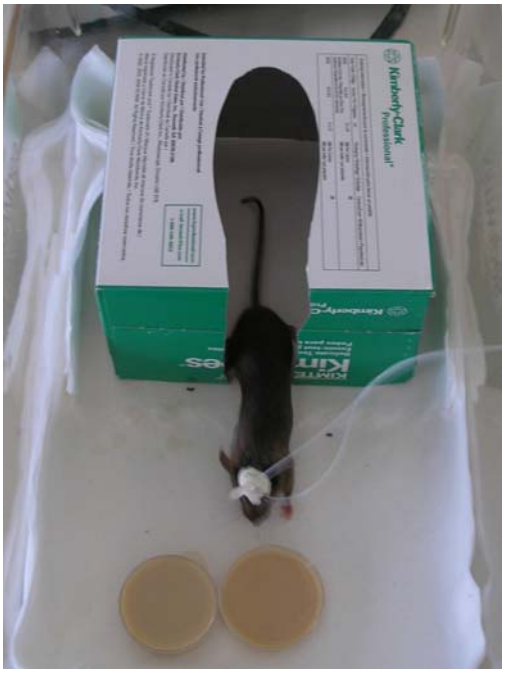


Figure 3.5: Close-up of an AChE-deficient mouse during perfusion. Visible are the paper bedding, the liquid diet provided in Petri dishes, the cardboard house, and the perfusion tubing. The mouse in the picture just emerged from her rest in the house to take a sip of food.

In the case of the APP transgenic animals, change of treatment was initiated by unplugging the inlet tubing from the head of animals, flushing it with the new solution, and reattaching it to the probe. This allowed for minimizing the lag time between switch of perfusate and onset of effect. For the AChE-project, however, in order to minimize distress for AChE-knockouts, treatments were initiated by simply switching the syringe at the pump. Experiments in wild type control mice were conducted accordingly. The resulting additional lag due to travel time of fluid from syringe to probe was accounted for.

Several mice were used after microdialysis for assessment of high-affinity choline uptake (see below, section 3.4.). Those mice were sacrificed on the third day after surgery, and the left brain hemisphere – the one that did not contain the microdialysis probe – was used to extract cortex and hippocampus for this assay. All other mice were sacrificed at the end of the second day of microdialysis by decapitation after isoflurane overdose. Brains (in case of animals used for choline uptake measurement, the right hemispheres) were placed into buffered formaldehyde solution. After fixation for 24 hours, they were cut by hand with a razor blade to verify the probe location within the hippocampus.

With the exception of intraperitoneal injections of choline chloride, all drug treatments were applied locally by dissolving the drugs at appropriate concentrations in the perfusion fluid. Choline chloride for i.p. injection was dissolved in DI water and administered in a dose of 20mg/kg and a volume of 1ml/kg body weight, which amounts to an isotonic solution.

3.2.5. Calibration of microdialysis probes

Probes from different batches were used for calibration purposes. To this end, probes were placed in an unstirred solution containing ACh and Ch at a concentration of 1μM each and were dialyzed with perfusion fluid, just as in the animal experiments. Dialysate concentration after equilibration was expressed as percentage of the initial concentration in the sample. This percentage is the *in vitro* recovery of the probe.

An additional approach was taken for the AChE-deficient mice: After implantation, probes were perfused at different flow rates. Non-linear regression of the dialysate concentrations after equilibration at the different flow rates yielded the theoretical concentration at a flow rate of zero. At zero flow, dialysate and tissue concentration should be identical, due to unlimited diffusion time between compartments.

3.3. High-performance liquid chromatographic detection of acetylcholine and choline

3.3.1. Principle of separation and detection

Analytes of interest were acetylcholine and choline. They were assessed using a high-performance liquid chromatographic (HPLC) method with electrochemical detection, similar to the one described by Kehr and co-workers (1998). Due to the small volumes available by microdialysis, it was necessary to use a microbore system with column inner diameters of 1mm, which allows for better separation and use of smaller volumes compared to conventional HPLC systems. After injection of aliquots (5µl) of microdialysis samples, analytes were separated on a cation exchange column. The alkaline pH of the mobile phase used (see below) guaranteed that only permanent cations, such as the quarternary amines ACh and Ch, will interact with the cation exchanger, whereas all other relevant basic compounds will be uncharged at this pH and not bind to the column. After passing the separation column, analytes reach an immobilized enzyme reactor (IMER) containing both acetylcholinesterase and choline oxidase (ChOx; E.C.1.1.3.17). Acetylcholine was hydrolyzed by the esterase, yielding choline and acetate. Choline was stoichiometrically broken down by choline oxidase in the presence of traces of oxygen in the mobile phase, yielding betaine and hydrogen peroxide. Hydrogen peroxide finally was detected by its oxidation at a platinum electrode with a potential of +0.5V relative to a silver/silver chloride reference electrode. The resulting current was recorded. Quantification was accomplished by comparison of peak heights with those of external standards, ACh and Ch, injected in known concentrations after

every fifth sample; this procedure allowed for correction of fluctuations in system performance within one day.

3.3.2. Instrumentation and mobile phase

HPLC equipment was manufactured by Bioanalytical Systems, Inc. (BASi, West Lafayette, IN). It consisted of a pulsation free low flowrate pump (PM-80), electrochemical detector (LC-4C) and a cross-flow electrode cell (platinum working electrode, and silver/silver chloride reference electrode, RE-5B). Separation columns with an undisclosed cation exchanger (UniJet microbore, 1x530mm) and IMER with immobilized AChE and ChOx (UniJet microbore, 1x50mm) were also from BASi.

Temperature controlled autoinjectors were used to increase the throughput of samples by allowing for unsupervised overnight operation. The type predominantly used was CMA/200 (Carnegie Medical A/B, West Chelmsford, MA). Some runs were performed by an autoinjector XL231, coupled to syringe pump 402 (Gilson Inc., Middleton, WI).

The mobile phase consisted of monobasic sodium phosphate (29mM) and sodium acetate (22mM, both HPLC grade, from EM Science, Gibbstown, NJ) and disodium EDTA (Fisher Scientific, Fair Lawn, NJ), dissolved in HPLC-grade water. Kathon CG, a mixture of 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-4-isothiazolin-3-one (Rohm and Haas, Croydon, UK) was added at a final concentration of 0.015% as a preservative to discourage microbial growth. A 5N solution of sodium hydroxide (Fisher) was used to adjust pH to 8.4.

At a flow rate of 120µl/min, retention time for ACh and Ch was 10.3 and 12.1 minutes, respectively. Limit of detection was at 3-6fmol/5µl.

3.4. High-affinity choline uptake – biochemical assay

3.4.1. Tissue preparation

Animals were lightly anesthetized with isoflurane and subsequently decapitated. The skull was carefully cut open and the brain was quickly removed and placed on a pre-

wetted filter paper, kept on ice. All mice used for the high-affinity choline uptake assay underwent microdialysis before, so the right brain hemisphere, which had contained the microdialysis probe, was removed and processed as described above (3.2.4). Only the left hemisphere was used for the uptake assay. From the left hemisphere, cerebellum, brain stem, striatum and thalamus including smaller adjacent structures, as well as olfactory bulb were removed, leaving only cortex and hippocampus. Cortex and hippocampus were placed into a 15ml Dounce tissue homogenizer (Bellco, Vineland, NJ) containing 3ml of ice cold 0.32M sucrose solution, buffered with 10mM HEPES, saturated with a mixture of 95% oxygen and 5% carbon dioxide and adjusted to pH 7.2-7.4. Tissue was homogenized with 10 strokes with a tight-fitting pestle and transferred to centrifuge tubes. In a J2-HS centrifuge (Beckman Coulter, Fullerton, CA), homogenates were spun at a temperature of 4°C for 10 minutes with a JA-14 rotor at a speed of 2550 rpm, creating a force of 1000g. The supernatant from each tube was distributed onto two centrifuge tubes and underwent a second round of centrifugation, this time at 10600 rpm or 17000g for 10 minutes. One pellet (P2 fraction) from this spin was rinsed with 200µl of Krebs-Henseleit buffer (KHB; NaCl, 115mM, KCl, 7.1mM, $CaCl_2$, 1.2mM, $MgSO_4$, 1.2mM, $NaHCO_3$, 25mM, Na_2HPO_4, 1.5mM, Glucose, 12.8mM; saturated with 95% oxygen and 5% carbon dioxide and adjusted to pH 7.2-7.4). The other pellet was rinsed with KHB containing hemicholinium-3 at a concentration of 1µM. After rinsing, the pellets were resuspended in 200µl of the respective solution and three aliquots of 50µl were transferred into microcentrifuge tubes.

3.4.2. Choline uptake assay

Two aliquots, filled up to 250µl with the respective solution, were used for parallel incubations. The third aliquot was put aside for protein determination (Bradford assay, Kit from Sigma, St. Louis, MO). Aliquots were warmed up to 30°C in a dry heating bath (Fisher Isotemp) for 10 minutes. After this time, tubes were placed on ice, [^{3}H]-choline (Specific Activity 60-90Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) was added to a final concentration of 25nM. Tubes were transferred back to the heating

bath for exactly 5 minutes. Tracer uptake was terminated by placing the tubes on ice and adding 1ml of ice cold KHB to the aliquots. After immediate centrifugation at 1400g at 4°C for 10 minutes (Centrifuge 5415D, Eppendorf, Hamburg, Germany), supernatant was removed. The pellet was washed three times by adding 600µl of ice cold KHB, homogenization and subsequent centrifugation. After washing, the remaining pellet was suspended in 0.5ml of methanol and transferred into 7ml liquid scintillation vials. After the addition of 4ml of liquid scintillation cocktail (Scintisafe 30%; Fisher), the samples underwent scintillation counting (Beckman Coulter LS 6000).

3.5. Histology

Histology of brains was performed by our collaborators at Bayer Healthcare, Inc. Wuppertal, Germany. In brief, coronal sections (40µm) were stained with either thioflavin S or the antibody 4G8 to quantify amyloid plaques. Abundance of amyloid peptides was assessed after extraction of brain tissue and subsequent binding to specific antibodies in a liquid phase chemoluminescence assay. Additionally, sections of the septum were stained with a specific antibody for choline acetyltransferase. Stereologic counting of sections was used to quantify the number of cholinergic cell bodies in the septum.

3.6. Representation of data and statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). When individual data points are illustrated, they are obtained from individual animals.

Comparisons of two sets of data were done by unpaired two-tailed t-test; Welsh's correction for unequal variances was performed as necessary. Comparisons of more than two sets of data were performed by One-way ANOVA.

CHAPTER IV
APP-PROJECT – RESULTS AND DISCUSSION

4.1. Results

It was the aim of this project to characterize the impact of Alzheimer's disease related amyloidosis on the function of septohippocampal cholinergic neurons. The study compared APP_{SL} mice, $APP_{SL}xPS1_{wt}$ mice and $APP_{SL}xPS1_{M146L}$ mice. For the sake of readability, the mouse strains will henceforth be referred to as APPsl, PSwt and PSmut, respectively. Center piece was a microdialysis study in the dorsal hippocampus. Furthermore, activity of the high-affinity choline uptake in corticohippocampal synaptosomes was determined as a marker of ACh turnover. Additional histochemical studies were performed to quantify amyloid load and number of amyloid plaques in cortex and hippocampus as well as the number of choline acetyltransferase-positive neurons in the septum. Finally, correlation analysis between different of the above parameters was performed to test for interrelationships between these.

4.1.1. Animals

For the histochemical studies, mice of several ages were used. The number of animals varied between three and ten per age group.

For microdialysis, 9-13 animals per genotype were used. Their mean age and body weight at the day of probe implantation and their gender is displayed in Table 4.1. PSmut mice were significantly younger than the other groups ($F=12.46$, $df=34$, $p<0.0001$). No significant difference in body weight was observed ($F=1.09$, $df=34$, $p=0.35$).

Table 4.1: Comparison of age, gender and body weight between mouse strains.
Included are animals used for microdialysis and high-affinity choline uptake assay.

	APPsl	PSwt	PSmut
Age [days] ± SEM	836 ± 33	797 ± 27	601 ± 45
Body weight [g] ± SEM	36 ± 1.8	36.2 ± 1.8	40.2 ± 3.0
male/female [n]	11/2	8/3	9/2

4.1.2. Histochemical characteristics

The histochemistry and amyloid quantification was performed by collaborators at the CNS research department of Bayer Healthcare Inc., Wuppertal, Germany (Hartmann et al., 2004).

Amyloid load was determined by immunohistochemistry, using the specific antibody 4G8. Plaques were also visualized using the thioflavin S staining procedure (Wisniewski et al., 1989). Representative sections are presented in Figures 4.1. and 4.2.

The antibody staining demonstrated the complete absence of amyloid deposits in the brains of APPsl and PSwt mice, up to an age of 26 months. In the PSmut mice, first plaques were visible at 14 months of age; their number increased to 400 plaques per coronal section at 26 months of age (Figure 4.3.B), causing intense staining in cortex and hippocampus (Figure 4.1. and 4.2.).

Amyloid levels were also assessed after extraction of brain tissue and quantification of amyloid peptides by a luminescence assay. Analysis of the time course of amyloid pathology revealed increased amounts of soluble amyloid in PSmut mice at the age of 10 months. These amounts rose steadily until 20 months and then slowly leveled off, reaching more than 10nmol/g wet weight at 29 months, the last data point taken (Figure 4.3.A).

Investigation of the number of ChAT positive cell bodies in the septum revealed an increased number of those neurons in the septum in PSmut mice: However, this increase failed to reach significance (data not shown). Furthermore, no apparent changes of neuronal morphology were evident after Nissl staining.

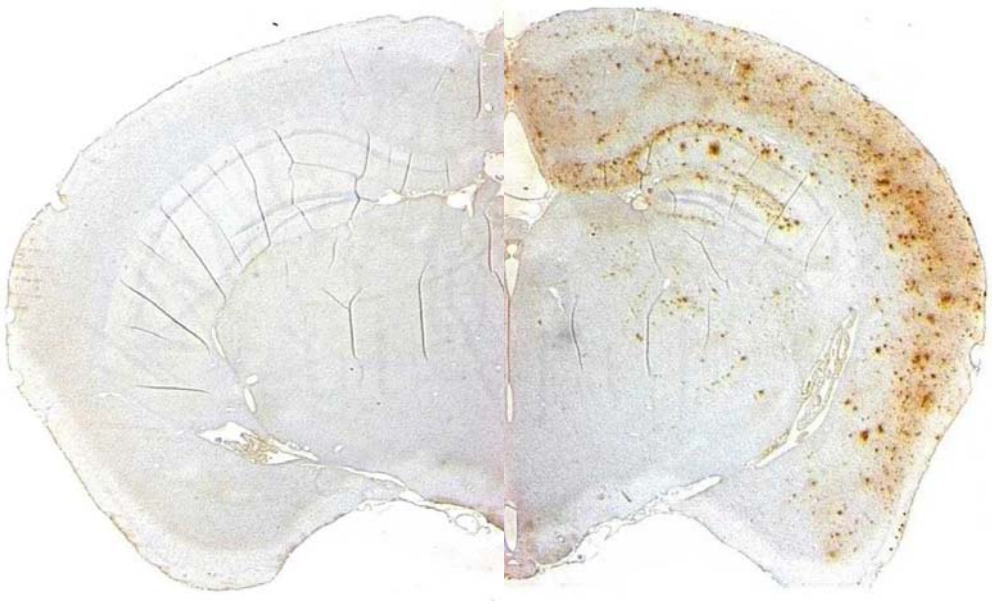


Figure 4.1: Amyloid deposits, stained with antibody 4G8. Left panel: $APP_{SL}xPS_{wt}$ mouse at 26 months. Right panel: $APP_{SL}xPS_{M146L}$ mouse at 26 months. No staining is visible in $APP_{SL}xPS_{M146L}$ mice. Courtesy of Dr. Christina Erb, Bayer Healthcare Inc.

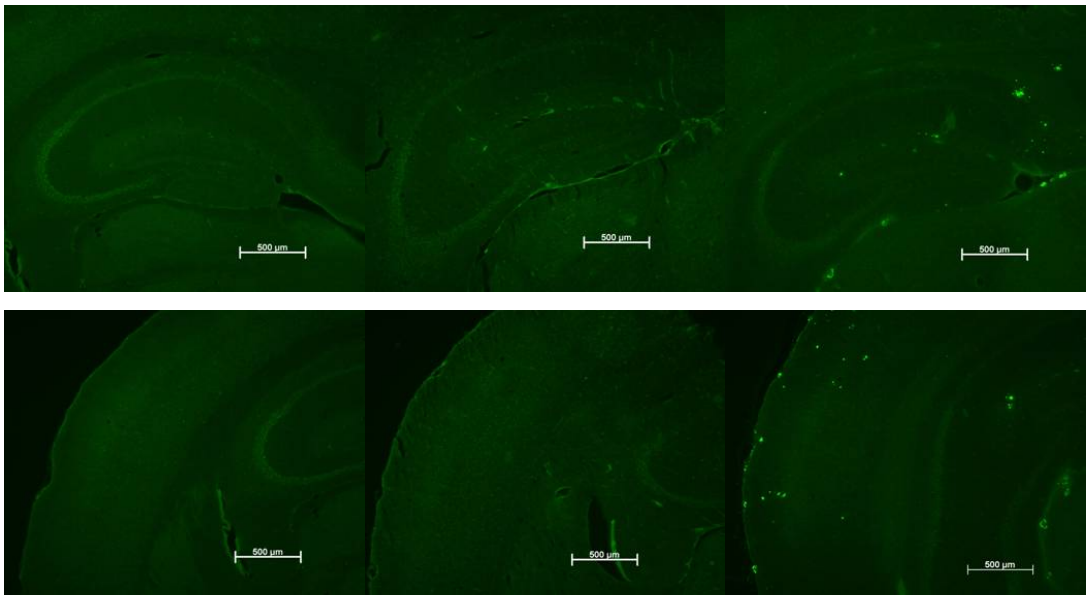


Figure 4.2: Amyloid plaques, visualized after thioflavin S staining. Top: hippocampus, bottom: cortex. Left: APP_{SL} mouse, middle, $APP_{SL}xPS_{wt}$ mouse, right, $APP_{SL}xPS_{M146L}$ mouse. No staining is visible in either APP_{SL} or $APP_{SL}xPS_{M146L}$ mice. Courtesy of Dr. Christina Erb, Bayer Healthcare Inc.

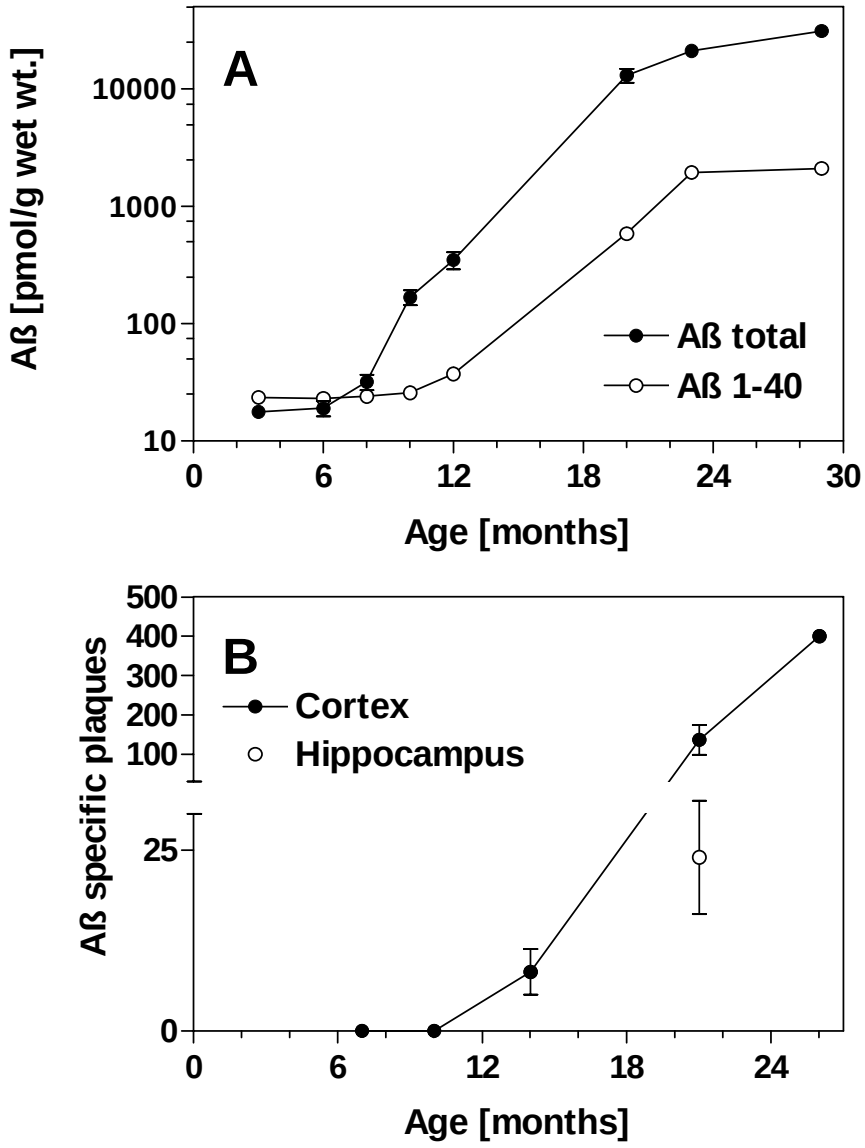


Figure 4.3: Amyloid abundance and plaque number.
A: Amount of amyloid peptide in tissue. Levels in APPsl and PSwt mice were not measurable
B: Count of amyloid plaques as detected by the 4G8 antibody. No plaques were detected in APPsl or in PSwt mice.
Data are mean ± SEM from seven to 10 mice per group. Some error bars were smaller than the symbol representing the mean value.

4.1.3. Microdialysis

4.1.3.1. Baseline values

Baseline values of acetylcholine, averaged over both experimental days, are displayed in Figure 4.4. Average values were: for APPsl, 50.9 ± 5.9fmol/5µl (n=13); for PSwt, 65.4 ± 6.1fmol/5µl (n=11) and for PSmut, 46.3 ± 6.4fmol/5µl (n=11). There was no significant difference in baseline values between animal groups (F=2.52, df=34, p=0.096).

After obtaining baseline values, hippocampal ACh release was stimulated. This was done behaviorally, by placing mice into a novel environment (open field), and pharmacologically, by local application of the muscarinic receptor antagonist, scopolamine.

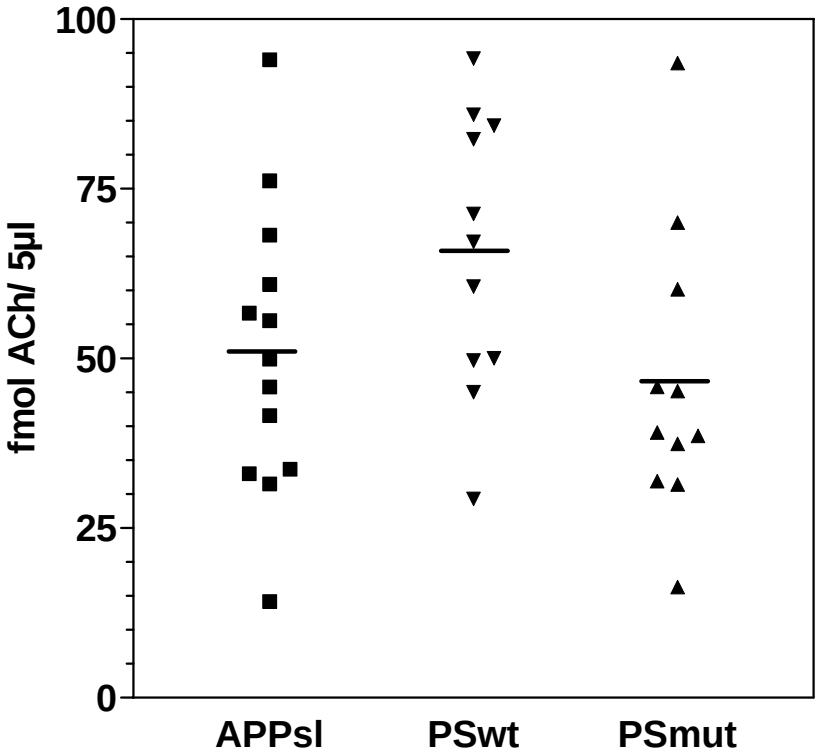


Figure 4.4: Baseline values of acetylcholine levels in dialysate from hippocampus in the presence of 1µM neostigmine. Each data point indicates the average baseline levels from one individual animal. Bars represent means. Mice were between 441 and 972 days old and PSmut mice showed prominent amyloid pathology.

4.1.3.2. Behavioral stimulation of ACh release

When mice were exposed to a novel environment, extracellular levels of acetylcholine in hippocampus increased immediately and declined towards baseline for the remaining time in the open field, as well as after return to their homecage (Figure 4.5.). Maximum dialysate concentrations were 104 ± 24fmol/5µl (APPsl; n=12), 142 ± 24fmol/5µl (PSwt; n=9) and 103 ± 21fmol/5µl (PSmut; n=10). The area under the time-effect curve above baseline after exposure of animals to the open field was 336 ± 76AU (APPsl), 421 ± 136AU (PSwt) and 216 ± 63AU (PSmut). These values were not significantly different ($F=1.154$, $df=30$, $p=0.33$).

When the same results are expressed relative to baseline concentrations, the following effects are observed: The maximum of ACh levels in APPsl mice was 188 ± 20% (n=12), in PSwt mice, it was 234 ± 45% (n=9), and in PSmut mice, a maximum was reached at 232 ± 33% (n=10). Similarly, when the area under the time-effect curve above baseline after placement of animals into the open field was calculated, values of 629 ± 93AU, 699 ± 211AU, and 526 ± 85AU were obtained, respectively (APPsl, PSwt, PSmut). These values did not show significant differences ($F=0.40$, $df=30$, $p=0.67$).

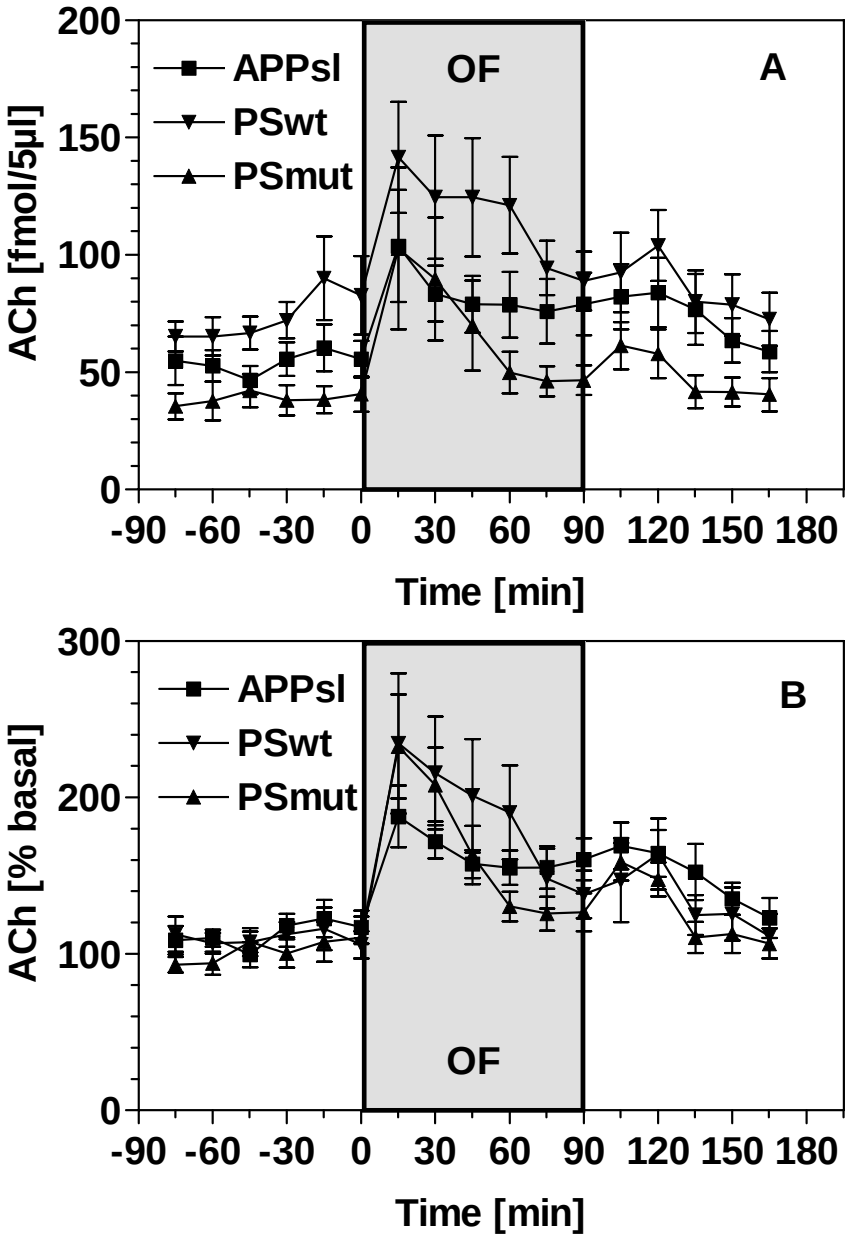


Figure 4.5: Effect of behavioral stimulation on hippocampal ACh release. Mice were exposed to a novel environment (open field, OF) for 90 minutes, starting at time 0.
A) Effects expressed in absolute dialysate concentrations; B) Effects expressed relative to baseline levels. All PSmut animals showed prominent amyloid pathology at the time of experiments, as confirmed *post mortem*. No amyloid pathology was observed in either of the other transgenic lines.

4.1.3.3. Pharmacologic stimulation of ACh release

Results from pharmacologic stimulation of hippocampal ACh release by blockade of inhibitory M_2/M_4 autofeedback-receptors with scopolamine, applied with the dialysis fluid in a concentration of 1µM, are displayed in Figure 4.6. Upon initiation of scopolamine-perfusion, extracellular levels of ACh immediately started to rise, reaching a maximum after 60-75 minutes, and started to decline slowly after cessation of scopolamine-perfusion. Maximum dialysate concentrations were 269 ± 36fmol/5µl (APPsl; n=11), 337 ± 58fmol/5µl (PSwt; n=11) and 235 ± 56fmol/5µl (PSmut; n=10). The area under the time-effect curve above baseline after exposure of animals to the open field was 1478 ± 232AU (APPsl), 1195 ± 314AU (PSwt) and 1146 ± 261AU (PSmut). These values were not significantly different ($F=0.44$, $df=31$, $p=0.65$).

When the same observations are expressed relative to baseline concentrations, the following results are obtained: Maximum ACh release was, relative to baseline: 505 ± 69% in APPsl (n=11), 337 ± 58% in PSwt (n=11) and 475 ± 71% in PSmut (n=10). Similarly, when the area under the time-effect curve above baseline after scopolamine application was calculated, values of 2640 ± 351, 1520 ± 225, and 2520 ± 481AU were obtained, respectively (APPsl, PSwt, PSmut). These values did not show significant differences ($F=2.99$, $df=31$, $p=0.07$).

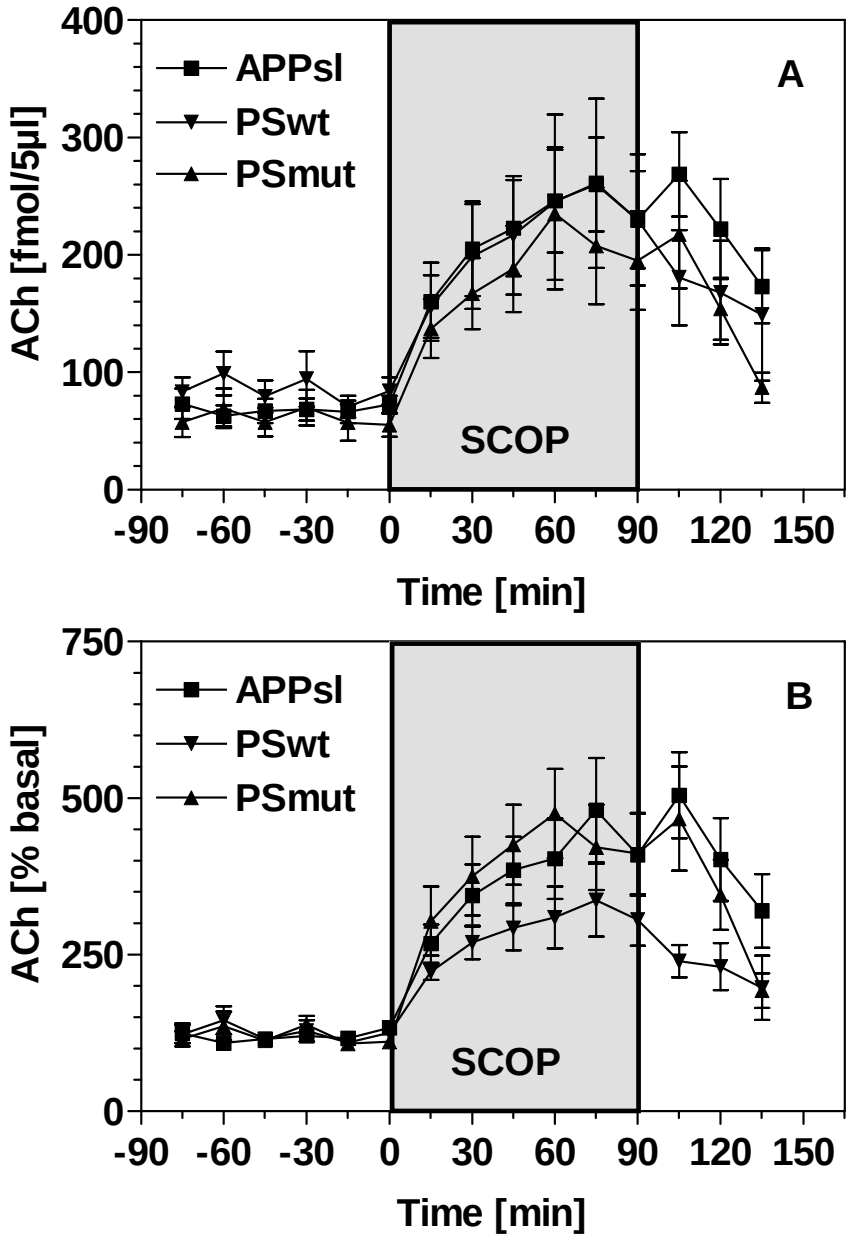


Figure 4.6: Effect of pharmacologic stimulation on hippocampal ACh release. Mice were exposed to 1µM scopolamine, applied through the microdialysis probe, for 90 minutes, starting at time 0.
A) Effects expressed in absolute dialysate concentrations; B) Effects expressed relative to baseline levels. All PSmut animals showed prominent amyloid pathology at the time of experiments, as confirmed *post mortem*. No amyloid pathology was observed in either of the other transgenic lines.

4.1.4. High affinity choline uptake assay

In a synaptosomal preparation from combined cortex and hippocampus, the activity of hemicholinium-3-sensitive and -insensitive [^{3}H]choline uptake was assessed. The results are represented in Figure 4.7.

High affinity choline uptake, defined as [^{3}H]choline uptake sensitive to incubation with hemicholinium-3 at a concentration of 1μM, reached values of 73.6 ± 4.9dpm/μg protein/5 minutes in APPsl (n=14), 79.1 ± 10.9dpm/μg protein/5 minutes in PSwt (n=9), and 60.2 ± 12.4dpm/μg protein/5 minutes in PSmut (n=10). These values did not differ significantly between genotypes (F=0.95, df=32, p=0.40).

Protein content per incubation did not differ significantly between genotypes and reached 296 ± 13.6μg in APPsl, 246 ± 31.8μg in PSwt and 252 ± 21.8μg in PSmut (F=1.57, df=32, p=0.18; data not illustrated).

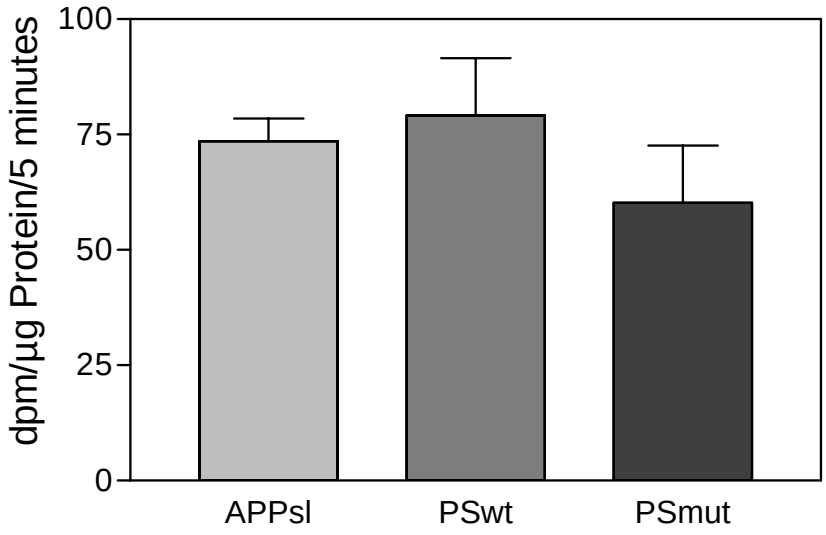


Figure 4.7: Activity of high-affinity choline uptake. Synaptosomes from combined cortex and hippocampus were exposed to [^{3}H]choline for exactly five minutes in the presence or absence of the specific inhibitor, hemicholinium-3 (1μM). High-affinity choline uptake is the hemicholinium-sensitive portion of total [^{3}H]choline-uptake. All PSmut animals showed prominent amyloid pathology at the time of experiments, as confirmed *post mortem*. No amyloid pathology was observed in either of the other transgenic lines.

4.1.5. Correlation analysis

Since the average age of PSmut mice was significantly lower than that of the other transgenic lines (Table 4.1.), and since amyloid pathology occurs age-dependently (Figure 4.3.), it was investigated whether any of the parameters measured in PSmut mice correlated with either age or plaque density. The correlations that have been calculated are:

- basal levels of ACh versus age of animal
- area covered by amyloid plaques versus basal levels of ACh
- age versus AUC in open field experiments
- age versus AUC in scopolamine experiments
- age versus maximum increase of ACh in open field experiments
- age versus maximum increase of ACh in scopolamine experiments.

None of them reached statistical significance ($p>0.1$; data not shown and Figure 4.8).

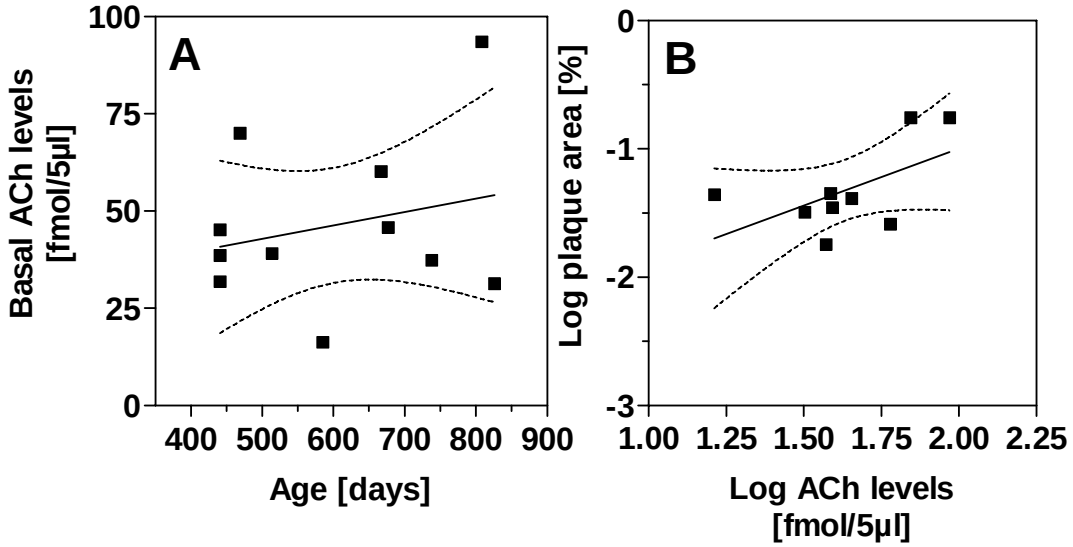


Figure 4.8: Correlation of selected parameters in PSmut mice. A) Basal ACh levels vs. age of animals; B) Area covered by amyloid plaques vs. basal ACh levels. There is no correlation in either case (A: $R^2=0.06$, $p>0.4$; B: $R^2=0.32$, $p>0.1$).

4.2. Discussion

The first part of this thesis is concerned with central nervous cholinergic function in transgenic mice expressing mutated human forms of both the amyloid precursor protein (APP) and presenilin-1 (PS1). The study was performed to clarify the effects of Alzheimer's disease (AD)-like dysfunction of amyloid metabolism on the function of cholinergic neurons in the hippocampus. Numerous studies in autopsy samples have clearly shown that deficits of the basal forebrain cholinergic system are a consistent and pronounced feature of AD. The functional approach chosen in our study promised to give clear-cut information on the overall balance of probable amyloid-induced damages and compensatory mechanisms.

4.2.1. Mouse models

4.2.1.1. Our mice

In our mice, genetic information for a form of human amyloid precursor protein (APP) associated with familial AD was inserted into the genome, replacing the endogenous murine *App* gene. As a consequence, mutated APP will be expressed at endogenous levels. For the other transgenic lines, these mice were crossed with mice carrying a wild-type or mutated human gene encoding presenilin-1 (*PSEN1*). Measurable amyloid levels and plaque deposits are absent both in APPsl and PSwt mice, but develop in an age-dependent manner in PSmut mice (Figure 4.1, 4.2, 4.3). This demonstrates that the presence of mutated human APP at physiologic levels is not sufficient to cause amyloid pathology in mice. The additional presence of a mutated presenilin-1 is absolutely required to generate detectable levels of amyloid and its deposition in plaques, which is in agreement with the observation that PS-1 mutations cause the majority of familial cases of AD.

In our mice, amyloid deposits are observed to occur in a pattern resembling that seen in humans suffering from AD, although the density of amyloid plaques might be slightly lower than that seen in humans (Köhler et al., 2005).

Overall, amyloid pathology in PSmut mice can be considered to closely resemble that seen in the human disease. The other transgenic lines, especially the PSwt mice, are an excellent control group, because they differ from PSmut mice only in the one mutation induced into the gene encoding PS-1.

4.2.1.2. Models described in the literature

Many studies in transgenic models of brain amyloid mismetabolism employed one of the first available mouse lines, termed Tg2576. This line expresses human APP with the "Swedish" double mutation (K670N/M671L) under control of the hamster prion promoter, causing fivefold overexpression (Hsiao et al., 1996). Compared to this often used amyloid mouse, amyloid generation and deposition in PSmut mice is delayed and slightly less prominent (Köhler et al., 2005).

Another frequently employed model for amyloid deposition is the PDAPP mouse. These mice are overexpressing the "London" mutation (V717F) of APP under the strong platelet-derived growth factor promoter, causing more than 10-fold increased expression of APP (Games et al., 1995). These mice develop amyloid pathology as early as 4 months of age. The area in brain sections covered by amyloid plaques reaches up to 90%, which is dramatically more than the 4-8% seen in humans at advanced stages of the disease (Kraszpulski et al., 2001; Liu et al., 2004).

4.2.2. Cholinergic markers

4.2.2.1. Our results

The functional testing of the cholinergic system in our mice was done at an age when amyloid generation has been going on for several months (Table 4.1, Figure 4.3). The average age of PSmut mice used for the microdialysis study was significantly lower than that of mice from the other transgenic lines (Table 4.1). Due to organizational limitations, this was inevitable. Nevertheless, the conducted correlation analyses did not reveal age-dependent changes in any of the parameters analyzed (Figure 4.8). In addition, at their average age of 20 months, plaque pathology was already prominently

developed in these mice and there was ample time for amyloid to exert its purported synapto- and neurotoxic effects (Figure 4.3; Hardy, 2002; Hardy and Selkoe, 2002). It seems safe to assume that, although better age-matching of transgenic lines would have been desirable, no major confounding effect on the investigated observations should be expected from this discrepancy.

Baseline levels of ACh

Our results show that baseline levels of acetylcholine in the extracellular space of the dorsal hippocampus did not differ significantly between transgenic lines (Figure 4.4). This finding implies unchanged transmitter release, although it cannot be excluded that amyloid did cause damage in the PSmut mice, e.g. reduced number of synapses, which then was masked by compensatory changes, e.g. increased density of exocytotic vesicles in the remaining synapses. Determination of baseline levels alone is not able to discriminate between these possibilities (but see below).

Stimulation of ACh release

However, evidence against compensatory changes in PSmut mice was seen in the following results: Baseline acetylcholine output can be stimulated considerably with both behavioral and pharmacological challenges (Figure 4.5 and 4.6). The respective experiments, exposure of animals to a novel environment and blockade of presynaptic inhibitory autofeedback receptors by application of a muscarinic antagonist, here scopolamine, are commonly used for stimulation of septohippocampal fibers both in rats and mice (Köppen et al., 1997; Kopf et al., 2001; Tzavara et al., 2003) and the magnitude of the effect in the present study is comparable to that seen in the cited studies. The ability of these fibers to respond to such stimuli with increased output of acetylcholine is indicative of large functional reserves in the septohippocampal cholinergic neurons, which would not be expected if some compensatory mechanisms for an amyloid induced dysfunction would be in place even under baseline conditions.

High-affinity choline uptake

Additionally, high-affinity choline uptake, which is considered to be a marker for the firing rate of cholinergic neurons and an important marker of cholinergic nerve function (Kuhar and Murrin, 1978; Jope, 1979), is also unchanged between transgenic lines (Figure 4.7). High affinity choline uptake activity has repeatedly been shown to be increased in compensation for deficits in cholinergic neurotransmission. This included increased ACh hydrolysis (Erb et al., 2001), where increased HACU was the only obvious neurochemical adaptation; increase in HACU was also seen in mice haploinsufficient for the ACh-synthesizing enzyme, ChAT (Brandon et al., 2004) and in mice haploinsufficient for CHT-1, the protein conducting high affinity choline uptake itself (Ferguson et al., 2004). In addition, amyloid has inhibitory properties towards HACU *in vitro* in low nanomolar concentrations (Kar et al., 1998), so it would have been expected to observe an impact on this neurochemical process early on. Moreover, animals were investigated at a rather high age, well past mid age. It would have been expected that any defect in cholinergic neurochemistry would have become evident by that time.

Correlation with age

Finally, neither basal ACh release, nor open field nor scopolamine induced ACh release did show any negative correlation with age over the age span covered by the mice in our experiments, which would have been expected if there was ongoing deterioration of cholinergic function (Figure 4.8).

Conclusion

In conclusion, an overall lack of major functional compromise of septohippocampal neurons is firmly established in PSmut mice. The conclusion can be drawn that amyloidosis in the living animal can occur in the absence of overt cholinergic compromise.

4.2.2.2. Results reported in the literature

Tg2576 mice

As opposed to our functional approach, previous studies investigated several markers of the basal forebrain cholinergic system in different transgenic models and showed ambiguous results. In the Tg2576 mouse, a widely used model, two studies published in 2002 both demonstrated unchanged activity of ChAT and AChE in cortex and hippocampus. One study, investigating radioligand binding both to the vesicular ACh transporter, VAChT, and to the high-affinity choline uptake carrier, CHT-1, failed to observe differences in aged transgenic mice compared to controls (Gau et al., 2002). The other study, however, observed a significant 35% decrease in high-affinity choline uptake in hippocampus of 17 month old animals, but not in other brain regions or at younger ages (Apelt et al., 2002). Moreover, this study revealed decreased radioligand binding for the M_1 and M_2 receptor, the latter one only at advanced age. The M_1 results were interpreted as a sign of deleterious effects of soluble amyloid, because tissue levels of soluble amyloid were already detectable at the first observation at 8 months of age, but no deposition of amyloid in plaques had occurred by then. However, no data point was taken at an earlier age, thus leaving the possibility that a decreased M_1 receptor density was already present during development. In their discussion, the authors cite another study (Cha et al., 2001) that failed to find decreased muscarinic receptor binding in Tg2576 mice at 4 months using the unselective radioligand, [^{3}H]-quinuclidinyl benzilate. However, that same study also failed to detect such a deficit at 15 months of age, adding to the inconclusive body of evidence for cholinergic dysfunction in this transgenic line.

The study by Ikarashi and colleagues (2004) demonstrates reduced tissue levels of ACh in Tg2576 mice. The logical next step, performing microdialysis to assess its levels in the extracellular space, which is the relevant site for functional activity, was not done by this group, despite earlier publications utilizing this technique.

Microdialysis in Tg2576 mice was presented after our study had been published (Watanabe et al., 2005). The authors did not observe changes in baseline ACh levels in the ventral hippocampus at any age investigated, but reported an attenuation of

potassium-evoked ACh release in middle aged and old transgenic mice compared to controls. However, inspection of the poster at the Society for Neuroscience meeting in 2005 cast serious doubt on the validity of the authors' observations that could not be dispelled by the poster presenter. For one, baseline levels of ACh were reported to be around 40μM, more than 1000-fold higher than in our experiments. Although some discrepancy could be explained by different microdialysis probes, flow-rate, use of esterase inhibitor, etc., a difference of that magnitude seems impossible, although there is a chance that the stated concentration was a typographical error and should be 40nM instead; the poster presenter could not comment on that. Furthermore, the reported composition of the perfusion fluid for the potassium-depolarization indicated a highly hypertonic solution (potassium chloride was simply increased by 100mM without compensatory changes in sodium). Again, information whether this might be a typographical error was not available from the poster presenter. Additionally, the potassium induced increase in ACh release did not change in Tg2576 mice with age, but increased wildly from young (4-6 months) to middle-aged (9-11 months) controls and less dramatic in aged (14-16 months) control mice, for which no explanation was given either, thus generating significant differences at the middle-aged and aged groups of mice. One has to doubt the validity of these data; at least the study did not help to clarify whether there is a cholinergic deficit in these mice or not.

PDAPP mice

A microdialysis study in PDAPP mice was published very recently (Bales et al., 2006). At 4-6 months of age, these mice showed significantly lower baseline levels of ACh in hippocampus compared to controls. In addition, an almost twofold increase of hippocampal HACU with no change in CHT-1 mRNA was reported. Moreover, scopolamine-induced increases were dampened and open field-induced increases were accentuated in these mice, reminiscent of the results in the M_2/M_4-deficient mice investigated in the same lab (Tzavara et al., 2003). Unfortunately, the authors did not compare and contrast their results with the results of our study, which was published

almost two years earlier. However, considering the enormously exaggerated production of amyloid in the PDAPP mouse (which still fails to cause overt neurodegeneration or neurofibrillary tangles, Games et al., 1995), an impact of the sheer mass of amyloid on the central nervous system is no surprise. One has to question the rationale behind the use of such a drastic model in which one protein is overexpressed so drastically (and furthermore, one might also question the decision to test an experimental therapeutic, based only on its effectiveness in this model, in human trials; Schenk et al., 1999; McGeer and McGeer, 2003). The only valid conclusion, it seems, is that amyloid, no matter how much of it is present, will not cause major neurodegeneration or neurofibrillary tangles in mice.

CHAPTER V
ACHE-PROJECT – RESULTS AND DISCUSSION

5.1. Results

It was the aim of this project to assess central cholinergic function in genetically modified mice deficient for acetylcholinesterase (AChE) and to compare it to wild-type control mice (henceforth designated as KO – for knockout – and wt, respectively). The study focused on the dorsal hippocampus and employed predominantly microdialysis, but also synaptosomal [^{3}H] choline uptake.

5.1.1. General characteristics of animals

KO mice are smaller than their wt litter mates (Figure 3.1.). The mice undergoing surgeries had an average body weight of 17.0 ± 0.3g (KO, n=62) and 30.9 ± 0.7 (wt, n=68), respectively ($p=1.54*10^{-30}$). Age at time of probe implantation was 86 ± 5d (KO, n=62) and 169 ± 9d (wt, n=68), respectively ($p=1.33*10^{-12}$). Male and female mice were used in this study; the gender distribution was 31male/31female in KO and 47male/21female in wt.

5.1.2. Calibration of microdialysis probes

In vitro calibration was performed with five probes from different batches. Average *in vitro* recovery was 27.37 ± 1.74% for ACh and 35.93 ± 2.94% for Ch.

Extrapolation to zero flow was performed assuming a first-order relationship between dialysate concentrations and flow-rate. Theoretically, dialysate concentrations should approach zero with increasing flow-rate, thus the line-fitting was adjusted to reach a plateau of zero at time ∞. With these assumptions, the correlation coefficients for non-linear regression obtained for individual animals ranged from 0.9761 to 0.9923 for ACh and from 0.9740 to 0.9951 for Ch.

5.1.3. Basal values of acetylcholine and choline

Baseline concentrations of acetylcholine (ACh) in the microdialysate from dorsal hippocampus are presented in Figure 5.1.A. Average dialysate concentrations were 838 ± 51fmol/5µl (168nM) in KO (n=47) and 12.1 ± 1.1fmol/5µl (2.4nM) in wt (n=58) ($p=1.67*10^{-20}$).

Baseline dialysate concentrations of choline (Ch) were 3.91 ± 0.31pmol/5µl (0.78µM) in KO (n=47) and 7.73 ± 0.28pmol/5µl (1.55µM) in wt (n=40) ($p=3.03*10^{-14}$).

When these values are corrected for *in vitro* recovery, average concentrations of ACh are estimated to be 614nM in KO and 8.8nM in wt. Average concentrations of Ch are estimated to be 2.18µM in KO and 4.31µM in wt.

Extrapolation to zero flow in KO mice (Figure 5.1.B) estimates extracellular concentrations to be 674nM for ACh and 2.6µM for Ch.

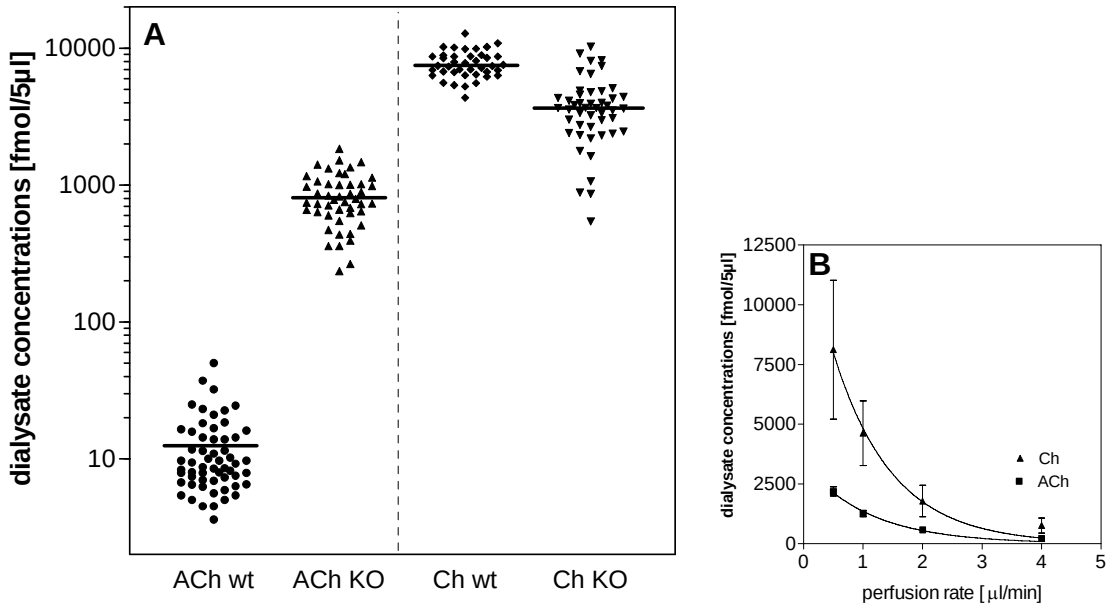


Figure 5.1: Baseline values of ACh and Ch in dialysate from mouse hippocampus. A) Baseline values at a perfusion rate of 1µl/min. Each point represents one individual mouse. Bars represent means. B) Extrapolation to zero flow. Each point represents the average of four individual mice

5.1.4. Effect of selective inhibitors of AChE

Application of the selective inhibitor of AChE, BW284c51, through the dialysis probe caused dose-dependent, up to 16-fold increases in extracellular levels of ACh in wild-type animals (Figure 5.2A). Similar results were obtained with another selective inhibitor of AChE, tolserine (Figure 5.2.C).

In contrast, neither compound was able to considerably alter levels of ACh in AChE-deficient mice (Figure 5.2.B,D).

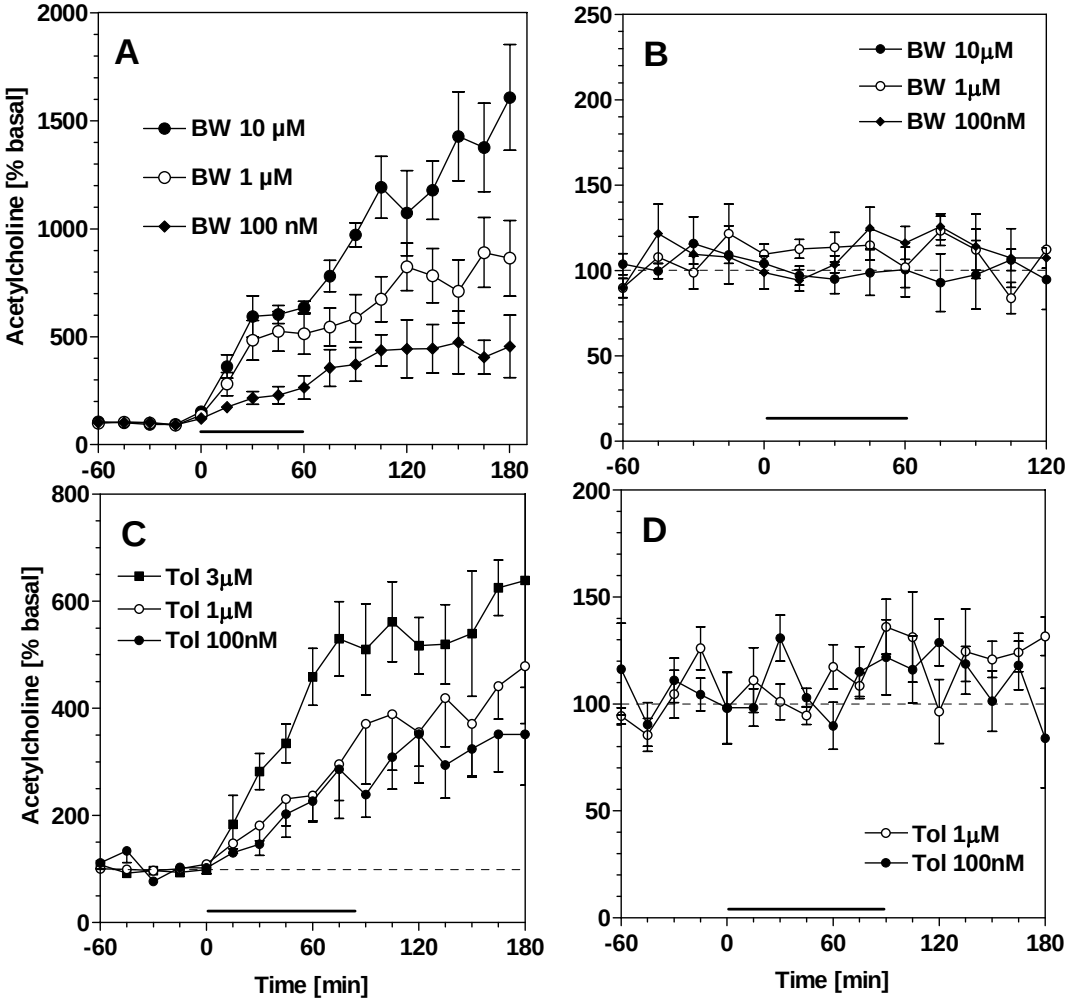


Figure 5.2: Effect of AChE inhibition on ACh levels. A) BW284c51 wt; B) BW284c51 KO mice; C) tolserine wt; D) tolserine KO. Inhibitors were added to perfusion fluid for the time indicated by horizontal bars. Both inhibitors cause concentration-dependent increases of ACh levels in wt, but fail to alter ACh levels in KO mice. N=4-8.

5.1.5. Effect of selective inhibitors of BChE

Application of the selective inhibitor of BChE, bambuterol, through the dialysis probe did not cause effects on either ACh or Ch levels in wild-type animals. (Figure 5.3.A) In contrast, bambuterol caused dose-dependent, up to five-fold increases in extracellular levels of ACh in KO mice, with concomitant decreases in extracellular levels of Ch to 50% of baseline (Figure 5.3.B). The hydrolysis product of bambuterol, the β_2 agonist terbutaline, did not affect ACh levels in KO mice at a concentration of 1μM (B)

Similarly, no effect was observed with another BChE-selective inhibitor, bisnorcymserine (BNC) in wild-type mice (Figure 5.3.C), whereas BNC increased extracellular levels of ACh in KO mice (Figure 5.3.D).

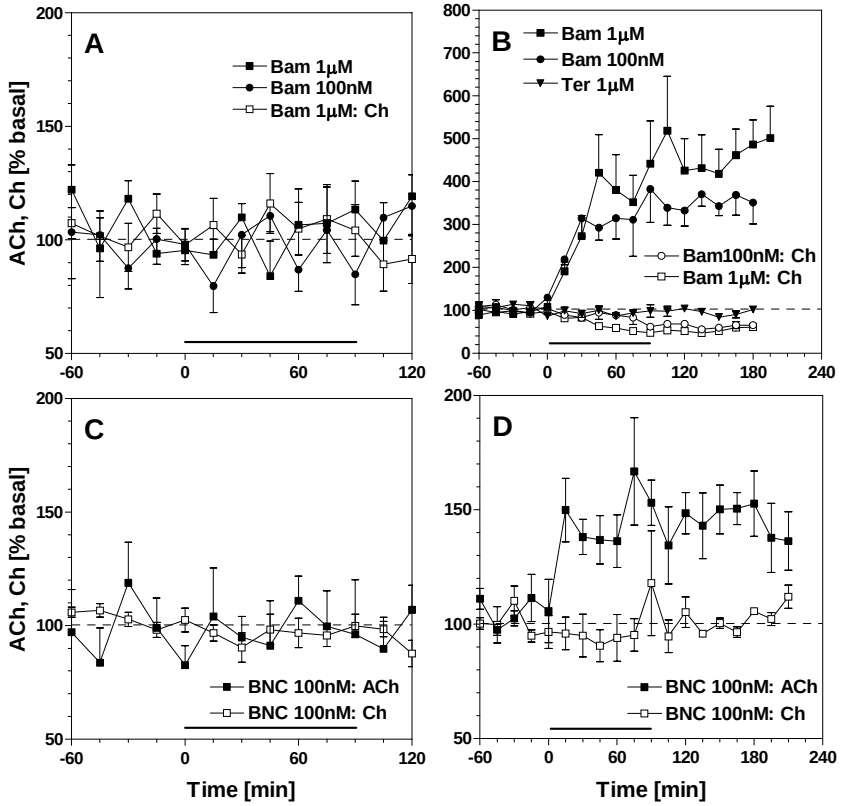


Figure 5.3: Effect of BChE inhibition on extracellular levels of ACh and Ch.
A) bambuterol wt; B) bambuterol KO; C) bisnorcymserine (BNC) wt; D) BNC KO.
Both inhibitors fail to alter levels of ACh in wt, but cause distinct elevations of ACh levels in KO mice. Terbutaline, the hydrolysis product of bambuterol, is ineffective (B). N=4-8.

5.1.6. Interruption of neurotransmission

When microdialysis probes were perfused with a calcium-free solution (Figure 5.4.A,B), levels of ACh in both wt and KO mice dropped immediately to 50% of baseline. Upon return to regular perfusate, levels immediately returned back to baseline.

When the blocker of voltage gated sodium channels, tetrodotoxin (TTX) was added to the perfusion fluid at a concentration of 1µM (Figure 5.4.C,D), ACh levels dropped rapidly to 10% of baseline in wt and 5% of baseline in KO mice and recovered slowly after cessation of TTX application.

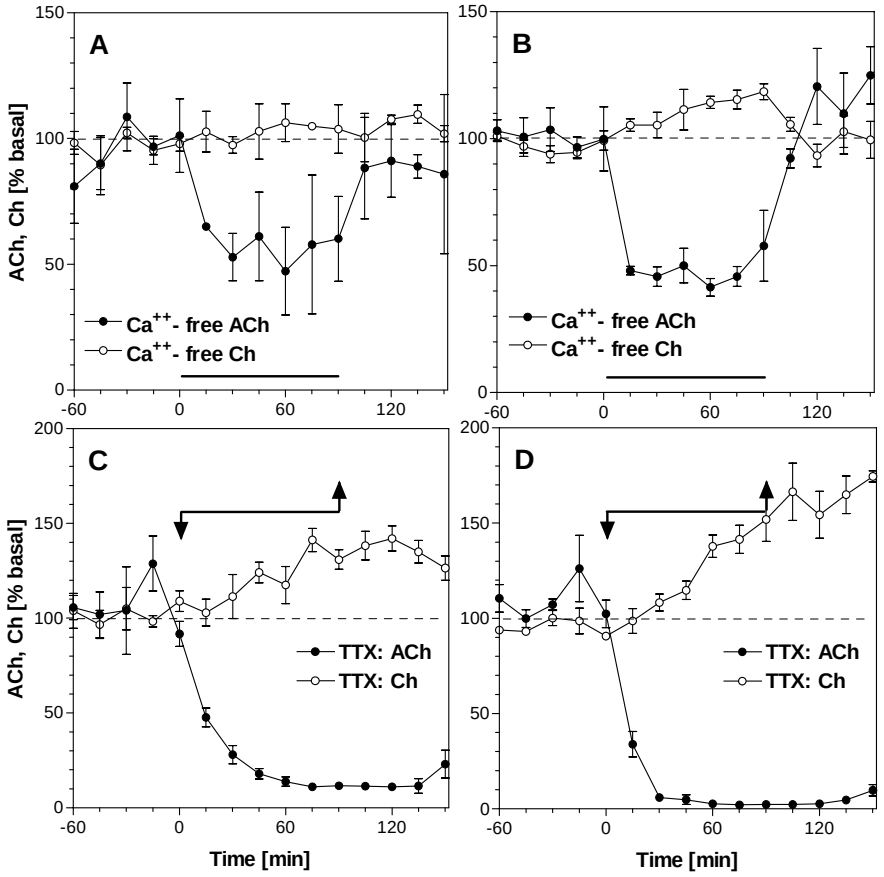


Figure 5.4: Effect of inhibition of neurotransmission.
A) Calcium-free in wt; B) Calcium-free in KO; C) TTX in wt; D) TTX in KO. Both treatments cause a drop of ACh levels which is comparable between genotypes. N=4-6. To enable detection of decreases of ACh-levels in wt mice, aCSF for these experiments contained the unselective cholinesterase inhibitor neostigmine at a concentration of 3µM

5.1.7. Synaptosomal choline uptake

As illustrated in Figure 5.5, total uptake of [^{3}H]-choline into corticohippocampal synaptoneurosomes accounted for 100.6 ± 12.5dpm/μg protein/5min in wt and 171.7 ± 29.8dpm/μg protein/5min in KO ($p<0.05$). Uptake in the presence of hemicholinium-3 at a concentration of 1μM, which is defined as high-affinity choline uptake, was 57.6 ± 9.7dpm/μg protein/5min in wt and 135.9 ± 25.0dpm/μg protein/5min in KO ($p<0.05$). Hemicholinium-insensitive uptake of [^{3}H]-choline was 43.0 ± 4.0dpm/μg protein/5min in wt and 35.8 ± 5.3dpm/μg protein/5min in KO ($p=0.38$). As a consequence, the contribution of the high affinity uptake to total [^{3}H]-choline uptake was 56.0 ± 2.5% in wt mice and 77.7 ± 2.8% in KO mice ($p<0.0001$).

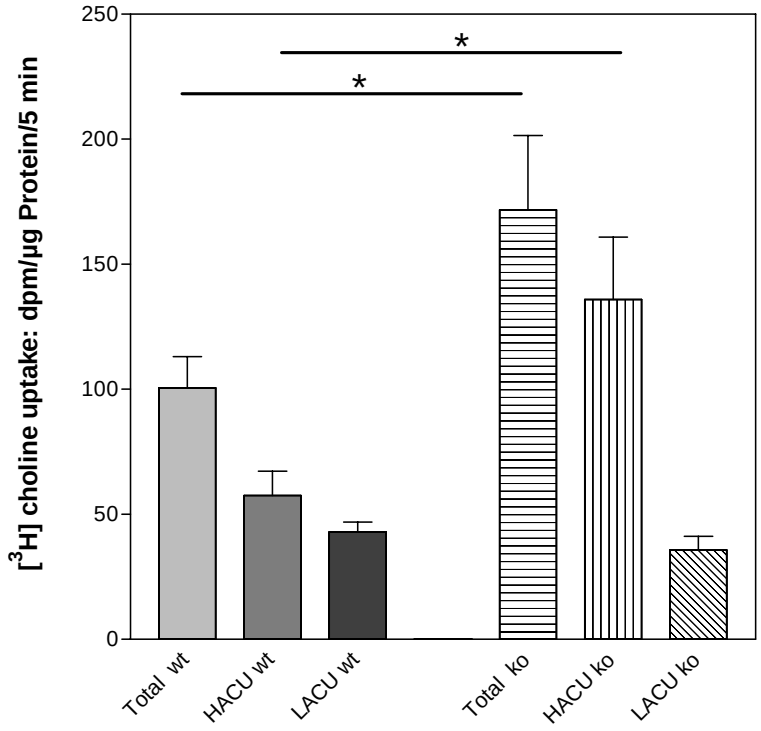


Figure 5.5: [^{3}H]choline uptake into corticohippocampal synaptosomes. Total uptake in KO mice is increased due to increased high-affinity choline uptake, whereas low-affinity uptake is indistinguishable between genotypes. N=6 (wt) and 11 (KO)

5.1.8. Blockade of high-affinity choline uptake *in vivo*

Hemicholinium-3 is a selective blocker of high-affinity choline uptake. As illustrated in Figure 5.6, perfusion of microdialysis probes with the selective inhibitor of high-affinity choline uptake, hemicholinium-3, at a concentration of 10μM caused a drop of extracellular levels of ACh to 40-50% of baseline in wt and 5-10% in KO. At the same time, Ch levels in wt increased by up to 30%, and doubled in KO.

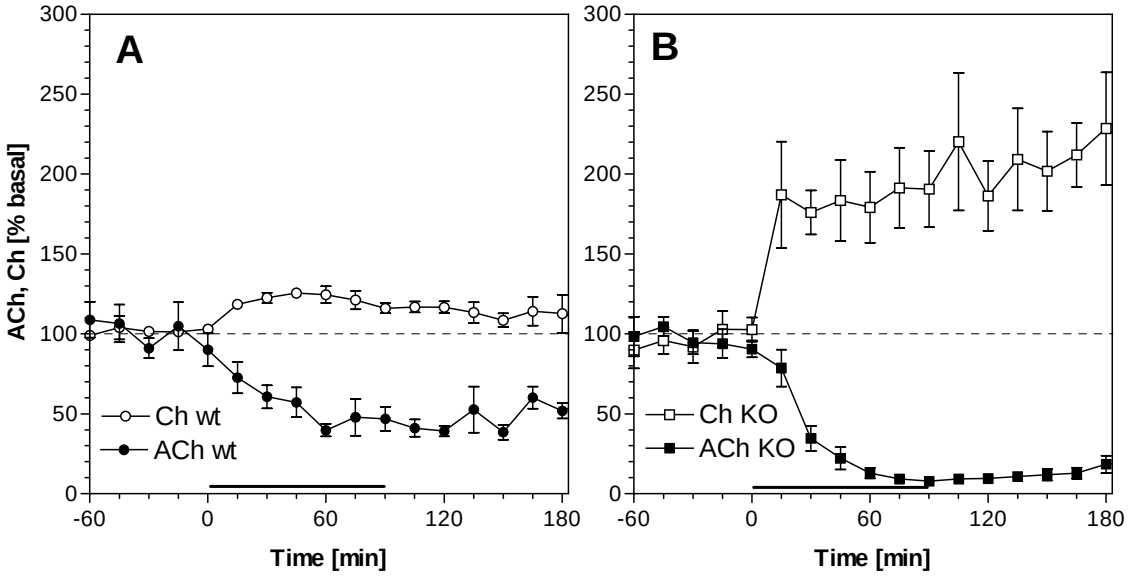


Figure 5.6: Inhibition of high-affinity choline uptake *in vivo*. The selective inhibitor, hemicholinium-3 (10μM), was added to the perfusion fluid for the time indicated. A) wt, B) KO mice. ACh levels in wt mice dropped to 40-50% of baseline, whereas they dropped to 5-10% of baseline in KO mice. N=8 (wt) and 5 (KO).

To facilitate observation of decreases in extracellular levels of ACh in wt mice, aCSF for these experiments contained the unselective cholinesterase inhibitor neostigmine at a concentration of 300nM.

5.1.9. Supplementation with choline

When choline chloride was injected intraperitoneally in a dose of 20mg/kg and a volume of 1ml/kg body weight, extracellular hippocampal levels of Ch increased shortly by 50% in both wt and KO, and returned to baseline immediately afterwards (Figure 5.7.A,B). Extracellular levels of ACh also increased by 50% in both wt and KO, returning to baseline immediately afterwards. Control injections with saline (1ml/kg) caused no changes in Ch, but 50% increases in ACh both in wt and KO (N=3; not illustrated).

Application of choline chloride in a concentration of 10µM, dissolved in the perfusion fluid, did not change extracellular levels of ACh in wt mice, but caused a more than twofold increase in KO (Figure 5.7.C,D).

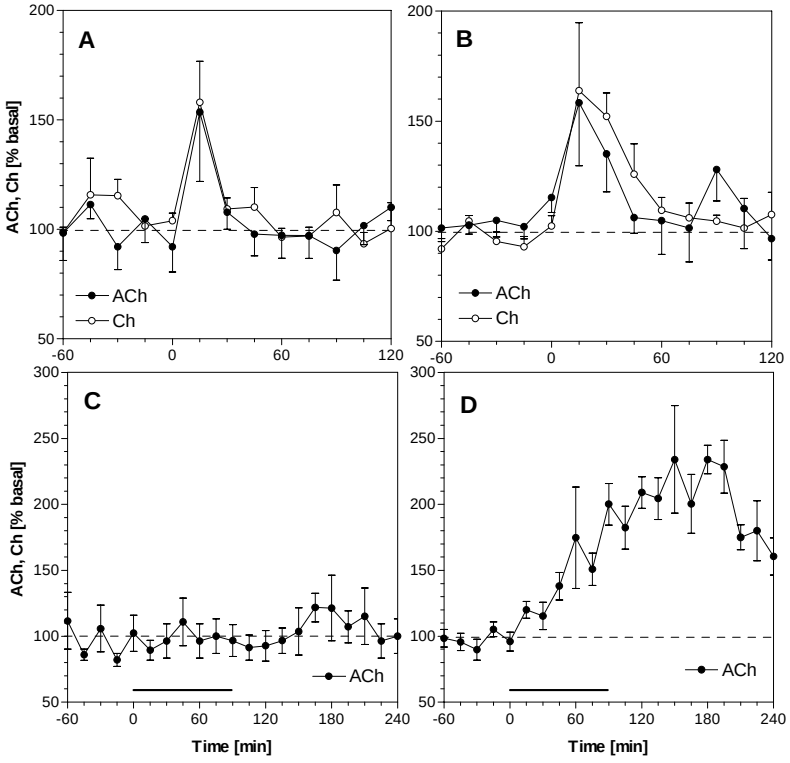


Figure 5.7: Supplementation with choline.
A) wt, 20mg/kg i.p. B) KO, 20mg/kg i.p. C) wt, 10µM locally, D) KO, 10µM locally
Systemic application of Ch caused short increases hippocampal Ch levels in either genotype. Local application of Ch caused increases in ACh levels only in KO mice. N=6-7(wt) and 4-5 (KO).

5.1.10. Blockade of presynaptic muscarinic receptors

Perfusion of microdialysis probes with the unselective muscarinic receptor blocker scopolamine at a concentration of 1μM caused an almost five-fold increase of ACh levels in wt mice and a 50% increase in KO mice (Figure 5.8). This increase was slowly reversible in both cases.

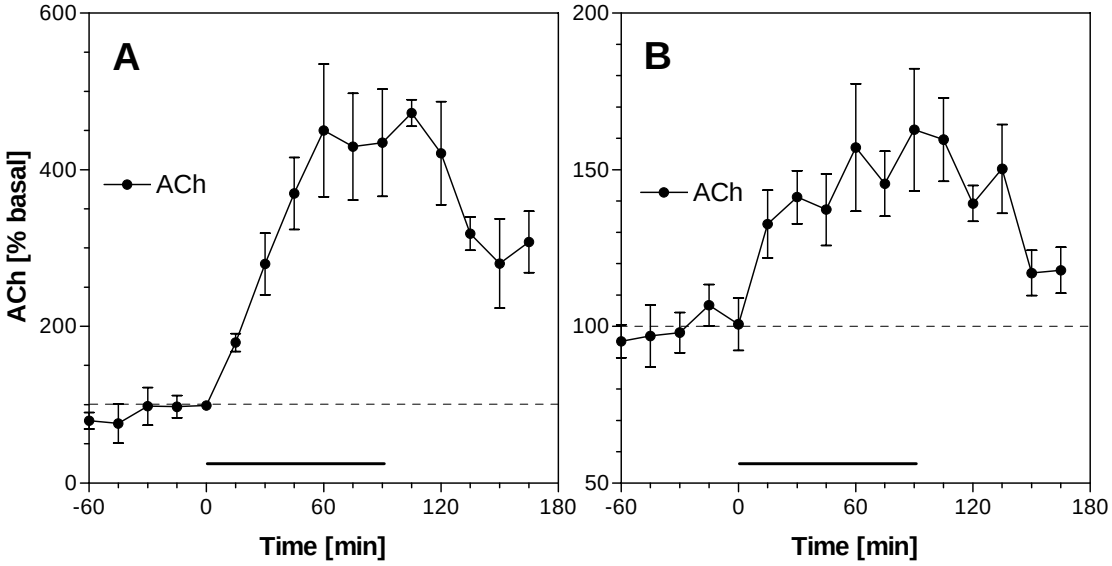


Figure 5.8: Stimulation of ACh release by scopolamine. A) wt, B) KO. Perfusion with scopolamine (1μM) for the time indicated increased ACh levels in wt mice four- to fivefold relative to baseline. In KO mice, the same treatment increased ACh levels by 50%. N=5 (wt) and 6 (KO).

To make increases in extracellular ACh levels detectable, aCSF for the wt experiments contained the selective AChE-inhibitor tolserine at a concentration of 100nM.

5.1.11 Correlations

Due to the fact that in KO mice, ACh and Ch responded to several treatments with mirror-like changes, statistical analysis was performed to check for significant correlations between baseline extracellular levels of ACh and Ch. Neither genotype showed significant correlations (wt: R^2=0.0013, p=0.79; KO: R^2=0.0001, p=0.95; data not shown).

Because KO mice were significantly younger than wt mice (see section 5.1.1.), statistical analysis of a possible correlation between baseline extracellular levels of ACh and age of animals showed no correlation for either wt (R^2=0.0068, p=0.54; data not shown) or KO (R^2=0.0115; p=0.44; Figure 5.9.A), even when the two oldest animals were omitted from the KO data set (R^2=0.0227; p=0.28; not illustrated).

Statistical analysis of a possible correlation between baseline extracellular levels of Ch and age of animals revealed no significant correlation in wt mice (R^2=0.0071, p=0.66; data not shown). In KO mice, a weak correlation was observed that failed to reach significance (R^2=0.0767; p=0.054; Figure 5.9.B). However, when the two oldest animals were omitted from the data set, this correlation reached significance (R^2=0.1406; p<0.01; not illustrated).

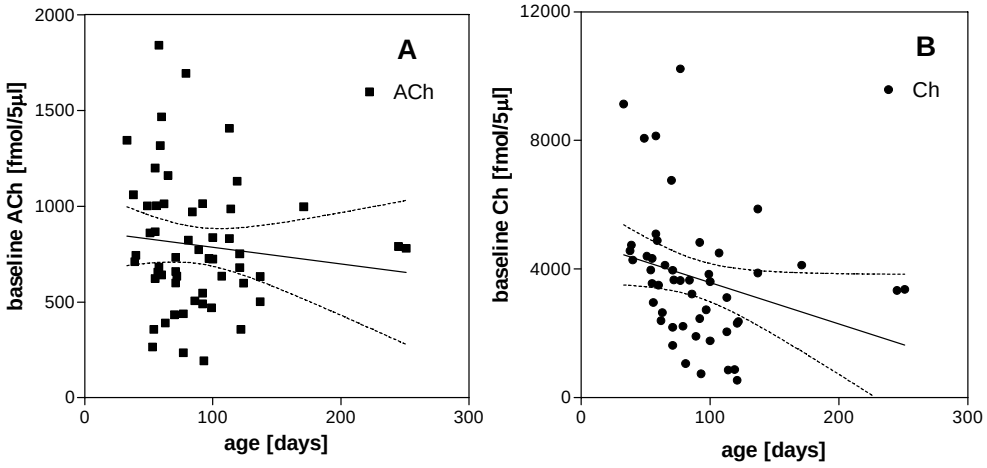


Figure 5.9: Correlation analysis in AChE-deficient mice. A) Baseline ACh vs. age. (R^2=0.0115; p=0.44); B) Baseline Ch vs. age (R^2=0.0767; p=0.054). Each point represents one individual mouse. Solid lines represent regression lines; dotted lines represent 95% confidence interval for regression lines.

5.2. Discussion

The second part of this thesis aimed to characterize the septohippocampal cholinergic system in mice deficient for acetylcholinesterase (AChE). The study was undertaken, because acute AChE-inhibition is lethal, and the survival of these mice leads one to suspect that compensatory mechanisms are in place to make their survival possible. With respect to human health, long-term inhibition of AChE is sustained during treatment for Alzheimer's disease (AD), where cholinesterase-inhibitors are still first line treatment. The study in AChE-deficient mice might give clues to possible changes such long term treatments might bring upon the cholinergic system.

5.2.1. Baseline levels of ACh

The first observation made in these animals was a vast increase of extracellular ACh levels in dorsal hippocampus, a finding that was expected in the absence of AChE, although the magnitude of the difference, 60-fold, was unexpected – selective AChE inhibition in control mice caused increases of up to 16-fold (Figure 5.1 and 5.2).

Both methods of calibration, *in vitro* recovery and extrapolation to zero flow, produced very similar (±10-20%) results for baseline concentrations of ACh and Ch in KO mice (614nM vs. 674nM for ACh, 2.18μM vs. 2.6μM for Ch). It seems appropriate to assume that the values obtained for wt mice with *in vitro* recovery (8.8nM ACh, 4.31μM Ch) are similar to those that would have been obtained by extrapolation to zero flow, which was not applied due to analytical limitations for the determination of ACh. The values from wt mice are comparable to results reported for rat and mouse extracellular space (Löffelholz, 1998; Laplante et al., 2004; Parikh and Sarter, 2006).

5.2.2. Role of AChE and BChE

The first idea coming to mind to explain increased levels of ACh at baseline is a lack of ACh hydrolysis, allowing the transmitter to build up in the extracellular space. However, evidence was found that there is still ACh hydrolysis occurring: In our hands,

selective inhibition of butyrylcholinesterase (BChE), another enzyme capable of ACh hydrolysis (Darvesh et al., 2003), by bambuterol (Tunek and Svensson, 1988) and bisnorcymserine (Yu et al., 1999) further increased extracellular levels of ACh (Figure 5.3.B,D). This is the first clear-cut *in vivo* observation indicating that BChE is able to hydrolyze ACh in the absence of AChE. However, due to the temporal resolution of microdialysis, no statement can be made to the speed of ACh hydrolysis. BChE *in vitro* is known to hydrolyze ACh less efficiently than AChE, so the possibility exists that transmitter cleavage *in vivo* in knockouts is considerably slower than in control mice. At the same time, the failure of BChE to modulate extracellular levels of ACh in the presence of AChE was demonstrated (Figure 5.3.A,C).

These findings, together with the increased baseline levels of ACh in AChE-deficient mice, are in line with the generally held assumption that AChE is the key-enzyme in ACh hydrolysis and that BChE does not play a significant role in this process under physiologic conditions (Soreq and Seidman, 2001; Darvesh et al., 2003).

5.2.3. Origin of extracellular ACh

Since ACh-hydrolysis in knockouts still occurred to a significant extent, increased release of ACh by depolarization-independent mechanisms was considered as a possibility to explain vastly elevated baseline levels (Descarries, 1998). To test for the dependence of extracellular ACh levels on neurotransmission, mice were dialyzed with a calcium-free perfusion fluid and with a fluid containing tetrodotoxin (TTX), a potent blocker of voltage gated sodium channels and thus a blocker of neurotransmission (Figure 5.4). As demonstrated previously for ACh and other neurotransmitters, both these approaches, as well as application of calcium channel blockers or perfusion with magnesium rich perfusate will decrease extracellular levels of ACh (Westerink et al., 1988; Williams et al., 1994; Kiewert et al., 2004). Our results show that ACh levels in knockouts and wild-type mice drop to 50% of baseline under calcium-free perfusion, whereas they drop to 5% (KO) and 10% (wt), respectively, of baseline under TTX, values

similar to those in the above cited literature. Therefore, the vast majority of extracellular ACh in knockouts originates from classical neuronal activity.

5.2.4. Turnover of ACh

However, to explain the vast amounts of extracellular ACh in knockouts compared to controls, it seemed necessary to postulate even an increased turnover of ACh in knockouts. Evidence for this was obtained by assessment of the high-affinity choline uptake (HACU) in synaptosomes, which was increased by 70% in KO compared to wt (Figure 5.5). HACU is considered to be kinetically coupled to ACh-synthesis. It is a recurrent observation that HACU activity, measured *ex vivo*, increases and decreases with the firing rate of cholinergic neurons at the time of sacrifice of the animal (Kuhar and Murrin, 1978; Jope, 1979). An explanation for this behavior might be the localization of CHT-1, the protein mediating HACU, on a subset of exocytotic vesicles (Ferguson et al., 2003). Our results of doubled HACU in KO mice support the assumption of an increased turnover of ACh. This is in agreement with previous observations in striatum of these mice, which demonstrated 60% increased plasma membrane localization of CHT-1, the protein mediating HACU (Volpicelli-Daley et al., 2003a). Moreover, as elaborated above, increased HACU is a frequently observed compensatory mechanism in basal forebrain cholinergic neurons, so alterations in this process were considered a prime candidate for compensatory changes.

5.2.5. Muscarinic autofeedback receptors

In the scenario sketched by the above experiments, i.e. increased input of ACh into the extracellular space (increased turnover) and vastly decreased output (decreased hydrolysis rate), the question arose whether there are additional compensatory changes in the cholinergic system. The studies by Volpicelli-Daley and colleagues (2003a,b) demonstrated that M_1, M_2, and M_4 receptors in striatum, cortex and hippocampus are largely internalized in knockouts. An experiment to assess the functional consequence of this changed localization would be to test the ability of muscarinic antagonists to

stimulate ACh release by blockade of the presynaptic inhibitory feedback receptors of the M_2 and M_4 type (Kopf et al., 2001; Tzavara et al., 2003). We chose to add scopolamine, an unselective blocker of all muscarinic receptors, to the perfusion fluid, to exclude peripheral effects and hypersensitive response in knockouts (Xie et al., 2000; Li et al., 2003). Our results (Figure 5.8) demonstrate that the relative increase in extracellular ACh levels in knockouts caused by scopolamine is less pronounced (+50%) compared to that seen in wild-type mice (+400%), which in turn was comparable to literature reports (Erb et al., 2001; Kopf et al., 2001). This lack of effect is comparable to observations of the same response after systemic application of scopolamine in mice deficient for one or both types of the autofeedback inhibitory receptors (Tzavara et al., 2003). However, the possibility exists that the low magnitude of the scopolamine effect in KO is due to a ceiling effect; inhibition of presynaptic muscarinic receptors might simply not be able to further stimulate ACh release, when baseline release is already as high as it is in KO mice. The observation of reduced scopolamine sensitivity parallels the literature reports on impaired activation of second messengers by muscarinic agonists in hippocampus of these mice (Volpicelli-Daley et al., 2003b) and on lacking sensitivity to the pharmacological effects of muscarinic agonists as well as hypersensitivity to muscarinic antagonists (Li et al., 2003). It can be concluded that function of the presynaptic autofeedback inhibitors is compromised in AChE deficient mice. On the other side, in the study by Tzavara and colleagues (2003), absence of M_2 and M_4 receptors caused an increase of baseline ACh levels in hippocampus. So, the functional compromise of these receptors in AChE knockouts might contribute to the increased baseline levels of extracellular ACh as well.

5.2.6. Extracellular levels of choline

At the same time as AChE-deficient mice display increased extracellular levels of ACh, their extracellular levels of Ch were decreased by 50%. An indication that these changes are interrelated came from observations that most treatments that increased or

decreased ACh in KO mice had the opposite effect on Ch, a trend that was absent or at least much less pronounced in wt mice (Figure 5.3, 5.4, 5.6).

In addition, it was observed that extracellular levels of Ch correlate negatively with age, at least when animals older than 200 days were excluded (Figure 5.9). This might indicate that the constant requirement of choline illustrated by the increased high-affinity choline uptake leads to a deprivation of the tissue with time.

When the experiments utilizing TTX-infusion are presented differently (Figure 5.10), it becomes apparent that cessation of neurotransmission returns the altered levels of both ACh and Ch almost back to wild-type levels, indicating that increased synthesis and release of ACh is the single major cause for the imbalance between ACh and Ch, as observed in the KO hippocampus.

Similar, but not as clear-cut results are obtained when the respective analysis is conducted with data from infusion of hemicholinium-3, the blocker of HACU (Figure 5.10). This might be due to incomplete inhibition of CHT-1 by the concentration of hemicholinium-3 that reaches the tissue across the dialysis membrane. However, this is unlikely, since microdialysis experiments in rat striatum have demonstrated that infusion with 100μM did not produce larger changes in ACh and Ch than infusion with 10μM, the concentration used in the study presented here (Ikarashi et al., 1997).

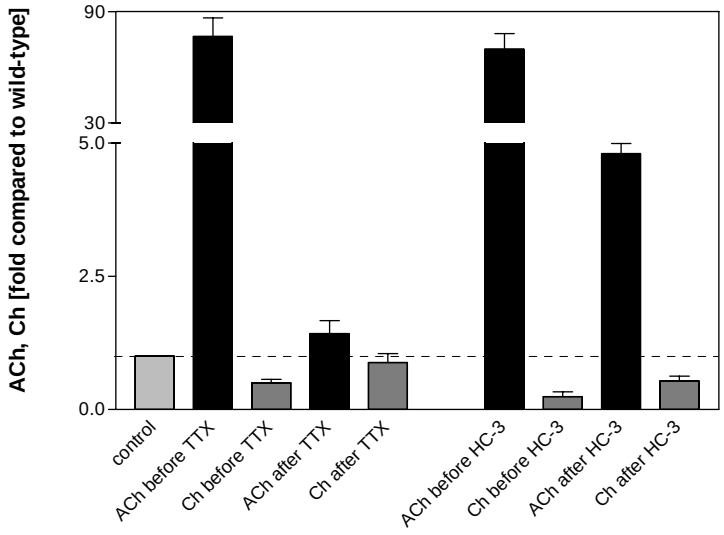


Figure 5.10: Effect of TTX and HC-3 infusion on the imbalance between ACh and Ch.

Alternatively, since ACh extracellular levels do not decline as strongly after hemicholinium-3 as they do after TTX, one might speculate that alternative sources of Ch can be employed for residual ACh synthesis and release, e.g. low-affinity choline uptake or phospholipase-mediated release of choline from choline-containing phospholipids (Klein, 2005).

5.2.7. Supplementation with choline

After establishing the impact of extracellular ACh levels on Ch levels, the interaction in the opposite way was investigated by supplying additional Ch.

Choline supplementation was performed in two different ways: By systemic injection (i.p.) of 20mg/kg choline chloride, and by addition of choline to the perfusion fluid in a concentration of 10μM.

Injection of choline chloride, both in KO and in wt, causes a short 50% increase in hippocampal extracellular levels of Ch, which rapidly dropped back to baseline. This finding was expected in wt, since extracellular Ch concentrations are known to be held in a tightly controlled concentration range (Löffelholz, 1998). Obtaining virtually identical results in KO mice indicates that the homeostatic processes responsible for removal of excess Ch from the extracellular space, i.e. cellular uptake and subsequent phosphorylation, are largely preserved in AChE-deficient mice.

Concomitant with the increase in extracellular Ch levels, ACh rises shortly to 150% of baseline in both mouse lines. Comparison with saline injections reveals that this ACh peak is due to arousal caused by the injection itself and not a consequence of the application of choline. Again, this is consistent with literature reports demonstrating an extraordinary sensitivity of hippocampal ACh levels to external stimuli like handling, startling, on/off-switching of light or presentation of palatable food (reviewed by Day et al., 2001).

Local application of Ch via the microdialysis probe was performed in a low concentration of 10μM. This is not much higher than the extracellular concentrations in the tissue, which are approximately 3-5μM (Figure 5.1; Klein et al., 2002; Parikh and

Sarter, 2006) and clearly below the concentrations required to stimulate α_7 nicotinic receptors (EC_{50}=1.5mM; Alkondon et al., 1997). Moreover, as indicated by the probe calibration by *in vitro* recovery, it would be expected that only approximately one third of surplus Ch present in the aCSF would permeate the dialysis membrane and reach the tissue. Thus, the expected increase in extracellular levels of Ch caused by this manipulation would be even more modest.

Wild-type mice respond to this treatment without any changes in extracellular levels of ACh, indicating that baseline levels of Ch are sufficient to maintain the necessary level of ACh synthesis and -release. In KO mice, however, even this small amount of additional Ch caused an increase of extracellular ACh levels to more than twofold, which was reversible upon cessation of Ch-perfusion. This result indicates that Ch availability becomes rate-limiting for the synthesis and release of ACh under the high-demand conditions present in those mice, i.e. lack of regeneration of Ch from ACh hydrolysis and increased cellular uptake. This is in agreement with earlier studies that showed an effect of choline application on stimulated, but not basal levels of extracellular ACh in hippocampus both in rat (Köppen et al., 1997) and mouse (Kopf et al., 2001).

An attempt was also made to decrease tissue Ch levels by intraperitoneal injection of the Ch-metabolizing enzyme, choline oxidase (Ikarashi et al., 1993, 1994, 2000). However, initial results (not shown) indicated that up to four hours after injection, extracellular levels of Ch and ACh in hippocampus were unaltered. No further experiments, e.g. sampling after later time points, were conducted.

5.2.8. Summary

Taken together, the following scenario could be developed to explain the investigated neurochemical alterations: Absence of AChE causes a decrease in the hydrolysis rate of ACh, although hydrolysis does not completely disappear, due to BChE activity. As a consequence, extracellular levels of ACh will rise (Figure5.1.). In the short term, this will stimulate muscarinic receptors, the postsynaptic ones and the presynaptic inhibitory autofeedback receptors, the latter effect causing an inhibition of ACh release. With time,

however, these autofeedback receptors will be internalized and less functional, allowing again an increase in ACh-release (Figure 5.8.). Alternatively, regular M_2 signaling might increase the fraction of CHT-1 remaining inside cells, and failure of M_2 signaling would allow CHT-1 to be increasingly localized at the cell membrane. However, this proposed mechanism is so far not supported by experimental data. The increased Ch demand for ACh synthesis will be supplied by the high-affinity choline uptake, whose activity is increased, partly due to an increase of the fraction of CHT-1 located at the plasma membrane (Figure 5.5; Volpicelli-Daley et al., 2003a). Increased uptake of Ch into cholinergic neurons, together with reduced regeneration of Ch from ACh hydrolysis, will cause extracellular levels of Ch to decline (Figure 5.1.). Extracellular levels of Ch are reduced to such an extent that they become rate-limiting for further release of ACh and thus limit the imbalance between ACh and Ch (Figure 5.7.). As demonstrated by the TTX experiments, this mechanism explains almost the entire imbalance between ACh and Ch (Figure 5.4. and 5.10.).

CHAPTER VI
GENERAL DISCUSSION

The data presented in this thesis has been obtained in different strains of mice. Since mouse strains are known to differ in brain anatomy (Franklin and Paxinos, 1997) as well as physiology or response to drugs (e.g. Kehr et al., 2001; Anonymous, 2005; Duysen and Lockridge, 2005), care should be taken not to generalize the findings obtained here and transfer them uncritically to other mouse strains or even species. Along the same line, most of the background presented in chapter II refers to data obtained predominantly from experiments and observations in rat, but also in guinea pig, non-human primates and human. Cohesive data on the respective characteristics in mouse, or even in a specific mouse strain, is barely available due to the fact that most neurophysiology and neuropharmacology work was performed in rat before the advent of transgenic mice (as an example: A PubMed search in April 2006, looking for "acetylcholine" and "hippocampus" and "microdialysis" and "mouse" yielded 21 hits; the same search for the species "rat" yielded 296 hits). However, it seems prudent to assume that most physiologic characteristics differ only slightly between the closely related rodent species of rat and mouse, especially those that were confirmed to be similar in those other organisms mentioned above.

6.1. Methodological discussion

6.1.1. Microdialysis

Microdialysis is the method of choice to obtain samples that reflect the composition of the extracellular space (Westerink, 1995; de Lange et al., 1997; Day et al, 2001). In fact, for acetylcholine, there is no other assay available than sampling in the tissue and subsequent analysis.

6.1.1.1. Alternatives to microdialysis

Microelectrodes for choline became available recently, and it is claimed that, with appropriate control experiments, also ACh can be determined with these electrodes (Parikh et al., 2004). However, uncertainties about true baseline concentrations of analytes and the fact that so far only one lab has published results obtained with these electrodes lessens the enthusiasm to use those. Moreover, the detection limit has been reported as approx. 200nM, thus, the method is not sensitive enough to determine ACh in the concentration range required for our studies (Parikh and Sarter, 2006). Finally, the size of these electrodes, which were so far used only in rat brain, seems to be too large for useful experiments in mouse brain.

Non-invasive approaches, including magnetic resonance imaging (MRI), are not a viable alternative for the experiments presented here. First, the spatial resolution of these approaches is still limited; Brockmann et al. (2006) report a resolution of 120x120μm. Second, no discrimination between intracellular and extracellular compartments can be made, since both contribute to the signals recorded by this technique (de Lange et al., 1997; Klein, 2000).

6.1.1.2. Limitations of microdialysis

In microdialysis, limiting factors for the recovery of extracellular components in the dialysate are solubility and molecular weight of the compound of interest and the cut-off of the dialysis membrane, permeability across the membrane, membrane exchange length and perfusion flow rate. Compounds of our interest, acetylcholine (ACh) and choline (Ch) were repeatedly measured after microdialysis experiments (reviewed by Day et al., 2001). Both are highly water soluble, and the molecular weight of the permeating species (146.2 and 104.2Da, respectively) is well below the molecular weight cut-off of the membranes used (10kDa). Experiments aiming at the recovery of genuine ACh and Ch from unstirred solutions at the flow rate used in the animal experiments, i.e. 1μl/min, showed a prominent dialysate recovery of 27 and 36%, respectively, of the original concentration of analytes. Effects after pharmacological treatments were rapidly

reflected in dialysate concentrations, leading to assume that permeation across the membrane was rapid. One literature example of a compound with good physical characteristics, but limited membrane permeability is serotonin (de Lange et al., 1997). When dialyzed with the same membranes as used here, changes in serotonin concentrations in the dialysate after pharmacologic challenge were delayed and less pronounced than those obtained with other membranes, whereas no such complication was observed with ACh.

Another concern with microdialysis is the tissue damage caused by probe implantation. It is known that a disruption of the blood-brain barrier occurs during probe implantation. However, the barrier function for low molecular weight compounds has been reported to be restored after some hours, a factor taken into consideration by allowing the animals to recover over night after probe implantation (de Lange et al., 1997). Reactive gliosis, covering the dialysis membrane, becomes prominent 48-72 hours after probe insertion (Westerink, 1995; Day et al., 2001). Experiments were performed on the first and second day after probe implantation, so no major interference from this process is expected. This is also consistent with literature recommendations on the conduction of microdialysis experiments to avoid both early tissue damage and delayed tissue response (Day et al., 2001).

Although it has to be acknowledged that microdialysis is performed in traumatized tissue, the responses seen in neurotransmitter levels are those expected to occur after given stimulations. The conclusion seems valid to assume close to normal transmitter neurochemistry in the vicinity of the microdialysis probe.

6.1.1.3. Advantages of microdialysis

Microdialysis carries one major advantage over other approaches: It allows for local application of treatments through the dialysis probe. This feature was used extensively for the studies presented here, especially for the AChE-project. AChE-deficient mice would likely have succumbed to the effects of several compounds used in this study after systemic application, as evidenced by their hypersensitivity to bambuterol and atropine

(Xie et al., 2000; Li et al., 2003). Moreover, local application of compounds allows for narrowing down the site of action of those treatments, which is near impossible with systemic application. When microdialysis is used for the local application of drugs, only the drug concentration in the perfusate is known. The concentration of drug that will cross the membrane and act in the tissue will not only depend on solubility and membrane permeability, but also on its kinetic behavior in the extracellular space, its distribution between intra- and extracellular compartments and its removal from brain interstitial fluid into blood. A rough estimate suggests that compounds with a molecular weight of <500Da, such as the pharmacological tools used in this study, will reach tissue concentrations equivalent to 10% of the concentration of drug present in the perfusate.

6.1.1.4. Other considerations

Anesthesia will have an impact on brain neurochemistry. This was taken into account in two ways. First, during the sampling experiments, animals were conscious, awake or asleep, according to their biorhythm, and allowed to move freely. Second, to exclude long lasting effects from the implantation surgery, volatile anesthetics (halothane or isoflurane, nitrous oxide) were used. As opposed to injection anesthetics, it is safe to assume that most of these volatile drugs would be out of the animals' bodies by the time of the experiments.

Other investigators have reported problems with the attachment of dialysis probes to the skull. Kehr and colleagues (2001) reported that the dental cement probe supports frequently got loose in their mice (129/sv strain and galanin-transgenics in the same background, the same strain as in our AChE study); they chose to anchor the cement with the help of screws inserted into the skull. Since we did not lose more than an occasional animal (1-2%) due to a loose probe support and since insertion of a screw into the delicate mouse skull seems to be a very sensitive and potentially trouble evoking task, we chose not to follow this literature example.

With microdialysis, there has been a discussion on the best way to illustrate the changes of transmitter levels after stimulation, either by displaying the absolute dialysate

concentrations, or the changes relative to baseline (e.g. Day et al., 2001). This is of particular importance when two groups of animals are compared and their baseline levels differ slightly. Just this is the case in the first part of this study (Figure 4.5. and 4.6.). Both ways of representation of the data have been given here, revealing quantitative, but not qualitative differences. In the second part of this thesis, however, only relative changes were given. This was due to the immense differences in baseline levels between animal groups. In fact, the baseline fluctuation in the AChE-deficient mice is larger than the largest increase in ACh levels in control mice, making it unreasonable to compare absolute changes between these groups.

6.1.2. Analysis of microdialysis samples

Microdialysis typically produces small quantities (10-30µl) of dialysate per aliquot. Highly sensitive methods are necessary to analyze compounds of interest with such limited volumes. For ACh and Ch, a microbore high-performance liquid chromatographic (HPLC) method with enzymatic cleavage of analytes and electrochemical detection of resulting hydrogen peroxide has been developed and used successfully in numerous microdialysis studies by other investigators (Kehr et al., 1998; Chang et al., 2006) and by our lab (Erb et al., 2001; Hilgert et al., 2003; Kiewert et al., 2004). Selectivity for ACh and Ch and removal of interfering compounds was obtained in two ways: First, separation of samples on a cation exchange column at a basic pH of 8.4 fractionated only those compounds that are cationic at this pH, such as quarternary compounds like ACh, Ch, betaine, carnitine, etc. Second, enzymatic hydrolysis employed highly specific enzymes, acetylcholinesterase and choline oxidase, which all but excludes the transformation of compounds other than ACh and Ch. Background noise of other redox active compounds at the platinum electrode was typically not a significant problem, because the process of microdialysis provides purified samples, as opposed to analysis of tissue homogenates.

We were able to run HPLC analysis with a low detection limit of 3-6fmol per 5µl injection volume, sensitive enough to measure the basal concentrations of ACh without

artificial enhancement by addition of cholinesterase inhibitors to the perfusate, as almost always done in microdialysis experiments aiming at measuring this analyte (Day et al., 2001; Chang et al., 2006). Thus, we can exclude any potential artifact resulting from this subtle pharmacologic manipulation and estimate true baseline values. For the first part of the study, the APP-project, we nevertheless chose to add the cholinesterase inhibitor neostigmine to the perfusion fluid in a low concentration of 1μM. This was done for several reasons, primarily because stimulation of hippocampal ACh release by scopolamine and open field, as performed in this study, is known to produce reliable and dose-dependent responses only in the presence of a cholinesterase inhibitor, thus the addition of neostigmine was necessary for these experiments (Chang et al., 2006). Furthermore, this procedure facilitated routine sample analysis, since optimum performance of the HPLC-system is not a given and often needs to be regained by intensive technical care.

6.1.3. High-affinity choline uptake assay

Synaptosomes are small presynaptic vesicles that are formed during tissue homogenization by spontaneous resealing (Whittaker et al., 1964). They have been used in numerous studies aiming at elucidating the physiology of presynaptic events. Moreover, many of the initial studies investigating choline uptake and acetylcholine turnover utilized this system (reviewed in Kuhar and Murrin, 1978; Jope, 1979). Alternative approaches would have been uptake measurements in brain slices or in neuronal/neuron-like cell culture. Cell culture was not chosen due to the lack of persistent release of ACh from cultured neurons. The choice to take freshly made tissue preparations allowed us to perform choline uptake assays in tissues from the same population of animals that had undergone the microdialysis experiments. Brain slices would have had no advantage over synaptosomes; they also would have been prepared acutely and would suffer from some degree of trauma due to preparation.

6.2. Implications for Alzheimer's disease

The results obtained in the projects presented here have bearing for our understanding of the pathophysiology of Alzheimer's disease (AD) and for possible long-term effects of current pharmacotherapy utilized for alleviation of symptoms in this disease.

6.2.1. Pathobiology

As sketched in chapter II, the etiology of sporadic cases of AD is still unknown. Hereditary cases have been linked to mutations in the genes encoding presenilin-1, but also *APP* and other genes, and susceptibility for the sporadic form is associated with the Apo E genotype. The connection between these genetic observations and the neurochemical deficits observed in the disease, where amongst other neurons, predominantly cholinergic fibers originating in the basal forebrain degenerate heavily, is not immediately evident. Starting from the mismetabolism of APP, induced by mutations in the protein itself or in presenilin-1, a component of the γ-secretase complex, or due to unknown factors in the sporadic forms of the disease, overt amounts of the amyloid β peptide are formed. Amyloid has been demonstrated to be synapto- and neurotoxic in cell culture and in *in vivo* models (Mattson, 1997; Hardy and Selkoe, 2002; Selkoe, 2002). Thus the amyloid hypothesis of AD has been formulated which puts amyloid at the core of the pathophysiological processes in the disease. However, no compelling *in vivo* evidence has been provided so far that would link amyloid to the two other major hallmarks of the disease, neurofibrillary tangles due to hyperphosphorylation of the protein tau, and neurodegeneration.

The first project presented here aimed at investigating the interrelationship between amyloid mismetabolism and cholinergic function. Although not the first study addressing this point (cf. Cha et al., 2001; Apelt et al., 2002; Gau et al., 2002 and other studies), it was the first major approach which investigated several functional aspects of the basal forebrain cholinergic system in an animal model of central amyloidosis, using extracellular levels of ACh as endpoint. Our results demonstrate that amyloidosis,

occurring at a degree similar to that seen in AD patients, can occur without causing cholinergic dysfunction. Recent studies in mouse models of a more drastic progression of amyloidosis, using similar approaches as we did, have demonstrated functional cholinergic deficits, thus generating conflicting data to ours (Watanabe et al., 2005; Bales et al., 2006). Methodological uncertainties about one of these studies have been mentioned above. A major reason for the observed discrepancy might be the differing degree of amyloid generation and disposition in the mouse strains under investigation. However, it should be kept in mind that overexpression of APP is a model to generate amyloid pathology in mice, which do not naturally develop amyloidosis. In contrast, APP expression in diseased humans is on baseline levels. In addition, most of the mutations responsible for familial forms of AD occur in the gene encoding presenilin-1, part of the γ-secretase complex, not in *app*, indicating that altered cleavage of APP is a major driving force for the development of increased amyloid burden. This is mirrored in our mouse model, where mutated APP is expressed at baseline levels, and the co-expression of a mutated presenilin-1 is required to cause amyloid pathology (PSwt vs. PSmut mice; Figure 4.1.). This implies that the animal model chosen by us more closely mirrors the human disease. Taken together, it becomes clear that cholinergic dysfunction is not a necessary consequence in mouse models of altered amyloid metabolism. This confirms earlier notions that mice with excessive amyloid production are not a good model of AD. In fact, AD encompasses much more than just amyloid metabolism (Schwab et al., 2004). In consequence, effects of potential treatments for the human disease that have proven useful to lower amyloid burden in these models must also be tested in other models to further evaluate their action. Efficacy in amyloid models can not substitute for testing on other parameters, for example neurodegeneration, memory function and learning behavior.

6.2.2. Cholinergic Treatment

The first treatment strategies devised for AD targeted the cholinergic deficit, the first clearly characterized and consistent pathophysiologic marker in AD (Bartus et al., 1982).

To date, four FDA-approved cholinesterase inhibitors are used to this end: Tacrine (Cognex®), donepezil (Aricept®), rivastigmine (Exelon®) and galantamine (Razadyne®; Reminyl® in Europe). Additionally, the N-methyl-D-aspartate (NMDA)-receptor channel blocker memantine (Namenda®; Ebixa® and Axura® in Europe) has been approved fairly recently, the first drug which does not primarily target cholinergic dysfunction, but the neurodegenerative process. Other treatment strategies are under investigation, including interruption of amyloid generation (α-secretase stimulation, β- and γ-secretase inhibition), increased clearance of amyloid (vaccination approaches), antioxidative, anti-inflammatory, antiapoptotic agents, interference with cholesterol homeostasis and neurotrophic treatments, including gene therapy with NGF-encoding vectors (Mattson, 2004; Pietrzik and Behl, 2005).

Cholinergic enhancement in AD was developed as a treatment strategy in analogy to the successful supplementation of the dopamine precursor, L-DOPA, in Parkinson's disease. It should be stated, however, that simply replenishing the amount of extracellular ACh will not necessarily compensate for the loss of controlled, physiologic stimulation of cholinergic receptors in cortex and hippocampus and can only be a patch to diminish the problem, not its solution. On the other hand, whichever additional treatment options might find their way to clinical use, AD treatment without an attempt to correct the cholinergic problem is bound to be very limited in its effectiveness.

It is acknowledged that genetic ablation of AChE is not the same as degeneration of cholinergic fibers with subsequent loss of this enzyme and that consequently our model will not mimic all the characteristics of the diseased brain. In particular, a complete loss of AChE activity in the brain is not expected to occur, neither by the disease process, nor by the application of an inhibitor; maximum inhibition of brain AChE with clinical doses of approved drugs is estimated to be not more than 50-60% (Giacobini, 2000).

Our results indicate that the use of cholinesterase inhibitors will not simply elevate synaptic concentrations of ACh by blocking its hydrolysis. Rather, long-term effects of such cholinergic enhancement on the cholinergic system, although not thoroughly investigated yet, have to be expected. The first finding, BChE's capability in the absence

of AChE to hydrolyze ACh and control its extracellular levels *in vivo*, seems to be of particular importance to current AD pharmacotherapy, because AChE activity in AD brain declines by up to 85% in severe cases, whereas BChE activity increases to some extent, shifting the AChE/BChE-ratio from around 0.5 to up to 11 (Giacobini, 2000).

The currently approved cholinesterase inhibitors can be distinguished according to their selectivity for any of the esterases: Donepezil and galanthamine are selective inhibitors of AChE, whereas rivastigmine inhibits both esterases with similar potency. Tacrine, which is barely used anymore due to its hepatotoxicity and due to its inconvenient four times a day dosing regimen, is slightly selective for BChE (Giacobini 2000). In the light of our experiments, it seems useful to target both cholinesterases to achieve maximum inhibition of ACh breakdown and thus maximum cholinergic stimulation. At late stages of the disease, it might even be prudent to target BChE selectively, since AChE inhibition in the gastrointestinal tract is the major reason for adverse drug effects observed with cholinesterase inhibitors, leading to intestinal cramps, diarrhea, and nausea and vomiting in a significant portion of patients. These side effects are severe enough to jeopardize patient compliance or even cause the patients/caregivers to discontinue therapy and prevent dosages that could probably further improve cognitive function. Selective BChE inhibition would not be expected to cause these symptoms beyond the level seen with placebo treatment, since peripheral AChE levels are unchanged in AD and BChE has no impact on ACh hydrolysis in the presence of AChE.

Increased cholinergic neurotransmission will invariably also stimulate presynaptic autoreceptors. The inhibitory autoreceptors (M_2/M_4) are considered to limit further increases in synaptic ACh levels once they are stimulated. Our results together with literature reports (Li et al., 2003; Volpicelli-Daley et al., 2003a, b) indicate that, although this might hold true in the short term, long term stimulation of these receptors will cause their internalization and a decrease of their activity. This, in turn, would further support the increase in extracellular levels of ACh intended by cholinesterase inhibitor treatment. However, considering that postsynaptic muscarinic receptors will also be functionally downregulated (Volpicelly-Daley, 2003b) and thus postsynaptic responses to elevated

levels of ACh will most likely be diminished, one should expect that at some point, it might not be possible to gain further therapeutic benefit by simply increasing the degree of cholinesterase inhibition. This is in agreement with bell-shaped dose effect curves obtained in human trials with some, but not all cholinesterase inhibitors (reviewed by Giacobini, 2000). These studies also state that maximum cognitive improvement is obtained when cholinesterases in red blood cells or plasma (as surrogates for the non-accessible brain enzymes) are inhibited by about 30-60%.

At the same time, the observation that ACh levels in the absence of AChE can be further increased by choline supplementation, could be perceived as an additional therapeutic approach for AD. However, this effect was only seen after local application of choline, not after systemic treatment, although the effects of long-term fortification of the mouse diet with choline was not investigated. Nevertheless, trials in human failed to demonstrate a benefit of choline (or choline precursor) supplementation on cognitive function in AD patients (Bartus et al., 1982).

6.3. Outlook

6.3.1. Amyloid transgenic mice

It would be helpful to be able to closely compare the results obtained in different transgenic lines developing amyloidosis. To that end, additional studies in our mouse lines, including muscarinic receptor binding and quantification of high-affinity choline uptake sites, are desirable. Additionally, the capability of postsynaptic receptors to convey their stimulation to a second messenger response would be highly desirable.

6.3.2. AChE-deficient mice

It would be helpful to determine the degree of AChE-deficiency required for the observed extracellular changes. Therefore, baseline extracellular levels of ACh and Ch in AChE-haplodeficient mice should be established.

In an effort to develop a model of age-related or sudden onset loss of AChE-activity, conditional AChE-deficient mice would be an interesting model.

Finally, to delineate the role of AChE in amyloid generation and deposition, it would be helpful to generate mice deficient (or haplodeficient) for AChE and simultaneously expressing mutated human APP and/or presenilin-1.

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