Selective lesion of the basal forebrain cholinergic system as a model for Alzheimer’s disease: a morphological analysis

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Ph.D. Thesis

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Publications involved in the present thesis


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AcSh</td>
<td>shell of nucleus accumbens</td>
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<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
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<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
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<tr>
<td>BFB</td>
<td>basal forebrain</td>
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<tr>
<td>CB</td>
<td>calbindin D-28k</td>
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<td>ChAT</td>
<td>choline-acetyltransferase</td>
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<tr>
<td>CR</td>
<td>calretinin</td>
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<tr>
<td>Cy</td>
<td>carbocyanine</td>
</tr>
<tr>
<td>GP</td>
<td>globus pallidus</td>
</tr>
<tr>
<td>HDB</td>
<td>horizontal diagonal band of Broca</td>
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<tr>
<td>MBN</td>
<td>magnocellular nucleus basalis/nucleus basalis of Meynert</td>
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<tr>
<td>MS</td>
<td>medial septum</td>
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<tr>
<td>NCa</td>
<td>caudate nucleus</td>
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<tr>
<td>NMERA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
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<tr>
<td>p75&lt;sup&gt;NTR&lt;/sup&gt;</td>
<td>low-affinity neurotrophin receptor p75</td>
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<tr>
<td>PB</td>
<td>phosphate buffer</td>
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<tr>
<td>PV</td>
<td>parvalbumin</td>
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<tr>
<td>Pu</td>
<td>putamen</td>
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<tr>
<td>SI</td>
<td>substantia innominata</td>
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<tr>
<td>SP</td>
<td>substance P</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-BSA</td>
<td>Tris-buffered saline containing 2% bovine serum albumin</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine-hydroxylase</td>
</tr>
<tr>
<td>VDB</td>
<td>vertical diagonal band of Broca</td>
</tr>
<tr>
<td>VP</td>
<td>ventral pallidum</td>
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INTRODUCTION

The basal forebrain cholinergic system and its implication in Alzheimer's disease (AD)

The basal forebrain (BFB) refers to a heterogeneous collection of structures located close to the ventro-medial surfaces of the cerebral hemispheres. It contains heterogeneous cell populations e.g. cholinergic, GABAergic and peptidergic neurons. Although the cholinergic corticopetal projection neurons represent only a small part of the whole neuron population in these areas, it has received particular attention. The cholinergic system is involved in the regulation of a broad range of brain functions (Rasmusson, 2000; Semba, 2000). In particular, tonic cholinergic input to the cerebral cortex and hippocampus contributes to learning processes and memory consolidation and retrieval (Everitt and Robbins, 1997; Sarter and Bruno, 1999; Rasmusson, 2000). In addition, cholinergic neurons of the BFB also participate in the control of attention (Everitt and Robbins, 1997) as well as in the sleep/wake cycle (Semba, 2000). Multiple levels of evidence from both human and animal studies indicate that damage to cholinergic projection systems correlates with the development of cognitive impairment (Schliebs, 1998; Berger-Sweeney et al., 2001). Moreover, one of the earliest pathological events in AD is thought to be the degeneration of cholinergic neurons of the basal forebrain (Davies et al. 1999; Whitehouse et al. 1982; Coyle et al. 1983). Indirect support for basal forebrain cholinergic system involvement in the cognitive deficits accompanying AD comes from considerable psychopharmacological evidence indicating that systemic administration of cholinergic receptor antagonists (e.g. scopolamine) interferes with the acquisition and performance of a variety of memory tasks in both rodents and non-human primates (Aigner 1993; Fibiger 1991). Support for this "cholinergic hypothesis" of cognitive impairment in AD also comes from the fact that drugs that potentiate central cholinergic function have thus far proven to be the most effective forms of therapeutic treatment against the disease. However, such cholinomimetic replacement strategies have only met with limited success (Giacobini, 2000) in reducing the cognitive deficits in AD, emphasizing the need for a better understanding of the way in which changes in central cholinergic systems are related to both AD symptomatology and disease progression. Beside the degeneration of cholinergic projection neurons in the basal forebrain nuclei, other morphological hallmarks of AD arise in the target cortical areas: neuritic plaques containing the highly toxic beta-amyloid and hiperphosphorylated protein-tau expressing neurons (e.g. Arendt et al. 1985). Moreover, beta-amyloid depositions are found within the basal forebrain nuclei that contain the cholinergic somata (e.g. Arendt et al., 1988). Numerous, but not all,
post-mortem studies have demonstrated that ChAT activity in many cortical areas of AD brains shows negative correlation with the density of amyloid plaques (Mountjoy et al. 1984). Moreover, there is a small (or insignificant) correlation between ChAT levels and density of amyloid plaques, whereas another study reported a significant association between tangle density and cholinergic neurodegeneration in many (but not all) cortical and hippocampal areas of the AD brain (Mountjoy et al. 1984; Zubenko et al. 1989 and Ransmayr et al. 1992). It is still highly elusive whether the neurotoxical beta-amyloid peptide causes the dysfunction and loss of cholinergic system, or vice versa: the cholinergic malfunction results in amyloidogenesis. Huge amount of experiments focused on the physiological effects of beta-amyloid administration (for review see Auld et al. 2002). Interestingly, cholinergic basal forebrain neurons were found to be selectively vulnerable to the neurotoxic effect of beta-amyloid peptides, meanwhile GABAergic neurons were relatively resistant (Harkany et al. 1995). The correlation between the amyloid-deposites and cholinergic malfunction were mostly approached in the aspect of in vivo application of synthetic beta-amyloid peptides.

The effect of cholinergic lesion on beta-amyloid plaque generation was not as firmly investigated as physiological effects of beta-amyloid administration. This niche in scientific investigation is caused by the lack of an appropriate model, which would exclusively mimic the loss of cholinergic neurons in basal forebrain on beta-amyloid expression in targeted areas. With fimbria/fornix transection not only cholinergic, but all non-cholinergic inputs arising from basal forebrain are damaged, moreover, ascending pathways from locus coeruleus (Harley et al. 1989), raphe nucleus (Assaf and Miller 1978) and the submammillary nucleus are also, at least in part, destroyed.

Development of general models for selectively demolishing the basal forebrain cholinergic system has been awaited till the beginning of the '90s, when R.G. Wiley (1992) reported the neural lesioning with the help of monoclonal antibodies coupled with ribosome-inactivating protein saporin. The target cell can internalize the specific antibody, directed against an extracellular epitope of a membrane protein, and the coupled saporin can block protein synthesis, which results in cell death. This type of immunolesion can be successfully used in elimination of BFB cholinergic neurons, since most cholinergic cells projecting to the cortex and hippocampus express exclusively the p75 low affinity neurotrophin receptor.

Rabbit as a model system for AD.

The highly toxic beta-amyloid peptides are derived by proteolytic processing of a larger amyloid precursor protein (APP). APP is highly conserved among mammalian species.
(Johnstone 1991), but experimental studies in rat are often hampered by the humble APP-processing in the amyloidogenic pathway and by the inability of rodent beta-amyloid peptides to form higher molecular aggregates such as soluble oligomers and insoluble beta-amyloid plaques, which are hallmarks of AD. Thus, there is need for in vitro and in vivo model systems that allow identification of factors that increase amyloidogenic APP processing and accelerate beta-amyloid plaque formation and testing the potency of pharmacological manipulations to ameliorate beta-amyloid load in brain. Transgenic mice that overexpress human APP containing AD-associated mutations (the so called Swedish mutation) that favor the amyloidogenic pathway of APP processing represent such a model (Holcomb et al. 1998). However, mutations of the APP gene are not frequent in AD and, therefore, the mechanisms of beta-amyloid plaque formation, the composition of beta-amyloid plaques, and the accompanying tissue response in brain of these animals may be different from that in AD. In contrast, rabbits express beta-amyloid peptides of the human sequence and appear to represent a more physiological model to examine the long-term effects of experimental manipulations on APP processing and beta-amyloid plaque formation in vivo.

There are several other reasons, which make the transgenic animal models inappropriate for modeling AD. For example, as a member of a mammalian order separated phylogenetically from most of other orders for about 70 millions of years, the rat and mice magnocellular basal forebrain complex differs in several cytological characteristics from non-related species. As already known, peculiarities in the expression of various proteins and additional neuronal constituents were also revealed in representatives of other orders such as primates and carnivores. Important species-specific differences were identified, e.g. the relative size of MS and VDB; appearance of septum pellucidum; topological organisation of the VP and the MBN/SI complex; and localization of the capsules interna, externa, and extrema. From a neurochemical point of view:

1) selective expression of the low-affinity neurotrophin receptor p75 (p75<sup>NTR</sup>) in cholinergic neurons of all mammalian species (Mufson et al., 1989, Maclean et al., 1997, Tremere et al., 2000);

2) the presence of calbindin D-28k (CB) in cholinergic neurons of primates, including humans (Chang and Kuo, 1991; Cote and Parent, 1992; Geula et al., 1993; Ichitani et al., 1993); and

3) coexpression of neuronal (constitutive) nitric oxide synthase (nNOS) in subsets of rat BFB cholinergic neurons (Schober et al., 1989; Brauer et al., 1991; Geula et al., 1993).
As a consequence, the generalization of findings or concepts drawn from structural and cytochemical analyses of the basal forebrain cholinergic system that are elaborated in only one mammalian species, hold the risk of misinterpretations of experimental data or of useless strategies. Thus, in our set of experiments beside the rat as the most conventional laboratory animal we used rabbit as a possible model for AD. Recent experimental data indicate that the rabbit brain might be in several respects appropriate for modeling multiple neurochemical characteristics of AD, inasmuch as the rabbit APP is identical with the human APP, and feeding with diets containing high cholesterol concentrations was shown to trigger the formation of senile-plaque-like beta-amyloid deposits in the rabbit cerebral cortex (Sparks et al., 1994, 1995, 2000).

Regarding to the cholinergic BFB lesion and its possible effect on amyloidogenesis, this thesis tries to answer three questions:

1. Are the cholinergic projection neurons selectively vulnerable to NMDA induced excitotoxicity?

Previously cholinergic BFB neurons were shown to be highly sensitive to excitotoxicity as was indicated by robust loss of their marker enzyme cholinergic acetyltransferase (ChAT) (Harkany et al. 2000, 2001b). In contrast, little is known about the reactivity of subpopulations of non-cholinergic basal forebrain neurons to neurotoxic insults. Although particular attention was directed towards the identifications of a role for calcium-binding proteins (CaBPs) in GABAergic neurons, that may serve as a potential means to rescue nerve cells from Ca$^{2+}$ overload associated with acute excitotoxicity (Mattson et. Al. 1991, D’Orlando et al. 2001) data are lacking as to whether acute excitotoxicity affects equally the cholinergic and non-cholinergic neuron populations in the BFB complex, or the CaBPs containing non-cholinergic neurons are protected.

In the first experiment (NMDA LESION IN RAT MBN) the number of cholinergic and non-cholinergic neurons were determined using multiple-labeling immunocytochemistry under control conditions as well as 4, 24, 48h after NMDA infusion in the rat MBN. Cholinergic neurons were identified by their immunoreactivity for ChAT. Neuron-specific nuclear protein (NeuN) was introduced as a pan-neuronal marker to visualize neuronal perikarya irrespective of their neurotransmitter contents in the MBN.
2. How comparable is the rabbit BFB system with human and higher mammalian species in neuroanatomical and neurochemical point of view?

In spite of the growing interest in using the rabbit for modeling cholinergic disfunction, detailed neuroanatomical studies on the organization of cholinergic components of the rabbit BFB are still missing. In fact, only a few neuroanatomical descriptions based on classical histological techniques (Winkler and Potter, 1911; Monnier and Gangloff, 1961; Urban and Richard, 1972) and immunohistochemical studies (McGeer et al., 1974; Kan et al., 1978; Chao et al., 1982; Baisden et al., 1984) have addressed the localization of cholinergic nuclei in rabbit brain. These studies fell short, however, because of either the only superficial localization of cholinergic nuclei or partly insufficient ChAT immunolabelling (as described in detail by Rossier, 1981). In the present thesis, therefore, sensitive immunoperoxidase labeling of ChAT and p75NTR were performed on serial sections spanning all major cholinergic BFB nuclei as well as cerebral cortical and dorsal hippocampal projection areas to map their structural organization and to measure the number and density of cholinergic nerve cells and of their cortical and hippocampal projections. Moreover, additional series of sections were double-labeled for ChAT and p75NTR, the calcium-binding proteins parvalbumin (PV), calretinin (CR), calbindin (CB), neuronal nitric oxide synthase (nNOS), tyrosine hydroxylase (TH), or substance P (SP) to identify the neurochemical heterogeneity and spatial relationships of cholinergic BFB neuron populations. Although in this part of the thesis the primary goal was to investigate the BFB cholinergic nuclei, the striatal regions were also studied, where large cholinergic interneurons exhibit some differences in their p75NTR expression in some animal species investigated to date (Kokaia et al., 1998; Ferreira et al. 2001).

3. Is it possible to induce amyloid-plaque generation by selective lesion of cholinergic projection neurons?

Beside the well-known cholinergic cell markers (Choline-acetyltransferase, vesicular acetylcholine transporter), an additional cholinergic BFB marker the p75NTR was demonstrated in the rat, racoon, monkey and human BFB cholinergic projection neurons. This unique expression pattern of the p75NTR located on the cell surface equipped scientist to target selectively the cholinergic BFB neurons with the help of in vivo application of 192IgG monoclonal antibody, directed against the extracellular epitope of the rat p75NTR. For in vivo visualization of the cholinergic cell bodies, fluorochrome carbocyanine (Cy3) were coupled
with the antibody, and for elimination of the targeted cells, saporin were coupled to the antibody prior the \textit{in vivo} application. The internalized antibodies form clusters in secondary lysosomes (Kacza et al. 2000) and either cause cell death (saporin conjugated) (Wiley, 1992, Rossner et al. 1997) or unaffected the electrophysiological and pharmacological properties, but enable the cholinergic cells to be visualized \textit{ex vivo} brain slice preparation containing the medial septum-diagonal band region (Cy3 coupled) (Wu et al. 2000).

However, 192IgG conjugates recognize $p75^{NTR}$ only in the rat precluding their application in other mammalian species. In contrast, the monoclonal antibody ME20.4 raised against an extracellular epitope of the human $p75^{NTR}$ was demonstrated to crossreact with $p75^{NTR}$ also with rabbit, monkey, racoon, pig, dog and cat (Tremere et al. 2000).

Since our main goal was to eliminate only the cholinergic projection neurons from the rabbit BFB, first we verified the selectivity of the model-system by injecting the fluorochromated ME20.4 antibody into the lateral ventricles of the rabbit brain, then the possible colocalization of labeled neurons with cholinergic and non-cholinergic markers was studied. To investigate the effect of cholinergic denervation on the cortical and hippocampal amyloidogenesis, highly sensitive immunohistochemical methods were exploited, which was successfully used in the investigation of other, beta-amyloid plaque generating species (Hartig et al., 2000).
MATERIALS AND METHODS

NMDA LESION IN RAT MBN:

Young adult male Wistar rats (250-300g, n=12; n=4/group) were caged individually and kept on a normal laboratory diet and tap water ad lib. The rats were deeply anesthetized with isoflurane (2% in 70%N₂O/30% O₂; 1,5l/min flow rate) and their head position was secured in a stereotaxic frame. 1μl NMDA (60mM; Sigma) was injected in the right MBN (AP-1,5mm; L3,2mm; DV 6,5mm) at an infusion rate of 0,1μl/min. NMDA was freshly dissolved in PBS. As dissolving of NMDA considerably decreases the pH value of its vehicle, pH equivalent PBS (1μl; pH=4,0) was used as control and injected in the contralateral (left) MBN of each animal in a manner identical to the NMDA infusion procedure. Short-term effects of NMDA infusion on MBN neurons were studied after a post-lesion delay of 4, 24 and 48h.

Immunocytochemical procedures, quantification and statistical analysis

Fixation of the brains was carried out under deep sodium pentobarbital anesthesia by transcardial perfusion with 0,1M phosphate buffer (PB, pH7,4; 50ml) than with 350ml fixative composed of 4% paraformaldehyde in PB. Brains were postfixed for 2h in the same fixative and subsequently cryoprotected by overnight storage in 30% sucrose in PB at 4°C. Coronal sections were cut on a cryostat microtome at a 30μm thickness and collected in PBS containing 0,1% sodium azide. Immunofluorescence double labeling of ChAT and NeuN was performed as described in „Experiments with rabbit” section.

At least three sections spanning the intermediate MBN in each animal were used to investigate the effects of vehicle and NMDB infusions. The number of all immunoreactive neurons was determined in both the ipsilateral and contralateral MBN. Care was given not to include fragmented neuronal profiles. Total cell numbers for ChAT and NeuN were estimated using the disector formula. The degree of colocalization of ChAT and NeuN was determined in a semi-quantitative fashion. To this end, the double-labeled sections were employed and inspected using a Zeiss Axioplan fluorescence microscope equipped with appropriate double band pass filter (Zeiss, Jena, Germany). The effect of NMDA lesion on the number of neurons, as well as on the degree of colocalization of the cellular markers at the particular post-lesion time points was statistically evaluated using paired t-test (SPSS for Windows, SPSS Inc., Chicago, IL, USA). Significance was defined as an overall effect on the entire
MBN region sampled. p<0.05 was taken as indicative of statistical significance. Data were expressed as percentages and presented as means.

EXPERIMENTS WITH RABBITS

Preparation of Cy3-ME 20.4

Cy3-ME 20.4 was prepared by coupling the antibody with a chemically activated ester of Cy3 under reaction conditions proved to maintain the antigen-binding properties of ME 20.4. Four hundred micrograms of ME 20.4 (pure IgG; AB-N07, Advanced Targeting Systems, San Diego, CA, USA) were reacted with a monofunctional Cy3-N-hydroxysuccinimide ester (Antibody Cy3 Labeling Kit, Amersham Pharmacia Biotech, Freiburg, Germany) according to the instruction of the manufacturer at pH 9.3 for 1 h, but with doubled dye:protein ratio. Thereafter, the reaction mixture was dialyzed against phosphate-buffered saline (3 × 4 h) and the concentration of Cy3-ME 20.4 was adjusted to approximately 0.5 mg/ml prior its use for intracerebroventricular (i.c.v.) injections.

Surgical procedure

New Zealand white rabbits of either sex [n = 22 (7 for the neuroanatomical study, 5 for in vivo labeling and 10 for selective immunolesion experiments); 2.3–5.1 kg; 4–21 months of age] were used in this study. The rabbits were anesthetized with a mixture of ketamine (50 mg/kg; Exalgon, Merck, Hallbergmoos, Germany) and xylazine (4 mg/kg; Rompun, Bayer AG, Leverkusen, Germany). Both in immunolesion and in vivo labeling experiments heads of the rabbits were mounted in a stereotaxic frame. Injection co-ordinates were at AP = 0.0 mm, L = 2.2 mm and DV = 9.0/7.5 mm relative to bregma. Two animals received unilateral i.c.v. injections, and three animals were injected bilaterally with 16 µl Cy3-ME 20.4 at an infusion speed of 0.5 µl/min with a Hamilton microsyringe (Hamilton, Bonaduz, Switzerland). 10µl (30µg) PBS dilution of saporin-conjugate ME20.4 antibody (Advanced Targeting System) were unilaterally applied into the lateral ventricle in 10 animals. All efforts were made to minimize animal suffering throughout the experiments including administration of the analgetic Metamizol (150 mg/kg; Berlosin, Berlin-Chemie, Germany) immediately after operation. The antibiotic Enrofloxacin (10 mg/kg daily; Baytril, Bayer AG) was given orally throughout the post-injection period. Two days, six months (in vivo labeling; immunolesion experiments, respectively) post-injection and the control rabbits were transcardially perfused under deep anesthesia with 800ml 4% paraformaldehyde containing 0.1% glutardialdehyde in
phosphate buffer (PB; 0.1 M, pH 7.4) that was preceded by a prerinse with ice-cold physiological saline (150 ml). Whole brains were removed from the skull, divided into fore- and hindbrain regions, and postfixed in 4% paraformaldehyde overnight.

Two Cy3-ME20.4 injected forebrains were coronally sectioned on a vibratome at 50μm for the electronmicroscopical investigations (see below), the other brains were cryoprotected by equilibration with 30% sucrose in PB. Thirty-micrometer-thick coronal sections were cut on a freezing microtome and collected individually in Tris-buffered saline (TBS; 0.1 M, pH 7.4). Sectioning started at the anterior pole of the MS and spanned the entire BFB, including the caudal pole of the MBN/SI complex, yielding about 250 sections in each individual case. To ensure standard cross-sectional distances throughout the quantification procedures in the untreated, control animals, each sixth section was immunolabeled for ChAT; all consecutive sections were processed for p75\textsubscript{NTR} [both with nickel-enhanced 3,3'-diaminobenzidine (DAB) as chromogen], whereas the successive series was used for Nissl staining. A fourth series of sections was applied to the immunofluorescence double labeling of ChAT and p75\textsubscript{NTR} and was pretreated with Sudan black B according to Schnell et al. (1999) to quench autofluorescence of the tissue and to reveal the myelination of fibers. To assess quantitatively the possible colocalization of CB and nNOS in cholinergic neurons, the fifth and sixth series of sections in two male and one female rabbits were used for double-immunofluorescence detection of CB/ChAT and nNOS/ChAT, respectively. For the carbocyanine (Cy) double-fluorescence staining of ChAT and a panel of additional neuronal markers (see Table 1), sections from the fifth series of one male rabbit and selected sections from two female animals were used without Sudan black B pretreatment. According to this sampling protocol, the cross-sectional distance amounted to 180 μm.

For electron microscopic investigation of the intracellular fluorescent label, Cy3 was photoconverted into an electron-dense reaction product. Therefore, Cy3-ME20.4-labelled tissue was primarily incubated for 20–30 min in cold 0.1 M phosphate buffer, pH 7.4, containing DAB and potassium cyanide (1.5% each). Next, the sections were placed in a closed conversion chamber connected with a recirculator (National Lab, Mölln) keeping the temperature at about 5°C. The subsequent photoconversion was performed according to Kacza et al. (2000) by using a HBO 100-W mercury vapour lamp and rinsing the chamber with pure oxygen in order to facilitate the photooxidation of DAB which usually lasted 15–20 min. Following three rinses in cold buffer for 15 min each, the sections were reacted with 1% osmium tetroxide for 45 min, dehydrated in an ascending series of ethanol (thereby block-
stained with 1% uranyl acetate) and embedded in Durcupan (Fluka, Darmstadt). The ultrastructural observation of photoconverted neurons was performed with a Zeiss Electron Microscope 912 Omega.

**Immunohistochemical procedures**

All immunohistochemical methods were performed on free-floating sections. Before immunoperoxidase staining, endogenous tissue peroxidase activity was abolished by treatment with 0.6% H₂O₂ in TBS for 30 minutes. For the immunoperoxidase labeling of ChAT, sections were then preincubated in 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA) in TBS containing 0.3% Triton X-100 for 1 hour. Subsequently, tissue samples were incubated in affinity-purified goat anti-ChAT (Li and Furness, 1998; Reiche and Schemann, 1999; Chemicon International, Hofheim, Germany; 1:100 in TBS containing 0.3% Triton X-100 and 5% normal donkey serum) for 16 hours at room temperature. Subsequently, sections were thoroughly rinsed and exposed to biotinylated donkey anti-sheep IgG [highly cross-reacting with goat IgG (Jackson Immunoresearch); 1:500 in TBS containing 2% bovine serum albumin (TBS-BSA)] for 1 hour. Finally, sections were processed with preformed complexes of streptavidin and biotinylated peroxidase and stained with nickel-enhanced DAB as chromogen (Härtig et al., 1995).

For the immunodetection of p75<sub>NTR</sub>, nonspecific binding sites were blocked in 5% normal goat serum (Jackson Immunoresearch) in TBS containing 0.3% Triton X-100 for 1 hour, followed by incubation of the sections in monoclonal mouse anti-p75<sub>NTR</sub> antibody [clone ME 20.4, 1:5 (Ross et al., 1984); donated by Dr. A. Fine] in the blocking solution overnight. After extensive rinsing, sections were reacted with biotinylated goat anti-mouse IgG (Jackson Immunoresearch; 2.5 μg/ml in TBS-BSA) for 1 hour. Thereafter, tissue samples were processed according to a visualization protocol identical to that described for the immunodetection of ChAT (Härtig et al., 1995). For the immunofluorescence double labeling of ChAT and other markers, sections were preincubated in TBS containing 0.3% Triton X-100 and 5% normal donkey serum for 1 hour. Sections were then exposed to a mixture containing both goat anti-ChAT and one of the primary mouse or rabbit antibodies listed in Table 1 in TBS containing 0.3% Triton X-100 and 5% normal donkey serum for 16 hours. The goat anti-ChAT antibody was applied in a 1:25 dilution and subsequently visualized by Cy2-conjugated donkey anti-goat IgG combined with the immunolabeling of p75<sub>NTR</sub>, SP, and TH with Cy3-tagged secondary antibodies. In contrast, goat anti-ChAT antibody was used at a dilution of 1:50 and localized with Cy3-tagged donkey anti-goat IgG when CB, CR, PV, and
nNOS were simultaneously revealed with Cy2 immunostaining (Table 1). All secondary antibodies were applied at a concentration of 20 μg/ml TBS-BSA for 1 hour and were obtained at the highest available purity (Jackson Immunoresearch).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Host</th>
<th>Dilution</th>
<th>Source</th>
<th>Fluorochromated secondary antibody</th>
<th>Reference</th>
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<tr>
<td>Choline acetyltransferase</td>
<td>Goat</td>
<td>1:25</td>
<td>Chemicon, Hofheim, Germany</td>
<td>Cy2 donkey anti-goat</td>
<td>Li and Furness, [1998]</td>
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<tr>
<td></td>
<td></td>
<td>1:50</td>
<td></td>
<td>Cy3 donkey anti-goat</td>
<td>Reiche and Schemann, [1999]</td>
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<tr>
<td>p75 Neurotrophin receptor</td>
<td>Mouse (clone ME 20.4)</td>
<td>1:2</td>
<td>Dr. A. Fine, Halifax, NS, Canada</td>
<td>Cy3 donkey anti-mouse</td>
<td>Ross et al., [1984]</td>
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<tr>
<td>Calbindin D-28k</td>
<td>Mouse (clone CL-300)</td>
<td>1:200</td>
<td>Sigma, Deisenhofen, Germany</td>
<td>Cy2 donkey anti-mouse</td>
<td>Celio et al., [1990]</td>
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<tr>
<td>Calretinin</td>
<td>Rabbit</td>
<td>1:300</td>
<td>SWant, Bellinzona, Switzerland</td>
<td>Cy2 donkey anti-rabbit</td>
<td>Schwaller et al., [1993]</td>
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<tr>
<td>Parvalbumin</td>
<td>Mouse (clone PA-235)</td>
<td>1:300</td>
<td>SWant, Bellinzona, Switzerland</td>
<td>Cy2 donkey anti-mouse</td>
<td>Celio et al., [1988]</td>
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<td>Nitric oxide synthase, neuronal</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Laboserv, Giessen, Germany</td>
<td>Cy2 donkey anti-rabbit</td>
<td>Alm et al., [1993]</td>
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<td>Substance P</td>
<td>Rabbit</td>
<td>1:1,000</td>
<td>DiaSorin, Stillwater, MN</td>
<td>Cy3 donkey anti-rabbit</td>
<td>Gall, [1988]</td>
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<td>Tyrosine hydroxylase</td>
<td>Rabbit</td>
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<td>Chemicon, Hofheim, Germany</td>
<td>Cy3 donkey anti-rabbit</td>
<td>Haycock, [1987]</td>
</tr>
</tbody>
</table>

Table 1. List of markers used for double-immunofluorescence labeling

To determine the specificity of in vivo labeling, Cy3-ME 20.4 pre-labeled sections were applied to the concomitant immunolabeling of ChAT and the calcium-binding protein calretinin. Non-specific binding sites for the immunoreagents were primarily blocked with 5% normal donkey serum in 0.1 M Tris-buffered saline (TBS) containing 0.3% Triton X-100 for 1 h. Subsequently, the sections were incubated with a mixture of affinity-purified goat anti-ChAT (1:25) and rabbit anti-calretinin (1:300) diluted in the blocking solution for 16 h. Next, rinsed sections were reacted with a cocktail of Cy2-tagged donkey anti-goat IgG and Cy5-conjugated donkey anti-rabbit IgG (20 μg/ml; Dianova, Hamburg, Germany) diluted in TBS containing 2% bovine serum albumin for 1 h.
To confirm the successfulness of the saporin-ME20.4 treatment, we performed immunoperoxidase labeling of ChAT on sections containing the MS-DB region of control and lesionated animal, as it was detailed previously. In order to visualize the potential beta-amyloid deposits in hippocampal and cortical areas, we performed highly sensitive immunoperoxidase staining. Sections were routinely treated with 90% formic acid for 10 min before the according to Kitamoto et al. (1987) which is known to improve the detectability of beta-amyloid. Sections were incubated with a mouse monoclonal antibody with a specificity for an epitope residing in the sequence $\beta_{8-17}$ (Dako, Hamburg; 1:50 in TBS-T-NGS) and with a rabbit antiserum generated against the amino acids 17–42 of human and monkey beta-amyloid (1:2000; generous gift of Prof. Dr. Kalaria, Newcastle) overnight at room temperature or for about 2 days at 4°C. After brief wash with TBS the sections were exposed to biotinylated secondary antibodies, than developed with the previously detailed ABC method (Hartig et al. 1995).

Omission of the primary antibodies in control experiments caused the absence of any cellular staining. Moreover, switching of the fluorophores for the detection of ChAT and any other relevant markers resulted in labeling patterns identical to those yielded in the serial staining experiments. After the histochemical procedures, sections were extensively washed in TBS, dipped in distilled water, mounted on fluorescence-free slides, air dried, and coverslipped. The sections were covered either with Entellan in toluene (Merck, Darmstadt, Germany) or, when pretreated with Sudan black B, with glycerol/gelatin (Sigma, Deisenhofen, Germany).

**Low-magnification mapping of BFB nuclei**

Mapping of cholinergic BFB nuclei was carried out on ChAT (Figure 1) at 2.5× primary magnification using a Leica LB100T microscope fitted with a high-resolution camera module (DC-100) that was attached to a Quantimet computerized image-analysis platform (Leica, Cambridge, United Kingdom). All sections were captured under identical illumination at 600 dpi resolution. Sections were systematically sampled to allow complete overview of the BFB, and composite images were assembled using Paint Shop Pro (v. 7.01; Jasc Software Inc., Eden Prairie, MN).

**High-magnification microscopy**

High-power photomicrographs of ChAT-ir neuron populations were prepared using a Zeiss Axioplan microscope equipped with an AxioCam high-resolution camera (Zeiss) at 1,030 × 1,300 pixel resolution. Captured images were uniformly processed for contrast and brightness
adjustment using Paint Shop Pro (v. 7.01). Tissue samples double-labeled for ChAT and p75NTR, CB, CR, PV, TH, nNOS, SP, or NeuN were first inspected using a Zeiss Axioplan fluorescence microscope equipped with appropriate single- and double-bandpass filters (Nos. 15, 09, and 24). Images of selected specimens were then obtained using a Zeiss 510 confocal laser scanning microscope. After color coding, ChAT immunolabeling always appears in red (the only exception is Figure 5.), whereas structures immunoreactive for the simultaneously stained marker are shown in green. Panels of images were generated by CorelDraw (v. 10.0; Corel Corp., Ottawa, Ontario, Canada).

Quantitative image analysis

Morphometric analysis of major cholinergic nuclei, such as the MS, VDB, HDB, NCa, nucleus putamen (Pu), olfactory tubercule, accumbens nucleus, GP, VP, and MBN/SI complex was performed using a Leica Q-600HR computerized image-analysis system. Complete series of sections stained for ChAT with nickel-enhanced DAB detection from three animals (two males and one female) were systematically sampled (35-40 sections/brain); brain regions of interest were manually delineated, and their surface area was determined (mm²). Subsequently, the number of neuronal perikarya with clearly discernible nuclei was counted in each brain region, whereas clearly fragmented nerve cell profiles were excluded from the sampling protocol (Harkany et al., 2001a,b). Because cholinergic BFB neurons are distributed in a largely inhomogeneous manner, posing a potential bias in disector-based cell counts (West, 1993), disector analyses were not carried out. It is worth noting that recent studies revealed no considerable difference in the number of cholinergic MBN/SI neurons when analyzed by either disector or exhaustive counting protocols (Horvath et al., 2002). To correct for the possibility of overestimation of cell numbers, a Floderus correction factor was introduced as described by Palkovits et al. (1971). Briefly, the average size of cholinergic BFB nuclei was determined in all forebrain regions of interest, and a clearly discernible nucleus was considered as 8.9 μm in diameter (a, mean value). Insofar as intact neurons contained nuclei with an average radius of 5.7 μm (r), the Floderus correction factor was calculated as $f = 0.8077$ in 30-μm-thick sections ($T_1$), given that $f = T_1/[T_1 + \sqrt{r^2 - (a/2)^2}]$. The probability of double cell counts, based on the 180-μm cross-sectional distance, was considered negligible. Thereafter, the average surface area of each brain region as well as the average number of ChAT-immunoreactive (ir) nerve cells was computed. Finally, by taking the length of each brain region along the z-axis, expressed as the number of sections spanned × 30 μm, into account, the volume of individual brain areas and the corresponding number of
ChAT-ir neurons were estimated (Horvath et al., 2000, 2002). Neuron density was calculated as the ratio of the number of neurons and the volume of the particular brain region and expressed as cell number per cubic millimeter. An identical quantification protocol was applied when assessing the frequency of CB/ChAT and nNOS/ChAT colocalization, except that cell counts were performed using an appropriate double-bandpass filter (No. 24; Zeiss) for simultaneous detection of Cy2 and Cy3 signals.

The density of ChAT-ir projection fibers was determined in the cingulate cortex and in layer V of the retrosplenial cortex, forelimb, and hindlimb representations of the somatosensory cortex and in the parietal and occipital cortices (Fleischhauer et al., 1980; Zilles, 1985). Moreover, the density of ChAT-ir terminals in the dorsal hippocampus was also recorded, in which hippocampal subfields were defined according to Freund and Buzsáki (1996). Image analysis was performed using a standard protocol on a Quantimet Q-550IV image-analysis system (Harkany et al., 2000, 2001a,b) in all sections employed for quantification of cell numbers. Briefly, after background subtraction and gray-scale threshold determination, the surface area of skeletonized ChAT-ir fibers ([the area covered by ChAT-positive cholinergic fibers]/[the total sampling area], given as percentages) was computed in each section using a 470-nm emission filter. Data on the volume of cholinergic nuclei, total numbers and density of the nerve cells, percentage of colocalization for CB/ChAT and nNOS/ChAT, and area density of cortical cholinergic projections were expressed as mean ± SEM.

Nomenclature

Cholinergic nuclei were identified according to the brain maps of Winkler and Potter (1911) and Urban and Richard (1972). In cases in which the anatomical description of a particular brain structure was not available, it was identified based on the corresponding structure in rat brain (Paxinos and Watson, 1986). Our nomenclature conformed to that introduced by Mesulam et al. (1983a,b) for the rhesus monkey and rat, but with some modifications because of interspecies differences (Butcher and Semba, 1989). Importantly, ChAT immunocytochemistry alone during the quantification process did not allow unequivocal delineation of the anatomical boundaries of VP, MBN, and SI, the latter made up of p75<sup>NTR</sup>-immunonegative cholinergic neurons, so we subdivided these territories as VP and the largely diffuse MBN/SI complex. Delineation of cortical areas adhered to mapping studies in the cerebral cortex of rabbit (Fleischhauer et al., 1980) and rat (Zilles, 1985).
RESULTS

NMDA LESION IN RAT MBN:
Quantitative analysis of ChAT-ir nerve cells 4, 24 and 48h after vehicle infusion in the MBN revealed $2167 \pm 383$, $2531 \pm 109$ and $2618 \pm 294$ neurons, respectively. All ChAT-positive neurons also displayed NeuN immunoreactivity. In contrast, only ~50% of NeuN-positive nerve cells were also immunoreactive for ChAT (Table 2). Thus, the population of NeuN-ir but ChAT-immunonegative MBN neurons corresponds with intermingled non-cholinergic neurons in the MBN. Accordingly, estimation of the number of NeuN-positive neurons in the sham-lesioned MBN indicated $4541 \pm 434$, $4441 \pm 351$ and $5071 \pm 108$ neurons 4, 24 and 48h post surgery. NMDA infusion resulted in a massive loss of ChAT immunoreactivity that became apparent as early as 4h postlesion and persisted throughout the survival period investigated. As Table 3 shows, a significant reduction of the ChAT-ir neuron population (cell numbers: $1283 \pm 352$, 4h; $885 \pm 104$, 24h; $1236 \pm 266$, 48h) was evident relative to the contralateral MBN. In parallel with the NMDA-induced cholinergic breakdown, the number of NeuN-ir neurons also exhibited a significant decline at each time-point investigated (cell-numbers: $2407 \pm 361$, 4h; $2060 \pm 166$, 24h; $2656 \pm 523$, 48h; Table 2). Interestingly, NMDA infusion did not alter the ratio of ChAT-ir and NeuN-ir neurons in the damaged MBN (Table 2).

Table 2. Cellular colocalization of neuron-specific nuclear protein (NeuN) and choline-acetyltransferase (ChAT) immunoreactivities 4, 24, and 48h after NMDA infusion in the rat MBN. Note that the NMDA infusion did not influence the percentage colocalization of the markers investigated, compared to sham-operated conditions at any of the post-lesion time-points investigated. Data are expressed as means ±s.e.m. (n=4/group).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Hemisphere</th>
<th>NeuN+/ChAT+ (%)</th>
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<tr>
<td></td>
<td>Sham lesioned</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NMDA lesioned</td>
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<td></td>
<td></td>
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<tr>
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<td></td>
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<td>40,7±10,5</td>
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<td>48</td>
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<td>NMDA lesioned</td>
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</table>
Table 3. Short-term effect of NMDA infusion in the MBN on the loss of NeuN and ChAT immunoreactivities. The number of nerve cells immunoreactive for NeuN and ChAT was determined in both the sham-and NMDA-lesioned MBN 4, 24 and 48 h post-surgery. The relative value of cell loss for each cellular marker was calculated as the percentage difference between the numbers of immunoreactive neurons at NMDA- and sham-lesioned sides of the brain. Statistically significant differences between the NMDA-lesioned and control MBN at particular time-points: **p<0,01, *p<0,05 (paired t-test). Data are expressed as means ± s.e.m. (n=4/group).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>ChAT+ (% loss of neurons)</th>
<th>NeuN+ (% loss of neurons)</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>48</td>
<td>54,4±8,1**</td>
<td>46,9±11,9*</td>
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EXPERIMENTS WITH RABBITS

General considerations

Rostrally, the MS appears as a small, compact nucleus in the midline of the brain and is surrounded by the lateral septal nuclei (Fig. 1A,A',B,B'). After gradual expansion, the MS reaches its maximal extent approximately at the coronal plain of bregma (Urban and Richard, 1972; Fig. 1B,B'), whereas the fornix, as its major output pathway innervating the hippocampus, appears more caudally (Fig. 1C,C',D,D'). While the dorsal and lateral boundaries of the MS are clearly discernible, a more dispersed group of cholinergic neurons, termed the VDB, neighbors the MS ventrally (Fig. 1B,B'). Because of the close proximity of these nuclei, they can be designated as the MS/VDB complex. Similarly to the organization of the rat BFB, the HDB is located lateroventrally from the VDB. Along the longitudinal axis of rabbit brain, the HDB extends laterally (Fig. 1B,B',C,C'). Subsequent to the disappearance of the compact group of cholinergic HDB neurons, the MBN forms the most caudal part of cholinergic BFB projection neurons in rabbit forebrain (Fig. 1D-F'). Tract-tracing studies in the rat BFB revealed that three, namely, the anterior, intermediate, and posterior, MBN subdivisions can be distinguished (Luiten et al., 1987; Gaykema et al., 1990). In the present study, we used the nomenclature of Gaykema et al. (1990) and identified the anterior MBN as extending caudally to the disappearance of the anterior commissure (Fig. 1D,D'), the intermediate MBN subdivision adjacent to the ventromedial surface of the GP (Fig. 1E,E'), and the compact posterior MBN subdivision (Fig. 1F,F'). A diffuse population of cholinergic neurons situated ventromedially from the intermediate MBN forms the SI. Because ChAT
immunolabeling does not provide a clear demarcation between these nuclei, they were further assigned as the MBN/SI complex.

Figure 1. Distribution of cholinergic BFB nuclei in rabbit forebrain. A-F represents schematic maps of major BFB nuclei that were prepared using sections stained for ChAT (A'-F'). Details of the imaging procedure and particular anterior-posterior coordinates of the captured planes are referred to in the text. Nuclei were identified and termed according to the nomenclature introduced by Winkler and Potter (1911) and Urban and Richard (1972), in conjunction with particular designations of Paxinos and Watson (1986) in rat brain. Numbered territories (2A-2J) correspond to the high-power images shown in Figure 2. 1, Central amygdaloid nucleus, capsular subdivision; 2, central amygdaloid nucleus, medial subdivision; 3V, third ventricle; AcA, comissura
The capsula interna served for anatomical separation between the NCa and the Pu, which contained large cholinergic interneurons (Fig. 1A'-F'). Interestingly, a well-separated ventral subregion of the Pu, referred to as the ventral putamen (Fig. 1C,C'), could also be identified. In conjunction with the disappearance of the core and shell (AcSh) divisions of the nucleus accumbens, which are located ventromedially from the NCa and are often assigned as parts of the ventral striatum, the medial segment of the Pu borders the GP. The GP extends in the caudal direction and is enveloped by cholinergic neurons of the MBN (Fig. 1D,D',E,E').

**Medial septum and vertical limb of the diagonal band of Broca**

Dense clusters of cholinergic neurons of the MS/VDB complex become already visible with low magnification (Fig. 1A',B'). Within the boundaries of the MS, ChAT-ir neurons are concentrated along the midline raphe of the septum and the outer edge of the nucleus and form mirror-symmetrical, shell-like cell assemblies. The highest density of cholinergic cells can be found within some 100 μm from midline (Figs. 1B', 2A). This laminar organization was less apparent in the VDB (Fig. 2B). Whereas the absolute number of ChAT-ir cells in the MS is approximately twofold higher than that in the VDB, the above-mentioned nonhomogeneous distribution of ChAT-ir neurons in the MS results in a lower mean density of nerve cells (Table 2). Cholinergic projection neurons in the MS and VDB were of intermediate size and appeared as bipolar or multipolar nerve cells (Fig. 2A,B). Double-immunofluorescence labeling for ChAT and p75NTR revealed a virtually complete colocalization of the two cholinergic markers (Fig. 3A,A',B,B'). None of the additional markers was coexpressed in cholinergic perikarya (see, e.g., Fig. 4C,D,H).
Figure 2. Distribution of ChAT-ir neurons in the rabbit BFB (A-H) and of cholinergic terminals in the somatosensory cortex (hindlimb area; I) and dorsal hippocampus (J). Clusters of multipolar cholinergic nerve cells were demonstrated in the medial septum (A) and vertical (B) and horizontal (C; HDB) diagonal bands of Broca. Note that C depicts spindle-shaped ChAT-ir neurons in the posterior subdivision of the HDB. Cholinergic neurons in the MBN (D) and substantia innominata (SI; E) formed a largely interrelated neuronal network in which MBN neurons appeared larger than SI neurons. It is worth noting that MBN neurons were intermingled with densely stained passing cholinergic fiber bundles (D). ChAT-ir neurons were frequently found in cell bridges between the nucleus caudatus (NCa) and putamen (Pu, F). Moreover, multipolar cholinergic interneurons of similar size and density were observed in NCa (G) as well as in Pu (H). A dense ChAT-ir fiber network was...
visualized throughout the cerebral cortex and exhibited a clear laminar distribution pattern. The highest density of cholinergic fibers was seen in layer V of the cerebral cortex (I). Cholinergic terminals in the hippocampus also showed strict topographical distribution. J is a reconstruction of the dorsal hippocampus, with the highest ChAT-ir terminal density in the stratum suprapyramidale (Sp) of the CA1 and CA3 subfields. Note the frequent presence of ChAT-ir interneurons (arrows). IML, inner molecular layer of the dentate gyrus; Ip, stratum infrapyramidale; OML, outer molecular layer of the dentate gyrus; Or, stratum oriens; Rad, stratum radiatum. Scale bar in H = 150 μm for A-H; bar in I = 50 μm; bar in J = 280 μm.

Horizontal limb of the diagonal band of Broca

In general terms, the cytoarchitecture of the HDB resembles that of the VDB, in which a dense cluster of multipolar ChAT-ir neurons is present (Fig. 1B', C'). High-power microscopy in the posterior region of the HDB - corresponding to Figure 1C' - often revealed spindle-shaped ChAT-ir perikarya (Fig. 2C). Quantitative analysis of cholinergic cell numbers in the HDB demonstrated the highest density of ChAT-ir neurons among the cholinergic BFB nuclei investigated (Table 4). Similarly to the case in the MS and VDB, all ChAT-ir neurons exhibited p75NTR immunoreactivity (data not shown), whereas none of the other markers showed colocalization with cholinergic HDB neurons (see, e.g., Fig. 4H).

Pallidum

The rostral expansion of the VP is emerging already at the level of the first appearance of the MS (Fig. 1A,A') and is often interrupted by striatal cell bridges between the olfactory tubercule and the AcSh. The neuropil is less densely stained for ChAT than the surrounding striatal regions (Fig. 1A'). In parallel with the disappearance of AcSh, the VP expands under the commissura anterior, whereas more caudally it seems to become identical with the SI. Cholinergic neurons situated in the VP region are similar in their morphological characteristics, density, and size to those of the AcSh (Table 4). Double-immunofluorescence labeling, however, revealed that ChAT-ir neurons in the VP, but not in the AcSh, express p75NTR. The GP itself lacks any cellular ChAT labeling and, concomitantly with its caudal extension, becomes encircled by a ribbon-like cluster of cholinergic magnocellular neurons that make up the MBN (Fig. 1D', E').

Magnocellular nucleus basalis/substantia innominata complex

Concomitantly with the disappearance of the commissura anterior, the anterior MBN subdivision can be distinguished, followed by the major - intermediate - part that transits into the compact posterior MBN subdivision. MBN neurons form clusters consisting of 5-20
ChAT-ir cells that are surrounded by densely stained fiber bundles (Fig. 2D). These cholinergic cell clusters exhibit a heterogeneous distribution pattern; e.g., some MBN-related neurons are also visible in the internal lamina between the Pu and GP as well as in the capsula interna (Fig. 3C). Whereas subsets of ChAT-ir neurons of different sizes can be observed in this region, ChAT immunolabeling alone did not allow the unambiguous delineation of definite boundaries between the MBN and the SI, the latter being generally found ventromedially from the MBN in other mammalian species.

Figure 3. Coexpression of ChAT (A-C) and p75<sup>NTR</sup> (A′-C′) in the MS, VDB, and MBN as visualized by combined immunolabeling using Cy2 (green)- and Cy3 (red)-tagged secondary antibodies, respectively. Note the overlap of the two signals in the MS and VDB, the presence of cholinergic cells in the capsula interna (Ci), and the ring-like pattern of MBN neurons surrounding the globus pallidus (GP). p75<sup>NTR</sup>-negative cholinergic neurons were found on the putamen (Pu)/MBN border (arrows). Box in C indicates a high magnification image of the territory shown in Figure 4B. Scale bar in B′ = 200 µm for A,B,A′,B′; bar in C′ = 500 µm for C,C′.

A dorsal-to-ventral descending gradient of p75<sup>NTR</sup>-ir neurons was present in the intermediate MBN/SI. Double-immunofluorescence labeling for ChAT and p75<sup>NTR</sup> confirmed the presence of neurochemically distinct subpopulations of cholinergic neurons (Fig. 3C,C′). Whereas virtually all large cholinergic nerve cells in the dorsolateral MBN/SI complex were immunoreactive for p75<sup>NTR</sup>, a subpopulation of medium-sized cholinergic neurons (Fig. 2E) was immunonegative for p75<sup>NTR</sup> in the ventromedial subdivision (Fig. 4A). The difference in p75<sup>NTR</sup> immunoreactivity, thus, provides a firm basis for defining a border between the MBN and SI (Fig. 1D,D′,E,E′). Interestingly, cholinergic neurons of the posterior MBN also contained CB (Fig. 4F) and nNOS (Fig. 4I), whereas ChAT was not found in cells containing PV (Fig. 4E) or CR (data not shown).
Figure 4. Immunofluorescence labeling of ChAT (appears red after color coding) combined with selected neurochemical markers (color coded in green) in the rabbit basal forebrain. A and B demonstrate partial colocalization of ChAT with the p75<sup>NTR</sup> in substantia innominata (SI) and putamen (Pu). The calcium-binding proteins calbindin D-28k (CB), calretinin (CR), and parvalbumin (PV) did not colocalize with ChAT in the medial septum (MS; C), vertical diagonal band of Broca (VDB; D), and magnocellular nucleus basalis (MBN; E), respectively. Interestingly, however, a portion of ChAT-ir neurons contained CB in the posterior subdivision of MBN (F). Low-power photomicrograph of the pallidal region (VP) depicts strong TH-positive input in the vicinity of cholinergic neurons (G). Also, substance P-ir fibers surround and terminate on cholinergic neurons of the horizontal diagonal band of Broca (HDB; H). Nitric oxide synthase (nNOS) was infrequently coexpressed in some cholinergic MBN neurons (I), but colocalization of nNOS with cholinergic perikarya in other investigated nuclei was not observed. Colocalization of the particular markers appears in yellow/orange. Scale bars = 100 µm in A,D, 50 µm in B,F,I, 200 µm in C,E, 250 µm in G, 20 µm in H.
Nucleus caudatus, putamen, and nucleus accumbens

As shown in Figure 1, the capsula interna provides structural separation between the NCa and Pu in the rabbit BFB. Although the capsula interna appears to be immunonegative for ChAT, it is often interrupted by striatal cell bridges that provide connections between the NCa and the Pu (Fig. 2F). Both the NCa and the Pu contain significant numbers of cholinergic interneurons. However, the pattern of neuronal density, as a function of the rostrocaudal axis, differs markedly (Table 4). Whereas a clearly descending anterior-to-posterior gradient in the density of ChAT-ir neurons was demonstrated in NCa, a more even distribution of cholinergic interneurons is characteristic for Pu. All cholinergic neurons of the NCa and Pu are large, round, and multipolar (Fig. 2G,H), resembling cholinergic striatal interneurons of other animal species (Sofroniew et al., 1982; Geula et al., 1993), and those situated in striatal cell bridges intruding into the capsula interna form a fusiform subset of ChAT-ir neurons (Fig. 2F). Whereas the NCa was devoid of any cellular p75\textsuperscript{NTR} labeling, p75\textsuperscript{NTR}-ir cells were found scattered in the ventromedial and laterodorsal segments of Pu (Fig. 4B). None of the other neurochemical markers (calcium-binding proteins, nNOS, TH, and SP) exhibited cellular colocalization with striatal cholinergic perikarya.

The accumbens complex could be divided into two subdivisions, by means of ChAT immunolabeling, namely, the lightly immunoreactive core and the densely stained AcSh nuclei. The core only exhibited diffuse ChAT-ir fiber staining. In contrast, AcSh not only consisted of a denser neuropil staining for ChAT but also contained, though in a relatively low density, cholinergic nerve cells (Table 4). Based on their multipolar appearance and size similarity to cholinergic neurons in Pu, these ChAT-ir nerve cells could also be regarded as cholinergic interneurons (data not shown).

Distribution of calcium-binding proteins, NOS, TH, and SP and their colocalization with cholinergic neurons

Although our primary objective was to determine whether cholinergic neuron populations coexpress the calcium-binding proteins CB, CR or PV, attention was also paid to the general distribution pattern of calcium-binding protein-containing neurons in the rabbit BFB. Relatively few CB-ir cells were localized in the midline of MS and VDB, where they were surrounded by the cholinergic cell columns or intermingled with ChAT-positive perikarya (Fig. 4C). Interestingly, CB-ir cholinergic neurons were present in the posterior MBN (Fig. 4F) but not in the anterior or intermediate MBN subdivisions. Quantitative analysis
demonstrated that 19.1% ± 0.4% of cholinergic neurons in the posterior MBN subdivision exhibited CB immunoreactivity. CR-positive neuronal perikarya were also present mainly in the proximity of the MS midline. In comparison with the distribution pattern of CB-ir neurons (Fig. 4C), however, a significantly broader zone of intermingled CR-ir and ChAT-ir neurons was evident in the MS and VDB (Fig. 4D). The dorsal subdivision of the NCa and Pu exhibited intense CB and CR immunoreactivity, where both cellular and neuropil labeling for these calcium-binding proteins was present, and virtually all striatal principal neurons expressed CB. Although CR-containing cells were scattered in the rabbit VP and MBN/SI complex, numerous CR-ir passing fibers, though with heterogeneous densities, were visualized. Surprisingly, a relatively low intensity of PV-ir neuronal structures was observed in the MS and VDB relative to other BFB nuclei. In contrast, the NCa and Pu displayed a dense subset of PV-positive neurons that outnumbered the cholinergic neurons (data not shown). Similarly to the case in the MS, all three calcium-binding proteins were also present in the HDB and formed neuronal populations distinct from those of cholinergic nerve cells. Irrespective of their particular calcium-binding protein content, noncholinergic neurons usually appeared to be smaller than cholinergic cells in rostral nuclei of the BFB. In fact, PV-ir neurons showed a wide range of somatic diameters, being largest in the GP, where they seemed to be as large as ChAT-ir neurons. Likewise, large PV-positive neurons were frequently observed in the VP and MBN/SI complex (Fig. 4E).

TH-ir projection fibers were localized in cholinergic areas that receive innervation from dopaminergic midbrain nuclei (Fig. 4G). These TH-positive fibers were of very thin appearance. Prominent TH immunosignal was present throughout the NCa and Pu as well as the AcSh. The distribution of TH-positive fiber projections was found as a distinctive marker between the AcSh and the VP, inasmuch as the VP exhibited significantly lower TH immunoreactivity, which became evident only in passing fibers (Fig. 4G). A few passing fibers were also visible in other cholinergic nuclei, such as MS, VDB, and MBN/SI complex (data not shown).

In parallel with TH-ir fibers, SP-containing projections were also found in the rabbit BFB that might originate in midbrain nuclei (Csillik et al., 1998). Similarly to the case in the rat (Bolam et al., 1986), SP-ir fibers were found predominantly in pallidal territories, whereas a lower density of SP-ir input was demonstrated in the NCa and Pu. However, other cholinergic nuclei, including the MS, HDB, and MBN, also received SPergic innervation. Interestingly, the MS was relatively weakly labeled for SP, whereas the lateral septum exhibited strong SP immunoreactivity. Similarly, cholinergic neurons in the HDB were embedded in a dense
network of SP-ir fibers that seemed to form synaptic terminals on ChAT-ir neurons (Fig. 4H). In contrast to our data on TH immunoreactivity in the AcSh and VP, SP-positive fibers were abundantly present in VP, but not in AcSh, making these two markers ideal for the demarcation of these nuclei (data not shown).

nNOS-ir neurons were found only in striatal (NCa, Pu and AcSh) and pallidal (VP) subregions, where nNOS labels a group of noncholinergic small to medium-sized neurons. Other BFB nuclei were mostly devoid of nNOS immunoreactivity. Interestingly, a few cells coexpressing ChAT and nNOS were observed in the posterior MBN subterritory (Fig. 4I), with a frequency of <1%.

**Cholinergic terminals in the neocortex and hippocampus**

Similarly to the case in other animal species, the rabbit cerebral cortex displayed a clear laminar distribution of cholinergic projection fibers. ChAT-ir fibers formed a dense network of fine axons (Fig. 2I) with apparently highest densities in layers I and V of the cerebral cortical areas investigated. Morphometric analysis was performed on layer V of the cerebral cortex because of its clear, distinctive borders with adjacent cortical laminae and demonstrated a largely homogeneous distribution of cholinergic terminals irrespective of the particular cortical subfield investigated (Table 4). It is worth noting that the adult rabbit neocortex contained only a few, faintly immunoreactive cholinergic interneurons, primarily in the somatosensory and retrosplenial areas. Similarly, a strictly organized cholinergic fiber pattern was apparent in the dorsal hippocampus. Determination of the surface area density of cholinergic axons revealed the highest density of ChAT-ir innervation in the suprapyramidal layer of both the CA1 and the CA3 subfields (Fig. 2J). It is worth noting that homogeneous distribution of ChAT-ir fibers was found in the inner and outer molecular layers of the dentate gyrus (Table 4). Multipolar ChAT-ir nerve cells were frequently observed in the dorsal hippocampus (Fig. 2J).
Table 4. Density of ChAT-ir terminals in cerebral cortex and hippocampus. Distribution of ChAT-ir projections, originating in cholinergic BFB nuclei, was determined in layer V of the rabbit neocortex and dorsal hippocampus. Divisions of cerebral cortical areas were identified and termed as introduced by Zilles (1985), whereas those of the dorsal hippocampus correspond to the description of Freund and Buzsáki (1996). Fiber density is presented as the mean surface area covered by skeletonized ChAT-ir projections. Data are expressed as means ± SEM. FL, forelimb representation; HL, hindlimb representation; DG IML, inner molecular layer of the dentate gyrus; DG OML, outer molecular layer of the dentate gyrus.

In vivo labeling and immunolesion in the basal forebrain

Two days following the i.c.v. infusion of Cy3-ME 20.4 there was a strong puctate labeling in many cells of the MSDB (Figure 5) that became less intense in the MBN. In comparison to
rabbits unilaterally infused with Cy3-ME 20.4, bilaterally injected animals appeared to contain more in vivo-labeled nerve cells in both hemispheres (not shown).

In vivo labeling of Cy3-ME 20.4 was exclusively found in cholinergic neurons, as revealed by their immunoreactivity for ChAT (Figure 5a). In contrast, in vivo labeling was never observed in non-cholinergic, calretinin-immunoreactive nerve cells (Figure 5b) and in magnocellular, parvalbumin-containing GABAergic BFB neurons (not shown). Electron microscopic analysis elucidated that Cy3-ME 20.4 was predominantly located intracellularly in secondary lysosomes (Figure 6a, b).

Figure 5. The confocal laser-scanning micrograph (a) shows red punctate Cy3-ME 20.4 in vivo labeling in cholinergic neurons of the rabbit medial septum. Selectivity of Cy3-ME 20.4 for cholinergic neurons was demonstrated by colocalization with ChAT that appears green (Cy2). (b) Merged carbocyanine triple fluorescence staining of Cy3-ME 20.4 (red clusters) and immunoreactivities for ChAT (Cy2, green) and calretinin (Cy5, color-coded in blue) in the border zone between the medial and lateral septum confirmed the specificity of in vivo labeling for cholinergic neurons and its absence in non-cholinergic, calretinin-containing nerve cells. Bar = 25 μm.

The efficacy of immunolesion was demonstrated by ChAT immunostaining (Figure 7.). Interestingly, the lesion in the MSDB was almost complete, but in the NBM/SI complex remained several group of cholinergic cells (not shown).
Figure 6. Electron micrographs of cholinergic neurons in the medial septum (a) and, at higher magnification, in the diagonal band of Broca (b) following photoconversion of internalized Cy3-ME 20.4. The *in vivo* label was predominantly present in secondary lysosomes (arrows) after two days of survival. In comparison to the photoconverted material, non-labeled primary lysosomes (arrowheads) are different in their size, shape and electron density. Bar = 500 nm (a), 250 nm (b).

The effect of the selective cholinergic immunolesion on beta-amyloid plaque deposits in targeted areas was investigated with highly specific antibodies. None of the antisera generated against the amino acids 8-17 and 17-42 of human beta-amyloid showed any kind of detectable beta-amyloid deposition even after half year survival time neither in neocortex nor in the hippocampus (not shown).

Figure 7. Immunoperoxidase labeling of ChAT in control (left) and lesioned (right) rabbit MSDB region. The control rabbit septal region is abundantly stained for ChAT ir cells, but half year after the selective immunolesion immunoreactive neurons can be found only scarcely. Scale bar: 400μm
DISCUSSION

NMDA lesion in rat MBN
Major novel findings of this study include that all cholinergic MBN neurons are positive for NeuN, while double-immunofluorescence labeling with ChAT and NeuN can also be utilized to visualize non-cholinergic nerve cells in the basal forebrain. Interestingly, the ratio of NeuN and ChAT colocalization was ~50%. The NeuN-labeled non-cholinergic neuron population of the MBN was as sensitive to acute excitotoxicity as the cholinergic neurons when exposed to 60mM NMDA. The simultaneous loss of ChAT and NeuN immunoreactivities suggests that at least a subset of non-cholinergic neurons is as selective to acute excitotoxicity as cholinergic nerve cells. This result corroborates the need of immunolesion experiments as the only possible way to eliminate selectively the cholinergic projection neurons in the BFB.

Experiments with rabbits
This part of the thesis demonstrates that, as in other classes of mammals, including rodents and primates, cholinergic neurons form a confluent series of nuclei in the BFB of the rabbit, in which two classes of ChAT-ir nerve cells were identified: projection neurons of MS, VDB, HDB, and MBN/SI and interneurons of NCa, Pu, VP, whereas the GP was devoid of cholinergic cells. Projection neurons of the rabbit BFB provided dense, topologically organized innervation to the cerebral cortex and hippocampus. Interestingly, cholinergic interneurons were frequently seen in the hippocampus but were scarce in neocortical areas. Analysis of the neurochemical heterogeneity of cholinergic cell populations in rabbit BFB revealed the sparse occurrence of CB (~19%) and nNOS (<1%) in cholinergic neurons of posterior MBN, a finding that provides a phylogenetic niche for the rabbit forebrain cholinergic system between rodents (with partial nNOS coexpression; Schober et al., 1989; Brauer et al., 1991; Geula et al., 1993) and primates (with partial CB coexpression; Chang and Kuo, 1991; Côté and Parent, 1992; Geula et al., 1993; Ichitani et al., 1993; Härtig et al., 2002). This assumption is further supported by recent phylogenetic concepts indicating that the rabbit, a member of the mammalian order Lagomorpha, has a distant relationship to members of the orders of both Rodentia and Primates (Benton, 2000).
Distribution of ChAT-ir neurons

The distribution of ChAT-ir BFB projection neurons adheres well to the nomenclature introduced by Mesulam et al. (1983a,b). In particular, the rabbit MS may correspond to the “Ch1” subdivision, the VDB may be regarded as the “Ch2” cell group, and the HDB and the MBN/SI complex can be assigned as the “Ch3” and “Ch4” regions, respectively. However, the mammalian species investigated so far exhibit marked differences in the complexity of BFB nuclei (Butcher and Semba, 1989). Increasing complexity of the BFB system, in parallel with cortical evolution, might be exemplified by the MBN. In the rat, the MBN can arbitrarily be divided into anterior, intermediate, and posterior subterritories, as was revealed by tract tracing studies (Luiten et al., 1987; Gaykema et al., 1990) that demonstrated distinct cortical projection patterns of ChAT-ir cholinergic MBN neurons. In contrast, the complexity of MBN gradually increases in primates and humans and can be subdivided into five and six subsectors, respectively (Mesulam and Geula, 1988). Here, we defined three - anterior, intermediate, and posterior - MBN subsectors based on the lack and/or presence of adjacent cholinergic nuclei and found these to be similar to those of rodents. Importantly, however, extensions of the MBN could also be observed in the lamina between the GP and the Pu, and ChAT-ir neurons coexpressing p75NTR in the capsula interna and adjacent to the basis of NCa could also be assumed to be parts of this nucleus (Maclean at al., 1997).

Stereology-based image analysis was used to determine the numbers of ChAT-ir nerve cells in BFB nuclei. In general terms, the total number of cholinergic neurons in rabbit BFB was approximately 37,000, ~22,000 being cholinergic projection neurons in “Ch1”-“Ch4” nuclei. To date, only a few studies have estimated the numbers of cholinergic neurons in BFB nuclei. In consideration of the findings of Smith et al. (1993, 1995) and Leanza (1998) and of Wu et al. (2000), who showed 1,000-2,000 cholinergic neurons in the rat HDB/MBN vs. approximately 55,000 ChAT-ir cells in the BFB of common marmoset, respectively, our data lend further support to the hypothesis that the complexity of the BFB cholinergic system can be regarded as a phylogenetic correlate of the differentiation of the cerebral cortex. In addition, the rabbit HDB consists of approximately the same amount of ChAT-ir cells as the MS, which is a hallmark of macrosmatic rodents (Brashear et al., 1986) and carnivores (Gritti et al., 1998), whereas the relative number of HDB cholinergic cells is much lower in the microsmatic primates (Mesulam et al., 1984). In contrast, the dorsal striatum (NCa and Pu) closely resembles that of primates, inasmuch as the capsula interna provides a clear structural separation between the two nuclei. Taken together with these data, our morphological analysis supports the theory of an increased complexity of cholinergic BFB nuclei in the rabbit.
compared with those of rodents (Leanza, 1998; Smith and Booze, 1995). It is likely that Old
Word primates, such as apes and humans, with a large brain size, may bear even many more
ChAT-ir neurons. As strict quantitative analysis of cholinergic BFB cells is as yet lacking, so
accurate interspecies comparisons of absolute or relative numbers of the BFB cholinergic
neurons cannot be made.

The density and distribution of cholinergic neurons in the BFB are not uniform, which is due
predominantly to the alternating density of intermingled noncholinergic - primarily
GABAergic cells (Freund, 1989). For example, cholinergic neurons in the MS and VDB are
situated in the midline and in compact lateral cell columns that are intermingled with
noncholinergic cell populations, providing an onionskin-like structure to the nucleus (Kiss et
al., 1990; Brauer et al., 1991; Jakab and Leranth, 1995). Similarly, the parallel existence of
cholinergic and noncholinergic neuron populations could also be observed in the HDB and
MBN/SI complex, but with considerable differences in the relative densities of cholinergic
and noncholinergic nerve cells (Sarter and Bruno, 2002).

Distribution of p75\textsuperscript{NTR}-ir neurons

In the present study, a high density of p75\textsuperscript{NTR}-ir neurons was demonstrated throughout the “
Ch1”-“Ch4” regions of the rabbit BFB. Double-immunofluorescence labeling experiments
showed abundant colocalization of ChAT and p75\textsuperscript{NTR} in these nuclei. Whereas virtually all
cholinergic neurons expressed both markers in the MS, VDB, and HDB regions, a
subpopulation of cholinergic cells located in SI was devoid of p75\textsuperscript{NTR} immunoreactivity. We
assume that these neurons correspond to p75\textsuperscript{NTR}-negative cholinergic neurons in the rat that
are known to project to the amygdala (Heckers et al., 1994). It is worth noting that, in contrast
to the case in the rat, the VP contains a few large cholinergic projection neurons that
coexpress p75\textsuperscript{NTR} (see also Riedel et al., 2002). Importantly, p75\textsuperscript{NTR}-ir cells without ChAT
coexpression were not detectable in the rabbit BFB.

Detailed assessment of the distribution of p75\textsuperscript{NTR}-ir neurons in the BFB of the rabbit also
revealed some differences from other mammalian species. For example, a unique ribbon-like
distribution of large cholinergic neurons can be observed around the GP, often extending into
the capsula interna. Similar, though not identical, localization of cholinergic neurons is known
in mouse (Roßner et al., 2000), rat (Riopelle et al., 1987; Springer et al., 1987; Kiss et al.,
1988), nonhuman primates (Riopelle et al., 1987; Wu et al., 2000), carnivores (Tremere et al.,
1998, 2000), artiodactyles (Tremere et al., 2000), marsupials (our unpublished observations),
and humans (Hefti et al., 1986; Mufson et al., 1989). We classified these cells as MBN-related
projection neurons based on their spatial relationships to other cholinergic neurons. p75\textsuperscript{NTR}-positive cholinergic interneurons were found only in the Pu and not in the NCa, as was also described for sheep (Ferreira et al., 2001) and rat (Kokaia et al., 1998), whereas widespread distribution of p75\textsuperscript{NTR}-ir cells in the NCa, Pu, and nucleus accumbens was seen in the common marmoset (Maclean et al., 1997).

**Neurochemical heterogeneity of cholinergic neurons**

From a neurochemical point of view, cholinergic neurons of the rabbit BFB form a relatively homogeneous cell population. Although some cholinergic neurons coexpressed CB or nNOS in the posterior part of the MBN, colocalization of ChAT and any of the other neurochemical markers investigated was not demonstrated.

In the rat, distinct subpopulations of cholinergic projection neurons are immunoreactive for nNOS (Schober et al., 1989; Brauer et al., 1991; Geula et al., 1993). In contrast, nNOS is entirely absent in cholinergic projection cells of primates (Geula et al., 1993; Wu et al., 2000), where ChAT- and nNOS-positive neurons form nonoverlapping populations throughout the "Ch1"-"Ch4" regions. nNOS immunoreactivity is absent in cholinergic neurons of the rabbit "Ch1"-"Ch3" cell groups, although some nNOS-containing cholinergic neurons are present in the posterior MBN subdivision. It is noteworthy that this nNOS immunoreactivity is apparently weaker than that of cortical neurons.

In the rabbit, CB expression was confined to cholinergic MBN neurons, whereas all other cholinergic cells were devoid of CB immunoreactivity. Comparative studies demonstrated that CB immunoreactivity is absent in cholinergic neurons of the rat BFB; conversely, almost all ChAT-ir neurons contain CB in the primate forebrain (Chang and Kuo 1991; Chang et al., 1991; Geula et al., 1993; Ichitani et al., 1993; Härtig et al., 2002). Moreover, Ichimiya et al. (1989) reported that CB-containing cholinergic projection neurons are first affected in Alzheimer's disease and are more severely damaged than cortical CB-ir nerve cells (Ferrer et al., 1993; Wu et al., 1997).

All BFB areas known to receive dopaminergic innervation contained strong TH-ir fiber labeling. No cell population with TH immunoreactivity was visible within the rabbit BFB. Although this finding corresponds to findings for nearly all other investigated species, TH immunoreactivity was reported in "Ch4" cholinergic cells in the common marmoset (Wisniowski et al., 1992; Wu et al., 2000).
Distribution of cholinergic terminals in the cerebral cortex and hippocampus

Similarly to the case in other mammalian species, corticopetal and septohippocampal cholinergic projections appear as a fine fiber network in target areas. ChAT-ir projections were present in all layers of the neocortical territories and hippocampal subfields investigated. In the cerebral cortex, the highest density of cholinergic projections was observed in layer V, followed by layers I-II = IV-VI > III. Quantitative analysis of the area density of ChAT-ir cholinergic projections in layer V, which provides information on the percentage area covered by skeletonized cholinergic fibers in a particular sampling field, demonstrated a relatively even distribution of cortical cholinergic projections, with peak density values in the forearm representation (FL); and cingulate cortices. Interestingly, layer V of the FL and cingulate areas received the densest cholinergic innervation also in the rat (unpublished observations), which may point to at least partial similarities in the cortical innervation of these species. Likewise, the hippocampal innervation of the rabbit also resembled that of the rat (Freund and Buzsáki, [1986], inasmuch as the CA1 and CA3 strata suprapyramidale and infrapyramidale and the inner molecular layers and outer molecular layers of the dentate gyrus are most abundantly covered by cholinergic fibers.

In vivo labeling and lesion of cholinergic projection neurons

The granular cellular labeling of neurons in the rabbit BFB with intracerebroventricularly applied Cy3-ME20.4 resembled that of Cy3-192IgG in the rat BFB (Hartig et al. 1998). The counting of Cy3-192IgG positive cells in the rat BFB revealed a medial septum > horizontal limb of the diagonal band > nucleus basalis magnocellularis gradient of labeling efficacy (Harkany et al. 2001 a). An apparently similar gradient was observed after the in vivo labeling with Cy3-ME20.4 (not shown). The predominant locations of Cy3-ME20.4 in the cells are the secondary lysosomes, as revealed by electron microscopy. This finding also corresponds with the observation that lysosomes are preferred targets for internalized nerve growth factor, the natural ligand of p75NTR, which might be mimicked by fluorochromated antibodies raised against p75NTR (Kasaian et al. 1988). The successful immunolesion of cholinergic projection neurons in the MSDB did not result in amyloidogenesis in the neocortex and hippocampus.
CONCLUSIONS

Answering our first question, in our first set of experiments we provided evidence that support the hypothesis that not the excitatory-inhibitory imbalance causes the selective loss of cholinergic projection neurons in AD. Although calcium-binding proteins are thought to be protective agents against high intracellular calcium level which arises during excitotoxicity, in our experiment with rat – where the CaBPs can be visualized only in non-cholinergic BFB neurons – non-cholinergic neurons seem to be as vulnerable for NMDA infusion as cholinergic projection neurons. Moreover, in human those cholinergic neurons are affected first in AD which contain CaBPs (Ichimiya et al. 1989). Thus, symptoms of AD cannot be mimicked, or modeled with excitotoxicity. These data query the role of CaBPs in AD progression and also emphasize the need of \textit{in vivo} model animals in which the BFB resembles the human as much as possible concerning the chemoarchitecture.

In the second part of experiments we found the rabbit to be a good remedy for this problem. Rabbits express the same APP sequence as humans and – relatively to mouse and rat – its BFB represents an evolitional stage which resembles more to primates than do rodents: the rabbit cholinergic projection neurons do not contain NOS, but partially express calbindin. Moreover, we provided detailed anatomical data about BFB and striatal cholinergic nuclei regarding their volume, ChAT-ip cell number and density.

Intracerebroventricular infusion of fluorochromated antibodies directed against an extracellular epitope of the human p75$^{NTR}$ revealed that the saporin conjugate of the same antibody is able to selectively eliminate the cholinergic projection neurons in the BFB of the rabbit. We utilized this method to investigate the effect of cholinergic lesion on amyloid-plaque formation in cortical and hippocampal regions. Our experiments did not show any immunohistochemically detectable amyloid-plaque even after half-year survival time. This result suggests that although the cholinergic loss is one of the earliest pathological events in AD, it cannot indicate itself the $\beta$-amyloid deposition.
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