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**PhD thesis**

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# A Pig Model for Studies of Serotonergic Mechanisms in Memory Disorders

Neurobiology Research Unit
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Ph.D. Thesis

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## Preface

The present thesis is based on experimental work performed at several different research facilities primarily at the Neurobiology Research Unit and Center for Integrated Molecular Brain Imaging, Copenhagen University Hospital Rigshospitalet. This work would not have been possible without the generous help and assistance from a substantial number of people, whom I would like to thank here.

I am grateful to all the people at the Neurobiology Research Unit. Most importantly to my supervisor Gitte Moos Knudsen for your never failing support through the years. Your scientific understanding, vision and courage are truly impressing and inspiring. A special thank also to the entire NRU staff, to Susana Aznar and Claus Svarer, to Pia Farup and Dorthe Givard, and to the never ending list of master and PhD-students. It has been a privilege to work with you all. A special thanks to Mette Søgaard Hansen for expert technical assistance and for your pleasant being, to Christine Janssen and Hans Jørgen Jensen for making the lab run smoothly, to Karin Stahr, Inge Møller, Anja Pedersen, Kirsten Hornsyld, Liselotte Jacobsen, Anita Dole, Lene Rottensten, and Søren Hasbirk for all taking your turn with the pig blood samples, and to Daniel Tolnai for counting some of the many cells. Thanks to Lisbeth Marner for help and valuable discussions on PET kinetic modelling and to Kristin Sjølie Thygesen for many enjoyable hours in the pig behaviour facilities. I am especially grateful to Thomas Rune Nielsen for realizing the pig T-maze with great enthusiasm, and also to Anders Ettrup for equal enthusiasm in continuing the line of pig research at NRU.

The pigs were scanned at the PET and Cyclotron Unit, Copenhagen University Hospital Rigshospitalet. I am sincerely grateful to Liselotte Højgaard, Annika Loft and Kate Pedersen for allowing pigs into the facility. The pig scans in this thesis could not have been done without the scanner operators Elisabeth Abrahamsson and Susanne Svalling. Thank you both for working late hours, for your kindness and helpfulness. I would also like to thank the radio chemists Nic Gillings, Szabolcs Lehel, and Jacob Madsen for producing radioligand, and physicist Søren Holm for making difficult concepts easy to understand. Last but not least, thanks to Flemming Andersen for teaching me how to do PET data analysis and for always being cheerful.

The viral vector study was performed in collaboration with Brain Repair And Imaging in Neural Systems, Department of Experimental Medical Science, Lund University. It has been a great pleasure to work with Deniz Kirik and his group. My gratitude to Deniz Kirik for making this possible. I admire your scientific understanding and your meticulousness when preparing manuscripts. Thanks to everybody at the lab for making me feel very welcome, and especially to Liselijn Wisman for your hospitality, to Ulla Jarl and Ulrika Sparrhult-Björk for expert technical assistance, to Stephan Hermening for producing the vectors, and to Bengt Mattsson for the beautiful images.

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The viral vector experiments were performed at the Department of Veterinary Disease Biology, Faculty of Life Sciences, Copenhagen University where Karin Kold and Jens Fletcher have been outstanding in their ability to solve all kinds of practical problems. Also I would like to thank Pia Adolfsen and Charlotte Kyhn for expert technical assistance. Thank you all for taking on any challenge I could come up with.

Several hindrances where further overcome by the kind and generous help from Nanna Grand (from Ellegaard Göttingen Minipigs A/S), Jacob Jelsing (from Research Laboratory for Stereology and Neuroscience, Bispebjerg Hospital), Tim Dyrby (from Danish Research Centre for Magnetic Resonance, Hvidovre Hospital), and Aage Kristian Olsen, Arne Møller and Paul Cumming (all from Aarhus PET Center Denmark). Thank you all.

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*Birgitte Rahbek Kornum*, Copenhagen, 2009

# 1 Summary

Serotonin modulates certain aspects of memory function and serotonergic functioning is adversely affected in various memory disorders, including Alzheimer's disease (AD). It is, however, currently unclear to what extent the serotonergic deficits observed in, e.g., AD are causally related to the memory disturbances.

The serotonergic receptor subtype 5-$HT_4$ has several interesting functions related to memory and memory disorders. The 5-$HT_4$ receptor is expressed in abundance in hippocampal, cortical and striatal regions, it is known to regulate acetylcholine release and to modulate long-term potentiation and depression, and further, it regulates the processing of amyloid precursor protein with possible implications for AD.

Pigs are emerging as a valuable species within neuroscience research. Compared to the rodent brain, the pig brain more closely resembles the human brain both in terms of anatomy and biochemistry, which gives the pig a higher translational value. Further, the size of the pig brain facilitates surgical procedures, lesion studies, and stereotaxic injections, and also permits the identification of cortical and subcortical structures by imaging techniques such as magnetic resonance imaging, and positron emission tomography (PET). The aim of this thesis was to develop a basis for using the Göttingen minipig as a model of serotonergic changes in memory disorders.

In the present thesis, we quantified the brain distribution of the 5-$HT_4$ receptor in the minipig brain with both *in vitro* and *in vivo* methods and found a distribution that resembles that of humans.

Many behavioural responses of the pig are similar to those described for rodents and primates. Still, the cognitive abilities of the pig have not been fully explored, and behavioural testing paradigms for assessing pig learning and memory are few and not all are thoroughly validated. For this thesis, we chose to implement and validate the spontaneous object recognition task and to develop a delayed non-match to sample (DNMS) task in a T-maze for minipigs. We found both tasks to be applicable to pigs, and shoved that performance depends on the length of the inter-trial interval. Further, scopolamine decreased performance in the DNMS task.

Finally, we developed a method for transgene delivery to the minipig brain using recombinant adeno-associated viral (rAAV) vectors, and validated different vector subtypes with respect to their diffusion pattern and cell tropism in the minipig brain. We report that the rAAV5 subtype injected into the neonatal striatum gives neuronal transduction efficiencies around the injection site of up to 49.8 % decreasing with distance. Transduced cells were also detected in large areas of the cortex. Only a few (< 2.4%) of the transduced cells were glia cells, the rest being neurons.

With this work we have developed a methodological basis for future studies of serotonergic mechanisms both in normal learning and memory and in memory disorders using the pig. We conclude that it is indeed possible to apply techniques traditionally used for rodents to the pig model, and that this model has a potential high translational value in neuroscience research.

# 2 Dansk resumé

Serotonin er en vigtig modulator af visse hukommelsesfunktioner, og dysfunktion af dette transmittersystem ses bl.a. ved hjernesygdomme, hvor hukommelsen er påvirket, herunder ved Alzheimer's sygdom (AD). Hvorvidt disse serotonerge forstyrrelser er medvirkende til hukommelsesforstyrrelserne, eller om de to fænomener optræder parallelt, er imidlertid usikkert.

Den serotonerge receptor subtype 5-$HT_4$ har flere funktioner, der knytter sig til hukommelse og hukommelsesforstyrrelser: 5-$HT_4$ receptoren findes i høj koncentration i hippocampus og cortex, den regulerer acetylcholin-frigivelse og modulerer synaptisk plasticitet, og endelig er den involveret i processeringen af amyloid precursor protein, således så stimulation af receptoren har en mulig beskyttende effekt ved AD.

Gennem de senere år er grisen i tiltagende grad med succes blevet anvendt ved hjerneforskning. Dette skyldes at grisehjernen, sammenlignet med gnaverhjernen, både anatomisk og biokemisk ligner menneskets hjerne bedre. Grisehjernen har desuden en størrelse, der lettere tillader invasive indgreb så som præcise læsioner og injektioner, og ydermere gør det muligt at identificere kortikale og subkortikale strukturer med billededannende teknikker som f.eks positron emissions tomografi (PET).

Målet med denne afhandling var at udvikle et metodologisk grundlag for at anvende grisen som model for det serotonerge systems betydning ved hukommelsesforstyrrelser.

Afhandlingen kortlægger for første gang fordelingen og mængden af 5-$HT_4$ receptorer i grisens hjerne, og det vises med såvel *in vivo* som *in vitro* teknikker, at grisens 5-$HT_4$ receptordistribution ligner menneskehjernens.

Adfærdsmæssigt findes der ligheder mellem grisen og både gnavere og primater, men grisens kognitive egenskaber er stadig ikke særligt velbeskrevede, og der findes kun ganske få velvaliderede kognitive paradigmer, der har været anvendt i grisen. I denne afhandling beskrives valideringen af en spontan objekt-genkendelsesopgave og udviklingen og valideringen af en delayed non-match to sample (DNMS) opgave i en T-formet labyrint. Vi viser, at begge opgaver kan udføres af grise, og at antallet af korrekt udførte opgaver afhænger af længden af tidsrummet over hvilken hukommelsen er aktiveret. Vi viser også, at skopolamin nedsætter evnen til at løse DNMS opgaven.

Endelig har vi udviklet en metode til transgen ekspression i grisehjernen ved injektion af rekombinante adeno-associerede virale (rAAV) vektorer, og valideret forskellige vektorsubtyper med henblik på deres celletype-specificitet og spredningsmønster. Vi finder, at injektion af rAAV5 subtypen i striatum hos neonatale grise medfører en transduktionseffektivitet på op til 49.8 % ved injektionsstedet faldende med afstanden til dette. Vi observerede også transducerede celler i store områder af cortex. Kun ganske få af de transducerede celler (<2.4 %) var gliaceller, resten var neuroner.

Med denne afhandling er der dermed udviklet et metodologisk grundlag for at anvende grisen til studier af serotonerge mekanismer ved hukommelsesforstyrrelser. Det er således muligt at overføre traditionelle gnaver-baserede teknikker til grisen, og dette vil i fremtiden formentlig vise sig som et værdifuldt redskab for translationel hjerneforskning.

# 3 Table of contents

# 4 Abbreviations

| | |
|---|---|
| 1-TC | one-tissue compartment |
| 2-TC | two-tissue compartment |
| 5-HT | 5-hydroxytryptamin (serotonin) |
| AAV | adeno-associated virus |
| Aβ | amyloid beta |
| ACh | acetylcholine |
| AD | Alzheimer's disease |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| APC | adenomatous polyposis coli |
| APP | amyloid precursor protein |
| BP | binding potential |
| CA | Cornu Ammonis |
| cAMP | cyclic adenosine monophosphat |
| CNS | central nervous system |
| CT | computerised tomography |
| DNA | deoxyribonucleic acid |
| DNMS | delayed non-match to sample |
| fAD | familial Alzheimer's disease |
| GABA | gamma-aminobutyric acid |
| GFAP | glial fibrillary acidic protein |
| GFP | green fluorescent protein |
| $K_D$ | dissociation constant |
| LTD | long-term depression |
| LTP | long-term potentiation |
| MCI | mild cognitive impairment |
| MRI | magnetic resonance imaging |
| mRNA | messenger ribonucleic acid |
| MRTM | multilinear reference tissue model |
| nAChR | nicotinergic acetylcholine receptor |
| NeuN | neuronal nuclei |
| pCPA | para-clorophenylalanine |
| PET | positron emission tomography |
| PFC | prefrontal cortex |
| PKA | protein kinase A |
| PS | presenillin |
| rAAV | recombinant adeno-associated virus |
| ROI | region of interest |
| SERT | serotonin reuptake transporter |
| SOR | spontaneous object recognition |
| SRTM | simplified reference tissue model |
| TPH | tryptophan hydroxylase |
| $V_S$ / $V_T$ | specific / total volume of distribution |

# 5 List of papers

This thesis is based on the following papers and manuscripts:

I Birgitte R. Kornum, Nanna M. Lind, Nic Gilling, Lisbeth Marner, Flemming Andersen, and Gitte M. Knudsen. Evaluation of the novel 5-$HT_4$ receptor PET ligand [$^{11}$C]SB207145 in the Göttingen minipig. *Journal of Cerebral Blood Flow and Metabolism*, 2009:29:186-196.

II Birgitte R. Kornum, Kristin S. Thygesen, Thomas R. Nielsen, Gitte M. Knudsen, and Nanna M. Lind. The effect of the inter-phase delay interval in the spontaneous object recognition test for pigs. *Behavioural Brain Research*, 2007:181:210-217.

III Thomas R. Nielsen, Birgitte R. Kornum, Anette Moustgaard, Anders Gade, Nanna M. Lind, and Gitte M. Knudsen. A novel spatial delayed non-match to sample (DNMS) task in the Göttingen minipig. *Behavioural Brain Research,* 2009:196: 93-98.

IV Birgitte R. Kornum*, Simon R.W. Stott*, Bengt Mattsson, Liselijn Wisman, Anders Ettrup, Stephan Hermening, Gitte M. Knudsen, and Deniz Kirik. Adeno-associated viral vectors targeted to neonatal rat and pig striatum induce widespread transgene expression (manuscript).
* Equal contributions.

## INTRODUCTION

Research from the Neurobiology Research Unit and other groups has during the last decades explored the involvement of the serotonergic system in memory deficits. It is evident that serotonin controls certain aspects of memory function and that serotonergic functioning is adversely affected in several memory diseases such as Alzheimer's disease (see sections 6-8). What still remains to be elucidated, however, is whether there is a functional link between the serotonergic deficits and the memory disturbances in these diseases, or if these phenomena are occurring in parallel.

One approach to this question would be to perform in vivo imaging of serotonergic receptor levels and correlate these to cognitive performance in patients with different memory deficits. This kind of research is currently being conducted at Neurobiology Research Unit in human subjects, but to establish a causal link and to gain more detailed knowledge on how serotonergic malfunctioning influences learning and memory, it will be necessary to benefit from the possibilities of working with animal models.

Since the specificity of the serotonergic system is very much determined by the presence of multiple serotonergic receptor subtypes, one possible approach is to study how serotonergic malfunctioning influences learning and memory by investigating correlations between memory performance and serotonergic receptor levels in the same animals, and further to examine the effects of pharmacological manipulations. Modelling of memory disorders in the same animals would then further add valuable information.

With studies of psychological entities, such as learning and memory, and their biological basis, there is always the question of how results from non-human animal models translate to human biology. The choice of an animal model must therefore comprehend considerations regarding several aspects such as: available models, translational value, species specific methods, and not least practicalities regarding housing and costs.

Pigs are emerging as an exciting species within neuroscience research. The pig brain more closely resembles the human brain in general anatomy and biochemistry, than the smaller laboratory animals (see section 9). This potentially gives the pig a higher translational value. Further, the size of the pig brain facilitates surgical procedures (Dall *et al.*, 2002; Dalmose *et al.*, 2004; R: Bjarkam *et al.*, 2008), lesion studies (Eriksson *et al.*, 2002; Manley *et al.*, 2006), and stereotaxic injections (Felix *et al.*, 1999; Bjarkam *et al.*, 2004; Study IV), and also permits the identification of cortical and subcortical structures by imaging techniques such as magnetic resonance imaging (MRI, Watanabe *et al.*, 2001; Jelsing *et al.*, 2005) and positron emission tomography (PET, Stalnacke *et al.*, 1982; Poulsen *et al.*, 1997; Kornum *et al.*, 2009 - Study I).

The aim of this thesis was therefore to develop a basis for using the pig as a model for studying serotonergic function in relation to memory and memory disorders.

# THEORETICAL BACKGROUND

The literature on learning and memory, the serotonergic system, and interactions between these entities are immense. To focus this thesis, I have chosen to concentrate on the serotonergic receptor subtype 5-$HT_4$, since this receptor has several interesting functions related to memory and memory disorders. First, the 5-$HT_4$ receptor is expressed in abundance in hippocampal, cortical and striatal regions. Second, it is known to regulate acetylcholine (ACh) release, third it modulates long-term potentiation and depression, and fourth, it regulates the processing of amyloid precursor protein (APP) with implications for AD.

In the following sections, I will provide the theoretical framework for studying the 5-$HT_4$ receptor in learning and memory and in particular in AD using the pig. The introductions to the serotonergic system and to learning and memory will be limited to what is reasonably well accepted, and I will only present more speculative data with respect to the 5-$HT_4$ receptor. Because of the vast literature on these topics, for citations I rely on a mixture of primary research papers and reviews. To avoid confusion regarding this, I have marked reviews with R, when they are cited in the text.

## 6 The Serotonergic System

The serotonergic system plays a key modulatory role in the central nervous system (CNS). Even though the serotonergic system is present in multiple areas of the brain, its influence is by no means diffuse and non-selective. Multiple receptor subtypes and region specific innervations from various brainstem nuclei result in a complex pattern of modulatory control over various processes. These processes include: Affective states such as anxiety, fear, depression, and aggression; control of sleep; regulation of cognitive performance; modulation of ingestive behaviour; and influence on reward circuits that mediate for example motivation (R: Barnes and Sharp, 1999; R: Ursin, 2002; R: Schmitt *et al.*, 2006)

### 6.1 Serotonin

The neurotransmitter of the serotonergic system is serotonin, also called 5-hydroxytryptamin (5-HT, Rapport *et al.*, 1948; Rapport, 1949; Erspamer and Asero, 1952). The precursor of serotonin is the amino acid tryptophan, and tryptophan hydroxylase (TPH) catalyses the rate-limiting step in the serotonin synthesis (Jequier *et al.*, 1967). TPH is expressed by serotonergic neurons in the brainstem raphe nuclei and the melatonergic neurons in the pineal gland (Lovenberg *et al.*, 1967; Jequier *et al.*, 1969; Joh *et al.*, 1975; Austin and O'Donnell, 1999). Serotonergic neurons project from the raphe nuclei and ramify extensively in the CNS (R: Jacobs and Azmitia, 1992). After release from presynaptic serotonergic terminals, serotonin is free to interact with the various 5-HT receptors. Serotonin present in the synapse is inactivated by uptake into the presynaptic terminal by the serotonin reuptake transporter (SERT, Horschitz *et al.*, 2001).

## 6.2 Serotonergic receptors

Up until now 14 structurally and pharmacologically distinct mammalian 5-HT receptor subtypes have been described. These are assigned to one of seven families, 5-$HT_{1-7}$, based on their structure, affinity for different ligands, and second messenger activation (Hoyer *et al.*, 1994). All 5-HT receptors, except the 5-$HT_3$ receptor, are G-protein coupled seven transmembrane domain receptors. The 5-$HT_3$ receptor is a ligand-gated ion channel. The 5-$HT_1$ receptor subgroup includes five different receptors (5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1D,}$ 5-$ht_{1E}$[i], and 5-$HT_{1F}$), that all couple negatively to the adenylate cyclase via $G_{i/o}$-protein. The 5-$HT_2$ receptors (5-$HT_{2A}$, 5-$HT_{2B}$, and 5-$HT_{2C}$) all couple preferentially to $G_{q/11}$ to increase inositol phosphates and cytosolic $Ca^{2+}$, and the 5-$HT_{4-7}$ receptors (5-$HT_4$, 5-$HT_6$, and 5-$HT_7$) all couple to $G_s$ and promote cyclic adenosine monophosphat (cAMP) formation. The function of the 5-$ht_5$ (5-$ht_{5A}$ and 5-$ht_{5B}$) receptors is not established, neither is their preferential G-protein coupling (R: Hannon and Hoyer, 2008).

Autoradiographic studies and immunohistochemistry have demonstrated that each receptor subtype has its own highly distinct pattern of distribution in the CNS, and it is known that each receptor exerts its own specific actions in the brain, even though much still remains to be elucidated (R: Barnes and Sharp, 1999; R: Fink and Gothert, 2007; R: Hannon and Hoyer, 2008). In the next section, I will give a more detailed description of the 5-$HT_4$ receptor.

### *6.2.1 The 5-$HT_4$ receptor*

The 5-$HT_4$ receptor couple positively to adenylate cyclase via $G_s$-protein and activation thus increases intracellular levels of cAMP (Dumuis *et al.*, 1988; Bockaert *et al.*, 1990; Fagni *et al.*, 1992). 5-$HT_4$ stimulation increase neuronal excitability by reducing the $Ca^{2+}$-evoked $K^+$-currents responsible for after-hyperpolarisation, a process that involves protein kinase A (PKA) (Torres *et al.*, 1995; Ansanay *et al.*, 1995).

The expression of 5-$HT_4$ receptors has been examined in the brains of several species including guinea pig (Waeber *et al.*, 1993), rat (Waeber *et al.*, 1994; Jakeman *et al.*, 1994), mouse (Waeber *et al.*, 1994), pig (Kornum *et al.*, 2009 - Study I), monkey (Jakeman *et al.*, 1994), and human (Bonaventure *et al.*, 2000; Varnäs *et al.*, 2003) with a fairly consistent pattern across species. Minor differences have however been reported (Vilaro *et al.*, 2005). Receptor density is highest in the basal ganglia, olfactory tubercle, and hippocampus (granule cell layer of the dentate gyrus and pyramidal cell layer of the Cornu Ammonis (CA) areas and subiculum), with moderate levels in hypothalamus, septum, and amygdala, and lower levels in thalamus, neocortex, and the raphe nuclei. Comparison of mRNA distribution and radioligand binding has shown that the receptor is located on both somato-dendritic and axon terminal regions (Vilaro *et al.*, 1996; 2005). Lesion studies indicate that, in striatum, the receptor is present on interneurons as well as striatonigral and striatopallidal projection neurons and not on dopaminergic

[i] No direct evidence of functional native 5-$ht_{1E,\ 5A,\ and\ 5B}$ receptors exists and hence, in accordance with recommendations from the IUPHAR receptor nomenclature committee, lower case letters are used (Hoyer *et al.*, 1994; Hannon and Hoyer, 2008).

or serotonergic axon terminals (Patel *et al.*, 1995; Compan *et al.*, 1996; Vilaro *et al.*, 2005). There is further functional evidence that 5-$HT_4$ receptors might be present on cholinergic terminals in several brain areas (see section 7.2.1).

Acute stimulation of the 5-$HT_4$ receptor, via systemic injection of an agonist, increases the mean firing rate of serotonergic neurons in the raphe nuclei, while inhibition via antagonism decreases the firing rate (Lucas and Debonnel, 2002). This latter finding suggests the presence of a continuous endogenous stimulation of the 5-$HT_4$ receptor. Interestingly, the 5-$HT_4$ receptor further shows a rather high constitutive activity when expressed in cell cultures (Blondel *et al.*, 1998; Claeysen *et al.*, 1999).

The 5-$HT_4$ receptor gene is highly complex. Is has 38 exons and generates a number of C-terminal splice variants. Ten different splice variants have been reported so far, with distinct expression profiles and minor variations across species (Gerald *et al.*, 1995; Claeysen *et al.*, 1998; R: Bockaert *et al.*, 2008), little is however known on the functional differences between these splice variants, and in this thesis the 5-$HT_4$ receptor will be treated as one entity.

# 7 Learning and memory

Learning and memory are separate but not independent processes. Learning is the acquisition of new information, while memory is the process by which that information is encoded, stored and later retrieved. Memory is typically divided into different subtypes based on either the duration of the memory or the content.

In this chapter, I will give a short introduction to the major biological principles of learning and memory, and further briefly mention some of the processes involved at the cellular and molecular level. Finally, I will review the influence of the 5-$HT_4$ receptor in learning and memory.

## 7.1 Memory classifications and anatomical basis

Memory can be classified in a number of ways, depending on the criterion used. Memory classifications are still a subject of great debate, and here I will try to give a short review of the most important theories.

With duration as the criterion, three different types of memory seem to exist. A very brief sensory kind of memory during perception, a short-term memory often included in or equalled to working memory, and long-term memory.

### *7.1.1 Perception*

Perception can be described as the retrieval and processing of sensory information. In the process of perception the information provided by the senses is retained accurately but very briefly. These fleeting perceptual representations have to be consolidated into working memory to survive the presentation of new sensory inputs. This process is very fast – a recent suggestion is 50 ms/item (Vogel *et al.*, 2006).

### *7.1.2 Working memory*

Working memory seems to be a central battleground of cognitive psychologists, and a myriad of definitions have been proposed. The distinction between working memory and short-term memory is further clouded by different investigators using different terms. Here I will however try to give a short description of this very important concept, that by some researcher are even taken to be at the very centre of consciousness (The global workspace theory, Baars and Franklin, 2003).

Short-term memory (sometimes called primary memory) refers to the capacity for holding a small amount of information in an active, readily available state (Atkinson and Shriffin, 1968; R: Cowan, 2008). An often cited storage capacity is 7±2 elements (Miller, 1956), but recent data suggests the best estimate of short-term memory to be about three pieces or chunks of information (Chen and Cowan, 2009). Short-term memory is classically seen as memory that decays over short time spans and is sensitive to interference (R: Cowan, 2008). Most researchers today use the concept of working memory to replace or include the older concept of short-term memory, thereby marking a stronger emphasis on the notion of manipulation of information instead of passive maintenance. Working memory has been defined in at least three slightly different

ways: as memory used actively to plan and carry out behaviour (Miller *et al.*, 1960), as the combination of multiple components working together to hold and manipulate information in short-term memory (Baddeley and Hitch, 1974; Baddeley, 2000; Cowan, 2008), and as a reference only to the attention-related aspects of short-term memory (Engle *et al.*, 1999).

According to the famous Baddeley and Hitch multi-component model two "slave systems" (the phonological loop and the visuospatial sketch pad) and the episodic buffer are responsible for short-term maintenance of information, and a "central executive" is responsible for directing attention to relevant information, and for coordinating cognitive processes (Baddeley and Hitch, 1974; Baddeley, 2000). Cowan (2008) supports the notion of a multi-component system, but regards working memory not as a separate system, but included in long-term memory as activated memory representations of which a capacity limited subset is at the focus of attention. Results from both animal research (Fuster, 1973), brain lesion studies (Ghent *et al.*, 1962; D'Esposito and Postle, 1999; R: Muller and Knight, 2006) and functional brain imaging in humans (Jonides *et al.*, 1993; Wager and Smith, 2003) have suggested that the prefrontal cortex (PFC) is the most important substrate for working memory. Since the PFC has been found to be active in a variety of tasks that require executive functions, some researchers argue that the role of PFC in working memory is in controlling attention, selecting strategies, and manipulating information in working memory, but not in maintenance of information. The maintenance function is attributed to more posterior areas of the brain, including the parietal cortex (Kane and Engle, 2002; R: Curtis, 2006).

### *7.1.3 Long-term memory*

Long-term memory is memory that is stored for a shorter while and up to an entire life time. Long-term memory is divided into two main types: declarative and non-declarative memory, based on whether the memory's content can be expressed verbally (Figure 1). Declarative or explicit memory is the conscious acquisition of knowledge about people, places and things, and non-declarative or implicit memory is the non-conscious learning of motor skills and other tasks (R: Squire, 2004).

Declarative memory has two major subdivisions: Episodic memory refers to memory for specific events in time and semantic memory refers to knowledge about the world independent of time and place (Tulving, 1983). The medial temporal lobe has been a site of major interest in understanding declarative memory processes and, in particular, episodic memory, since the famous case-study of the unfortunate patient H.M. who was subjected to a bilateral medial temporal lobectomy (Scoville and Milner, 1957). As a consequence he developed severe amnesia, but had no deficits in for example procedural learning and memory (Milner, 1962; Squire, 2009).

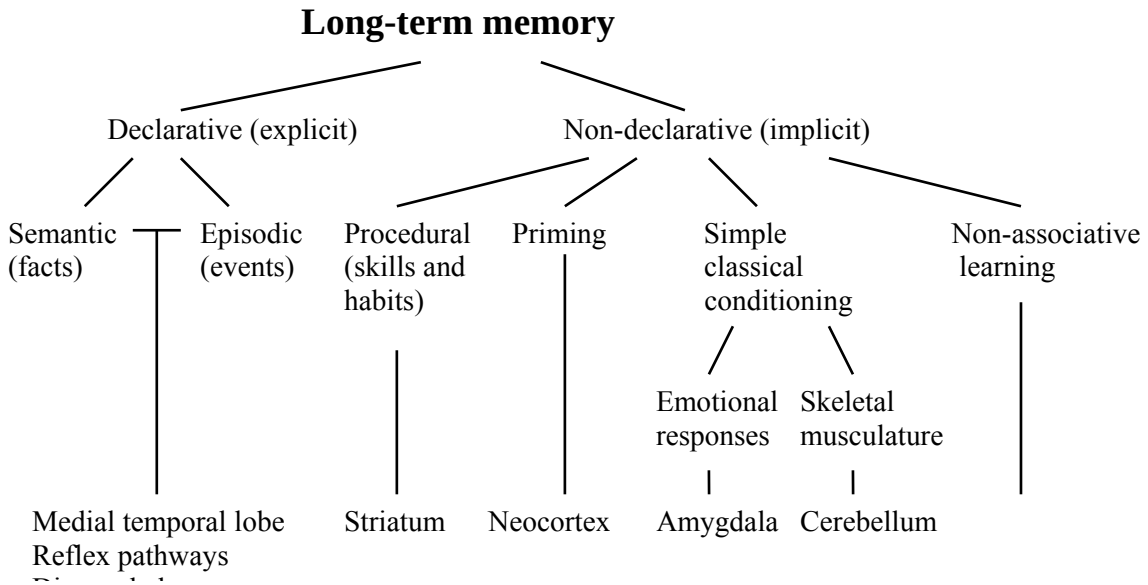


**Figure 1:** A taxonomy of mammalian memory systems listing the brain structures thought to be especially important for each form of declarative and non-declarative memory.
Modified from Squire 2004.

Knowledge stored as declarative memory is first acquired through processing in one or more of the three polymodal association corticies (prefrontal, limbic, and parieto-occipital-temporal cortices) that synthesize visual, auditory, and somatic information. From there information is conveyed to the medial temporal lobe where it passes through parahippocampal and perirhinal cortices, then the entorhinal cortex, the dentate gyrus, CA3, CA1, the subiculum, and finally back to the entorhinal cortex. From here information is sent back via the parahippocampal and perirhinal cortices to the association areas in neocortex where long-term memory is probably stored (R: Squire *et al.*, 2004; R: Eichenbaum *et al.*, 2007). Memory impairments associated with the entorhinal cortex are particularly severe, and this is also the site of early pathological changes in AD (see section 8.1.2). The perirhinal cortex appears to be of particular importance for object recognition memory, whereas the hippocampus is critical in spatial memory (Broadbent *et al.*, 2004; Forwood *et al.*, 2005; R: Winters *et al.*, 2008). Consensus regarding the specific role of the hippocampus has however still not been reached. According to Squire (2004) information that has been encoded in long-term memory for a lengthy period of time no longer requires the hippocampus, but activates instead the frontal and temporal cortices. Others believe that both recent and remote episodic memories depend on the hippocampus, whereas semantic memory does not (R: Moscovitch *et al.*, 2005). Recognition memory is often divided into two distinct processes: recollection, which corresponds to episodic memory, and familiarity, which is the ability to recognize an item without retrieving the context in which the item was encountered (R: Yonelinas, 2002). Several experimental data support the theory that while recollection is dependent on the hippocampus, familiarity is supported by the surrounding parahippocampal region (Eichenbaum *et al.*, 1994; Brown and Aggleton, 2001; Fortin *et al.*, 2004; R: Eichenbaum *et al.*, 2007).

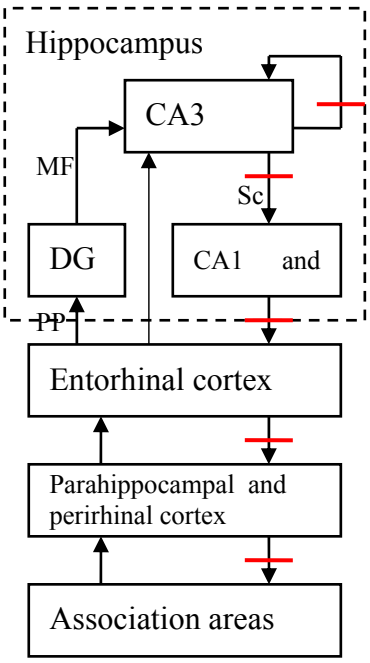


**Figure 2.** Schematic drawing of declarative memory networks. During encoding information from the neocortex flow to the hippocampus. Meanwhile acetylcholine suppresses the feed back connections, thus preventing interference from previous encoded memories (red bars). DG: dentate gyrus, CA: Cornu Ammonis area, Sb: subiculum, Sc: Schaffer collaterals, MF: mossy fibers, PP: perforant pathway. Modified from Hasselmo et al., 2006.

Declarative memory results from at least four related but distinct types of processing: acquisition, consolidation, storage, and retrieval. Acquisition is the process by which new information is attended to and processed. Consolidation refers to those processes that alter the newly stored and still labile information to make it more stable for long-term storage. Retrieval permits recall and use of the stored knowledge. Here different kinds of information stored separately are brought together.

The other major form of long-term memory is non-declarative memory (R: Squire, 2004). It is also known as implicit memory, because it is expressed by means other than words. Non-declarative memory is divided into several sub-categories. The best known of the various types of non-declarative memory is procedural memory, which enables people to acquire motor skills and gradually improve them. Procedural memory is associated with the cerebellum, the basal ganglia, and the motor cortex, all of which are involved in motor control. Non-declarative memory also comprises priming and conditioned reflexes. Some very intense personal memories that bring conditioned emotional responses, sometimes called emotional memory, into play also classify as non-declarative and involve the amygdala (R: Phelps and LeDoux, 2005). The associative learning that constitutes the basis for these forms of memory can take place without the intervention of the conscious mind.

### *7.1.4 The 5-HT$_4$ receptor in learning and memory*

There is increasing evidence showing that, among its many other functions, serotonin is associated with cognitive functions especially learning, memory, and attention. These functions are not independent from each other, and serotonin can thus modulate memory in both direct and indirect ways. Here I will describe how serotonin via the 5-HT$_4$ receptor can influence different aspects of memory.

Behavioural experiments have generally confirmed a pro-cognitive effect of 5-HT$_4$ receptor agonists with several aspects of memory performance being modulated. In rats, memory acquisition is enhanced following injection of 5-HT$_4$ agonists in several tasks including social olfactory memory (Letty *et al.*, 1997), olfactory associative memory (Marchetti-Gauthier *et al.*, 1997; Marchetti *et al.*, 2000), autoshaping (Meneses and Hong, 1997), place and object recognition (Lamirault and Simon, 2001; Orsetti *et al.*, 2003), and the Morris water maze (Fontana *et al.*, 1997; Lelong *et al.*, 2001).

Stimulation of 5-$HT_4$ receptors in both younger and older macaques enhances delayed matching performance (Terry, Jr. *et al.*, 1998). These results suggest that the 5-$HT_4$ receptor has an important role in both working memory and long-term declarative memory. One study has shown that antagonists alone can interfere with olfactory associative memory (Marchetti *et al.*, 2000)

5-$HT_4$ receptors seem to have differing effects on the different stages of memory processing. In most studies the 5-$HT_4$ agonist is injected before the acquisition phase, and here a clear positive effect is found as described above. The effect on memory consolidation is less clear. Consolidation does not seem to be affected by systemic injection of the partial agonist RS67333 whereas local injections in the nucleus basalis magnocellularis enhances it (Lamirault and Simon, 2001; Orsetti *et al.*, 2003) and negative effects on consolidation has also been reported (Meneses and Hong, 1997). More recently it has been suggested that both 5-$HT_4$ mRNA and receptor protein increases during memory consolidation most notably in cortical areas (Manuel-Apolinar *et al.*, 2005; Perez-Garcia *et al.*, 2006). There does not seem to be an effect of the 5-$HT_4$ receptor on memory retrieval (Lamirault and Simon, 2001).

5-$HT_4$ agonists further reverse scopolamine induced deficits in different cognitive paradigms, suggesting that the 5-$HT_4$ receptor has a regulatory effect on cholinergic neurotransmission (Matsumoto *et al.*, 2001; Cachard-Chastel *et al.*, 2008; see also next section).

5-$HT_4$ knock-out mice have been developed, and the mice survive to adult-hood (Compan *et al.*, 2004). These mice are however not ideal for studies of the 5-$HT_4$ receptor in cognitive functions, since their dorsal raphe serotonergic neurons exhibit reduced spontaneous activity associated with diminished tissue levels of serotonin (Conductier *et al.*, 2006).

## 7.2 The cholinergic system and memory

The cholinergic system has been widely studied in connection with memory and memory dysfunction boosted by the formulation of the ‘cholinergic hypothesis of geriatric memory dysfunction’ (Bartus *et al.*, 1982).

ACh is known to enhance both encoding and attention by enhancing the influence of sensory input relative to the internal processing of stimuli (Figure 2; R: Hasselmo and Giocomo, 2006). Since the early discovery by Drachman and Leavitt (1974), that muscarinic receptor antagonism could induce amnesic-like deficits in healthy subjects, a number of subsequent studies have shown that antagonism of both muscarinic receptors and nicotinic receptors can induce deficits in a range of cognitive domains including attention and memory (R: Bartus, 2000).

Numerous memory studies demonstrate that blockade of muscarinic ACh receptors by systemic administration of the drug scopolamine interferes with the encoding of new information, while having little effect on retrieval of previously stored information (R: Hasselmo and Giocomo, 2006). Scopolamine appears to primarily affect episodic memory, while sparing semantic and procedural memory (Atri *et al.*, 2004).

Cholinergic agonists (both muscarinic and nicotinic) has also been shown to enhance activity-dependent synaptic plasticity, such as long-term potentiation at a number of different synaptic pathways including the perforant pathway input to the dentate gyrus and the Schaffer collateral input to region CA1 (Huerta and Lisman, 1993; Sawada *et al.*, 1994; R: Kenney and Gould, 2008)

Even though the cholinergic system plays an important role in memory and learning other modulatory neurotransmitter systems also affects these processes, and interactions are further known to occur between the different neurotransmitter systems (R: Decker and McGaugh, 1991; R: Fink and Gothert, 2007).

### *7.2.1 The 5-HT$_4$ receptor modulates acetylcholine release*

There are several reports demonstrating the ability of 5-HT$_4$ receptors to modulate the release of various neurotransmitters including ACh. A global stimulation of 5-HT$_4$ receptors increase both hippocampal and frontal cortex ACh release in freely moving rats (Consolo *et al.*, 1994; Matsumoto *et al.*, 2001). This effect is at least partly mediated by local 5-HT$_4$ receptors situated on the cholinergic nerve terminals (Yamaguchi *et al.*, 1997; Siniscalchi *et al.*, 1999). In addition, local 5-HT$_4$ stimulation in the hippocampus also increases ACh release (Siniscalchi *et al.*, 1999; Matsumoto *et al.*, 2001). Of notice the 5-HT$_4$ receptor has also been shown to modulate GABA release in the hippocampus (Bianchi *et al.*, 2002) and dopamine release in the striatum (Bonhomme *et al.*, 1995), both of these modulatory effects are however indirect and mediated by the release of ACh and glutamate, respectively (Steward *et al.*, 1996; Bianchi *et al.*, 2002). As further evidence to the interaction between 5-HT$_4$ receptors and the cholinergic system, it has been shown that sub-efficacious doses of 5-HT$_4$ agonists and cholinesterase inhibitors enhance spatial memory and object recognition synergistically (Moser *et al.*, 2002; Mohler *et al.*, 2007; Cachard-Chastel *et al.*, 2008), and two 5-HT$_4$ receptor partial agonists (SL 65.0155 and VRX-03011) are currently in phase II clinical trials for the treatment of Alzheimer's disease (Moser *et al.*, 2002; Mohler *et al.*, 2007).

## 7.3 The molecular basis of memory and learning

Using biological terms memory can also be divided into different forms according to the underlying molecular processes at the synapse. The fastest (milliseconds) synaptic action stem from ion flux through transmitter-gated ion channels. Various neurotransmitters (such as serotonin) can also mediate slower synaptic activity such as regulation of transmitter release (minutes) and long-term changes (days – years) via activation of gene expression leading to structural changes in the synapse and growth of new synaptic connections (R: Kandel, 2001). In *Aplysia* sensory neurons, serotonin induces both a short-term enhancement of synaptic strength via a cAMP/PKA induced enhanced transmitter availability and release (Brunelli *et al.*, 1976), and a long-term change in synaptic architecture specifically targeted to the stimulated synapses via local protein synthesis (Casadio *et al.*, 1999). In the following sections, I will introduce some

of the molecular processes that underlie memory formation and their modulation by 5-$HT_4$ receptor activation.

### 7.3.1 Long-term potentiation and long-term depression

The Spanish neuroanatomist Santiago Ramón y Cajal was the first to suggest a mechanism of learning that did not require the formation of new neurons. In 1894, he proposed that memories might instead be formed by strengthening the connections between existing neurons to improve the effectiveness of their communication (Croonian Lecture, retold in: Jones, 1994). This has turned out to hold true.

At the cellular level, long lasting changes in synaptic strength, also called synaptic plasticity, occurs in response to both electrical and neurotrophic stimulation. The most studied forms of synaptic plasticity in mammals are long-term potentiation (LTP) and long-term depression (LTD) (R: Malenka and Bear, 2004). Since the original discovery of LTP in the rabbit hippocampus (Bliss and Lomo, 1973; Douglas and Goddard, 1975), LTP and LTD has been observed in a variety of other species and brain areas, including the cerebral cortex, striatum (Uno and Ozawa, 1991), amygdala (Rogan *et al.*, 1997), and many others. LTP and LTD in general occurs as a result of synaptic activity and a subsequent up or down regulation of AMPA receptor activity. The synaptic modulations can be either short- or long-lasting, the latter being dependent on new protein synthesis. The mechanism behind LTP and LTD in different areas is however not the same, and it is therefore more useful to conceptualize LTP as a general class of cellular and synaptic phenomena. Current research are still trying to work out the details in the signalling pathways responsible for LTP, and it has become clear that a multitude of different forms exist (R: Malenka and Bear, 2004).

### 7.3.2 The 5-$HT_4$ receptor in long-term potentiation and depression

Accumulating evidence indicates that the 5-$HT_4$ receptor modulates synaptic plasticity. For instance, recently systemic injection of the partial 5-$HT_4$ receptor agonist SL65.0155 has been shown to potentiate learning-induced dendritic spine growth in the mouse hippocampus (Restivo *et al.*, 2008). 5-$HT_4$ stimulation has also been shown to modulate both LTP and LDP in the hippocampus and amygdala.

Systemic injection of the 5-$HT_4$ agonists increase LTP in the dentate gyrus and CA1, while antagonists accelerate the late depotentiation of LTP, thus decreasing the synaptic strengthening (Matsumoto *et al.*, 2001; Marchetti *et al.*, 2004). Three studies from an other group report that the partial 5-$HT_4$ agonist RS67333 inhibits synaptic LTP in the dentate gyrus and LDP in CA1, and further that it inhibits depotentiation of LTP in both of these areas suggesting a rather complex modulatory effect of 5-$HT_4$ receptor stimulation (Kulla and Manahan-Vaughan, 2002; Kemp and Manahan-Vaughan, 2004; 2005), in these studies the 5-$HT_4$ receptor agonist was however given via intraventricular injection, and this makes it difficult to separate direct effects on the hippocampus from indirect effects via e.g. the raphe nuclei. In the 2002 study, the authors show that RS67333 depresses basal synaptic activity in the hippocampus, a phenomenon that in a brain slice study have been found to be locally mediated by 5-

$HT_{1A}$ receptors (Huang and Kandel, 2007). This study was however performed on amygdala slices, and here they further reported that local stimulation of the 5-$HT_4$ receptor induces long-lasting LTP. This was blocked by inhibitors of either PKA or mitogen-activated kinase, and also by the actin inhibitor cytochalasin D, suggesting that a cytoskeletal rearrangement is involved in the synaptic strengthening. A study by Matsumoto and colleagues (2001) showed that the 5-$HT_4$ receptor agonist SC 53116 augments LTP in the CA1 field at least partly via the release of ACh. Together these studies suggest that stimulation of the 5-$HT_4$ receptor locally increases LTP. Further studies are however needed to firmly establish the role of the 5-$HT_4$ receptor in LTP and LDP.

# 8 Memory disorders

Several different types of memory disorders occur in humans. Minor everyday slips and lapses of memory are fairly commonplace, and may increase naturally with age, when ill, or when under stress (Reason, 1995). More serious problems with memory generally occur due to traumatic brain injury or neurodegenerative disease, such as Alzheimer's disease (AD), and learning and memory impairments are also seen in disorders such as addictions, anxiety, depression, and schizophrenia.

In this section I will however keep my focus on AD, and describe the current knowledge of this disease and how both the cognitive deficits seen in AD and the molecular events behind this disease are influenced by 5-$HT_4$ receptors.

## 8.1 Alzheimer's disease

### *8.1.1 Definition and Ethiology*

AD is a chronic brain disorder characterized by the loss of cognitive functions and by specific pathological changes in the brain. The definite diagnosis of AD is based on the neuropathologic abnormalities originally reported by Alois Alzheimer in 1907, who noted the occurrence of numerous senile plaques and neurofibrillary tangles in the cerebral cortex of a demented patient (Alzheimer *et al.*, 1995). The first cognitive symptoms in AD are usually a decline in memory and an inability to learn new material. Episodic memory is severely affected by the disease, and recent research also suggests semantic memory to be disturbed early in the disease, but this is still controversial (Vogel *et al.*, 2005). Later on, memory disturbances become more severe, including a worsening of remote memory. Clinically, the diagnosis of probable Alzheimer's disease is made when symptoms have an insidious onset, and there is progression of dementia, and no other systemic or brain disease can account for the dementia (McKhann *et al.*, 1984).

Besides memory disturbances, behavioral and psychological symptoms of dementia occur in 50-90% of AD-patients. They can occur at any point in the disease process, but the overall number and severity of behavioral problems increase with worsening of cognitive impairment. The most common symptoms include delusions, hallucinations, depression, insomnia, aggression, and restlessness (R: Parnetti *et al.*, 2001).

The disease cases are divided in two groups: late-onset AD, also known as spontaneous AD, and the rarer early-onset AD or familial AD (fAD). In the clinic, a preclinical stage called mild cognitive impairment (MCI) is characterized by memory complaints and impairments on formal testing but no other cognitive deficits (Petersen *et al.*, 1999). Patients with MCI have an increased risk of progression into AD, but not all patients that meet the criteria for MCI develop AD (Busse *et al.*, 2006; R: Petersen and Negash, 2008).

### *8.1.2 Pathology*

The pathological hallmarks of AD fall in two groups: extracellular plaques and intracellular tangles. Both phenomena are complex protein aggregates and a lot is

already known about their structure, the proteins involved, and the way they develop (see section 8.1.3).

Tangles, in particular, display a characteristic distribution pattern that allows disease progression to be divided into six stages known as the Braak-stages I to VI. During these stages tangles develop first in the perirhinal and entorhinal cortices and then the hippocampal formation. From here the lesions extend into the high order sensory association areas of the neocortex, then the frontal and occipital cortex and finally the secondary and primary sensory motor areas of the neocortex become involved. In all stages the lesions in previously involved sites worsen. The development of plaques also follows a specific pattern, although the distribution seems to vary widely between individuals. Low densities of amyloid deposits first develop in the basal portions of the frontal, temporal, and occipital lobes, while the primary sensory and motor fields remain free of plaques. The hippocampal formation is gradually involved. In the final stage, virtually all isocortical areas have densely packed deposits, and a gradual involvement of numerous subcortical structures is seen (Braak and Braak, 1991). Using a combination of five in vivo imaging techniques (structural and functional MRI, PIB-, $H_2O$- and FDG-PET), Buckner and colleagues (2005) have suggested that the areas first affected in AD are in fact more posterior to the medial temporal lobe (see description of the default network theory in section 8.1.5).

A large pool of research has tried to pin point the pathological feature that correlates most precisely with the clinical observations in AD (e.g. Arnold *et al.*, 1991; Hyman and Gomez-Isla, 1994; Duyckaerts *et al.*, 1997; Delacourte *et al.*, 1999; Thal *et al.*, 2004), with results pointing at cortical tangles. In a recent study, the character, abundance, and distribution of the different lesions were correlated with clinical signs of MCI and AD. The authors conclude that the memory deficits correlate most closely with an abundance of tangles in CA1 of the hippocampus and in the entorhinal cortex (Markesbery *et al.*, 2006). There are some discussions in the literature regarding the extent of cortical cell loss in AD (R: Stark *et al.*, 2005), with studies showing from no cell loss (Regeur *et al.*, 1994) to a striking reduction (Bussiere *et al.*, 2003), but, in contrast, there is a large pool of firm evidence for a progressive loss of neurons in both nucleus basalis of Meynert and hippocampus (especially the CA1 field) (Davies and Maloney, 1976; Whitehouse *et al.*, 1981; Davies *et al.*, 1992; West *et al.*, 1994).

### *8.1.3 Molecular mechanisms and their modulation by the 5-HT$_4$ receptor*

It is out of the scope of this thesis to give a full account on the current knowledge on the molecular events leading to AD. To be able to fully appreciate the intimate connection between the 5-$HT_4$ receptor and AD pathology, it is however necessary to briefly introduce the reader to some of the most well understood concepts.

The main component of plaques is the small peptide amyloid-beta (Aβ) (Glenner and Wong, 1984a; 1984b; Wong *et al.*, 1985; Masters *et al.*, 1985a; 1985b). Aβ has the ability to self-aggregate and form dimers, oligomers, and larger aggregates. It is believed that Aβ-oligomers seed the formation of plaques and further that these small

molecules are highly neurotoxic, and a large pool of research is therefore centred on the Aβ peptide.

It is becoming more and more evident that oligomers of Aβ are the major cause of memory disturbances in early AD, while tangles cause the neurodegeneration in the late stages of the disease. Plaques may contribute as an activator of the immune system, but also serve a role as non-toxic deposits of Aβ (R: Clippingdale *et al.*, 2001; R: Walsh and Selkoe, 2004; R: Goedert and Spillantini, 2006; R: Hardy, 2006).

Aβ is formed by proteolytic cleavage of the membrane bound protein amyloid precursor protein (APP) (Kang *et al.*, 1987). A very recent study has shown that a n-terminal fragment of APP can mediate cell death, but it remains to be shown whether this has any relevance in AD (Nikolaev *et al.*, 2009). The protein-complexes responsible for the formation of Aβ are called beta-secretase and gamma-secretase. The gamma-secretases are also known as presenillin 1 and 2 (PS1 and PS2). APP further has a cleavage site situated within Aβ for a secretase called the alpha-secretase. Cleavage by this secretase is therefore often referred to as the non-amyloigenic pathway. Stimulation of the 5-$HT_4$ receptor has been shown to modulate APP processing by increasing the activity of the alpha-secretase via cAMP production and the exchange factor Epac (Lezoualc'h and Robert, 2003). In line with this, it has also been shown that the 5-$HT_4$ receptor agonist RS67333 inhibits the generation of Aβ in primary cortical neurons (Cho and Hu, 2007). This effect on APP processing is however not unique to the 5-$HT_4$ receptor, since increased alpha-secretase cleavage of APP also has been observed following agonist stimulation of muscarinic M1 and M3 receptor subtypes (Nitsch *et al.*, 1992).

### *8.1.4 Neurotransmitter deficits in AD*

Impairment of cholinergic transmission and decreased numbers of nicotinic binding sites are well-known features accompanying the cognitive dysfunction seen in AD (R: Bartus, 2000; R: Davies, 2006). Basal forebrain cholinergic neurons project to the hippocampus and cortex and these neurons are severely atrophic in AD brains (Davies and Maloney, 1976; Whitehouse *et al.*, 1981). A consistent, significant loss of nAChRs has also been observed in post mortem AD brains relative to age-matched healthy subjects, and it is furthermore believed that the nAChR deficits occur early in the course of the disease (R: Nordberg, 2001). It has been observed that both the decreased choline acetyltransferase activity, and the cortical nAChR deficits correlate well with the degree of cognitive impairment in AD (Wilcock *et al.*, 1982; Nordberg *et al.*, 1995). It is also known that Aβ binds with high affinity to the nAChR subunit alpha7 (Wang *et al.*, 2000; Liu *et al.*, 2009). The cholinergic deficit is however only an incomplete account of the total AD pathology, and even though acetylcholinesterase inhibitors are the current standard treatment of AD, they have only modest clinical benefits and no effect on the overall progressive deteoriation of the illness (R: Birks, 2006).

In AD, the decline of several receptor systems seems accelerated compared to normal aging (R: Knudsen, 2003). A high number of post mortem studies of AD brains find decreases in an equally high number of different neurotransmitter receptors. This

reflects the devastating cause of the disease, and can be seen as an unspecific destruction of eventually all important brain systems with death as a consequence. To understand more about the early disease mechanisms, a growing number of studies take advantage of the non-invasive technique positron emission tomography (PET, described in more detail in section 10.2). A considerable body of evidence from PET studies has confirmed the early decrease in cholinergic markers (R: Nordberg, 2001), and considerable interest has also been devoted to the dopaminergic (R: Tatsch, 2008) and serotonergic systems. Only a few serotonergic markers can be imaged by PET, and among these a decrease in 5-$HT_{1A}$ (Kepe *et al.*, 2006) and 5-$HT_{2A}$ (Blin *et al.*, 1993) receptors has been reported in AD patients. A study of patients with MCI has further suggested that the decrease in 5-$HT_{2A}$ receptors is an early event in the development of AD (Hasselbalch *et al.*, 2008), whereas the 5-$HT_{1A}$ receptor has been observed to be up-regulated in MCI patient brains (Truchot *et al.*, 2007).

The 5-$HT_4$ receptor has also been studied in post mortem AD brain, and reports suggests decreased levels in hippocampus (Reynolds *et al.*, 1995), but conflicting results regarding the frontal and temporal cortices (Reynolds *et al.*, 1995; Lai *et al.*, 2003). Neurobiology Research Unit is currently conducting a PET study with the purpose of clarifying 5-$HT_4$ receptor changes in AD.

Evidence also exists of reduced serotonin levels in AD brains. Decreased serotonin concentration and increased 5-$HT_{1A}$ receptor density in the post-mortem frontal cortex has been associated with a faster rate of cognitive decline (Lai *et al.*, 2002). The negative correlation between serotonin concentration and 5-$HT_{1A}$ receptor density may be due to an up-regulation of the receptors in response to serotonin deficits, and this hypothesis also offers an explanation to the up-regulation of 5-$HT_{1A}$ receptors in MCI patient brains. AD also affects serotonergic cells, and in post mortem AD brains tangles are found not only in cholinergic perikarya, but also in the serotonergic cells in the raphe nuclei (Rub *et al.*, 2000).

### *8.1.5 What triggers AD development?*

An emerging theory regarding the distribution of AD pathology links default brain activity with amyloid-deposition. Buckner and colleagues (2009) have identified a set of hubs in the posterior cingulate, lateral temporal, lateral parietal, and medial/lateral prefrontal areas of the cortex, whose activity highly correlate with multiple other parts of the brain. The distribution of hub activity is significantly correlated with amyloid deposition. Interestingly, the five core hubs lie in the default mode network, a set of interconnected brain areas that are most active when the brain is not challenged by mental tasks or sensory stimulation (Raichle *et al.*, 2001). It has previously been showed that the default network is a preferential site for amyloid deposition (Buckner *et al.*, 2005), and an other study has established that disruption of this network is a marker for early AD (Greicius *et al.*, 2004). These results led to the speculation that the high level of background activity in the default network could account for the preferential accumulation of Aβ in those regions as a result of activity-dependent processing of APP. In support of this hypothesis it has been shown experimentally, using an in-vivo

combined field potential recording and microdialysis system, that increasing synaptic activity drives up levels of Aβ in the interstitial fluid (Cirrito *et al.*, 2005). Even though this hypothesis is gaining increasing popularity it is still unclear why some brains eventually accumulate Aβ while others do not.

## 8.2 Other memory disorders

A myriad of memory disorders exist and serotonergic functioning has been studied in several of these. Very little is however known about the involvement of the 5-$HT_4$ receptor in dementia conditions besides AD.

Some neurodegenerative diseases, such as Parkinson's or Huntington's disease, primarily affect the basal ganglia, and reduced striatal 5-$HT_4$ receptor levels have been reported in Huntington's patients (Reynolds *et al.*, 1995). In these diseases procedural memory rather than declarative memory is affected. One single study describes the treatment of Parkinson's patients with a 5-$HT_4$ agonist. In this study mosapride was found to increase gastric motility and improve motor functions in Parkinson's patients, the authors did, however, not look at cognitive effects (Asai *et al.*, 2005).

Since activation of the 5-$HT_4$ receptor increases memory functions (as described in section 7.1.4), it is tempting to speculate, that 5-$HT_4$ agonists in general could be used as cognition enhancers, although gastrointestinal side-effects may limit their use (R: Kamm, 2002). Clinical studies are needed to test this hypothesis, and the pharmaceutical industry is indeed currently developing 5-$HT_4$ compounds for CNS indications.

## HYPOTHESIS

As described in the previous chapters, activation of the 5-$HT_4$ receptor increases memory acquisition and consolidation possibly via modulation of LTP and LDP and also via a facilitatory effect on ACh release. Activation of the 5-$HT_4$ receptor further increases the "non-pathogenic" APP processing pathway. Since the 5-$HT_4$ receptor has a high constitutive activity, we speculated whether variations in the basal level of 5-$HT_4$ receptors in the brain and especially in the hippocampus could account for individual differences in memory performance. We also speculated whether high numbers of constitutively active 5-$HT_4$ receptors could increase the threshold for developing amyloid-related pathology, in other words if individual "AD-resistance" is partly determined by the basal level of 5-$HT_4$ receptors.

Since Göttingen minipigs are relatively out bred compared to rats (Brandt and Möllers, 1999), they provide a feasible model for studies of the functional consequence of individual variations in 5-$HT_4$ receptor levels. Göttingen minipigs offer further advantages, as will be described in next section, but a clear disadvantage is the lack of well validated methods for studies of brain receptors and cognition in this species.

We therefore decided to develop a methodological basis for studies of individual 5-$HT_4$ receptor levels in pigs and their relevance for memory performance and AD susceptibility.

# METHODOLOGICAL CONSIDERATIONS

## 9 The pig as an experimental animal

The use of pigs (*Sus scrofa*) in biomedical research is well established in particular in surgical and physiological research (Tumbleson, 1986). Within brain research the large bulk of results are attributable to rodents and invertebrates, but there is a growing interest in large animal brain research using pigs and also other species, like cat, dog, sheep, and primate.

Compared to non-human primates, the pig is affordable, it is easily housed and its use may potentially avoid some of the ethical dilemmas concerning the use of primates as laboratory animals (Goodman and Check, 2002). Compared to rodents and invertebrates the larger brain size enables the use of human clinical equipment, e.g. conventional brain scanners, and further the greater complexity of the pig brain enables a more direct translation to human brain function. Neuroscience studies using a large animal such as the pig therefore complement promising small animal basic studies by bridging small animal brain research to understanding of the human brain.

### 9.1 The pig brain

The adult pig brain weighs 80-180 g depending on the strain and is, like the human brain, gyrencephalic in contrast to the lisencephalic rodent brain (Herre, 1936; Hofman, 1985). The gyri and sulci patterns of the pig brain have been described in several cytoarchitectural studies (Campbell, 1905; Stephan, 1951; Kruska, 1970), however the assessments of gyri and sulci homologies to humans have been rather inconsistent. This is further complicated by the apparent inter-individual variability in pigs, and variability between strain and gender (Herre, 1936; Stephan, 1951; Kruska and Rohrs, 1974). The organisation of the main cortical lobes is in general somewhat different to that of primates, the most obvious difference being the insular lobe, which is observed as a large region on the lateral side of the brain overlying the claustrum. In primates, the insular is buried in the depths of the lateral sylvian sulcus concealed by the temporal lobe. Subcortical pig brain anatomy is quite well defined (Yoshikawa, 1968; Felix *et al.*, 1999). The striatal part of the pig brain has distinct caudate and putamen structures divided by the internal capsule (Matsas *et al.*, 1986), like in the primate, but in contrast to the single caudate-putamen complex in the rodent brain. The topology of the pig hippocampus has been extensively studied, and indicates a degree of encephalisation in the pig that lies between that of rodents and primates (Holm *et al.*, 1990; Holm and Geneser, 1991a; 1991b; Holm and West, 1994).

The pig brain has been used extensively to model human brain development (R: Niblock *et al.*, 2005). The brain growth spurt for pigs is similar to that in humans, extending from late prenatal to early postnatal life (Dickerson and Dobbing, 1967; Pond *et al.*, 2000), whereas neurogenesis of rodents occurs largely in the postnatal period (Dobbing and Sands, 1979). The pig brain further matures in a human like manner with

respect to both myelination, biochemistry, and electrical activity (Pampiglione, 1971; Flynn, 1984; Thibault and Margulies, 1998; Fang *et al.*, 2005).

## 9.2 The pig serotonergic system

The regional distribution of neurotransmitters and their receptors in the pig brain has been described in several studies using e.g. immunohistochemical techniques, high-performance liquid chromatography (HPLC), autoradiography, or PET. High concentrations of serotonin are primarily observed in the brain stem and hypothalamus similar to the distribution in other mammals (Meunier-Salaun *et al.*, 1991; Adeola *et al.*, 1993). The distribution of SERT also resembles the human distribution (Cumming *et al.*, 2001). Among the serotonergic receptors the following subtypes have been identified in pig brain tissue: 5-$HT_{1A}$ (Hoyer *et al.*, 1985), 5-$HT_{1B}$ (Waeber *et al.*, 1988; Heald *et al.*, 1994), 5-$HT_{1D}$ (Waeber *et al.*, 1988; Bhalla *et al.*, 2000), 5-$HT_{1F}$ (Bhalla *et al.*, 2002b), 5-$HT_{2A}$ (Hoyer *et al.*, 1985), 5-$HT_{2C}$ (Pazos *et al.*, 1984), 5-$HT_3$ (Fletcher and Barnes, 1996), 5-$HT_4$ (Schiavi *et al.*, 1994), 5-$HT_6$ (Schoeffter and Waeber, 1994), and 5-$HT_7$ (Bhalla *et al.*, 2002a).

The 5-$HT_4$ receptor is present in high density in pig caudate nucleus homogenate, and several 5-$HT_4$ compounds have similar affinities for pig and human 5-$HT_4$ receptors (Schiavi *et al.*, 1994). This suggests a high similarity between 5-$HT_4$ receptors from pig and human tissue. We provided the first quantitative description of the regional distribution of the 5-$HT_4$ receptor in the pig brain (Kornum *et al.*, 2009 - Study I ) and found that the Göttingen minipig has a 5-$HT_4$ receptor distribution that resembles the human distribution as reported by Varnäs and colleagues (2003) and others.

In conclusion, despite incomplete characterisation of the pig brain, it can be said to resemble the primate brain with respect to morphology, histology, brain development, and the serotonergic system, and may thus present advantages over the use of rodents for studying the 5-$HT_4$ receptor in learning and memory, and human dementias.

## 9.3 The Göttingen minipig

The work in this thesis was performed using the Göttingen minipig. This minipig race was developed in 1961-1962 at the University of Göttingen. A coloured miniature swine, originating from a crossing between the Minnesota minipig and the Vietnamese potbelly swine, was used in a back-crossing with the white German Landrace. The offspring, the Göttingen minipig, combined the type and calm temperament of the Minnesota minipig with the fertility of the Vietnamese potbelly pig, and the white phenotype of the German Landrace (R: Glodek, 1986).

Compared to pigs bred for meat production, Göttingen minipigs offer several advantages: they are purpose-bread, standardised laboratory pigs with highly controlled microbial status, they have a low daily weight gain and a low adult body weight (Kohn *et al.*, 2007), and despite this they have the same brain size as conventional pigs (Watanabe *et al.*, 2001).

# 10 Methods for studying pig brain 5-$HT_4$ receptors

## 10.1 *In vitro* receptor studies

The aim of this thesis was to develop a basis for using the pig as a large animal model of memory and memory disorders, with special emphasis on looking at 5-$HT_4$ receptor function. We therefore wanted to establish and evaluate methods for quantifying the concentration of the 5-$HT_4$ receptor in the pig brain.

When studying receptors it is important to consider the different states and cellular compartments a receptor can be present in. The cellular regulation of G-protein coupled receptor signalling is very complex and includes several different conformational states of the receptor (Kenakin, 2004; Vauquelin and Van Liefde, 2005). Briefly, the receptor molecule exists in a conformational equilibrium between active and inactive states. The binding of an agonist to the receptor shifts the equilibrium toward the active receptor states, whereas antagonists are ligands that binds to both active and inactive receptors and prevents agonist binding without affecting the equilibrium. The number of receptors available on the cell surface is regulated by endocytotic trafficking (R: Moore *et al.*, 2007; R: Ulloa-Aguirre and Conn, 2009).

By use of specific radiolabelled ligands, receptor density ($B_{max}$) and the affinity of the ligand ($1/K_D$) can be measured in brain tissue homogenate. The same radiolabelled ligands can also be used for autoradiography, where receptors are detected in thin tissue sections mounted on glass slides. It is believed that these methods detect receptor molecules on both the cell surface and in intracellular compartments. Western blotting is another very well known and widely used method for determination of protein levels. This semi-quantitative technique detects total protein content in tissue homogenate relative to a household protein or relative to control tissue by use of specific antibodies. With western blotting you therefore in reality quantify the number of antibody epitopes, which does not necessarily reflect the number of available receptors.

To quantify the number of receptors available for G-protein signalling, a functional assay is needed. The [$^{35}$S]GTPγS assay is one such assay that can be applied to both brain homogenates and tissue slices. The assay measures the level of G-protein activation following agonist binding to a G-protein coupled receptor. The great advantage of this assay is that it measures a functional consequence of receptor occupancy at a very early cell signalling event (Harrison and Traynor, 2003). Cecilie Löe Licht, Ph.D. from the Neurobiology Research Unit, tried to develop a protocol for 5-$HT_4$ stimulated [$^{35}$S]GTPγS binding, but despite a substantial effort it was not possible to detect a response, probably because of the high constitutive activity of the receptor (Licht, 2009). Other possible functional assays include quantifying other down-stream messengers, such as Arc, following 5-$HT_4$ agonist treatment.

## 10.2 *In vivo* positron emission tomography (PET)

PET is an important tool for studies of the living brain. It has been used for studies of molecular (i.e. glucose) metabolism (Phelps *et al.*, 1979; Friedland *et al.*, 1983; Hasselbalch *et al.*, 1999; Foster *et al.*, 2008), blood-brain barrier transport (Kessler *et*

*al.*, 1984; Josserand *et al.*, 2006; Bartels *et al.*, 2008; Syvanen *et al.*, 2009), neurotransmitter release (Dewey *et al.*, 1992; Piccini *et al.*, 2003), and cerebral blood flow (Iida *et al.*, 2000). The latter is, however, increasingly being measured non-quantitatively by MRI (Carroll *et al.*, 2002).

PET utilizes the physical phenomenon of positron annihilation. When a positron is emitted from a radioisotope and thereafter encounters an electron, the positron and electron annihilate producing a pair of 511 keV gamma photons. The photons are detected when they reach a scintillator in the scanner, and since they are emitted at 180 degrees to each other, it is possible to localize their source to a straight line and from an image reconstruction then estimate the density of the radioisotope in a 2- or 3-dimensional area. The isotopes typically used in PET include $^{11}C$, $^{13}N$, $^{15}O$, and $^{18}F$. These can be incorporated into compounds where their non-radioactive counterparts are present, such as glucose analogues and receptor ligands. Such labelled compounds are called radiotracers or radioligands, and in this thesis we have utilized [$^{11}$C]SB207145 that binds selectively to the 5-$HT_4$ receptor (Gee *et al.*, 2008; Marner *et al.*, 2009).

The sequence of PET images obtained during a dynamic PET investigation provides a quantitative estimation of the radiotracer concentration in a region of interest (ROI) over time, often displayed as a time-activity curve. By use of kinetic modelling principles these data can be used to calculate the distribution volumes ($V_T$, see below) for the ROIs as a measure of, in our case, receptor concentration in the ROI. For this kind of data to be valuable the ROIs must be carefully defined. The recent development of dual modality scanners, e.g. PET combined with computerised tomography (CT), has enabled the generation of images displaying both the radiotracer distribution and the anatomical structures of the tissue, and this can be used as an aid when defining ROIs. In study I, we used a combined PET/CT scanner (Discovery LS scanner, General Electric) and co-registered the CT-image from each pig to a Göttingen minipig brain MRI-based atlas with 34 predefined brain regions (Figure 3; Watanabe *et al.*, 2001). This was done to avoid the possible bias from manually defined ROIs.

Several different models for kinetic modelling of PET data exists, and compartmental analysis with arterial parent compound as input function is considered the gold standard for quantification of radioligand binding. From a PET scanning with arterial blood sampling, and parent compound metabolite analysis the concentration of

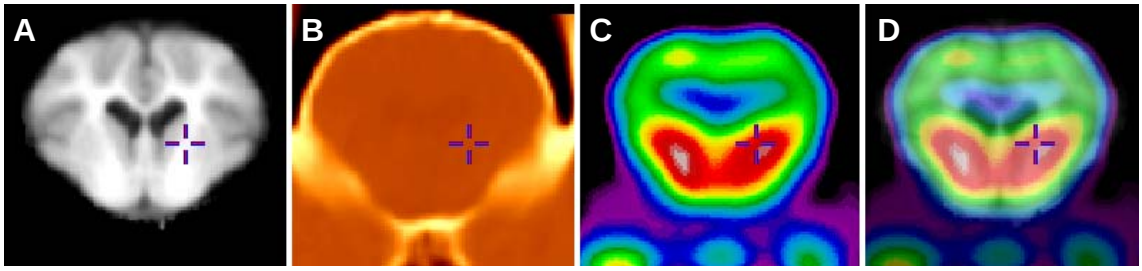


**Figure 3.** Illustration of co-registration to the MRI-based Göttingen minipig atlas. All sections are coronal images of the same level in the pig brain. The cross marks the same x-y-z coordinate in each picture. A: MRI-based atlas (Watanabe *et al.*, 2001). B: CT image obtained in the same session as the PET image and co-registered to the MR image in A. C: [$^{11}$C]SB207145-PET image. D: PET image overlaid on the MR image.

radioligand in a volume of tissue relative to the plasma concentration can be calculated. This is termed the total volume of distribution ($V_T$), and its calculation using a one- or two-tissue compartment model can be found in paper I. From a single PET scanning with the radioligand in tracer dose the binding potential (BP) of the ligand to the receptor can further be calculated. BP is calculated as a ratio between the concentration of specifically target-bound ligand and a reference concentration. This reference concentration can be either the total parent compound plasma concentration ($BP_P$, equal to the specific volume of distribution $V_S$), the free tissue concentration, which is equalled to the free plasma concentration ($BP_F$), or the concentration of non-displaceable ligand in tissue ($BP_{ND}$) (Innis *et al.*, 2007).

If a brain region exists where radioligand uptake represents only the non-specific uptake, then the binding potential ($BP_{ND}$) of the radioligand in any ROI can be calculated as the difference between the $V_T$'s of the target and reference region divided by $V_T$ of the reference region.

$BP_{ND}$ can also be calculated using a reference tissue model. In general, reference tissue models benefit from not requiring arterial blood sampling often at the expense of a bias. Several different reference tissue models have been published, the most often used are: the multilinear reference tissue model (MRTM) (Ichise *et al.*, 2003), the simplified reference tissue model (SRTM) (Lammertsma and Hume, 1996; Wu and Carson, 2002) and the Logan non-invasive model (Logan *et al.*, 1996). One can not from the structure of the ligand predict what kinetic model will most precisely fit the data. When working with a new tracer it is thus important to evaluate the feasibility of the different kinetic models.

## 10.3 Serotonin depletion

A widely used method for studying central serotonergic dysfunction is serotonin depletion. Many experimental serotonin depletion regimes are available and widely used in rats, monkeys, and humans. These regimes are based on specific serotonergic degeneration, tryptophan depletion, or inhibition of serotonin synthesis. Para-chlorophenylalanine (pCPA) is a specific inhibitor of TPH well-known to produce extensive and selective serotonin depletion across a variety of species (Gradwell *et al.*, 1975; O'Connell *et al.*, 1991; Kornum *et al.*, 2006). Ph.D.-student Anders Ettrup at the Neurobiology Research Unit has, supervised and assisted by the author of the present thesis, developed and validated a pig model of sub-chronic serotonin depletion. We administered pCPA (50 mg/kg or 100 mg/kg i.m.) to young landrace pigs (n=4 /group) for four consecutive days, and on day five homogenates from seven brain regions were extracted, covering from the frontal cortex to the rostral brain stem. All regions showed decreased tissue levels of serotonin relative to control (61 ± 14 %) as detected by quantitative HPLC-analysis. We further showed that 5-$HT_4$ receptors were significantly down regulated in this model, but that SERT, 5-$HT_{1A}$ and 5-$HT_{2A}$ receptor levels were unaffected by the treatment (unpublished data).

This model provides an excellent tool for future studies of serotonergic neurotransmission regulation.

# 11 Behavioural studies of pig learning and memory

To be able to study pig learning and memory we obviously need to implement one or more behavioural paradigms that target the type of memory we are interested in, and that are suitable for pigs. A wide range of cognitive paradigms have been developed to assess particular aspects of learning and memory in both humans and animals. In this section, I will first introduce some behavioural tasks that target working memory and declarative memory, and then I will give a description of the behavioural paradigms that have been developed for pigs so far.

## 11.1 Behavioural tests for assessing working memory

Several attempts have been made at defining animal working memory, and the literature is clouded by the lack of consensus regarding the definition of human working memory (see section 7.1.2). A useful definition for discussing behavioural paradigms has been proposed by Dudchenko (2004): *Animal working memory is a short-term memory for an object, stimulus, or location that is used within a testing session, but not typically between sessions.* This is distinguished from long-term memory referred to in this context as reference memory. Reference memory is e.g. memory for the rules of a given task, and it is typically acquired with repeated training and would persist from days to months. Working memory, in contrast, is typically a delay-dependent memory of stimuli that are used to guide behaviour within a task. This type of memory is sensitive to interference and decays over time, like human short-term memory (R: Dudchenko, 2004). In early literature, tasks requiring this type of memory are often referred to as delayed reaction paradigms. Some of these studies are reviewed by Dudchenko (2004) and will not be dealt with here. Some of the first studies that formally addressed working memory in rats used an eight-arm radial maze (Olton and Samuelson, 1976; Olton *et al.*, 1979). It was observed that rats during a single session were able to remember which arms they had already visited during that session, and this did not depend on olfactory or other intramaze cues. Eight-arm radial maze tasks are still widely used for studies of working memory (e.g. Bolhuis *et al.*, 1986; Ogasawara *et al.*, 1995; Laughlin *et al.*, 1999; Micale *et al.*, 2006).

Another class of tasks very often used to test working memory is delayed non-match to sample (DNMS) tasks. In these tasks the animal must remember a stimulus over a delay period in which that stimulus is no longer present, and then choose the alternative stimulus in the test situation. This type of task requires that the animals are first trained to learn the non-matching rule. DNMS tasks have been developed in several different settings using stimuli such as objects (Mumby *et al.*, 1990; Kesner *et al.*, 1993), odours (Otto and Eichenbaum, 1992; Wood *et al.*, 1999), and spatial directions (Murray *et al.*, 1989; Markowska *et al.*, 1989).

Several studies have tried to determine which brain areas mediate performance in these tasks. The picture that emerges is that depending on the type of stimuli and length of the delay different areas are involved. Short delays target the prefrontal cortex, while longer delays also requires an intact medial temporal lobe system (Floresco *et al.*, 1997;

Porter *et al.*, 2000; Lee and Kesner, 2003). Further, spatial tasks are critically dependent on the hippocampus, while object recognition tasks are not (R: Mumby, 2001; Broadbent *et al.*, 2004; Clark *et al.*, 2007).

## 11.2 Behavioural tests for assessing declarative memory

Certain characteristics of declarative memory allow the term to be extended to experimental animals. Declarative memory encodes relationships among items and events and can guide performance under a wide range of test conditions. In contrast, non-declarative memory is expressed through performance rather than recollection.

The Morris water maze is one of the most frequently used laboratory tools in behavioural neuroscience (Morris *et al.*, 1982; R: D'Hooge and De Deyn, 2001). In the water maze a rodent learns to locate a hidden rescue platform in a large pool by creating a spatial map using extra-pool cues. The Morris water maze is typically used to test declarative memory, but working memory versions of the task have also been developed (Steele and Morris, 1999).

Spontaneous object recognition (SOR) measures how much the animal explores a novel object compared to a familiar one. In its simplest form, developed by Ennaceur and Delacour (1988), one or two identical objects are presented for a brief exploration. After a delay the original object is presented again with a novel object. This task exploits an animal's innate preference for novelty, and a preference for exploration of the novel object compared to the familiar object indicates that the familiar object is remembered. Since performance in this task is dependent on the memory of an object that has only been presented once, it has been suggested that this corresponds to memory of an episode, like in human episodic memory. It is however often questioned whether one-trial learning resembles episodic learning since the prior does not necessarily include explicit encoding and recall of the event (Morris, 2001; Crystal, 2009). One of the big questions in animal cognitive research has indeed been whether non-human animals have a capacity for episodic memory. The first evidence that this is indeed the case came from Clayton and Dickinsons (1998) famous experiments using scrub jays. They introduced the term episodic-like memory to emphasize that behavioural testing only indirectly assess an animal's ability to remember specific episodes. Later they developed some behavioural criteria for studying episodic-like memory, namely to evaluate whether an animal can discriminate *what* occurred, *where* it took place and *when* it happened (Clayton *et al.*, 2003). Several approaches have been used toward examining episodic-like memory in animals (R: Crystal, 2009), but only one approach has been applied to pigs. This method is briefly described here:

Based on the SOR task, Eacott and Norman (2004) developed a way to test integrated memory for what (object), where (location), and when (context). In this task the animal are exposed to two different configurations of two distinct objects within two different locations and contexts. The animal is then tested on its ability to distinguish a previous encountered configuration from a novel configuration. Both rats and pigs are capable of making this discrimination, and this has been interpreted as evidence of

episodic-like memory in these species (Eacott and Norman, 2004; Kouwenberg *et al.*, 2009).

## 11.3 Pig behaviour

Several well-known behavioural paradigms are available for studying affective pig behaviour (R: Lind *et al.*, 2007). These assess modalities such as anxiety and aggressiveness and include the open field test (Kratzer, 1971; Andersen *et al.*, 2000), the elevated plus maze (Andersen *et al.*, 2000; Janczak *et al.*, 2002), and resident-intruder confrontations (Erhard and Mendl, 1997; D'Eath *et al.*, 2005).

For studies of cognitive behaviours often the animal has to be trained on a given task. For this, task requirements should be in accordance with the sensory abilities of the chosen species. Visual accuracy of pigs is inferior to that of primates, and the colour vision of the pig is dichromatic, such that blue is the only colour it can distinguish from grey (Neitz and Jacobs, 1989; Tanida *et al.*, 1991; Tanaka *et al.*, 1998). Auditory sensitivity of pigs is similar to that of primates (Heffner and Heffner, 1990), and the olfactory discrimination and sensitivity is by far superior to primates (Meese *et al.*, 1975; Kristensen *et al.*, 2001). Pigs can be trained on different types of tasks with the same effort as has to be put into rodent or monkey training. Still only relatively few studies have addressed cognitive functions such as learning and memory in pigs, and most of these have focused on the implications for livestock production (R: Held *et al.*, 2002; R: Lind *et al.*, 2007).

Mendl and colleagues were the first to investigate pig spatial memory (1997). In a foraging arena with 10 enclosed areas the pigs were able to learn the location of food (win-stay task), and the memory for this was found to be sensitive to longer retention intervals, irrelevant disturbing stimuli and mild stressors. This was later confirmed by using a win-shift task in a radial eight-arm maze (Laughlin *et al.*, 1999). Pigs also show memory of familiar objects (Moustgaard *et al.*, 2002; Kornum *et al.*, 2007 - study II), and learn visual and spatial discriminations as well as their reversals (Moustgaard *et al.*, 2004; Bolhuis *et al.*, 2004). A recent study tested pigs with a modified version of the test developed by Eacott and Norman (2004), where an integrated recall of object, location and context are hypothesised to test episodic-like memory. The authors rapport that pigs are indeed capable of recalling what (object), where (location) and when (context) (Kouwenberg *et al.*, 2009). Pigs further display rather complex social cognitive abilities resembling primates more than rats (Held *et al.*, 2000).

Because of its simplicity and the fact that object recognition is improved in rats when treated with 5-$HT_4$ agonists (see section 7.1.4), we first decided to implement the SOR task for pigs in our lab. This task had already been applied to Göttingen minipigs (Moustgaard *et al.*, 2002), but the authors noted a very high between-subjects variability, and they did not validate sensitivity to either delay or scopolamine. We therefore decided to address the questions of delay and scopolamine sensitivity, and further to implement an automatic tracking system for scoring of exploration times (Lind *et al.*, 2005). As described in further detail in result section 14.1, we observed that the test under our conditions was very sensitive to interference, and that the test-retest

variability was high. Since we further wanted a behavioural task that targeted the hippocampus, we decided to implement a spatial memory task. It would have been interesting to develop a water maze task for pigs comparable to the Morris water maze, but the practical issues regarding this made the idea less attractive. Instead we chose to develop a spatial DNMS task in a T-like maze (Figure 4) originally introduced for rats by Ladieu (1944). Spatial DNMS tasks have been shown to be sensitive to hippocampal damage (Murray *et al.*, 1989; Markowska *et al.*, 1989; Shaw and Aggleton, 1993; Clark *et al.*, 2000; Vago and Kesner, 2008), and further their wide use allows for comparison between species. We chose a maze based task, since pigs previously with success have been tested in different mazes (Mendl *et al.*, 1997; Laughlin *et al.*, 1999; Bolhuis *et al.*, 2004). Again we addressed the sensitivity of the task to time delay and scopolamine. The further details of these behavioural studies can be found in paper II and III, and the results are described in section 14.

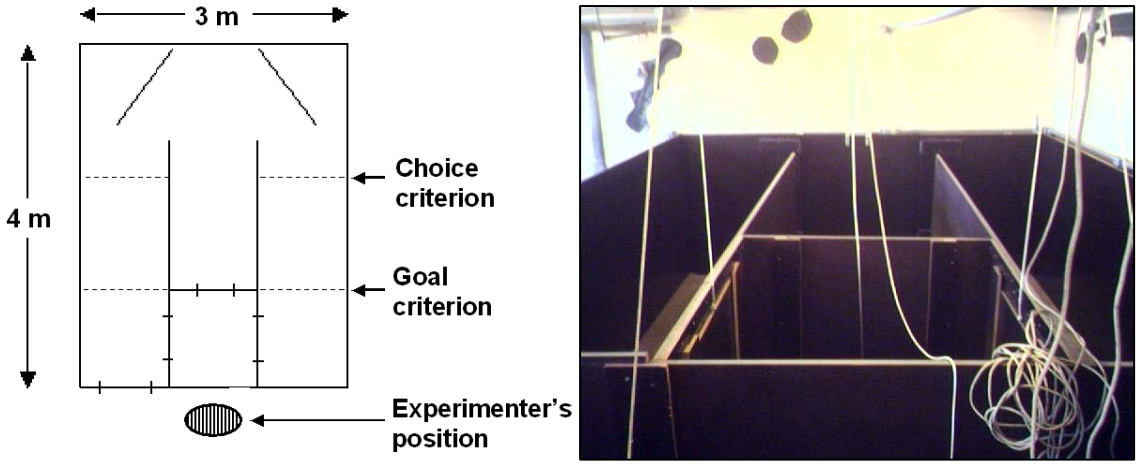


**Figure 4**. A: Illustration of the T-maze. B: Photograph showing a view of the maze from the position of the experimentor. Robe for opening guillotine dors are seen in the foreground, and in the back extra-maze cues can be seen. By courtesy of T.R.Nielsen.

# 12 Transgene expression in the pig brain

Genetic manipulation offers a lot of possibilities for studies of receptor function and disease mechanisms, but only very few studies report of genetic manipulations of the pig brain. Here, I will describe two different methods used for transgene expression in the brain, and how they apply to the pig brain. These techniques have already been applied for studies of 5-$HT_4$ receptor function in mice (knock-out, Compan *et al.*, 2004), and rats (local protein over expression, Lucas *et al.*, 2005).

## 12.1 Traditional transgenesis

A transgenic animal is developed from an early stage embryo with a gene modification introduced. Modifications include removal of a gene (knock-out), replacement of a gene or introduction of a gene with a dominant mutation (knock-in). A transgenic model is typically established using the mouse, but several other species are also being used including the pig (Petters *et al.*, 1997; Uchida *et al.*, 2001; Kragh *et al.*, 2005). Despite ongoing progress in technique development, the production of transgenic pigs remains a demanding task (R: Wheeler and Walters, 2001; R: Niemann and Kues, 2007).

A research group at University of Aarhus is currently trying to develop a transgenic pig model of AD. So far they have reported a successful expression of APPsw in pig brain tissue, but no occurrence of AD-related pathology in one-year old transgenic pigs (Kragh *et al.*, 2009). They are currently studying the longer-term consequences of the transgene.

## 12.2 Viral vectors as transgene carriers

Viral vectors are replication deficient viruses capable of transferring their genetic material to a host cell. The mechanisms behind gene delivery to the host cell are plentiful and will not be reviewed here, but a key point is that the affinity of the capsid or envelope proteins for different cell surface proteins determines which cell types are infected most efficiently. A viral genome has two fundamental elements: the *trans*-elements that encode the viral proteins; and the *cis*-elements that are responsible for replication and packaging of the viral genome within the host cell. By replacing the entire *trans*-element with a foreign gene of interest, and via transfection, a host cell can be made to express any protein of interest with the limitation that the size of the corresponding gene must not significantly exceed the size of the *trans*-element (R: Davidson and Breakefield, 2003).

The spectrum of vectors used in basic research applications is large comprising those with simple capsid virions (e.g. adenovirus and adeno-associated virus (AAV)), and viruses with enveloped virions (e.g. lentivirus and herpes simplex virus). The transduction properties and transgene capacity of the different viral vectors vary, and thus different types are suited for different purposes. In this study we chose to work with recombinant AAV (rAAV) vectors for reasons listed below.

The AAV is a small (20-30 nm), non-enveloped, single stranded DNA animal virus. It belongs to the *Parvoviridae* family, which are characterized by their inability to

replicate their genome without the presence of a helper virus (such as adenovirus). Nine subtypes of the virus have been described (AAV1-9), the most common being AAV2. Humans are the primary host, but AAVs have never been associated with any human diseases. AAVs are able to infect both dividing and non-dividing cells, and show a high preference for transducing neurons. A disadvantage of the AAV vector is the relatively small transgene capacity (4-5kb) (Mayor and Melnick, 1966; R: Tenenbaum *et al.*, 2004; R: Vasileva and Jessberger, 2005).

The defective replication and non-pathogenic nature of wild-type AAV triggered the rapid development of rAAVs derived from AAV2. rAAVs are ideal for modelling brain diseases, as they provide long-term transduction with little toxicity or immune reaction (Chamberlin *et al.*, 1998; Lo *et al.*, 1999; Mastakov *et al.*, 2002; Peden *et al.*, 2004; Reimsnider *et al.*, 2007). Numerous serotypes of the rAAV have been introduced with varying tropism and transduction patterns, and *in vivo* delivery of these vectors has been shown to result in very efficient transduction in the adult brain (Xiao *et al.*, 1999; Davidson *et al.*, 2000; Burger *et al.*, 2004; Gao *et al.*, 2005; Cearley and Wolfe, 2006; Broekman *et al.*, 2006; Cearley *et al.*, 2008). To achieve a widespread gene delivery to the brain, Passini and colleagues successfully injected AAV2 vectors into the ventricular space of the neonatal mouse brain (Passini and Wolfe, 2001), and several studies have since benefited from this idea (Passini *et al.*, 2003; Burger *et al.*, 2004; Broekman *et al.*, 2006; Cearley *et al.*, 2008). Three non-rodent species (cat, dog, and monkey) have been examined for AAV vector transduction in brain tissue (Kirik *et al.*, 2003; Vite *et al.*, 2003; Ciron *et al.*, 2006; Ciron *et al.*, 2009), and it is evident from these studies that different rAAV serotypes displays species-specific transduction efficiencies and patterns. Study IV is the first study to explore transgene expression after injection of viral vectors into the pig brain.

Because of the complexity of the brain, with its various cell types and connections between these, it is a complicated target for genetic manipulation using viral vectors. Since the current knowledge of vector tropism and retrograde and anterograde transport properties has its gaps, an initial evaluation of several potential vector subtypes for a particular experiment is therefore necessary. We decided to evaluate the cell tropism and transduction pattern of rAAV subtypes 1, 2, 5, and 6, when targeted to the Göttingen minipig brain. To achieve a widespread transduction in the brain, we decided to use neonatal minipigs. Very few studies (Jelsing *et al.*, 2006; Dyrby *et al.*, 2007) report stereotaxic guided injections to the neonate Göttingen minipig brain, and in these cases injection parameters were deduced from trial and error experiments. We guided our injections using a MRI of a neonatal minipig brain where a drop of oil had been injected 2 cm below bregma, and decided to target the striatum and the hippocampus. We utilized vectors encoding for the green fluorescent protein (GFP) marker gene, and assessed the efficiency of transduction and phenotype of GFP positive cells six weeks post-surgery. The details of this study can be found in paper IV, and the results are described in section 15.

# RESULTS AND DISCUSSION

## 13 The serotonergic 5-HT$_4$ receptor in the pig brain

We have determined the 5-HT$_4$ receptor concentrations in different pig brain areas with both tissue homogenate binding and autoradiography (Table 1 and Figure 6). With both methods we find the highest binding in the striatum, low binding in cortex, and no specific binding in the cerebellum. In the hippocampus, we observed that the 5-HT$_4$ receptors were mostly found in the pyramidal cell layer. This resembles the distribution in human brain tissue (Bonaventure *et al.*, 2000; Varnäs *et al.*, 2003).

For several reasons, it is difficult to compare results obtained with tissue homogenate binding and autoradiography. First, for tissue homogenate binding larger tissue areas are often used whereas for autoradiography thin brain slices are used. This makes it difficult to compare the exact same areas. Second, both procedures include a washing step where excess radiolabelled ligand is removed. In this step there is a risk that specifically bound ligand is also washed away, and this step obviously differs between the procedures. Third, with tissue homogenate, the measurement are related to the total protein concentration in the sample, while in autoradiography the radioactivity concentration is converted to fmol per mg estimated wet-tissue-equivalent via [$^3$H]microscales. We suggest that measuring receptor binding in tissue homogenates is more precise in large areas, if these are accurately dissected, whereas autoradiography is the superior method for investigating regional differences, and smaller ROIs.

**Table 1.** 5-HT$_4$ concentrations (mean ± S.D.) in different brain regions as determined by autoradiography or homogenate receptor binding with [$^3$H]SB207145.

| **Brain Region** | **Brain homogenates (n = 8)** | | **Autoradiography (n = 3)** | **Autoradiography Human data [a]** |
|---|---|---|---|---|
| | Kd (nM) | Bmax (fmol/mg prot) | Bmax (fmol/mg t.e.) | Bmax (pCi/mm$^2$) |
| Striatum | 0.39 ± 0.06 | 97.3 ± 2.6 | 40.2 ± 4.9 | ND |
| - caudate nucleus | ND | ND | 42.4 ± 4.3 | 32 ± 5.3 |
| - putamen | ND | ND | 38.0 ± 5.5 | 26 ± 2.8 |
| Hippocampus | 0.45 ± 0.08 | 52.3 ± 1.6 | 26.8 ± 5.0 | ND |
| - CA3 | ND | ND | 38.5 ± 4.2 | 30 ± 11 |
| Mesencephalon | 0.68 ± 0.38 | 21.6 ± 2.1 | ND | ND |
| Cortex | 0.71 ± 0.43 | 12.7 ± 1.3 | ND | ND |
| Frontal cortex | 0.35 ± 0.28 | 12.5 ± 1.9 | 19.3 ± 1.0 | 18/12 ± 6.2/3.5[b] |
| Cerebellum | No fit | 4.1 ± 6.0* | -0.01 ± 1.09* | 0.35 ± 0.4* |

For calculations of $B_{max}$, $K_D$ was fixed to 0.39 nM. [a]As comparison are shown data obtained from human tissue using the antagonist [$^{125}$I]SB207710 (Varnäs *et al.* 2003). [b]Values are determined in external and internal layers respectively.
ND: Not determined, t.e.: tissue equivalent, * not significantly different from zero.

## 13.1 *In vivo* assessment of pig 5-HT$_4$ receptor distribution volume

When evaluating [$^{11}$C]SB207145 as a possible PET radioligand, Gee and colleagues (2008) performed *in vivo* PET scans in the Yorkshire and Danish Landrace crossbreed pig. They reported an overall distribution similar to the one presented in paper I, but they did not report any quantitative measurements.

In study I, we evaluated different kinetic models for quantification of 5-HT$_4$ receptors with [$^{11}$C]SB207145, and found that the one-tissue compartment (1-TC) model was superior to the two-tissue compartment (2-TC) model, and the Logan graphical analysis (Logan *et al.*, 1990). Goodness-of-fit was evaluated using the Akaike Information Criterion (Akaike, 1974), and we also compared the calculated $V_T$ for the ROIs, since it is well known that for many PET-ligands $V_T$ is a more stable outcome parameter than the individual rate constants (Carson *et al.*, 1993).

In our *in vitro* assays, we did not detect any specific binding in the cerebellum with [$^3$H]SB207145, supporting that cerebellum serves as a suitable reference region in the pig for this radioligand. This notion is also in line with data from *in vivo* blocking studies (Gee *et al.*, 2008; Marner *et al.*, 2009). Using cerebellum as reference region we compared the 1-TC data to different reference tissue models. Both the MRTM and the SRTM are sensitive to noisy data. To improve modelling we therefore fixed k2 to a global mean value k2' for all regions (MRTM2 and SRTM2) (Wu and Carson, 2002). As demonstrated by Ichise et al. (2003) using a single-tissue ROI to estimate k2 potentially induces bias in $BP_{ND}$ values because of noise. To reduce this noise we estimated k2 for several ROIs and used a mean of these values as k2'. Both MRTM and MRTM2 were found to underestimate the binding potential, and MRTM also significantly overestimated k2'. This is equivalent to a previous study showing that bias in k2' estimation transfers to bias in the binding potential (Ichise *et al.*, 2003). With SRTM2 we found a very good correlation to 1-TC model data with no bias (Figure 5), and also we found that using SRTM to estimate k2' gave a result very similar to k2 estimated from the 1-TC model. This is consistent with one of the major assumptions of the SRTM approach: that the kinetics follow a 1-TC model, the other being that the level of non-displaceable binding in the reference and the target regions is similar (Lammertsma and Hume, 1996).

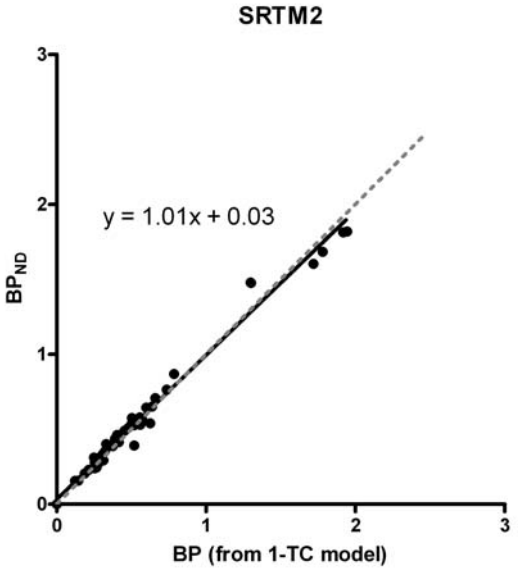


**Figure 5.** Correlation between $BP_{ND}$'s calculated from [$^{11}$C]SB207145 PET data using a 1-TC model and SRTM2. $R^2 = 0.98$.

In conclusion, we suggest that SRTM2 with a fixed k2 is employed as the standard analysis in future studies of [$^{11}$C]SB207145 uptake in pig brain. Generally, to get robust estimates of k2', it should be modelled over a large high binding region or taken as the averaged value from several high binding regions.

## 13.2 *In vivo* and *in vitro* receptor measurements compared

*In vitro* receptor binding can be described by the Michaelis-Menten equation:

$$B = \frac{B_{max} \cdot F}{K_D + F}$$

where B is the concentration of receptor bound ligand and F is the concentration of free ligand. For *in vivo* PET studies with the radioligand in tracer dose $F << K_D$ thus:

$$\frac{B}{F} = \frac{B_{max}}{K_D} = BP_F$$

From this it follows that it is possible to compare *in vitro* and *in vivo* data by comparing $B_{max}/K_D$ to $BP_F$. We used this approach to evaluate the reliability of [$^{11}$C]SB207145 PET measurements in predicting 5-HT$_4$ concentration in a ROI. As discussed above, homogenate receptor binding is an appropriate method for determining receptor concentrations in larger brain regions, and we determined the 5-HT$_4$ receptor concentrations in tissue homogenates from six different brain areas (striatum, hippocampus, mesencephalon, frontal cortex, rest of cortex, and cerebellum) from eight pigs and compared the outcome to $BP_{ND}$ calculated from PET data from the same animals. In our study we measured the same affinity of SB207145 for the 5-HT$_4$ receptor in all brain regions examined, and we therefore fixed $K_D$ to the mean striatal value to get more stable $B_{max}$ calculations. Because of this we decided to compare $B_{max}$ directly to $BP_{ND}$ and thus expected a positive correlation but not numerically similar results. In single brain regions (e.g. striatum) we did not see any correlation between the PET data and the homogenate binding data. This finding is not entirely unexpected

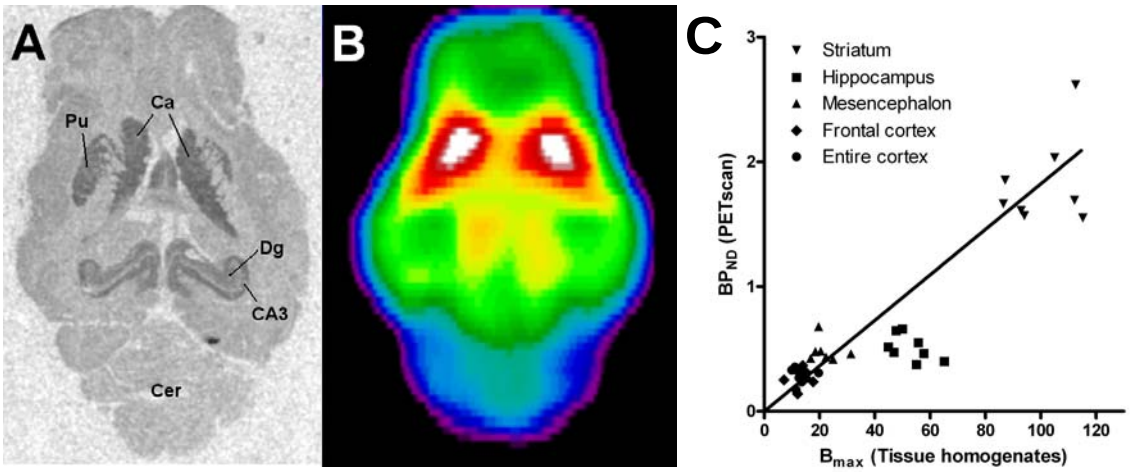


**Figure 6.** A and B: Horizontal sections of the Göttingen minipig brain showing the distribution of the 5-HT$_4$ receptor as detected by A) [$^3$H]SB207145 and B) [$^{11}$C]SB207145. In A) light gray represents low radioactivity signal and dark gray represents high signal. In B) purple and blue represents low radioactivity signal and red to white represents high signal. In both cases a high radioactivity signal indicates a high 5-HT$_4$ receptor concentration. Abbreviations: Ca, caudate nucleus; CA3, Ammon's horn of the hippocampus; Cer, cerebellum; Dg, dentate gyrus; Pu, putamen. C: Comparison of 5-HT$_4$ concentrations in brain tissue homogenates and 5-HT$_4$ $BP_{ND}$ measured with PET. The PET data were modelled using SRTM2. The outcome of a linear regression analysis (without hippocampal data) is shown. $R^2$=0.92.

since both measurements, particularly PET, are noisy. Further, with *in vitro* receptor studies using homogenate, all receptors are available for detection, but *in vivo* only a subset of these receptors will be available for binding since some may be compartmentalized or occupied by endogenous transmitter. When data from all regions, except hippocampus, were pooled we found a high correlation between the two measurements ($R^2$=92). [$^{11}$C]SB207145 PET measurements did however significantly underestimate hippocampus receptor density (Figure 6). The hippocampus is a quite small region and further, as we show with autoradiography, binding in this region is confined to the narrow band of pyramidal cells. On top of this it lies adjacent to the lateral ventricles where no binding is present. This makes it very difficult to quantify accurately with PET because of partial volume effects. This phenomenon is caused by the limited spatial resolution of PET, which leads to an underestimation of regional radioactivity distribution in regions with high radioactivity concentration relative to the surroundings, and also to the converse situation with overestimation in regions with low radioactivity concentration (Hoffman *et al.*, 1979). With a co-registered magnetic resonance image it is possible to correct for these effects of partial volume (Muller-Gartner *et al.*, 1992), and this is a possibility in future studies with [$^{11}$C]SB207145 if accurate binding potentials are to be obtained from the hippocampus and other small regions. Another possibility is to use a PET scanner with a higher spatial resolution such as the high resolution research tomography (HRRT) scanner (Heiss *et al.*, 2004).

# 14 Development of behavioural paradigms for studying pig memory

We chose to implement and validate two different behavioural paradigms: the SOR test and a DNMS task in a T-maze as described in section 11.3. The results are presented in the following sections.

## 14.1 Spontaneous object recognition in pigs

It has been shown that pigs, like rats, prefer to explore novel objects over familiar ones (Wood-gush and Vestergaard, 1991), and based on the principles of the rodent SOR test, a SOR test has been developed for pigs by Moustgaard and colleagues (2002). The SOR test for pigs consists of a sample phase during which the pig is placed in an open arena in the presence of two similar objects and a test phase during which the pig explores the arena in the presence of a familiar object (previously explored) and a novel object. Comparing the time spent in exploring the novel versus the familiar object during the test phase assesses recognition memory. One important aspect of the test is the length of the inter-phase delay interval. In rodents, an increase of the inter-phase delay leads to decreased habituation to the familiar object and to decreased discrimination between the familiar and the novel object (Ennaceur and Delacour, 1988; Dodart *et al.*, 1997). This is interpreted as a weakening of the memory of the familiar object. The aim of study II was to implement the SOR test at our facility and further to determine the sensitivity to increasing inter-phase delay intervals and to scopolamine. Further, we refined the test by applying the automatic tracking system PigTrack (Lind *et al.*, 2005).

With a 10-minute sample phase, we found that the pigs were able to discriminate between the familiar and a novel object after a 10-minute inter-phase delay interval, but not after delays of 1 hour and 24 hours (Figure 7). In comparison, Moustgaard et al. (2002) observed a significant discrimination between novel and familiar objects after a 1-hour inter-phase interval. We were not able to replicate this finding but did confirm the high between-subject variation reported in that study. The discrepancy in finding a

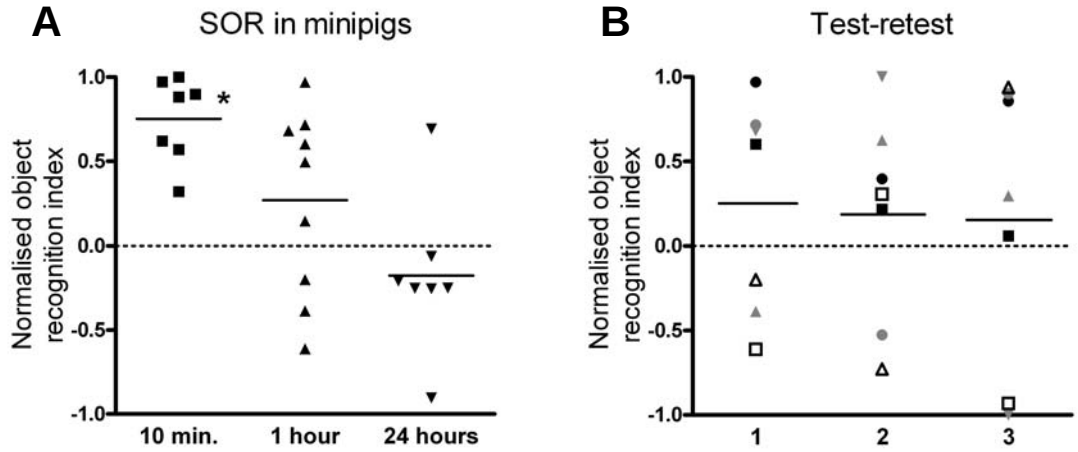


**Figure 7**. A: Normalised object recognition index with different inter-phase delay intervals. Shown are individual pigs and the mean. * Significantly different from chance level (dotted line), $p < 0.001$. B: Test-retest results using a 1-hour inter-phase delay. Individual pigs are shown with different symbols.

significant discrimination between the two studies could be due a slightly different methodology for data acquisition. Alternatively, it can be speculated that one hour is approximately the limit for memory of an object for young adult Göttingen boars. In the present study all pigs had positive scores after the 10-minute delay pointing at a very strong recollection of the familiar object at this time point.

A recent study addressed the question of how inter-phase delay intervals affect object recognition in production piglets (Gifford *et al.*, 2007). Here significant preference of the novel object was neither observed at inter-phase intervals of 1 hour, 3 hour, or 6 days. However, the two studies are not directly comparable as the animals differ by breed and age, and further a different study design was used (Gifford et al. used home pens in the sample phase and pair wise testing in the test phase). The effect of the delay interval on a pig's retrieval of information has also previously been studied in spatial memory tasks. A significant recollection of the location of food rewards has been seen after a 10-minute delay in an eight-arm radial maze (Laughlin *et al.*, 1999), and in a similar, but simpler task, the location of a food reward could be retrieved after 10 minutes as well as 2 hours (Mendl *et al.*, 1997).

In the SOR test recognition is interpreted from the difference in response towards a novel and a familiar object (Ennaceur and Delacour, 1988). Consequently, positive discrimination scores in the SOR test are interpreted as recognition of the familiar object. This measure is, however, sensitive to differences in the inherent preference between the two objects comprising a set. We therefore tested twelve different sets of objects and did indeed find different exploration times for the different objects (See paper II for details). These data illustrate the importance of addressing object bias when implementing the SOR test.

We wanted to test the scopolamine sensitivity of the test, but unfortunately the control group in this setup (1-hour inter-phase interval) did not perform over chance level, and the results were therefore inconclusive. Due to limited access to the test facilities we could not repeat the study with a different inter-phase interval. The large between-subjects variation made us worry about the test-retest stability of task performance especially at the intermediate 1-hour inter-phase interval. We therefore tested seven pigs three times on different days with a 1-hour inter-phase interval. We obtained similar results between the three groups ($p = 0.98$, one-way ANOVA), but observed highly varying results at the individual level (Figure 7). This is probably reflecting a high sensitivity to irrelevant stimuli of the spontaneous one-trial learning that is the basis of the test.

## 14.2 A spatial delayed non-match to sample task for pigs

In study III the ability of Göttingen minipigs to learn a spatial DNMS task was evaluated. The DNMS task is a well-validated memory test, and studies have been performed in many different species and modalities. This is, however, the first study of DNMS in pigs.

The DNMS task in our study consisted of a sample phase during which the pig was placed in a maze and forced to go either into the right or left arm. After a fixed time interval a test phase followed in which the pig was allowed to solve the maze without constraints and was rewarded for choosing the novel direction in the maze. The number of correct choices in a series of trials was interpreted as a measure of spatial memory. In the first 30 trials the pigs performed at chance level and then their performance gradually increased. On average the pigs required 144 trials and 60 errors to learn the task (Figure 8). Only one pig required more than 200 trials. Although it is not possible to make a direct comparison because of slightly different experimental setups and procedures, this level of learning is similar to macaque monkeys' performance on a DNMS task in a T-maze. In a study by Murray and colleagues (1989) macaque monkeys required a mean of 152 trials and 68 errors to attain the same criterion. Rats and marmoset monkeys, on the other hand, have been shown to have a significantly better performance on the task (Markowska *et al.*, 1989; Easton *et al.*, 2003).

The performance of saline injected animals decreased from 90% to 66.5% and 56% correct choices as the delay interval increased from 60 seconds to 5 minutes and 15 minutes (Figure 9). This delay dependent reduction in performance is also found in rats and dogs solving spatial DNMS tasks (Head *et al.*, 1995; Clark *et al.*, 2001). The time dependent reduction in performance is believed to indicate reduced memory rather than other types of problems such as problems in motivation, perception, motor control, etc. (Markowska *et al.*, 1989).

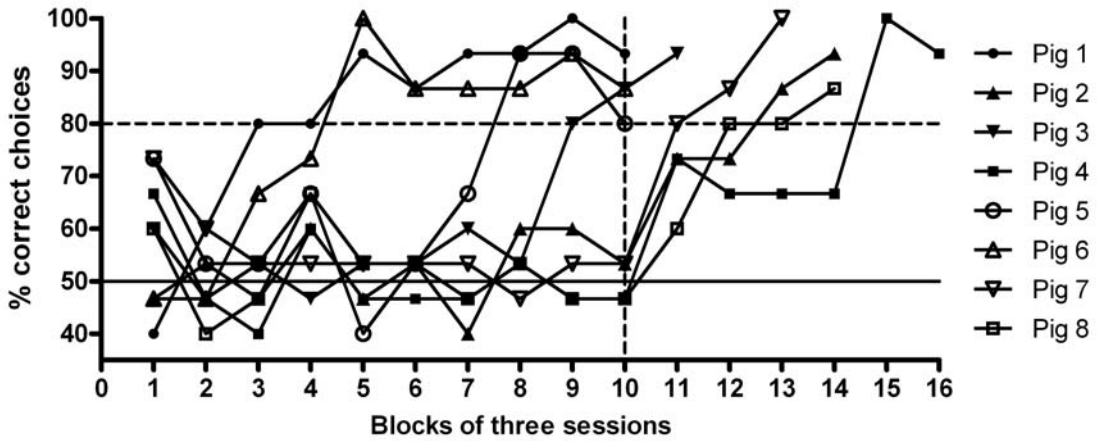


**Figure 8.** Individual learning curves shown as percent correct choices per block of three sessions (15 runs in total, 5 per session). Horizontal full line shows chance level, horizontal dotted line marks the criterion for learning. Vertical dotted line marks the point where correction training was initiated (see further explanation in paper III).

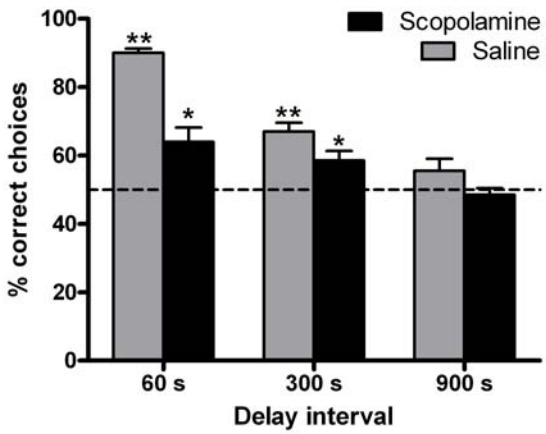


**Figure 9.** Percent correct choices at different delay intervals and the effect of scopolamine (0.04 mg/kg) on performance. There was a significant effect of both time and treatment ($p<0.0001$, two-way ANOVA). At 60 s and 300 s both control pigs and scopolamine treated pigs performed significantly better than chance level (* $p<0.05$, ** $p<0.001$), but at the 900 s delay the performance was not significantly different from chance level. N = 8.

Scopolamine reduced the mean performance to 58.5% with the 5-minute delay interval and to 48.5% with the 15-minutes delay interval (Figure 9). With the 60 second delay interval scopolamine reduced the mean performance to 64%. This was a highly significant decrease, but unfortunately the dose of scopolamine in this experiment is uncertain in the range 0.04–0.05 mg/ml. These results are in accordance with earlier studies in other experimental animals where similar reductions in performance were found (Ogasawara *et al.*, 1995; Taffe *et al.*, 1999; Araujo *et al.*, 2004). Based on results from earlier studies in different experimental animals and the results of the present study, we conclude that the spatial DNMS task is a valid tool for measuring spatial memory in the pig.

Numerous studies in rats have shown that they have a natural tendency to alternate in their choices in spatial tasks (Tolman, 1925; Dudchenko, 2001). This might be the explanation for the interspecies difference in the initial performance level in the T-maze. However, in a study of foraging strategies in pigs by Laughlin and Mendl (2000) they showed that pigs also have a tendency to alternate in their choices in a spatial task in a radial arm maze. The results from the present study do not confirm that pigs have this tendency, as they did not spontaneously alternate in their choices of arm in the training trials but had to learn this strategy. As the pigs were rewarded for choosing the correct arm in the test phases the results can, however, not be taken as a direct measure of spontaneous alternation.

Rats are in general superior at solving spatial tasks, whereas monkeys are superior in visual tasks. Since pigs have superior olfactory abilities (section 9.3), it could be interesting to develop a memory task based on olfactory cues. A previous study has already shown that pigs are capable of using olfactory cues to locate a food resource (Croney *et al.*, 2003).

### 14.3 Do 5-HT$_4$ receptor levels determine memory performance?

In the seven pigs that were used for addressing test-retest stability in the SOR test we also measured 5-HT$_4$ receptor density in hippocampus, frontal cortex, and striatum. Striatum was included as a negative control, since it is probably not involved in SOR performance. Based on the three SOR tests with 1-hour inter-phase intervals the pigs were divided into "low", "medium", or "high" memory performance (1, 2, or 3 positive discrimination scores). We found a positive correlation between memory performance and basal 5-HT$_4$ receptor concentration in the hippocampus (non-parametric Spearman correlation p=0.024) but no correlation to frontocortical or striatal 5-HT$_4$ receptor levels (p=0.66). Using the more conservative one-way ANOVA test instead, we did not find a statistical significant difference between the groups (p=0.11). The hippocampal data are shown in figure 10. The difference found in hippocampal 5-HT$_4$ receptor density between the three groups could support our hypothesis that basal 5-HT$_4$ receptor levels partly determine memory performance, but with only seven animals and no replications or manipulations of the system this result is very premature and needs to be confirmed and expanded in future studies.

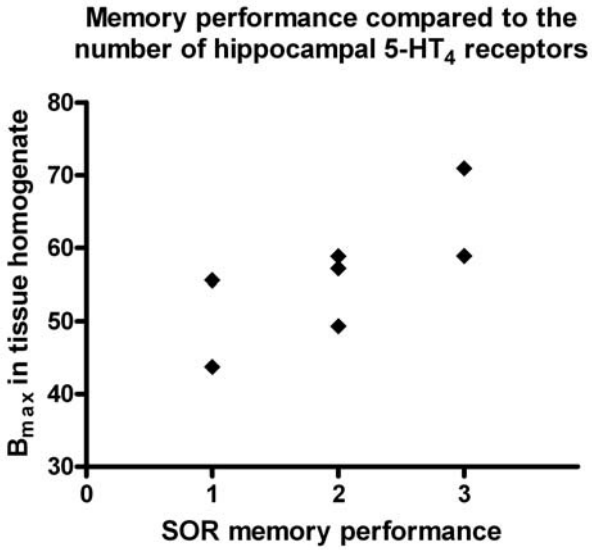


**Figure 10.** Preliminary data from comparing memory performance and 5-HT$_4$ receptor levels in the Göttingen minipig. The seven pigs were tested three times in the SOR task, and the x-axis shows the number of positive scores in these three tests. No pigs had zero positive scores. The y-axis shows the number of 5-HT$_4$ receptors measured in tissue homogenate from individual hippocampi (fmol/mg tissue).

## 15 Transgene delivery to the pig brain

Since transgene delivery to the pig brain using viral vectors, to our knowledge, has never been reported before, and it is well known that different vector subtypes displays different cell tropism in different species, we wanted to test whether we could apply this technique to the pig brain, and we also wanted to address the efficacy of different vector subtypes.

In study IV, we delivered rAAV vectors to the neonatal pig striatum by stereotaxic surgery and assessed the efficiency of transduction and phenotype of transduced cells within the forebrain. We also tried to target the hippocampus, but unfortunately we did not succeed. The tested vectors were carrying rAAV1, 2, 5, or 6 capsids containing the rAAV2 genome (denoted below as rAAV1 and rAAV5) and encoded the reporter transgene GFP.

The first data indicated that high transduction frequency was achieved from rAAV5 and lower frequencies with rAAV1, 2 and 6. Since rAAV2 and 6 only transduced cells in a small area confined to the injection site, we decided to only include rAAV1 and 5 in the final analysis. To achieve a denser cortical transduction than with intracerebroventricular injections we injected the rAAV vectors directly into the striatum of newborn piglets. We also included a group of animals that were injected seven weeks old. The pig brain is not fully myelinated until at least six months post natal (Fang *et al.*, 2005), and we speculated that a widespread transduction could still be achieved at this time point. This was however not the case (Figure 11), and the results reported below are from viral injections in newborn animals.

We found that rAAV1 primarily transduced cells in the caudate nucleus and putamen, but few GFP-positive (GFP+) cells were also found in most parts of the cortex and in thalamus. The rAAV5 serotype displayed a much more widespread distribution of GFP expression. We saw a dense GFP expression around the injection site in striatum. Further, GFP expressing cells were seen in striatal input areas such as substantia nigra pars compacta, thalamus, and layer V neurons in sub regions of frontal, temporal and parietal cortex, and in the output areas globus pallidus and substantia nigra pars reticulata. GFP expression was also observed in several other areas such as the amygdala. With both vector subtypes some diffusion or active transport of the vectors to the contralateral side of the brain took place. This was most evident with rAAV5 (Figure 11).

Detailed quantitative microscopic analysis of the GFP+ cells in the striatum and frontal cortex were performed using both stereological cell counts for unbiased quantification and confocal microscopy to provide phenotypic characterization of the transduced cells at six weeks after the operations. In the striatum both vector subtypes gave rise to numerous GFP+ cells with the highest density around the injection site, and decreasing cell numbers 3-4 mm in both rostral and caudal directions (Figure 12). The average numbers of GFP+ cells in caudate nucleus and putamen in the rAAV1 group were $1.42 \times 10^5$ and $0.98 \times 10^5$, respectively, and in the rAAV5 group the caudate nucleus contained $12.61 \times 10^5$ GFP+ cells while putamen contained $6.64 \times 10^5$ GFP+ cells.

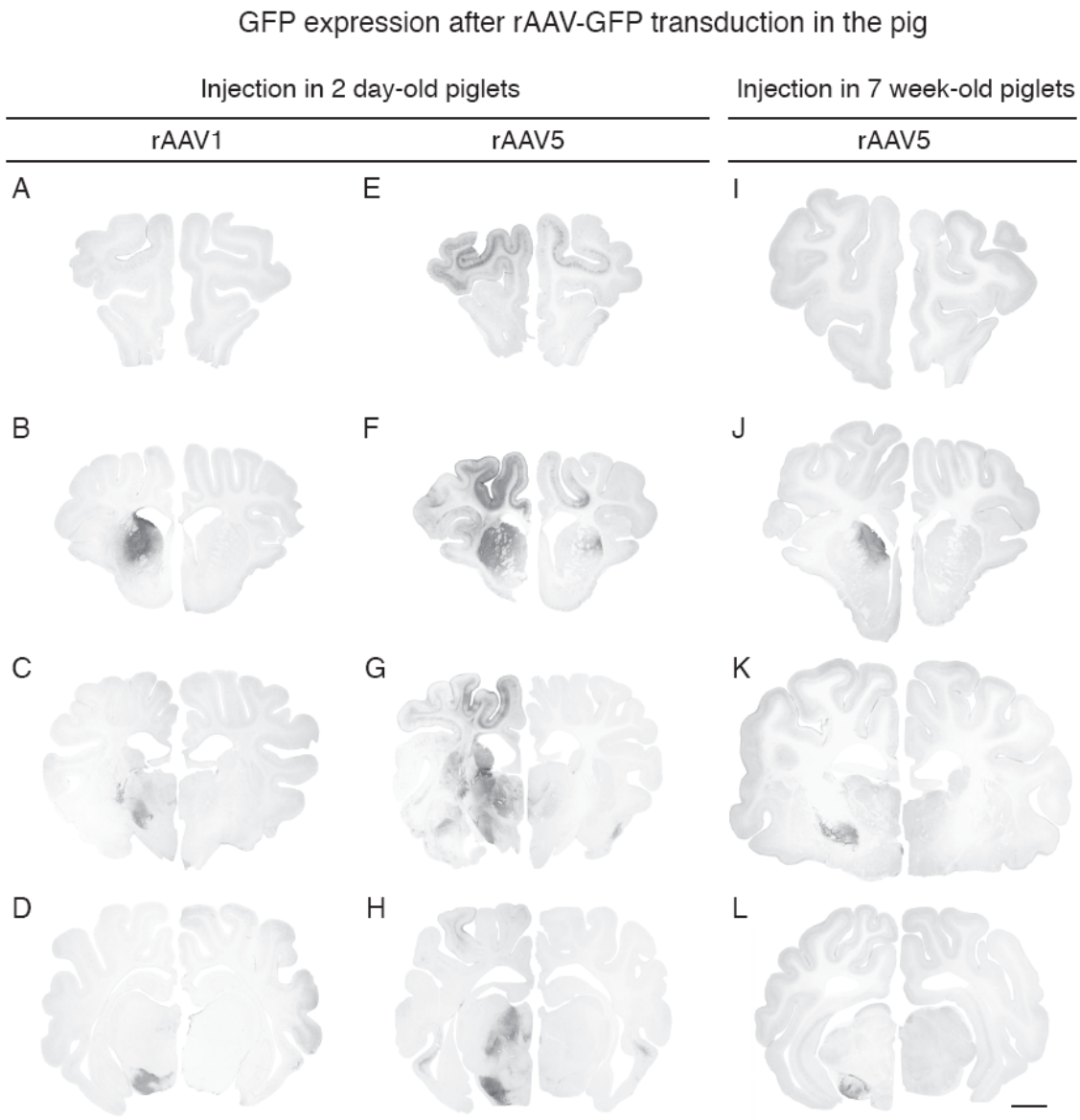


**Figure 11**. Low power photomicrographs showing coronal views of sections from the pig brain immunostained against the GFP protein after neonatal injection of rAAV1 (first column) or rAAV5 (second column) or rAAV5 injection in seven-week old animals (third column) from animals killed at 6 weeks after transduction. Injection site was approximately at the level of sections B, F, and J. The scale bar represents 5 mm

In parallel sections we estimated the total number of neurons using the neuronal marker NeuN (Mullen et al., 1992) and found an average number of NeuN+ cells in the caudate nucleus of 7,897,344 ± 386,465 and in putamen of 7,003,968 ± 434,438 (n=6). This gives transduction frequencies of 1.4% (caudate nucleus) and 0.9% (putamen) for rAAV1, and 15.6% (caudate nucleus) and 9.6% (putamen) for rAAV5.

Following rAAV5 injections we also quantified the transduction frequency in frontal lobe grey matter and in an area approximately corresponding to area frontalis agranularis (Brodmann area 6), where the transduction frequency was highest. In the entire frontal lobe the total number of GFP+ cells was 711,168 ± 142,947, with the majority of cells (286,310 ± 39,984) found in area 6 (Figure 12). Over the entire frontal lobe we found a transduction frequency of 1.7 %.

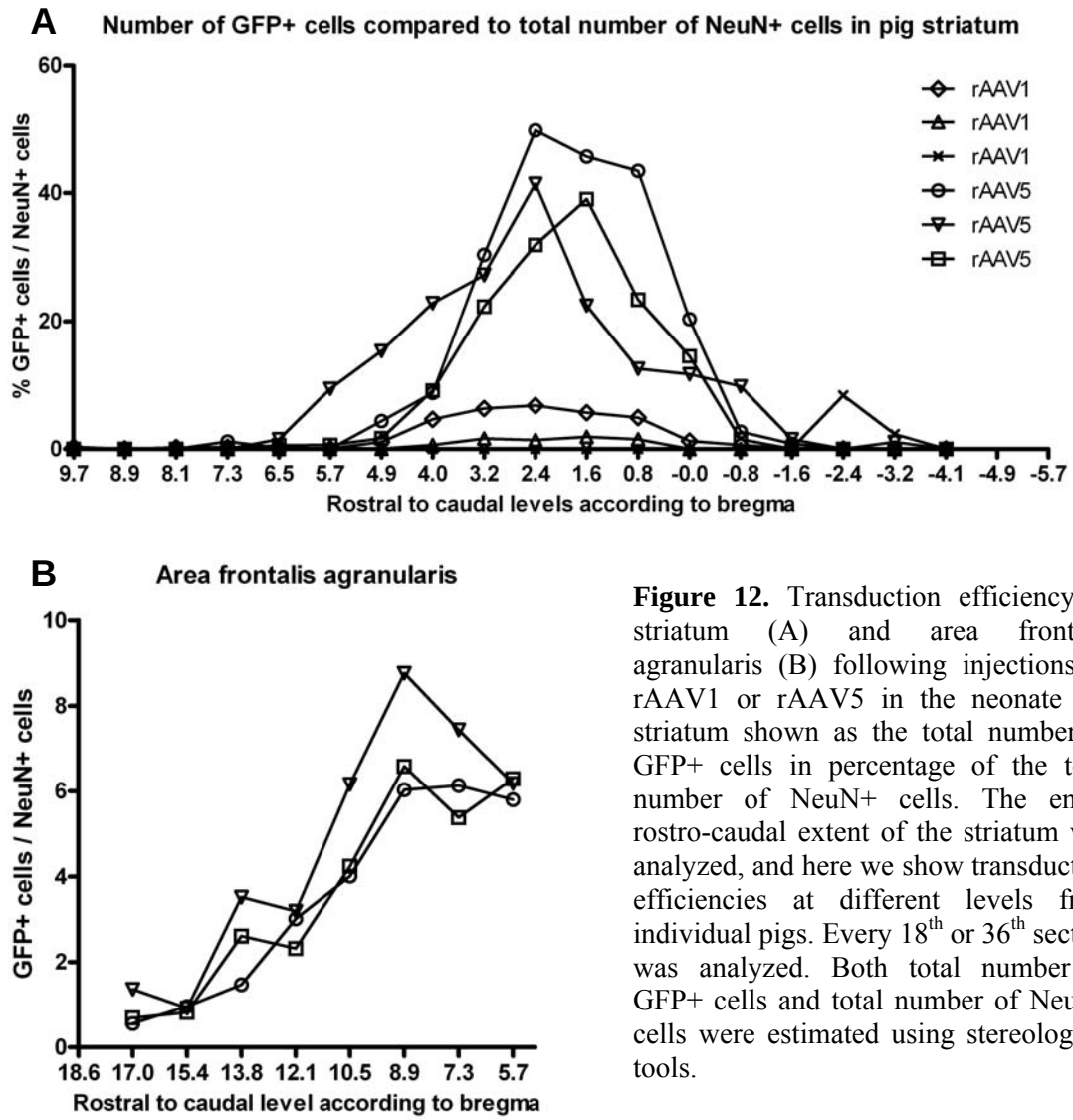


**Figure 12.** Transduction efficiency in striatum (A) and area frontalis agranularis (B) following injections of rAAV1 or rAAV5 in the neonate pig striatum shown as the total number of GFP+ cells in percentage of the total number of NeuN+ cells. The entire rostro-caudal extent of the striatum was analyzed, and here we show transduction efficiencies at different levels from individual pigs. Every 18$^{th}$ or 36$^{th}$ section was analyzed. Both total number of GFP+ cells and total number of NeuN+ cells were estimated using stereological tools.

The first qualitative observations on the morphology of the GFP+ cells in striatum suggested that a small number of glial cells were also transduced. Further analysis of the GFP+/NeuN− cells in the confocal series indicated that in the rAAV5 group about 2.4% of the cells in striatum were non-neuronal. This fraction was <0.5% in the rAAV1 group. In pig frontal cortex, less than 0.5 % of the total population of GFP expressing cells were GFP+/NeuN−.

In order to reveal the phenotype of the other cell types that were transduced by rAAV5 vectors, we performed triple labeling immunohistochemistry against GFP and the two astroglial markers glial fibrillary acidic protein (GFAP) and S100β (Lyck *et al.*, 2006) and double labeling against GFP and the oligodendrocyte marker adenomatous polyposis coli (APC) tumor suppressor protein (Bhat *et al.*, 1996). In pig caudate and putamen, 97.6% of the GFP+ cells were NeuN-positive, 1.8% of GFP+ cells were APC-positive, while <0.5% of the cells co-expressed GFP and GFAP or S100β. In frontal cortex 99.6% of the GFP+ cells were NeuN-positive, and <0.5% co-expressed GFP and APC, GFAP, or S100β (Figure 13).

In summary, we have found that the neuronal transduction efficacy is highest with rAAV5, and with this serotype we also saw a widespread transduction of cortical cells in frontal lobe grey matter. Our results from this method validation show that striatal delivery of rAAV5 vectors to the neonatal pig brain represents a useful tool to express genes of interest not only in the basal ganglia, but also in the neocortex.

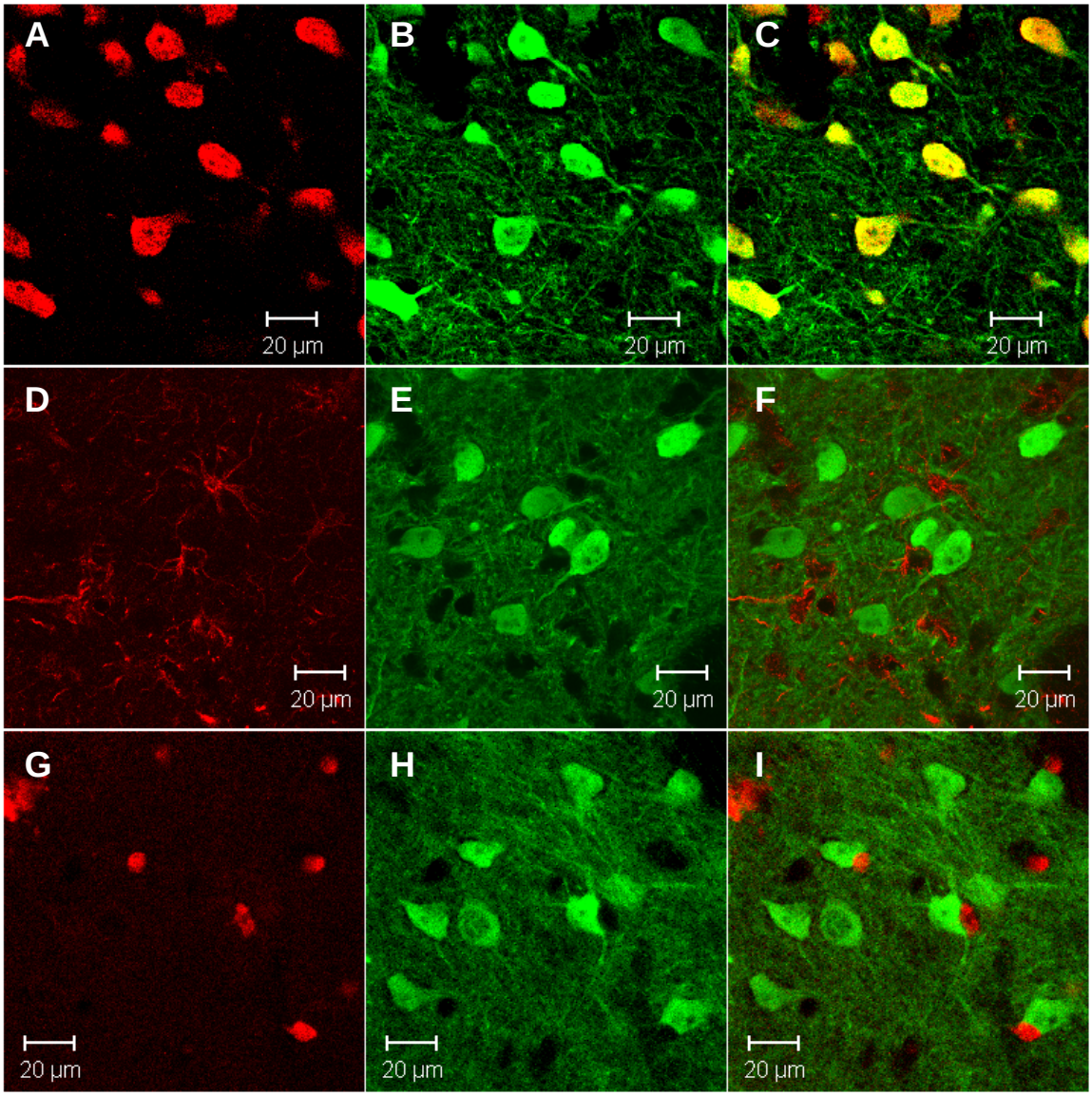


**Figure 13.** Phenotypic analysis of transduced cells (GFP+, see B,E,H) was conducted by performing double- or triple-fluorescent immunohistochemical stainings and analyzing the co-labeling frequency by use of confocal microscopy. Neurons were measured as NeuN+ cells (A), astrocytes as GFAP+ and S100β+ cells (D), and oligodendrocytes as APC+ cells (G). In the rAAV5 injected animals, in particular, large fractions of NeuN+ cells were transduced. Panels C, F, and I shows the images to the left overlaid and yellow indicates co-expression.

## CONCLUSIONS

This thesis presents different neuroscience methods and their application to the Göttingen minipig brain. From these studies we can conclude the following basic points about the Göttingen minipig:

- The 5-$HT_4$ receptor has the same overall distribution in the Göttingen minipig brain as in the human brain.
- The pig brain 5-$HT_4$ receptor distribution can be studied *in vivo* using [$^{11}$C]SB207145-PET and a suitable kinetic model is the SRTM2.

- Both object and spatial one-trial memory decays rather fast in this species. Approximately within the first hour.
- The Göttingen minipig can learn a non-match to sample rule and maintain a high task performance level over a longer period of time when food-rewarded.

- The viral vector subtype rAAV5 targeted to the neonate Göttingen minipig striatum induces transgene expression in neurons in both the basal ganglia and parts of the neocortex, with highest levels around the injection site.

We can not yet confirm or reject the hypothesis regarding 5-$HT_4$ receptor functions proposed in this thesis, but by using the methods presented here, it should be possible to come closer to an understanding of the 5-$HT_4$ receptor and its involvement in learning and memory and the memory disorder Alzheimer's disease.

The work presented in this thesis is basically a translation of different well established rodent methods to the pig model. We show that it is possible to perform both pharmacological and behavioural studies using the pig, and that it is possible to genetically modify neurons in the pig brain. From this and the current literature reviewed in the theoretical parts of this thesis we conclude that the pig is indeed a valuable species in translational neuroscience research.

## Perspectives and future studies

From the studies presented in this thesis emerge two future lines of research.

The first is based on the modulatory effect of the 5-$HT_4$ receptor on normal memory, and aims at confirming or rejecting the hypothesis that basal 5-$HT_4$ receptor levels in the hippocampus partly determines memory performance in relevant tasks. Encouraged by the result from the pilot study described in this thesis, where a correlation between memory performance in the SOR task with a 1-hour inter-trial interval and 5-$HT_4$ receptor concentration in hippocampal tissue is suggested, we will try to confirm this in a bigger sample, testing each animal more times. The performance after systemic injection of a 5-$HT_4$ agonist and an inverse agonist will also be determined. In such a study it is not possible to distinguish between direct and indirect effects on e.g. hippocampal functioning. To determine the direct effect of 5-$HT_4$ agonism/inverse agonism on the hippocampus it will be necessary to inject the drug directly into the hippocampus using stereotaxic implanted canullas. Another interesting study to perform would be to over express 5-$HT_4$ receptors or induce a knock-down through small interfering RNA (siRNA) expression in hippocampal neurons using viral vector technology. Animals subjected to this kind of manipulation could then be tested in both the SOR and the DNMS task. It could also be interesting to see if the altered levels of 5-$HT_4$ receptors could be detected with PET. Here the same animals could be scanned before and after injection of viral vectors. To avoid partial volume problems a co-registered MRI should also be obtained or the animals should be scanned in a HRRT scanner. It would be worth vile to consider whether some of these experiments should be performed using rats, and then only expanded into pigs if positive results are obtained from the rat studies.

The second line of research evolves around the idea of inducing Aβ accumulation in the pig brain via viral vector technology. This could be done by injections of rAAV5 vectors carrying a mutated version of APP. We have already shown that we can transduce neurons in large parts of the cortex even though at a rather low transduction frequency. We speculate that by injecting a larger volume and possibly in more than one site a higher cortical transduction frequency could be achieved. An interesting alternative would be to try to target the hippocampus. If we could succeed in manipulating the pig brain into accumulating Aβ, it would be very interesting to assess the memory performance of these animals and also to see if AD-like pathology would develop over time in the pig brain tissue. In the context of this thesis, it would be extremely interesting to determine the levels of 5-$HT_4$ receptors in the brains of these animals and to correlate the receptor density to the Aβ concentration. If a change in receptor density after the manipulation is detected, it would be very valuable to determine the time course of this change in relation to the emergence of memory disturbances and different pathological markers.

The pig is an interesting species in neuroscience research and, hopefully, the work presented in this thesis will inspire several new lines of research with the pig brain as a model system.

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# Appendices

I Evaluation of the novel 5-$HT_4$ receptor PET ligand [$^{11}$C]SB207145 in the Göttingen minipig.

II The effect of the inter-phase delay interval in the spontaneous object recognition test for pigs.

III A novel spatial delayed non-match to sample (DNMS) task in the Göttingen minipig.

IV Adeno-associated viral vectors targeted to neonatal rat and pig striatum induce widespread transgene expression (manuscript).