

Novel insights towards memory restoration

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NOVEL INSIGHTS TOWARDS MEMORY RESTORATION

Sarah-Anna Hescham

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Novel insights towards memory restoration.

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NOVEL INSIGHTS TOWARDS MEMORY RESTORATION

DISSERTATION

To obtain the degree of Doctor at Maastricht University,
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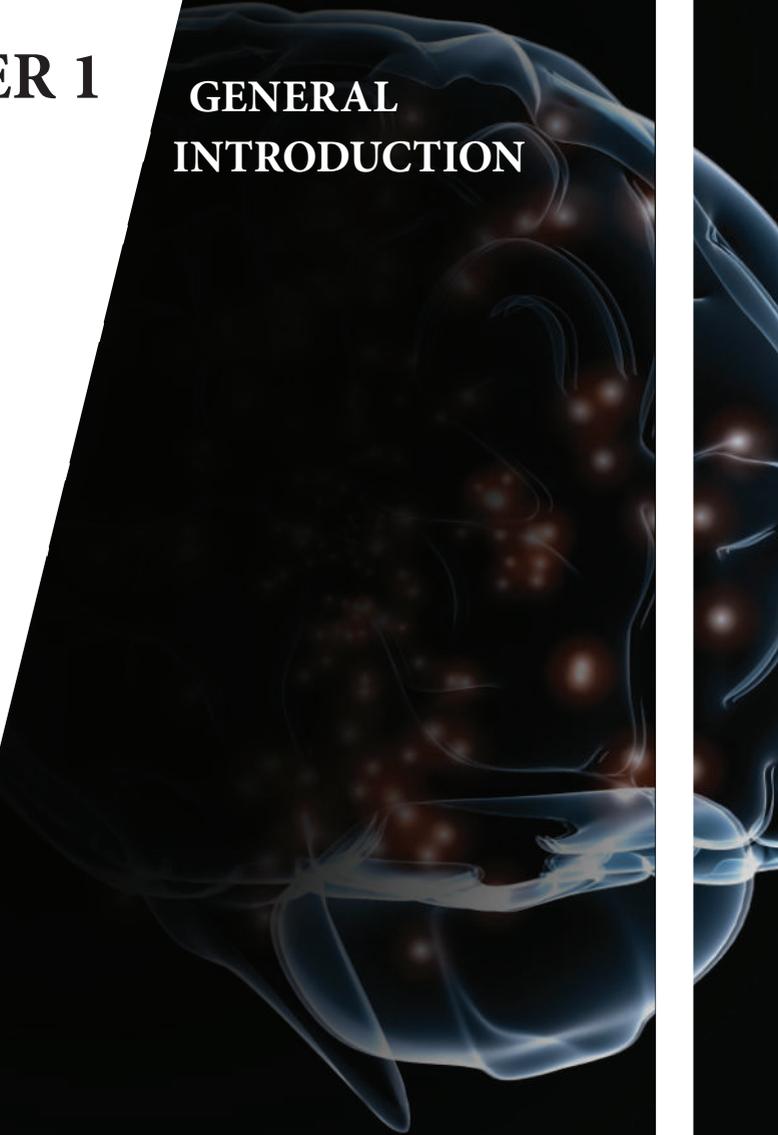
To my beloved grandmothers

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CHAPTER 1

GENERAL INTRODUCTION



MEMORY

Memory refers to the organism's ability to encode, retain and recall experiences and information. Failure of memory consequently results from failure of adequately storing the memory, failure to retain the information or failure to retrieve the information later. The first and best-studied case of memory dysfunction was the 27 year old patient called H.M., who underwent a surgery for the relief of intractable epilepsy in 1953 [1]. In this surgery portions of the temporal lobe were removed bilaterally. H.M.'s seizures were better controlled after surgery, but the removal of the medial temporal lobes left him with a devastating memory deficit. In fact, H.M. showed normal short-term memory, had a good command of language and his IQ remained unchanged, but he lacked the ability to transfer new information into long-term memory [1, 2].

TYPES OF MEMORY

Memory formation and recall is essential for higher cognition and survival. One can differentiate between sensory, short- and long-term memories, which are defined by the duration for which a memory is expected to last. Sensory memory corresponds to sensory (visual, auditory, gustatory, olfactory or tactile) recollection 200-500 μ s after perceiving the information. In short-term memory the efficiency with which nerve impulses pass across synapses is increased for several seconds to minutes. Long-term memory requires that new synaptic connections are formed, inducing a permanent change in the neural circuit [3]. Various forms of long-term memory can furthermore be classified as either explicit (declarative) or implicit (non-declarative) and be assigned to different structures of the brain. Explicit memory requires conscious thought and can be associated with the medial temporal lobe, while implicit memory does not require conscious recollection and involves structures like the striatum, amygdala, cerebellum and the neocortex [4].

One major pathway of the brain which is primarily involved in the cortical control of emotion and in storing memory is the circuit of Papez. It plays a role in consolidating short-term to long-term memory after receiving emotional and motivational inputs from structures such as the hypothalamus, amygdala or orbital cortex. In this circuit the entorhinal cortex projects to the hippocampus, which efferents are bundled in the fornix and reach the mammillary bodies. In fact, the fornix connects the hippocampus and subcortical structures in a bi-directional manner. From the mammillary bodies, the information proceeds to the anterior nucleus of the thalamus via the mammillo-thalamic tract. The circuit is completed by projections of the anterior thalamic nuclei

to the cingulate gyrus which in turn transfers the information via the cingulum to the parahippocampal gyrus of the entorhinal cortex.

The role of the Papez circuit has been the subject of many investigations designed to elucidate the neural basis of memory. As seen in the case-study of H.M. the hippocampus is essential for the consolidation of information from short-term to long-term memory, although it does not seem to store information itself [2]. In addition, structures like the hippocampus are required for spatial learning and memory.

MEMORY LOSS

Different aspects of memory are affected by damage to distinct brain regions [5]. Aging, for example, impairs hippocampal and prefrontal-cortex dependent memory and cognition. In an aging society, dementia has emerged as one of the leading health problems of our time and has been recently recognized as one of the major threats to world population [6]. Worldwide 35.6 million people suffer from dementia, a number that is anticipated to triple by 2050. The most common form of dementia is Alzheimer's Disease (AD), accounting for 60-80% of all cases. Every year 6 million new cases of AD are diagnosed, imposing a tremendous burden on families and caregivers, as well as financial cost to society. To date, five drugs are approved for the treatment of AD in North America and most European countries, including four acetylcholinesterase inhibitors (Tacrine, Donepezil, Galantamine and Rivastigmine) and an N-methyl-D-aspartate (NMDA) receptor antagonist (Memantine) [7]. These pharmacological treatments, however, only provide limited and short-lasting symptomatic relief. In some patients substantial side-effects such as nausea and muscle weakness are seen [8].

Yet, the localization of diverse memory processes in different brain areas and the understanding of their biochemical and physiological underpinnings has opened the doors to novel therapeutic approaches, which do not require drugs. One approach, which is currently being explored, is deep brain stimulation.

DEEP BRAIN STIMULATION

Deep brain stimulation (DBS) is a reversible, minimal invasive neurosurgical intervention in the brain, aimed at treating neurological disorders. It consists of three components: the implanted pulse generator, the electrodes, and the extension. The pulse generator is a battery-powered neurostimulator, which sends electrical pulses to the brain to interfere with neural activity at the target site. The electrode is a coiled wire insulated in polyurethane with four platinum iridium contact sites and is placed in

different nuclei of the brain. It is connected to the pulse generator by the extension, which is an insulated wire placed subcutaneously.

Worldwide more than 130,000 patients have been implanted with DBS electrodes. In fact, this procedure has become a routine second-tier therapy in patients with specific neurological disorders, including Parkinson's Disease [9], essential tremor [10, 11] and dystonia [12]. In patients with severe epilepsy [13], Tourette syndrome [14] and obsessive-compulsive disorder [15, 16], DBS has been shown to have good therapeutic effects. In the past years, studies have begun to explore the applicability of DBS in a widening array of psychiatric conditions including disorders of mood and cognition. DBS within the limbic system, for example, has shown positive effects in treatment-resistant depression [17-19]. Recently, DBS has been proposed as a potential tool to enhance memory functions. A first evidence for this emerged when Hamani and colleagues [20] stimulated the fornix/hypothalamus area in a patient suffering from morbid obesity. In this specific case, DBS generated detailed autobiographical memories in the patient and an electroencephalographic source localization elucidated that hypothalamic stimulation evoked activity in medial temporal lobe structures including the hippocampus. Based on this case-observation, the same group performed a phase-I trial in patients with AD [21]. Patients with mild AD were implanted with electrodes in the area of the fornix/hypothalamus and after an intraoperative evaluation of stimulation, patients received chronic DBS for a period of 12 months. The hypothesis of this study stated that it might be possible to use DBS of the fornix to drive its activity and to modulate the circuits mediating memory functions. Indeed, memory tests confirmed possible improvements and/or slowing in the rate of cognitive decline in some patients following chronic DBS.

Despite the encouraging results of the clinical trials presented above, basic neural and chemical mechanisms underlying DBS are still debated [22]. One approach to address these issues is to investigate the effects by stimulating homologous regions in experimental animal models [23].

EXPERIMENTAL DBS IN ANIMAL MODELS

In order to investigate which neural processes are modulated by DBS or which stimulation parameters or surgical targets should be used, preclinical animal studies can be of great value [23]. The development of experimental DBS in animal models has been a major breakthrough for preclinical research. With the use of translational hardware concerning electrode compositions, preclinical studies can advance the application of clinical DBS. A DBS device can be constructed for unilateral as well as bilateral stimulation. For the latter, a bilateral electrode has to be assembled, whereas the inter-

electrode distance depends on the brain target of stimulation. Stimulation parameters (frequency, pulse width and amplitude) can be defined according to the hypothesis. Of note, stimulation amplitudes in animal research are relatively high compared with those used in human DBS. This is because in preclinical studies, stimulation is usually acute and thus side effects are less obvious. When high amplitudes (i.e. $\geq 200 \mu\text{A}$) are applied in chronic stimulation paradigms, substantial tissue damage can be observed [23]. Only through understanding the anatomical, physiological and neurochemical substrates of DBS, this therapy can be fine-tuned to produce the best possible symptom relief in patients.

AIM OF THIS THESIS

The overall aim of this thesis is to investigate whether DBS can be used as a tool to restore memory loss and what the mechanisms of action are.

First, state-of-the-art application of DBS in psychiatric conditions will be described (Chapter 2). Following this, I will primarily focus on dementia-related disorders and speculate on potential mechanisms of action (Chapter 3). In order to investigate restoration of memory loss, an experimental rat model of dementia needs to be verified. I chose for the scopolamine-induced rat model of dementia and explored effects on various brain regions (Chapter 4). Using this rat model I assessed restoration of memory loss by means of DBS in different target regions using behavioral tasks (Chapter 5 and 7). Since dementia occurs with aging, I was also engaged in a collaborative project, in which various behavioral tasks with DBS were performed in an aged animal model (Chapter 6). Finally I investigated mechanisms of action of DBS with regard to memory restoration. First, I examined the effects of DBS on dopamine levels in the VTA and substantia nigra (Chapter 8). Next, I investigated whether DBS had an effect on neural activity within certain brain regions as well as neurotransmitter release (Chapter 9). I also assessed whether DBS has any long-term effects on memory, which can be linked to neurogenic processes (Chapter 10). Finally, the main findings of this thesis will be summarized and discussed and the overall conclusion of the research will be provided (Chapter 11).

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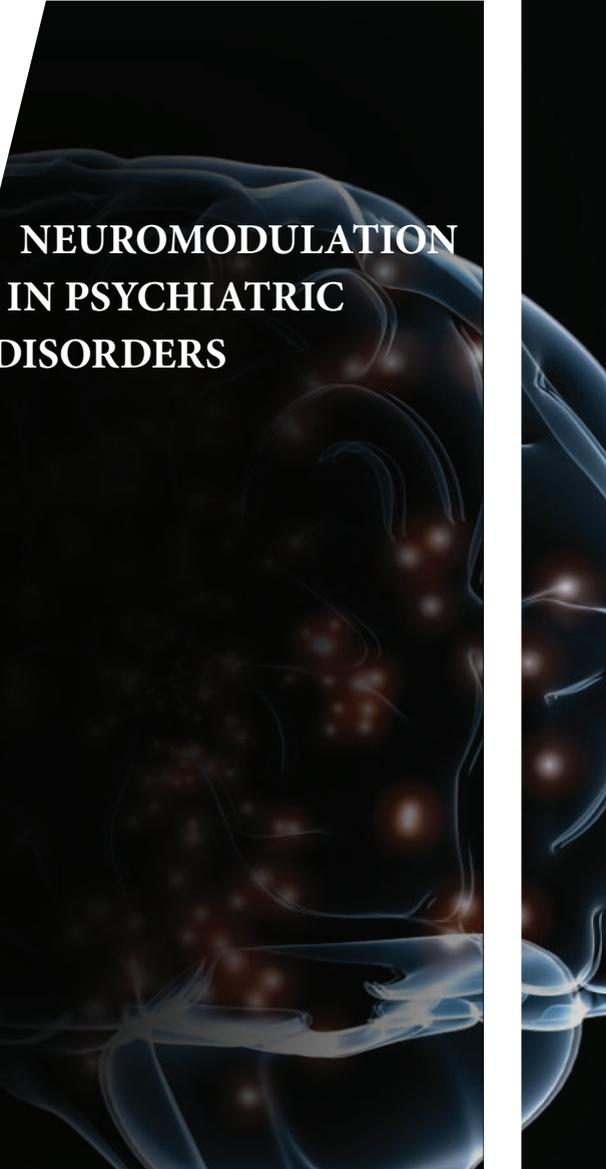
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CHAPTER 2

NEUROMODULATION IN PSYCHIATRIC DISORDERS

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Adapted from *International Review of Neurobiology*, 2012;107:283-314.



ABSTRACT

Psychiatric disorders are worldwide a common cause of severe and long-term disability and socio-economic burden. The management of patients with psychiatric disorders consists of drug therapy and/or psychotherapy. However, in some patients, these treatment modalities do not produce sufficient therapeutic effects or induce intolerable side-effects. For these patients neuromodulation has been suggested as a potential treatment modality. Neuromodulation includes deep brain stimulation, vagal nerve stimulation, and transcranial stimulation. The rationale for neuromodulation is derived from research identifying neurobiologically localized substrates for refractory psychiatric symptoms. Here, we review the clinical data on neuromodulation in some of the major psychiatric disorders. Relevant data from animal models will also be discussed to explain the neurobiological basis of the therapy.

INTRODUCTION

Psychiatric disorders are characterized by alterations in thinking, mood or behaviour. They cause an enormous economic burden on societies and on quality of life of individuals and families [1]. Current treatment options of the patients with psychiatric disorders include drug therapy and/or psychotherapy. However, in some patients, these treatment modalities do not produce sufficient therapeutic effects or induce intolerable side-effects. For these patients, ablative surgery and neuromodulation therapies have been proposed. In the early and mid 20th century, refractory psychiatric (“mentally ill”) patients have mainly undergone ablative surgery and in some cases experimental electrical brain stimulation. The rationale for these procedures was to counteract the dysfunctional neuronal activity of specific parts of the limbic and associative brain circuits. Although this approach seems hypothesis-driven, there was only limited scientific evidence to support this view at that time. The outcome of these procedures was unfortunately not extensively documented and some patients experienced major side-effects. The latter together with advances in the field of medical ethics resulted in stopping most of the “psychosurgery” programs, except for some centres which continued.

In the late 20th century, the neurosurgical treatment of refractory psychiatric disorders underwent a revival with the application of deep brain stimulation (DBS). This technique is actually a modernized and advanced version of the previous electrical brain stimulation paradigms, also applied in human subjects [2]. Nevertheless, there are major differences between the present and past. At present, we have supporting clinical and experimental evidence, extensive medical ethical considerations and the availability of advanced stimulation technology. Recent studies have consistently shown that DBS has promising therapeutic effects in selected cases and as a result this field of Neuroscience is rapidly emerging. The rationale for DBS is related to the neurobiological mechanisms underlying psychiatric disorders, which will be explained in more detail in the sections below.

Neurobiological mechanisms underlying psychiatric disorders have been considered for a long time to depend on solitary systems such as neurotransmitters and specific cortical areas. Although these models might still explain some of the symptoms, more recent findings from both clinical and experimental studies [3] suggest a dysfunction of interrelated networks involving cortical and subcortical structures and various neuroactive substances [4, 5]. For instance, symptoms of Tourette syndrome (TS) and obsessive-compulsive disorder (OCD) have been linked to non-motor elements of the basal ganglia-thalamocortical pathways, including the nucleus accumbens (NAc), ventral pallidum and medial parts of the thalamus, in combination with a dysfunctional mesolimbic dopaminergic system [5, 6]. The dysfunctional network hypothesis

provided a basis to explore DBS in psychiatric disorders. DBS is, however, not the only neuromodulation-based therapy for refractory psychiatric disorders. Others include vagal-nerve stimulation, transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS). Electroconvulsive therapy, used for a long-time to treat depression for instance, is also a neuromodulation-based therapy, but is beyond the scope of this article. Here, we review the clinical data on DBS in some of the major psychiatric disorders in an alphabetic order. We will briefly discuss other neuromodulation techniques as well. Relevant data from animal models in specific sections will also be discussed to explain the neurobiological basis of the therapy.

Neuromodulation in addiction

Addiction or substance dependence is a chronic relapsing disease characterized by a strong psychological and physical dependence, resulting in a withdrawal syndrome when the use of the drug is stopped. Interestingly, like in other disorders, ablative surgery of certain brain areas, including the cingulate gyrus, the substantia innominata, ventromedial hypothalamus and the NAc have been performed to treat patients with addiction [7-12]. The rationale was to interfere with a dysfunctional neuronal circuit responsible for the symptoms. To our knowledge, no randomized controlled trials have been published thus far to test the effect of DBS on refractory addiction. Some case reports and series have been reported in which the effects of DBS of the subthalamic nucleus (STN) (Figure 1) or NAc (Figure 2) are described.

Deep brain stimulation

Recently, the effect of DBS of the NAc in three patients with alcohol addiction was described [14]. In these patients, craving fully disappeared after DBS. Two patients remained abstinent during the one year follow up, and the other reduced his alcohol consumption. In one patient also the number of cigarettes smoked per day was reduced. In this patient a hypomanic episode of two weeks was reported, which resolved after adjusting the stimulation parameters. In another publication, a case of DBS within the NAc in a patient with heroin addiction was described [15]. After surgery this patient remained abstinent from drugs during the six-year follow up. He also reduced his smoking to 75%, with 10 cigarettes a day. Interestingly, the stimulator was turned off 2 to 3 years after surgery and was later removed. The improvement persisted.

In a patient with severe agoraphobia, DBS of the NAc had almost no effect on the phobia, but alcohol consumption was substantially reduced [16]. One patient who underwent DBS of the NAc for OCD decided to stop smoking 10 months after surgery, while she had many unsuccessful attempts to stop before surgery [17]. Although, the case descriptions demonstrate beneficial effects, it is still hard to draw final conclusions from these uncontrolled studies. This view is also supported when considering the vari-

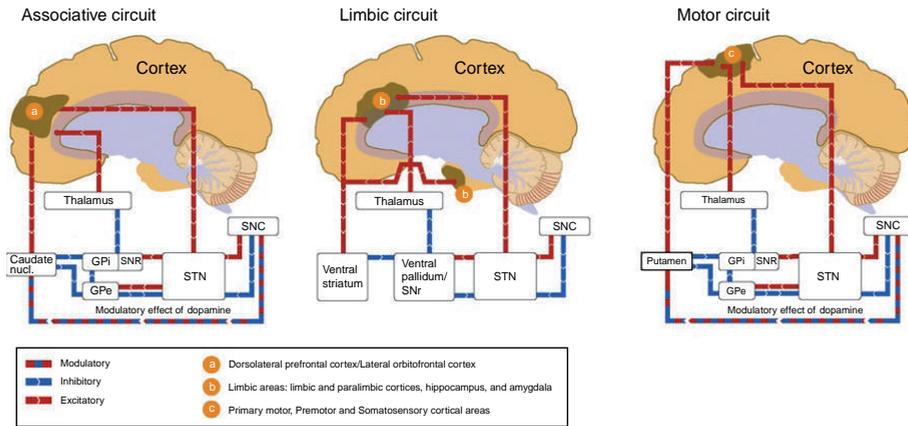


Figure 1: The subthalamic nucleus (STN) receives, integrates and projects at least three types of information: motor, limbic and associative. These are illustrated in this schematic illustration of the primate basal ganglia-thalamocortical associative, limbic and motor circuits. *Associative circuit:* The two associative circuits are the dorsolateral prefrontal circuit (DPC) and lateral orbitofrontal circuit (LOC), which are initiated from the dorsolateral prefrontal cortex (DLPC), and lateral orbitofrontal cortex (LOFC), respectively. The second station is the dorsomedial head and the rostrocaudal axis of the caudate nucleus. From here, this circuit is directed to the dorsomedial part of the GPI and to the rostral region of the SNr and to the anterior parts of the GPe. The GPI and SNr project to the ventroanterior (VA) and centromedian nucleus (CM) nuclei of the thalamus and the DPC is closed by the thalamocortical pathway back to the DLPC and the LOC back to the LOFC. This pathway is also known as the direct pathway. From the GPe, a projection to the STN and GPI/SNr exists. This pathway is also known as the indirect pathway. The STN is anatomically connected with both direct, through its projection to the GPI and SNr, and indirect pathway, through its projection to the GPe. *Limbic circuit:* Projections from the hippocampus, the amygdala, limbic and paralimbic cortices are primarily concentrated at the level of the ventral striatum. The ventral striatum consists basically of the nucleus accumbens, ventromedial part of the caudate-putamen and the medium-celled portion of the olfactory tubercle. The ventral striatum projects in turn to the ventral pallidum (VP). From here the limbic circuit is directed to the MD nucleus of the thalamus. This circuit is closed by a thalamocortical pathway to the anterior cingulate area and medial orbitofrontal cortex. The STN has reciprocal connections with the ventral pallidum. The ventral pallidum is considered to be the major limbic circuit output region. *Motor circuit:* The cortical input to the motor circuit originates mainly from the primary motor, premotor and somatosensory areas. This glutamatergic input is largely directed to the putamen which projects topographically to the motor parts (ventrolateral (VL) and posterior) of the GPe and GPI, and the SNr. From the GPe, a pathway projects to the STN. The STN, mainly projects to the GPI and SNr. The SNr and the GPI serve as the output nuclei of the basal ganglia. The thalamic areas involved in the motor circuit are mainly the VL, VA and the CM. This loop is closed by means of the thalamic projection (glutamatergic) to the cortical areas. Adopted from Temel *et al.* [6].

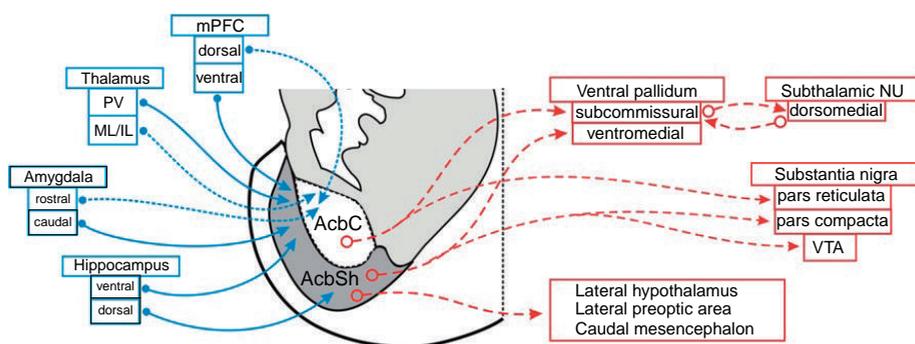


Figure 2: The nucleus accumbens is part of the ventral striatum and a popular target for DBS in several psychiatric disorders. Here we illustrate its connections with other brain areas. Schematic drawing of the inputs and outputs of the shell (AcbSh) and core (AcbC) of the nucleus accumbens. Dopaminergic, serotonergic, and noradrenergic inputs have been omitted from the drawing. Note that virtually all structures (left hand side of the scheme), although via different subdivisions or subnuclei, project to both shell and core. This also holds true for the outputs of both subdivisions (right hand side of the scheme), but it must be noted that there are also main differences. Both shell and core reach pallidal and nigral areas, albeit different parts of these basal ganglia structures. The shell, in addition projects to preoptic and hypothalamic areas and the caudal mesencephalon.

Abbreviations: ML/IL, midline and intralaminar thalamic nuclei; mPFC, medial prefrontal cortex; PV, paraventricular thalamic nucleus; VTA, ventral tegmental area. Adopted from Basar et al. [13]

able effects of NAc DBS on nicotine addiction in a case series in which only 3 out of 10 patients improved [18].

The STN is targeted for DBS in patients with advanced Parkinson's disease (PD). The STN has strong motor, associative and limbic connections (Figure 1) [6]. Interestingly, in PD patients, STN DBS resolved their addictive behaviour [19-22], however opposite effects have been found as well. In a case report, a patient without any history of addiction became a pathologic gambler after STN DBS [23]. Other studies found also varying effects of STN DBS, some patients improved, while in others the addictive behaviour remained the same or even worsened [24]. The underlying mechanisms are unclear.

Transcranial stimulation

TMS and tDCS show promising results on addictive behaviour [25-34]. Several studies have shown that TMS is effective in reducing the level of cravings for smoking, alcohol, and cocaine when applied at high frequencies to the dorsolateral prefrontal cortex [26]. TMS to the dorsal anterior cingulate cortex reduced alcohol craving and consumption as well [34].

In rodents, TMS increases dopamine in the dorsal hippocampus [35] and the NAc [35, 36]. TMS in the rat has also been shown to change the expression of proteins involved in the synthesis of γ -aminobutyric acid (GABA) by two isoforms of glutamate decarboxylase [37]. The effect of TMS on addiction is not explained by local changes, but by plastic and structural changes in the neural networks involved in addiction. Animal data show that the STN is connected with the reward circuitry (ventral tegmental area and NAc) [38-41]. STN lesions or high frequency stimulation (HFS) reduces motivation to obtain cocaine while increasing the drive to attain food rewards [38, 39]. From the animal studies we can conclude that the STN plays a complex role in the reward circuit and is therefore an unpredictable target to treat addiction.

Animal studies on DBS of the NAc showed a significant reduction of drug related behaviour of either core or shell stimulation [42-45]. Also lesions of the medial NAc shell in animal models reduced addictive behaviour and decreased ethanol consumption [46].

Neuromodulation in Alzheimer's disease

Alzheimer's disease (AD) is the most frequent age-dependent form of dementia. In the early stages of AD, the most common symptom is the inability to acquire new memories. In further progression of the disease, patients suffer from confusion, agnosia, aphasia, apraxia and finally long-term memory loss. Despite the devastating impact, there are currently no known treatments to prevent, cure or delay the progression of AD. To date only symptomatic treatments are available. Pharmacological interventions that are approved for treatment of AD in North America and most European countries include: Memantine (an NMDA receptor antagonist) for severe AD and acetylcholinesterase inhibitors for mild to moderate AD: Tacrine, Donepezil, Galantamine and Rivastigmine [47]. These pharmacological treatments, however, have limited effects which are usually short-lasting. Neuromodulation takes a completely different approach to treat AD from current medication. Researchers are currently exploring the applicability of DBS [48, 49], TMS [50-53] or tDCS [54, 55] to halt or reduce the progression of memory loss in AD and to improve the quality of life of patients and caregivers.

Deep brain stimulation

Recently, Laxton et al. [48] performed the first DBS study in which six patients with mild AD were implanted with electrodes in the vicinity of the fornix/hypothalamus. After an intraoperative evaluation of stimulation to survey for recollective experiences and adverse effects, patients received chronic high frequency DBS for a period of 12 months. The authors have found that the application of DBS in the hypothalamus/fornix vicinity was safe and triggered neural activity in the memory circuit, including the entorhinal and hippocampal areas. PET scans showed an early and striking reversal of

the impaired glucose utilization in the temporal and parietal lobes that was maintained after 12 months of continuous stimulation. Evaluation of the Alzheimer's Disease Assessment Scale cognitive subscale and the Mini Mental State Examination suggested possible improvements and slowing in the progression of memory loss at 6 and 12 months, especially in patients that were less severely affected at the time of surgery. After 1 year of continuous DBS, the functional connectivity analysis demonstrated increased cerebral metabolism in cortical-subcortical and cortical-hippocampal networks. In similar cortical regions, both a higher baseline metabolism and an increase after 1 year of DBS were correlated with less decline or improvement in global cognition, memory, and quality of life [49]. When considering animal studies, DBS of the forniceal region (Figure 3), improves spatial memory performance in the object location task in a rat model of experimental dementia [56]. Memory enhancement was substantial and became comparable to the performance of controls. The current hypothesis states that this effect is accomplished by driving the fornix activity, both orthodromically as well as antidromically [56-58]. This is supported by the view that large myelinated axons produce excitatory responses upon electrical stimulation [59].

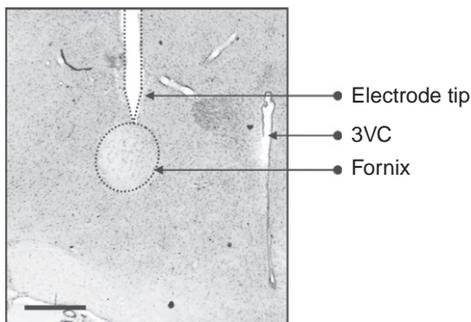


Figure 3: The fornix is part of the circuit of Papez and is considered a target for DBS in AD. Here, we illustrate the placement of the electrode in the vicinity of the rodent fornix. DBS of this area improved memory impairment in a rat model of dementia. Illustrative coronal section showing the histological verification of the electrode location in the vicinity of the fornix (bar = 400 μm). Abbreviations: 3VC, third ventricle. Adopted from Heschem et al. [56].

Transcranial stimulation

To date, there are few reports about the effects of TMS and tDCS on memory investigating focal and non-focal neuroplasticity changes in subjects with AD [50-55]. Cotelli et al. [52] investigated the effects of TMS over the left or right dorsolateral prefrontal cortex on mild to moderate AD patients. Magnetic pulses were delivered for 600 ms from the onset of the stimulus presentation of a picture naming task. Repetitive TMS

(rTMS) of both left and right dorsolateral prefrontal cortex resulted in an improvement in action naming in the mild AD group. To investigate the cumulative effects of repeated consecutive sessions and possible long-lasting effects, Cotelli and colleagues [51] assessed ten moderate AD patients before TMS treatment and at 2, 4 and 12 weeks after the onset of TMS intervention. The authors showed a significant effect of TMS on auditory sentence comprehension and language improvement. Moreover, they found long-lasting improvement in sentence comprehension eight weeks after the end of TMS treatment. Ferruci et al. [55] on the other hand, compared the effects of anodal, cathodal and sham tDCS over the temporo-parietal junction in 10 patients with mild AD. The authors found that anodal tDCS significantly improved verbal recognition memory, while cathodal tDCS significantly decreased accuracy in the word recognition task. Boggio et al. [60] also found an improvement of AD patients on an object recognition memory task during temporal and prefrontal cortex anodal tDCS, respectively.

In summary, there is initial evidence that DBS, TMS and tDCS can induce cognitive enhancement in different cognitive domains such as memory, executive functions, language, visuo-spatial abilities and attention in AD patients. The neurobiological basis is probably related to driving the neuronal activity of the memory circuit, which is affected by the pathology in AD. Further clinical studies are needed in order to replicate and extend the initial findings and to explore different targets of stimulation.

Neuromodulation in depression

Major depression is the most common of all psychiatric disorders and is ranked as the fourth leading cause of societal burden among all diseases. Depression can be treated effectively by either medication, and/or psychotherapy. Nevertheless, approximately 20% fail to respond to these standard therapies and nearly 60% may not achieve adequate response. For these patients, combinations of drugs and electroconvulsive therapies are often required. In patients who remain severely depressed, the so-called condition of treatment resistant depression (TRD), ablative surgeries were performed [61], similarly to the situation in other psychiatric disorders. Nowadays, this treatment modality is largely abandoned. The introduction of DBS of the subgenual cingulate gyrus [62] and later of the NAc [63] as a potential therapy in patients with TRD has redirected research in this field and neuromodulation-based therapies regained interest.

Deep brain stimulation

In 2005, Mayberg and coworkers presented their first results of DBS of the subgenual cingulate gyrus in TRD [62]. Their aim was to silence the subgenual cingulate cortex, which showed abnormal activity in chronically depressed patients. Six patients suffering from TRD received stimulating electrodes at the level of the subgenual cingulate gyrus. Striking and sustained remission of depressive symptoms was observed in four

out of six patients. Clinically, improvement in mood was accompanied with an increase in energy, interest, psychomotor speed and a decrease of apathy and anhedonia. In addition, positron emission tomography (PET) images demonstrated normalization of the metabolic activity of the stimulated area. Recently, the same group published their long-term findings in 20 patients with TRD and confirmed the beneficial effects [64]. No significant side-effects were seen. However, two patients died by suicide during depressive relapses.

Schlaepfer and associates performed DBS of the NAc and reported beneficial effect on depressive symptoms in their three patients [63]. PET imaging showed enhanced metabolic activity in the NAc, amygdala, and dorsolateral and dorsomedial prefrontal cortices, and reduced metabolism in the ventral and ventrolateral medial prefrontal cortices. In their next two publications, the same group described more patients with a longer follow-up period [65, 66]. In their last publication, a follow-up of up to four years for 11 patients was described. Their response criterion was at least 50% improvement in the Hamilton Depression Rating Scale (HDRS). Five out of 11 patients met the criterion of being a responder. The responders showed a strong and sustained therapeutic effect of DBS. From the non-responder group, on the other hand, one patient committed suicide and one patient attempted suicide [66].

In 2009, Malone and colleagues performed a study of DBS of the ventral capsule/ventral striatum for TRD [67]. Fifteen patients, with TRD were included to this study. Interestingly, the response rates were 40% at 6 months, and 53.3% at the last follow-up (up to 4 years) examination. No serious adverse effects were reported. Jimenez and co-workers described the effects of DBS of the inferior thalamic peduncle region in a 49-year-old woman. The patient had a history of recurrent episodes of major depression for over 20 years and DBS resulted in substantial reduction of the depressive symptoms [68]. Kosel and associates described the case of a 62-year-old woman with TRD with comorbid neuroleptic-induced tardive dyskinesias. DBS of the globus pallidus internus (GPi) reduced not only the dyskinesias, but also the HDRS-score. The latter was reduced by circa 50% [69].

In another case report, the authors described the therapeutic effects of DBS of the lateral habenula in a patient with TRD. Interestingly, depressive symptoms reappeared when DBS was switched off as a result of an incidental bicycle accident. After turning on the stimulator, depressive symptoms were reduced substantially [70].

Transcranial stimulation

In 2008, TMS was approved by the FDA for the treatment of TRD. Recent clinical studies showed that TMS of the left dorsolateral prefrontal cortex can produce antidepressant effects [71-73], varying from moderate to substantial [73, 74]. TMS can also be used as an adjuvant therapy to enhance the effect of antidepressant drugs [75-78]. It

is not yet clear, what the exact place of TMS and also tDCS treatment is in TRD, since DBS, a potentially more effective therapy, is gaining interest.

Vagal nerve stimulation

Vagal nerve stimulation (VNS) was first introduced as an experimental anticonvulsant procedure in dogs [79, 80] and few years later in patients with refractory epilepsy [81]. After it became an established therapy in refractory epilepsy, VNS was proposed as a treatment for TRD at the end of the 1990's [82, 83]. The rationale was based on the observation that epileptic patients with VNS showed an improvement of mood, without having a clear explanation for the underlying mechanism [84-86]. Studies with long-term follow up have shown that VNS can be beneficial in patients with major depression, while the therapeutic effect at short-term can be less pronounced [83, 87, 88]. The mechanisms underlying this antidepressant effect are under investigation. Further clinical trials are needed to confirm its efficacy. See also [89, 90] for reviews on VNS in depression.

Data from animal and clinical studies suggest that the antidepressant effect of neuromodulation involves at least the following mechanisms; driving the neuronal activity of prefrontal limbic circuits [91], enhancing central levels of 5-hydroxytryptamine (5-HT; serotonin) [92, 93] and stimulating the production of neurotrophins such as brain-derived neurotrophic factor (BDNF) [94-96].

Neuromodulation in other psychiatric indications

Some of the major psychiatric disorders in which neuromodulation has been applied, are described above. In case reports and case series neuromodulation-based therapies have been used to treat patients suffering from schizophrenia and attention deficit hyperactive syndrome (ADHD). These will be briefly reviewed here.

Schizophrenia

Recently, series of well-conducted clinical trials have shown that TMS applied over the left temporal regions induces clinically meaningful effects on schizophrenia [97-101]. Besides TMS, DBS of hippocampus and NAc have been suggested as targets for neurosurgical intervention in schizophrenia [102], but no large well-designed clinical trials are available. Plewnia et al., has reported a case study, in which a patient with intractable OCD and residual symptoms of schizophrenia was treated with unilateral DBS of the right NAc [103]. This patient showed a substantial reduction of obsessions and compulsions as well as improvement of psychosocial functioning with DBS.

Attention deficit hyperactive syndrome

A double-blind, sham-controlled clinical trial showed that 10 Hz TMS applied to the right prefrontal cortex for 10 days was effective in reducing the symptoms in a young adult with severe ADHD [104]. More recently, a double-blind randomized and controlled study demonstrated that high frequency TMS of the right prefrontal cortex improved attention in ADHD patients [105]. A significant amount of TS patients also suffer from comorbid ADHD, and DBS in these patients did not only reduce tics, but had also some beneficial effect on ADHD symptoms.

CONCLUSION

The treatment of psychiatric disorders has entered a new era with advances in neuromodulation technology. The developments in neuromodulation therapies can be summarized into two categories, non-invasive and invasive ones. The non-invasive neuromodulation therapies include mainly transcranial stimulation methods. TMS and tDCS can produce beneficial effects on refractory psychiatric symptoms on the short-term. However long-term controlled studies still need to show what the exact efficacy of the treatment modality is.

A main disadvantage seems to be that transcranial stimulation methods mainly target cortical structures and not deeply situated brain regions. For these latter ones, an invasive procedure is required, and the most frequent one is DBS. In our opinion, this is a highly promising neuromodulation therapy. Important reason for this is the availability of scientific and clinical evidence justifying the clinical use of DBS.

At present, data accumulates on the safety and efficacy of DBS, showing significant therapeutic effects in patients with refractory psychiatric disorders. Good evidence is available for the use of DBS in TS, OCD and depression. For other indications, research is still ongoing and preliminary data are encouraging. Nevertheless, a major concern is that the clinical application of DBS in psychiatric illnesses is moving faster than the scientific evidence supporting or discouraging the application of this approach. The field is no longer waiting for case reports of DBS in a new and exciting brain region, but for well-designed clinical trials backed up with robust scientific evidence. This increases the likelihood of a therapy to become successful, as illustrated by the history behind the successful application of DBS in the treatment of severe PD. Finally, we are witnessing the revival of neurosurgery in the field of psychiatric illnesses. Perhaps with a collaborative effort of neurosurgeons, psychiatrists, scientists and ethicists, the 'negative' societal reputation of psychosurgery which has originated from experiences in the 20th century can be changed to the benefit of severely ill patients and their caregivers.

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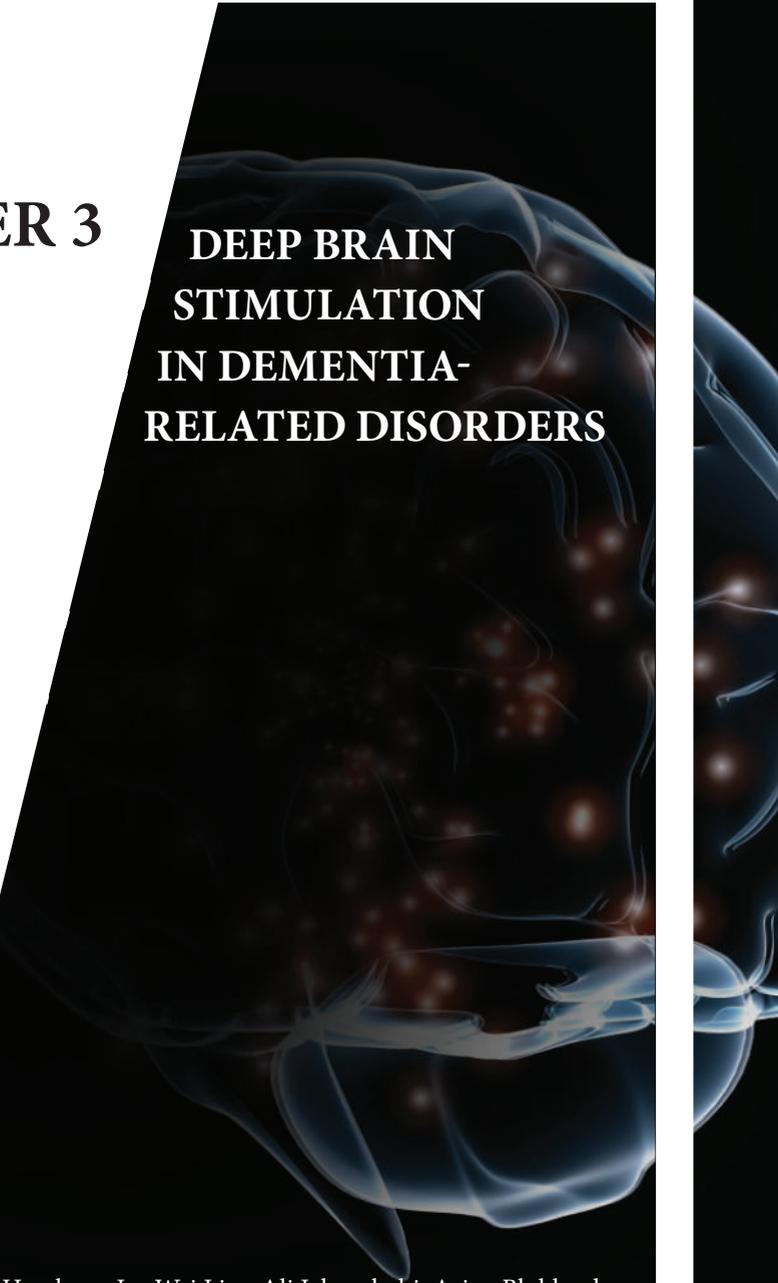
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CHAPTER 3

DEEP BRAIN STIMULATION IN DEMENTIA- RELATED DISORDERS

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ABSTRACT

Memory loss is the key symptom of dementia-related disorders, including the prevalent Alzheimer's disease (AD). To date, pharmacological treatments for AD have limited and short-lasting effects. Therefore, researchers are investigating novel therapies such as deep brain stimulation (DBS) to halt or reduce the progression of memory impairment. Clinical and preclinical studies have been performed and stimulations of the fornix, entorhinal cortex and nucleus basalis of Meynert have been carried out. The results of these studies suggest that DBS has the potential to enhance memory functions in patients and animal models. The mechanisms underlying memory enhancement may include the release of specific neurotransmitters and neuroplasticity. Some authors suggest that DBS might even be disease-modifying. Nevertheless, it is still premature to conclude that DBS can be used in the treatment of AD, and the field will wait for the results of ongoing clinical trials.

INTRODUCTION

Dementia is the condition of severely impaired cognitive functioning in various domains and has a substantial negative effect on patients, families and caregivers. There are different types of dementia, amongst others Alzheimer's disease, vascular dementia, Parkinson's disease dementia, Huntington's disease, alcohol-related dementia and Creutzfeldt-Jakob disease. The most prevalent cause of dementia is Alzheimer's disease (AD), which accounts for an estimated 50-80% of all cases. AD is a progressive neurodegenerative disease, which has a detrimental impact on the quality of life of patients. The age-standardized prevalence of people aged 65 years or older of population-based studies in Europe suggests that 4.4 % suffer from AD [1]. In the United States, the study of a national representative sample of people aged more than 70 years provided a prevalence for AD of 9.7 % [2]. In the early-stage of AD, cognition and the ability to acquire new memories are impaired. In later stages, symptoms include progressive cognitive deterioration, long-term memory loss, aphasia, apraxia and finally the inability to perform activities of daily living. Also behavioural and psychological symptoms, i.e. agitation, depression and aggressive behaviour, occur. Structural and functional imaging studies have shown a generalised cerebral atrophy and a fluorodesoxyglucose - positron emission tomography (FDG-PET) measured glucose hypometabolism in AD patients, which is often more focused on the frontal regions, the medial temporal lobe and the parietal regions [3]. The dysfunction and death of neurons is associated with cytoskeletal abnormalities, such as neurofibrillary tangles, as well as amyloid plaques [4, 5]. The mean life expectancy following diagnosis is approximately seven years [6]. Currently only symptomatic treatments are available for AD. There are no known treatments that cure or delay the progression of this neurodegenerative disease. Pharmacological therapies that are approved for treatment of AD in North America and most European countries include memantine (an N-methyl-D-aspartate receptor antagonist) for severe AD and few acetylcholinesterase inhibitors for mild to moderate AD such as tacrine, donepezil, galantamine and rivastigmine [4].

These pharmacological treatments, however, are not effective for every patient and only improve symptoms temporarily. In some patients substantial side-effects such as gastrointestinal symptoms (nausea, vomiting, diarrhea), eating disorder/weight loss, dizziness and muscle cramps are seen [7]. Therefore, researchers are currently exploring the applicability of novel non-drug based therapies, such as deep brain stimulation (DBS) [8-10], transcranial magnetic stimulation (TMS) or transcranial direct current stimulation (tDCS) [11] to reduce or halt the progression of memory loss in AD and ultimately to improve the quality of life of patients and their caregivers.

Deep brain stimulation (DBS) is a minimal invasive surgical treatment involving the implantation of electrodes, which deliver electrical impulses to specific parts of

the brain. It has been shown that DBS has substantial therapeutic effects in a range of neurological disorders, including Parkinson's disease, Tourette's syndrome and severe forms of epilepsy [12-16]. In the past years, the applicability of DBS in psychiatry has been evaluated in affective disorders. DBS of 'key' regions within the limbic system resulted in therapeutic effects in patients with treatment-resistant depression [17-19] and obsessive-compulsive disorder [20]. In this respect, recent clinical [9, 10] and preclinical [8, 21, 22] studies have suggested that DBS can be used as a tool to enhance memory functions. TMS and tDCS, on the other hand, are non-invasive techniques that can induce significant and long-lasting changes in cognitive function in both healthy volunteers and patients with neurological disease [23-26]. To date, there are few reports about the effects of rTMS and tDCS on memory. Most of them investigate focal and non-focal neuroplasticity changes in subjects with mild AD disease [27-29]. For a detailed review on TMS and tDCS on Alzheimer's disease see Boggio et al. [11]. Here, we will focus on DBS and address the question whether there is a place for DBS as a treatment of memory-related disorders. We will review relevant preclinical and clinical literature.

OUTLINE OF THE REVIEW

This review was based on articles identified by a PubMed search with the terms "Deep brain stimulation", "dementia" and "memory" as the main keywords. Relevant articles were also identified from the reference lists of articles, review papers, and book chapters. Only original data is described in this review, giving preference to behavioral studies investigating memory performance of subjects. Review papers were utilized for the explanation of DBS mechanisms.

The outline of the review is as follows: in the first section, the neuroanatomical circuit responsible for memory functions is summarized in order to provide background information for the choice of the different targets for DBS. In the second section, preclinical studies, experimental findings in humans and clinical studies which have applied DBS to modulate memory functions are outlined. Finally, we provide an overall discussion of the evidence available thus far.

The memory circuit

The selection of the brain regions for DBS is mainly based on the so-called memory circuit of the brain. The major pathway for memory, including long-term storage and recognition memory, is located in the medial temporal lobe (i.e. hippocampus, rhinal cortices and amygdala) and diencephalic structures (i.e. mammillary bodies, thalamus). In the classical memory circuit, the entorhinal cortex projects to the hippocampus via

the perforant pathway. The perforant pathway is considered the main afferent pathway to the hippocampus, where glutamatergic fibers from the entorhinal area reach the granule cell layer of the dentate gyrus. Moreover, the perforant pathway also projects to the subiculum as well as to the CA3 and CA1 subfield of the hippocampus [30]. From the dentate gyrus connections are made to the pyramidal neurons in the CA3 subfield via mossy fibers. Lastly, CA3 neurons project to pyramidal neurons in the CA1 through Schaffer collaterals. It is known that pyramidal neurons contain glutamatergic and GABAergic synapses [31].

From the hippocampus the information proceeds through the subiculum to the fimbria and the fornix. The precommissural branch of the fornix projects amongst others to the anterior cingulate cortex via the septal nuclei and ventral striatum. Cholinergic fibers from the basal forebrain, including the septal nuclei and the nucleus basalis of Meynert (NBM), run through the fornix. Some fibers from the fornix also pass through the anterior commissure to the contralateral hippocampus. The postcommissural branch of the fornix projects to the anterior nuclei of the thalamus and the mammillary bodies. Because the mammillothalamic tract couples the mammillary bodies and the anterior thalamic nucleus, the hippocampus can have a direct as well as indirect effect on the thalamus [32, 33]. Findings from several electrophysiological studies indicate that the anterior nucleus of the thalamus is the primary source of glutamatergic input to cingulate neurons [34, 35]. Thus, the postcommissural branch of the fornix reaches the cingulate gyrus through the anterior thalamic nucleus. This memory circuit is completed by projections of the cingulate gyrus to the entorhinal cortex of the parahippocampal region.

Evolving experimental approaches have contributed to a nuanced view of understanding the different structures of the memory circuitry, since lesions in the memory circuit can mimic typical memory deficits seen in ageing and dementia. Hippocampal and anterior thalamic nuclei lesions predominantly affect episodic [36, 37] and spatial memory [38, 39]. To some extent also recognition memory can be attributed to these structures [40-43]. Fornix, cingulum and mammillary bodies, on the other hand, can be assigned merely to spatial memory functions [44-48], and memory recall [49]. Finally, the parahippocampal gyrus and entorhinal cortex are involved in both, spatial [50-53] and recognition memory [54, 55]. There is evidence that glutamatergic and GABAergic neurons of the hippocampus are severely affected in AD and degeneration of these neurons might be an early event in the pathogenesis of this disease in humans [56] and animal models of AD [57]. Degeneration of cholinergic neurons in the basal forebrain, which includes the NBM and the associated loss of cholinergic neurotransmission in the cerebral cortex and other areas significantly contribute to the deterioration in cognitive functions [58]. In AD, dysfunctions of the noradrenergic locus coeruleus and the serotonergic dorsal raphe nucleus have also been reported [59]. There seems to

be a multifocal disease pathology in dementia-related disorders. While some of the memory-circuit related regions are involved in the cognitive aspects of dementia, others are linked to the affective aspects such as the monoaminergic systems [60].

Modulating memory through brain stimulation

Thus far only a few studies have tried to stimulate structures of the memory circuit (Table 1) and have found beneficial effects when applying DBS correspondingly to the fornix [8-10, 62, 63], entorhinal cortex [21, 64] and NBM [65, 66]. The fornix and entorhinal cortex are both directly connected to the hippocampus (Fig. 1). The NBM has strong projections not only to the neocortex including primary and secondary visual, auditory, somatosensory, and higher association cortex, but also the hippocampus [67].

Preclinical studies

The use of animal models has proven its importance in the development and refinement of DBS as a therapy in neuropsychiatry [68, 69]. The evaluation of different target structures for DBS can be performed in rodents to exclude structures which are less effective. Besides, various stimulation parameters can be tested in animals to define the most optimal stimulation settings producing therapeutic benefits. Moreover, potential side-effects, as well as the underlying mechanisms of DBS can be investigated utilizing animal models.

In line with this, rats treated with corticosterone, a known suppressor of neurogenesis, were stimulated in the anterior nucleus of the thalamus for 1 h with 2.5 V, 130 Hz and 90 μ s pulse width while being under general anaesthesia [70]. The authors chose this model, in order to investigate, whether high frequency stimulation can restore the disruption of neurogenesis, which may occur as a consequence of pathological conditions. Stimulation was bilateral as well as bipolar. The voltage of 2.5 V, however, is unusually high and it is also more common to use current-based stimulations in animal research. Stimulations with voltage do not guarantee a constant current over time, due to the changes in impedance of the electrodes. In preclinical research it is important to deliver a controlled amount of current, in order to eliminate variations between the subjects. The group size of the stimulated animals was also rather small in this study (n=4). Nevertheless, the authors found that with high frequency stimulation, cell division in the subgranular layer of the hippocampus significantly increased 28 days after the last BrdU injection. Stimulation was therefore able to reverse the suppression of neurogenesis in corticosterone-treated animals when applied sufficiently in advance. Since interventions that increase hippocampal neurogenesis have been associated with enhanced behavioural performance, the authors hypothesized that it may be possible to use electrical stimulation to treat conditions associated with memory impairment.

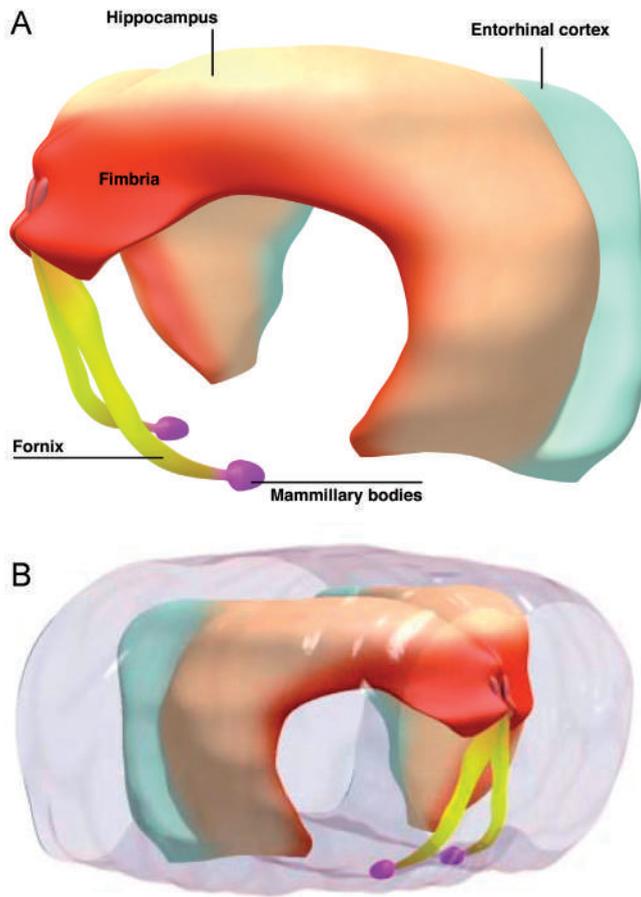


Figure 1: (A) This picture illustrates the anatomy of the mammillary bodies, fornix, fimbria, hippocampus and entorhinal cortex in the rat brain according to the rat brain atlas of Paxinos and Watson (1998). These structures are important components of the Papez circuit and are considered to be involved in the neuronal basis of memory. Especially structures like the hippocampus are involved in spatial learning and memory. Projections within this circuit are mostly cholinergic and glutamatergic. (B) 3D web-animation of (A). The classical idea of the Papez circuit is that the entorhinal cortex projects to the hippocampi, which pass the information on to the fimbria. Efferents are then bundled in the fornices and reach the mamillary bodies. Through the mammillothalamic tract information reaches the anterior nucleus of the thalamus, which is not illustrated here. A functional interaction between hippocampus and anterior thalamic nucleus is required for spatial memory and conditional learning [61]. See (A) for the colour coding of the structures.

Table 1: Table of stimulation studies organized by target structures. The table shows the subjects involved, type of stimulation, memory task and the behavioral outcome of the surgical intervention. ADAS-cog, Alzheimer's Disease Assessment Scale- cognitive subscale; OLT, object location task; DBS, deep brain stimulation; LTP, long-term potentiation; MMSE, mini-mental state examination

Structure	Subject	Type of stimulation	Memory task	Effect	Reference
Fornix	Human (morbid obesity patient) N=1	Bilateral, 3 - 5 V, 130 Hz and 60 μ s pulse width, continuous for 3 weeks	Neuropsychological tests, e.g. verbal learning test, WAIS attention index, spatial associative learning, etc.	Significant improvements on the California Verbal Learning Test and Spatial Associative Learning Test	Hamani (2008)
	Human (AD patients) N=6	Bilateral, 3.0 - 3.5 V, 130 Hz, and 90 μ s pulse width, continuous for 12 months	ADAS-cog, MMSE	Possible improvements and/or slowing in the rate of cognitive decline at 6 and 12 months in some patients	Laxton (2010)
	Human (AD patient) N=1	Bilateral, 2.5 V, 130 Hz and 210 ms pulse width, continuous for 12 month	ADAS-cog, MMSE, Free and Cued Selective Reminding Test	Cognitive scores worsened after 6 months but returned to baseline after 12 months of chronic DBS.	Fontaine (2013)
	Rats (model of experimental dementia) N=10	Bilateral, 100 and 200 μ A, 10 and 100 Hz, 100 μ s pulse width, acute stimulation	OLT	Memory enhancement in high current densities (frequency-independent)	Hescham (2012)
Entorhinal cortex	Mice N=25	Bilateral, 50 μ A, 130 Hz and 90 μ s pulse width, for 1 hour during surgery	Morris Water Maze	water-maze memory was facilitated 6 weeks after stimulation due to hippocampus-dependent neurogenesis	Stone (2011)
	Human (epilepsy patients) N=7	Bilateral, 0.5 to 1.5 mA, 50-130 Hz and 300 - 450 μ s pulse width, cycle of 5 seconds on and 5 seconds off	Virtual memory task	Memory enhancement and theta-phase resetting	Suthana (2012)

Table 1: Table of stimulation studies organized by target structures. The table shows the subjects involved, type of stimulation, memory task and the behavioral outcome of the surgical intervention. ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; OLT, object location task; DBS, deep brain stimulation; LTP, long-term potentiation; MIMSE, mini-mental state examination (continued)

Structure	Subject	Type of stimulation	Memory task	Effect	Reference
Ncl. Basalis Meynert (NBM)	Human (senile dementia of Alzheimer's type patient) N=1	Unilateral, 3 V, 50 Hz and 210 μ s pulse width, cycling between 15 seconds on and 12 minutes off throughout the day and night, repetitive for 9 months		No clinical effect, but increased cerebral glucose metabolism	Turnbull (1985)
	Human (Parkinson patient) N=1	NBM: Bilateral, 1 V, 20 Hz, and 120 μ s pulse width STN: Bilateral, 3.5-4.2V, 130 Hz and 60 μ s pulse width	Neuropsychological tests, e.g. clock drawing, letter-number- span, auditory verbal learning, etc.	Combined bilateral stimulation lead to improvement in attention, concentration, alertness, drive, and spontaneity	Freund (2009)
	Rats N=10	Unilateral, 200 μ A, 50 Hz and 0.5 ms pulse width, duration of 100 min		In adult, but not aged rats, NGF levels were significantly increased	Hotta (2009)
Anterior thalamic nucleus	Rats N=4	Bilateral, 2.5 V, 130 Hz and 90 μ s pulse width, duration 1 hour		High-frequency stimulation of the AN restores corticosterone- suppressed hippocampal neurogenesis	Toda (2008)
	Rats N=12	Bilateral, 500 μ A, 130 Hz and 90 μ s pulse width, acute stimulation	Contextual fear conditioning, spatial alternating test	High frequency stimulation of 500 μ A disrupted the acquisition of contextual fear conditioning and impaired spatial memory	Hamani (2010)
	Rats N=17	Bilateral, 2.5 V, 130 Hz and 90 μ s pulse width, duration 1 h	Non-Matching-to-Sample and Delayed Non-Matching-to-Sample	ATN stimulation administered to corticosterone-treated rats one month prior to testing improved performance on a delayed non-matching to sample task and increased hippocampal neurogenesis	Hamani (2011)
Hippocampus	Human (epileptic patients) N=12	Bilateral, 4-6 mA, single pulse, 1 ms pulse width	Computerized recognition test	Bilateral stimulation was associated with a pronounced decrease in memory scores	Lacruz (2010)

Therefore, in their next study corticosterone-treated rats were again stimulated with the stimulation parameters mentioned above [22]. The authors showed that DBS of the anterior thalamic nuclei applied one month prior to testing improved memory performance on a delayed non-matching to sample task. In contrast, no changes in this task were observed in animals that were tested a few days after surgery, suggesting that stimulation of the anterior thalamic nuclei only induces long-term plastic changes. However, these results should be treated with care, since the aforementioned voltage-based stimulation parameters do not ascertain constant current amplitudes.

In an earlier publication, the same research group showed that acute bilateral, monopolar stimulation of the anterior nucleus of the thalamus, at a relatively high current (500 μA), 130 Hz and 90 μs pulse width, disrupted the acquisition of contextual fear conditioning and impaired performance on a spatial alternating task (four-arm maze) in rats [71]. Testing started 2 weeks after surgery and even with parameters generating a charge density that approximated the one used in clinical practice (100 μA), memory performance of DBS rats was not enhanced. The authors suggest that stimulation with too high current density causes a depolarization block. This finding should be carefully considered in light of the use of anterior thalamus DBS in patients for memory enhancement. It could also be argued that the time between surgery and testing was not sufficient for long-term plastic changes to occur, since stimulation effects on neurogenesis are only observed 4 weeks after surgery [22, 70].

Contrary to this, animal studies have also highlighted DBS targets producing clear memory enhancing effects. For example, in a rat model of experimental dementia bilateral DBS of the fornix region, enhanced spatial memory performance in the object location task [8]. Thus, DBS reversed the memory impairing effects of scopolamine when compared to sham rats in high current densities (both 200 μA and 100 μA were effective and independent of frequency, 100 μs pulse width). The bilateral electrodes were all implanted in the vicinity of the fornix. No differences between the ventrally, medially or laterally placed electrodes were found in terms of behavioral outcome and stimulation settings did not produce histological damage or anxiety-related side effects. For the fornix the current hypothesis states that this effect is accomplished by driving the fornix activity through DBS, both orthodromically (away from the soma) as well as antidromically (towards the soma). It was suggested that large myelinated axons usually produce excitatory responses upon electrical stimulation [72].

Another stimulation site inducing memory enhancing effects is the entorhinal cortex. In rodents, the effects of entorhinal cortex DBS have thus far only been reported after chronic stimulation [21]. The entorhinal cortex is strongly connected to the dentate gyrus via the perforant pathway. Either 1.5 or 6.5 weeks after bilateral stimulation of the entorhinal cortex for 1 hour at 50 μA , 130 Hz and 90 μs pulse width (while being under general anaesthesia), they evaluated the effects on neurogenesis and memory

performance. The current density of 50 μA is considered as rather low. With the help of the proliferation marker BrdU, the authors found that DBS increased proliferative activity in the dentate gyrus after 6.5 weeks. These newly generated cells survived for several weeks and acquired normal dentate granule cell morphology. Using immunohistochemical approaches, the authors found that, once sufficiently mature, these stimulation-induced neurons integrated into hippocampal circuits, which was associated with an enhanced performance of the stimulated rats in a spatial water maze task. Moreover, because beneficial effects of entorhinal cortex stimulation could be inhibited by blocking neurogenesis with the DNA alkylating agent Temozolomide, a causal relationship between stimulation-induced promotion of adult neurogenesis and enhanced spatial memory can be assumed.

These findings suggest that neurogenesis can be enhanced by stimulation of different regions in the memory circuit (i.e. entorhinal cortex and anterior thalamus). No studies have been conducted in which neurogenesis markers were evaluated after fornix stimulation.

In the context of AD-associated degeneration of the basal forebrain and the dysregulations in nerve growth factor (NGF) levels [73], it seems to be of particular interest to examine the effects of DBS within one of the basal forebrain structure, such as the NBM. It is acknowledged that survival of cholinergic basal forebrain neurons is dependent upon NGF and its high- and low-affinity receptors. Hotta and colleagues [74] therefore applied unilateral electrical stimulation of the NBM (200 μA , 50 Hz and 0.5 ms pulse width for a duration of 100 min) in anesthetized rats. NGF levels were measured via a microdialysis probe in the parietal cortex. NGF is known to be essential not only for the survival but also maintenance of neurons and is especially down-regulated in the NBM in AD [73]. The authors found that in adult, but not aged rats, NGF levels were significantly increased ipsilaterally to the stimulation, confirming a potential effect of DBS on neurotrophin release [74]. The question here is whether such a substantial response can also be seen in an animal model of dementia, in which the NGF levels are already affected.

Experimental findings in humans

The hippocampus is a central structure in the so-called memory circuit and one would assume that hippocampal stimulation would facilitate memory performance. In epileptic patients, the hippocampus was stimulated using 1 ms single pulse electrical stimulation, intensity ranging between 4-6 mA of bilateral implanted intracerebral depth electrodes [75]. This stimulation paradigm was enforced in order to identify human epileptogenesis and to mimic the physiological stimulation, where cortical networks receive one stimulus before returning to their resting state. Computer-controlled electrical stimulation and recognition memory tests (which included written words,

geometrical drawings and faces) were performed while patients were connected to multi-channel video-telemetry. One ms single pulses of electrical stimulation were time-locked to item presentation on a computer screen during encoding, recognition or both. However, major deficits were found after bilateral hippocampal stimulation in episodic memory. The findings confirm that localized and brief 1 ms electrical pulses can disrupt memory function and further support the notion that hippocampal structures are highly relevant to processing episodic memory. The hippocampus, therefore, does not seem to represent a good target structure for DBS in memory-related disorders.

On the contrary, when stimulating the in-/output of the hippocampus, namely the fornix, investigators found an immediate effect on memory [9]. In a case study the authors bilaterally stimulated the fornix/hypothalamic area in a patient suffering from morbid obesity. Stimulation resulted immediately in the generation of detailed autobiographical memories. Also significant improvements on the California Verbal Learning Test and Spatial Associative Learning Test were observed after 3 weeks of continuous stimulation. Electroencephalographic (EEG) source localization elucidated that in this specific case DBS with 3-5 V, 130 Hz and 60 μ s pulse width evoked neuronal activity in the medial temporal lobe structures like the hippocampus. Following this interesting finding, the authors have performed a phase I trial of fornix DBS in AD patients (see section on clinical studies).

The entorhinal cortex, which provides the major input to the hippocampus, has also been stimulated in a recent human study with seven pharmacoresistant epilepsy patients. Bilateral intracranial depth electrodes were implanted in the entorhinal cortex region in order to define the area of seizure onset [64]. When subjecting these patients to cognitive testing, Suthana and colleagues found that spatial learning in humans was enhanced in a virtual spatial memory task. Stimulation was acute with biphasic rectangular pulses set below the threshold for afterdischarge. In addition, stimulation parameters were bipolar at a frequency of 50-130 Hz and a pulse width of 300-450 μ s with a 5 s on and a 5 s off cycle. The current densities ranged from 0.5 to 1.5 mA. Although the cyclic stimulation settings are not often applied in clinical practice of DBS, the authors found that entorhinal stimulation with 50 Hz led to a theta phase resetting measured through hippocampal depths electrodes. The theta rhythm (3-8 Hz) is a large EEG potential, which can be recorded from the hippocampus of humans and rodents. During working memory tasks, stimulus presentation induces shifts in the phase of the hippocampal theta-band oscillation and it is hypothesized that resetting theta activity facilitates long-term potentiation by allowing the best possible encoding of relevant incoming stimuli. A potential mechanism for this effect could be *neural hijacking*. The evidence for the phenomenon of neural hijacking has been extensively described for the microstimulation of the motor cortex in rhesus monkeys (see review

[76]). The hypothesis states that DBS eliminates natural cortical output activity and replaces it with stimulus-evoked activity.

Taken together, these experimental findings suggested that the fornix and the entorhinal cortex may be considered as interesting targets for dementia, which corresponds to the findings from experimental animal studies. On the other hand, the hippocampus appeared to be a less appealing structure for DBS to enhance memory performance.

Clinical studies

Laxton et al. [10] performed the first DBS study in patients with AD. Six Patients with mild AD were implanted with electrodes in the area of the fornix/hypothalamus and after an intra-operative evaluation of stimulation, patients were subjected to chronic high frequency DBS for a period of 12 months. Subjects were stimulated with 3.0-3.5 V, 130 Hz and 90 μ s pulse width. The authors found that the application of DBS in the hypothalamus/fornix triggered neural activity in the entorhinal and hippocampal areas. PET scans revealed an early and striking reversal of the impaired glucose metabolism in the temporal and parietal lobes that was maintained after 12 months of chronic stimulation. After evaluating the AD Assessment Scale cognitive subscale (ADAS-cog) and the Mini Mental State Examination (MMSE), the authors concluded that especially patients with mild cognitive impairments might benefit from this therapy. They showed possible improvements and slowing the progression of memory loss at 6 and 12 months, respectively. When comparing the rate of decline in the 11 months preceding surgery to the 11 months after surgery a decrease in the rate of decline from a mean rate of 2.8 to 0.8 points in the MMSE across the 6 patients can be observed. After 1 year of continuous DBS, the functional connectivity analysis demonstrated increased cerebral metabolism in cortical-subcortical and cortical-hippocampal networks. This increase after 1 year of DBS was correlated with less decline in global cognition and memory as measured with the ADAS-cog [62]. The authors reported adverse effects only in their first publication [10], but did not mention them in their follow-up study [62]. Side effects were autonomic and cardiovascular in nature and occurred merely in high stimulation settings above 7 V. Chronic stimulation was therefore chosen as 50 % of the voltage threshold for adverse effects. No patient developed sleep disturbances, weight changes or evidence of hypothalamic dysfunction [10]. Since only 6 patients were tested in this first trial, new clinical trials are currently implemented in the ADvance study, which is sponsored by the Functional Neuromodulation Ltd company and is being conducted at sites across the U.S. and in Canada. The study will involve 20 mild AD patients, with DBS electrodes implanted in the fornix. The stimulation device will be turned on immediately for half of the patients and for the other half only after 12 months. It is a double-blind randomized controlled feasibility study to evaluate acute and long-term safety. Efficacy outcomes will be measured at 12 months

and identify improvements in ADAS-cog, clinical dementia rating and changes in the glucose metabolism assessed by FDG-PET.

A prospective pilot study in France has already investigated the feasibility of applying DBS of the fornix in AD patients [63]. Since inclusion criteria are strict, e.g. patients must be under 70 years of age, have a MMSE between 20 and 24 (mild cognitively impaired), show a predominant impairment of episodic memory and fulfil the AD DSM IV criteria for less than 2 years, only 9 out of 110 recruited patients were found suitable to enter the study. In the end, only one patient agreed to undergo surgery. This number is very low, considering the fact that alternative efficient treatments are currently lacking.

Attention has also been drawn to the NBM as a potential target structure for DBS in AD, since it has wide cholinergic projections to the neocortex and the hippocampus. In the pathogenesis of AD the NBM degenerates, leading to decreased cholinergic transmission and ultimately to cognitive decline in patients [77]. In this regard, Turnbull and colleagues [66] used chronic, cyclical, unilateral, monopolar stimulation of the left NBM in a patient with mild to moderate AD. It has been shown that low-frequency stimulation has excitatory actions [78] and neuromodulation might therefore be a strategy for enhancing residual NBM cholinergic output. Stimulation parameters were set to 3 V, 50 Hz, and 210 μ s, cycling between 15 seconds on and 12 minutes off throughout the day and night. The authors found that the patient had no clinical response to the stimulation 8 months after the procedure. However, the NBM was targeted indirectly using atlas coordinates and electrode placement was only verified by a computed tomography (CT) scan, in which the NBM was not visible. Another issue of debate is the choice for specific stimulation parameters: unilateral, short-lasting and intermittent stimulation, which was delivered for a total of 30 minutes every 24 hours. No rationale for these stimulation parameters was provided by the authors. By today's standard these settings are highly unusual, given that nowadays stimulation is often chronically applied to patients. Nevertheless, stimulation had an effect on cerebral glucose metabolism. Using the patient's unstimulated contralateral hemisphere as a control, Turnbull and colleagues [66] were able to compare his pre- and postoperative FDG-PET scans. The preoperative scan was obtained 4 months before surgery and the postoperative scan was obtained 2 months after the initiation of stimulation, respectively. The FDG-PET scans of the right hemisphere showed that glucose metabolism in the frontal, temporal, parietal, and occipital lobes decreased by 21%, 24%, 10%, and 7.5%, respectively. In contrast, glucose use in the stimulated left hemisphere had decreased by only 12% in the frontal lobe and 4.1% in the occipital lobe. No change was found in the parietal lobe and glucose use was increased by 1.5% in the temporal lobe.

Freund and colleagues [65] have also stimulated the NBM (in addition to the subthalamic nucleus) in a patient suffering from Parkinson's disease dementia. Imaging and

post-mortem studies have identified that degeneration of the NBM appears early in the pathogenesis and worsens progressively [79]. With bilateral stimulation settings of 1 V, 20 Hz and 120 μ s the patient showed sustained improvement in various aspects of cognitive functioning, such as concentration, attention, alertness, apraxia, ataxia and memory. No adverse effects were reported in this study. The authors also hypothesized that DBS of this structure induces neuroprotective effects through the release of NGF into the NBM [80]. However, NGF was not measured in this study. A clinical study is currently being performed with six mild AD patients at the Uniklinik Köln in Germany (NCT01094145). In this study, two weeks after DBS electrodes in the NBM, the conventional stimulation will take place as a doubleblind, randomized change between on and off stimulation periods. Throughout the one-year observation-period, the patients will be followed closely to monitor the effects of DBS on their cognitive abilities, psychopathological well being, the quality of life, praxia and nutritional condition using standardized neurological and psychiatric rating scales.

DISCUSSION

DBS as a potential therapy for memory-related disorders is currently being explored in clinical trials. Especially DBS of the fornix and the NBM are investigated as potential treatments for dementia. Based on the limited data that are available it is assumed that DBS of the fornix might provide symptomatic relief for verbal recollection, recall and recognition as well as episodic memory, while NBM stimulation might modulate apraxia and alertness. Promising results have also been observed in entorhinal cortex stimulation, even though no clinical trials are examining the effects of entorhinal cortex DBS as memory enhancer at present.

An important issue that needs to be raised is that up to now, most DBS studies in psychiatric disorders were first conducted in humans. Since the clinical data and the findings from animal studies show similarities, animal models can be of important value to find potential new DBS targets and settings for memory enhancement (see review [68]). From present DBS studies it is evident that structures within the brain show different responses to stimulation parameters. Moreover, clinical case studies and animal studies have shown that DBS of different target structures may enhance memory functions by different mechanisms of action (Fig. 2).

The fornix, for example, is not dependent on frequency but rather on current densities. In preclinical research relatively high current densities of 100 or 200 μ A produced beneficial effects on memory [81]. In clinical trials AD patients have been stimulated with 2.5-3.5 V [10, 63], which is generally accepted as moderate-sized voltage in the treatment of psychiatric disorders with DBS. The investigators of the clinical studies

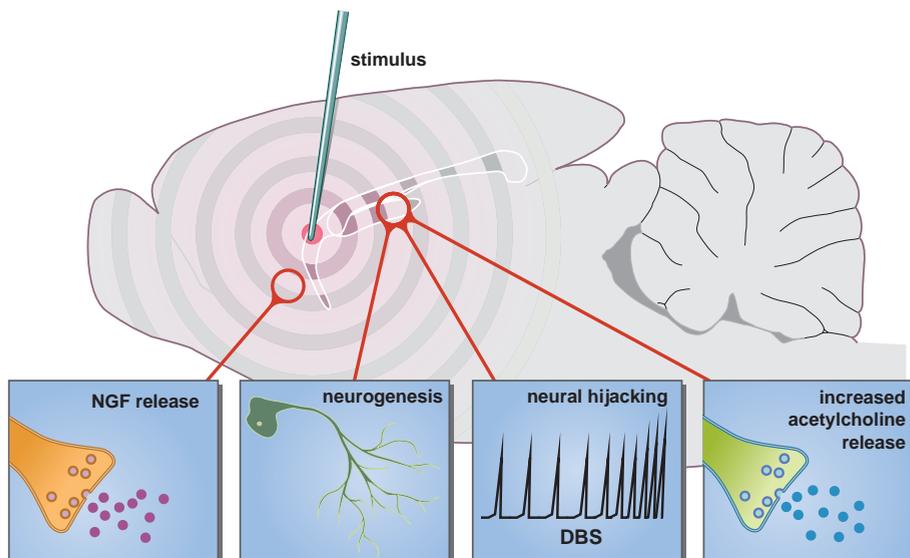


Figure 2: Schematic representation of the potential mechanisms involved in enhancing memory functions by deep brain stimulation. Stimulation of a target area within the memory circuit (e.g. fornix) can provoke NGF release in the NBM, hippocampal-dependent neurogenesis, neural hijacking by resetting theta activity and increased acetylcholine release within the hippocampal region.

chose these parameters in order to abolish adverse effects. With these settings the authors described possible improvements and/or slowing in the rate of cognitive decline at 6 and 12 months in some patients [10, 63]. The reason why the fornix is not dependent on frequency can be related to its morphology. The fornix consists of a bundle of myelinated fibres, known as white matter. This white matter is activated upon stimulation, regardless whether stimulation is applied with high or low frequency, and produces an excitatory response. As seen in the rodent study, relatively high current is needed to produce beneficial effects on memory, most probably because the fornix is a small target area and electrodes were only located in its vicinity [8]. In the same study, memory deficits induced by the muscarinic acetylcholine receptor antagonist scopolamine were reversed after fornix stimulation. Thus, DBS of the fornix might increase acetylcholine release in the hippocampus.

The NBM, on the other hand, is rather dependent on frequency. To the best of our knowledge, no animal study has been conducted, in which memory was assessed after NBM stimulation. It has been shown, however, that low frequency stimulation of 50 Hz induces increased NGF release from the NBM in anaesthetized rats, a neurotrophin responsible for survival and maintenance of neurons [74]. Since typical neuronal discharge rates of the NBM are of low frequency and it is known that cell bodies are usually activated by low frequency, stimulations with 50 Hz [66] and 20 Hz [65] were

applied in demented patients. In fact, in a demented Parkinson's patient the combined bilateral stimulation of subthalamic nucleus and NBM led to improvement in attention, concentration, alertness, drive and spontaneity [65]. Nevertheless, this has only been shown in a single case study and more evidence is needed. Similar to the observations of the rodent study, the authors hypothesized that an increase of neurotrophin release from the NBM upon stimulation correlates with improved memory performance [65, 80].

Next, stimulation of the entorhinal cortex demonstrates effects on neurogenesis, as well as theta-phase resetting within the hippocampus when stimulated with low current densities [21, 64]. Rodents were stimulated in the entorhinal cortex with 50 μ A [21] and humans with 0.5-1.5 mA [64], both considered as relatively low current densities. With these stimulation parameters increased neurogenesis in the rodent study was associated with enhanced spatial memory in the water maze [21], while in the human study hippocampal theta-phase resetting was directly related to enhanced memory performance in a virtual memory task [64]. Besides, both studies utilized high frequency stimulation. For the entorhinal cortex it can be hypothesized that DBS with low current and high frequency has an effect on neighbouring axons. Extensive evidence supports the speculation that axons have a lower threshold for activation by electrical stimulation than cell bodies and usually respond to high frequency stimulation. The fibres from the perforant pathway might be activated upon stimulation inducing neurogenesis in the dentate gyrus and neural hijacking might take place. Since alterations in neurogenesis have been extensively described in animal models of AD and its decline is to some extent responsible for learning and memory impairments (see review [82]), the entorhinal cortex might be an interesting structure for future DBS studies.

Finally, the anterior nucleus of the thalamus seems to be a target producing contradictory stimulation effects on memory. On the one hand, effects on neurogenesis have been observed following high frequency stimulation of 130 Hz [22, 70], but on the other side application of high current densities (500 μ A) within this structure produced memory disruption [71]. Interestingly this nucleus is being targeted for treating severe epilepsy, based on the promising data from the SANTE (Stimulation of the Anterior Nucleus of the Thalamus for Epilepsy) trial. In this study the safety and effectiveness of bilateral stimulation of the anterior nucleus of the thalamus is examined as adjunctive therapy for reducing the frequency of seizures in adults diagnosed with epilepsy (NCT00101933). The 110 participants of this study were randomized to relatively high stimulation at 5 V or no stimulation at 0 V (control), using 90 μ s pulses, 145 pulses/s, 1 min on, and 5 min off. Indeed, this study demonstrated a beneficial and sustained effect of DBS on seizure frequency. Neuropsychological testing showed no group differences on cognitive functions, however significantly more participants of the stimulated group reported depression and memory problems as adverse events [83]. These behavioral

effects are partially in line with findings from animal studies, in which stimulation with a relatively high current density of 500 μ A also disrupted memory performance.

Similarly, experimental findings in epileptic patients indicated that hippocampal single pulse stimulation might impair rather than facilitate memory functions [84, 85]. Stimulations within the hippocampus might cause an acute depolarization block, which possibly disrupts formation and recall of recent episodic memory.

From this we can conclude that consideration of ethics in clinical DBS research is of growing importance. The cost/benefit ratio should be carefully evaluated for each patient. For the treatment of dementia-related disorders, data suggests that DBS of the fornix is most beneficial for patients who are less severely affected [10]. DBS might therefore be more suitable for patients in the early stages of the pathogenesis of memory impairment, and not after drug treatment fails. This might be contrary to DBS treatment in movement disorders, where DBS applied to treatment-resistant patients has already been shown to be an effective therapy in alleviating motor symptoms. Since at the moment not much data is available showing clear beneficial effects of DBS in AD, patients might not be motivated to undergo this therapy. Up to now, DBS studies in memory-related disorders were mainly safety studies, with small sample sizes and no control group. Furthermore, suitable cognitive re-evaluations were lacking in some studies [65, 66]. Since no clear conclusion can be drawn from these findings, we expect that patients favour drug treatment above undergoing brain surgery. The difficulty in finding participants, who fulfil the strict inclusion criteria for this type of studies has been discussed earlier [63].

Since DBS in Parkinson's disease patients has been applied for more than a decade now, a lot of evidence has already been reported about adverse effects. For example, it has been shown that approximately 0.5 % of patients implanted with DBS electrodes within the subthalamic nucleus commit suicide due to the development of a major depression [86]. For AD or other demented patients, very few adverse effects have been described so far. Since the target regions of DBS to enhance memory functions are different from the ones used in movement disorders, also no speculations can be made. Nevertheless, one major advantage of DBS is its reversible nature, since stimulation settings can be modulated in a way to keep adverse effects at a minimal level.

Besides showing therapeutic effects, another question is whether DBS can be disease-modifying. In the context of extensive experience of DBS in Parkinson's patients, evidence that DBS influences the underlying neurodegenerative process is up to now very weak. In animal research, however, studies in both rats and monkeys have demonstrated that DBS of the subthalamic nucleus can prevent the degeneration of nigral dopamine neurons from the insult produced by dopamine depleting neurotoxins [87-90]. Nevertheless, animal models of neurodegeneration following the administration of

toxins do not adequately reflect the neurodegenerative processes in humans and these results should not be over-interpreted.

Up to now, contributions of lesion studies within the memory circuit have provided solid groundwork in understanding the neuroanatomy of memory [37-39, 42, 52, 91, 92]. The next challenge is to use these findings and derive target structures for the application of DBS in diseases characterized by dementia. The good predictive and construct validity of some animal models for understanding the effects and neural substrates underlying DBS suggest that preclinical animal models are an important tool for accelerating and validating clinical application of DBS. Nevertheless, it is still premature to conclude that DBS can be used in the treatment of AD, and the field will wait for the results of ongoing clinical trials.

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CHAPTER 4

A NEUROANATOMICAL ANALYSIS OF THE EFFECTS OF A MEMORY IMPAIRING DOSE OF SCOPOLAMINE IN THE RAT BRAIN USING CYTOCHROME C OXIDASE AS PRINCIPLE MARKER

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ABSTRACT

Acetylcholine plays a role in mnemonic and attentional processes, but also in locomotor and anxiety-related behavior. Receptor blockage by scopolamine can therefore induce cognitive as well as motor deficits and increase anxiety levels. Here we show that scopolamine, at a dose that has previously been found to affect learning and memory performance (0.1 mg/kg i.p.), has a widespread effect on cytochrome c oxidase histochemistry in various regions of the rat brain. We found a down-regulation of cytochrome c oxidase in the nucleus basalis, in movement-related structures such as the primary motor cortex and the globus pallidus, memory-related structures such as the CA1 subfield of the hippocampus and perirhinal cortex and in the anxiety-related structures like the amygdala, which also plays a role in memory. However choline acetyltransferase levels were only affected in the CA1 subfield of the hippocampus and both, choline acetyltransferase and c-Fos expression levels were decreased in the amygdala. These findings corroborate strong cognitive behavioral effects of this drug, but also suggest possible anxiety and locomotor-related behavioral changes in subjects. Moreover, they present histochemical evidence that the effects of scopolamine are not ultimately restricted to cognitive parameters.

INTRODUCTION

Acetylcholine has been implicated as a neurotransmitter involved in learning and memory. Therefore, a vast number of studies have demonstrated that the anticholinergic drug scopolamine produces cognitive deficits in both humans [1-4] and rodents [5-9]. Scopolamine is a non-selective muscarinic acetylcholine receptor antagonist [10], which binds the receptor with an affinity of 1 nM in the rat brain [11]. There are five different muscarinic acetylcholine receptors, all of which are G-protein coupled: M1, M3 and M5 are linked to G_q, while M2 and M4 are autoreceptors linked to G_{i/o} [12]. Studies investigating the localization and distribution of these receptors have elucidated that M1 and M5 receptors in particular are located in the central nervous system, while M2, M3 and M4 are evenly distributed in the central and peripheral nervous system [13, 14]. Due to its non-selective binding properties scopolamine has effects on anxiety levels, locomotor behavior and cognition [15, 16].

In general cholinergic neurons can be classified as projection neurons located mainly in the forebrain and upper brain stem, and interneurons, which are mainly found in the caudate-putamen, nucleus accumbens, hippocampus, cerebral cortex, hypothalamus and spinal cord. The basal forebrain cholinergic complex is composed of the septum, horizontal and vertical diagonal band of Broca, and nucleus basalis of Meynert. This complex provides the major cholinergic projections to the cerebral cortex and hippocampus [17]. In particular, neurons located in the medial septum predominantly innervate the hippocampus, while those of the vertical and horizontal diagonal band project to the anterior cingulate cortex and olfactory bulb, respectively [18]. The hippocampal formation and the medial prefrontal cortex play an important role in cognitive functioning. Cholinergic neurons of the nucleus basalis of Meynert provide efferents to the amygdala and habenular nuclei, both of which process memory and emotional reactions. Apart from this, primate and human pathological studies show that the nucleus basalis of Meynert also sends substantial efferent projections to a number of diencephalic structures, including the nucleus caudatus, putamen and thalamus, which are involved in motor behavior [19, 20]. The pontine cholinergic nuclei, act mainly through thalamic intralaminar nuclei, but also through the reuniens nucleus and the anterior dorsal nucleus, and only provide minor innervation of the cerebral cortex [21].

Doses higher than 0.1 mg/kg of scopolamine have been shown to impair performances related to non-mnemonic processes, such as anxiety [22, 23]. A dose of 0.1 mg/kg scopolamine, on the other side, appears to have only memory impairing properties as seen in various behavioral tasks in rodents, such as object location and recognition task, water maze and fear conditioning [6, 24-26]. Since this dose of scopolamine is used extensively in preclinical testing as a model evaluating new substances to treat memory dysfunction, we here provide the first overview of the histochemical effects in

the rat brain when a cognitive impairing dose is administered. Hence, we can evaluate whether only cognitive regions are affected, or whether some side effects contribute to the drug-induced cognitive impairments. The present study used molecular markers of neural activity to test the hypothesis that the dose of 0.1 mg/kg has a widespread effect on the brain. The principle marker used was the endogenous metabolic enzyme cytochrome c oxidase (COX), supplemented by measurements of choline acetyltransferase (ChAT) and c-Fos (K-25) immunoreactivity. COX is an enzyme responsible for oxidative metabolism and therefore a marker for neural activity. Metabolic mapping is based on the principle that energy utilization in brain tissue is determined by the overall functional activity of neurons [27]. A decrease in COX levels can thus be associated with decreased neuronal activity at regional levels. C-Fos (K-25) can be used to map long-term neuronal activation at the cellular level, since it recognizes Fos, Fos-B, Fra-1 and Fra-2 [28, 29]. We examined regions which are related to memory, emotions and motor behavior due to the various behavioral effects of scopolamine.

MATERIALS AND METHODS

Subjects

All procedures involving animals were carried out with approval of the Animal Experiments and Ethics Committee of Maastricht University. Adult male Sprague Dawley rats (body weight 300-350 g) from the Central Animal Facility (Maastricht University, the Netherlands) were housed in pairs in Makrolon cages, with rat chow and water available ad libitum. The colony room was maintained at a temperature of 21 ± 1 °C and on a reversed 12:12 h light:dark cycle. Experimental manipulations were conducted during the dark phase under red light.

Experimental groups

Rats were randomly assigned to the following groups: saline (n=12) and scopolamine (n=12).

Drugs

Scopolamine hydrobromide (Acros Organics BVBA, Geel, Belgium) was dissolved in vehicle (saline; 0.9% NaCl) and administered (i.p.) at a dose of 0.1 mg/kg (in 1 ml/kg) once daily over a time period of 5 days. This time period was chosen in order to resemble the time course of a behavioral task, in which scopolamine is administered repeatedly [9, 30, 31]. On the fifth day, rats were injected 60 min before decapitation or perfusion.

Histological analysis

At the end of the experiment 6 rats of the saline group and 6 scopolamine treated animals were decapitated without anesthesia. Brains were rapidly removed, frozen in isopentane (- 45 °C) and stored in - 80°C. Subsequently, the brains were cut serially on a cryostat (Leica, Wetzlar, Germany) into 30 µm coronal sections, thaw mounted on gelatine-coated slides and again stored at - 80 °C. To determine the metabolic activity of different brain structures, a cytochrome c oxidase (COX) staining was performed on the freshly frozen brain slides. For the COX staining, sections were incubated in a solution of Hepes buffer (0.1 M) containing 4.5% sucrose, 0.12% 3,3'-diaminobenzidine-4HCl (DAB), 0.125% ammonium nickel sulfate and 0.023% cytochrome C type III (C2506, Sigma-Aldrich, Seelze, Germany), for the duration of 1 hour at 37 °C. To stop the reaction the tissue was fixated in a solution of neutral buffered formaldehyde for 10 minutes. Finally sections were rinsed, dehydrated, and cover slipped with depex mounting medium.

The remaining 6 saline treated and 6 scopolamine treated animals were transcardially perfused first with Tyrode (NaCl, KCl, CaCl₂, MgCl₂, NaH₂PO₄, NaHCO₃, glucose, distilled water) followed by Somogyi solution (4% paraformaldehyde, 15% picric acid, 0.05% glutaraldehyde in 0.1M phosphate buffered saline (pH 7.6)). To prevent the development of postperfusion artifacts, brains were fixed in fresh fixative (same content as in the Somogyi solution but lacking glutaraldehyde) at 4 °C. After 2 h, the brains were gently removed and stored in 1% NaN₃ in 4 °C. Brains were embedded in 10% gelatin from porcine skin (Sigma-Aldrich, Zwyzndrecht, The Netherlands), and cut into 30 µm slices in the frontal plane using a vibratome (Leica®, Wetzlar, Germany). Slices were immediately transferred into 1% NaN₃. Significant findings of the previous COX staining were further evaluated with ChAT and c-Fos (K-25) immunohistochemistry. In brief, sections were incubated overnight with polyclonal rabbit anti-c-fos (K-25) (1:2000; Santa Cruz Biotechnology Inc, Santa Cruz, USA) or choline acetyltransferase (H-95) (1:250; Santa Cruz Biotechnology Inc, Santa Cruz, USA) primary antibody and then, after washing, biotinylated donkey anti-rabbit secondary antibody (1:800; Jackson Immunoresearch Laboratories Inc., Westgrove, USA) followed by avidin-biotin peroxidase complex (1:800, Elite ABC-kit, Vestastatin, Burlingame, CA, USA). Both stainings were visualized by DAB/NiCl₂. Finally sections were mounted on glass, dehydrated, and cover slipped with pertex mounting medium.

Digital photos of COX, ChAT and c-Fos in the different regions were taken using the Cell P software (Olympus Soft Imaging Solutions, Münster, Germany) from a Olympus U-CMAD-2 digital camera connected to an Olympus AX 70 microscope (Olympus, Zoeterwoude, the Netherlands). For COX and ChAT densitometry (Image J software version 1.38x; NIH, Bethesda, USA) was performed for 2-3 antero-posterior levels of both right and left hemispheres. Histological data are presented as mean optical density

(OD) ratio \pm S.E.M, which equals optical density/area of interest in mm^2 . Regions of interest were always delineated in their entirety, i.e. hippocampal subfields included pyramidal and dendritic layers. Cells immunopositive for c-Fos were counted manually, whereas the mean number of cells was corrected for surface area and expressed as cells/ mm^2 . Optical density measures of COX and ChAT were corrected by being subtracted from background measures of white matter. Bregma levels according to the rat brain atlas of Paxinos and Watson are as follows: prefrontal cortex + 3.72 mm, primary and secondary motor cortex +3.72 mm, Ncl. Accumbens shell/core +0.72 mm, entorhinal and perirhinal cortex -7.56 mm, subthalamic nucleus -3.36 mm, hippocampus -3.36 mm, lateral habenula -3.36 mm, amygdala -2.16 mm, globus pallidus and caudate putamen -1.72 mm, diagonal band of Broca and septum +0.72 mm, Ncl. Basalis -0.48 mm, anterior thalamus -1.92 mm, dorsolateral periaqueductal gray (PAG) and dorsal raphe nucleus (DRN) -7.56 mm. As mentioned before, only significant histochemical findings of the COX staining were further investigated with ChAT and c-Fos (K-25) immunohistochemistry.

Statistical analysis

Optical densities and cell densities from the left and right hemispheres, as well as similar slides per structure were pooled and corrected individually for background. Slides containing cryodamage or other tissue damage were excluded in the analysis. An independent samples t-test was performed for the different targets and to supplement these findings partial eta-squared were individually reported as an estimate of the effect size. All p-values ≤ 0.05 were considered significant.

RESULTS

Our findings showed a differential pattern of COX (de)activation in the brain (see Table 1). We found a significant downregulation of oxidative energy metabolism due to scopolamine injection in the following structures: CA1 (Fig.1 A and B), globus pallidus and nucleus basalis of Meynert (Fig. 1 C and D), primary motor cortex (Fig. 1 E and F), perirhinal cortex (Fig. 1 G and H) and amygdala (Fig. 1 I and J). No effects of scopolamine treatment were found in these brain regions: diagonal band of Broca and medial septum (Fig. 1 K and L), secondary motor cortex, nucleus accumbens core and shell, caudate putamen, subthalamic nucleus, lateral habenula, dorsolateral PAG, prefrontal cortex, CA3, dentate gyrus, entorhinal cortex, anterior/dorsal (AD) and anteroventral-ventral lateral (AVVL) thalamus and DRN.

ChAT was only downregulated in the CA1 ($t(10) = 2.724$, $p = 0.02$, $\eta^2 = 0.426$; Fig. 2 A and B) and the amygdala ($t(10) = 2.692$, $p = 0.02$, $\eta^2 = 0.420$; Fig 2 C and D). No

Table 1: Summary of cytochrome c oxidase optical density measures for different structures of the brain when comparing scopolamine to saline treated animals. Structures are organized according to their primary function and optical density measures are presented as mean (SEM). The statistical outcome includes the effect size (partial eta squared) and significant brain-depressant effects are presented in bold.

Structure	Optical density	Statistical outcome
Diagonal band of Broca	Saline: 776.8 (37.3) Scopolamine: 754.1 (62.1)	t(9) = 0.296, n.s., $\eta^2 = 0.010$
Ncl. Basalis	Saline: 523.1 (31.2) Scopolamine: 311.3 (62.8)	t(9) = 2.828, p = 0.020, $\eta^2 = 0.470$
Medial Septum	Saline: 248.4 (63.0) Scopolamine: 165.9 (45.2)	t(10) = 1.065, n.s., $\eta^2 = 0.102$
Primary Motor Cortex	Saline: 472.7 (42.4) Scopolamine: 300.2 (25.1)	t(9) = 3.314, p = 0.009, $\eta^2 = 0.550$
Secondary Motor Cortex	Saline: 346.2 (35.4) Scopolamine: 273.6 (13.8)	t(8) = 1.909, n.s., $\eta^2 = 0.313$
Ncl. Accumbens Core	Saline: 1036.5 (122.8) Scopolamine: 658.8 (129.7)	t(9) = 2.084, n.s., $\eta^2 = 0.325$
Ncl. Accumbens Shell	Saline: 1373.8 (235.1) Scopolamine: 917.5 (168.5)	t(9) = 1.616, n.s., $\eta^2 = 0.225$
Caudate Putamen	Saline: 848.9 (88.9) Scopolamine: 714.6 (42.3)	t(9) = 1.275, n.s., $\eta^2 = 0.153$
Globus Pallidus	Saline: 288.0 (14.8) Scopolamine: 152.5 (26.5)	t(8) = 4.457, p = 0.002, $\eta^2 = 0.713$
Subthalamic nucleus	Saline: 797.0 (97.3) Scopolamine: 521.2 (110.0)	t(10) = 1.878, n.s., $\eta^2 = 0.261$
Lateral Habenula	Saline: 588.7 (83.4) Scopolamine: 398.6 (59.5)	t(10) = 1.856, n.s., $\eta^2 = 0.256$
Amygdala	Saline: 313.3 (22.8) Scopolamine: 190.4 (36.1)	t(10) = 2.877, p = 0.016, $\eta^2 = 0.453$
Dorsal Raphe Nucleus	Saline: 1024.9 (100.8) Scopolamine: 957.6 (86.6)	t(10) = 0.506, n.s., $\eta^2 = 0.025$
Dorsolateral PAG	Saline: 793.5 (48.7) Scopolamine: 699.7 (35.3)	t(10) = 1.561, n.s., $\eta^2 = 0.196$
Prelimbic Cortex	Saline: 436.7 (64.5) Scopolamine: 350.6 (27.6)	t(9) = 1.140, n.s., $\eta^2 = 0.126$
Infralimbic Cortex	Saline: 335.8 (69.5) Scopolamine: 233.3 (20.5)	t(9) = 1.296, n.s., $\eta^2 = 0.157$
Cingulate Gyrus	Saline: 424.7 (52.4) Scopolamine: 376.3 (28.1)	t(9) = 0.765, n.s., $\eta^2 = 0.061$
CA1	Saline: 939.1 (25.2) Scopolamine: 791.2 (53.8)	t(9) = 2.323, p = 0.045, $\eta^2 = 0.375$
CA3	Saline: 776.3 (96.7) Scopolamine: 607.4 (69.0)	t(9) = 1.458, n.s., $\eta^2 = 0.191$
Dentate Gyrus	Saline: 2196.1 (112.7) Scopolamine: 2263.2 (38.3)	t(8) = 0.564, n.s., $\eta^2 = 0.038$
Entorhinal cortex	Saline: 377.0 (21.0) Scopolamine: 289.6 (42.9)	t(10) = 1.832, n.s., $\eta^2 = 0.251$
Perirhinal cortex	Saline: 451.0 (20.8) Scopolamine: 342.6 (41.0)	t(10) = 2.358, p = 0.040, $\eta^2 = 0.357$
AD Thalamus	Saline: 2453.0 (144.7) Scopolamine: 2681.6 (99.8)	t(8) = 1.158, n.s., $\eta^2 = 0.144$
AVVL Thalamus	Saline: 1723.8 (152.6) Scopolamine: 1501.1 (183.6)	t(10) = 0.933, n.s., $\eta^2 = 0.080$

changes were found in the nucleus basalis, primary motor cortex, globus pallidus and perirhinal cortex (Fig. 2 E). C-Fos expression was downregulated only in the amygdala ($t(9) = 3.860$, $p = 0.004$, $\eta^2 = 0.623$; Fig. 3 C and D), while the expression of c-Fos in the CA1 subfield of the hippocampus (Fig. 3 A and B), nucleus basalis, primary motor cortex, globus pallidus and perirhinal cortex did not differ from control animals (Fig. 3 E).

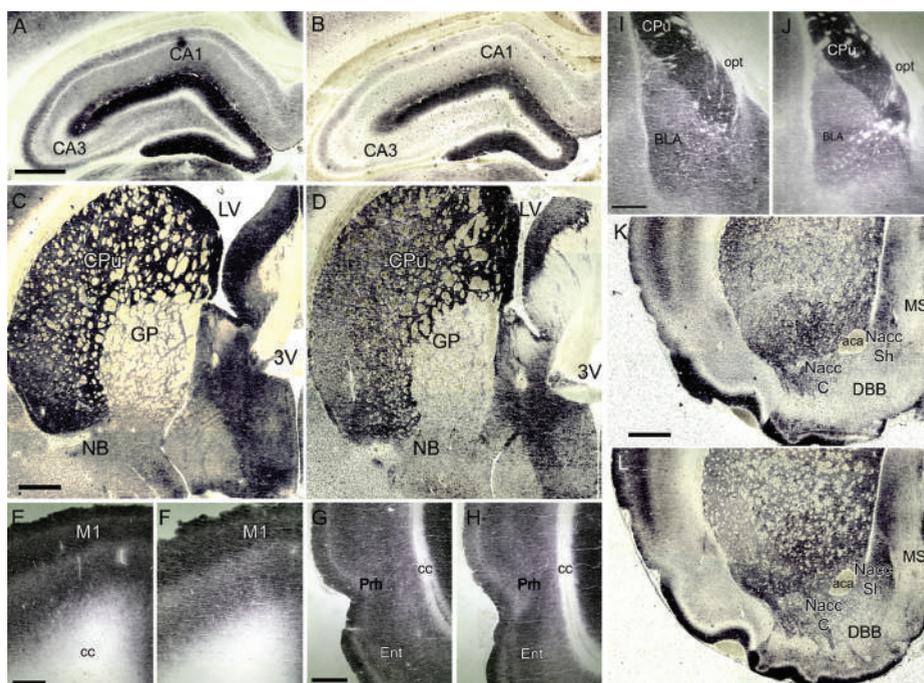


Figure 1: Representative low-power photomicrographs of coronal brain sections stained for COX showing the hippocampus (A and B), globus pallidus (C and D), primary motor cortex (E and F), perirhinal cortex (G and H), amygdala (I and J) and diagonal band of Broca (K and L) of saline (left) and scopolamine (right) treated rats. Anatomical levels are according to the rat brain atlas of Paxinos and Watson (1998). (A and B) Hippocampus CA1 and CA3 at Bregma -3.36 mm. Scale bar = 1000 μ m. (C and D) Globus pallidus (GP), caudate putamen (CPu) and nucleus basalis (NB) at Bregma -0.48 mm. Scale bar = 1000 μ m. (E and F) Primary motor cortex (M1) at Bregma +3.72 mm. Scale bar = 500 μ m. (G and H) Perirhinal (Prh) and entorhinal (Ent) cortex at Bregma -7.56 mm. Scale bar = 500 μ m. (I and J) Amygdala (BLA) at Bregma -2.16 mm. Scale bar = 500 μ m. (K and L) Diagonal Band (DBB), medial septum (MS) and nucleus accumbens core (Nacc C) and shell (Nacc Sh) at Bregma +0.72 mm. Scale bar = 1000 μ m.

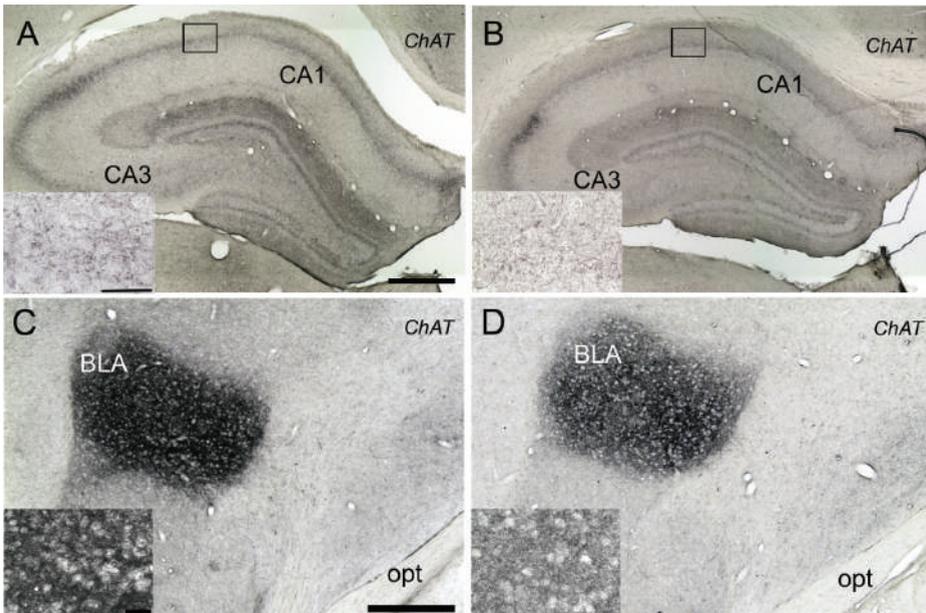


Figure 2: Representative low-power photomicrographs of coronal brain sections stained for choline acetyltransferase (ChAT) showing the hippocampus (A and B) and the amygdala (C and D) of saline (left) and scopolamine (right) treated rats. The high-power photomicrograph insets in the lower left corner show the CA1 subregion of the hippocampus and the amygdala, respectively. (A and B) Hippocampus CA1 and CA3 at Bregma -3.36 mm. Scale bar = 500 μ m, scale bar of inset = 50 μ m. (C and D) Amygdala (BLA) at Bregma -2.16 mm. Scale bar = 500 μ m, scale bar of inset = 50 μ m.

DISCUSSION

Cholinergic neurotransmission is essential for many forms of learning and memory. It has been claimed that the anticholinergic action of scopolamine may more readily impact the hippocampus than other structures [25, 32-34]. Here we provide histochemical and immunohistochemical evidence that both COX and ChAT are down-regulated in the CA1 region of the hippocampus and the amygdala after chronic scopolamine treatment. With c-Fos (K-25) we only found a significant decrease in Fos positive cells in the amygdala and no difference in the CA1 region of the hippocampus between scopolamine and saline injected animals.

The hippocampus and the amygdala both belong to the limbic system and are linked to two independent memory systems, which constantly interact with each other [35]. The hippocampus is critical for episodic memory and correlates with subsequent memory for neutral items. However, variables such as context and prior experience affect the way in which information is retained and the accuracy with which the retention occurs. In fact, the amygdala can modulate these memories through emotional

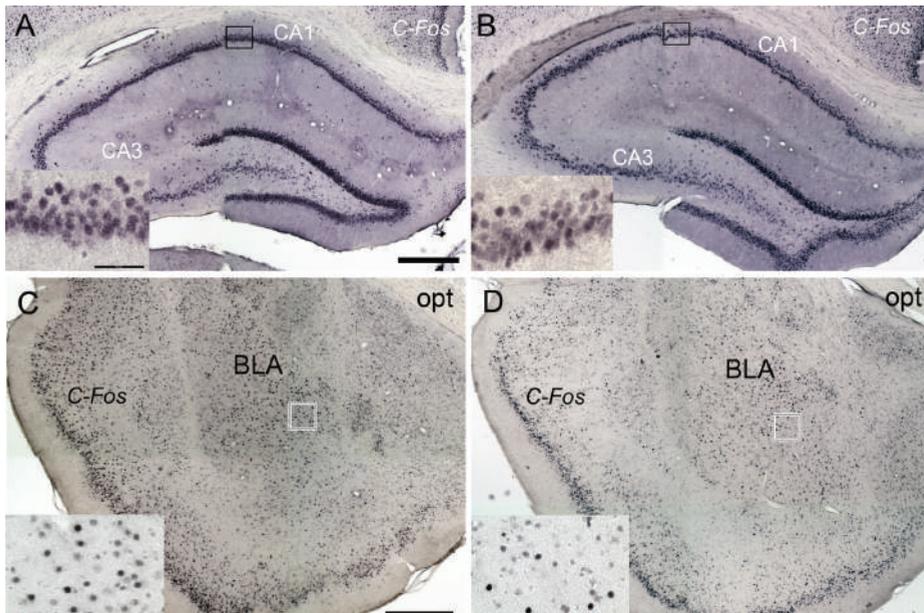


Figure 3: Representative low-power photomicrographs of coronal brain sections stained for c-Fos showing the hippocampus (A and B) and the amygdala (C and D) of saline (left) and scopolamine (right) treated rats. The high-power photomicrograph insets in the lower left corner show the CA1 subregion of the hippocampus and the amygdala, respectively. (A and B) Hippocampus CA1 and CA3 at Bregma -3.36 mm. Scale bar = 500 μ m, scale bar of inset = 50 μ m. (C and D) Amygdala (BLA) at Bregma -2.16 μ m. Scale bar = 500 μ m, scale bar of inset = 50 μ m.

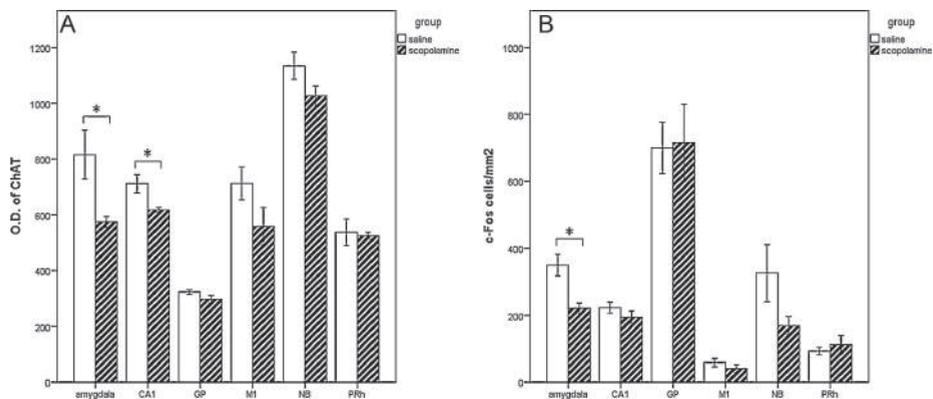


Figure 4: Cumulative data showing the means \pm SEM of (A) choline acetyltransferase (ChAT) optical density levels and (B) c-Fos expression, measured in the amygdala, CA1 subregion of the hippocampus, globus pallidus (GP), primary motor cortex (M1), nucleus basalis (NB) and perirhinal cortex (Prh). Note that, scopolamine treated animals have decreased ChAT activity in the amygdala and CA1. C-Fos cells were only declined in the amygdala of the scopolamine treated animals. Statistically significant difference ($P < 0.05$) is indicated by an (*).

responses. Thus, damage to the amygdala results in a profound impairment of learning abilities in tasks that require the subject to make a connection between environmental stimuli and strong emotional responses [36]. The down-regulation of c-Fos (K-25), ChAT and COX levels in the amygdala could explain the effects of scopolamine on impaired context and cued conditioning [15]. Future studies should investigate structures belonging to the major pathway mediating emotional/anxiety-related effects on memory in more detail.

With the dose of 0.1mg/kg scopolamine there was no effect on ChAT and c-Fos levels in the motor and basal forebrain structures, suggesting that the aforementioned dose only acts on the memory system. However, the effects of scopolamine at the dose applied might have been too subtle to be detected by immunohistochemistry in these brain regions.

Using a different approach, namely COX histochemistry, we were able to detect additional effects on the brain of the relatively low dose of scopolamine. We showed a significant down-regulation of COX activity with an injection dose of 0.1 mg/kg scopolamine in the CA1 subregion of the hippocampus. However, this was not found for the CA3 subfield or dentate gyrus. It has been shown before, that M1 and M4 receptors are enriched in the CA1 region when compared to the CA3 [37]. The CA3 subfield of the hippocampus rather contains more M2 receptors, while m1-m4 immunoreactivities are differentially localized in neuronal populations and neuropil in the dentate gyrus [37]. We also found a clear reduced COX level in the perirhinal, but not the entorhinal cortex. The anteriodorsal and anterioventral nuclei of the thalamus have also been implicated in cognitive functions. In these regions mainly M3 receptor subtypes are expressed [38]. However, scopolamine did not affect the COX level in the anteriodorsal and anterioventral nuclei of the thalamus.

Since scopolamine has effects on anxiety related behavior we examined some regions that are implicated in this behavioral domain. As mentioned before, we found a decrease of COX activity in the amygdala, but no effects in the periaqueductal gray. The periaqueductal gray contains predominantly M1 and M2 receptors. It has been found that activation of M1 and M2 receptor subtypes usually inhibit GABAergic and glutamatergic synaptic transmission in this structure [39]. Furthermore, there were no effects on the lateral habenular nuclei, which contain high levels of M3 receptor subtypes.

Besides, we found a strong COX down-regulation in the nucleus basalis but not in the medial septum or diagonal band of Broca. This implies that at a dose of 0.1 mg/kg the effects of scopolamine are mainly mediated via nucleus basalis and less likely via the medial septum or diagonal band of Broca. BIRTHELMER and colleagues have also suggested that acetylcholine from the medial septum and the diagonal band is not derived from intrinsic neurons but rather from the lateral septum or from terminals of neurons located outside the septal region [40]. Another possibility might include compensation

mechanisms. Inactivation or lesion of neurons in the medial septum-diagonal band of Broca completely abolishes theta oscillation in the hippocampus, demonstrating a critical role of these two structures [41]. In fact, the median raphe nucleus projects the efferent serotonergic pathway to γ -aminobutyric acid (GABA) interneurons of the medial septum – diagonal band complex, which suppresses the generation of hippocampal theta waves [41-43]. Therefore the medial septum and the diagonal band possess more complex functions besides cholinergic transmission.

We did not find any significant changes in COX levels in the medial prefrontal cortex, i.e. the cingulate gyrus, prelimbic and infralimbic cortex. Therefore, we can conclude that the injection dose of 0.1 mg/kg scopolamine does not affect medial prefrontal cortex activity. The primary motor cortex, however, showed decreased COX activity when compared to the secondary motor cortex. Scopolamine also had clear effects on COX levels of the globus pallidus, a structure mainly involved in motor functions. Within the striatum and accumbens, mainly M2 receptors and to a lesser extent M1 and M4 receptors are expressed [44, 45]. COX activity was unaffected in regions like the caudate putamen, STN, nucleus accumbens core and shell. The reduced COX activity in some of the motor regions supports previous studies showing decreased locomotor activity after scopolamine treatment [5, 46-48]. It should be noted that a few studies have reported no effects or an increase in motor activity after scopolamine injections [15, 49]. This may at least be explained by differences in experimental factors such as strain, test and dose.

Since scopolamine was found to reduce COX activity in different regions it implies that scopolamine decreases neuronal activity in various structures of the brain. Of note, there were no brain regions with an increased COX activity.

Several muscarinic receptor subtypes might underlie the cognitive effects of scopolamine. However, muscarinic receptor subtypes are also present in brain structures not primarily involved in learning and memory, but locomotion and anxiety. Muscarinic receptors can furthermore be found in the periphery. In this study we showed that chronic scopolamine administration indeed has a widespread effect on brain depressant activity which corroborates the variety of behavioral effects of this drug (e.g., anxiety, motor activity). We have, however, not investigated peripheral effects of scopolamine, nor can we speculate whether these results would be different following an acute administration paradigm, although cognitive impairments are prominent after a single administration. Due to its non-selective binding properties and its widespread effects on behavior, scopolamine may not be an ideal drug to induce cognitive deficits.

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CHAPTER 5

DEEP BRAIN STIMULATION OF THE FORNICEAL AREA ENHANCES MEMORY FUNCTIONS IN EXPERIMENTAL DEMENTIA: THE ROLE OF STIMULATION PARAMETERS

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ABSTRACT

Deep brain stimulation (DBS) is currently being evaluated as a potential therapy in improving memory functions in Alzheimer's disease. The target for DBS and the stimulation parameters to be used are unknown. Here, we implanted bilateral electrodes in the vicinity of the fornix, a key element of the memory circuitry, and applied DBS with different stimulation frequencies and amplitudes in an experimental model of dementia. Rats received scopolamine, a muscarinic acetylcholine receptor antagonist, to mimic memory impairment. Rats were then tested in the object location task with the following conditions: (i) with attachment of stimulation cable (off stimulation), and (ii) with DBS at various amplitudes (50 μA , 100 μA and 200 μA), 100 μs pulse width and 100 Hz or 10 Hz stimulation frequency. DBS reversed the memory impairing effects of scopolamine when compared to sham rats. We found that the fornix is not sensitive to the frequency of stimulation, but rather to current levels. With the most optimal stimulation parameter, we found no side-effects on anxiety levels and general motor activity. These findings identify the fornix as a key region in controlling spatial memory functions. DBS of this region, using tailored stimulation parameters, has the potential to improve memory functions in conditions characterised by memory impairment.

INTRODUCTION

In the past years, the applicability of deep brain stimulation (DBS) in psychiatry has been evaluated in disorders of mood and thought. DBS of 'key' regions within the limbic system resulted in therapeutic effects in patients with treatment-resistant depression [1-3] and obsessive-compulsive disorder [4]. In this respect, recent clinical studies have suggested that DBS can be used as a tool to enhance memory functions. For instance, Hamani and colleagues [5] stimulated the fornix/hypothalamus area in a patient suffering from morbid obesity. Remarkably, DBS in this patient generated detailed autobiographical memories. Electroencephalographic source localization elucidated that DBS evoked activity in medial temporal lobe structures like the hippocampus. Because of this case-observation, the same group performed the first study in patients with Alzheimer's disease (AD) [6]. Six patients with mild AD were implanted with electrodes in the area of the fornix/hypothalamus and after an intraoperative evaluation of stimulation, patients received chronic DBS at 3 to 3.5 Volts with the frequency set at 130 Hz and the pulse width at 90 μ s for a period of 12 months. Although being a safety study, the central hypothesis was to use DBS to drive the activity of the fornix and obtain beneficial effects on memory functions. Indeed, the authors have found that the application of DBS in the hypothalamus/fornix vicinity was safe and triggered neural activity in the memory circuit, including the entorhinal and hippocampal areas. PET scans showed an early and striking reversal of the impaired glucose utilization in the temporal and parietal lobes that was maintained after 12 months of continuous stimulation. Evaluation of the AD Assessment Scale cognitive subscale and the Mini Mental State Examination suggested possible improvements and/or slowing in the rate of cognitive decline at 6 and 12 months, especially in patients that were less severely affected at the time of surgery.

The fornix is part of the so-called circuit of Papez. The circuit of Papez is one of the major pathways of the limbic system and is primarily involved in the cortical control of emotions and in storing memory [7]. Information is considered to circulate within the circuit of Papez for a certain time and is strongly linked with internal states (emotional as well as motivational) before being processed for long-term storage. In this circuit, the entorhinal cortex projects to the hippocampus, whose efferents are bundled in the fornix and reach the mamillary bodies. In fact, the fornix constitutes a major inflow and output pathway from the hippocampus and medial temporal lobe. It provides a direct source of input from the hippocampal formation to the anterior thalamic nuclei [8, 9] and in turn, the anterior thalamic nuclei project to the subicular and postsubicular cortical regions [10]. Furthermore, cholinergic fibers from the basal forebrain, including the septal nuclei and the nucleus basalis of Meynert run through the fornix.

Lesioning the fornix in experimental animals and also in humans results in major memory deficits [10-12].

In the present study, we addressed three research questions. Does DBS of the fornix in an animal model of memory impairment result in therapeutic benefits in memory functions? Does it result in behavioural side-effects? Which stimulation parameters deliver the most substantial outcome? In order to examine potential memory improvements by DBS, we injected rats with scopolamine, a muscarinic acetylcholine receptor antagonist, to induce memory dysfunction. Justification for this purpose has been provided by the cholinergic hypothesis of geriatric memory impairment [13-17]. The scopolamine model is used extensively for preclinical testing of new substances designed to treat cognitive dysfunctions [13-16]. We evaluated the effects of DBS on memory functions using the object location task (OLT) [18] and potential behavioural side-effects, on anxiety or general motor activity, using the open field (OF) test. The effects of different stimulation parameters, including high and low frequency, as well as high and low current intensities, were examined.

MATERIALS AND METHODS

Subjects

All procedures involving animals were carried out with approval of the Animal Experiments and Ethics Committee of Maastricht University. Adult male Sprague Dawley rats (body weight 300-350 g at the time of surgery) from the Central Animal Facility (Maastricht University, the Netherlands) were individually housed in Makrolon™ cages, with rat chow and water available ad libitum. The colony room was maintained at a temperature of 21 ± 1 °C and on a reversed 12:12 h light:dark cycle. All experimental manipulations were conducted during the dark phase under red light, when rodents are most active.

Experimental groups

Rats were randomly assigned to one of the following groups: Sham (n= 11) or Fornix DBS (n = 10).

Surgical Procedure

For the Fornix DBS group, stimulation electrodes were implanted bilaterally in the fornical region using a rodent stereotact (Stoelting, Wood Dale, IL, USA, model 51653). We aimed not to enter the fornix to prevent damage to the white matter, and placed our electrodes in close vicinity to the fornix. Rats were anesthetized with Isoflurane (IsoFlo®, Abbott Laboratories Ltd, Berkshire, Great Britain) throughout the procedure.

After exposing the skull, the rat received two burrholes at the level of the fornix in order to implant a bilateral electrode construction (coordinates from Bregma according to the Rat Brain atlas of Paxinos and Watson [19]: AP -1.88, ML 1.3, DV -8.2). In addition, five holes were then drilled into the skull and stainless steel screws were placed to provide support in anchoring the electrode with dental acrylic (Paladur, Heraeus Kulzer GmbH, Hanau, Germany). Stimulations were applied bilaterally by using two gold-plated coaxial electrodes with an inner wire of a platinum–iridium combination (250 μm shaft diameter, 50 μm tip diameter; Technomed, Beek, The Netherlands). More details about the electrode construction and surgery have been published before [20, 21]. Sham rats underwent the same surgical procedure with electrode implantation. They were, however, not stimulated.

Drugs

Scopolamine hydrobromide (Acros Organics BVBA, Geel, Belgium) was dissolved in vehicle (saline; 0.9% NaCl) and administered (i.p.) at a dose of 0.1 mg/kg (in 1 ml/kg) 30 min before the first trial.

Deep brain stimulation

Rats were tested with the following conditions: (i) with attachment of stimulation cable (off stimulation), and (ii) with DBS at various amplitudes (50 μA , 100 μA and 200 μA), and 100 Hz or 10 Hz stimulation frequency. The pulse width was set at 100 μs . The mode of stimulation was bipolar in all cases (the inner platinum-iridium wire functions as the negative contact and the outer stainless steel electrode as the positive contact). Stimulation was performed in a descending manner ranging from 200 μA , 100 μA and 50 μA to investigate the effect of a relatively high, medium, and low current density. Taking into account the electrode diameter and exposed surface area, the charge density per phase generated by currents in the 50 – 300 μA approximates that used in humans. It was shown before that 200 μA was the most effective parameter for mood improvement [22, 23]. The frequency applied was low (10 Hz) and high (100 Hz) in order to determine whether changes were dependent on the stimulation frequency. Stimuli were delivered using a World Precision Instrument digital stimulator (DS8000, WPI, Berlin, Germany) and stimulus isolators (DLS100, WPI, Berlin, Germany). Stimulation started 2 min before the test and lasted for the duration of each test session, as it was shown that changes in behavior can occur immediately after the onset of stimulation [5, 21, 23, 24]. Each animal had at least a 24 h stimulation-off period before the next session started.

Behavioural Testing

Object Location Task

Following the first week of recovery animals were handled daily for one week. Handling involved weighing and procedures of injection. The rats were also allowed to explore the arena of the OLT and its objects in the same week. The apparatus consisted of a circular arena, 83 cm in diameter, in which half of the 40 cm high walls was made of grey and the other half of transparent polyvinyl chloride. The arena was placed in such a manner that the light intensity was equal in the different parts of the apparatus (approximately 20 lux). The experimental paradigm was adapted from Rutten and colleagues [18].

Two pre-tests with vehicle injections but without stimulation were carried out before the actual experiment. This was done to habituate the rats to the testing procedures. Objects were presented in a counterbalanced order according to a fully-balanced design, in which each rat obtained a different order of objects to avoid preferences for particular objects or locations.

In the first trial (T1) two objects were placed in a symmetrical position about 10 cm away from the walls. The different objects were: (A) a standard 1 L transparent glass bottle (diameter 10 cm, height 22 cm) filled with water, (B) plastic cups (diameter 7.8 cm, height 9 cm), (C) aluminium containers (7.5 cm x 7.5 cm x 10 cm) and (D) ceramic pyramids (11.5 cm x 11.5 cm x 9.5 cm). Objects were filled with regular sand cement so that the animals could not move the objects. Rats were given scopolamine injections 30 min before T1 in which the animal was allowed to explore the two identical objects for 3 min. A rat was always placed in the apparatus facing the wall at the middle of the transparent segment. Two minutes before placing the rat into the testing arena stimulation started in a separate box and continued while the rats were in the arena. Sham rats underwent the same procedures but without being stimulated. After T1 the rat was put back in its home cage and the second trial (T2) followed after 90 min. In T2 one of the objects presented before was moved 10 cm to the front or back of the arena. Again the rats were stimulated 2 min before as well as during T1. Six different stimulation parameters in the following order were used in 6 consecutive sessions: 100 Hz + 200 μ A, 10 Hz + 200 μ A, 100 Hz + 100 μ A, 10 Hz + 100 μ A, 100 Hz + 50 μ A and 10 Hz + 50 μ A. Each animal had at least a 24 h stimulation-off period before the next session started.

The times spent in exploring each object in T1 and T2 were recorded manually using a personal computer. Exploration was defined as follows: directing the nose to the object at a distance of no more than 2 cm or touching the object with the nose. Sitting on top of the object or biting it was not considered as exploratory behaviour. In order to avoid the presence of olfactory cues the objects were cleaned with an ethanol solution before each trial.

Total exploration time during T1 and T2 (e1 and e2, respectively) was considered as the sum of time spent at both objects. Discrimination performance (d2) was calculated as follows (time at object at novel position – time at objects in old position)/e2. Side bias was determined by the time at the left or right object, independent of the novel or old position of the object.

Open Field

The OF consisted of a clear Plexiglas square arena measuring 100 x 100 cm with 40 cm high walls and a dark floor as described in Lim et al. [25]. The test measures time spent and number of entries into the inner and outer zone. More time spent in the centre, indicates increased exploration, and is defined as reduced anxiety. The distance travelled provides an indication of the rat's locomotor activity. Animals were individually placed in the centre of the OF arena and were allowed to move freely in the arena for 5 min. DBS with the most optimal stimulation parameters derived from the OLT (100 Hz and 100 μ A) was performed 2 min before testing as well as 5 min throughout the OF session. Sham animals were attached to cables, but not stimulated. The behavior of each rat was recorded on a computer using Ethovision tracking software (Ethovision, Noldus Information Technology, Wageningen, The Netherlands). The software automatically calculated and analyzed data including the locomotion/distance moved and time spent in the different areas of the open field. The inner zone of 70 x 70 cm was demarcated manually on the computer, leaving a surrounding outer zone of ca. 15 cm width. After each trial the OF was cleaned with an ethanol solution to diminish odours of other rats.

Histological analysis

At the end of the experiments an overdose of pentobarbital (Apotheek Faculteit Diergeneeskunde, Utrecht, The Netherlands) was given and rats underwent perfusion-fixation first with Tyrode solution (NaCl, KCl, CaCl₂, MgCl₂, NaH₂PO₄, NaHCO₃, Glucose, distilled water) followed by Somogyi solution (4% Paraformaldehyde, Picric Acid, PBS, Glutaraldehyde, distilled water). To prevent the development of postperfusion artifacts, brains were fixed in fresh fixative (same content as in the Somogyi solution but lacking Glutaraldehyde) at 4 °C. After 2 hours, the brains were gently removed and stored in 1% NaN₃ in 4 °C.

Brains were embedded in 10% gelatin from porcine skin (Sigma-Aldrich, Zwyndrecht, The Netherlands), and cut into 30 μ m slices in the frontal plane using a vibratome (Leica®, Wetzlar, Germany). Slices were immediately transferred into 1% NaN₃. We collected the sections containing the electrode trajectories, mounted on gelatine coated slides and air dried overnight. Subsequently, we performed a standard hematoxylin-eosin staining. After dehydration, sections were coverslipped with Pertex mounting medium (Histolab Products AB, Gothenburg, Sweden) and photographed under bright field microscopy (Olympus®).

Statistical analysis

For the OLT, e1 and e2 for the vehicle (no stimulation) were analyzed by a paired sample t-test in order to assess a priori differences between the two groups. For the scopolamine sessions a mixed three-way ANOVA with Current and Frequency as within-subjects factor and Group (sham and DBS fornix) as a between-subjects factor was used. In order to test whether the discrimination performance, d_2 , deviated from zero, a one-sample t-test was performed for each observation. Side bias in the second trial was evaluated within groups using a paired samples t-tests. For the OF test, the data were analyzed using an independent samples t-test. All p-values < 0.05 were considered significant.

RESULTS

Histological evaluation of electrode tips

There were 10 rats in the DBS fornix group in total, so we verified 20 electrode tips. We lost no animals. The bilateral electrodes were all implanted in the vicinity of the fornix (Figs. 1A and 1B). The electrodes were situated ventral, medial or lateral to the

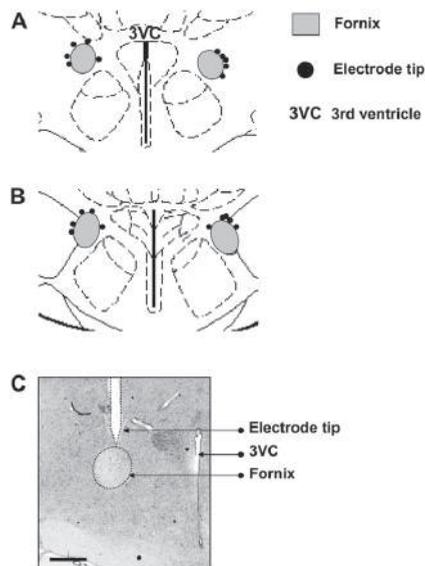


Figure 1: Histological evaluation of electrode tips. Anatomical locations of bilateral stimulation sites close to the fornix at (A) bregma level 1.60 mm (24) and (B) 1.80 mm (24). Sites were localized by microscopic examination of histologically prepared tissue. C) Illustrative coronal section showing the histological verification of the electrode location in the vicinity of the fornix (bar = 400 μm).

fornix. We found no differences between the ventrally, medially or laterally placed electrodes in terms of behavioural outcome. With the current stimulation setting, we found no evidence for histological damage observable with a hematoxylin-eosin staining (Fig. 1C).

Object Location Task

There were no significant a priori differences between sham and DBS fornix rats in the exploration levels e1 and e2 of the first and second trial in the vehicle and off stimulation condition. Only one rat in the fornix DBS group had to be excluded due

Table 1: Mean exploration times (in s) of rats with sham surgery and DBS on the objects in T1 (e1) and T2 (e2). In T2 the time the animals spent on the right and left side was recorded to examine possible side bias. Both groups received vehicle and scopolamine injections as indicated, but only the rats with fornix DBS were stimulated. Entries represent mean (SEM). Bold values characterize significant effects.

		Sham	Fornix	e1	e2	Side Bias (left vs right)
Vehicle	e1	43.6 (2.9)	Off stim	42.6 (2.9)	$\underline{e1}$: $t(19) = 0.23$, n.s.	sham:
	e2	42.9 (2.7)		46.4 (3.6)	$\underline{e2}$: $t(19) = 0.81$, n.s.	$t(10) = 0.52$, n.s.
	time left	22.3 (2.5)		23.7 (3.4)		DBS fornix:
	time right	20.5 (2.0)		22.8 (2.5)		$t(9) = 0.19$, n.s.
Scopolamine session 1	e1	44.2 (3.3)	100Hz	38.0 (5.5)	$\underline{e1}$	sham:
	e2	42.8 (3.6)	200 μ A	38.4 (3.5)	\underline{curr} :	$t(10) = 1.25$, n.s.
	time left	16.1 (1.8)		18.3 (3.6)	$F(2;36) = 0.051$, n.s.	DBS fornix:
Scopolamine session 2					$\underline{curr * group}$:	$t(9) = 1.22$, n.s.
	e1	40.3 (3.5)	10Hz	33.7 (4.2)	$F(2;36) = 0.512$, n.s.	sham:
	e2	34.9 (4.2)	200 μ A	40.2 (4.1)	\underline{freq} :	$t(10) = 0.05$, n.s.
	time left	17.4 (2.3)		17.3 (3.3)	$F(1;18) = 1.42$, n.s.	DBS fornix:
Scopolamine session 3					$\underline{freq * group}$:	$t(9) = 1.64$, n.s.
	e1	35.5 (4.3)	100Hz	36.7 (5.9)	$\underline{curr * freq}$:	sham:
	e2	30.4 (2.0)	100 μ A	43.3 (4.4)	$F(2;36) = 7.15$, $p < 0.01$	$t(10) = 0.76$, n.s.
	time left	15.9 (1.3)		25.2 (3.4)	$\underline{curr * freq * group}$:	DBS fornix:
Scopolamine session 4					$F(2;36) = 0.91$, n.s.	$t(9) = 1.37$, n.s.
	e1	28.5 (2.7)	10Hz	31.6 (3.5)	$\underline{e2}$	sham:
	e2	24.1 (3.4)	100 μ A	28.9 (2.8)	\underline{curr} :	$t(10) = 0.43$, n.s.
	time left	11.7 (1.6)		15.2 (1.7)	$F(2;36) = 0.81$, n.s.	DBS fornix:
Scopolamine session 5					$\underline{curr * group}$:	$t(9) = 0.53$, n.s.
	e1	37.3 (2.9)	100Hz	36.4 (3.8)	$F(2;36) = 2.42$, n.s.	sham:
	e2	35.1 (3.9)	50 μ A	35.7 (3.6)	\underline{freq} :	$t(10) = 1.7$, n.s.
	time left	15.8 (1.4)		16.2 (2.0)	$F(1;18) = 2.36$, n.s.	DBS fornix:
Scopolamine session 6					$\underline{freq * group}$:	$t(9) = 1.02$, n.s.
	e1	29.9 (3.0)	10Hz	32.5 (2.4)	$\underline{curr * freq}$:	sham:
	e2	28.3 (3.4)	50 μ A	27.9 (4.0)	$F(2;36) = 4.59$, $p < 0.02$	$t(10) = 3.02$, $p < 0.02$
	time left	12.2 (1.7)		11.3 (1.8)	$\underline{curr * freq * group}$:	DBS fornix:
	time right	16.1 (1.9)		16.6 (2.4)	$F(2;36) = 0.54$, n.s.	$t(9) = 3.45$, $p < 0.01$

to low exploration levels and was not considered in the following analysis. All current, frequency and interactions within groups were not significant for exploration times during subsequent scopolamine and stimulation sessions (all F 's < 0.9, n.s.). Only one interaction between current density and frequency was found in e1. There were no side biases in the second trial in all sessions, except for the last scopolamine session (Table 1).

For the time ratio of discrimination between the displaced and the familiar objects, d_2 , we found a significant effect of current ($F(2;36) = 3.61$, $p < 0.05$). There was no significant effect of frequency or the interaction between frequency, current and groups (all F 's < 2.05, n.s.). However, the fornix stimulated group performed significantly better on the memory task than the sham group (Group effect: $F(1,10) < 18.14$, $p < 0.001$, Fig. 2). Analysis of the individual stimulation conditions revealed that the 10 Hz + 200 μ A and both 100 μ A conditions significantly improved the memory performance (all t 's (8) < 2.85, $p < 0.05$). In the sham group it was found that only in the fifth scopolamine session the discrimination performance was below zero ($t(10) = 2.96$, $p < 0.02$).

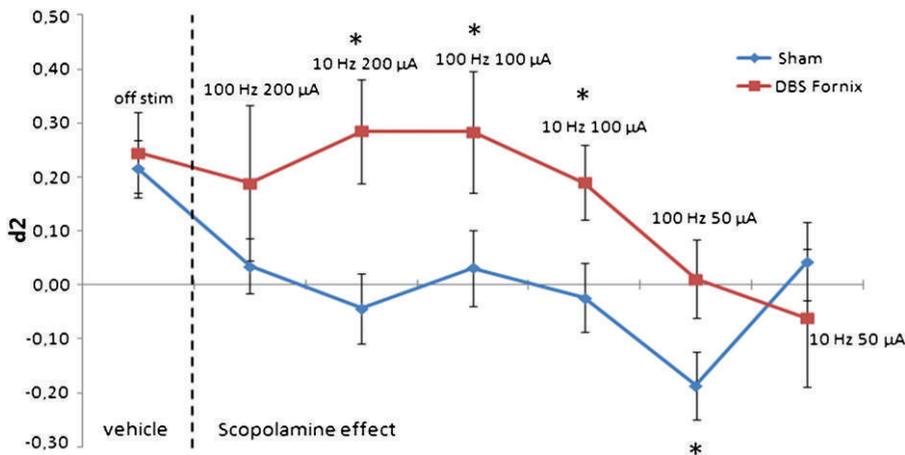


Figure 2: Ratio time spent at the new object for sham and Fornix DBS rats in the OLT. In the first session stimulation was off and saline was injected in all rats. In the following 6 sessions scopolamine was injected and rats in the fornix DBS group were stimulated with the parameters mentioned above. Sham rats only received scopolamine and were not stimulated. There was a current effect in the DBS fornix group, with the most optimal effects at 100 (and 100 Hz) and 200 μ A (and 10 Hz). In the sham group the discrimination ratio was significantly below zero at the fifth scopolamine session, indicating a particular bad memory performance on this day. * indicates a p -value < 0.05, showing a significant difference.

Open field

There was no significant difference between fornix DBS and sham rats for the times spent in corners, walls, centre or distance moved in the OF ($t(19) = 1.99$, n.s., Table 2).

Table 2: Time spent in the different areas of the Open Field and the total distance moved. There was no significant difference between sham operated rats and rats with fornix DBS. Data are presented as mean \pm SEM.

	Corners [s]	Walls [s]	Centre [s]	Distance moved [cm]
Sham	123 \pm 6	132 \pm 4	44 \pm 3	3653 \pm 245
DBS Fornix	104 \pm 7	140 \pm 5	54 \pm 8	3329 \pm 279

DISCUSSION

We found that DBS of the fornical region improved impaired spatial memory performance, which was induced by injections of scopolamine in order to produce a rat model of experimental dementia. This memory improvement was substantial and became comparable to the performance of controls. In the OF we found no side-effects on locomotor and anxiety-related behaviours. Stimulation of parts of the limbic system is known to induce strong effects on anxiety levels since it is interconnected with regions such as the periaqueductal gray, amygdala, hypothalamus and the nucleus accumbens. Stimulation of the first region induces fear and anxiety behaviour in animals [26] while stimulation of the latter produces anxiolytic effects in humans [1].

Interestingly, for the time ratio of discrimination between the displaced and the familiar objects, d_2 , we found a significant current density threshold rather than a frequency effect, since both 10 Hz and 100 Hz were efficient. However, in the case of 10 Hz, we needed higher currents to reach therapeutic effects than when 100 Hz was applied. These findings suggest a total energy/current density threshold rather than a frequency effect. The latter is well-known for DBS of the subthalamic nucleus in patients with Parkinson's disease as well as corresponding animal models, where only higher frequencies produce therapeutic effects. The major difference is that in Parkinson's disease and in most of the other neurological disorders grey matter (cell bodies, dendrites, and small axons) is usually stimulated to cause an overall inhibition [27, 28], although some excitation also occurs. Furthermore, in some DBS-implanted epilepsy patients as well as in animal models high frequency in combination with high current stimulation of the anterior thalamic nucleus, impairs, rather than facilitates cognitive function due to an overall inhibition of the limbic structures [22, 29]. Note that all structures mentioned above, consist of grey matter and high frequencies are used to cause an overall

inhibition. In our study, we aimed to stimulate white matter (large myelinated axons) and found memory enhancement. Our hypothesis is that this effect is accomplished by driving the fornix activity, both orthodromically as well as antidromically. This is supported by the view that large myelinated axons produce excitatory responses upon electrical stimulation [30].

AD is a prototypical neurodegenerative disease and the most common form of dementia. It is characterized by a series of abnormalities in the neocortex, the entorhinal area, hippocampus, amygdala, nucleus basalis of Meynert, anterior thalamus and several brain stem monoaminergic nuclei [31]. The distribution and spread of neural abnormalities in AD follow characteristic patterns that are area-specific and even cell-specific. Besides degeneration of glutamatergic pyramidal neurons and interneurons, cholinergic neurons in the nucleus basalis of Meynert, the medial septal nucleus, and the diagonal band of Broca which provide the principal cholinergic pathways to the neocortex and hippocampus, are also destroyed [31, 32]. These lesions have profound clinical consequences such as memory impairments. Abnormalities of the entorhinal cortex, hippocampus and in the association areas of the neocortex, which are believed to be related to alterations in the basal forebrain cholinergic systems, contribute to the memory difficulties and attention deficits of this disease [31, 32]. In our study, we applied a cholinergic amnesia model and injected scopolamine to induce memory impairment. It has been shown that cholinergic activity in the hippocampus is associated with memory and systemic administration of scopolamine impairs hippocampal-dependent learning and memory in rats [33-35]. We are aware that the model applied in our study does not resemble human AD, for instance it lacks AD specific neuropathology and the progressive nature of the disease. Nevertheless, it is an adequate and widely used model to evaluate the potency of experimental therapies on cognitive functions.

As mentioned before, cholinergic fibers from the basal forebrain and from the septo-hippocampal system run through the fornix, which in turn establishes a connection between the hippocampus and the anterior thalamic nucleus. The hippocampus itself contains muscarinic acetylcholine receptors and it is probable, that the blockade of these receptors through scopolamine administration leads to impaired memory performance in the OLT [33]. Since DBS of the fornix is thought to drive its output, it reinforces the connection between the hippocampus and the anterior thalamic nucleus and thus counteracts the effects of scopolamine receptor blockade in the hippocampus. A functional interaction between hippocampus and anterior thalamic nucleus is necessary for spatial memory and conditional learning as seen in the study of Dumont et al. [36].

To date only two studies have been published, which investigated DBS within the fornix vicinity in patients suffering from AD and morbid obesity [5, 6]. Using imaging techniques, both authors have found that stimulation activated medial temporal lobe

structures like the hippocampus and the parahippocampal gyrus and thus improved hippocampus-dependent memory performance. This is in line with our hypothesis that stimulation of the fornix drives its output and triggers the activity of the hippocampus and/or the anterior nucleus of the thalamus. One way of action of DBS might be increasing neurotransmitter release and local or transsynaptic modulation of neural activity at the cellular level [37] as well as restore basal activity levels within dysregulated brain regions at circuit levels [6, 38]. Stimulation of the entorhinal cortex, for instance, has been shown to induce hippocampal neurogenesis [39, 40] and to improve performance in cognitive tasks [41]. Our hypothesis requires more investigation and further studies are needed to clarify inhibitory as well as excitatory responses to DBS and their implications in memory enhancement.

Our findings identify the fornix as a key region in controlling spatial memory functions. DBS of this region, using tailored stimulation parameters, has the potential to improve memory functions in conditions characterised by memory impairment.

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CHAPTER 6

DEEP BRAIN STIMULATION IN AN AGED ANIMAL MODEL RESTORES MEMORY FUNCTIONS



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Under preparation for submission

ABSTRACT

Background: Age is the biggest risk factor for developing dementia and with the anticipated increase in life expectancy, dementia is becoming one of the greatest medical challenge. To date, there is no known cure that can halt or delay the progression of memory loss. Researchers are currently exploring the applicability of deep brain stimulation (DBS) as potential treatment.

Methods: We have used an aged animal model and implanted bilateral DBS electrodes in the fornix and entorhinal cortex. Rats have performed several behavioral tasks: elevated plus maze, light-dark box, open field, water maze and object recognition task. In both memory tasks, we investigated effects on recent as well as remote memory.

Results: We found no indication for anxiety-related behavior following DBS. Fornix DBS rats showed an increased memory performance when we assessed recent memory in a spatial memory task, the water maze. Contrary to this, entorhinal cortex DBS rats showed beneficial effects for remote memory in the object recognition task.

Conclusion: DBS of the fornix and entorhinal cortex has an impact on different memory systems. Yet it seems that fornix DBS is more relevant to improve symptoms of age-dependent dementia.

INTRODUCTION

Ageing constitutes one of the major health challenges faced by modern societies. People of old age suffer from cognitive and functional decline. In this respect, the prevalence of dementia also increases with age, from 10 % at 65 years to about 50 % over age 85 [1]. The World Health Organization estimates that 35.6 million people suffer from dementia, a number that is anticipated to triple by 2050 (World Health Organization, 2012). Demented behavior can include wandering, problems with communicating and reasoning, aggression, depression and changes in personality. Alzheimer's disease and vascular dementia are the most common types of dementia, but there are many other conditions that can cause symptoms of dementia. Currently, there have only been advances for symptomatic treatments, which were mostly palliative in nature [2, 3], while cures or treatments that delay or reverse memory dysfunctions are unknown. These pharmacological treatments may produce severe side effects and only work for a limited number of patients. Moreover, their effect usually wears off over time [4].

Therefore, researchers have been exploring the application of non-drug based therapies as alternative treatments to alleviate symptoms of dementia-related disorders, such as deep brain stimulation (DBS). The basic principle of this treatment includes electrical stimulation via implanted electrodes in the desired regions of the brain. These stimulation electrodes are connected to an internal pulse generator through insulated wires [5, 6]. At stimulation settings commonly used in clinical practice, DBS decreases spontaneous firing of neuronal populations and drives axonal projections near the electrodes. Thus, DBS can modulate local and distant targets.

So far only a few studies have investigated the effects of DBS on memory and favorable outcomes were observed following fornix [7-11], entorhinal cortex [12, 13] and nucleus basalis of Meynert DBS [14-16]. However, up to now most studies were conducted in humans, although the use of animal models has played a substantial role in the development and refinement of DBS as a therapy [6, 17, 18]. Potential side effects as well as mechanisms of action can be explored in animals. Currently, there have been a few preclinical studies investigating memory effects following DBS. In an experimental model of dementia, for instance, fornix DBS was able to reverse scopolamine-induced memory deficits in the object location task when certain stimulation parameters were used [9]. Another study, applied entorhinal cortex DBS in mice (1 hour at 130Hz, 90 μ s pulse width and 50 μ A) and also found improvements in spatial memory performance reflected by enhanced water maze scores. Six and a half weeks after surgery, behavioral improvement was accompanied by an observed increase in proliferation in the dentate gyrus, a hippocampal structure maintaining strong connections with the entorhinal cortex [12].

Limitations of preclinical studies up to now, however, include the use of young, healthy animals. In the present study we therefore focused on an aged animal model in which the natural aging process leads to cognitive decline. When considering the life span of a laboratory rat, which approximates a maximum of 2.5- 3 years, our aged animals of 2 years seem to be comparable to 60 year old humans [19]. These aged rats may yield more representative data and thus be more translatable to humans. We have implanted DBS electrodes in the fornix and entorhinal cortex and evaluated memory performance, but also anxiety-related side effects in a behavioral task battery. The fornix and entorhinal cortex have been chosen based on the aforementioned studies and in order to stimulate the output and input of the hippocampus, respectively.

METHODS

Subjects

Male Sprague Dawley rats 23 months of age were used, their weight ranging between 500-650g at time of surgery. The temperature of the colony room was maintained at a temperature of 25 ± 1 °C and rats were individually housed in standard cages with rat chow and water available ad libitum. Furthermore, the rats were exposed to a reversed 12:12 h light dark cycle. All experimental procedures were approved by the Institutional of Animals Care and Use Committee of Nanyang Technological University, Singapore (ARF-SBS/NIE-A 0169 AZ).

Experimental groups

Rats were randomly assigned to one of the following experimental groups: Sham (n=12), Fornix DBS (n=11), and entorhinal cortex DBS (n=7). The exact timeline of the experimental procedures can be seen in Figure 1.

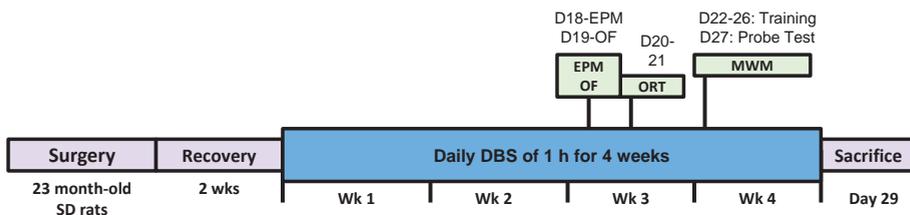


Figure 1: Timeline of the experimental procedures. Please note, DBS was always applied 1 h before behavioural testing took place. Sham animals were only attached to cables and not stimulated.

Surgical procedure

DBS electrodes were implanted bilaterally in the target areas making use of a rodent stereotactic apparatus (Vernier Stereotaxic Instrument, Leica Biosystems, Nussloch, Germany) and Isoflurane of 5% concentration was used as induction anesthesia and 2.5% concentration as continuous maintenance. After exposure of the rat skull, two burr holes were made either at the level of the fornix (coordinates from Bregma according to Rat Brain Atlas of Paxinos and Watson [20]: AP: -1.92 mm; ML: 1.5 mm; DV: -8.4 mm), or entorhinal cortex (AP:-6.84 mm; ML: 4 mm; DV: -8.6 mm). This construct was anchored on the rat skull through stainless steel screws and dental acrylic (Paladur, Heraeus Kulzer GmbH, Hanau, Germany). More detailed information about electrodes and surgical procedures is given in previous publications from our group [21, 22]. Sham rats underwent the same surgical procedure with electrode implantation.

Deep brain stimulation

Stimulation was performed using a World Precision Instrument digital stimulator (DS8000, WPI, Berlin, Germany) and two stimulus isolators (DLS100, WPI, Berlin, Germany). Rats were attached to cables and the stimulator was switched on 1 hour per day before testing. In the sham group the cable was also connected but this group was not stimulated. Based on previous studies, fornix and entorhinal cortex DBS rats were stimulated with 100 Hz, 100 μ A and 100 μ s pulse width [9, 12].

Behavioral testing

Elevated Plus Maze

The elevated plus-maze apparatus consisted of two opposing open arms (50 x 10 cm) with a 10 cm high surrounding wall and two opposing enclosed arms (50 x 10 cm) with a 50 cm high surrounding wall. The arms extended from a central platform (10 x 10 cm). The floor of the open arms, the central platform, and the closed arms were made of black Plexiglass, while the surrounding walls of the arms were made of clear Plexiglass. The maze was elevated 50 cm from the floor. A rat was always placed on the central platform facing one of the open arms. The cumulative time spent in the open and closed arms was measured for a 5 min period using Noldus Ethovision. The maze was thoroughly cleaned with 70% ethanol after each session.

Open Field

The Open Field (OF) consisted of a square arena measuring 40 x 40 cm with 40 cm high walls. The center of 20 x 20 cm was demarcated manually on the computer, leaving a surrounding outer zone of 20 cm width. The behavior of each rat was recorded under

low light condition using a computer with Ethovision tracking software (Ethovision, Noldus Information Technology, Wageningen, The Netherlands).

Morris Water Maze

The Morris water maze consisted of a black circular pool, which was 128 cm in diameter and 60 cm deep and was filled with 30 cm water. The pool was divided into four quadrants and a 10 cm circular escape platform was submerged 25 cm from the wall in the target quadrant of the pool. The position of the platform was kept constant for the duration of the 5 day acquisition period. Water temperature was maintained between 23°C and 25°C.

The water maze spatial learning test took place over a period of seven days. The first five days constituted the training phase in which the rats learned where the hidden platform is. Immediately 1 h after the last training session on the 5th day, the first probe trial followed in order to assess recent memory. Rats were again trained on the 6th day and 24 h later subjected to a second probe trial to assess remote memory.

The training period consisted of four trials per day, starting the rat at four different positions in the water maze, to avoid left and right navigation to the platform. Each trial began with the rat in the pool facing the sidewalls and ended when the rat found the platform or after 60 s; in both cases the rat was allowed to stay on the platform for 10 s. On the 5th and 7th day a probe trial of 60 s was performed after the escape platform had been removed. Time spent in the peripheral and target quadrants was analyzed using Ethovision tracking software.

Object Recognition Task

The apparatus consisted of a square arena measuring 40 x 40 cm with 40 cm high walls. In total 3 different objects were used (A) a glass bottle, (B) a round glass jar and (D) a square plastic drinking bottle. In the first trial (T1) two identical glass bottles were placed in two different corners of the arena and the rat was allowed to explore these objects for 3 min. A rat was always placed in the apparatus facing the wall. After T1 the rat was placed back in its home cage and after an intertrial delay of 1 h, the second trial (T2) followed. In T2 one of the glass bottles presented before was replaced by a novel object, a glass jar. Again rats were allowed to explore these two objects for 3 min. The third trial (T3) was evaluated after a 24 retention interval. In this trial the other glass bottle was replaced by a plastic drinking bottle. The duration of T3 was 3 min. Entorhinal cortex and fornix DBS rats were stimulated with 100 Hz, 100 μ A and 100 μ s 30 min before T1, T2 and T3, while sham rats were only attached to cables and not stimulated.

The time spent exploring each object in T1, T2 and T3 was monitored using a personal computer. Discrimination performance was calculated by subtracting the total

time at familiar objects from the time at the novel object and dividing it by the total exploration time in T2 or T3. Side bias was determined by the time spent at the left or right object, which was independent of the novel or old position of the object.

Statistical analysis

Statistical analysis for all behavioral tasks was performed using one-way ANOVA, followed by an LSD post-hoc test. P-values ≤ 0.05 were considered significant.

RESULTS

Elevated Plus Maze

The performance of entorhinal cortex DBS, fornix DBS and sham rats did not differ in the elevated plus maze. In particular, time spent in center, open or closed arms was similar between the groups (all F 's < 1.377 , n.s., Fig. 2).

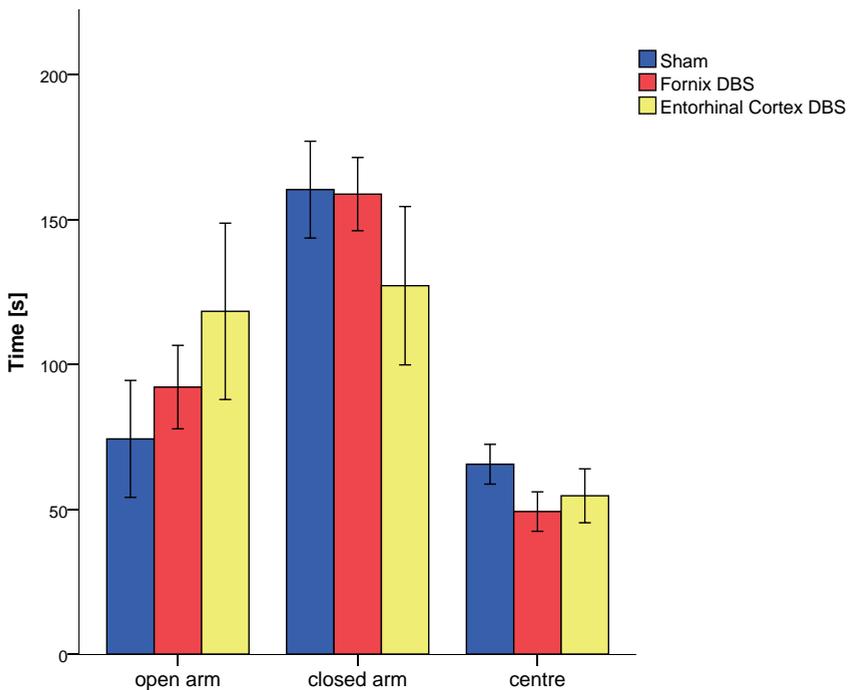


Figure 2: Time spent (in s) in different compartments of the elevated plus maze: open arms, closed arms, center. There was no statistical difference between sham, fornix DBS or entorhinal cortex DBS rats. Data represents mean \pm S.E.M.

Open Field

We found no significant difference for time spent in corners, walls and center of the open field between DBS groups and sham operated animals (all F 's < 0.624, n.s., data not shown). The total distance moved did also not differ between the groups ($F(2,25) = 0.368$, n.s., data not shown).

Morris Water Maze

Fornix and entorhinal cortex DBS rats reached the target faster than sham animals in the first and third training session (all F 's ≥ 4.791 , $p \leq 0.02$). Fornix DBS animals also learned quicker in the second training session when compared to sham ($F(2,28) = 5.864$, $p = 0.02$, Fig. 3).

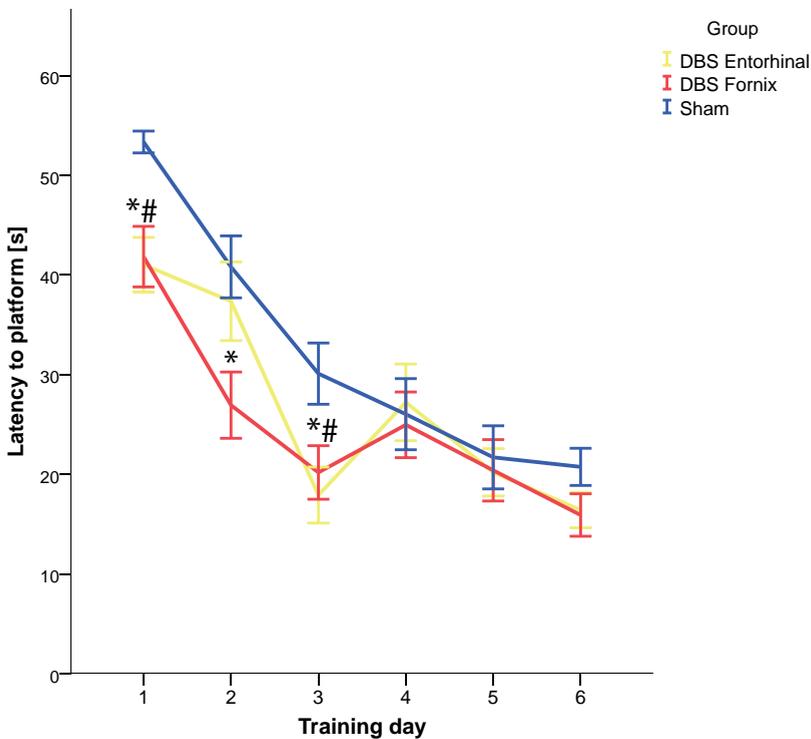


Figure 3: Latency to reach platform in [s] on training day 1-6. Fornix and entorhinal cortex DBS rats acquired the position of the platform faster than sham rats on training days 1-3. * indicates $p < 0.05$ for fornix DBS and # indicates $p < 0.05$ for entorhinal cortex DBS rats. Data are represented as mean \pm S.E.M.

In the first probe test 1 h after training only fornix stimulated animals spent significantly more time in the target quadrant when compared to sham ($F(2,24) = 8.252$, $p = 0.001$, Fig. 4). The post-hoc test did not reveal statistical significance for entorhinal cortex stimulated animals ($p = 0.06$). In the second probe test 24 h after training, there was no difference for time spent in target quadrant between DBS animals and sham ($F(2,25) = 3.066$, n.s.). Entorhinal cortex stimulated animals spent significantly more time in the quadrant opposing the target quadrant ($F(2,25) = 3.851$, $p = 0.03$, data not shown).

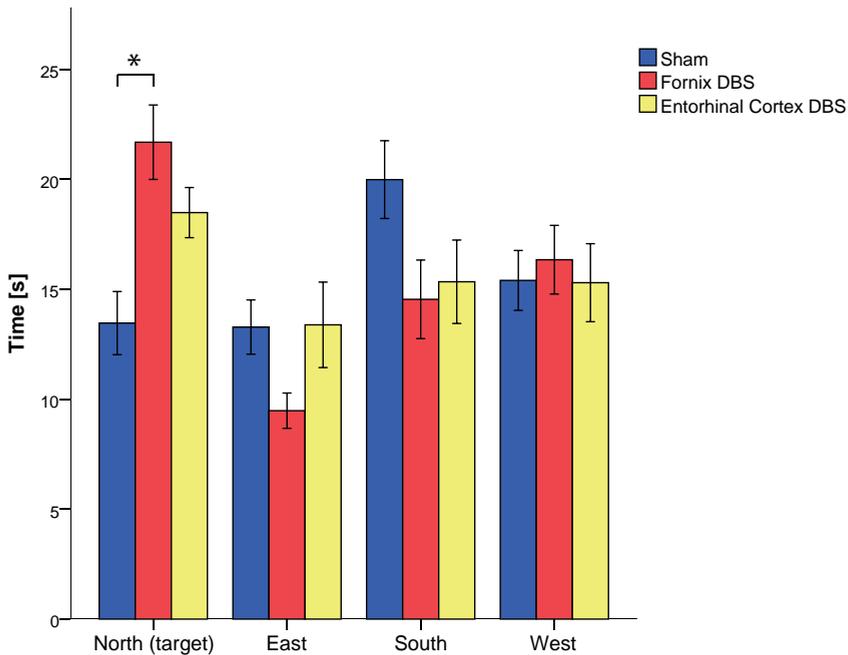


Figure 4: Time spent (in s) in different zones of the water maze after a 1h retention interval. Fornix DBS rats spent significantly more time in the target quadrant when compared to sham. * indicates $p < 0.05$. Data represents mean \pm S.E.M.

Object Recognition Task

After the 1 h retention interval we found no differences between DBS groups and sham ($F(2,25) = 0.616$, n.s.). At the 24 h retention interval only entorhinal cortex DBS rats showed improved memory performance when compared to sham. ($F(2, 23) = 3.999$, $p = 0.01$, Fig. 5).

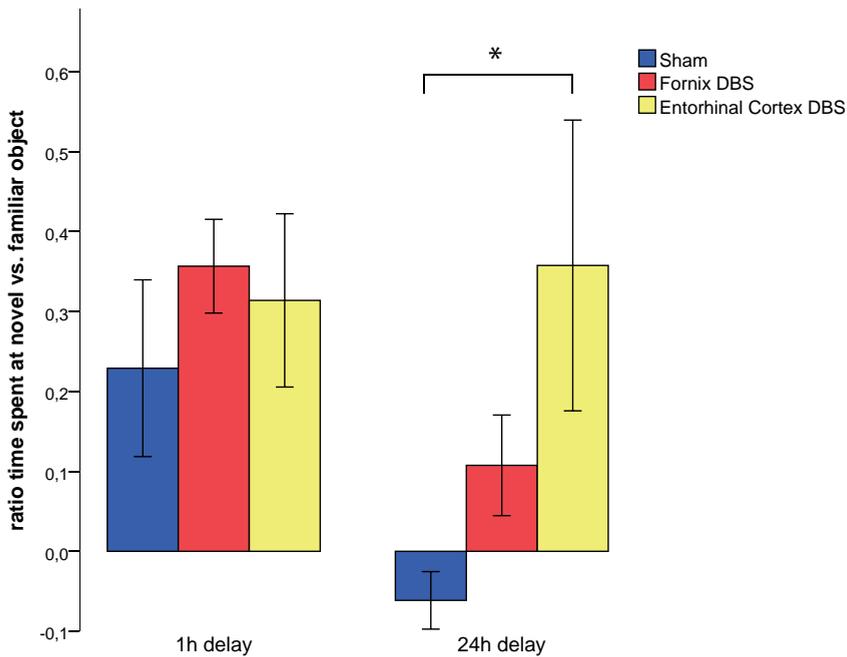


Figure 5: Ratio time spent at novel vs. familiar object for sham, fornix DBS and entorhinal cortex DBS groups. At the 1h retention interval there was no significant difference between the groups. All animals show intact recognition memory. At the 24h retention interval only entorhinal cortex DBS rats spent significantly more time at the novel object when compared to sham. * indicates $p < 0.05$. Data represents mean \pm S.E.M.

DISCUSSION

In an aged animal model, DBS of the fornix and entorhinal cortex resulted in beneficial memory effects in different domains. DBS of the fornix restored impaired spatial memory performance in the water maze, when compared to sham treated animals and animals with entorhinal cortex DBS. In particular, rats with fornix DBS spent significantly more time in the target quadrant when the probe trial was carried out immediately after the last training session. Thus, DBS of the fornix improved short-term memory. Contrary to this, DBS of the entorhinal cortex improved recognition memory in the ORT after a 24 hour delay.

The fornix carries numerous projections to and from the hippocampal formation. Close to the anterior commissure, the fornix divides into a precommissural and postcommissural branch. The precommissural fornix innervates the prefrontal cortex and the ventral striatum, while the postcommissural fornix either connects to the anterior thalamus directly or indirectly through the mammillary bodies [23]. Lesions to the fornix have been shown to affect spatial memory [23-25], while recognition memory

has been spared [24]. Previously, DBS of the fornix has been shown to improve spatial memory in an experimental rat model of dementia [9]. More specifically, fornix DBS was able to restore scopolamine-induced amnesia in the object location task when a combination of certain stimulation parameters was used. Beneficial effects were observed with acute stimulations of 10 Hz and 200 μA or 100 Hz and 100 μA during the trials [9]. Here, we demonstrated that spatial memory deficits naturally occurring through aging might also be improved through fornix DBS.

The entorhinal cortex has strong reciprocal connections with the hippocampal formation and constitutes the major gateway between the hippocampal formation and the neocortex. In fact, the entorhinal cortex has been found to be one of the first structures to degenerate in AD [26]. Several studies have reported a role of the entorhinal cortex in spatial memory. It was reported that the entorhinal cortex contains a spatial map, consisting of grid cells, which contribute to spatiotemporal orientation during behavior [27]. Previously, it was found that entorhinal cortex DBS of 1 hour at 130 Hz, 90 μs pulse width and 50 μA in mice also showed enhanced spatial memory performance in the water maze. Six and a half weeks after stimulation, behavioral improvement was accompanied by an increase in BrdU positive cells in the dentate gyrus, a hippocampal structure maintaining strong connections with the entorhinal cortex [12]. In the present study we did not find any significant spatial memory improvement in the water maze, neither at the immediate nor at the remote retention interval when stimulating with 100 Hz, 100 μs pulse width and 100 μA . It might be possible that the applied current density of 100 μA was too high, because the current hypothesis states that low current applied in the entorhinal cortex has an effect on neighboring axons, thus activating fibers of the perforant path, which in turn induce neurogenesis in the dentate gyrus [17]. Another possibility is that the 24h retention interval was not sufficient to produce effects on spatial memory. In the aforementioned study, mice were tested in the water maze six and a half weeks after stimulation, allowing the maturation of new-born neurons [12].

Contrary to this, we found enhanced recognition memory following entorhinal cortex DBS at a 24h retention interval in the object recognition task. There was no difference between the groups at the 1h retention interval, which is commonly used to test short-term memory. These findings suggest that recognition short-term memory is intact in all groups. The 24h retention interval is used to test natural forgetting, since young and healthy rats usually do not discriminate anymore between novel and familiar object after such an interval [28]. Here, we found that entorhinal cortex DBS was able to reverse natural forgetting. Although most studies, which lesioned the entorhinal cortex found impaired spatial memory, some also reported (mild) deficits in recognition memory [29, 30]. In fact, one has to distinguish different roles of the medial and lateral entorhinal cortex. The medial entorhinal cortex contributes to spatial processing and

the lateral to non-spatial memory [31]. Besides the hippocampus, it has been suggested that the lateral entorhinal cortex is connected to the perirhinal and postrhinal cortices [32]. The perirhinal and postrhinal cortices have been shown to play a strong role in recognition memory [33, 34]. Therefore, it might be possible that entorhinal cortex DBS activated the rhinal cortices and thereby induced enhanced recognition memory.

Taken together, these results suggest that the fornix and entorhinal cortex are employed in different memory domains. The fornix seems to play a major role in spatial memory and the entorhinal cortex in recognition memory.

ACKNOWLEDGEMENTS

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CHAPTER 7

BEHAVIORAL EFFECTS OF DEEP BRAIN STIMULATION OF DIFFERENT AREAS OF THE PAPEZ CIRCUIT ON MEMORY- AND ANXIETY-RELATED FUNCTIONS

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Lim, Arjan Blokland, Yasin Temel

Behavioural Brain Research, 2015; 292:353-60.

ABSTRACT

Deep brain stimulation (DBS) has gained interest as a potential therapy for advanced treatment-resistant dementia. However, possible targets for DBS and the optimal stimulation parameters are not yet clear. Here, we compared the effects of DBS of the CA1 sub-region of the hippocampus, mammillothalamic tract, anterior thalamic nucleus and entorhinal cortex in an experimental rat model of dementia. Rats with scopolamine-induced amnesia were assessed in the object location task with different DBS parameters. Moreover, anxiety-related side effects were evaluated in the elevated zero maze and open field. After sacrifice, we applied c-Fos immunohistochemistry to assess which memory-related regions were affected by DBS. When comparing all structures, DBS of the entorhinal cortex and CA1 sub-region was able to restore memory loss when a specific set of stimulation parameters was used. No anxiety-related side effects were found following DBS. The beneficial behavioral performance of CA1 DBS rats was accompanied with an activation of cells in the anterior cingulate gyrus. Therefore, we conclude that acute CA1 DBS restores memory loss possibly through improved attentional and cognitive processes in the limbic cortex.

INTRODUCTION

Dementia, is a debilitating neurological condition with deterioration in more than one domain of cognitive function. The most prevalent cause of dementia is Alzheimer's disease (AD), accounting for 50-80 % of all cases. In the United States, 9.7 % of the population over 70 years of age, on average, suffers from AD [1]. In Europe the age-standardized prevalence of people aged 65 years or older of population-based studies suggests that 4.4 % is affected [2]. Symptoms include loss of memory, problems with communicating and reasoning, as well as changes in personality and behavior. After diagnosis the expected life span is approximately seven years [3]. Whilst there have been advances for symptomatic treatments for AD [4, 5], there are currently neither cures nor treatments that delay or reverse the effects of AD. Thus, care and pharmacological interventions for dementia are mainly of palliative nature [6, 7].

Against this background of limited progress in dementia treatment, researchers have been exploring non-drug based therapies as alternative treatment strategies to reduce or delay the progression of memory loss in AD. One such treatment is deep brain stimulation (DBS). The basic principle of modern DBS is the use of implanted electrodes connected to an internal pulse generator to deliver electrical stimuli to specific brain regions [8, 9]. This method has mainly been applied in the field of Parkinson's disease in the last two decades, but has been found to produce therapeutic effects in a wide range of neurological [10-12] and psychiatric diseases [13-16]. In line with these developments, evidence from recent preclinical and clinical case studies suggests that DBS of the fornix, entorhinal cortex and nucleus basalis of Meynert might have memory improving properties [17-22].

The rationale for choice of brain regions to improve memory functions in dementia using DBS is based on the classical circuit of Papez [23]. In this circuit, memory-related information flows through the perforant pathway from the entorhinal cortex to fields of the hippocampal formation, including the dentate gyrus, subiculum, CA1 and CA3. The information is then carried through the fornix to the mammillary bodies and thereafter to the anterior nucleus of thalamus via the mammillothalamic tract. The anterior nucleus of the thalamus in turn projects to the cingulate gyrus, and the memory circuit is completed by projections of the cingulate gyrus to the entorhinal cortex of the parahippocampal region [23].

Much of the above circuitry makes a relevant target for DBS. Although we already reported beneficial memory effects following fornix DBS [19], we wanted to explore the applicability and efficacy of other target regions within the circuit of Papez. Therefore, we compared the memory enhancing effects of DBS of the CA1 sub-region of hippocampus, mammillothalamic tract, anterior nucleus of thalamus and entorhinal cortex in a spatial memory task and several anxiety tasks. In addition, we mapped memory-

related brain regions affected by DBS using early the immediate gene c-Fos (K-25). C-Fos is expressed when neurons fire action potential and can be even used to map long-term activation. In particular, we investigated areas related to memory processes i.e. the medial prefrontal cortex and hippocampus.

MATERIALS AND METHODS

Subjects

Sprague Dawley (SD) rats from Charles River (Sulzfeld, Germany) were used, their weight ranging between 300-350 g at the time of surgery. The temperature of the colony room was maintained at a temperature of 21 ± 1 °C and rats were individually housed in Makrolon™ cages with rat chow and water available ad libitum. Furthermore, the rats were exposed to a reversed 12:12 h light dark cycle. All experimental animal procedures were executed during the dark phase under red light.

Experimental groups

Rats were randomly assigned to one of the following experimental groups: Sham (n=11), CA1 DBS (n=10), mammillothalamic tract DBS (n=13), anterior thalamic nucleus DBS (n=14) and entorhinal cortex DBS (n=15).

Surgical procedure

DBS electrodes were implanted bilaterally in the target areas using a rodent stereotactic apparatus (Stoelting, Wood Dale, IL, USA, model 51653). Isoflurane (IsoFlo®, Abbott Laboratories Ltd, Berkshire, Great Britain) was used as inhalation anesthesia. After exposure of the rat skull, two burr holes were made either at the level of the CA1 (coordinates from bregma according to the rat brain atlas of Paxinos and Watson [24]: AP: -3.6 mm; ML: 1.8 mm; DV: -2.6 mm), mammillothalamic tract (AP:-1.8 mm; ML: 1 mm; DV: -6.2 mm), anterior thalamic nucleus (AP: -1.6 mm; ML: 1.5 mm; DV: -5.2 mm), or entorhinal cortex (AP:-6.7 mm; ML: 4 mm; DV: -8 mm). More detailed information about electrodes and surgical procedures is given in previous publications from our group [25, 26]. Shams also underwent electrode implantation in the same brain targets, but were never stimulated. All rats were given a 2 week recovery period after surgery.

Drugs

Scopolamine hydrobromide (Acros Organics BVBA, Geel, Belgium) was dissolved in vehicle solution (saline; 0.9% NaCl) and injected intraperitoneally at a dose of 0.1 mg/kg (in 1 ml/kg) 30 min before the first trial in 6 consecutive sessions of the Object

Location Task (OLT). The chosen dose of scopolamine has been described to affect spatial and recognition memory [27].

Deep brain stimulation

The following experimental conditions were tested: (i) saline injection with attachment of the stimulation cable (no stimulation) and (ii) scopolamine injection with DBS at various amplitudes (50 μ A, 100 μ A and 200 μ A) and frequencies (100 Hz or 10 Hz) at a pulse width of 100 μ s. Stimulation was bipolar and monophasic in all cases. A randomized stimulation paradigm was applied between animals on the different testing days of the OLT. Stimulation was performed using a World Precision Instrument digital stimulator (DS8000, WPI, Berlin, Germany) and two stimulus isolators (DLS100, WPI, Berlin, Germany). Each animal was given a 24 h stimulation-off period between testing sessions. Prior to perfusion, the animals were stimulated for 1 h and were given 1 h of rest before sacrifice in order to observe expression of c-Fos [28].

Behavioral testing

Object Location Task

A full description of the OLT can be found in our previous publication [19]. In short, rats were placed in a circular arena (83 cm diameter) with 40 cm high walls. Half of the surrounding walls were made of grey polyvinyl chloride and the other half of transparent polyvinyl chloride. In total four different sets of objects were used (A) a standard 1 L glass bottle (diameter 10 cm, height 22 cm) filled with water, (B) plastic cups (diameter 7.8 cm, height 9 cm), (C) aluminum containers (7.5 cm, 7.5 cm, 10 cm) and (D) ceramic pyramids (11.5 cm x 11.5 cm x 9.5 cm).

Objects were presented in a counterbalanced order to avoid preferences for particular objects or locations. In the first trial (T1) two identical objects were placed in a symmetrical position in the center of the arena and the rat was allowed to explore these objects for 3 min. After an inter-trial delay of 90 min, the second trial (T2) followed. In T2 one of the objects presented before was moved to a novel position (either left or right object was moved 10 cm to the front or to the back in a counterbalanced order). Using different sets of objects on each day per animal, as well as novel positions in T2 prevented habituation effects.

Two pre-tests with vehicle injections but without stimulation were carried out in order to establish a baseline. Following this, rats were given scopolamine injections 30 min before T1. Stimulation started 2 min before placing the rat into the testing arena (for both T1 and T2) and continued while the rats were in the arena for 3 min. Sham rats underwent the same procedures but were not stimulated.

Exploration time in T1 and T2 was measured manually by pressing keys on a custom-made computer program. During T1 and T2 total exploration time was considered as the sum of time spent at both objects. Discrimination performance was calculated by subtracting the total time at objects in old position from the time at object at novel position and dividing it by the total exploration time in T2. Groups, which showed an effect for particular stimulation parameters, were re-tested and the average discrimination performance was used for analysis. Side bias was determined by the time spent at the left or right object, which was independent of the novel or old position of the object.

Open Field

The Open Field Test (OFT) consisted of a clear Plexiglas square arena measuring 100 x 100 cm with 40 cm high walls and a dark floor. The centre of 70 x 70 cm was demarcated on a computer, leaving a surrounding outer zone of 15 cm width. DBS with the most optimal stimulation parameters derived from the OLT was applied for each DBS group starting 2 min before placing the rat into the arena as well as throughout the 5 min trial. Please note, most optimal stimulation parameter refers to the stimulation parameter, which was able to restore scopolamine-induced memory loss in the OLT or which produced the highest discrimination performance. The behavior of each rat was recorded with Ethovision tracking software (Ethovision, Noldus Information Technology, Wageningen, The Netherlands).

Elevated Zero Maze

The Elevated Zero Maze (EZM), consists of a circular runway (98 cm diameter, 10 cm path width, 70 cm above floor level), which was equally divided into two open and two parts enclosed with high side walls (50 cm). DBS was again applied using most optimal stimulation parameters from the OLT. Rats were stimulated 2 min before as well as throughout the 5 min trial. Time spent in the open and enclosed parts was recorded with Ethovision tracking software.

Tissue collection

At the end of the experiments, the rats were overdosed with pentobarbital (120-180 mg/kg I.P., Pharmacy Faculty of Veterinary Science, Utrecht, The Netherlands) and perfused, first with tyrode solution and then Somogyi fixative solution (4% paraformaldehyde, picric acid, PBS, glutaraldehyde). Brains were removed and placed in fresh fixative (identical composition as Somogyi solution, but lacking glutaraldehyde) at 4°C for 2 h. Subsequently brains were transferred to 1% NaN₃ at 4°C for long-term storage.

For the sectioning procedure, brains were embedded in 10% gelatin from porcine skin (Sigma-Aldrich, Zwijndrecht, The Netherlands), and then cut into 30 µm slices in

the frontal plane using a vibratome (Leica®, Wetzlar, Germany). Slices were immediately transferred into 1% NaN₃ and kept at 4°C.

Fos immunohistochemistry

For immunohistochemistry, sections of 6 random animals per group were selected. In brief, sections were incubated overnight with polyclonal rabbit anti-c-Fos (K-25) primary antibody (1:2000; Santa Cruz Biotechnology Inc, Santa Cruz, USA) and then with biotinylated donkey anti-rabbit secondary antibody (1:800; Jackson Immunoresearch Laboratories Inc., Westgrove, USA) followed by avidin-biotin peroxidase complex (1:800, Elite ABC-kit, Vestastatin, Burlingame, CA, USA). The staining was visualized by 3,3'-Diaminobenzidine (DAB) combined with NiCl₂ intensification.

For immunohistochemical analysis, always 2 similar bregma levels were photographed using a U-CMAD-2 digital camera connected to an Olympus AX70 brightfield microscope (analySIS; Imaging System, Münster, Germany) and evaluated with ImageJ (Image J software version 1.38x; NIH, Bethesda, USA). C-Fos cells in the medial prefrontal cortex (Bregma +3.2 mm ± 0.1 mm) and hippocampus (Bregma -3.3 mm ± 0.1 mm) were counted manually and the percentage increase or decrease when compared to sham was analyzed.

ELECTRODE VERIFICATION

Sections containing the electrode trajectories from all animals were collected and mounted on gelatin coated glass slides. A standard haematoxylin-eosin staining was carried out before sections were photographed under bright field microscopy.

Statistical analysis

For the OLT, the total exploration time in T1 was analyzed by a one-way ANOVA followed by a 2-sided Dunnett post-hoc test. Since the discrimination performance of a sham group can either be slightly positive or negative and this would lead to a Type I or II error, a virtual group was constructed. This statistical approach has been suggested previously [29, 30]. In short, a virtual group has a mean of zero and a S.E.M. comparable to the experimental groups. Therefore, comparisons to a virtual group have been suggested as the most valid manner to examine discrimination performance of experimental groups [29]. A one-way ANOVA was performed comparing individual groups to the virtual group and one-sided Dunnett's post-hoc test was applied [c.f. 29]. For the OFT and EZM test as well as the immunohistochemical data, a one-way ANOVA was employed, followed by 2-sided Dunnett post-hoc test, in which individual

DBS groups were compared to sham. All p -values < 0.05 for the aforementioned tests were considered significant.

RESULTS

Histological evaluation of electrode placements

For CA1, anterior thalamic nucleus and entorhinal cortex, all electrode tips were verified within the target area. We excluded one animal in the mammillothalamic tract group due to incorrect placement of the electrodes. With the current stimulation settings we found no evidence for histological damage as observed by a standard haematoxylin-eosin staining (Fig. 1 and 2).

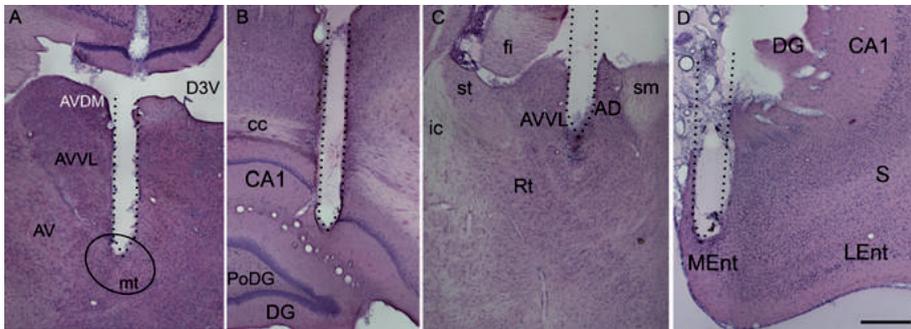


Figure 1: Histological evaluation of electrode tips for mammillothalamic tract (A), CA1 (B), anterior thalamic nucleus (C), and entorhinal cortex (D). Sites were localized by microscopic examination of histologically prepared tissue (bar = 500 μ m). AVDM: anteroventral thalamic nucleus, dorsomedial part; AV: anteroventral thalamic nucleus; AVVL: anteroventral thalamic nucleus, ventrolateral part; mt: mammillothalamic tract; D3V: dorsal third ventricle; cc: corpus callosum; DG: dentate gyrus; PoDG: polymorph layer of dentate gyrus; AD: anterodorsal thalamic nucleus; fi: fimbria; ic: internal capsule; st: stria terminalis; Rt: reticular thalamic nucleus; sm: stria medullaris of the thalamus; S: subiculum; LEnt: lateral entorhinal cortex; MEnt: medial entorhinal cortex.

Object Location Task

In the OLT, all groups had equal exploration levels in T1 in all sessions when compared to sham. Only entorhinal cortex DBS animals spent significantly less time exploring the objects than sham animals ($F(4,57) = 3.619$; $p < 0.01$; data not shown). However, entorhinal cortex DBS rats still met the requirements of the minimal exploration time (i.e. 10 s) by twofold and were therefore considered to be reliable for calculating discrimination performance. When vehicle was injected and stimulation was off, all groups significantly differed from the virtual group ($F(5,68) = 3.106$; all p 's ≤ 0.02). When

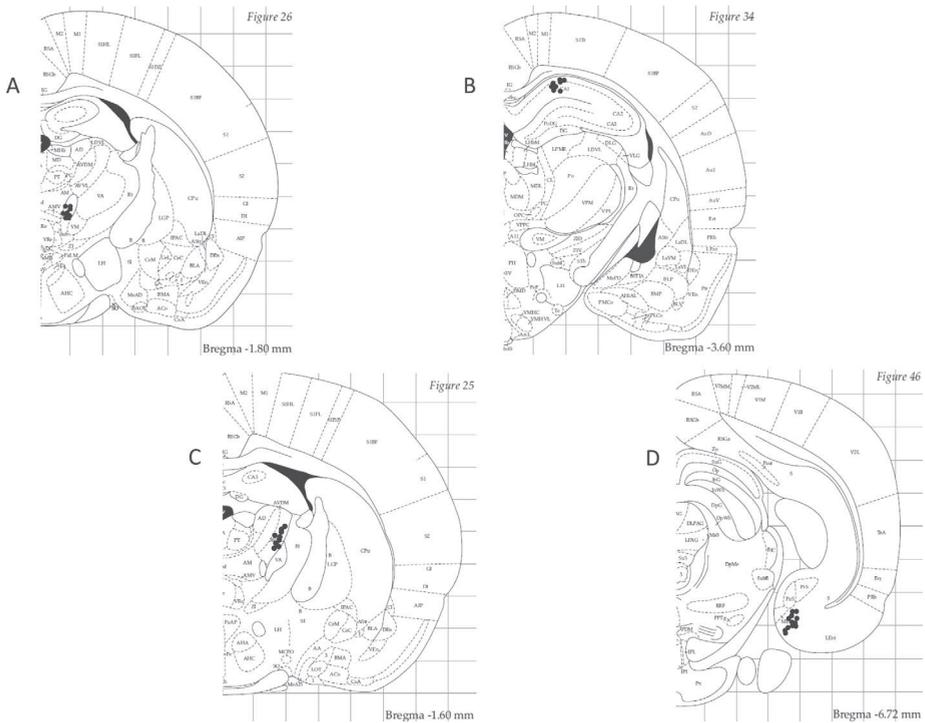


Figure 2: Location of electrode tips in mammothalamic tract (A), CA1 (B), anterior thalamic nucleus (C), and entorhinal cortex (D) shown schematically in one hemisphere (modified from the atlas of Paxinos and Watson [24]).

scopolamine was injected and DBS groups were stimulated, we found a significant restoration of memory for entorhinal cortex DBS rats at 10 Hz and 200 μ A ($F(5,66)=2.965$; $p < 0.03$). Although the ANOVA did not show an overall effect between all groups at 100 Hz and 100 μ A, the post-hoc test revealed significant memory restoration for CA1 DBS rats when compared to the virtual group ($p < 0.02$; Fig. 3).

Open Field and Elevated Zero Maze

The total distance moved in the OFT was similar between DBS groups and sham ($F(5,53) = 0.608$; n.s.; Table 1). When considering time spent in center or periphery of the OFT, the overall ANOVA indicated a statistical difference between the groups, however the post-hoc test revealed no significant differences between different DBS groups and sham (all F 's ≥ 2.516 ; n.s.; data not shown). There was also no difference in time spent in the open ($F(4,51) = 0.818$; n.s.) or closed arm ($F(4,51) = 0.860$; n.s.) of the EZM between the DBS groups and sham (Table 2).

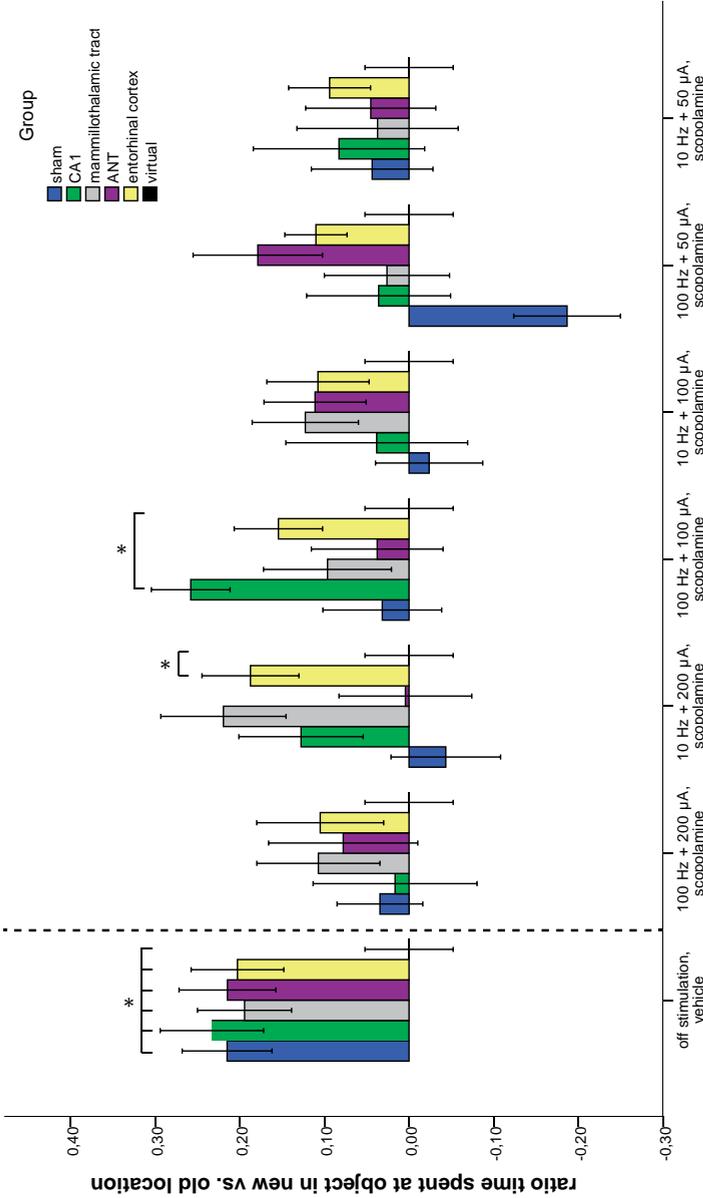


Figure 3: Ratio time spent at new object vs. old object for sham, CA1, mammillothalamic tract, anterior thalamic nucleus (ANT) and entorhinal cortex DBS groups. All groups were compared to a virtual group, which has a mean of zero and a S.E.M. comparable to the other groups (always last column, only S.E.M visible because mean is zero). In the first session vehicle was injected and stimulation was off (all groups differed from zero). In the following sessions scopolamine was injected and DBS groups were stimulated with the parameters mentioned below. Sham rats were attached to cables, but not stimulated. At 10 Hz + 200 μ A entorhinal cortex DBS and at 100 Hz + 100 μ A CA1 DBS rats significantly differed from the virtual group, suggesting enhanced memory performance. * indicates $p < 0.05$. Data represents mean \pm S.E.M.

Table 1: Total distance moved (in cm) in the open field \pm S.E.M. There was no significant difference between sham and DBS groups. ANT: anterior thalamic nucleus.

Group	Mean [cm] \pm S.E.M.
Sham	3653.8 \pm 245.6
CA1	3513.4 \pm 209.2
Mammillothalamic tract	3691.8 \pm 297.5
ANT	3506.0 \pm 224.9
Entorhinal cortex	3888.5 \pm 118.0

Table 2: Time spent in the open or closed arm of the elevated zero maze in seconds \pm S.E.M. There was no significant difference between sham and DBS groups. ANT: anterior thalamic nucleus.

Group	Open arm [s] \pm S.E.M.	Closed arm [s] \pm S.E.M.
Sham	146.8 \pm 6.0	153.0 \pm 6.0
CA1	162.5 \pm 10.4	137.3 \pm 10.4
Mammillothalamic Tract	150.0 \pm 15.7	149.3 \pm 15.7
ANT	172.2 \pm 12.6	126.9 \pm 12.3
Entorhinal cortex	158.5 \pm 8.7	138.0 \pm 9.1

Fos immunohistochemistry

In the medial prefrontal cortex we observed an increased Fos expression in the cingulate gyrus only for CA1 DBS rats ($F(4,30) = 6.966$; $p \leq 0.001$). Moreover, we found a significant increase of c-Fos levels in CA1 and mammillothalamic tract DBS animals in the infralimbic ($F(4,30) = 6.433$; $p \leq 0.015$) and prelimbic cortex ($F(4,30) = 3.563$, $p \leq 0.025$; Fig. 4).

In the hippocampus we found an increased c-Fos expression in the CA3 subregion for entorhinal cortex and anterior thalamic nucleus DBS animals when compared to sham ($F(4,27) = 4.293$; $p \leq 0.01$). There was no statistical difference between the different DBS groups and sham in the CA1 subregion and dentate gyrus (all F 's ≤ 2.409 ; n.s.; Fig. 5).

DISCUSSION

The present study examined the effect of DBS of different targets within the circuit of Papez in a rat model of memory deficit, specifically scopolamine-induced, spatial memory deficit in the OLT. We show that DBS in the CA1 and entorhinal cortex can reverse scopolamine-induced memory deficits. Moreover, we did not observe anxiety-related side effects of different DBS groups when compared to sham in the EZM and OFT.

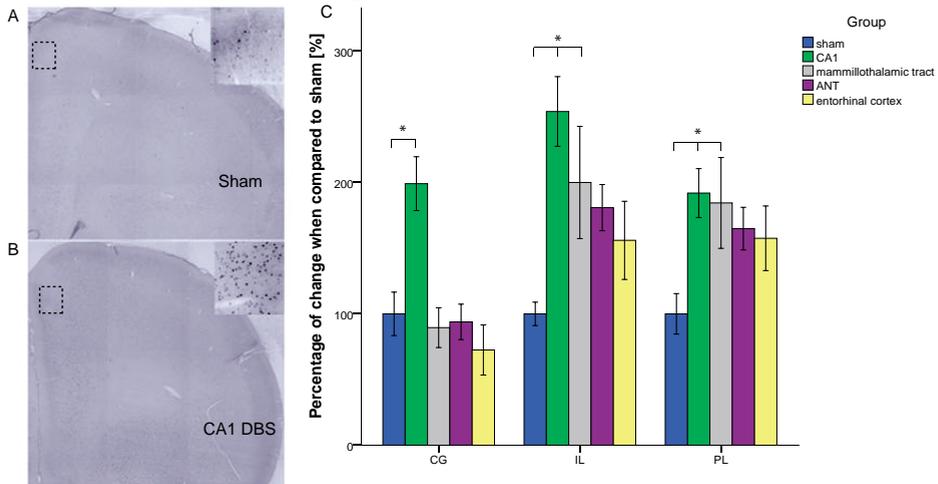


Figure 4: Representative low-power photomicrographs (scale bar = 1000 μm) of coronal brain sections stained for c-Fos (K-25) showing the medial prefrontal cortex of sham (A) and CA1 DBS animals (B). The high-power photomicrograph insets in the upper right corner show the anterior cingulate gyrus (scale bar = 50 μm). (C) Comparisons were made as percentage increase/decrease with regard to sham. CA1 DBS animals showed increased Fos expression in Cg, whereas CA1 DBS and mammillothalamic tract DBS rats had increased Fos levels in IL and PL. ANT: anterior thalamic nucleus; Cg: cingulate gyrus; IL: infralimbic cortex; PL: prelimbic cortex. * indicates $p < 0.05$. Data represents mean \pm S.E.M.

At present, the evaluation of hippocampal DBS has mainly focused on seizure frequency of epileptic patients [31, 32] and little is known about the secondary effects in memory processes. Studies in which neuropsychological tests for memory were included have reported no change in memory scores following hippocampal DBS [33, 34]. Single pulse stimulations of the hippocampus of epileptic patients were even found to impair rather than facilitate memory [35, 36]. In these studies, it was suggested that stimulation within the hippocampus acutely disrupts formation and recall of recent episodic memory. Here, we found beneficial memory effects following acute CA1 DBS at 100 Hz, 100 μA, and 100 μs pulse width. This favorable outcome might have been related to the site of stimulation. While in epileptic patients, DBS electrodes are usually not placed in particular subfields, we specifically targeted the CA1 subregion of the hippocampus. The CA1 subregion of the hippocampus is important for spatial memory [37] and is frequently used to study plasticity. It might be possible that DBS of the CA1 subregion induced activity-dependent plastic changes, although this needs further investigation, since we found no evidence for enhanced Fos activity in the CA1 region. Instead, we could link the beneficial memory effects to enhanced neuronal activation in the cingulate gyrus; a structure, which is engaged in attentional processes [38], but also memory consolidation and retrieval [39]. Other regions within the medial prefrontal cortex, namely the infralimbic and prelimbic cortex, exhibited elevated Fos expression

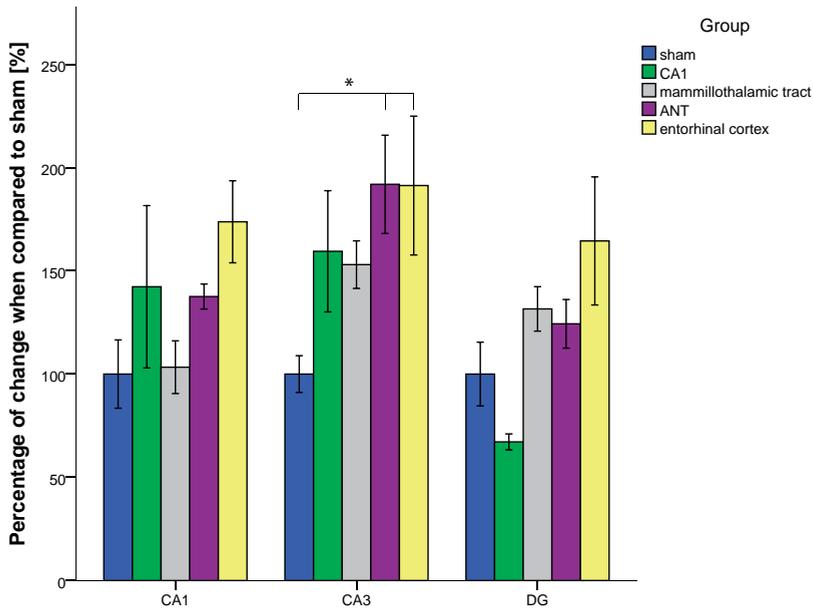


Figure 5: Comparisons of hippocampal c-Fos (K-25) were made as percentage increase/decrease with regard to sham. In the CA3 subregion, entorhinal cortex and anterior thalamic nucleus DBS rats show increased c-Fos expressions. There was no statistical difference between sham and DBS groups in the CA1 and dentate gyrus. ANT: anterior thalamic nucleus. * indicates $p < 0.05$. Data represents mean \pm S.E.M.

for CA1, but also for mammillothalamic tract DBS rats. Since mammillothalamic tract DBS animals did not show memory restoration in the OLT, these results can be interpreted as mere consequence due to stimulation or the activation of cell types, which do not play a role in memory.

We also found restoration of memory loss for entorhinal cortex DBS rats when stimulating with 10 Hz and 200 μ A. This is in line with findings from a clinical study in which seven pharmacoresistant epilepsy patients were implanted with bilateral DBS electrodes in the entorhinal cortex region [22]. The authors reported that spatial learning was enhanced by DBS in a virtual spatial memory task. Stimulation was acute and at a frequency of 50 Hz. Moreover, an intermittent stimulation paradigm with a cycle of 5 s on and 5 s off was applied. The current hypothesis states, that beneficial effects of entorhinal cortex DBS during the learning phase might be associated to theta phase resetting in the hippocampus, allowing the best possible encoding of stimuli [40]. The theta rhythm (3–8 Hz) can be recorded by electroencephalography (EEG) and has been strongly implicated in mnemonic processing. Stimulus presentation, for example, induces shifts in the phase of hippocampal theta-band oscillations and it has been suggested that resetting theta activity facilitates long-term potentiation [41]. In the present

study, we were not able to correlate beneficial memory effects of entorhinal cortex DBS to a selective neuronal activation in the hippocampus. In fact, we found increased c-Fos expression in the CA3 subfield of the hippocampus for both, entorhinal cortex and anterior thalamic nucleus DBS rats. The CA3 sub-region plays a role in encoding and retrieval of spatial location sequences [42]. This effect could be related to the improved memory performance of entorhinal cortex stimulated animals, but since DBS of the anterior thalamic nucleus did not reverse the scopolamine-induced memory deficit, these results may again be interpreted as mere stimulation effects, which did not contribute to the memory performance of the rats. There was no difference in c-Fos levels in the CA1 and dentate gyrus between DBS groups and sham.

We could not find beneficial memory effects following mammillothalamic tract or anterior thalamic nucleus DBS with any applied combination of stimulation. While mammillothalamic tract DBS has not changed memory scores in patients [43], anterior thalamic nucleus DBS has shown to disrupt rather than facilitate memory functions in rodents, at least when high current densities are used [44]. DBS was applied to this nucleus for the treatment of severe epilepsy in the SANTE (stimulation of the anterior nucleus of the thalamus for epilepsy) trial (NCT00101933). Although neuropsychological testing showed no group differences in cognitive functions, more participants of the stimulated group subjectively experienced depression and memory problems [45].

In the OFT and EZM, we found no clear evidence for anxiety-related side effects after DBS. It has been shown that structures within the circuit of Papez are involved in different experimental models of anxiety [46]. Although the present study did not reveal anxiogenic effects of DBS in various structures of the Papez circuit, it is our opinion that tests for anxiety should be included when applying DBS to different structures within this circuit in clinical trials [47].

Summarizing, our results suggest that DBS of the CA1 subfield of the hippocampus seems to reverse scopolamine-induced deficits by modulating neuronal activity of the cingulate gyrus. In terms of accessibility in the human brain, however, we foresee challenges in targeting the CA1 subregion for DBS. DBS of the entorhinal cortex did not induce increased neuronal activity in the medial prefrontal cortex or hippocampus, which could be linked to the improved memory performance. Therefore, further studies are indicated to explore circuit-wide effects.

Previously we have shown that fornix DBS is also able to restore memory loss in an experimental rat model of dementia [19]. Fornix DBS was able to reverse scopolamine-induced amnesia independent of frequency, but when high current densities were applied. The fornix consists of myelinated fibers and has been targeted in several clinical trials, which reported beneficial effects on memory [18, 44]. In a concurrent unpublished study, fornix DBS has shown to influence neuronal activity and neurotransmitter release in the hippocampus.

Altogether, our conclusions are twofold. Firstly, we have shown that within the circuit of Papez, only DBS of the CA1 subregion, entorhinal cortex and fornix is able to restore spatial memory-related functions. Secondly, however, from a stereotactic point of view, we feel that a strategic target should be chosen for therapy. The hippocampus and entorhinal cortex are large areas in rodents and primates and neuromodulation through a single electrode might be difficult. The fornix, on the other side, consists of a localized bundle of myelinated fibers and has an accessible size in both, rodents and primates. Thus, when considering the circuit of Papez, the fornix might consequently represent a preferred target for DBS therapy in dementia-related disorders.

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CHAPTER 8

INCREASED NUMBER OF TH- IMMUNOREACTIVE CELLS IN THE VENTRAL TEGMENTAL AREA AFTER DEEP BRAIN STIMULATION OF THE ANTERIOR NUCLEUS OF THE THALAMUS

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ABSTRACT

Dopamine (DA) has been long implicated with the processes of memory. In long-term memory, the hippocampus and ventral tegmental area (VTA) use DA to enhance long-term potentiation, while prefrontal DA D1 receptors are involved in working memory. Deep Brain Stimulation (DBS) of specific brain areas have been shown to affect memory impairments in animal models. Here, we tested the hypothesis that DBS could reverse memory impairments by increasing the number of dopaminergic cells in the VTA. Rats received DBS at the level of the mammillothalamic tract, the anterior nucleus of the thalamus, and entorhinal cortex before euthanasia. These regions are part of the so-called memory circuit. Brain sections were processed for c-Fos and tyrosine hydroxylase (TH) immunocytochemistry in the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc). C-Fos, TH and c-Fos/TH immunoreactive cells were analyzed by means of stereology and confocal microscopy. Our results showed that DBS of the anterior nucleus of the thalamus induced substantial higher numbers of TH-immunoreactive cells in the ventral tegmental area, while there were no significant differences between the experimental groups in the number of TH immunoreactive cells in the substantia nigra pars compacta, c-Fos immunoreactive cells and c-Fos/TH double-labelled cells in both the substantia nigra pars compacta and ventral tegmental area. Our findings suggest a phenotypic switch, or neurotransmitter respecification, of DAergic cells specifically in the ventral tegmental area which may be induced by DBS in the anterior nucleus of the thalamus.

INTRODUCTION

The neurotransmitter dopamine (DA) has been implicated in the process of learning and memory. DA has for example been linked to memory on different topics such as memory studies on sex and reinforcement [1], on drug addiction [2], and feeding preferences [3]. On a cellular level, DA has been shown to positively influence hippocampus dependent learning and memory by enhancing long-term potentiation (LTP) in the hippocampus through D1/D5 receptors (mainly the CA1 region) [4] by interacting with the ventral tegmental area (VTA). The hippocampus is also part of the circuit of Papez, which has been linked to episodic and spatial memory [5].

Memory impairments are the core features of the different types of dementia and researchers have been exploring the application of non-pharmacological therapies in order to reduce or delay the progression of dementia. One treatment currently being tested is deep brain stimulation (DBS). Findings from recent preclinical studies [6, 7] and clinical studies [8] have suggested that DBS can be used as a tool to enhance memory functions, including reversing memory impairments.

In addition to restoring memory loss, DBS is also used to treat other disorders, such as Parkinson's disease, Tourette's syndrome and several forms of epilepsy [9]. DBS has been proposed as an alternative to drug therapy and has the advantages of reversibility and adjustability in patients [10]. Changes in DA levels through DBS may have some impact in other types of disorders.

In the present study, DBS was implemented in different brain regions of rats with scopolamine-induced memory impairments. The current study focuses on the quantifications of the number of tyrosine hydroxylase (TH, a rate-limiting enzyme in the formation of DA) immunoreactive cells, the number of c-Fos (a proto-oncogene and marker for neuronal activation) immunoreactive cells and the co-localization of the two markers in the major sites of DA synthesis: the Ventral Tegmental Area (VTA) and the Substantia Nigra pars compacta (SNc). It is hypothesized that DBS in various stimulation sites [circuit of Papez structures: Entorhinal Cortex (EC), Mammillothalamic Tract (MTT) and the Anterior Nucleus of the Thalamus (ANT)] will have differential effects on the number of TH-immunoreactive cells or co-localized TH/c-Fos immunoreactive cells in the VTA and SNc after DBS and scopolamine-induced memory impairments.

MATERIALS AND METHODS

This is a summarized section. For a complete "Material and Methods" section, please see the online resource for this article.

Experimental groups

Sprague Dawley (SD) rats were assigned at random to receive DBS in one of several brain sites: the mammillothalamic tract (MTT, n=5), the entorhinal cortex (EC, n=6), the anterior nucleus of the thalamus (ANT, n=5) and a sham-stimulated group, without scopolamine injections (n=3) was made for a negative control.

Pre-perfusion experimental procedures

Scopolamine-model of memory impairments was used during a previous behavioral study. Before each behavioral task rats were injected with scopolamine and stimulated with various parameters during testing. For more information please see [7].

Deep brain stimulation

Before perfusion took place, three different experimental conditions were given: (a) DBS at an amplitude of 100 μ A in the EC (b) DBS at an amplitude of 50 μ A in the ANT and (c) an amplitude of 200 μ A in the MTT. The used stimulation frequency was 100 Hz and the pulse width was set at 100 μ s. All animals were stimulated for 1 h and allowed to rest for 1 h before sacrifice.

Immunohistochemistry

After perfusion, tissue was collected and cut into 30 μ m slices on a vibratome. A fluorescence protocol was conducted for the visualization of TH and c-Fos. First, primary antibodies of mouse anti-TH (1:100, Médimabs, Montreal, Canada) and rabbit anti- c-Fos (1:500, Santa Cruz, Santa Cruz California, USA) were incubated overnight. The next day, the slices had incubation of the secondary antibodies: Alexa 594 donkey anti-mouse (1:100, Invitrogen, Grand Island, NY, USA) followed by Alexa 488 Donkey anti-rabbit (1:100, Invitrogen, Grand Island, NY) for 2 h each.

Histological analysis and quantification

For analysis and cell counting of TH immunoreactive, c-Fos immunoreactive and c-Fos/TH double-labeled cells in the SNc and VTA of the brain sections, a spinning disk confocal microscope (Olympus DSU BX51WI, Pennsylvania, USA) was used. In all sections showing the SNc and VTA were delineated and the total numbers of TH containing cells were estimated with the optical fractionator. For more details about the stereologic counting procedure see [11]. On the settings and data of our current quantifications, see the online resource.

Statistical analysis

One-way ANOVA's were performed using SPSS (version18 for Apple, SPSS, Chicago, IL, USA), and post-hoc multiple comparisons test were performed with a Tukey's Hon-

est Significant Difference (HSD) test, due to the fact that it has the best all-possible pairwise comparisons when sample sizes are unequal. A p -value < 0.05 was considered to be significant.

RESULTS

Number of TH-immunoreactive cells in the VTA and SNc

Through qualitative visual inspection, it would be difficult to discern the difference in TH-levels just by looking at the brain sections (Figure 1), therefore stereology was conducted to analyze the data quantitatively. The one-way ANOVA indicated that DBS had a significant effect on the number of TH-immunoreactive cells in the VTA ($F(3,15)=5.902$, $p < 0.05$; Figure 2). Post hoc comparisons using the Tukey's HSD indicated that the mean number of TH-immunoreactive cells was significantly higher for the ANT-stimulated group ($M=24,773$, $SEM= 942$) as compared with the MTT-stimulated group ($M=18,884$, $SEM=1,586$), the EC-stimulated group ($M=20,694$, $SEM= 840$), and the sham-stimulated group ($M=16,648$, $SEM= 2,474$) (Figure 2A). No significant difference was found between the MTT-stimulated group, the EC-stimulated group and the sham-stimulated group. In addition, TH cell measurements in the VTA and VTA volume measurements were not significantly different throughout the four conditions. Lastly, no significant differences were found for the number of TH-immunoreactive cells in the SNc (Figure 2B).

c-Fos immunoreactive cells and double-labelled c-Fos/TH cells in the VTA and the SNc

One-way ANOVAs were conducted to compare the effect of c-Fos immunoreactive cells in both the VTA and the SNc of different DBS target structures. There were no significant differences found on c-Fos immunoreactive cells in the VTA, the SNc, or double-labeled c-Fos/TH cells between groups at the $p < 0.05$ level (Figure 3).

DISCUSSION

ANT-DBS increased the number of TH-immunoreactive cells in the VTA when compared to the EC-stimulated, MTT-stimulated and sham group, while no difference was found in the SNc. The c-Fos immunoreactive cells and the double-labelled c-Fos/TH cells were not found to be significantly different between the conditions. From the c-Fos and double-labeled results, it can be concluded that DA, or any other active

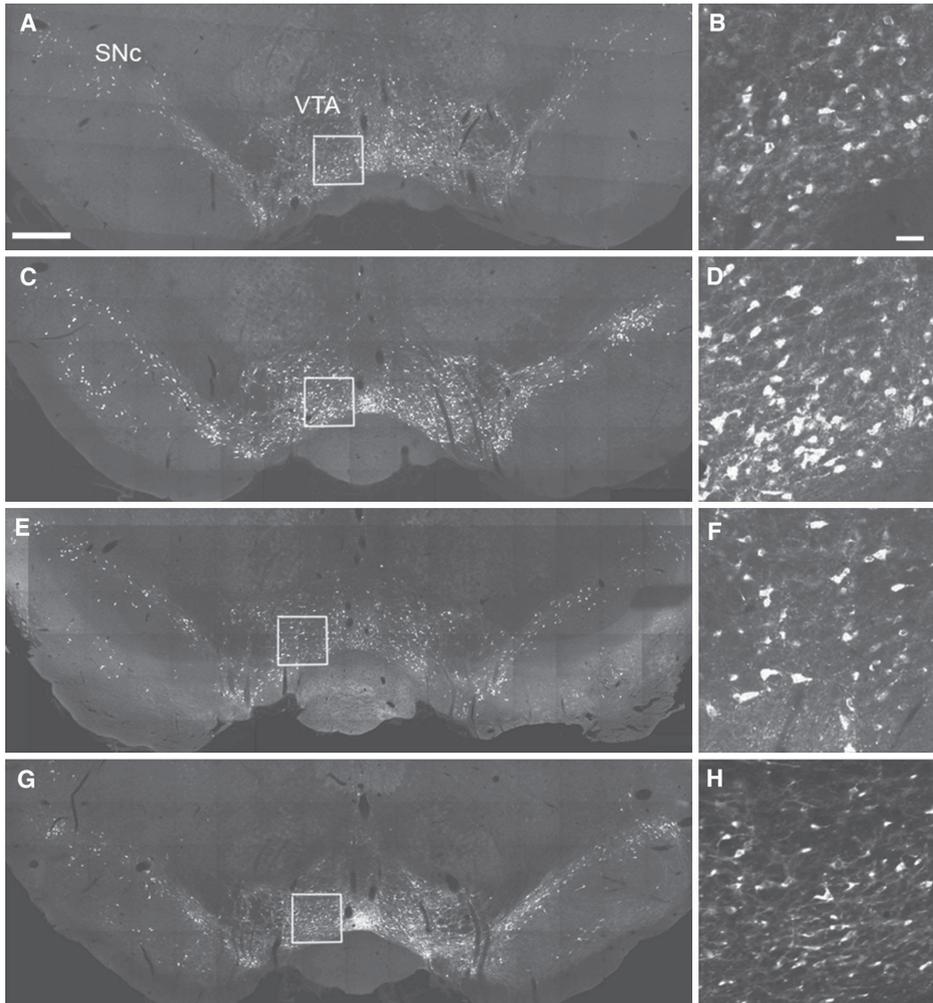


Figure 1: All pictures are around bregma level -6.00mm. A) Overview of TH immunoreactive cells in the VTA and SNc for a MTT stimulated rat. B) The VTA in a MTT stimulated rat. C) Overview for an ANT stimulated rat. D) The VTA in an ANT stimulated rat. E) Overview for an EC stimulated rat. F) The VTA in an EC stimulated rat. G) Overview for a sham rat. H) The VTA in a sham rat. Scale bars, A), C), E) and G) 500 μm ; B), D), F), H) 50 μm .

neurons in the VTA or SNc, were not involved in the activation of processes by DBS before being sacrificed.

One theory to explain the higher levels of TH is phenotypic switching, or neurotransmitter respecification. In a recent study, some populations of interneurons found in the adult rat hypothalamus switched between dopamine and somatostatin in response to exposure to short- and long-day photoperiods [12]. Furthermore, a following study found changes in TH levels in the SNc and VTA when exposed to differing manipula-

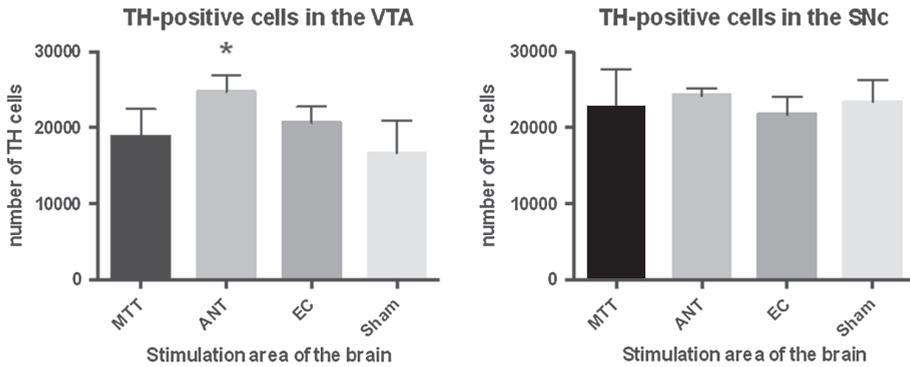


Figure 2: Number of TH-immunoreactive cells in the VTA and the SNc after DBS. A) The group that was stimulated in the Anterior Nucleus of the Thalamus (ANT, $n = 5$) was found to have significantly more TH-immunoreactive cells in the VTA than the groups stimulated in the Mammillothalamic Tract (MTT, $n = 5$), in the Entorhinal Cortex (EC, $n = 6$), and the sham stimulated group ($n = 3$). * $p < 0.05$, B) there was no significant difference found between the four groups in the SNc.

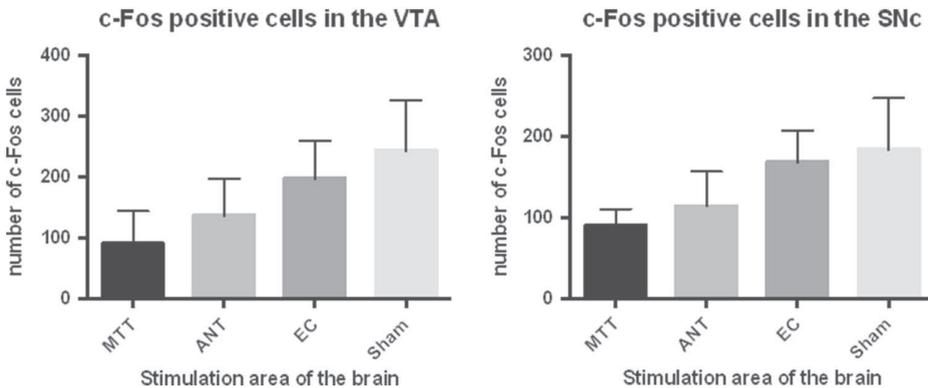


Figure 3: The number of c-fos cells in the A) VTA and B) SNc after DBS. No significant difference was found in the c-Fos positive levels between the groups stimulated in the Mammillothalamic Tract (MTT, $n = 5$), in the Anterior Nucleus of the Thalamus (ANT, $n = 5$), in the Entorhinal Cortex (EC, $n = 6$), and the sham stimulated group ($n = 3$).

tions of environmental stimuli [13, 14]. More TH-immunoreactive cells were found in environmentally enriched males, while this increase was lost after an infusion of a GABA receptor antagonist [14]. Recently, in postmortem human Huntington's disease (HD) tissue and in the transgenic rat model of HD, we found an increased number of dopaminergic neurons, which was accompanied by a decreased number of serotonergic neurons in the dorsal raphe nucleus [15], suggesting that a pathological condition such as genetic mutation can lead to neurotransmitter respecification as well. These studies

show that neurons, in the brain are not fixed, but respond to environmental stimuli and behaviors.

Dopaminergic neurons comprise 50-60% of all neurons in the VTA [16], while GABAergic neurons comprise a sizable population [17]. Presumably electrical stimulation in the ANT, and not the MTT or the EC, causes shifts in transcriptional level from one type of neurotransmitter cell (i.e. GABAergic cells) to a DA cell in an attempt to have non-invasive restoration of the normal functioning nervous system. One suggestion for a subsequent study would be to double-stain TH with GAD67, an antibody that tags GABA cell bodies to examine the neuronal compositions of the VTA between the four conditions of this study.

An alternative interpretation for the increase in TH cells in the VTA can be explained through neurogenesis. Stimulating the ANT might have modulated TH cell migration to the VTA. Due to the lack of TH/c-Fos colocalization, it can be speculated that these cells have not been functionally integrated at the time of sacrifice. DBS of specific target regions has been shown to cause neurogenesis. DBS of the EC [6] and ANT [18] for example, have shown to induce hippocampal neurogenesis in neurogenic regions, e.g. hippocampus. While brainstem neurogenesis has been reported earlier [19] later studies did not confirm those findings.

Interestingly, electrical stimulation of the ANT was found effective for treating refractory focal epilepsy in a clinical trial [20], while memory problems were reported as adverse effects [21], which may be in part due to its central connectivity [22]. In line with the finding from these studies, memory improvement was observed in rats stimulated in the EC during behavioral studies [6], while memory impairments were retained in rats stimulated in the ANT [18]. DBS effects in the MTT and memory are still unknown.

In conclusion, our findings suggest a phenotypic switch between GABA neurons and DA neurons in the VTA following ANT-DBS. The environmental stimuli of DBS in the ANT, but not DBS in the MTT or EC, may influence this increase in TH-immunoreactive cells in the VTA. As dopaminergic projections from the VTA mediate reward-seeking behavior (among other functions), our current findings may imply that ANT-stimulation with DBS, such as used in the treatment of epilepsy, may induce alterations in reward-seeking and other behavioral phenotypes.

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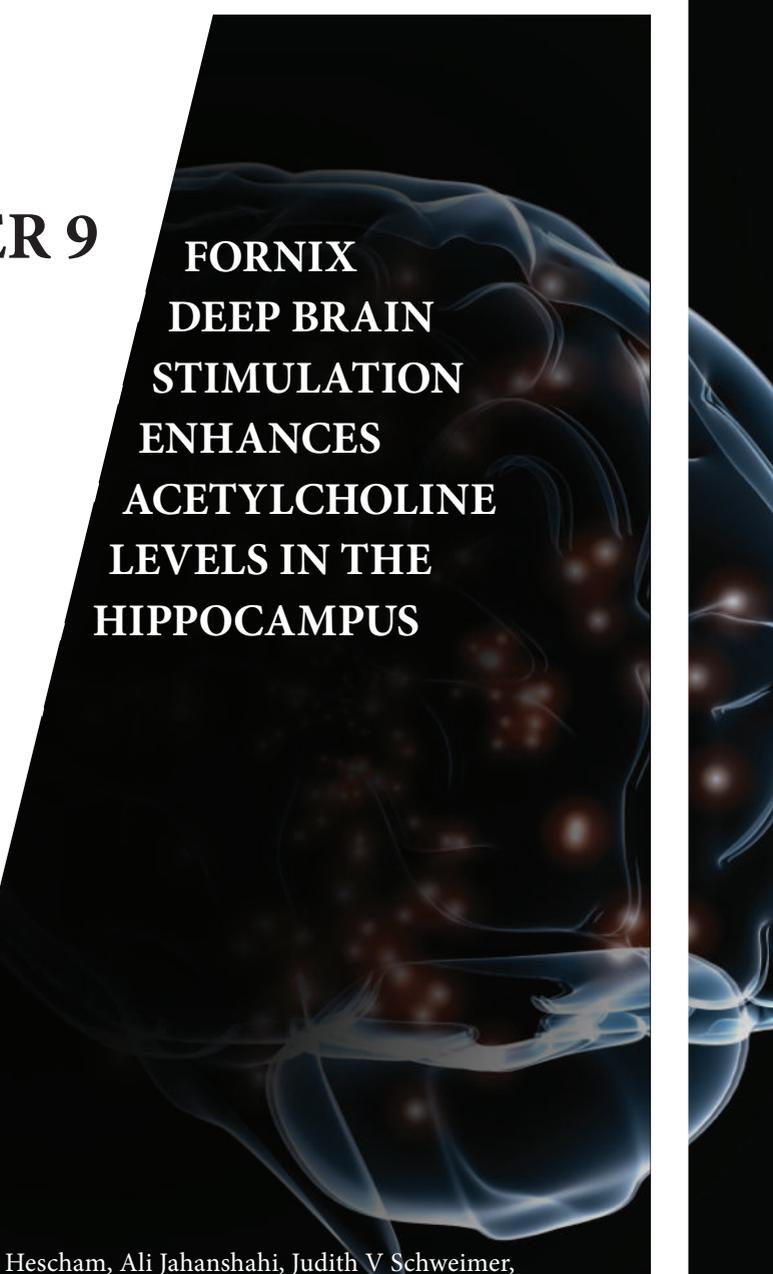
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CHAPTER 9

FORNIX DEEP BRAIN STIMULATION ENHANCES ACETYLCHOLINE LEVELS IN THE HIPPOCAMPUS

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ABSTRACT

Deep brain stimulation (DBS) of the fornix has gained interest as a potential therapy for advanced treatment-resistant dementia, yet the mechanism of action remains widely unknown. Previously we have reported beneficial memory effects of fornix DBS in a scopolamine-induced, rat model of dementia, which is dependent on various brain structures including hippocampus. To elucidate mechanisms of action of fornix DBS with regard to memory restoration, we performed c-Fos immunohistochemistry in the hippocampus. We found that fornix DBS induced a selective activation of cells in the CA1 and CA3 subfields of the dorsal hippocampus. In addition, hippocampal neurotransmitter levels were measured using microdialysis before, during and after 60 min of fornix DBS in a next experiment. We observed a substantial increase in the levels of extracellular hippocampal acetylcholine, which peaked 20 min after stimulus onset. Interestingly, hippocampal glutamate levels did not change compared to baseline. Therefore, our findings provide first experimental evidence that fornix DBS activates the hippocampus and induces the release of acetylcholine in this region.

INTRODUCTION

Dementia is a major threat to human population. The World Health Organization estimates that 35.6 million people suffer from dementia, a number that is anticipated to triple by 2050 [1]. The most common form of dementia is Alzheimer's disease (AD), accounting for 60-80% of all cases. So far there are no known treatments available that cure or delay the progression of AD. Current pharmacological treatments are not effective for every patient and only alleviate symptoms temporarily [2, 3].

Recently, the application of DBS to reduce or delay the progression of memory loss in AD has shown to be effective. In particular, the convergence of the latest observations in humans and rats pinpoints that DBS of the fornix can restore memory loss [4-6]. In our own experiments [5], fornix DBS reduced memory deficits induced by the muscarinic receptor antagonist scopolamine in a rat model. In this scopolamine-induced rat model of memory impairment various brain structures including the hippocampus are affected [7]. The mechanism of action of fornix DBS is not well understood but the latter suggests an involvement of the hippocampus and acetylcholine.

In the present study, we therefore investigated the effects of fornix DBS on the hippocampus with a dual approach. Firstly, we evaluated c-Fos expression in the dorsal hippocampus. The immediate early gene c-Fos is an indirect marker of neuronal activation and can be even used to map long-term activation [8, 9]. Secondly, we investigated the effect of fornix DBS on levels of hippocampal acetylcholine as well as glutamate; neurotransmitters which are widely acknowledged as important for memory functions [10, 11].

MATERIALS AND METHODS

Experimental design

This study consists of two experiments:

Experiment 1: Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) were either assigned to the fornix DBS ($n = 5$) or sham ($n = 5$) group. Electrode implantations were performed as described previously [5]. DBS electrodes were implanted at the site of the fornix (coordinates from bregma according to the rat brain atlas of Paxinos and Watson [12]: AP: -1.8 mm; ML: 1.3 mm; DV: -8.0 mm). It was found that fornix DBS reduced memory deficits induced by the muscarinic receptor antagonist scopolamine in a hippocampal-dependent rat model as reported elsewhere [5]. In these same animals, one week later (when scopolamine is no longer present), rats were stimulated at 100 Hz, 100 μ A and 100 μ s pulse width for 1 h and allowed to rest for 1 h.

Sham rats were only attached to cables, but not stimulated. Subsequently, animals were transcardially perfused, first with Tyrode's solution and then Somogyi fixative [13]. Animal procedures in experiment 1 were approved and carried out in accordance to the Animal Experiments and Ethics Committee of Maastricht University.

Experiment 2: In order to establish whether fornix DBS effects the neurochemistry in the hippocampus, microdialysis experiments were carried out in male Sprague–Dawley rats (Harlan, Bicester, U.K., fornix DBS $n = 13$ and sham $n = 6$) under urethane anaesthesia. Animal procedures in experiment 2 were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated Home Office guidelines, and with local ethical approval.

Immunohistochemistry

Following transcardial perfusion in experiment 1, brains were collected and cut into 30 μm slices on a vibratome (Leica®, Wetzlar, Germany). For immunohistochemistry, sections were incubated overnight with polyclonal rabbit anti-c-Fos primary antibody (1:2000; K-25, Santa Cruz Biotechnology Inc, Santa Cruz, USA) followed by biotinylated donkey anti-rabbit secondary antibody (1:800; Jackson Immunoresearch Laboratories Inc., Westgrove, USA) and avidin-biotin peroxidase complex (1:800, Elite ABC-kit, Vectastain®, Burlingame, CA, USA). The staining was visualized by 3,3'-Diaminobenzidine (DAB) combined with NiCl_2 intensification.

Images were taken at two similar bregma levels were taken using a U-CMAD-2 digital camera connected to an Olympus AX70 brightfield microscope (analySIS; Imaging System, Münster, Germany), and evaluated with ImageJ (Image J software version 1.38x; NIH, Bethesda, USA). C-Fos-positive cells in the medial prefrontal cortex and hippocampus were counted manually by an observer blind to treatment.

In-vivo microdialysis

For experiment 2, rats were anaesthetized with 25% urethane (Ethyl carbamate, Sigma) and mounted in a stereotaxic frame. DBS electrodes were implanted at the site of the fornix, and a single cannula microdialysis probe (CMA11, tip length 2 mm, CMA Microdialysis, Kista, Sweden) was implanted into the hippocampus (coordinates from bregma: AP: -4.8 mm; ML: 3 mm; DV: -4.2 mm). Microdialysis probes were perfused with artificial cerebrospinal fluid (141 mM NaCl, 5 mM KCl, 0.8 mM MgCl_2 , 1.5 mM CaCl_2) at a flow rate of 1.5 $\mu\text{l}/\text{min}$ (CMA/100, Carnegie Medicine) for 2 h before dialysate collection started. Dialysate samples were collected every 20 min, DBS stimulation time (100 Hz, 100 μA and 100 μs) was 1h. In total 10 samples were collected (4 baseline, 3 during stimulation and 3 after stimulation). Samples were immediately frozen on dry ice and later analyzed with liquid chromatography/mass spectrometry.

Liquid chromatography/mass spectrometry

All microdialysis samples were analyzed at Eli Lilly Research Centre, Windlesham, U.K. The high-performance liquid chromatography (HPLC) system consisted of a PAL HTC-xt autosampler (CTC Analytics AG Zwingen, Switzerland), a pair of Shimadzu LC-20AD XR pumps with a Shimadzu CBM-20A controller and Shimadzu CTO-20AD column oven (Shimadzu Ltd, Milton Keynes, U.K.). Injection volume was 10 μ l. The chromatographic retention was obtained by using an XBridge BEH Amide column (75 x 2.1 mm, i.d. 2.5 μ m; Waters Ltd, Elstree, U.K.) at 35°C. The gradient elution was carried out using acetonitrile and 2 mM ammonium formate, 95:5 and 5:95 (the pH of 3.0 was adjusted with formic acid). Detection was carried out by an AB Sciex Triple Quad API5500 mass spectrometer MS (AB Sciex UK Ltd, Warrington, U.K.), which operated in TurboIonSpray® mode using a Turbo V™ source and SRM analysis.

Acetylcholine was detected by monitoring the m/z 146.1 \rightarrow 87.0 transition and its D4 analogue internal standard at m/z 150.1 \rightarrow 91.0 (dwell time: 50 ms, collision energy: 20 V, collision cell exit potential: 4.2 V). Glutamic acid was detected by monitoring the m/z 148.0 \rightarrow 84.0 transition and its D5 analogue internal standard at m/z 153.0 \rightarrow 88.0 (dwell time: 50 ms, collision energy: 23 V, collision cell exit potential: 10 V). Samples were prepared by 1:10 dilution in internal standard and a single acetylcholine calibration curve between 0.10 and 20.0 nM and glutamic acid curve between 50 and 2000 nM was run at the end of each batch of samples for quantification.

Verification of DBS electrodes and microdialysis probes

Sections containing the electrode trajectories from all animals were collected and mounted on gelatin coated glass slides. A standard haematoxylin-eosin staining was carried out before sections were photographed under bright field microscopy.

Statistical analysis

For experiment 1, the number of Fos-positive cells was expressed as percentage of change when compared to sham (i.e. the average of Fos-positive cells in sham was chosen as reference value and defined as 100%). Raw values as well as percentage scores were normally distributed as assessed by the Shapiro Wilk's test. For better comparison between the groups, however, we chose to present the percentage scores.

An independent-samples t-test was employed and p-values < 0.05 were considered significant. Microdialysis data of experiment 2 were represented as percentage of the mean of 4 baseline samples prior to DBS. The effect of fornix DBS on extracellular acetylcholine and glutamate levels over time was analyzed by repeated-measures ANOVA followed by Fisher's least significant difference (LSD) post-hoc test.

RESULTS

Verification of DBS electrodes and microdialysis probes

The location of all DBS electrodes was verified within the fornix, and microdialysis probes were all correctly placed in the dorsal hippocampus. For the current stimulation settings used, we found no evidence for histological damage as observed by a standard haematoxylin-eosin staining.

Immunohistochemistry

We found increased *c-Fos* expression in the CA1 subregion for fornix stimulated animals when compared to sham ($t(8) = 4.285$; $p < 0.01$). In addition, increased levels of *c-Fos* cells in the CA3 subregion were observed in fornix DBS rats ($t(8) = 4.363$; $p < 0.01$). There was no statistical difference between levels of *c-Fos* expression in the dentate gyrus of fornix stimulated and sham rats ($t(7) = 0.455$; n.s.; Fig. 1).

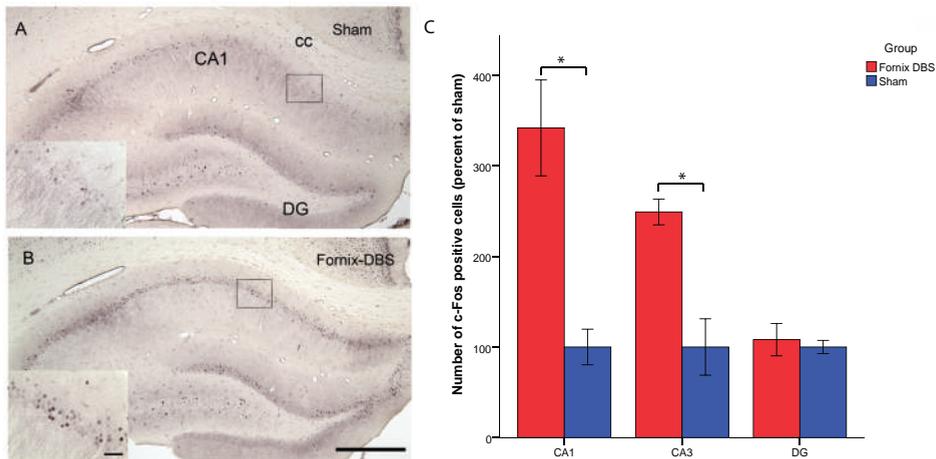


Figure 1: Representative low-power photomicrographs (scale bar = 500 μm) of coronal brain sections stained for *c-Fos* (K-25) showing the hippocampus of sham (A) and fornix stimulated animals (B). The high-power photomicrograph insets in the lower left corner show the CA1 subregion of the hippocampus (scale bar = 50 μm). C) Comparisons were made as percentage increase/decrease with regard to sham. Fornix DBS rats show increased *c-Fos* (K-25) expression in the CA1 and CA3 subregion of the hippocampus when compared to sham. There was no statistical difference between sham and DBS groups in the dentate gyrus. * $p < 0.05$, independent samples t-test for fornix DBS vs. sham rats. Data represents mean \pm S.E.M. DG, dentate gyrus; cc, corpus callosum.

In-vivo microdialysis

Fornix DBS for 60 min caused a significant increase in hippocampal acetylcholine levels in comparison to non-stimulated controls (repeated measures ANOVA: $F(1,17) = 6,608$; $p < 0.03$). This effect was evident in the first 20 min of fornix DBS as shown by the LSD post-hoc test ($p < 0.03$; Fig. 2A). Acetylcholine levels in other time points during stimulation as well as after stimulation did not differ between the groups. There was no statistical difference for hippocampal glutamate levels between fornix DBS and sham (repeated measures ANOVA: $F(1,16) = 0.038$; n.s.; Fig. 2B).

DISCUSSION

According to several studies, fornix DBS in AD patients leads to a decreased rate of cognitive decline [6, 14]; yet, a potential mechanism of action has not been found. In the present study, we found that beneficial memory effects of fornix DBS might be linked to an enhanced neural activity in the CA1 and CA3 subfield, suggesting increased activity of certain neurons in this region. The CA1 and CA3 subregions of the hippocampus are important for spatial memory [15, 16]. In particular, the CA3 subregion plays a role in encoding and retrieval of spatial location sequences and the CA1 contributes to memory encoding by binding cues to their temporal context, which in turn also enables retrieval of location sequences [15]. Interestingly, a substantial neuronal loss in the CA1 is observed in postmortem AD brains [17]. If fornix DBS is able to increase neural activity in the CA1 subfield, then it may compensate for reduced neuronal integrity of this region in AD.

In the etiology of AD, evidence exists for both cholinergic and glutamatergic involvement [18]. In the present study, microdialysis data revealed that hippocampal glutamate levels were not affected by fornix DBS. A clear connection between glutamate and memory-related processes is provided by the association of ionotropic glutamate receptors in the induction of long-term potentiation. Although memory can be disrupted by blockade of these receptors (e.g. N-methyl-D-aspartate, NMDA) [19], the enhancement of the glutamatergic signal may also have adverse effects on memory because high levels of glutamate are neurotoxic [20]. Our results suggest that fornix DBS does not influence glutamate-dependent long-term potentiation, but also does not induce excitotoxicity.

Contrary to this, fornix DBS did have an effect on extracellular hippocampal acetylcholine and it might be possible that increased c-Fos expression in the hippocampus was mediated by enhanced acetylcholine levels [21]. In an earlier study, we have shown that fornix DBS was able to restore scopolamine-induced memory deficits when stimulating

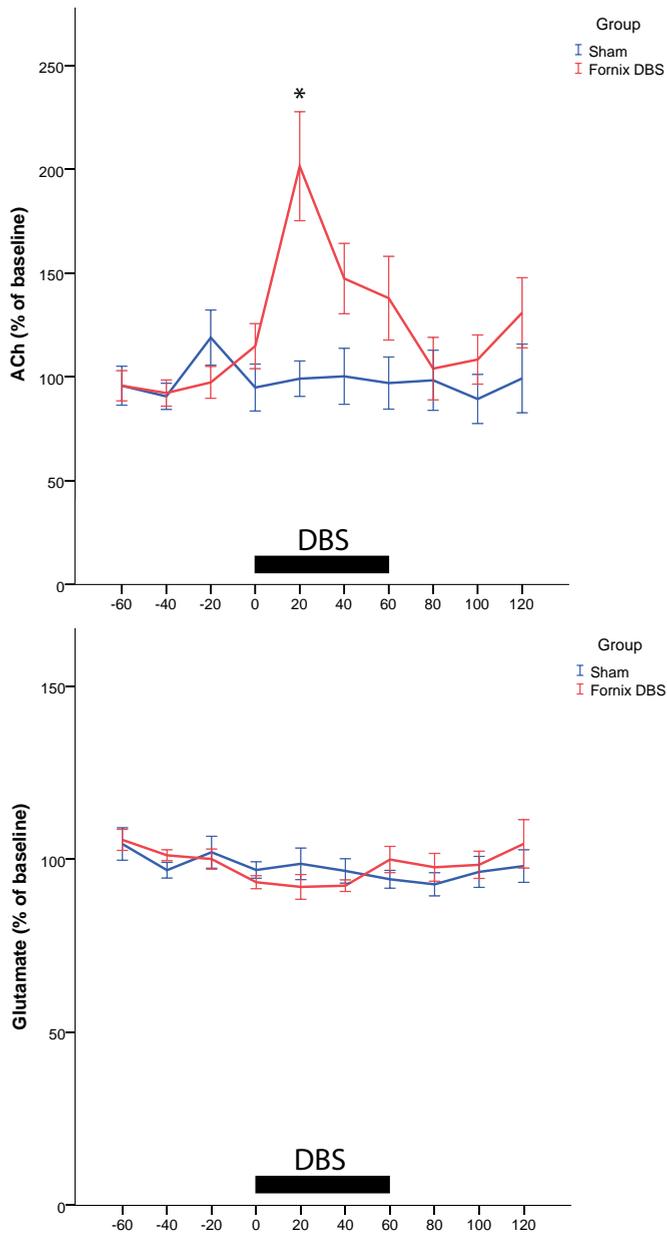


Figure 2: Microdialysate acetylcholine (ACh, A) and glutamate (B) levels of the dorsal hippocampus in anesthetized fornix DBS (n=13) and sham rats (n=6). Horizontal bar indicates stimulation period. Acetylcholine levels were significantly elevated in the fornix DBS group after 20 min of stimulation. No difference in glutamate levels was detected between fornix DBS and sham. Data points are mean \pm S.E.M. values expressed as a % of baseline. * $p < 0.05$, repeated measures ANOVA for fornix DBS vs. sham rats.

the fornix for 5 min during the acquisition and retention trial of the object location task, a test of hippocampal-dependent memory [5, 22]. Potentially, the increased hippocampal acetylcholine measured via microdialysis after 20 min, facilitated the enhanced memory performance. However, these results should be interpreted with caution, since we did not include any memory tests in the present study. The effect of DBS (20 min) on acetylcholine release seemed to decline with continued stimulation. This transient effect can be explained by exhaustion of the acetylcholine pools that may take place after an enhanced release for more than 20 min. Thus, chronic DBS might eventually be associated with acetylcholine depletion. In this case, either intermittent or closed-loop stimulation [23] when applying chronic DBS of the fornix might be necessary to maintain beneficial effects on memory functions.

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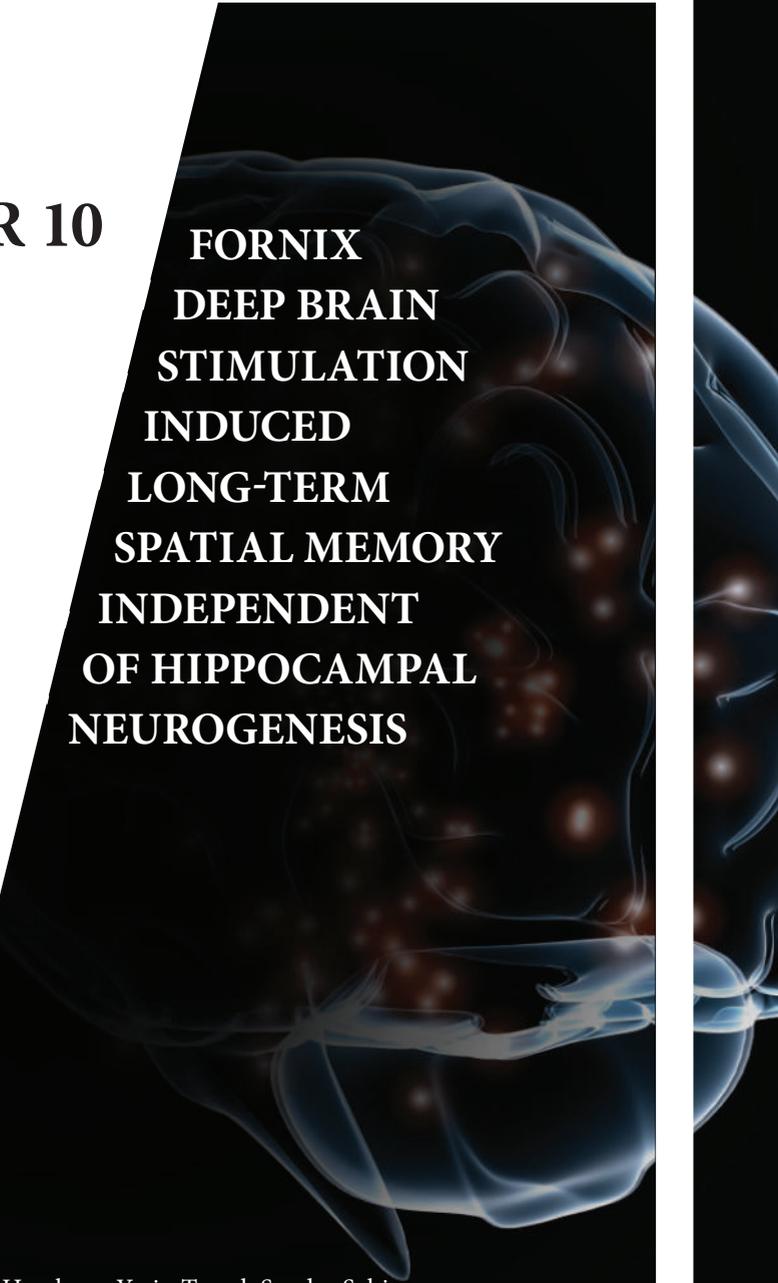
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CHAPTER 10

FORNIX DEEP BRAIN STIMULATION INDUCED LONG-TERM SPATIAL MEMORY INDEPENDENT OF HIPPOCAMPAL NEUROGENESIS

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Submitted



ABSTRACT

Deep brain stimulation (DBS) is an established symptomatic treatment modality for movement disorders and constitutes an emerging therapeutic approach for the treatment of memory impairment. In line with this, fornix DBS has shown to ameliorate cognitive decline associated with dementia. Nonetheless, mechanisms mediating clinical effects in demented patients or patients with other neurological disorders are largely unknown. There is evidence that DBS is able to modulate neurophysiological activity in targeted brain regions. We therefore hypothesized that DBS might be able to influence cognitive function via activity-dependent regulation of hippocampal neurogenesis. Using stimulation parameters, which were validated to restore memory loss in a previous behavioural study, we here assessed long-term effects of fornix DBS. In order to do so, we injected the thymidine analogue, bromodeoxyuridine (BrdU) after DBS and perfused animals 6.5 weeks later. A week prior to perfusion, memory performance was assessed in the water maze. We found that acute stimulation of the fornix improved spatial memory performance in the water maze when the probe trial was performed 1 hour after the last training session. However, no evidence for stimulation-induced neurogenesis was found in fornix DBS rats when compared to sham. Our results suggest, that fornix DBS improves memory function, independent of hippocampal neurogenesis, possibly through other mechanisms such as synaptic plasticity and acute neurotransmitter release.

INTRODUCTION

Deep brain stimulation (DBS) by permanently implanted electrodes in the brain is a popular treatment. Up to now, DBS has provided treatment for more than 130,000 patients, suffering from Parkinson's disease, essential tremor, and dystonia worldwide [1-3]. Despite its widespread use, the precise mechanism of action of DBS therapy remains unknown. Clinical applications of DBS in neuropsychiatric diseases including memory impairment are in the early stages or under investigation [4, 5]. The fornix has gained growing attention as potential DBS target to alleviate memory impairments [6-8]. In a phase 1 clinical trial, the effects of fornix stimulation in Alzheimer's disease (AD) patients were assessed [8]. Although some patients showed improvement and slowing in the rate of cognitive decline at 6 and 12 months, others did not respond to the therapy. It was reported recently, that in 2 AD patients with best clinical response of this study, bilateral fornix DBS of 130 Hz, 3-3.5 V and 90 μ s pulse width increased hippocampal volume [9]. This hippocampal volume change strongly correlated with hippocampal metabolism and a volume change in the fornix and mammillary bodies, suggesting a circuit-wide effect of stimulation.

Besides inducing morphological changes, fornix DBS might also have an effect on adult neurogenesis. Throughout life, new neurons are continuously generated in the granule cell layer of the dentate gyrus and in the subventricular zone. Most of these adult-generated dentate granule cell neurons are thought to contribute to the formation of hippocampus-dependent memory [10, 11]. A few studies in rodents have already provided evidence that DBS of the anterior thalamic nucleus and entorhinal cortex, promotes hippocampal neurogenesis by increasing the cell proliferation and survival of newly generated neurons in the dentate gyrus [12-14]. In these studies, rats or mice were first treated with electrical stimulation in the target region, then injected with the cell-proliferation marker bromodeoxyuridine (BrdU) and sacrificed after a few weeks. Increased neurogenesis could furthermore be linked to improved spatial memory in the water maze [13].

In the present study, we also investigated whether fornix DBS induces long-term neurogenic changes in the dentate gyrus, by injecting BrdU after DBS. Subsequent to the completion of a hippocampus-dependent memory task, we analyzed the hippocampi 6.5 weeks after fornix DBS and labeled them with antibodies to BrdU and the neuronal marker, NeuN.

MATERIALS AND METHODS

Subjects

Sprague Dawley (SD) rats from Charles River (Sulzfeld, Germany) were used, their weight ranging between 280-300 g at time of surgery. The temperature of the colony room was maintained at a temperature of 21 ± 1 °C. Rats were housed 2 or 3 per cage with rat chow and water available ad libitum in a reversed 12:12h light-dark cycle. For the time of experimental procedures (stimulation and BrdU injections) rats were housed individually, but were re-grouped thereafter. All experimental animal procedures were executed during the dark phase.

Experimental groups

Rats were randomly assigned to one of the following experimental groups: sham (n = 7) or fornix DBS (n = 10). We included more rats in the fornix DBS group in order to account for factors such as loss of electrodes or incorrect electrode placements.

Surgical procedure

The exact surgical procedure has been described elsewhere [15]. In short, DBS electrodes were implanted in the fornix using a rodent stereotactic apparatus (Stoelting, Wood Dale, IL, USA, model 51653). The rat skull was exposed under Isoflurane anaesthesia (IsoFlo®, Abbott Laboratories Ltd, Berkshire, Great Britain) and two burr holes for bilateral DBS electrodes were made at the level of the fornix (coordinates from bregma according to the rat brain atlas of Paxinos and Watson [16]: AP: -1.8 mm; ML: 1.3 mm; DV: -8.0 mm). The construct was anchored with dental cement (Paladur, Heraeus Kulzer GmbH, Hanau, Germany). Sham rats underwent the same electrode implantations, but were not stimulated.

Deep brain stimulation

DBS was applied for 4 h at 100 Hz, 100 μ A and 100 μ s pulse width using a World Precision Instrument digital stimulator (DS8000, WPI, Berlin, Germany) and two stimulus isolators (DLS100, WPI, Berlin, Germany). Sham animals were only attached to cables and not stimulated.

BrdU labeling

BrdU was used to identify cells that were born after DBS. BrdU (Sigma-Aldrich) was dissolved in 0.9% NaCl (pH 7.6) to 8 mg/ml. Three days after DBS, all rats were injected intraperitoneally twice daily (8 h apart) with 50 mg/kg BrdU for 3 consecutive days. The interval between DBS and onset of BrdU injection was chosen based on a previous

study, in which proliferative activity evaluated by BrdU in the dentate gyrus reached a plateau 3-5 days after DBS [13].

Water maze

The water maze consists of a circular black polyethylene tank (diameter 153 cm) with 63 cm high walls. The pool was filled with 40 cm of water, which was maintained at 22 ± 1 °C and made opaque by adding black, nontoxic paint. The black escape platform (diameter 11 cm), was submerged 1.5 cm below the surface of the water. A video camera was mounted in the center above the pool and registered movements of the rat (Ethovision Pro, Noldus, The Netherlands).

In general, the animals received 4 trials during 5 acquisition sessions, which were given on 5 consecutive days (total of 20 swim trials). Different start locations were used and tracing began when the animal was released into the pool facing the wall of the tank. Rats were given 60 s to reach the platform and if the rat failed to find the platform within 60 s, the experimenter guided the rat to the target. The time between subsequent trials was about 10 min. A probe trial was given 1 h and 48 h after the last trial on the 5th acquisition session.

In the reversal learning paradigm the platform was moved to a novel location and the rats were again trained for 2 consecutive days with 4 trials/day. Rats were subjected to the reversal probe trial, 1 h after the last training session.

Tissue collection

At the end of the experiments, the rats were overdosed with pentobarbital (Apotheek Faculteit Diergeneeskunde, Utrecht, The Netherlands). Transcardial perfusions with Tyrode and then Somogyi fixative solution (4% paraformaldehyde, picric acid, PBS, glutaraldehyde) were carried out. Brains were removed and placed in fresh fixative (identical composition as Somogyi solution, but lacking glutaraldehyde) at 4°C for 2 hours. Subsequently brains were transferred to 1% NaN₃ at 4°C for long-term storage.

For vibratome sectioning (Leica®, Wetzlar, Germany), brains were embedded in 10% gelatin from porcine skin (Sigma-Aldrich, Zwijndrecht, The Netherlands), and then cut into 30 µm slices in the frontal plane. Slices were immediately transferred into 1% NaN₃ and kept at 4°C.

Immunohistochemistry

A double-immunofluorescent BrdU/NeuN staining was performed for sections containing the hippocampus in 7 fornix DBS and 7 sham animals. For BrdU detection, DNA denaturation was conducted by incubating for 2 h in 50 % formamide at 65 °C, followed by washing and 30 min in 2N HCL at 37 °C. After blocking, sections were incubated with mouse monoclonal anti-BrdU (1:100; Sigma-Aldrich, Zwijndrecht,

Netherlands) overnight at 4 °C. Subsequently, donkey-anti-mouse secondary antibody (1:100; Alexa 488, Invitrogen, Carlsbad, CA) was applied. Incubation with biotinylated NeuN (1:100; Chemicon, Temecula, CA) was carried out for 3 days at 4 °C, followed by streptavidine 594 (1:1000 Invitrogen, Carlsbad, CA). Lastly, brain sections were mounted and coverslipped with 80% glycerol.

The number of BrdU/NeuN double-labeled cells was counted using the stereological procedure, optical fractionator. Counts were done using a confocal microscope (DSU, Olympus® BX51W1), a motorized stage, and the StereoInvestigator software (MicroBrightField, Williston, VT). The granule cell layer of the dentate gyrus was defined as the region of interest. All double-labelled BrdU/NeuN cells in an average of 6 sections, 300 µm apart were counted with a 60X objective. The chosen brain sections extended from bregma -3.1 mm to bregma -4.9 mm. The total number of positive cells was estimated as a function of the number of cells counted and the sampling probability [17].

Verification of electrode placements

Sections containing electrode trajectories from all animals were mounted on gelatin coated glass slides. A standard haematoxylin-eosin staining was employed in order to inspect the sections under bright field microscopy.

Statistical analyses

Statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA). Acquisition data of water maze trials were analyzed by repeated measures ANOVA. Probe trials and immunohistochemical findings were analyzed using an independent samples t-test. All p-values less than 0.05 were considered statistically significant.

RESULTS

Verification of electrode placements

DBS electrodes were all correctly located in the vicinity of the fornix. The electrode trajectory in one animal of the stimulated group was directed ventral of the fornix, but since the rat showed similar behaviour to other stimulated animals, we expected that the fornix was within the electric field and included the rat in the analysis.

Water maze

DBS rats received fornix stimulation and were trained in the water maze 8 days before perfusion took place. Sham rats were treated identically, except that no current was delivered. During the acquisition phase, latency to find the platform declined similarly in both groups ($F(1,66) = 0.658$; n.s., Fig. 1). One hour after completion of the train-

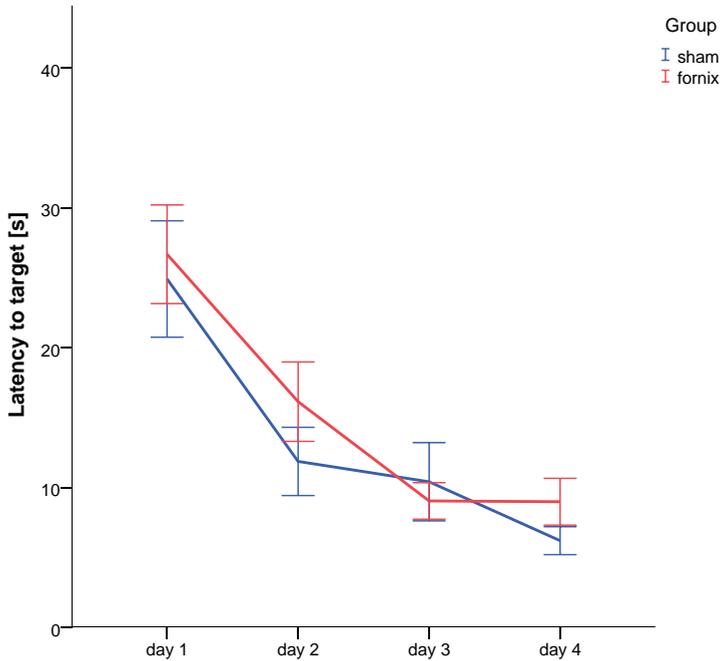


Figure 1: Acquisition trials of fornix DBS and sham animals. No difference in latency to find the platform was found between the groups. Data represents mean \pm S.E.M.

ing, spatial memory was assessed in a probe trial. In this test, both DBS and sham rats spent similar time in the target zone ($t(15) = 1.241$; n.s., Fig. 2A). However, only fornix DBS rats crossed the former platform location more frequently when compared to sham ($t(15) = 2.209$; $p < 0.05$, Fig. 2B), indicating that fornix stimulation facilitated short-term spatial memory. At the 48 h probe test, no difference between time spent and number of crossings to the target location was found. In the reversal training both groups performed equally well and there was also no statistical difference between the groups in the reversal probe trial which took place 1 h after the last reversal training session (all t 's ≤ 0.462 ; n.s.).

BrdU/NeuN cell counts

There were similar numbers of BrdU/NeuN double-labeled cells in both fornix DBS and sham groups ($t(12) = 0.522$, n.s.), and no additional differences between the anterior, medial and posterior portions of the dentate gyrus (all t 's ≤ 0.985 ; n.s., Fig. 3).

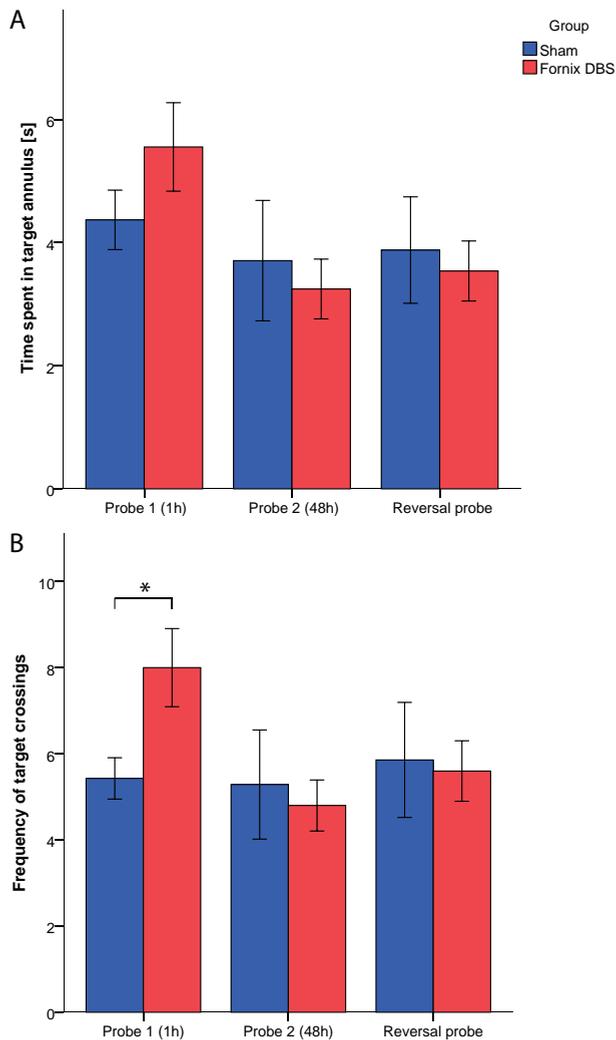


Figure 2: Effects of fornix DBS during the different probe trials (after 1 h or 48 h delay and in the probe trial 1 h after the reversal training) when compared to sham. There was no difference between the groups in time spent in the target annulus (A). Fornix DBS rats, however, crossed the target annulus more often than sham animals (B). * indicates $p < 0.05$. Data represents mean \pm S.E.M.

DISCUSSION

Adult hippocampal neurogenesis has long been implicated as important for learning and memory [11]. Consequently, the role of neurogenesis in the adult brain might open up important opportunities for the development of therapeutic interventions, which could moderate or delay disorders with memory decline. Studies in rodents have

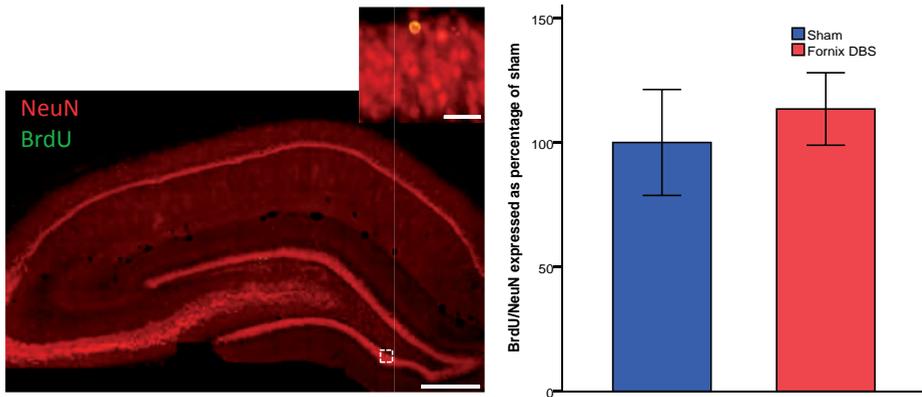


Figure 3: Representative picture (scale bar = 500 µm) of a hippocampal section stained for NeuN (red) and BrdU (green) with a high-power inset on the top right showing a double-labeled cell (scale bar = 50 µm). Graph represents number of double-labeled BrdU/NeuN cells in the dentate gyrus expressed as percentage of sham \pm S.E.M. There was no significant difference between the groups.

demonstrated that DBS of the anterior thalamic nucleus or entorhinal cortex increases cell proliferation in the dentate gyrus [13, 14]. These targets, however, have not yet been explored as potential treatment for dementia. DBS of the fornix, on the other hand, has already been reported in a case study [7] and phase I clinical trial [8], to slow down progression of memory loss in individuals with early Alzheimer's disease. The exact mechanisms of action are yet to be defined. Previously, we have also found superior memory performance of fornix DBS rats in an experimental model of dementia [18]. We were able to link restoration of memory loss to an increased neuronal activation in the CA1 and CA3 subfield of the dorsal hippocampus, as well as increased hippocampal acetylcholine levels (chapter 9). The hippocampus receives abundant cholinergic innervation from the basal forebrain and it has been shown that selective neurotoxic lesion of the basal forebrain reduces neurogenesis in the dentate gyrus and thus impairs spatial memory [19]. In the same study systemic administration of the cholinergic agonist physostigmine was able to increase neurogenesis in the dentate gyrus. In contrast to these studies, we did not find any evidence for enhanced neurogenesis following fornix DBS. Although no increased neurogenesis was observed, the stimulated rats visited the site of platform location more frequently than sham animals in the 1 h probe trial delay.

It has been shown that hippocampal acetylcholine primarily influences proliferation and/or short-term survival rather than long-term survival or differentiation of new neurons [19]. It might therefore be possible, that our experimental paradigm, which allowed new-born cells to mature for 6 weeks, was too long to depict differences between fornix DBS and sham animals. Indeed, in an unpublished data set, we stained brains of fornix DBS and sham animals from a previous behavioral study [18] for doublecortin

and found clear evidence for enhanced proliferative activity in the dentate gyrus of stimulated rats. Nonetheless, short-term neurogenic differences might not explain the enhanced memory performance after 6 weeks of stimulation. We therefore speculate that fornix DBS might have had an effect on other neuroplastic mechanisms, for instance synaptic potentiation. It has been suggested before, that changes in proliferative activity is not necessarily a key factor determining the efficacy of synaptic potentiation [20]. In line with this, it was shown that synaptic potentiation was restored within 6 weeks in a transgenic mouse model of suppressed neurogenesis [21].

In conclusion, our results suggest that acute fornix DBS improves long-term spatial memory independently from DBS-induced neurogenesis, but possibly by increasing the connectivity among existing neurons. Further studies are indicated, which investigate the effects of fornix DBS on long-term potentiation and long-term depression.

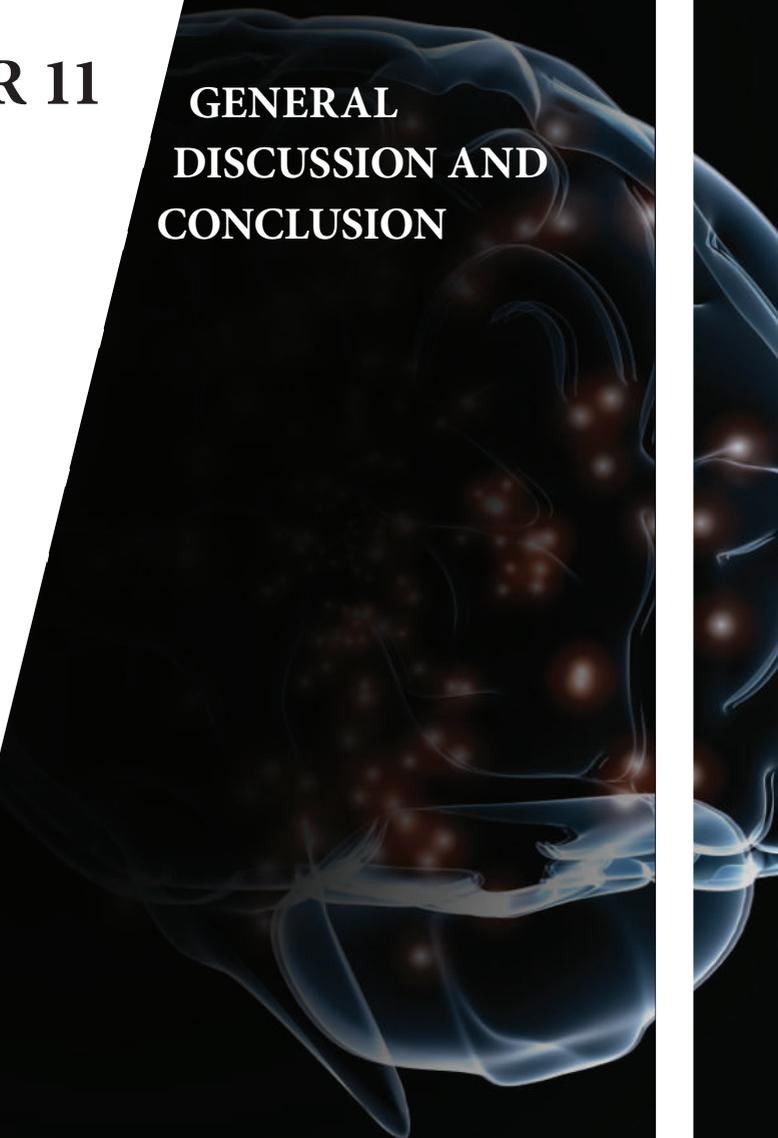
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CHAPTER 11

GENERAL DISCUSSION AND CONCLUSION



GENERAL DISCUSSION

Cognitive disorders are a major societal burden. In an ever-growing and aging population, these conditions demand urgent attention, in order to alleviate healthcare resources as well as provide a good quality of life for patients and caregivers. Although memory impairments are relatively common conditions in the elderly, few treatments are available to arrest or slow down the progression of memory loss. By targeting specific structures within known circuits, deep brain stimulation (DBS) can have beneficial effects across memory and cognitive networks, and is therefore a potentially promising avenue for non-drug based dementia treatments.

The application of DBS in psychiatry has emerged in the past 50 years [1-3]. To understand state-of-the-art applications of DBS in psychiatric conditions which potentially include memory impairments, I have reviewed relevant clinical studies on neuromodulation (Chapter 2). Non-invasive neuromodulation therapies, such as transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS), can produce short-term beneficial effects on refractory psychiatric symptoms [4, 5]. However, the effects on long-term treatment modalities are unknown. A main disadvantage seems to be that transcranial stimulation mainly targets cortical structures and does not reach deeply situated brain regions. Deep brain stimulation, on the contrary, enables focused stimulation of specific brain targets. At present, an increasing number of studies investigate the safety and efficacy of DBS, showing significant therapeutic effects in patients with different refractory psychiatric disorders [6].

With regard to memory-related disorders, DBS of the fornix and nucleus basalis of Meynert have been investigated as potential treatments for dementia in clinical trials (Chapter 3). The studies that were carried out, however, were mostly safety studies, with small sample sizes and no control group [7, 8]. As a result, this encouraging therapy has not been approved in the clinics, yet. In order to conduct standardized clinical trials, more preclinical research is warranted. Preclinical animal models are crucial to examine relevant brain targets and can accelerate the development of clinical applications of DBS. In addition, animal models are essential to increase our understanding of the underlying neural substrates of memory enhancement after DBS [3].

To further investigate the behavioral effects and mechanisms of DBS, we used a scopolamine-induced rat model of dementia. Scopolamine, a non-selective acetylcholine antagonist, has been used as a standard drug to induce memory impairments associated with aging and dementia in animals and humans [9]. Chronic scopolamine administration indeed has a widespread effect on brain depressant activity, which corroborates the variety of behavioral effects of this drug (Chapter 4). The dose I chose for the subsequent behavioral studies has been validated to impair recognition and spatial memory [9].

Thus, I have used the scopolamine-induced rat model of memory impairment and applied DBS to one of the potential targets in dementia-treatment, namely the fornix (Chapter 5). The fornices are white matter bundles, which reciprocally connect the bilateral hippocampi to the septal nuclei, nucleus accumbens (precommissural fornix) and hypothalamus (postcommissural fornix) [10]. In this study, I found that DBS of the fornix was able to restore memory loss in a spatial memory task when specific set of stimulation parameters was used. In particular, DBS of the fornix was not frequency dependent, but rather dependent on high current densities. With the investigated stimulation parameters, I found no evidence on anxiety-related side effects [11].

I have also investigated the efficacy of DBS in an aged animal model (Chapter 6). In particular, I aimed to investigate DBS of the input and output of the hippocampus and have stimulated the fornix and entorhinal cortex, respectively. In 24-months-old rats, I found that DBS of the fornix improved short-term memory in a spatial memory task. Entorhinal cortex DBS had beneficial effects on long-term recognition memory. Again no evidence for anxiety-related side effects were found. Taken together, these results support the notion that the fornix and entorhinal cortex are involved in different memory domains. This is in line with lesion studies, showing the involvement of the fornix in spatial [12-14] and the entorhinal cortex in recognition memory [15, 16], respectively.

Next, I assessed whether DBS can have beneficial memory effects when applied to other structures within the circuit of Papez (Chapter 7). Therefore, I implanted electrodes in the CA1 subregion of the hippocampus, the mammillothalamic tract, anterior thalamic nucleus and entorhinal cortex and again made use of the scopolamine-induced rat model of dementia. DBS of the CA1 and entorhinal cortex was able to restore memory loss when specific set of stimulation parameters were used. The conclusions, however, were twofold. Firstly, I have shown that within the circuit of Papez, only DBS of the CA1 subregion, entorhinal cortex and fornix is able to restore spatial memory loss. Nonetheless, secondly, from a stereotactic point of view, I concluded that a strategic target should be chosen for therapy. The hippocampus and entorhinal cortex are relatively large brain regions in rodents and primates [17]. Therefore, neuromodulation through a single electrode might be challenging, given the risk of not targeting the relevant region in a patient. Contrary to this, the fornix entails localized fiber bundles and its size is accessible in both, rodents and primates. Thus, within the circuit of Papez, the fornix might consequently represent a preferred target for the treatment of dementia-related disorders with DBS.

In the last 3 chapters of this thesis, I focused on mechanisms of action of DBS. In Chapter 8, I investigated brains of rats, which underwent DBS in the entorhinal cortex, mammillothalamic tract and anterior thalamic nucleus. When looking at dopamine, a neurotransmitter widely acknowledged as important for memory functions, I have not

found any difference between sham and entorhinal cortex DBS animals in the ventral tegmental area. Only DBS of the anterior thalamic nucleus, induced substantial higher numbers of TH-immunoreactive cells in the ventral tegmental area, while there was no difference in the number activated cells (measured by c-Fos). My findings suggest that DBS can induce a phenotypic switch, or GABA - dopamine neurotransmitter re-specification, in the ventral tegmental area [18].

With regard to fornix DBS, mechanisms of action were evaluated by immunohistochemistry and microdialysis experiments (Chapter 9). First, I performed c-Fos immunohistochemistry in the dorsal hippocampus and found that beneficial memory performance was accompanied with a selective activation of cells in the CA1 and CA3 subfields. In order to elucidate what types of neurons were activated in the hippocampus, additional rats were implanted with fornix DBS electrodes and hippocampal microdialysis probes. During stimulation, I observed a substantial increase in the levels of extracellular hippocampal acetylcholine in fornix DBS rats when compared to sham. Specifically, acetylcholine levels increased in the first 20 min of stimulation. No effects on hippocampal glutamate levels were found during or after DBS. Therefore, my findings provide first experimental evidence that beneficial memory effects following fornix DBS can be attributed to cholinergic modulation of the hippocampus.

Lastly, I assessed whether acute DBS of the fornix can produce long-term changes in memory and whether they can be linked to adult hippocampal neurogenesis (Chapter 10). Although, fornix stimulated rats showed superior memory performance 6 weeks after DBS when compared to sham, I did not find evidence for increased hippocampal neurogenesis. My findings suggest, that besides yielding immediate effects on memory (e.g., vivid autobiographical recall seen during initiation of stimulation in some clinical studies [8, 19] and improved memory performance in rodents [11]), fornix DBS might also induce long-term memory effects. These long-term effects, however, cannot be explained by neurogenesis and further studies are indicated, which investigate changes in other neuroplastic domains.

CONCLUSION

Clinical studies have shown that targeting key structures in memory and cognitive circuits for DBS therapy is safe and can activate these circuits. In particular, case studies or phase I trials of patients with Alzheimer's disease or other dementias suggest that DBS may be associated with stabilization or improvement in memory. In the present thesis, I have found the fornix to be the most optimal target structure within the circuit of Papez to improve short-term spatial memory. DBS of the fornix induces neuronal activation in the CA1 and CA3 subfields of the hippocampus and also enhances hippocampal

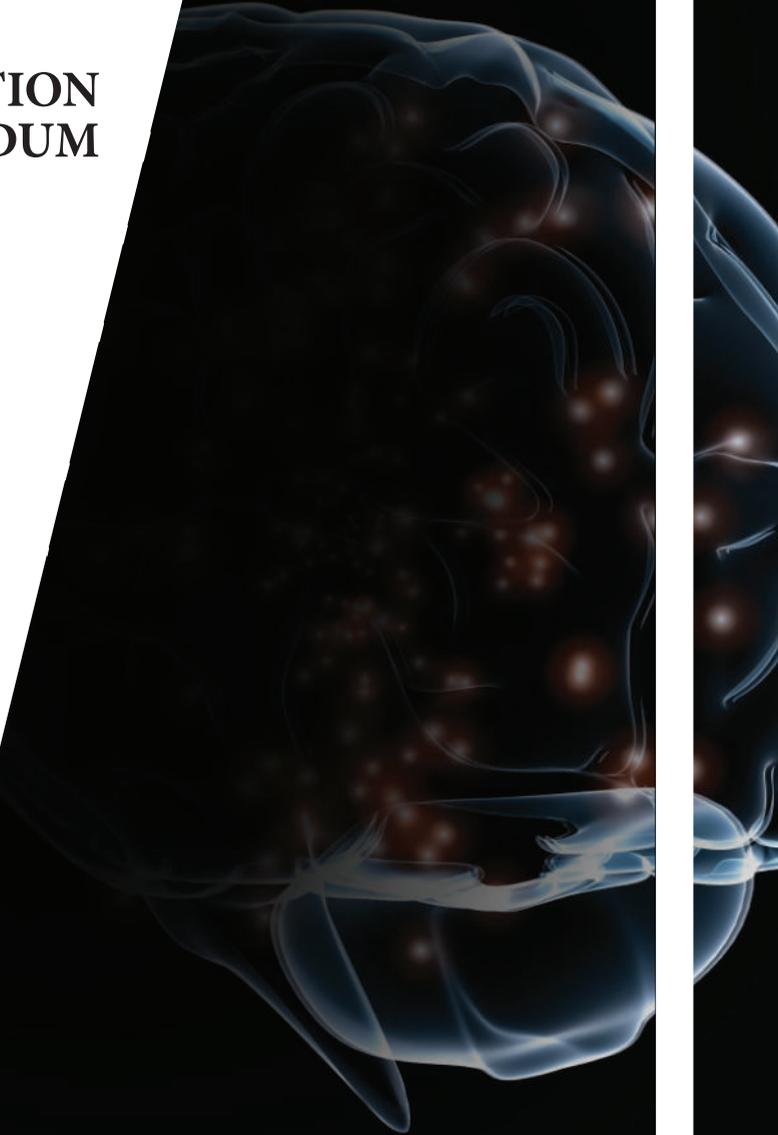
acetylcholine levels instantaneously. Moreover, acute fornix DBS produced long-term memory effects. Nonetheless, no evidence for stimulation-induced survival of newborn hippocampal neurons was found. Despite these findings, the exact mechanisms of action are yet to be explored. Moreover, the efficacy of nucleus basalis of Meynert DBS, which highlights another promising target in the treatment of Alzheimer's disease [20] needs further investigation. Also, the outcome of long-term DBS in animals could reveal interesting findings with respect to the effects on brain functions and the efficacy on memory enhancement. Ongoing preclinical work may help to find the most beneficial target structure and define the therapeutic value of DBS in dementia-related disorders. Ultimately, the outcomes of sham-controlled, randomized clinical trials will determine whether DBS will play a role in the management of memory and cognitive disorders.

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**VALORIZATION
ADDENDUM**



RELEVANCE FOR SOCIETY

Today, more than 44.4 million people suffer from dementia [1]. This number will increase to about 135.5 million in 2050. Every year 7.7 million new cases of dementia are diagnosed, implying that there is a new case of dementia somewhere in the world every four seconds. Dementia has emerged as one of the leading health problems of our time and has been recently recognized as one of the major threats to world population [2]. Symptoms include progressive loss of memory, impaired reasoning and judgement, difficulties paying attention, communication problems and various non-cognitive symptoms, ultimately leading to disability and need for care.

Most individuals diagnosed with dementia are 65 years or older, although there is a growing awareness of cases that start before the age of 65 [1]. Demographic ageing is a worldwide process resulting from constantly improving health care systems. The fastest growth in the elderly population is taking place in China, India, and their South Asian and Pacific neighbors. In 2010, the total estimated worldwide cost of dementia was US\$604 billion, which equals to around 1% of the world's gross domestic product [3]. About 70% of the costs occur in Western Europe and North America. Statisticians even claim, that if dementia care would represent a country, it would be the world's 18th largest economy, ranking between Turkey and Indonesia [1].

Unfortunately, despite decades of research, we are still in need of an effective therapy for dementia, symptomatic or curative. There are 5 approved drugs on the market, which either modulate the cholinergic system by inhibiting acetylcholinesterase or reduce glutamate by antagonising specific glutamate receptors [4]. These pharmacological interventions, however, have limited efficacy and severe-side effects for patients; therefore, we are in need of new, effective, and safe alternative treatment options.

Recently, deep brain stimulation (DBS) has shown to have beneficial effects across memory and cognitive networks. A first evidence for this emerged when Hamani and colleagues stimulated the fornix/hypothalamus area in a patient suffering from morbid obesity [5]. In this specific case, DBS generated detailed autobiographical memories in the patient. Based on this case-observation, the same group performed a phase-I trial in patients with Alzheimer's Disease (AD) [6]. Indeed, memory tests confirmed possible improvements and/or slowing in the rate of cognitive decline in some patients following chronic DBS of 12 months.

Despite the encouraging results of the clinical trials presented above, basic neural and chemical mechanisms underlying DBS are still debated [7]. One approach to address these issues is to investigate the effects by stimulating homologous regions in experimental animal models [8].

Therefore, the studies described in this dissertation aimed at investigating which DBS target structures and stimulation parameters produce the most beneficial effects in an

experimental model of dementia. In addition, the present dissertation also describes potential mechanisms of action of DBS with regard to memory restoration. Only through understanding the mechanisms, DBS therapy in dementia patients can be fine-tuned to produce the best possible symptom relief currently available.

TARGET GROUPS

Target groups of the research presented in this dissertation are broad.

The first target audience entails the patients and their caregivers. As described above, effective dementia treatment represents a major unmet medical need at present. The quality of life of millions of patients as well as their caregivers significantly decreases in the progression of the disease. The consequences usually include a substantial decline in family's health and emotional well-being. Unfortunately, drug-based research activities in the last years have not resulted in breakthroughs in treating patients with dementia. In this respect, DBS might open new ways for alternative, non-drug based treatment in dementia-related disorders. In line with this, by examining the effects of DBS in an experimental model of dementia, we might have established the groundwork of new treatment options for not only for patients with dementia, but also for patients suffering from other cognitive impairing CNS diseases. In fact, many neurological and psychiatric conditions are constituted by cognitive disorders, for example patients with schizophrenia, attention-deficit hyperactivity disorder, depression and addiction often suffer from comorbid cognitive impairment. Thus, the present dissertation provides primary findings, which help to elucidate how DBS could potentially improve cognitive functions in dementia and other neurological and psychiatric diseases.

Coherent to this, the general public forms another target audience. Dementia has been defined as the most costly disease of our time. Treatment costs are covered by health insurances, affected individuals, their families and private insurances. In this respect, alleviating symptoms of dementia by means of DBS, would not only improve the quality of life of patients and caregivers, it would also provide a major financial relief for health care costs. Thus, delaying institutionalisation of patients by means of DBS can have an impact on economy and society, because patients function independently for longer, i.e. they can work, consume and invest for longer.

The third target group comprises the scientific critical mass. The findings of which brain region target for DBS produces most beneficial effects with regard to memory restoration might be of interest to neurosurgeons. Out of all structures within the circuit of Papez, the studies described in this dissertation, and other studies indicate that the fornix is the most relevant one for dementia therapy [9, 10]. Finally, the academic community as well as neuromodulation companies might profit from findings within

this dissertation. For example, there is an increasing awareness for the necessity to develop adaptive DBS systems (i.e., closed-loop), which prevent side-effects caused by overstimulation. Closed-loop feedback systems are latest advances in DBS therapy for epilepsy, but have not yet been developed for AD.

ACTIVITY/PRODUCTS

The major product/finding which can be derived by studies in this dissertation is that DBS might constitute an alternative treatment option for patients with dementia-related disorders. Results of the current dissertation provide evidence, that the fornix is the most suitable DBS target structure within the circuit of Papez to restore memory loss in an experimental rat model of dementia. The effects of fornix DBS are accompanied by selective neural activation in the hippocampus as well as increased hippocampal acetylcholine levels and no long-term neurogenic changes. In fact, the experiments in Chapter 9 have indicated that adaptive mechanism-based DBS might be necessary to maintain beneficial memory effects.

Chapter 3 of this dissertation also sheds light on the fact that up to now, most DBS studies in psychiatric disorders were first conducted in humans. Since the clinical data and the findings from animal studies show similarities, animal models can be of important value to find potential new DBS targets and settings for memory restoration. Close collaboration between basic scientists and clinicians has led to a successful implementation of preclinical findings to clinical DBS over the past years. For a successful treatment, such as DBS in patients with Parkinson's disease, a solid scientific base is needed. Increased understanding of basal ganglia function from studies in translational Parkinson's disease models [11, 12] paved the way for the clinical application of this method [13] and became the most successful application of DBS thus far.

INNOVATION

The studies of this dissertation have been innovative for several reasons. This work comprised in-depth investigation of the recently introduced concept of treating demented patients with DBS. This is a novel concept that might provide more rapid and robust therapies for patients with dementia. Furthermore, a multi-level, interdisciplinary approach was applied: bilateral DBS in freely moving animals, behavioral assessment, microdialysis and histology. This multi-level approach helped to understand if and how memory can be improved by DBS, in particular what the potential mechanisms

of action are. Novel mechanisms of DBS were identified, which might facilitate the treatment and management of patients with dementia in the future.

IMPLEMENTATION

In line with the abovementioned relevance of this project for patients, society and the scientific community, the implementation of the knowledge generated by this dissertation is again at various levels. Therefore, novel insights will be shared with patient organizations, health care professionals and scientific societies. From an academic perspective, results have been or will be published in peer-reviewed international journals and are presented at national and international conferences.

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BIOGRAPHY

Sarah-Anna Hescham was born on 8 July 1987 in Tönisvorst, Germany. After graduating from the Albertus-Magnus Gymnasium, Viersen in 2006, she went to study Neuroscience at the University of Cologne, Germany. She completed her practical semester and Bachelor's project at the Neuroscience Department, University of Cape Town, South Africa, under the supervision of Prof. Vivienne Russell. This research stay resulted in 2 publications in international peer-reviewed journals and Sarah received her B.Sc. degree in 2009. Intrigued by Neuroscience, Sarah decided to continue her studies at the University of Göttingen, Germany, where she pursued a M.Sc. degree in Neuroscience in 2011. For her Master's project, she worked at the Centre of Clinical Research in Neuropsychiatry, Perth, Western Australia under the supervision of Prof. Mathew Martin-Iverson. Following this, she started her doctoral research at the School for Mental Health and Neuroscience at Maastricht University, The Netherlands, supervised by Prof. Yasin Temel. As a PhD student, Sarah spent 7 months in the laboratory of Prof. Trevor Sharp from the Pharmacology Department of Oxford University, U.K. The presented work in this thesis received various awards for best poster or best publication at scientific meetings. In June 2015, Sarah has been awarded the Kootstra Talent Fellowship of Maastricht University to continue her scientific career in the arena of cognitive neuroscience.

THESIS DEFENSES FROM MHENS - SCHOOL FOR MENTAL HEALTH AND NEUROSCIENCE

2013

- Rob Havermans: **Bipolar disorder in daily life; Mood and cortisol responses to naturally occurring events.** Supervisor: Prof.dr. M. de Vries; Co-Supervisor: Dr. N. Nicolson.
- Véronique Moers-Hornikx: **Deep brain stimulation and the cerebellum.** Supervisors: Prof.dr. J. Vles / Prof. dr. Y. Temel; Co-Supervisor: Dr. G. Hoogland.
- Nicole Veldhorst-Janssen: **Intranasal delivery of rapid acting drugs.** Supervisors: Prof.dr. M. Marcus / Prof. dr. C. Neef; Co-Supervisor: Dr. P.H. van der Kuy.
- Stéphanie Knippenberg: **Vitamin D and Multiple Sclerosis: immunological and clinical outcome.** Supervisor: Prof.dr. J. Cohen-Tervaert; Co-Supervisors: Dr. J. Damoiseaux / Dr. Y. Bols.
- Erik D. Gommer: **Dynamic Cerebral Autoregulation: from methodology towards clinical application.** Supervisors: Prof.dr. W.H. Mess / Prof.dr. R.B. Panerai, UK; Co-Supervisor: Dr.ir. J.P.H. Reulen.
- Olga A.H. Reneerkens: **Can PDE inhibition improve cognition ? Translational insights.** Supervisor: Prof. dr. H.W.M. Steinbusch; Co-Supervisor: Dr. J. Prickaerts
- Lyzel S. Elias-Sonnenschein: **Clinical and biomarker correlates of genetic risk factors for Alzheimer's disease.** Supervisor: Prof.dr. F.R.J. Verhey; Co-Supervisor: Dr. P.J. Visser.
- Diego F. Mastroeni: **Epigenetic Dysregulation and the Pathophysiology of Alzheimer's Disease.** Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. P.D. Coleman, Sun City, Arizona; Co-Supervisors: Dr. B.P.F. Rutten / Dr. D.L.A. van den Hove.
- Leonidas Chouliaras: **Epigenetic Regulation in Aging and Alzheimer's disease: A translational perspective.** Supervisor: Prof.dr. H.W.M. Steinbusch; Co-Supervisors: Dr. B.P.F. Rutten / Dr. D.L.A. van den Hove.
- Liesbeth Knaepen: **Perinatal events and altered pain sensitivity in later life.** Supervisors: Prof.dr. E.A.J. Joosten / Prof.dr. D. Tibboel, EUR; Co-Supervisor: Dr. J. Patijn.
- Marisela Martinez-Claros: **Hippocampal plasticity and corticosterone: From dendrites to behaviour.** Supervisor: Prof.dr. H.W.M. Steinbusch; Co-Supervisors: Dr. J.L. Pawluski / Dr. J. Prickaerts.
- Marcus D. Lancé: **A circle of improvement in bleeding management: from laboratory to clinic and back.** Supervisors: Prof.dr. M.A.E. Marcu / Prof.dr. J.W.M. Heemskerck; Co-Supervisor: Dr. Y.M.C. Henskens.
- Hilde Braakman: **Imaging the brain; neuronal correlates of cognitive impairment in children with frontal lobe epilepsy.** Supervisors: Prof.dr. A.P. Aldenkamp / Prof.dr. J.S.H. Vles; Co-Supervisors: Dr.ir. W.H. Backes / Dr. P.A.M. Hofman.
- Willem H. van Zwam: **Aneurysmal subarachnoid hemorrhage: imaging strategies and cost-effectiveness aspects in diagnostic work-up and post-therapeutic follow-up.** Supervisors: Prof.dr. J.T. Wilmink / Prof.dr. J.E. Wildberger; Co-Supervisor: Dr. P.A.M. Hofman.
- Klara De Cort: **The Pathogenesis of Panic Disorder.** Supervisors: Prof.dr. I. Myin-Germeys / Prof.dr. E.J.L. Griez; Co-Supervisors: Dr. K.R.J. Schruers / Dr. I. Van Diest, Leuven.
- Kim van Wijck: **Mind the Gap; experimental studies on splanchnic hyperfusion and gastrointestinal integrity loss in man.** Supervisors: Prof.dr. W.A. Buurman / Prof.dr. C.H.C. Dejong; Co-Supervisor: Dr. K. Lenaerts.
- Yvette Roke: **Antipsychotic-induced hyperprolactinemia in children and adolescents with mainly autism spectrum disorders. Prevalence, symptoms, clinical consequences and genetic risk factors.**

- Supervisors: Prof.dr. P.N. van Harten / Prof.dr. J.K. Buitelaar (RUN); Co-Supervisor: Dr. A. Boot (UMCG).
- Fleur Goezinne: **Retinal detachment surgery: pre and postoperative prognostic factors.** Supervisors: Prof. dr. F. Hendrikse / Prof.dr. C.A.B. Webers; Co-Supervisor: Dr. E.C. La Heij (Amsterdam).
- Ralph L.J.G. Maassen: **The Merits of Videolaryngoscopy during Glottic Visualisation for Endotracheal Intubation.** Supervisors: Prof.dr. M. Marcus / Prof.dr. A. van Zundert (University of Queensland).
- Maria J. de Sousa Guerreiro: **The role of sensory modality in age-related distraction.** Supervisor: Prof.dr. C.M. van Heugten; Co-Supervisor: Dr. P.W.M. van Gerven.
- Ine Rayen: **Effects of developmental fluoxetine exposure on neurobehavioral outcomes.** Supervisor: Prof. dr. H.W.M. Steinbusch; Co-Supervisors: Dr. J.L. Pawluski / Dr. T.D. Charlier (Ohio University, USA).
- Nynke M.G. Bodde: **Psychogenic non-epileptic seizures; a separate disorder or part of a continuum?** Supervisors: Prof.dr. R. van Oostenbrugge / Prof.dr. K. Vonck (UZ Gent); Co-Supervisors: Dr. R. Lazeron / Dr. A. de Louw (Epilepsiecentrum Kempenhaeghe, Heeze).
- Alejandro M. Gomez: **Novel strategies for making myasthenia less gravis: targeting plasma cells and the neuromuscular junction.** Supervisor: Prof.dr. M.H. De Baets; Co-Supervisors: Dr. M. Losen / Dr. P. Martinez-Martinez.
- Mohammad S. Rahnama'i: **Prostaglandins and Phosphodiesterases in the Urinary Bladder Wall.** Supervisors: Prof.dr. Ph. Van Kerrebroeck / Prof.dr. S. de Wachter (Universiteit Antwerpen); Co-Supervisor: Dr. G. van Koeveringe.
- Mariken B. de Koning: **Studying biomarkers in populations at genetic and clinical high risk for psychosis.** Supervisors: Prof.dr. T. Amelvoort / Prof.dr. J. Booij (AMC).
- Fabien Boulle: **Epigenetic regulation of BDNF/TrkB signaling in the pathophysiology and treatment of mood disorders.** Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. L. Lanfumey (Universiteit Parijs); Co-Supervisors: Dr. D. van den Hove / Dr. G. Kenis.

2014

- Iris Nowak-Maes: **Tinnitus; assessment of quality of life & cost-effectiveness.** Supervisors: Prof.dr. M. Peters / Prof.dr. B. Kremer; Co-Supervisors: Dr. M. Joore / Dr. L. Anteunis.
- Marjolein Huijts: **Cognitive function in patients with cerebral small vessel disease.** Supervisor: Prof.dr. R.J. van Oostenbrugge; Co-Supervisors: Dr. A.A. Duits / Dr. J. Staals.
- Markus Gantert: **Fetal inflammatory injury as origin of long term disease: Lessons from animal models.** Supervisors: Prof.dr. B. Kramer / Prof.dr. L. Zimmermann; Co-Supervisor: Dr. A. Gavilanes.
- Elke Kuypers: **Fetal development after antenatal exposures: Chorioamnionitis and maternal glucocorticoids.** Supervisors: Prof.dr. B.W. Kramer / Prof.dr. H.W. Steinbusch / Prof.dr. Suhas G. Kallapur (University of Cincinnati, Ohio, USA).
- Pieter Kubben: **Ultra low-field strength intraoperative MRI for Glioblastoma Surgery.** Supervisor: Prof.dr. J.J. van Overbeeke; Co-Supervisor: Dr. H. van Santbrink.
- Laura Baijens: **Surface electrical stimulation of the neck for oropharyngeal dysphagia in Parkinson's disease: therapeutic aspects and reliability of measurement.** Supervisor: Prof.dr. B. Kremer; Co-Supervisor: Dr. R. Speyer, Townsville.
- Janneke Hoeijmakers: **Small fiber neuropathy and sodium channels; a paradigm shift.** Supervisor: Prof.dr. R.J. van Oostenbrugge; Co-Supervisors: Dr. C.G. Faber / Dr. I.S.J. Merckies.
- Stephanie Vos: **The Role of biomarkers in preclinical and prodromal Alzheimer's disease.** Supervisor: Prof.dr. F.R. Verhey; Co-Supervisor: Dr. P.J. Visser.
- Muriël Doors: **The Value of Optical Coherence Tomography in Anterior Segment Surgery.** Supervisors: Prof.dr. R.M. Nuijts / Prof.dr. C.A. Webers; Co-Supervisor: Dr. T.T.J.M. Berendschot.

- Anneke Maas: **Sleep problems in individuals with genetic disorders associated with intellectual disability.** Supervisors: Prof.dr. I. Curfs / Prof.dr. R. Didden.
- Sebastiaan van Gorp: **Translational research on spinal cord injury and cell-based therapies; a focus on pain and sensorimotor disturbances.** Supervisors: Prof.dr. B. Joosten / Prof.dr. M. van Kleef; Co-Supervisors: Dr. J. Patijn / Dr. R. Deumens, KU Leuven.
- Andrea Sannia: **High risk newborns and brain biochemical monitoring.** Supervisor: Prof.dr. J.S.H. Vles; Co-Supervisors: Dr. D. Gazzolo, Alessandria, Italy / Dr. A.W.D. Gavilanes.
- Julie A.D.A. Dela Cruz: **Dopamine mechanisms in learning and memory; Evidence from rodent studies.** Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. R.J. Bodnar, New York; Co-Supervisor: Dr. B.P.F. Rutten.
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- Catherine van Zelst: **Inside out; On stereotype awareness, childhood trauma and stigma in psychosis.** Supervisors: Prof.dr. Ph. Delespaul / Prof.dr. J. van Os.
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- Pamela Kleikers: **NOXious oxidative stress: from head toe too and back.** Supervisors: Prof.dr. H.H.H.W. Schmidt / Prof.dr. H.W.M. Steinbusch; Co-Supervisor: Dr. B. Janssen.
- José Luis Gerardo Nava: **In vitro assay systems in the development of therapeutic interventions strategies for neuroprotection and repair.** Supervisors: Prof.dr.med. J. Weis / Prof.dr. H.W.M. Steinbusch; Co-Supervisor: Dr. G.A. Brook, RWTH Aachen.
- Eva Bollen: **Cyclic nucleotide signaling and plasticity.** Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. R. D'Hooge, KU Leuven; Co-Supervisor: Dr. J. Prickaerts.

2015

- Jessica A. Hartmann: **A good laugh and a long sleep; Insights from prospective and ambulatory assessments about the importance of positive affect and sleep in mental health.** Supervisor: Prof.dr. J. van Os; Co-Supervisors: C.J.P. Simons / Dr. M. Wichers.
- Bart Ament: **Frailty in old age; conceptualization and care innovations.** Supervisors: Prof.dr. G.I.J.M. Kempen / Prof.dr. F.R.J. Verhey; Co-Supervisor: Dr. M.E. de Vugt.
- Mayke Janssens: **Exploring course and outcome across the psychosis-continuum.** Supervisor: Prof.dr. I. Myin-Germeys; Co-Supervisor: Dr. T. Lataster.
- Dennis M.J. Hernau: **Dopayours is not dopamine: genetic, environmental and pathological variations in dopaminergic stress processing.** Supervisor: Prof.dr. I. Myin-Germeys; Co-Supervisors: Prof.dr. F.M. Mottaghy / Dr. D. Collip.
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- Francesco Riso: **Urinary and salivary S100B monitoring in high risk infants.** Supervisor: Prof.dr. J.S.H. Vles; Co-Supervisors: Dr. D. Gazzolo, Genoa, Italy / Dr. A.W.D. Gavilanes.
- Alessandro Borghesi: **Stem and Progenitor Cells in Preterm Infants: Role in the Pathogenesis and Potential for Therapy.** Supervisor: Prof.dr. L. Zimmermann; Prof.dr. B. Kramer; Co-Supervisors: Dr. D. Gazzolo, Genoa, Italy / Dr. A.W.D. Gavilanes.
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- Martine van Nierop: **Surviving childhood new perspectives on the link between childhood trauma and psychosis.** Supervisors: Prof.dr. I. Myin-Germeys / Prof.dr. J. van Os; Co-Supervisor: Dr. R. van Winkel.

- Sylvia Klinkenberg: **VNS in children; more than just seizure reduction.** Supervisors: Prof.dr. J. Vles / Prof. dr. A. Aldenkamp; Co-Supervisor: Dr. H. Majoie.
- Anouk Linssen: **Considerations in designing an adult hearing screening programme.** Supervisor: Prof.dr. B. Kremer; Co-Supervisors: Dr. L. Anteunis / Dr. M. Joore.
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- Kimberly Cox-Limpens: **Mechanisms of endogenous brain protection; Clues from the transcriptome.** Supervisors: Prof.dr. J. Vles / Prof.dr. L. Zimmermann; Co-Supervisor: Dr. A. Gavilanes.
- Els Vanhoutte: **Peripheral Neuropathy outcome measures; Standardisation (PeriNomS) study part 2: Getting consensus.** Supervisors: Prof.dr. C. Faber / Prof.dr. P. van Doorn; Co-Supervisor: Dr. I. Merckies, Spaarne ziekenhuis Hoofddorp.
- Mayienne Bakkers: **Small fibers, big troubles; diagnosis and implications of small fiber neuropathy.** Supervisors: Prof.dr. C. Faber / Prof.dr. M. de Baets; Co-Supervisor: Dr. I. Merckies, Spaarne ziekenhuis Hoofddorp.
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- Esther Bouman: **Risks and Benefits of Regional Anesthesia in the Perioperative Setting.** Supervisors: Prof.dr. M. van Kleef / Prof.dr. M. Marcus, HMC, Qatar / Prof.dr. E. Joosten; Co-Supervisor: Dr. H. Gramke.
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- Saskia Ebus: **Interictal epileptiform activity as a marker for clinical outcome.** Supervisors: Prof.dr. A. Aldenkamp / Prof.dr. J. Arends, TUE / Prof.dr. P. Boon, Universiteit Gent, België.
- Inge Knuts: **Experimental and clinical studies into determinants of panic severity.** Supervisor: Prof.dr. I. Myin-Germeys; Co-Supervisor: Dr. K. Schruers
- Nienke Tielemans: **Proactive coping post stroke: The Restored4Stroke Self-Management study.** Supervisors: Prof.dr. C. van Heugten / Prof.dr. J. Visser-Meily, UMC Utrecht; Co-Supervisor: Dr. V. Schepers, UMC Utrecht.
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- Rohit Shetty: **Understanding the Clinical, Immunological and Genetic Molecular Mechanisms of Keratoconus.** Supervisors: Prof.dr. R.M.M.A. Nuijts / Prof.dr. C.A.B. Webers.
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wenberg; Dr. H. Tange.
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- Marc Hilhorst: **Crescentic glomerulonephritis in ANCA associated vasculitis.** Supervisors: Prof.dr. J. Cohen-Tervaert; Co-supervisor: Dr. P. van Paassen
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- Bart Biallostorski: **Structural and functional aspects of sensory-motor interaction in the urinary bladder.** Supervisors: Prof.dr. Ph. Van Kerrebroeck; Prof.dr. S. De Wachter, UvAntwerpen; Co-supervisors: Dr. G. van Koeveringe; Dr. M. Rahnama'i.
- Alexandra König: **The use of information and communication technologies (ICT) for the assessment of patients with Alzheimer's Disease and related disorders.** Supervisors: Prof.dr. F. Verhey; Prof.dr. Ph. Robert, Nice, Fr; Co-supervisors: Dr. P. Aalten; Dr. R. David, Nice, Fr.
- Micheline Chenault: **Assessing Readiness for Hearing Rehabilitation.** Supervisors: Prof.dr. M.P.F. Berger; Prof.dr. B. Kremer; Co-supervisor: Dr. L.J.C. Anteunis.
- Anand Vinekar: **Retinopathy of Prematurity. Recent advances in tele-medicine screening, risk factors and spectral domain optical coherence tomography imaging.** Supervisor: Prof.dr. C.A.B. Webers; Co-supervisor: Dr. N.J. Bauer
- Fleur van Dooren: **Diabetes and Depression: exploring the Interface between Pathophysiological and Psychological factors.** Supervisors: Prof.dr. F.R.J. Verhey; Prof.dr. J.K.L. Denollet, UvT; Prof.dr. F. Pouwer, UvT; Co-supervisor: Dr. M.T. Schram.
- Gabriëlla Pons van Dijk: **Taekwondo and physical fitness components in middle-aged healthy volunteers; the Sekwondo study.** Supervisors: Prof.dr. J. Lodder; prof.dr. H. Kingma; Co-supervisor: Dr. A.F. Lenssen.
- Yara Pujol-Lopez: **Development and psycho neuro immunological mechanisms in depression.** Supervisor: Prof.dr. H.W.M. Steinbusch; Co-supervisors: Dr. G. Kenis; Dr. Aye Mu Myint, München.