

Deep brain stimulation and the cerebellum

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Deep brain stimulation
and
the cerebellum

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Deep brain stimulation and the cerebellum

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in het openbaar te verdedigen
op vrijdag 1 maart 2013 om 14:00 uur

door

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Chapter 1

Introduction

The cerebellum

Anatomy

The cerebellum is a remarkable organ that is located in the posterior fossa. It has a uniform cellular structure across the whole cortex and contains about 50 billion neurons, approximately half of all neurons in the brain. This suggests that the cerebellum is capable of powerful information processing¹. Comparative anatomical studies have shown that the gross anatomy of the cerebellum in mammals is more or less similar². The cerebellar surface shows numerous horizontal folds called folia. These are interrupted by fissures. The deep primary fissure divides the cerebellum in an anterior lobe and a posterior lobe, while the posterolateral fissure separates the flocculonodular lobe from the posterior lobe (Figure 1.1a). In the posterior lobe there is a clear division in a central vermis and two lateral hemispheres. In the anterior lobe no clear vermis can be discerned, although slight indentations can be seen caused by the paravermian vein². Shallower fissures lead to a division of the vermis and corresponding parts of the hemispheres into ten lobules which have received names from several anatomists but are now often denoted by roman numerals as proposed by Larsell³ (Figure 1.1b).

The cerebellar cortex has a specific cellular organisation which is uniform across the whole cerebellum (Figure 1.1c). It is divided into three layers, containing five cell types. Most conspicuous are the Purkinje cells, large cells arranged side by side in a single layer between the molecular layer and the granule cell layer. Their dendrites are highly branched and located in the molecular layer in one plane perpendicular to the direction of the folia. Each Purkinje cell has one axon which traverses the white matter to the deep cerebellar nuclei, where they exert an inhibitory influence on the nuclear cells. The Purkinje cells receive two main inputs. Indirect output comes from the pontine nuclei in the form of mossy fibres that synapse with the granule cells in the granule cell layer. These small glutamatergic cells make up about 95% of all cerebellar neurons. They send their axons into the molecular layer where they branch and travel in two directions parallel to the direction of the folia. These axons, which are called parallel fibres, form synapses with the dendritic trees of the Purkinje cells, exerting an excitatory influence. The other input to the Purkinje cells derives directly from the inferior olive. These climbing fibres each form multiple excitatory synapses with the dendritic tree of a single Purkinje cell. The only neurons located in the molecular layer are the stellate cells, a subtype of which is the basket cell. These inhibitory interneurons receive input from the granule cells and pass this on to the Purkinje cells. Another type of interneuron is the Golgi cell, which has its cell body in the granule cell layer and, like the Purkinje cells, has a branched dendrite tree in the molecular layer. Their dendrites are contacted mostly by parallel fibres. The axons of the Golgi cells remain in the cerebellar cortex and contact the dendrites of granule cells.

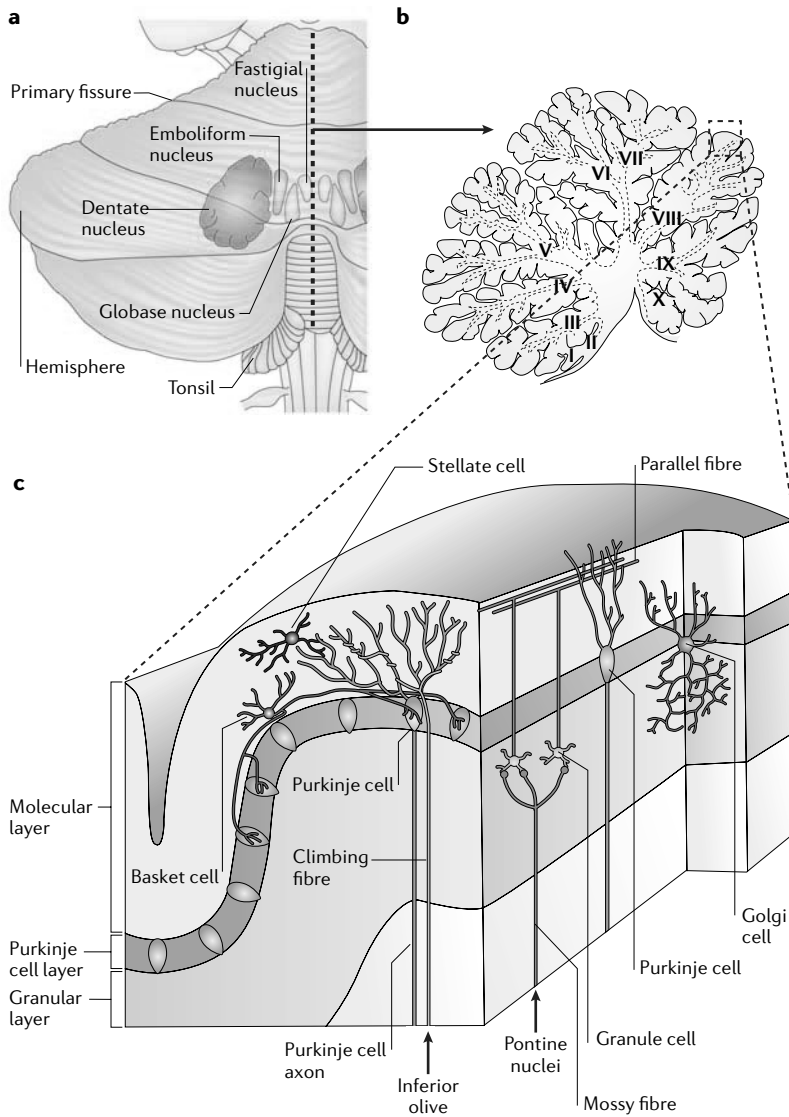


Figure 1.1 Anatomical architecture of the cerebellum. a: Posterior view of the human cerebellum, showing the cerebellar nuclei embedded below the cerebellar cortex. b: Drawing of midsagittal cross-section through the human cerebellum (dotted line indicates the plane of section), showing lobular organization. Each of the ten lobules is demarcated by a Roman numeral (I–X). c: The microstructural organization of the cerebellar cortex. Cut-away illustration of an individual cerebellar cortical lobule, indicating the presence of three layers. The figure shows the relative positions of Purkinje cells and their main inputs (parallel and climbing fibres). Reprinted with permission from Ramnani N. *Nat Rev Neurosci*. 2006.

Deep in the white matter several clusters of neurons form the cerebellar nuclei (Figure 1.1a). These structures form the output channels of the cerebellum. They receive input mainly from the Purkinje cells in the cerebellar cortex; however, both mossy fibres and climbing fibres send collaterals directly to the deep cerebellar nuclei. In humans there are four cerebellar nuclei: most medial is the fastigial nucleus, followed by the small globose nucleus and emboliform nucleus. Most lateral is the dentate nucleus. In most mammals four nuclei can also be identified: a medial (or fastigial) nucleus, a posterior interpositus nucleus, an anterior interpositus nucleus and a lateral (or dentate) nucleus.

Function

The function of the cerebellum has been extensively investigated in the past centuries. In the beginning of the nineteenth century Flourens was the first to propose that the cerebellum is involved in motor coordination^{4,5}. This was confirmed by succeeding research. Gradually it has become clear that the cerebellum plays a role in many other processes. Not only motor coordination but also motor learning has been located to the cerebellum⁶. It has been shown to influence autonomic function such as heart rate, blood pressure and micturition⁷. Independent of the influence on autonomic function is a role in fear and fear conditioning^{8,9}. Clinical observations and studies have implicated the cerebellum in several psychiatric disorders¹⁰, and lesions of the cerebellum have been shown to cause a variety of cognitive and behavioural symptoms. The latter has been called the cerebellar cognitive affective syndrome (CCAS)¹¹. A role for the cerebellum in epilepsy was suspected in the beginning of the twentieth century when Hunt described coexistence of myoclonic activity and cerebellar atrophy in patients¹². Many studies have analysed the effect of cerebellar lesions and stimulation on seizure generation and spread, with inconsistent results^{13,14}.

Traditionally the cerebellum was thought to integrate input from multiple cortical areas leading to a single output to the primary motor cortex¹⁵. Parallel to the elucidation of the complex functions of the cerebellum it has been shown that the cerebellum projects to many more cortical and subcortical areas. In the research on its role in autonomic functions reciprocal connections between the cerebellum and the hypothalamus have been shown¹⁶. Projections from the cerebellum to the periaqueductal grey have been shown in the rat, connecting the cerebellum with the fear network¹⁷. It has become increasingly clear that the connections between the cerebellum and the cortex are also reciprocal, forming separate closed loop circuits which likely subserve different functions¹⁸. Besides connections with the primary motor cortex, such closed loop circuits have also been shown between the cerebellum and the premotor, prefrontal and parietal cortical areas, supporting a role of the cerebellum in cognitive functions¹⁸. Projections from the cerebellum to the amygdala have been suspected based on behavioural changes due to cerebellar lesions, but an anatomical pathway has never been shown¹⁹. Recent tracing experiments have shown

that there is a closed loop between the dentate nucleus of the cerebellum and the basal ganglia which is independent of cortical input²⁰.

The deep cerebellar nuclei are the output channels of the cerebellum. Each nucleus is thought to have its specific connections and function. Lesion studies have shown that only the fastigial nucleus is involved in heart rate conditioning^{8,21,22}. Lesions of the dentate and interpositus nucleus interfere with learning of conditioned responses but have no effect on heart rate conditioning⁸. In rats, projections to the hypothalamus arise from all three nuclei¹⁶, although the majority arise from the dentate nucleus^{16,17}. Historically the dentate nucleus was considered to be the only cerebellar nucleus to project to the cerebral cortex^{15,23}. However, it was shown that in non-human primates all three nuclei project to cerebral cortex through the ventrolateral thalamic nucleus (VL), the centrolateral thalamic nucleus (CL) and the mediodorsal thalamic nucleus (MD)^{23,24}. The cerebellar-recipient areas of these thalamic nuclei have been shown to project to the primary motor cortex in a somatotopic fashion²⁵. Projections to the prefrontal cortex originate in the dentate nucleus^{26,27}. The projections from the dentate nucleus to the premotor cortex derive from a different part of this nucleus than the projections to the primary motor cortex^{28,29}, suggesting the existence of motor and non-motor domains in the dentate nucleus. This was already suggested by Leiner *et al.* based on the existence of two anatomically different subdivisions in the dentate nucleus²⁴ and is supported by the finding that the relative increase in size of the ventral part of the dentate nucleus when comparing humans to great apes is disproportionately large compared to the dorsal part of the dentate nucleus³⁰. In relation to epilepsy both the fastigial nucleus and the dentate nucleus have been implicated³¹.

Cerebellar activity during deep brain stimulation in Parkinson's disease

Deep brain stimulation (DBS) has become an accepted treatment option for several movement disorders and is being investigated as a potential treatment option in psychiatric disorders³². The experience with DBS is largest in patients with Parkinson's disease (PD). The subthalamic nucleus (STN), the internal segment of the globus pallidus (GPi) and the ventrointermediate nucleus of the thalamus (VIM) have all been targeted with good effects on tremor, rigidity and bradykinesia. Recently the pedunculo-pontine nucleus (PPN) has also been targeted with promising effects on axial symptoms³³. The mechanisms underlying the therapeutic effects of DBS are under investigation. In patients functional imaging using PET and SPECT provides a method to investigate the effects of DBS *in vivo*. In these studies changes of activation are frequently seen in the cerebellum, as summarized below.

Subthalamic nucleus

In studies reporting changes in cerebellar blood flow or metabolism during STN DBS at rest, results are inconsistent. Several groups report decreased regional cerebral blood flow (rCBF) or glucose metabolism³⁴⁻³⁸, while others show increases³⁹⁻⁴⁷. Changes are reported in the cerebellar hemispheres as well as in the vermis. One group showed increased glucose metabolism in the anterior cerebellar cortex and decreases in the posterior cortex^{48,49}. Functional imaging during STN DBS while performing a movement task shows increased activation in the cerebellum ipsilateral to movement and decreases contralateral to movement^{50,51}.

Pallidal complex

One group reported changes in activity in the cerebellum during DBS of the internal segment of the globus pallidus (GPi)⁵². They describe an increase of rCBF bilaterally in the cerebellum using FDG-PET in rest. In the same patients improvement of several aspects of a motor task correlated with increased activity in both cerebellar hemispheres on H₂¹⁵O-PET⁵³. In another study examining the effect of unilateral GPi and GPe DBS, no changes were seen in the cerebellum during GPi stimulation⁵⁴. However, GPe stimulation led to decreased blood flow in the cerebellum ipsilateral to the side of stimulation.

Ventrolateral nucleus of the thalamus

Two studies performing H₂¹⁵O-PET during unilateral DBS of the ventrolateral nucleus of the thalamus (VIM) show decreased activity in the contralateral cerebellar hemisphere^{55,56}. Deiber *et al.* showed decreased rCBF in the anterior vermis and anterior part of the intermediate zone of the cerebellar hemispheres⁵⁷.

Pedunculopontine nucleus

The pedunculopontine nucleus (PPN) has recently been added as a possible target for DBS in PD, with effects especially on axial symptoms. In a case report, Strafella *et al.* show increased rCBF in the ipsilateral cerebellum during unilateral PPN DBS⁵⁸. In a subsequent study of three patients the same group showed rCBF increase bilaterally in the cerebellum⁵⁹. In a case report of a patient with freezing of gait PPN DBS led to an increase of connectivity with the cerebellum as measured by diffusion tensor imaging (DTI), restoring a pattern that resembles that of healthy controls⁶⁰.

Cerebellar changes following deep brain stimulation: animal research

Functional imaging in patients is an indirect method to examine the effects of DBS on the brain. Animal models provide a method to further examine local and distant effects of DBS permitting histological as well as functional analysis. Animal models using DBS have been used to analyse functional connectivity of the stimulated structures as well as to elucidate the mechanism of DBS. Furthermore, stimulation of several forebrain structures leads to the development of epileptic seizures. This process called kindling is used as an animal model to study the pathophysiology of epilepsy and the effect of treatment.

Literature search

We searched the available literature for animal studies analysing changes in the cerebellum following deep brain stimulation including kindling. We used the following search terms and various combinations of these terms: deep brain stimulation, DBS, high frequency stimulation, HFS, cerebellum, cerebellar nuclei, dentate nucleus, interpositus nucleus, fastigial nucleus, epilepsy, amygdala, kindling, subthalamic nucleus, STN, thalamus, ventrolateral nucleus, mediodorsal nucleus, periaqueductal grey, PAG. Results were limited to animal research and only papers written in English were included. References of relevant papers were screened to find additional papers. We found 14 papers which fit our objective. The results described in these papers are summarized below.

Substantia nigra

In a study using a quantitative autoradiographic 2-deoxy-D-[1-¹⁴C]glucose method (¹⁴C-DG) Savaki *et al.* analysed the local glucose utilisation in rats following unilateral stimulation of the substantia nigra (SN)⁶¹. They found a significant change in both ipsilateral and contralateral dentate nucleus (DN) and interpositus nucleus (IN) of the cerebellum. Additional changes in the vermis were attributed to motor behavioural manifestations seen in the conscious stimulated animals since these changes in metabolic activity following stimulation were not found in halothane anesthetized animals. Changes in metabolic activity in the DN and IN following SN stimulation were thought to be the result of orthodromic activation of direct efferents of the SN to the deep cerebellar nuclei (DCbN).

Subthalamic nucleus

Using the same autoradiographic method mentioned above, Tzamourissakis *et al.*⁶² found no significant alterations of glucose metabolism in the molecular or granular layer of the cerebellum following unilateral DBS of the STN in rats.

Thalamus

Raos *et al.* analysed local cerebral glucose utilization following unilateral stimulation of the intralaminar centrolateral thalamic nucleus (CL) in rats⁶³. They found a tendency to increased metabolism in the contralateral fastigial and interpositus cerebellar nuclei. These results showed significant correlation with the extent of locally induced CL-stimulation effect. Gao *et al.* showed that neither unilateral nor bilateral DBS of the anterior thalamic nucleus (ATN) leads to changes in glucose uptake levels in the cerebellum in rats⁶⁴.

Amygdala

Kindling is a model of epilepsy in which repeated subthreshold electrical stimulation of a certain brain region leads to a build-up of seizure activity, culminating in full fledged epileptic seizures⁶⁵. A frequently used target is the amygdala. Amygdala kindling is used to study the pathophysiology of temporal lobe epilepsy and the effects of treatment with novel antiepileptic drugs as well as vagus nerve stimulation^{65,66}. Animal studies have shown that cerebellar lesions and cerebellar stimulation interfere with the kindling process^{31,67}. However, few groups have looked at the effects of amygdala kindling on the cerebellum. Of those studies that included the cerebellum in their analysis, most do not offer a rationale for this analysis or consider the cerebellum to be a control region. Studies analysing the glucose utilization⁶⁸, calcium binding protein (CaBP) levels and gene expression⁶⁹, neuronal degeneration and changes in astrocytes and microglia⁷⁰ found no changes in the cerebellum in partially and fully kindled rats. Several studies have analyzed the role of neurotransmitters in the kindling model. No changes in GABA levels or GAD gene expression and activity were found in the cerebellum of fully kindled rats^{69,71,72}. In contrast, Tietz and Chiu found a change in GABAergic function in the cerebellum⁷³. Synaptosomal uptake of taurine increased in the cerebellum of fully kindled rats⁷¹. Teskey *et al.* reported slightly enhanced fos expression in the cerebellum one hour after a stage 5 seizure in fully kindled rats⁷⁴. Seegers *et al.* examined the expression of multidrug transporter P-glycoprotein (PGP) using the cerebellum as a control region; their only significant finding was a larger PGP-labelled area in the contralateral cerebellum of kindled rats⁷⁵.

Entorhinal cortex

One group performed entorhinal cortex kindling and analysed cytochrome oxidase activity 24 and 48 hours after the last seizure in fully kindled rats. They found no changes in any brain region including the cerebellum⁷⁶.

Aims of the thesis

In this study, we investigated the role of the deep cerebellar nuclei in motor, cognitive and emotional behaviour. We performed a set of experiments in which we modulated motor, cognitive and emotional behaviour using DBS, and investigated the neuronal activities in the deep cerebellar nuclei, utilising animal models. Our main aim was to advance our understanding on the functional roles of the deep cerebellar nuclei in different forms of behaviour linked to different brain regions.

In **chapter 2** we report on the effect of DBS of the mediodorsal thalamic nucleus (MD) and the ventrolateral thalamic nucleus (VL) in rats on impulsive behaviour and on neuronal activity in the deep cerebellar nuclei (DCbN) and prefrontal cortex (PFC). The hypothesis that inhibition of the DCbN leads to abnormal behaviour is explored further in **chapters 3** through **6**. In **chapter 3** we describe the effect of STN DBS, which is known to enhance motor behaviour and to decrease impulsivity in rats⁷⁷, on neuronal activity in the DCbN. DBS of the dorsolateral periaqueductal grey (dIPAG) and the ventromedial hypothalamus (VMH) in rats has been shown to mimic panic attacks in humans^{78, 79}. The effect of this stimulation model on neuronal activity in the DCbN is described in **chapter 4**. In **chapter 5** we report the effect of conditioned panic attacks on neuronal activity in the DCbN. In **chapter 6** we describe the effect of amygdala kindling, a model of chronic epilepsy, on neuronal activity and metabolic changes in the DCbN, and correlate these results to kindling parameters. Finally, in **chapter 7** we present a summary of the findings in this thesis and the implications of these findings are discussed.

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Chapter 2

Cerebellar nuclei are involved in impulsive behaviour

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Abstract

Recent anatomical and clinical evidence has shown that the cerebellum, primarily considered a motor control structure, is also involved in higher cognitive functions and behavioural changes, such as impulsive behaviour. Impulsive behaviour has been shown in several studies to be increased by lesions of the mediodorsal (MD) thalamic nucleus. We performed deep brain stimulation (DBS) of the mediodorsal and ventrolateral (VL) thalamic nuclei in rats, clinically mimicking such a lesion, and tested them for changes in impulsive behaviour in a choice reaction time test. We then analysed the effects of this stimulation on c-Fos expression in both the deep cerebellar nuclei (DCbN) and the prefrontal cortex (PFC), and correlated these outcomes to the measured changes in impulsive behaviour. DBS of the MD thalamic nucleus increased impulsive behaviour without changing motor parameters. This was accompanied by a decrease in the c-Fos expression in all cerebellar nuclei; with a corresponding increase in c-Fos expression in the PFC. DBS of the VL thalamic nucleus caused no significant change in behaviour or c-Fos expression in either region. The present study demonstrates that impulsive behaviour involves the cerebellar nuclei, possibly through a decreased selective attention caused by a disruption of the cerebello-thalamo-cortical pathways through the MD thalamic nucleus.

Introduction

Since the first description of the cerebellum as a motor structure by Flourens in the beginning of the nineteenth century, the cerebellum has been regarded to be involved in motor coordination and control¹. Inputs from several brain regions including the motor, premotor, posterior parietal, cingulate and prefrontal cortex (PFC) were shown to converge on the cerebellar cortex and deep cerebellar nuclei (DCbN)^{2,3}. It was thought that the deep cerebellar nuclei project strictly to a specific region in the ventrolateral thalamic nucleus (VL), and from there to the primary motor cortex^{4,5}. The last two decades, however, there has been increasing attention for a possible non-motor role of the cerebellum. Leiner and associates first hypothesized that the disproportional increase in size of the phylogenetically newer parts of the cerebellum in anthropoid apes and humans might imply a role of the cerebellum in mental skills in the same manner as the phylogenetically older parts of the cerebellum contribute to motor skills⁶. Neuroanatomical research has shown that the cerebellum projects to the PFC through the VL and other thalamic nuclei including the mediodorsal thalamic nucleus (MD)^{7,8} and the reticular nucleus of the thalamus (RNT)⁹. These anatomical connections between the cerebellum and the PFC suggest that the cerebellum is involved in non-motor circuits.

Other findings supporting the theory of a non-motor role of the cerebellum come from the clinical setting. This was first pointed out by case reports and case series describing behavioural disturbances in patients with cerebellar lesions due to for example stroke or tumours¹⁰⁻¹³. Nowadays there is an increasing amount of information from human neuroimaging studies to support this view of a non-motor role of the cerebellum^{14,15}. In this respect, Schmahmann and colleagues have described a constellation of cognitive deficits in a group of patients with selective cerebellar damage which has been termed the cerebellar cognitive affective syndrome (CCAS)¹⁴. This syndrome consists of impairment of executive functions, difficulties with spatial cognition, changes of personality including disinhibited or inappropriate behaviour, and language deficits. A similar syndrome has been described in children after resection of cerebellar tumours. This cerebellar mutism syndrome (CMS) is characterized by mutism in combination with other neurological, cognitive and behavioural deficits including either decreased initiation of activity or disinhibition^{11,16-18}. It has been suggested that CCAS and CMS are in fact different presentations of the same syndrome^{11,18}. Additional evidence that the cerebellum plays a role in higher-order behaviour comes from imaging studies showing significantly smaller cerebellar volumes in patients with neuropsychiatric diseases such as ADHD and schizophrenia¹⁹⁻²³, and altered metabolism in the cerebellum in patients with obsessive compulsive disorder (OCD)²⁴⁻²⁷. There is also an abnormal cerebellar activation on functional MRI in autistic subjects during motor and cognitive tasks²⁸. Pathological findings in these subjects include loss of Purkinje cells (PC's)²⁹. Impulsivity is a core feature of these neuropsychiatric diseases³⁰. As described above, there have

been several reports of changes in impulsive behaviour as part of the CCAS or CMS in patients with cerebellar damage^{11,12,14}. In autism spectrum disorders the degree of explorative behaviour is correlated to the volume of vermal lobules VI-VII of the cerebellum³¹. Duchenne's muscular dystrophy has been shown to have a heightened association with ADHD, autism spectrum disorders and OCD³², which is speculated to be caused by the absence of dystrophin in the cerebellum³³. Furthermore, King and co-workers showed a change of activity in the cerebellar vermis in a rat model of ADHD after treatment with a 5-HT agonist that is known to relieve impulsivity³⁰.

These recent findings suggest a key role of the cerebellum in impulsive behaviour. In this study we have tested this hypothesis. Previous research has shown that lesions of the MD thalamic nucleus induce an increase of impulsivity in rats as measured by premature responding in a choice reaction time test³⁴. High frequency stimulation (HFS) has been shown to cause a net inhibition of the target region, an effect that is clinically comparable to a lesion³⁵, although the underlying mechanisms are probably different. The benefit of HFS above lesions is that it is reversible and adjustable. Therefore, we chose to perform electrical stimulation of the MD thalamic nucleus in rats as a behavioural model of impulsivity, in which premature responses serve as a measure of inhibitory control³⁶. To evaluate the specificity of the site of stimulation, we stimulated the VL thalamic nucleus. DBS of the VL thalamic nucleus is known to effectively reduce various types of tremor in patients³⁷. Case series show a subtle memory effect of VL thalamic DBS^{38,39}, but to the author's knowledge changes of impulsivity were never reported. Finally, activation in the DCbN and the PFC was evaluated using c-Fos immunohistochemistry. We focussed on the cerebellar nuclei as these are the output structures of the cerebellum. c-Fos was chosen as it is an immediate early gene which is considered to reflect neuronal activation^{40,41}. The expression of c-Fos is maximal about two hours after administration of a stimulus, and disappears again after four to eight hours even if the stimulus is continued⁴¹. Therefore the c-Fos expression found two hours after a specific stimulus can be considered to be the direct effect of this stimulus, and this expression can be used to identify brain areas directly influenced by this stimulus⁴⁰.

Materials and methods

Animals

We used male Lewis rats (12 weeks old, bred and housed at the Central Animal Facility of the Maastricht University, The Netherlands), with an average body weight of 300 g. The rats were housed individually in standard cages with sawdust bedding in an air-conditioned room (about 20°C) under a 12/12-h reversed light/dark cycle. During testing (5 days a week), the rats were given 10-20 g of food (standard laboratory chow; Hopefarms, Woerden, The Netherlands) a day; the other 2 days they were left to rest

and fed ad libitum. This schedule reduced their weight to approximately 90% of their free feeding weight during the week. The rats had free access to water at all times. All experiments were approved by the Animal Experiments and Ethics Committee of the Maastricht University.

Surgical procedure

Rats were randomly assigned to one of the following groups: control (no surgery; $n=8$), bilateral VL thalamic nucleus electrode implantation ($n=10$), or bilateral MD thalamic nucleus electrode implantation ($n=10$). A detailed description of the surgical procedure has been reported previously⁴². In brief, animals were anaesthetized throughout the entire procedure using a combination of ketamine (90 mg/kg) and xylazine (10 mg/kg) injected subcutaneously. Rats were placed in a stereotactic apparatus (Stoelting, Wood Dale, USA; model 51653). Two burr holes were made in the skull immediately above the targets to allow for insertion of electrodes in the MD thalamic nucleus (coordinates from bregma: AP=-2.8 mm, ML= ± 0.6 mm, V=-5.0 mm) or in the VL thalamic nucleus (coordinates from bregma: AP=-2.8 mm, ML= ± 1.5 mm, V=-6.5 mm). A construction of two stainless steel electrodes (Technomed, The Netherlands), both concentric and bipolar, with a tip diameter of 50 μm and a shaft diameter of 250 μm , was implanted in this experiment. The electrodes were fixed in position using dental cement (Heraeus Kulzer, Germany).

Deep brain stimulation

The stimulus was delivered using a World Precision Instrument (WPI, Berlin, Germany) accupulser (A310) and a stimulus isolator (A360). Real time verification of the parameters applied during stimulation was obtained for both electrodes of the bilateral construction, using a digital oscilloscope (Agilent Technologies, Agilent 54622D oscilloscope, The Netherlands). Stimulation started a few minutes before the test and lasted for the duration of each session. The rats had 12 to 72 hours without stimulation between sessions.

Behavioural evaluation

A choice reaction time task was performed as described previously³⁶. In summary, rats were tested in Skinner chambers (inner dimensions: 40 cm \times 30 cm \times 33 cm) equipped with two retractable levers with cue lights just above the levers. A food tray (5 cm \times 5 cm and 2.5 cm above the grid floor) was positioned equidistant between the two levers and could be accessed by pushing a hinged panel. The levers (4 cm wide) projected 2 cm into the conditioning chamber and were located 6 cm from both sides of the food tray and 12 cm above the grid floor. A light and a loudspeaker were fixed in the ceiling of the conditioning chamber. The test procedure was controlled by a personal computer and the data stored on disk at the end of a session (time precision=1 ms). During a trial, the rat had to insert its nose into the central panel until

a tone sounded. A high tone (10 kHz; 80 dB) required the rat to press the left lever and a low tone (2.5 kHz; 80 dB) required pressing of the right lever. The period (randomly chosen from 0.6 s to 1.5 s, with steps of 0.1 s) between nose insertion and tone was termed the hold duration. When a rat did not hold the panel for the entire hold duration, the same interval was repeated when the panel was next pushed. Depression of the lever resulted in food reward in only 50% of cases to increase the motivation of the animals for the task⁴³. The reinforcement was given independently of the reaction time. After pushing the panel there was a lapse of ten seconds before the next trial could be started. Each session lasted 30 min or until the completion of 60 trials. The following parameters were analysed:

Reaction time (RT) is defined as the latency between the onset of the tone and the release of the tray panel. It is generally accepted that RT's shorter than 100 ms are unlikely to be true reaction times, and RT's longer than 1500 ms should not be considered to be task-related; therefore these RT's were discarded⁴³.

Motor time (MT) is defined as the latency between the release of the tray panel and the lever press. It was assumed that MT's longer than 2 s did not reflect 'true' motor time; these were left out of the analysis⁴³.

Premature responses ratio (PRr) is defined as the total number of times the rat released the tray panel before the hold duration had elapsed, divided by the number of trials. After a premature response the rats had to start the same trial again by pushing the hinged panel.

Prior to surgery, the rats underwent a period of two weeks of preliminary training until the mean reaction time of performances was stabilized, after which the baseline behavioural performance was evaluated⁴³.

Experimental procedures

Rats were allowed to recover from surgery for 1 week after which they were retrained in the Skinner boxes (modified to allow for the attachment of a stimulation cable), until behavioural parameters were stabilized. We then tested all rats during stimulation using the following DBS parameters: a pulse width of 60 μ s, a frequency of 130 Hz (high frequency), and variable amplitudes of 150, 30, 3, and 1 μ A to investigate the effect of a relatively high, medium and low current density with respect to the human condition⁴⁴. In addition all rats were tested with the stimulation cable attached but without stimulation. The order of the various amplitudes was randomized using a Latin square design in which each condition was repeated two times. Following this, the average of the 2 sessions was calculated for each behavioural parameter.

Histological processing

One week after the final behavioural experiment all rats were stimulated in their home cage for 30 minutes using a pulse width of 60 μ s, a frequency of 130 Hz, and an amplitude of 30 μ A. One hour after the end of the stimulation, they were perfused

transcardially with Tyrode (0.1 M), followed by a fixative containing 4% paraformaldehyde, 15% picric acid, and 0.05% gluteraldehyde in 0.1 M phosphate buffer (pH 7.6). The brains were removed and postfixed for 2 h followed by immersion in 10% sucrose at 4°C until saturated. The concentration of sucrose solution was increased to 20% and finally to 30% each time the brains were saturated. Brain tissue was then quickly frozen with CO₂ and stored at -80°C.

The prefrontal regions of the brains were cut serially on a cryostat into 30 µm frontal sections and collected per 10 sections in a cup for free floating staining. The cerebellum was cut serially into 10 µm sections which were collected on gelatine-coated glasses. We used a previously published protocol for c-Fos immunohistochemistry⁴⁵, with some adjustments for the staining of sections on glass to achieve a similar staining intensity. In summary, this staining was carried out by incubating sections with a polyclonal rabbit anti-c-Fos antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (Free floating sections: dilution 1:20,000; incubation overnight. Sections on glass: dilution 1:600, incubation two days), followed by incubation with the secondary antibody (biotinylated donkey anti-rabbit, Jackson Immunoresearch Laboratories Inc., Westgrove, USA) (Dilution 1:400; free floating: 90 minutes incubation; on glass: overnight incubation). Subsequently, the sections were incubated with an avidin-biotin-peroxidase complex (Elite ABC-kit, Vectastatin; Burlingame, USA) for two hours. To visualize the immune complex of Horse Radish Peroxide reaction product, sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB)/ Nickel Chloride (NiCl₂) solution (5 ml DAB solution, 5 ml Tris/HCl, 50 µl NiCl₂ 8%, and 3.35 µl hydrogen peroxide 30%) for ten minutes. This reaction was stopped by rinsing all sections thoroughly with TBS. The free floating sections were then mounted on gelatine-coated glasses. After dehydrating, all sections were cover-slipped with Pertex (HistolabProducts ab, Goteborg, Sweden).

Additionally, for each animal one other series of sections from the PFC was stained with standard haematoxylin–eosin (Merck, Darmstadt, Germany) to locate the electrode tips.

Quantitative evaluation of c-Fos immunoreactive cells

Systematic cell counts were performed of c-Fos immunoreactive (c-Fos-ir) cells in the prefrontal cortex and in the deep cerebellar nuclei. Our quantification method was similar to a previously reported method⁴⁶, with minor modifications with respect to the magnification and the threshold value for a positive cell. These modifications were previously explained by Lamprea *et al.*⁴⁶ and by Lim *et al.*⁴⁷. In summary, photographs of the areas of interest were taken at 4x magnification using an Olympus DP70 camera connected to an Olympus AX70 bright-field microscope (analySIS; Imaging System, Münster, Germany). The same light intensity and threshold settings of the camera were employed for all sections. For each animal we selected four sections of the PFC (AP coordinates from bregma: 2.2 to 2.7) and four to six sections of the cerebellar nuclei (AP coordinates from bregma: -11.2 to -11.8) for quantification. The boundaries

of the areas of interest (PFC (defined by the prelimbic cortex and the infralimbic cortex), DN, IN and FN) were delineated and the area was measured. The counting of the c-Fos-ir cells was performed using the image analysis program 'Image J' (version 1.38, NIH, USA). A cell was counted as c-Fos-ir if its density was 75% higher than the mean background density of that section. Artefacts in the sections were excluded from analysis to ensure the accuracy of measurements. The number of c-Fos-ir cells per square millimeter was calculated for each area of interest.

Statistical analysis

For the analysis of the behavioural data the results during stimulation were compared to the results in the off stimulation setting using the Analysis of Variance (ANOVA) repeated measures analysis. The c-Fos expression in the prefrontal cortex was analysed using ANOVA. An LSD post hoc test was used in both cases to analyse group differences in more detail. The data of the c-Fos analysis in the deep cerebellar nuclei showed a skewed distribution and were therefore analysed using the Kruskal-Wallis test. A Mann-Whitney test was used to follow up significant findings. Correlation analysis was done using a bivariate analysis and is presented as Kendall's correlation coefficient tau. Data are presented as mean \pm S.E.M. p-values lower than 0.05 were considered significant.

Results

Histological evaluation of electrode localization

In 1 rat of the VL thalamic DBS group the electrodes were out of the target (centromedian nucleus of the thalamus) and 2 rats of the VL thalamic DBS group and 3 rats of the MD thalamic DBS group lost their electrode construction during the experiment. These rats were excluded from analysis. Electrode tips were located at the level of the VL thalamic nucleus in 5 rats and in the MD thalamic nucleus in 7 rats (Figure 2.1). No histological damage was observed except for the electrode trajectory, suggesting that repeated stimulation with the current stimulation settings did not cause observable tissue damage.

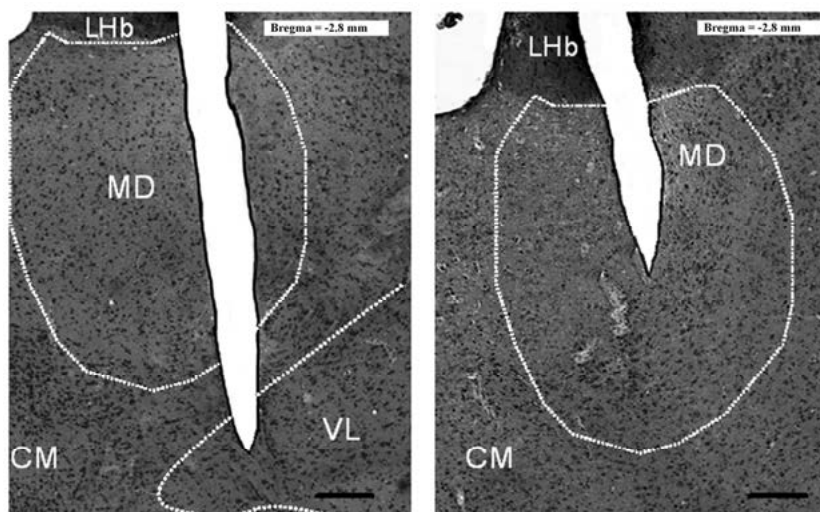


Figure 2.1 Representative low-power photomicrograph of a 30 μm -thick frontal section from the brains of rats with bilateral electrode implantation respectively in the ventrolateral (VL) and mediodorsal (MD) thalamic nuclei. The tips of the electrodes are situated within the respective targets. The white dotted lines show the boundaries of the structures and the dark lines delineate the boundaries of the electrodes trajectories. Scale bar=200 μm . LHb: lateral habenula; CM: centromedian thalamic nucleus.

Behavioural evaluation

The behavioural parameters never exceeded the time boundaries specified in the methods section; therefore all were used for the analysis. There was a significant increase in PRr after DBS of the MD thalamic nucleus as compared to the off stimulation setting ($F_{4,3}=11.061$; $p<0.05$) (Figure 2.2). Post hoc comparisons show an increase in PRr in the MD thalamic nucleus stimulated group at all stimulation parameters, however, only stimulation at $1\mu\text{A}$ caused a significant change compared to the off stimulation state. DBS of the MD thalamic nucleus had no effect on RT ($F_{4,3}=0.108$; $p=0.972$) or MT ($F_{4,3}=0.357$; $p=0.858$) compared to the off stimulation setting. There was no significant change of RT ($F_{3,2}=0.869$; $p=0.574$), MT ($F_{3,2}=4.524$; $p=0.186$) or PRr ($F_{4,1}=92.178$; $p=0.078$) (Figure 2.2) in the group with VL thalamic DBS as compared to the off stimulation setting. Comparison between the two groups revealed a significant difference with respect to PRr (stimulation*group; $F_{8,24}=2.502$ $p=0.39$) (Figure 2.2).

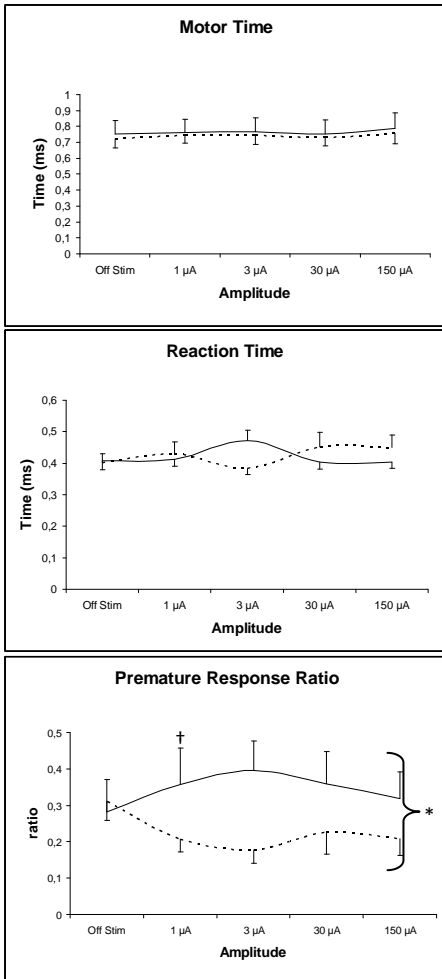


Figure 2.2 Behavioural results of rats subjected to different stimulation amplitudes using bilateral high frequency stimulation in either the MD thalamic nucleus (straight line) or the VL thalamic nucleus (dotted line). Data are shown as mean \pm SEM. A dagger (\dagger) indicates significant difference as compared to off stimulation within groups, an asterisk (*) indicates significant difference between groups. MD: mediodorsal thalamic nucleus; VL: ventrolateral thalamic nucleus.

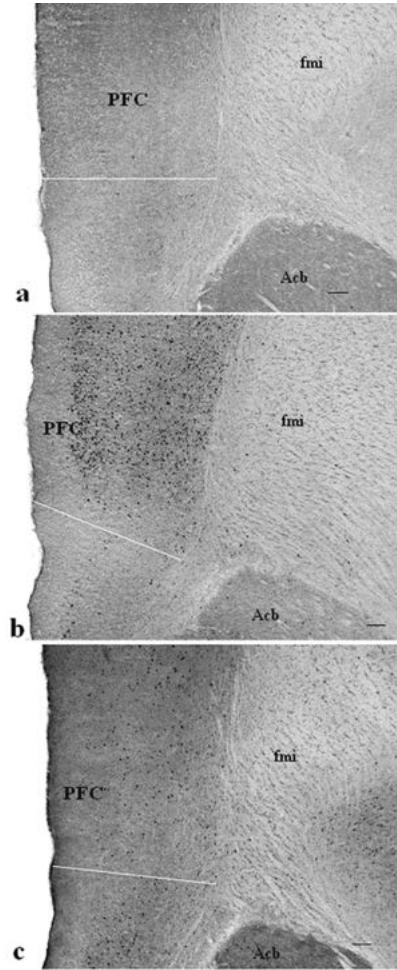


Figure 2.3 Representative low-power photomicrographs of a 30 μ m-thick frontal section at bregma level 2.20 mm showing the prefrontal cortex (PFC) of a control rat (a), a rat stimulated in the MD thalamic nucleus (b) and a rat stimulated in the VL thalamic nucleus (c). The small dark dots represent c-Fos-ir cells. White lines show the lower border of the PFC. Scale bar=300 μ m. fmi: forceps minor of the corpus callosum; Cg1: Cingulate cortex area 1; DP: dorsopeduncular cortex.

Evaluation of the number of c-Fos-ir cells

Since stimulation was performed bilaterally and no left/right difference was found in the number of c-Fos-ir cells, data from the left and right hemispheres were pooled. We found a significant increase in the number of c-Fos-ir cells in the prefrontal cortex ($F_{2,11}=4.494$, $p<0.05$; Figure 2.3) in the rats stimulated in the MD thalamic nucleus (21.92 ± 5.404 c-Fos-ir cells/ mm^2) as compared to control animals (7.603 ± 2.913 c-Fos-ir cells/ mm^2) and animals with DBS of the VL thalamic nucleus (9.188 ± 2.179 c-Fos-ir cells/ mm^2).

In the deep cerebellar nuclei a significant difference in the number of c-Fos-ir cells in the dentate nucleus ($H_2=6.057$, $p<0.05$), the interpositus nucleus ($H_2=7.214$, $p<0.05$), and in the fastigial nucleus ($H_2=7.419$, $p<0.05$) was found after thalamic DBS. The number of c-Fos-ir cells is lower in the MD thalamic nucleus -stimulated group compared to the VL thalamic nucleus -stimulated group and the control group in all three cerebellar nuclei (Figure 2.4 and Figure 2.5). Post-hoc analysis shows that this difference is significant between the MD thalamic nucleus -stimulated group and the control group in the dentate nucleus ($U=6.00$, $r=-0.59$); in the other nuclei there is a trend which does not reach significance (IN: $U=8.00$, $r=-0.52$; FN: $U=7.50$, $r=-0.54$). When comparing the MD thalamic DBS group and the VL thalamic stimulated animals there is a significant difference in the interpositus nucleus ($U=1.00$, $r=-0.77$) and in the fastigial nucleus ($U=1.00$, $r=-0.77$); in the dentate nucleus there is a trend to significance ($U=4.00$, $r=-0.61$). There is a negative relationship between the c-Fos expression in the PFC and the c-Fos expression in the DCbN in the animals that were stimulated in the MD thalamic nucleus ($\tau=-1.000$, $p<0.05$; Figure 2.6); a higher c-Fos expression in the PFC correlated with a lower c-Fos expression in the DCbN. No correlation was found in the animals with VL thalamic DBS ($\tau=0.200$, $p=0.624$). All results are summarized in Table 2.1.

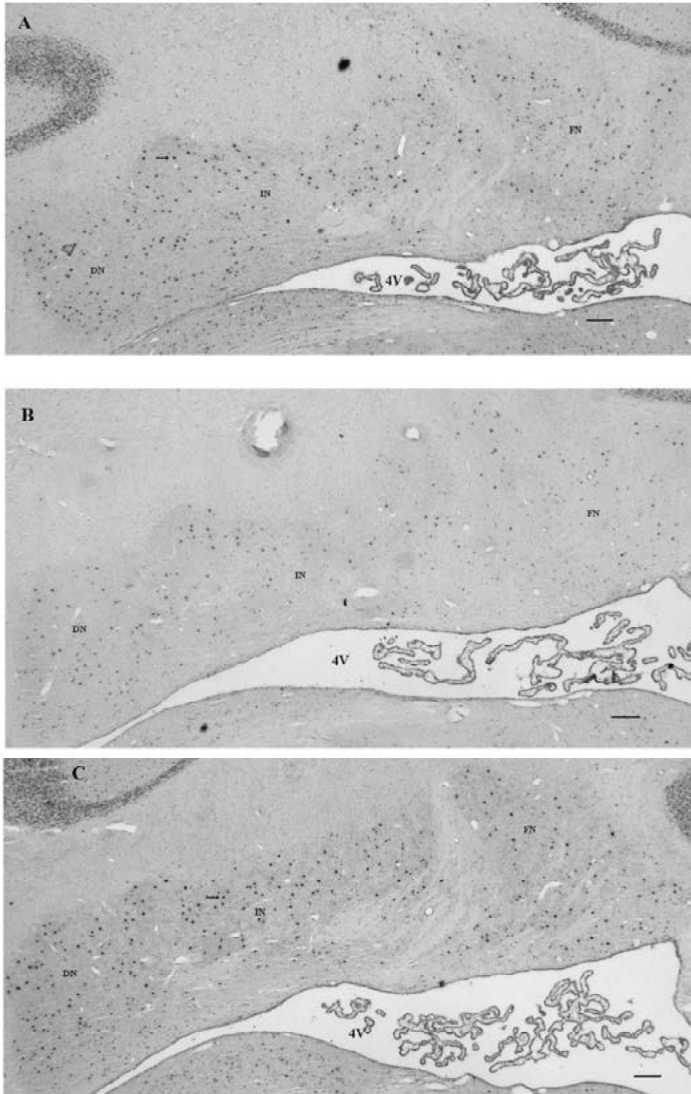


Figure 2.4: Representative low power photomicrographs of c-Fos expression in a 10 μ m thick frontal section of the deep cerebellar nuclei at bregma level -11.60 mm in a control rat (A), a rat stimulated in the MD thalamic nucleus (B), and a rat stimulated in the VL thalamic nucleus (C). The arrows point to a c-Fos immunoreactive (c-Fos-ir) cell. Cells are considered to be c-Fos-ir if their density is 75% above the background density of the section. In picture B there are no cells that meet this criterion. Scale bar=300 μ m. MD: mediodorsal thalamic nucleus; VL: ventrolateral thalamic nucleus; DN: dentate nucleus of the cerebellum; IN: interpositus nucleus of the cerebellum; FN: fastigial nucleus of the cerebellum; 4V: fourth ventricle.

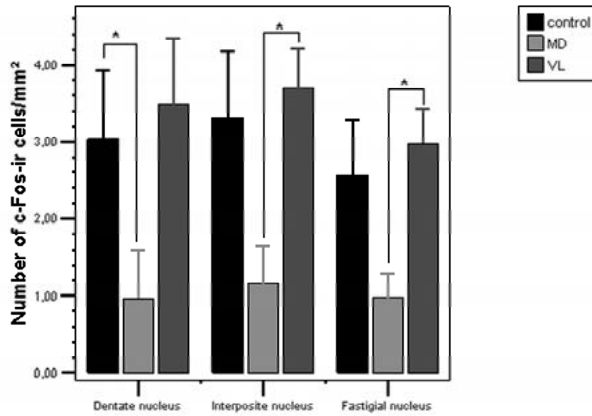


Figure 2.5 Grouped data of the c-Fos expression in the deep cerebellar nuclei. Data are represented as mean \pm S.E., showing control animals, animals with MD thalamic DBS and animals with VL thalamic DBS. An asterisk (*) indicates a significant difference between groups. MD: mediodorsal thalamic nucleus; VL: ventrolateral thalamic nucleus; DN: dentate nucleus of the cerebellum; IN: interpositus nucleus of the cerebellum; FN: fastigial nucleus of the cerebellum.

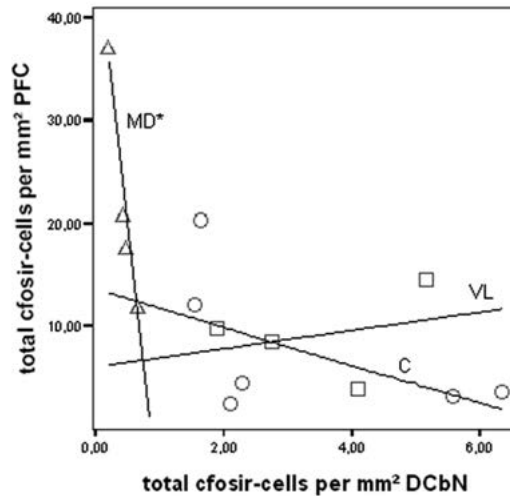


Figure 2.6 Correlation graph of c-Fos data in the DCbN and the PFC. The graph shows the amount of c-Fos-ir cells/mm² in the DCbN on the X-axis and the amount of c-Fos-ir cells/mm² in the PFC on the Y-axis. Circles represent control animals, triangles represent animals with MD thalamic DBS, and squares represent animals with VL thalamic DBS. Regression lines were fitted to the data to clarify the relationship between the parameters. An asterisk (*) indicates a significant correlation ($\tau < 0.05$). DCbN: deep cerebellar nuclei; PFC: prefrontal cortex; MD: mediodorsal thalamic nucleus; VL: ventrolateral thalamic nucleus.

Table 2.1 Summary of results.

Parameter	Behaviour			C-Fos expression			
				DCbN			
Stimulation Site (below)	PRr	RT	MT	PFC	DN	IN	FN
MD	↑	=	=	↑ *†	↓ *	↓ †	↓ †
VL	=	=	=	=	=	=	=

Upward arrows (↑) indicate a significant increase of the measured parameter; downward arrows (↓) indicate a significant decrease. An equation-sign (=) indicates no significant change was found. * indicates a comparison to control; † indicates a comparison between stimulation groups. The behavioural parameters were compared to the values in the same group when the animals were not stimulated. Significant changes in PRr were only found when stimulating with 1 μ A. No change was found in the motor parameters (RT and MT) irrespective of the stimulation parameters. PFC: prefrontal cortex; DCbN: deep cerebellar nuclei; DN: dentate nucleus of the cerebellum; IN: interpositus nucleus of the cerebellum; FN: fastigial nucleus of the cerebellum; PRr: premature response ratio; RT: reaction time; MT: motor time; MD: Mediodorsal thalamic nucleus; VL: ventrolateral thalamic nucleus.

Discussion

The present study shows that there is an increase in PRr in rats after MD thalamic DBS. This can be interpreted as an increase in impulsivity as PRr is thought to reflect an impairment in response inhibition⁴⁸. This is in line with previously described effects of lesioning of the MD thalamic nucleus on impulsive behaviour³⁴ and confirms that, at the behavioural level, high frequency DBS mimics that lesion.

The c-Fos expression in all deep cerebellar nuclei is lower after mediodorsal thalamic nucleus stimulation as compared to animals stimulated in the ventrolateral thalamic nucleus and controls; this is significantly correlated with an increase of c-Fos expression in the prefrontal cortex as compared to controls and animals stimulated in the VL thalamic nucleus: the higher the expression in the PFC, the lower the expression in the deep cerebellar nuclei. There is no change in motor parameters of the choice reaction time test after MD thalamic stimulation, which implicates that the lower c-Fos expression we found in this group is related to the increase in impulsivity. Nabeyama and co-workers recently showed that in OCD patients the activation on f-MRI while performing a Stroop test is significantly lower in the right cerebellar tonsil compared to control patients, and that this activation increases after successful behavioural treatment²⁷. They suggest that activation of the cerebellum and other regions related to the process of inhibition of irrelevant information might play a role in the improvement of symptoms in these patients. Others have also suggested that the cerebellum is involved in direction of selective attention, and that disruption of cerebello-thalamo-cortical connections leads to an impairment of selective attentional processes^{49,50}. Anatomical studies have shown that the DCbN project to the MD thalamic nucleus^{5,7}. In the rat, projections from the MD thalamic nucleus to the cortex are considered to define the rat PFC⁵¹. Retrograde tracing studies have shown that the cerebellar-recipient parts of the MD and VL thalamic nuclei project to the dorsolateral

PFC⁸. The same group has shown that specific areas of the cerebellar cortex that project to the PFC also receive projections from the same part of the PFC⁴. They hypothesize that there are several anatomically and functionally distinct cerebello-cortico-cerebellar loops. Our findings confirm that the MD thalamic nucleus is part of a cerebello-cortico-cerebellar loop through the PFC. Strick and co-workers show that the majority of the projections from the DCbN to the PFC arise from the dentate nucleus⁸, and that the projections from the dentate nucleus to the motor cortex originate only from 30% of the volume of the dentate nucleus⁵². They also show that these projections to the motor cortex arise from the dorsal part of the dentate nucleus, while the projections to the PFC arise predominantly from the ventral part of the dentate nucleus^{8,53,54}. Based on these different anatomical connections they suggest that different parts of the dentate nucleus subserve different functions. Matano showed that there is a larger increase in the volume of the ventral part of the dentate nucleus in comparison to the dorsal part when comparing humans to great apes⁵⁵. This also supports the conclusion that the ventral part of the dentate nucleus is involved in cognitive processing whereas the dorsal part subserves motor functions. In our study we found lower c-Fos expression in all cerebellar nuclei after MD thalamic DBS, without predominance in the dentate nucleus. However, if there are functionally distinct subdivisions of the dentate nucleus, an effect in one subdivision may not be evident when analysing the nucleus as a whole. Since we did not analyse the areas of the dentate nucleus separately, we cannot draw any conclusions about anatomically or functionally distinct subdivisions of the dentate nucleus. Furthermore, as discussed later, there are probably several underlying mechanisms by which deep brain stimulation can exert its influence on adjacent and remote brain structures. The exact underlying mechanisms, however, are not entirely clear. It is therefore as yet unknown whether these mechanisms pay respect to somatotopic organisation of neurons, especially when concerning remote effects. Additionally, there are authors who claim a more diffuse somatotopic organisation in the DCbN more consistent with a representation of muscle synergies⁵⁶, in contrast to the above mentioned point-to-point somatotopy^{5,52,53}.

In the animals stimulated in the VL thalamic nucleus we found no changes of behavioural parameters or c-Fos expression in the PFC or the DCbN as compared to control animals. The VL thalamic nucleus has always been seen as the primary target of cerebellar projections, and is still considered to be the main relay nucleus through which the cerebellum projects to the somatomotor cortex. VL thalamic DBS has been shown to be effective in reducing tremor in patients³⁷ and in an animal model of tremor⁵⁷. It is therefore very interesting that we found no changes in motor parameters after DBS of the VL thalamic nucleus. This does seem to correspond to the unaltered c-Fos expression in the DCbN. Possibly the lack of effect of VL thalamic DBS in our model is due to the fact that we stimulate naïve animals. To our knowledge, studies evaluating the mechanism of thalamic DBS in animals without tremor have never analysed effects on motor parameters. VL thalamic DBS in tremor patients is not

known to induce changes in impulsive behaviour; the lack of behavioural change in our model is in concordance with this. The absence of an effect of VL thalamic DBS on premature responding and on c-Fos expression in the DCbN supports the idea that the effects of MD thalamic stimulation we described are mediated by the prefrontal cortex and the cerebellum and are stimulation area dependent.

There is ongoing discussion about the underlying mechanism of electrical brain stimulation, despite extensive research. Nevertheless, some concepts have been generally accepted. Functionally, DBS mimics a lesion³⁵, suggesting that the main effect of DBS is caused by inhibition of the target area⁵⁸. However, the effect of DBS may be more complex: local inhibition of soma in combination with generation of an action potential in the surrounding axons^{35, 59}. Recently, the role of orthodromic versus antidromic action of DBS has gained interest. For instance, the effect that DBS of the rat nucleus accumbens has on the PFC is likely to be antidromic, whereas the effect of stimulation of the MD thalamic nucleus was more likely to be orthodromic⁶⁰. We hypothesize that in our model the activation of neurons in the PFC is caused by a combination of orthodromic and antidromic stimulation since we see increased c-Fos expression in all layers of the PFC. The projections from the MD thalamic nucleus to the PFC terminate mostly in layer III of the cortex and to a lesser degree in layer I, while the reciprocal corticothalamic projections from the PFC to the MD thalamic nucleus originate mostly in layer VI⁵¹. It is postulated that, depending on the myelo-architecture of the stimulation site, electrical stimulation leads to antidromic spikes in axons which either collide with ongoing spontaneous spikes or cause a low-frequency spike output^{61,62}, thereby functionally suppressing the neuronal message⁶³. Thus, MD thalamic stimulation possibly, via antidromic inhibition, resulted in a decrease in c-Fos expression in the DCbN. Alternatively, it is known that DBS can alter neurotransmitter release^{35,63}. Hence, orthodromic activation of the PFC may have altered the neurochemical message in the cerebellum, leading to a decreased activation of the DCbN.

In conclusion, this study supports the hypothesis that the cerebellum is involved in impulsive behaviour. High-frequency DBS of the MD thalamic nucleus has a strong effect on PRr and on c-Fos expression in the PFC and the DCbN, while VL thalamic stimulation has no significant effect on behaviour or c-Fos expression. The lack of effect of thalamic DBS on motor parameters implies that the role of the cerebellum may be a non-motor one, however, the exact nature of the place that the cerebellum has in the generation of impulsive behaviour remains to be elucidated. It is possible that the cerebellum plays a role in the process of inhibition of irrelevant information, thereby directing selective attention, and that the decreased c-fos expression we find implies a decrease of this inhibition, resulting in impulsive behaviour.

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Chapter 3

**Cerebellar nuclei are activated by high-frequency
stimulation of the subthalamic nucleus**

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Abstract

The cerebellum, primarily considered a pure motor structure, is increasingly considered to play a role in behaviour and cognition. In a similar manner, there is increasing evidence that the basal ganglia are involved in non-motor processes. Recently a direct connection between the cerebellum and the basal ganglia has been shown to exist. High-frequency stimulation (HFS) of the subthalamic nucleus (STN) has become an accepted treatment in advanced Parkinson's disease (PD). We performed HFS of the STN in rats to evaluate the neuronal activation in the deep cerebellar nuclei (DCbN) using c-Fos immunohistochemistry. We found an increased c-Fos expression in the DCbN. Previously, we have shown that STN HFS in rats leads to decreased impulsive behaviour and our findings now suggest a link with increased DCbN activity. This is in line with our previous work showing that decreased DCbN activity is accompanied by disruptive behaviour. We suggest that the DCbN play a role in the selection of relevant information on which a behavioural response is based. The connection between the cerebellum and the basal ganglia may imply a role for the cerebellum in behavioural aspects of disorders of the basal ganglia.

Introduction

The cerebellum was primarily considered to be predominantly involved in motor coordination and control¹. The last two decades there is increasing evidence for a role of the cerebellum in cognition and behaviour. This evidence comes from tracing studies showing a projection from the cerebellum to the prefrontal cortex^{2,3} and clinical studies in patients with cerebellar lesions showing cognitive and behavioural disturbances⁴⁻⁹. Furthermore imaging studies in patients with neuropsychiatric disorders such as schizophrenia¹⁰, attention-deficit-hyperactivity disorder¹¹⁻¹³, and anxiety disorders¹⁴⁻¹⁸ show altered anatomy and activation patterns in the cerebellum compared to controls. In a similar manner, the basal ganglia have classically been regarded as being predominantly involved in motor behaviour. Results from tracing studies imply that the basal ganglia are also involved in cognitive and behavioural processing³. Clinical evidence can be found in idiopathic Parkinson's disease (PD) which is characterised by motor as well as cognitive and psychiatric symptoms¹⁹.

A central feature of the pathophysiology in PD is hyperactivity of the subthalamic nucleus (STN) caused by a loss of dopaminergic cells in the substantia nigra²⁰. This leads to an increased activity of the output of the basal ganglia and subsequent inhibition of thalamocortical pathways. High-frequency stimulation (HFS) of the STN has become an important treatment option in advanced PD in the last three decades²⁰. A substantial portion of patients treated with STN HFS develop cognitive problems²¹. Moreover, STN HFS in animal models has been shown to alter cognitive functioning^{22,23}. Functional imaging is increasingly being used to analyse which structures are involved in PD and also to analyse the effect of STN HFS. These studies almost invariably show increased metabolism of the cerebellum in PD patients compared to control patients²⁴⁻²⁶, which can be reversed by levodopa treatment²⁷. Studies analyzing STN HFS show decrease as well as increase of metabolism in several regions of the cerebellum during therapeutic STN HFS^{25,27-33}. In some studies these changes in metabolism have been linked to motor improvement^{31,34}; however, in other studies this correlation was not found^{33,35}. Correlation with cognition or behaviour was not addressed in these studies. From an anatomical point of view, recent tracing experiments in nonhuman primates using a retrograde tracer have shown that there is a disynaptic projection from the STN to the cerebellum, and from the dentate nucleus of the cerebellum (DN) to the striatum, the input structure of the basal ganglia^{36,37}. These findings imply that there is a closed loop between the cerebellum and the basal ganglia, which seems to be independent of cortical input^{37,38}.

In this study we used HFS of the STN in a rat model to study the relationship of the STN with the cerebellum. Previously, we have demonstrated that HFS of the mediodorsal nucleus of the thalamus (MD) causes an increase in impulsive behaviour, which is accompanied by a decreased activation of the deep cerebellar nuclei (DCbN)³⁹. Furthermore we have shown that in rats in which panic-like behaviour is induced by HFS of the dorsolateral periaqueductal gray (dIPAG), there is again a decreased

activation of the DCbN⁴⁰. We hypothesize that the cerebellum plays a role in the selection of relevant information to coordinate appropriate behaviour, and that deactivation of the cerebellum may lead to an increase of inappropriate behaviour. It has been shown in a rat model that HFS of the STN in naïve animals causes a decrease in impulsive behaviour²³. We therefore expect an increased activation of the DCbN in the present study. To map the activation in the DCbN we used c-Fos immunohistochemistry. c-Fos is an immediate early gene which is considered to reflect acute neuronal activation^{41,42}.

Materials and methods

Subjects

Subjects were Lewis male rats (n=10, 10 weeks old, bred and housed at the Central Animal Facility of Maastricht University, Maastricht, The Netherlands) with an average body weight of 300 to 400 grams at the time of surgery. They were housed individually in standard cages with sawdust bedding in an air-conditioned room (about 20°C) under a 12/12-h reversed light/dark cycle. Standard laboratory chow (Hopefarms, Woerden, The Netherlands) and water were available *ad libitum*. All experimental procedures were approved by the Animal Experiments and Ethics Committee of Maastricht University.

Surgical procedure

Rats were randomly assigned to one of two experimental groups: “sham” (n=5) and “stimulation” (n=5). A detailed description of the surgical procedure was reported previously⁴³⁻⁴⁵. In brief, animals were anaesthetized throughout the entire procedure using a combination of ketamine (90 mg/kg s.c.) and xylazine (10 mg/kg s.c.). They were placed in a stereotactic apparatus (Stoelting, Wood Dale, USA; model 51653). After making a burr hole in the skull all rats received bilateral implantation of electrodes at the level of the STN (coordinates from Bregma : AP -3.8, ML 2.5 and V -8,0)⁴⁶. A gold-plated needle-like electrode with an inner wire of a platinum-iridium combination (Technomed, Beek, The Netherlands), with a tip diameter of 50µm and a shaft diameter of 250 µm was used. In the sham group the electrodes were inserted and immediately removed. In the stimulation group the electrodes were fixed in place using dental cement (Henaues Kulzer, Hanau, Germany).

Deep brain stimulation

Rats were allowed to recover from surgery for four days. On days five, six and seven postoperatively they received chronic stimulation during one hour each day. Stimulation was performed at 130Hz, 60µs and 30 µA. These are therapeutic

stimulation parameters⁴³. Stimuli were delivered using a World Precision Instrument (WPI, Berlin, Germany) accupulser (A310) and a stimulus isolater (A360). Using a digital oscilloscope (Agilent 54622D oscilloscope, Agilent Technologies, The Netherlands), real time verification was obtained for the parameters applied during stimulation for both electrodes of the bilateral construction. The mode of stimulation was bipolar in all cases (inner electrode negative and outer electrode positive).

Histological processing

On post-operative day 7 the rats were sacrificed two hours after their last stimulation session. The rats were deeply anaesthetized and transcardially perfused with Tyrode (0,1 M) and fixative containing 4% paraformaldehyde, 15% picric acid and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.6). Brains were removed and post-fixed for 2 h followed by overnight immersion in 15% sucrose at 4°C. Brain tissue was then quickly frozen with CO₂ and stored at -80°C. Subsequently, the cerebelli were cut serially on a cryostat into 10µm frontal sections. These were collected on gelatine-coated glasses and stored at -20°C. We used a previously published protocol for c-Fos immuno-histochemistry³⁹. In brief, sections were incubated for two days with the primary antibody (polyclonal Rabbit anti c-Fos; sc-52; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Subsequently sections were incubated overnight with the secondary antibody, followed by incubation with avidin-biotin-peroxidase complex for 2 hours. To visualize the immune complex sections were incubated with 3,3-diaminobenzidine (DAB) solution for 10 minutes. After dehydrating, all sections were coverslipped with Pertex (HistolabProducts ab, Goteborg, Sweden). A series of parallel sections was Nissl-stained to facilitate correct identification of the cerebellar nuclei. Additionally, for each animal the area of the STN was cut into 30 µm sections and stained with standard haematoxylin-eosin (Merck, Darmstadt, Germany) staining to locate the electrode tips.

Quantitative evaluation of c-Fos positive cells

Systematic cell counts of c-Fos immunoreactive (c-Fos-ir) cells were performed in the DCbN as previously described³⁹. For each animal four to ten sections with cerebellar nuclei (coordinates from Bregma AP -11.2 to -11.8) were analysed. In ImageJ (version 1.41, NIH, USA) the nuclei were delineated, the delineated area and the average grey value for each nucleus were measured. C-Fos-ir cells were counted in a semi-automatic fashion. We considered a cell to be c-Fos positive if its density was 65% higher than the average gray value of that nucleus. Artefacts in the sections were excluded from the delineations to ensure accuracy of the measurements. The number of c-Fos-ir cells per square millimeter was calculated for each nucleus.

Statistical analysis

The data of the c-Fos-ir cell density showed a non-normal distribution in Kolmogorov-Smirnov and Shapiro-Wilk tests. C-Fos expression in the DCbN was therefore analysed with the non-parametric Mann-Whitney tests.

Results and discussion

In the present study we performed high frequency stimulation of the subthalamic nucleus to analyse the relationship of the STN with the cerebellum. In all animals included in this study, the electrode tips were positioned correctly in the STN (Figure 3.1). No histological damage was observed except in the electrode trajectory, suggesting that repeated stimulation did not cause observable tissue damage. Our hypothesis was that there would be increased activation in the deep cerebellar nuclei of stimulated animals compared to controls. We found an increase in c-Fos expression in the rats stimulated in the STN compared to the sham-group in the right DN ($p < 0,001$), right interpositus nucleus (IN) ($p < 0,05$) and the right fastigial nucleus (FN) ($p < 0,05$) (Figures 3.2 and 3.3). In the left nuclei there was a trend towards a higher density in the stimulated rats, however, this did not reach significance. Both the STN and the cerebellum have motor functions as well as cognitive and limbic functions. In previous studies, we performed STN HFS and found no effects on motor function but a decrease in impulsive behaviour in naïve rats²³. In a rat model of Parkinson's disease using 6-OHDA-infusion in the striatum, these animals showed worsened motor function and an increase in impulsive behaviour, both of which were reversed by bilateral STN HFS⁴³. This suggests that the increase of c-Fos expression in the DCbN is accompanied by improved cognitive functioning during STN HFS. We have previously shown that increased impulsivity caused by HFS of the MD thalamic nucleus is accompanied by a decrease of c-Fos expression in the DCbN³⁹. In another study we showed a decrease in c-Fos expression in the DCbN of rats with DIPAG HFS induced panic-like behaviour⁴⁰. Altogether these results suggest a role for the cerebellum in non-motor behaviour, in which an increase of abnormal behaviour seems to be related to a decrease in activity of the DCbN and in which a decrease of abnormal behaviour is associated to an increase in DCbN activity. Interestingly, in contrast to the results of our previous behavioural studies in rats^{23,43}, PD patients treated with STN HFS often show an increase of impulsive behaviour [for review see Temel *et al.*²²]. On the other hand, several studies in PD patients treated with STN HFS show a decrease in obsessive compulsive behaviour^{47,48}.

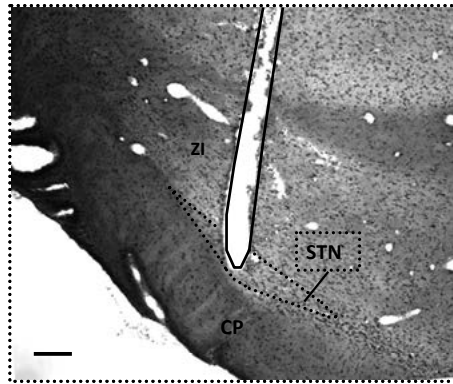


Figure 3.1 Representative low power photomicrograph of a 30 μm -thick frontal section of the brain of a rat subjected to stereotactic implantation of a concentric bipolar electrode in the subthalamic nucleus (STN) (scale bar = 400 μm) CP: cerebral peduncle; ZI: zona incerta.

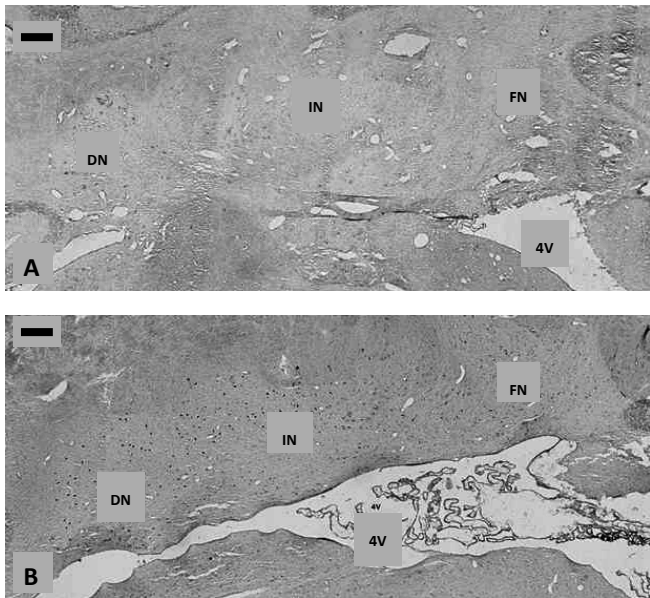


Figure 3.2 Representative low power photomicrographs of a 10 μm -thick horizontal section of the deep cerebellar nuclei (DCbN) at bregma -11.60 mm in a sham operated animal (A) and an animal stimulated in the subthalamic nucleus (STN) (B). Dark-gray cells represent c-Fos-immunoreactive (c-Fos-ir) cells. Note the higher density of c-Fos-ir cells in the DCbN of the animal stimulated in the STN. Scale bar=250 μm Abbreviations: DN: Dentate Nucleus, IN: Interpositus nucleus, FN: Fastigial Nucleus, 4V: fourth ventricle.

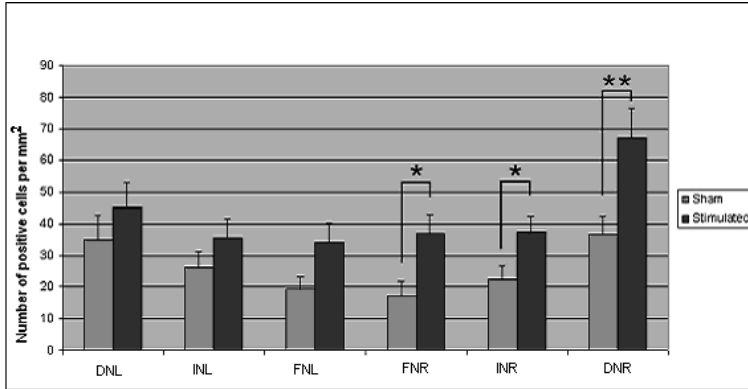


Figure 3.3 Density of c-Fos-ir cells in the deep cerebellar nuclei. Data are represented as means plus S.E.M. showing sham operated animals and animals stimulated in the subthalamic nucleus (STN). The asterisk (*) indicates a significant difference between the groups ($p < 0.05$); a double asterisk (**) indicates $p < 0.001$. Note the significantly higher density of c-Fos-ir neurons in the groups with animals stimulated in the STN. DNL: Dentate nucleus, left side, INL: Interpositus nucleus left side, FNL: Fastigial nucleus, left side, FNR: Fastigial nucleus right side, INR: Interpositus nucleus, right side, DNR: Dentate nucleus right side.

Functional imaging studies analysing the effect of STN HFS using FDG-PET^{25,27,28,31,32}, rCBPF-PET^{29,30,33}, ECD-SPECT³⁵ and [¹⁵O]H₂O-PET⁴⁹ show increased as well as decreased metabolic activity in the cerebellum during STN HFS. These changes were sometimes correlated to motor improvement^{31,34}, although other groups found no correlation^{33,35}. Correlation with cognitive or behavioural parameters was never analysed. Stefurak and coworkers describe a patient with bilateral STN-HFS who showed motor improvement and increased activity of the cerebellum on fMRI when the left STN was stimulated³⁴. When the right electrode, which was in the zona incerta, was stimulated she suffered from acute depressive dysphoria. There was no change in activity of the cerebellum when this electrode was stimulated.

These results imply that there may be a functional connection between the STN and the cerebellum. Aravamuthan and coworkers used probabilistic diffusion tractography (PDT) to analyse the topographical connections of the STN in humans⁵⁰. They found an anatomical connection between the STN and the mid-cerebellum. However, this technique does not give information about the direction or the excitatory or inhibitory nature of these connections. Recent tracing experiments in nonhuman primates using a retrograde tracer that is transported transneuronally show that there is a bisynaptic connection from the STN to the contralateral cerebellar cortex, and from the DCbN to the striatum, the input stage of basal ganglia processing³⁶⁻³⁸. These connections are topographically organised^{36,38}, and seem to be involved in integrating basal ganglia and cerebellar functions in motor as well as

nonmotor processing^{37,38}. It has already been shown that both the basal ganglia and the cerebellum form anatomically and functionally separate closed loops with several cortical areas^{2,3}. It was postulated that interaction between these loops only takes place at the level of the cortex. However, the current findings imply that the cerebellum and the basal ganglia may be linked together to form a functional network which is independent from the cortex^{37,38}.

We found a significant effect only in the right DCbN. Although a technical problem such as a slight difference in electrode positioning between the right and the left STN cannot be ruled out, it is possible that this is related to the different functions of the cerebellar hemispheres. Several studies have shown that injury to the cerebellum in preterm infants as well as adults leads to neuropsychological deficits, regardless of which cerebellar hemisphere is injured^{4,9}. However, there is a difference in the type of neuropsychological deficits that are seen: right-sided lesions were associated with deficits of logical reasoning and language processing; functions typically ascribed to the left cerebral hemisphere⁹. In contrast, left-sided cerebellar lesions were associated with deficits in right-hemispheric functions including attentional and visuospatial skills⁹. Behavioural and affective disturbances were seen almost exclusively in patients with right-sided cerebellar lesions⁹.

Conclusion

The present study adds to the evidence that there is a functional connection between the cerebellum and the basal ganglia, and that this connection may play a role in non-motor as well as in motor behaviour. STN HFS leads to an increased activation of the DCbN. Previous research has shown that STN HFS in naïve rats leads to a decrease of impulsive behaviour; furthermore we have previously shown that impulsive behaviour as well as panic-like behaviour is accompanied by de-activation of the DCbN. The DCbN may therefore play a role in the selection of relevant information on which a behavioural response is based. The connection between the cerebellum and the basal ganglia further implies a role for the cerebellum in behavioural aspects of basal ganglia disorders.

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Chapter 4

**Periaqueductal grey stimulation induced panic-like
behaviour is accompanied by deactivation of the deep
cerebellar nuclei**

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Abstract

Until recently the cerebellum was primarily considered to be a structure involved in motor behaviour. New anatomical and clinical evidence has shown that the cerebellum is also involved in higher cognitive functions and non-motor behavioural changes. Functional imaging in patients with anxiety disorders and in cholecystokinin tetrapeptide (CCK-4) induced panic-attacks show activation changes in the cerebellum. Deep brain stimulation (DBS) of the dorsolateral periaqueductal grey (dIPAG) and the ventromedial hypothalamus (VMH) in rats has been shown to induce escape behaviour, which mimics a panic attack in humans. We used this animal model to study the neuronal activation in the deep cerebellar nuclei (DCbN) using c-Fos immunohistochemistry. c-Fos expression in the DCbN decreased significantly after inducing escape behaviour by stimulation of the dIPAG and the VMH, indicating that the DCbN were deactivated. This study demonstrates that the DCbN are directly or indirectly involved in panic attacks. We suggest that the cerebellum plays a role in the selection of relevant information, and that deactivation of the cerebellar nuclei is required to allow inappropriate behaviour to occur, such as panic attacks.

Introduction

The cerebellum has long been considered to be predominantly involved in motor coordination and control^{1,2}. The last two decades, however, it has become increasingly clear that the cerebellum is also involved in behaviour and cognition^{3,4}. Schmahmann and Sherman described the cerebellar cognitive affective syndrome (CCAS) consisting of impairment of executive functions, difficulties with spatial cognition, changes of personality including disinhibited or inappropriate behaviour and language deficits⁵. Recent neuroanatomical and functional imaging research has pointed towards a role for the cerebellum in neuropsychiatric diseases such as autism, schizophrenia and mood and anxiety disorders⁶⁻¹¹. With respect to anxiety disorders, Levinson described the existence of cerebellar-vestibular dysfunction in 94% of patients with various types of anxiety disorders¹². De Bellis and Kuchibhatla showed that children and adolescents with post-traumatic stress disorder related to maltreatment have significantly smaller cerebelli than paediatric subjects with generalized anxiety disorder and healthy subjects¹³. Several groups have shown structural and functional differences in the cerebellum of patients with anxiety disorders compared to control subjects¹⁴⁻²². In patients with obsessive-compulsive disorder (OCD), a disorder characterised by anxiety, an increase in grey matter was found in the anterior cerebellum²². Using functional imaging, Busatto and colleagues found an increased activation in the superior cerebellum of OCD patients compared to control subjects¹⁴. Another group showed that after treatment, OCD patients showed a decreased activation in the cerebellum compared to pretreatment scans¹⁵. In contrast, Nabeyama and coworkers found decreased activation in untreated OCD-patients compared to control subjects, which increased after successful treatment of these patients¹⁶. In patients with panic disorder an increased activation of the cerebellum was found when compared to control patients²⁰, this activation decreased again after successful treatment of these patients²¹. In healthy individuals in whom panic attacks were induced by cholecystokinin tetrapeptide (CCK-4) an increased activation of the cerebellum was found, especially in the vermis²³⁻²⁶. However, the significance of these findings is not yet clear.

Panic disorder is one of the most frequently encountered anxiety disorders²⁷. Panic attacks consist of an acute, sometimes unexpected onset of intense fear, accompanied by a variety of physical symptoms²⁷. Treatment usually includes behavioural and pharmacological therapies, either alone or in combination. It is thought that panic attacks may be the result of an abnormally sensitive fear network²⁸. The periaqueductal grey (PAG) is known to be part of this fear network which further consists of the central nucleus of the amygdala, ventromedial hypothalamus (VMH), hippocampus, and other brainstem regions²⁸. Deep brain stimulation (DBS) in rats of the dorsolateral periaqueductal grey (dIPAG) and one of its target structures, the ventromedial hypothalamus has been shown to elicit a typical behaviour consisting of vigorous running and jumping²⁹. This behaviour, also known as escape behaviour, is

accompanied by strong emotional and autonomic activation, and thereby mimics panic attacks in humans^{29,30}.

In the present study, we used DBS of the dIPAG and the VMH as a model of panic attack to evaluate the involvement of the deep cerebellar nuclei (DCbN). In a previous study, we have found that increased impulsivity in rats was accompanied by a deactivation of the DCbN⁴. These findings suggested that the cerebellum plays a role in regulating appropriate behaviour. Based on their findings in a patient with pathological laughter and crying, Parvizi and co-workers hypothesized that the cerebellum adjusts behavioural responses to the correct situational context of a stimulus, and that when the cerebellum operates on the basis of incorrect information, this could lead to inappropriate or even chaotic behaviour³¹. We hypothesized that deactivation of the DCbN was needed for pathological behaviour to occur. In line with this, here we predicted to find again a deactivation of the DCbN in rats with panic-like behaviour. Panic attacks consist of an inadequate response to a stimulus. Possibly the cerebellum also plays a role in regulation of the response to these stimuli by selection of relevant information. Deactivation of the DCbN may then be linked to an inadequate response to a certain stimulus by disabling this selection process. To analyse activation patterns we have used c-Fos immuno-histochemistry. We focused on the DCbN as these are the output structures of the cerebellum. c-Fos was chosen as it is an immediate early gene which is considered to reflect neuronal activation^{32,33}. The level of c-Fos protein is maximal about two hours after administration of a stimulus, and disappears again after four to eight hours even if the stimulus is continued³². Therefore, the c-Fos expression found two hours after a specific stimulus in controlled conditions can be considered to result directly from the stimulus, and this expression can be used to identify brain areas influenced by this stimulus³³.

Materials and methods

Animals

The animals used were male albino Wistar rats (n=15, 12 weeks old, bred and housed at the Central Animal Facility of the Maastricht University, The Netherlands), with an average body weight of 300-350g at the time of surgery. Rats were housed individually in standard cages with sawdust bedding in an air-conditioned room under a 12/12-h reversed light/dark cycle. Food, standard laboratory chow (Hopefarms, Woerden, the Netherlands) and water were available *ad libitum*. All experiments were approved by the Animal Experiments and Ethics Committee of Maastricht University.

Surgical procedure

Rats were randomly assigned to one of the following three experimental groups: control (no surgery), dIPAG DBS or VMH DBS. A detailed description of the surgical

procedure was reported previously^{29,34}. In brief, animals were anaesthetized throughout the entire procedure using a combination of ketamine (90 mg/kg) and xylazine (10 mg/kg) injected subcutaneously. Rats were placed in a stereotactic apparatus (Stoelting, Wood Dale, USA; model 51653). After making a burr hole, rats received implantation of a unilateral electrode at the level of the right dIPAG (coordinates from Bregma: AP=-7.6 mm, ML=0.7 mm, V=-4.8 mm; approached with a coronal angle of 10 degree) and the right VMH (coordinates from Bregma: AP=-2.5 mm, ML=0.5 mm, V=-9.5 mm). A construction of one electrode with an inner wire of a platinum-irridium combination (Technomed, Beek, the Netherlands), with a tip diameter of 50 µm and a shaft diameter of 250 µm, was implanted in this experiment³⁵. The electrodes were fixed in position using dental cement.

Deep brain stimulation

After a recovery period of about one week, all animals underwent a first stimulation session to determine the escape threshold. The stimulation amplitudes were gradually increased until escape behaviour was observed. At each step, stimulation duration was 15 s followed by a period without stimulation of 45 s. The stimulation frequency was set at 50 Hz and pulse width at 0.1 ms based on previous experiments²⁹. A World Precision Instruments digital stimulator (DS8000, WPI, Berlin, Germany) and a stimulus isolator (DLS100, WPI, Berlin, Germany) were used to deliver the stimuli. Real time verification of the parameters applied during stimulation was obtained using a digital oscilloscope (Agilent 54622D oscilloscope, Agilent Technologies, Amstelveen, the Netherlands). After confirming the current amplitudes necessary to evoke escape behaviour, all rats had a period of two weeks without stimulation before the final stimulation session in the open field (OF) arena was performed. Control rats were handled and placed in the OF similarly.

Behavioural evaluation

Rats were evaluated in an OF test. For more details of this test, please see Lim *et al.*²⁹. In summary, rats were placed in the arena and were connected with an external stimulator through externalized leads. The stimulation started approximately 1 min after the rat was placed in the OF arena, using the current amplitudes previously eliciting escape behaviour. The behaviour of the rats was videotaped (Ethovision, Noldus Information Technology, Wageningen, the Netherlands).

Histological processing

Two hours after the final stimulation session, rats were deeply anesthetized and perfused transcardially with Tyrode (0.1M), followed by a fixative containing 4% paraformaldehyde, 15% picric acid and 0.05% gluteraldehyde in 0.1M phosphate buffer (pH 7.6). The brains were removed and postfixed for 2 h followed by immersion in 20% sucrose at 4°C until saturated. Brain tissue was then quickly frozen with CO₂ and stored

at -80°C . The cerebellum was cut serially into $10\text{-}\mu\text{m}$ sections which were collected on gelatine-coated glasses. We used a previously published protocol for c-Fos immunohistochemistry⁴. In summary, this staining was carried out by incubating sections two nights with a polyclonal rabbit anti-c-Fos antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). This was followed by overnight incubation with the secondary antibody (biotinylated donkey anti-rabbit, Jackson Immunoresearch Laboratories Inc., Westgrove, USA). Subsequently, the sections were incubated with an avidin–biotin–peroxidase complex (Elite ABC-kit, Vectastatin; Burlingame, USA) for two hours. To visualize the immune complex of horseradish peroxidase reaction product, sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB)/Nickel Chloride (NiCl_2) solution for ten minutes. After dehydrating, all sections were coverslipped with Pertex (Histolab Products ab, Goteborg, Sweden). Additionally, for each animal, the area of the dIPAG and VMH was cut into $30\mu\text{m}$ sections and stained with standard haematoxylin–eosin (Merck, Darmstadt, Germany) staining to locate the electrode tips.

Stereological quantification of c-Fos immunoreactive cells

Stereological analysis was performed with a stereology workstation (CAST-GRID-Computer Assisted Stereological Toolbox-Olympus, Denmark). After exactly tracing boundaries of the fastigial nucleus (FN) and dentate nucleus of the cerebellum (DN) on microscopic video images displayed on a monitor, numbers of c-Fos immunoreactive (c-Fos-ir) cells in both nuclei were evaluated with the modified Abercrombie cell counting method³⁶. All neurons of which the cell bodies gave a positive reaction within an unbiased counting frame distributed in a systematic-random fashion throughout the delineated regions were counted. Estimated numbers of neurons were calculated from the numbers of counted neurons and the corresponding sampling probability. The numerical density (N_V) of c-Fos-ir cells in the nuclei was estimated using the formula $N_V = (N_A/T) (T/(T+D))^{36}$. In this formula, N_A , T and D are the positive cell number in the unbiased counting frame, the section thickness and the mean particle diameter, respectively. To make an acceptable biological comment on any subject one should estimate total number of cells rather than numerical density of cells in the interested region. For this reason, we estimated the volume (V) of each cerebellar nucleus by multiplying the total area of each nucleus that is seen in the whole section of cerebellum (ΣA) with the mean section thickness (T), as shown in this formula $V = \Sigma A T^{37}$. The total number of cells in a nucleus was estimated by the formula $N = N_V V$.

Semiquantitative analysis of c-Fos-immunoreactive cells

Analysis of the cFos-ir cells in the interpositus nucleus of the cerebellum (IN) were performed using a previously described semiquantitative method⁴. In summary, photographs of the IN were taken at 4X magnification using an Olympus DP70 camera connected to an Olympus AX70 bright-field microscope (analySIS; Imaging System,

Münster, Germany). The same light intensity and threshold settings of the camera were employed for all sections. For each animal we selected two sections of the IN for quantification. The boundaries of the IN were delineated and the area measured. The counting of the c-Fos-ir cells was performed using the image analysis program 'Image J' (version 1.38, NIH, USA). A cell was counted as c-Fos-ir if its density was 65% higher than the mean background density of that section. Artefacts in the sections were excluded from analysis to ensure the accuracy of measurements. The number of c-Fos-ir cells per square millimeter was calculated.

Statistical analysis

The data of the c-Fos counts were analysed using the Kruskal-Wallis test for non-parametric data. Non-parametric testing was chosen due to small group sizes. A value of $p < 0.05$ was considered significant. Post hoc analysis was performed using the Mann-Whitney test. Since we expected a deactivation in the stimulated groups, we used a one-tailed procedure.

Results

Histological evaluation of the electrode localisation

In all animals included in this study, the electrode tips were positioned correctly in the dIPAG and VMH, respectively (Figure 4.1). No histological damage was observed except for the electrode trajectory with the standard haematoxylin–eosin staining, suggesting that stimulation with the current stimulation settings did not cause observable tissue damage.

Behavioural evaluation

The intensity of the electrical current applied to the dIPAG and VMH of the animals was based on the threshold shown to induce escape behaviour. All rats showed 'escape behaviour' characterized by rigorous and aimless running. The current amplitudes necessary for inducing escape behaviour were significantly different between rats with dIPAG- and VMH-DBS. ($F > 351.13$; $p < 0.00$). The mean current density applied to the dIPAG to induce escape behaviour was approximately 90 μA and for the VMH approximately 600 μA .

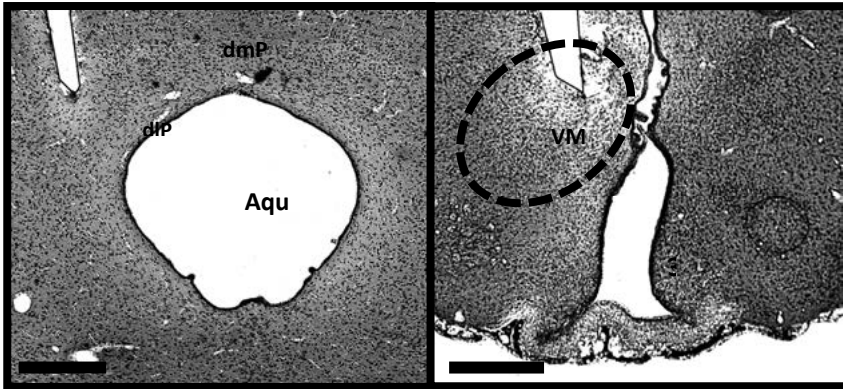


Figure 4.1 Representative low-power photomicrographs of 30 μm -thick frontal sections from the brain of a rat subjected to stereotactic implantation of a concentric bipolar electrode to stimulate the dIPAG (Figure 4.1a, scale bar=250 μm) and VMH (Figure 4.1b, scale bar=500 μm). The tips of the electrodes are situated within the respective targets. Aquad.: aqueduct of Sylvius, dmPAG: dorsomedial periaqueductal grey, dIPAG: dorsolateral periaqueductal grey, 3V: third ventricle, VMH: ventromedial hypothalamus

Evaluation of the number of c-Fos-immunoreactive cells

There was a significant decrease in the amount of c-Fos-ir cells in the dentate nucleus ($H_2=6.343$, $p<0.05$; Figure 4.2 and 4.3) and in the fastigial nucleus ($H_2=6.870$, $p<0.05$; Figure 4.2 and 4.3) after DBS. As expected, we found a lower amount of c-Fos-ir cells in the dentate and fastigial nucleus of the stimulated rats compared to the control rats. Post hoc analysis showed that in the rats stimulated in the dIPAG, the difference with control rats was significant for both nuclei. (DN: $U=1.00$, $r=-2.21$; FN: $U=0.00$, $r=-2.45$). When comparing VMH-stimulated rats to control rats this difference was significant only in the nucleus dentatus ($U=1.00$, $r=-2.02$); the difference in the fastigial nucleus showed a trend towards significance ($U=2.00$, $r=-1.73$). There was no significant difference in the amount of c-fos-ir cells between the stimulated groups (DN: $U=7.00$, $r=-0.74$; FN: $U=6.00$, $r=-0.98$).

Analysis of the interpositus nucleus (IN) showed a similar trend towards decreased c-Fos expression in the stimulated rats compared to controls (PAG: $U=19.00$, $r=-1.04$; VMH: $U=21.00$, $r=-1.37$). Again there was no difference between the stimulated groups ($U=36.00$, $r=-0.36$).

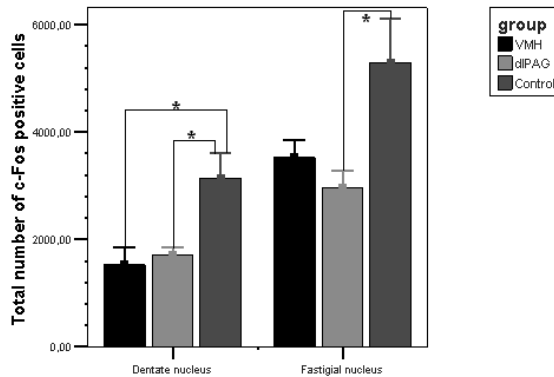


Figure 4.2 C-Fos expression in the dentate nucleus and in the fastigial nucleus of the cerebellum. Data are represented as means plus S.E.M. showing control animals, animals stimulated in the dorsolateral periaqueductal grey (dIPAG) and animals stimulated in the ventromedial hypothalamus (VMH). The asterisk (*) indicates a significant difference between groups. Note the significantly lower numbers of c-Fos immunoreactive neurons in the groups with animals showing panic attacks evoked by stimulation of the dIPAG and VMH.

Discussion

The objective of our experiment was to use electrical stimulation of the dorsolateral periaqueductal grey and of the ventromedial hypothalamus in rats as a model of panic attack to study the involvement of the deep cerebellar nuclei. We have found that panic-like behaviour was accompanied by a decrease of c-Fos-ir cells in the DCbN, indicating deactivation. c-Fos expression was significantly lower in the DN of both treatment groups and in the FN of the dIPAG DBS group when compared to controls. In the VMH DBS group, c-Fos expression in the FN was lower compared to controls, with a trend towards significance. Using a semiquantitative analysis, the IN showed a similar trend towards decreased c-Fos expression in the stimulated rats compared to controls. This should be considered a preliminary finding since the method of counting was not performed using stereological principles.

There is anatomical and functional evidence supporting the role of the cerebellum in panic. Sakai and colleagues found a significantly higher glucose uptake in the cerebellum of patients with panic disorder compared to control patients²⁰. After clinical improvement of these patients due to cognitive-behavioural therapy, the glucose uptake in cerebellum had decreased²¹. Several groups found increased activation of the cerebellum after CCK-4-induced panic attacks in healthy subjects^{24,25}. This increase was not seen in these subjects during anticipatory anxiety.

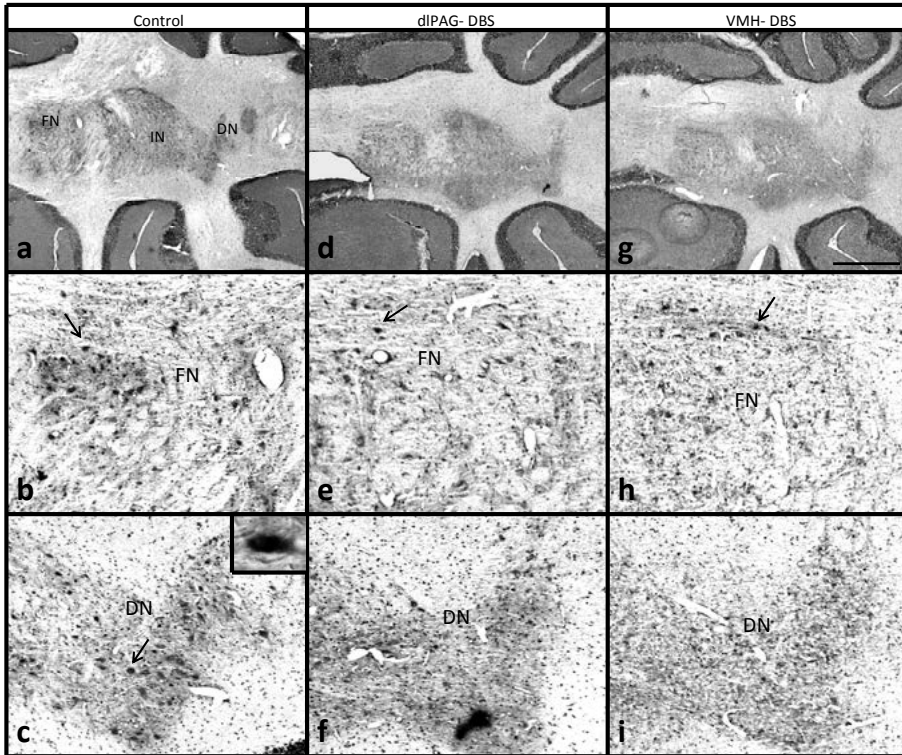


Figure 4.3 Representative low-power photomicrographs of c-Fos expression in a 10 μm -thick horizontal section of the deep cerebellar nuclei at bregma level -6.10 mm (according to the stereotactic rat brain atlas of Paxinos and Watson of 1998) in a sham rat (a, b, c), a rat stimulated in the dIPAG (d, e, f), and a rat stimulated in the VMH (g, h, i). Figures a, d, and g were taken at 2X magnification; and figures b, c, e, f, h, and i were taken at high-power magnification. The arrows point to a c-Fos-ir cell, represented by a small dark dot. The inset in c shows a representative high-power photomicrograph of a c-Fos-ir cell. Scale bar for 2X magnification power=1 mm. dIPAG: dorsolateral periaqueductal grey; VMH: ventromedial hypothalamus; DBS: deep brain stimulation; DN: dentate nucleus of the cerebellum; FN: fastigial nucleus of the cerebellum.

These studies were not initially designed to analyse changes in the cerebellum, and the changes found are usually unexpected. The findings are often simply mentioned as interesting or surprising, although several groups relate to the role of the cerebellum in fear conditioning as proposed by Sacchetti and others³⁸. Sacchetti and coworkers reviewed the evidence showing that the cerebellar vermis plays a role in the fear response and in fear conditioning, especially in fear consolidation. The most important changes are thought to take place at the level of the Purkinje cell (PC). It has been shown that several forms of fear conditioning lead to increased PC excitability and an increased firing rate of the PCs. In contrast, heterozygous Lurcher mice, which show early and complete apoptosis of cerebellar PCs, show reduced inhibition to

anxiety-provoking aversive areas³⁹. In summary, it seems that increased PC activity leads to more fear and decreased or absent PC activity to less fear. Functional imaging is designed to analyse activation changes in the cerebral cortex; therefore, it is likely that the activation seen in the cerebellum is also located in the cortex. Increased activation found in functional imaging studies may therefore reflect the increased activity of the PCs. Since PCs are known to have an inhibitory action on the DCbN, these findings are in line with deactivation that we found in the DCbN in the stimulated rats which show panic-like behaviour.

In the cerebellum, the vermis, projecting through the fastigial nucleus, seems to be the most important structure in fear and panic. In the cases described by Schmahmann and colleagues the vermis was always involved in patients with changes of affect⁵. In early experiments vermal lesions were shown to attenuate a variety of fear behaviour, whereas vermal stimulation leads to increased fear-related responses^{40,41}. Other animal research shows a selective role for the fastigial nucleus in heart rate conditioning^{40,42,43}. The vermis also contributes to consolidation of fear memory⁴⁴. There is also evidence of a role of the interpositus nucleus and the dentate nucleus in fear. In studies investigating several aspects of fear conditioning, animals with lesions of the dentate and interpositus nucleus do not acquire an aversive conditioned response, but they do acquire an appetitive conditioned response⁴⁵ and show unaltered heart-rate conditioning⁴² and vocalisation indicative of unspecific fear⁴⁶. Furthermore, there are clear bi-directional connections to the hypothalamus from (greatest to least concentration) the dentate nucleus, the interpositus nucleus and the fastigial nucleus⁴⁷⁻⁵⁰, supporting a role of all DCbN in autonomic processes, for example those related to fear. In addition, the DCbN have been shown to project to several parts of the fear network: Teune and colleagues injected tracers in the DCbN in rats and documented projections to the PAG from the fastigial nucleus and the dentate nucleus and, in lesser degree, from the interpositus nucleus⁵¹. Several groups have suggested projections from the fastigial nucleus to the amygdala, hippocampus, septal nuclei and the nucleus accumbens, based on behavioural changes following cerebellar stimulation or lesioning^{38,41,52}. Berntson and Torello, for example, showed that hyperemotionality caused by septal lesions was largely attenuated by lesions of the fastigial nucleus⁵³. An exact anatomical pathway has never been shown⁵⁴. However, these behavioural changes indicate that the involvement of the cerebellum in fear does not merely consist of regulation of an autonomic visceromotor response but that there is a place for the cerebellum in the network regulating fear processing³⁸. In a recent review, Stoodley and Schmahmann present a functional somatotopy of the non-motor functions of the cerebellum based on functional imaging⁵⁵. They conclude that the vermis of the posterior lobe seems to be specifically related to emotional processing, whereas activation in the posterior cerebellar hemispheres may be related to the decision-making aspects of the tasks used in the experimental setting⁵⁵. On the other hand, Timman and colleagues reviewed anatomical evidence for a role of the cerebellum in emotional and cognitive learning, and they conclude that with respect to

fear the vermis, projecting through the fastigial nucleus, contributes to the autonomic and somatic aspects, whereas the posterolateral cerebellar hemispheres, projecting through the dentate and interpositus nucleus, play a role in the emotional content of fear processing⁴⁰.

In summary there is ample evidence that all cerebellar nuclei are involved in fear, in which the fastigial nucleus possibly mediates a different aspect of fear than the dentate nucleus and the interpositus nucleus. This correlates with our findings in the present study showing a similar deactivation in the dentate nucleus and in the fastigial nucleus, and possibly also in the interpositus nucleus. Increased fear is associated with increased PC activity in functional imaging studies, which is hypothesised to lead to inhibition of the DCbN and therefore of cerebellar output. We speculate that the cerebellum plays a role in regulating appropriate behaviour in response to any stimulus, and that a decreased cerebellar output may play a role in emergence of an inappropriate response, such as a panic attack. This decreased output may be in response to incorrect input, as suggested by Parvizi and colleagues³¹; however, we suggest that the cerebellum also plays a direct role in the selection of relevant information on which an adequate behavioural response is based, and that deactivation of the DCbN then leads to inappropriate behaviour by inhibiting this selection process.

Conclusion

This study supports the hypothesis that the cerebellum is involved in panic attacks. Using DBS of the dIPAG and the VMH in rats as a model of a panic attack, we found that the activity of the DCbN as measured by c-Fos expression decreased significantly in the dentate nucleus and in the fastigial nucleus, and possibly also in the interpositus nucleus. In a previous study, we showed that the DCbN are de-activated in rats showing increased impulsivity. In conclusion, deactivation of the cerebellum is associated with inappropriate behaviour such as panic and impulsivity. We suggest that the cerebellum plays a role in the selection of relevant information, thereby inhibiting such inappropriate behaviour.

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Chapter 5

**c-Fos expression in the deep cerebellar nuclei in a rat
model of conditioned fear**

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Abstract

In contrast to what was previously believed, there is increasing evidence that the cerebellum is involved in emotional and cognitive behaviour. Preclinical and clinical research points towards a role of the cerebellum in impulsivity and panic. Deep brain stimulation (DBS) of the dorsolateral periaqueductal gray (dIPAG) in rats has proved to elicit escape behaviour directly, but can also be used for conditioning of context-dependant fear responses. We used this model to study the activation in the deep cerebellar nuclei (DCbN) after conditioned fear. For this purpose, we performed c-Fos immunohistochemistry in the DCbN.

We found no statistically significant difference in c-Fos expression in the DCbN between the subgroups in this animal model. We hypothesize that the lack of a significant difference in activation of the DCbN may be related to fear consolidation which takes place after dIPAG stimulation, after which retrieval of behaviour, whether adequate or inadequate, does not lead to a different activation of the DCbN. On the other hand the effect of conditioned fear may be smaller than the effect of panic behaviour elicited by DBS, requiring a larger study population.

Introduction

The cerebellum has been regarded for decades to be a neuroanatomical structure solely involved in the control of motor coordination¹⁻⁴. However, there is now more than enough evidence that the cerebellum also plays a prominent role in cognitive and behavioural processes⁵⁻⁷. The evolutionary newer regions of the cerebellum project to the prefrontal cortex (areas 9 and 46) and the inferior parietal lobe area 7 through the ventrolateral and other thalamic nuclei including the mediodorsal thalamic nucleus and the reticular nucleus⁸⁻¹³. The cerebellum also receives a wide range of input from structures involved in cognition and affect¹⁴⁻²¹. These structures form the so called cerebrocerebellar circuit for cognition and emotion^{9,22}. Behavioural disturbances resulting from isolated damage to the cerebellum comprise a constellation of symptoms which has been called the cerebellar cognitive affective syndrome (CCAS)²³⁻²⁵. These include impairment in executive functions, difficulties with spatial cognition, memory deficiencies, personality changes with disinhibited and inappropriate behaviour, language deficits, and mutism^{7,26-30}.

In our previous research we have shown that, in a rat model, impulsivity induced by deep brain stimulation (DBS) of the mediodorsal thalamic nucleus (MD) is associated with deactivation of the deep cerebellar nuclei (DCbN)³¹. In rats in which DBS of the dorsolateral periaqueductal grey (dIPAG) induces panic-like behaviour we have shown the same association³². In contrast rats stimulated in the subthalamic nucleus (STN), known to result in a decrease in impulsivity, show increased activation in the DCbN^{33,34}. We hypothesize that the cerebellum plays a role in the selection of relevant information on which a behavioural response is based, thereby coordinating an appropriate response to a stimulus. Deactivation of the cerebellar nuclei may then lead to inappropriate behaviour.

Aside from a role in acute panic, it has been shown that the cerebellum plays a significant role in fear consolidation and fear memory, and therefore also in conditioned fear^{35,36}. Lim et al. showed that the dIPAG stimulated rat is not only a good model for acute induced fear- and panic like behaviour, but can also be used for conditioning context dependent fear responses³⁷. In the same study this panic behaviour was also shown to be moderated on a behavioural level by escitalopram, with other research stating that similar antidepressants can reduce fos immunoreactivity in the periaqueductal grey^{37,38}. In the present study we performed DBS of the dIPAG in rats to induce conditioned fear. These rats were treated chronically with escitalopram or buspiron. We then determined the activation of the DCbN as measured by the amount of c-Fos immunoreactivity two hours after a conditioned fear response. As we have previously shown that panic behaviour induced by dIPAG stimulation is accompanied by a decrease of c-Fos expression in the DCbN, we now expected to find a decreased c-Fos expression in the animals showing a conditioned fear response compared to the animals that don't show this conditioned fear response^{32,37}.

Materials and methods

Subjects

Male albino Wistar rats (n=40, 12 weeks old, bred and housed at the Central Animal Facility of Maastricht University, the Netherlands). Animals were housed individually in standard cages on sawdust bedding in an air ventilated room (temperature approximately 20°C) under a 12/12-h reversed light/ dark cycle. Standard laboratory chow (Hopefarms, Woerden, the Netherlands) and water were available ad libitum. At the time of surgery the average body weight of the rats was 300-350 g. The present study was approved by the Animal Experiments and Ethics Committee of Maastricht University.

Experimental groups

Rats were randomly assigned to one of the following six experimental groups: A. dIPAG stimulation and treatment with saline (n=7); B. dIPAG stimulation and treatment with buspirone (n=7); C. dIPAG stimulation and treatment with escitalopram (n=7); D. dIPAG sham surgery and saline treatment (n=6); E. dIPAG sham surgery and buspirone treatment (n=6); and F. dIPAG sham surgery and escitalopram treatment (n=7).

Surgical procedures

A description of the surgical procedure has been previously reported³⁹⁻⁴¹. Rats were anesthetized using ketamine (90 mg/kg) and xylazine (10 mg/kg) injected subcutaneously (s.c.). By means of a stereotactic apparatus (Stoelting, Wood Dale, USA; model 51653) burr holes were made in the skull through which a gold-plated electrode with inner platinum- iridium combination was implanted (Technomed, Beek, the Netherlands) at the level of the right dIPAG (coordinates from Bregma: anteroposterior, -7.6 mm; mediolateral, +0.7 mm; and ventral, -4.8 mm; approached with a coronal angle of 10°; Paxinos and Watson, 1998). The electrodes were fixed in position using dental cement (Heraeus Kulzer, Hanau, Germany). After surgery the rats were injected with Temgesic (0.1 mg/kg, s.c.) for analgesia and allowed to recover for a period of two weeks.

Deep brain stimulation

Stimulation was performed at a frequency of 50 Hz and pulse width of 0.1 ms based on previous experience³⁷. The amplitude was gradually increased until escape behaviour was observed. Stimulation at each amplitude was performed during 15 s followed by a stimulation-off period of 45 s. Two consecutive positive responses of escape reaction were required to determine the escape threshold. The mean amplitude necessary to elicit an escape response was 60 µA. Animals which required stimulation intensities above 100 µA, suggesting incorrect positioning of the electrode, were discarded from

analysis. A World Precision Instruments (WPI) digital stimulator (DS8000, WPI, Berlin, Germany) and a stimulus isolator (DLS100, WPI, Berlin, Germany) were used to deliver the stimuli. After the threshold determination session, all rats were given a recovery period of two weeks. The sham animals were similarly connected to the stimulator but no current was delivered to the dIPAG.

Drug administration

Escitalopram oxalate (H. Lundbeck A/S, Copenhagen, Denmark) and buspirone hydrochloride (TOCRIS, Cookson Inc., Missouri, USA) were dissolved in saline 0.9%NaCl, as described previously³⁷. Habituation to the injection procedure was obtained by injection of 1 ml saline three times on alternating days one week before the actual experimental testing. The animals received 21 daily injections of either escitalopram (ESCIT, s.c., 10 mg/ml, 1ml/kg), buspiron (BUSP, s.c., 3 mg/ml, 1 ml/kg), or saline (SAL, 1 ml/kg), respectively. Chronic treatment was chosen as the maximal effect of these drugs is expected in the chronic phase^{37,42-46}. The final drug administration was performed 60 min (ESCIT, SAL) or 120 min (BUSP) before the test in the open-field.

Behavioural testing

Rats were tested in an open field (OF). Details of this test were published previously by Lim *et al.*³⁷. The OF consisted of a clear Plexiglas box (square: 100 cm x 100 cm, and height: 40 cm) with open top and dark floor⁴⁶. All open-field testing was conducted in the same room in a dimly lit condition. On the day of stimulation in the open-field, rats were placed in the open-field arena and connected with the external stimulator through externalized leads followed after 1 minute by electrical stimulation with parameters based on the previously determined escape threshold. Once the escape reaction was evoked, the cable connected to the rat was detached and the animal was left in the open-field for approximately 30 s. The electrical stimulation to elicit an escape reaction was always carried out between 20:00 and 22:00 h.

Twelve hours after the induced escape behaviour (between 08:00 and 10:00 h) animals were placed in the OF again during which the behaviour of each rat was recorded using an automated system consisting of a camera connected to a computer with the Ethovision tracking software (Ethovision, Noldus Information Technology, Wageningen, the Netherlands). The Ethovision software automatically calculated and analysed temporospatial data of the animal relative to the OF including locomotion / distance moved, velocity and time spent in the corner of the open field, each parameter on a per 2 minutes basis. A trial was stopped after 5 minutes and the rat was removed from the arena. Fear or freezing behaviour was defined by a decreased locomotion (immobility) and increased duration of time spent in the corner of the OF. Escape behaviour was characterized by rigorous and aimless running within the OF arena.

Histological processing

Two hours after the final testing of the rats in the OF they were placed under generalized anaesthesia with Nembutal (75 mg/kg) and transcardially perfused with Tyrode (0.1 M) and a fixative solution which consists of paraformaldehyde, picric acid and glutaraldehyde in phosphate buffer (pH 7.6). After two hours of post fixation the brains were cryoprotected by overnight sucrose and then quickly frozen with CO₂ and stored at -80°C. One series of brainstem sections per animal was stained with standard hematoxylin-eosin (Merck, Darmstadt, Germany) to examine the localization of the electrode tips.

The cerebelli were cut serially on a cryostat (MICROM, Walldorf, Germany) into 10 µm frontal sections which were collected on gelatin-coated slides and then stored at -30°C. We used a previously published protocol for c-Fos immunohistochemistry with minor modifications³². In short, we used a primary rabbit polyclonal IgG antibody against c-Fos (diluted 1:300, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for incubation at room temperature for two days on a constant shaker. Subsequently sections were incubated with the secondary antibody (diluted 1:400, biotinylated donkey anti-rabbit biotin; Jackson Immunoresearch laboratories Inc., Westgrove, USA) overnight. This was followed by incubation for 2 hours with an avidin-biotin-peroxidase complex (diluted 1:800, Elite ABC-kit, Vectastatin; Vector, Burlingame, USA). To visualize the immune complex of horseradish peroxidase (HRP) reaction product, the sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB)/nickel chloride (NiCl₂) solution. Finally sections were dehydrated and cover slipped with Pertex (HistolabProducts, Goteborg, Sweden).

A Nissl stain was conducted on a parallel series of 10 µm sections to facilitate delineation of the boundaries of the DCbN. This parallel series was first air-dried for 30 minutes and subsequently exposed to a buffer solution (40 ml 136.08 g/mol sodium acetate with 9.6 ml glacial acetic acid in a 1000 ml distilled water solution (milli-q)), followed by a 2% triton x-100 solution of 2.5 ml with 150 ml absolute alcohol and 50 ml distilled water, then the previously described buffer solution again, and finally, a Cresyl violet solution (0.1 g/1000 ml distilled water). Finally sections were rinsed with the buffer solution, dehydrated, and cover slipped with Pertex (HiStolabProducts, Goteborg, Sweden).

Quantative evaluation of c-Fos immunoreactive cells

Systematic cell counts of c-Fos immunoreactive (c-Fos-ir) cells were performed in the DCbN as previously described³⁴. Photography of both the c-Fos and parallel Nissl stained sections was performed at a 4X magnification on an Olympus AX70 bright-field microscope with an Olympus DP70 camera (analySIS; Imaging System, Münster, Germany). The bregma levels of the DCbN ranged from -10.52 to -11.80 mm, which was verified with the Paxinos and Watson Atlas of 1998⁴⁷.

In the c-Fos stained sections the delineation of the DCbN was difficult due to a weak background staining. As mentioned above, a parallel series of sections was stained with a Nissl-staining to aid in delineation. Using Image J (version 1.43), a digital subtraction of the DCbN as delineated in the Nissl stained sections were projected on the c-Fos stained slides thereby giving a more accurate delineation of the cerebellar nuclei. Minor deviations in this technique due to slight difference in bregma level were corrected for manually. Artefacts in the sections were excluded from analysis to ensure accuracy of measurements. A cell was defined as being c-Fos-immunoreactive (c-Fos-ir) if it had a 65% higher density than the corresponding background grey value of the DCbN. The number of c-Fos-ir cells per mm^2 was calculated for each nucleus.

Statistical analysis

Since we were primarily interested in the effect of conditioned fear on c-Fos expression in the DCbN, the treatment groups were pooled according to whether they did or did not show escape behaviour in the final test in the OF. The data of the c-Fos counts were analysed using the Mann-Whitney test for non-parametric data (Monte Carlo method) since the group sizes were relatively small and non-normally distributed. The significance level was set at $p < 0.05$. Furthermore we performed a Kruskal-Wallis test to analyse differences between stimulated and sham stimulated groups. Post hoc analysis to follow up significant findings from the Kruskal-Wallis test was performed using the Mann-Whitney test with a one-tailed procedure. The level of significance for the Kruskal-Wallis test was set at $p < 0.05$, and for the post-hoc Mann-Whitney tests it was set at $p < 0.0167$. Output is provided as the number of c-Fos ir cells per square millimeter with 2 standard error of the mean values (S.E.M).

Any outliers identified in the c-Fos data on a per rat per nucleus basis were corrected for by transforming the data into the maximal $2\text{SD} \pm$ the mean. Rats were excluded if their total average values exceeded the $2\text{SD} \pm$ the mean for the total of the groups of rats. Finally 12 rats showing freezing behaviour and 24 rats showing no freezing behaviour were included for statistical analysis.

To determine the amount of agreement concerning the semi-quantitative method of cell counting between RJH and VMH an intraclass correlation coefficient (ICC) was determined on previously stained c-Fos DCbN. This was done using the Statistical Package for the Social Sciences software (SPSS, version 17.0.2, 11th march 2009) in a two-way-mixed model of the exact type.

Results

Electrode localization

The histological evaluation of the electrode localization was previously described by Lim *et al.*³⁷. The electrode tip was correctly placed in the dIPAG in six animals per group for the sham-saline, sham-buspirone, stimulation-saline and stimulation-escitalopram

groups and seven animals for the stimulation-buspirone and sham-escitalopram group³⁷.

Behavioural results

Results of behavioural testing were published previously by Lim *et al.*³⁷. In summary electrical stimulation of the dIPAG evoked escape behaviour which was followed by decreased locomotion or a freezing response when animals were placed back in the OF 12 hours later; a behavioural pattern indicative of conditioned fear.

Escitalopram reduced the time in the corner squares of the OF in the stimulated animals but not in the sham animals ($F(2,33)=10.12$, $p<0.01$). Stimulation decreased the mean velocity of movements $F(1,33)=16.57$, $p<0.01$) while escitalopram corrected for this in the stimulated animals but not the sham animals.

Quantification of c-Fos-ir cells

There were no significant differences between the left and right DCbN, therefore the results from the left and right hemispheres were pooled for further analysis. There were no significant differences in c-Fos expression in the DCbN between rats that showed conditioned fear in the final OF session and those that did not. Furthermore there were no significant differences in c-Fos expression in the DCbN between the treatment groups. (Figures 5.1-5.3).

A few cells displayed a hyperintense cytoplasm. These were not considered to be the 'classically' stained c-Fos ir cells as often described in the literature and therefore not included in the data analysis.

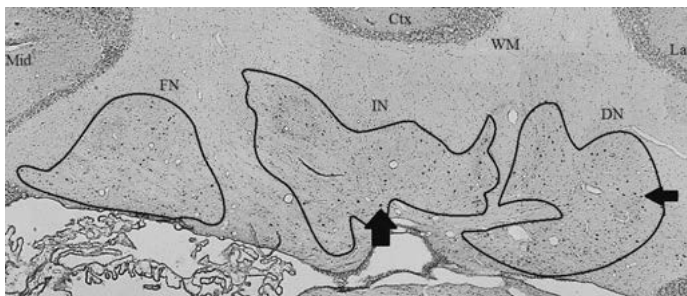


Figure 5.1 Representative low-power photomicrograph of a 10 μm -thick coronal section of the right cerebellar hemisphere at approximately bregma level -11.70 mm (-11.60 mm to -11.80 mm) showing c-Fos-ir cells in the DCbN. The DCbN are delineated by the black lines. The black arrows point towards a c-Fos positive cell. 4V: fourth ventricle; Ctx: cerebellar cortex; DN: dentate nucleus; FN: fastigial nucleus; IN: interpositus nucleus; Lat: lateral aspect of cerebellar hemisphere; Mid: midline; Pc: choroid plexus; Wm: white matter.

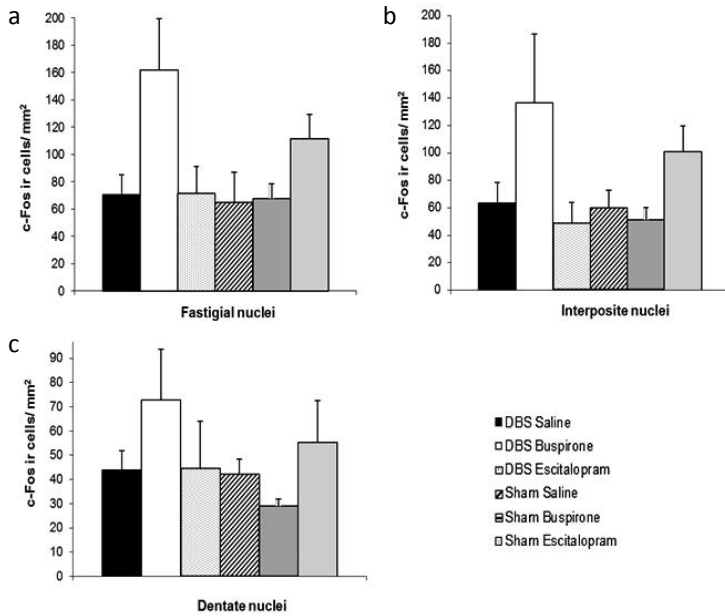


Figure 5.2 C-Fos expression in the deep cerebellar nuclei. Data are presented as mean plus S.E.M. showing six treatment groups: animals stimulated in the dorsolateral periaqueductal gray (dIPAG) and treated with respectively saline, buspirone or escitalopram, followed by animals with sham dIPAG stimulation again treated with respectively saline, buspirone or escitalopram. Data are presented for the fastigial nucleus of the cerebellum (FN; Figure 5.2a), the interpositus nucleus (IN; Figure 5.2b) and the dentate nucleus (DN; Figure 5.2c). Note the higher density of c-Fos-ir cells in the dIPAG-buspirone group and in the sham-dIPAG-escitalopram group which is consistently present in all nuclei, however, without reaching significance.

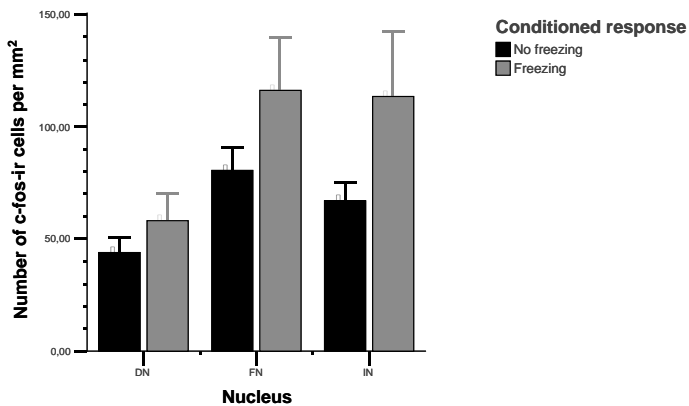


Figure 5.3 C-Fos expression in the deep cerebellar nuclei. Data are presented as mean plus S.E.M. showing the pooled treatment groups: animals showing freezing behaviour 12 hours after dorsolateral periaqueductal grey (dIPAG) stimulation and animals that don't show this freezing behaviour. Data are presented for the dentate nucleus (DN), the interpositus nucleus (IN) and the fastigial nucleus (FN).

Intraclass correlation coefficient

The intraclass correlation coefficient was determined based on the total c-Fos ir cells/mm² counted by RJH in comparison to VMH for all the DCbN in a previous experiment. The ICC for the total of the DCbN yielded a correlation of 0,795 (single value; 0.886 for average), which is a substantial to high level of agreement on the Landis & Koch scale.

Discussion

In the present study we analysed changes in activation in the deep cerebellar nuclei after a conditioned fear response 12 hours after dorsolateral periaqueductal grey-stimulation. We expected a decrease in activation in the animals which show this conditioned fear response in comparison to sham stimulated animals. In the animals treated with escitalopram which do not show a conditioned fear response after dIPAG stimulation we expected a normalisation of the deactivation of the DCbN. We were however not able to show a difference in activation in the DCbN.

The amygdala and the hippocampal complex are important structures in fear memory³⁵. There is also evidence for a substantial role of the cerebellum in the process of fear consolidation and retrieval. A key structure is the Purkinje cell (PC). Pairing of an unconditioned stimulus and a conditioning stimulus leads to long term depression (LTD) of the parallel fibre synapses with the PC's. This decreases the firing rate of the PC's, which allows the conditioned stimulus to be passed on to the DCbN^{35,36}. Fear conditioning is important for survival, therefore fear memory is rapidly acquired and long lasting³⁵. In classical Pavlovian conditioning several training sessions are usually necessary to elicit a conditioned fear response. In our study a short stimulation of the dIPAG in the open field (OF) is enough to elicit a conditioned fear response when rats are placed in the same OF arena 12 hours later³⁷. Possibly this strong stimulus already causes synaptical changes in the cerebellum. One could then hypothesize that in the rats with a conditioned fear response, retrieval of this conditioned behaviour does not cause a different activation than retrieval of normal behaviour in the non-conditioned rats or in the rats where the consolidation of this fear memory has been inhibited by treatment with escitalopram. This may account for our not finding any difference in activation in the DCbN between the different groups.

A different explanation is related to the stimuli themselves. In a previous study we found a decreased activation in the DCbN directly after panic behaviour caused by dIPAG-stimulation³². In the present study the delay between stimulation and sacrifice was 14 hours, in contrast to two hours in the previous study. Therefore we did not expect to see an effect of stimulation since the maximal expression of c-Fos takes place two to four hours after a stimulus⁴⁸. Electrical stimulation is a very strong stimulus. Possibly the conditioned fear is a weaker stimulus, as a result of which group sizes may not have been adequate to disclose a significant effect. This may furthermore account

for the low and variable signal intensity seen in the sections. The variability in signal intensity was not seen in the cerebellar cortical granule cell layer, therefore it could not be attributed to mere staining quality. There was no significant lateralization across the different treatment groups. This could be related to the suboptimal expression. It is also likely that the effects of dIPAG-stimulation are not confined to just one of the cerebellar hemispheres but have a more generalized effect (as claimed in the DBS background effect theory), since many structures in the cerebrocerebellar loop are (at least partially) bilaterally interconnected⁹.

Although not significant, there is a substantial increase in the amount of c-Fos ir/mm² in the DBS dIPAG- bupirone group compared to the sham-bupirone group (from 50 cells/mm² in the sham bupirone group to 124 cells/mm² in the dIPAG-bupirone group) and a decrease in the amount of c-Fos ir/ mm² in the dIPAG-escitalopram group compared to the sham-escitalopram group (from 68 cells/mm² in the sham-escitalopram group to 51 cells/mm² in the dIPAG-escitalopram group) (Figure 5.2). These changes did not show a correlation with the measured behavioural parameters: freezing and escape behaviour increased in the dIPAG stimulated group as a whole and this effect was normalised only by escitalopram in the stimulated group. A possible explanation could be that there is a mild combined permissive effect of DBS with bupirone treatment on c-Fos expression without any significant change in the measured behavioural parameters. No evidence was found in literature to back up this hypothesis.

In some of the sections we identified hyperintense cytoplasm in the absence of any nuclear staining with c-Fos. This was independent of the intensity of the staining. Some of these cells showed almost isointensity with adjacent c-Fos stained nuclei. This could be due to Fos-like immunoreactivity (Fos-LI) as has previously been described⁴⁹. Possibly there is an influence of deep brain stimulation induced electrochemical changes, altering electrical charges or even inducing cellular alterations considering the trafficking of proteins from diverse intracellular departments. These sparsely distributed cells were seen in only a few sections of the dIPAG stimulated group with escitalopram and sham with saline and escitalopram. Therefore there was no relation with the treatment group, which pleads against an influence of DBS. The number of these cells was too small to perform statistical analysis on. However, a vue microscopy did show that these cells were more often to be found in the lateral regions of the DCbN. This interesting finding could point towards several things. It could indicate that these cells are of a different subset of neurons or that the functional state of these neurons has changed (i.e. different metabolism or a pre-apoptotic state). The exact nature of this phenomenon remains a topic of discussion.

Conclusion

In this study we found no significant change in activation of the DCbN in rats that showed a conditioned fear response induced by dIPAG stimulation 12 hours before testing. We hypothesize that the lack of a significant difference in activation of the DCbN may be related to fear consolidation which takes place after dIPAG stimulation, after which retrieval of behaviour, whether adequate or inadequate, does not lead to a different activation of the DCbN. In our previous research we found deactivation of the DCbN associated with panic behaviour induced by dIPAG-stimulation. The conditioned fear response may have caused smaller changes in cerebellar activation which were not disclosed due to small group sizes.

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Chapter 6

Sustained reduction of cerebellar activity in experimental epilepsy

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In preparation

Abstract

Clinical and experimental evidence suggests a role for the cerebellum in seizure control, while no data are available on cerebellar activity between seizures. We hypothesized that interictal regional activity of the deep cerebellar nuclei is reduced in epilepsy, and tested this in an animal model by using Δ FosB and cytochrome oxidase (COX) (immuno)histochemistry. The expression of these two markers of neuronal activity was analysed in the dentate (DN), interpositus (IN) and fastigial (FN) nucleus of the cerebellum of fully amygdala kindled rats that were sacrificed 48 hours after their last seizure.

Cerebellar nuclei of kindled rats exhibited 22 to 28% less Δ FosB immunopositive cells than their respective counterpart in sham controls ($p < 0.05$). The density of Δ FosB immunopositive cells correlated negatively with the pre-kindling afterdischarge threshold (DN: $r = -0.726$; IN: $r = -0.635$; FN: $r = -0.664$, $p < 0.05$) and the change in afterdischarge threshold (DN: $r = -0.664$; IN: $r = -0.625$; $p < 0.05$). COX expression in the IN and FN of kindled animals was reduced by 32% compared to respective control values ($p < 0.05$).

These results indicate that an epileptogenic state is characterized by decreased activity of deep cerebellar nuclei, especially the FN. Possible consequences may include a decreased activation of the thalamus, contributing to further seizure spread. Restoration of FN activity by low frequency electrical stimulation is suggested as a possible treatment option in chronic epilepsy.

Introduction

The cerebellum plays a crucial role in the coordination and control of motor behaviour and cognitive processing¹. Studies in epilepsy patients and animals models of epilepsy have

demonstrated that the cerebellum takes part in the epileptogenic network as well. First of all, case reports have illustrated that the cerebellum can harbour the epileptic focus²⁻⁴. Furthermore, SPECT and fMRI studies in epilepsy patients have revealed that cerebellar blood flow and blood oxygen level-dependant (BOLD) signal, respectively, increase during secondary generalisation of a partial seizure⁵⁻¹¹. In rats, cerebellar glucose utilization increases upon administration of proconvulsant drugs¹².

At the cellular level it has been hypothesized that this ictally increased activity reflects increased GABA-ergic Purkinje cell firing¹³. Purkinje cells provide the purely inhibitory output of the cerebellar cortex¹⁴. These inhibitory projections modify the output of the cerebellum through the deep cerebellar nuclei, the dentate nucleus (DN), interpositus nucleus (IN) and fastigial nuclei (FN), which in turn project to the thalamus. This way, the cerebellum regulates activity of neurons elsewhere in the brain.

Activation of the Purkinje fibers by electrical stimulation of the cerebellar cortex has anticonvulsive effects in animal studies^{15,16}. It has therefore been investigated as an anticonvulsive therapy in humans as well, with varying results¹⁷⁻²⁰. The deep cerebellar nuclei are interconnected and therefore their output is affected by more than Purkinje cell firing alone²¹. Modification of this deep cerebellar network by electrical stimulation of the DN²²⁻²⁴ and FN^{25,26} has been performed in an attempt to treat epilepsy patients.

In summary, seizures are associated with increased activity of the Purkinje cells. Since these cells inhibit the deep cerebellar nuclei, it is likely that epilepsy is associated with decreased activity of these nuclei. It is not clear whether the different deep cerebellar nuclei are all inhibited to the same extent. The aim of the current study was therefore to assess the regional distribution of interictal neuronal activity in the deep cerebellar nuclei. To this end, we submitted cerebellar sections from fully amygdala kindled rats to (immuno)histochemistry of neuronal activity markers Δ FosB and cytochrome C oxidase (COX, a.k.a. complex IV), and quantified their expression in the DN, IN and FN.

Materials and methods

Animals

Experimental procedures were carried out in 12-weeks old male Sprague-Dawley rats (Harlan, Horst, The Netherlands). Animals were housed under controlled conditions, a 12 hour light/dark cycle (lights on 7 am), background noise, and food and water *ad libitum*. Adequate measures were taken to minimize pain and discomfort. All

experimental procedures were approved by the animal ethics committee of Maastricht University and complied with governmental legislation.

Amygdala kindling

Electrode placement

For amygdala kindling, rats were stereotactically implanted with an electrode in the left basolateral amygdala. The surgical procedure was performed under general isoflurane anesthesia (5% for induction and 2.5% for maintenance). Perioperative pain was minimized by administering 0.1 ml buprenorphinehydrochloride (Temgesic®, Schering-Plough Inc., Amstelveen, The Netherlands) subcutaneously 30 minutes before surgery. Rats were stereotactically implanted (Dual Manipulator Lab Standard Stereotact, Stoelting Inc., Wood Dale, USA) with a set of electrode designed and manufactured by the department of Instrument Development, Engineering and Evaluation of Maastricht University in collaboration with prof. dr. Y. Temel (Maastricht University Medical Center, Maastricht, The Netherlands). An electrode set consisted of a bipolar stimulating/recording electrode that was implanted in the left basolateral amygdala (coordinates relative to bregma: 2.5 mm posteriorly, 4.8 mm laterally, and 9.6 mm ventrally²⁷), and three monopolar stainless steel electrodes that were implanted in the cortex at 1 mm depth. One cortical electrode was used for EEG, one for reference, and one for ground. Connectors for the kindling/EEG electrodes were fixed on the skull using dental acrylic.

Afterdischarge threshold

Ten days after surgery the pre-kindling afterdischarge threshold (pre-KADT) was assessed in all rats by stimulating the amygdala with a series of pulses of increasing intensity starting at 10 μ A (2 seconds, 50 Hz, 0.2 ms blockpulse). Stimuli were delivered through a WPI Accupulser A310 connected to a WPI Stimulus Isolation Unit A360 (World Precision Instruments, Sarasota, FL, USA). EEG registrations from the amygdala and cortical electrodes were recorded from 1 min before the kindling stimulus till the end of the behavioural seizure using a Vanguard system (Vanguard Systems, Cleveland Clinics Foundation, Cleveland, USA). Recordings were made with a sample frequency of 200 Hz, a frequency band of 0.5–70 Hz and a 50 Hz notch filter. The afterdischarge threshold was defined as the stimulus amplitude necessary to elicit a two-second discharge with a high frequency and high voltage. One day after the last kindling stimulus, a post-kindling afterdischarge threshold (post-KADT) was assessed. The difference between pre-KADT and post-KADT (Δ ADT) was calculated by subtracting the respective values and was considered an indirect measure of excitability.

Amygdala kindling

Amygdala kindling started two weeks after surgery by administering two stimulations per day with an interstimulus interval of at least six hours. Each stimulus lasted two seconds and consisted of a 50 Hz, 400 μ A, 0.2 ms blockpulses. Seizure severity was assessed based on Racine's scale²⁸ (i.e. stage 1: freezing; stage 2: head nodding, blinking; stage 3: unilateral forelimb clonus; stage 4: bilateral forelimb clonus, rearing; stage 5: falling). Fully kindled animals were defined as animals that displayed a stage five seizure upon five consecutive amygdala stimulations. These fully kindled animals were subsequently stimulated once per day for two more weeks. Kindling rate was defined as the number of stimuli needed to reach the fully kindled state. Sham animals underwent the same surgical procedure and afterdischarge assessments, but were not subjected to daily stimulation.

Histological processing

Two days after the last seizures the rats received an overdose of pentobarbital (Nembutal), followed by perfusion with tyrode buffer (in mM: 136.9 NaCl, 2.7 KCl, 0.2 MgCl₂, 11.9 NaHCO₃, 0.3 NaH₂PO₄, 5.0 glucose, equilibrated with 5% CO₂/95% O₂), and then with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.6. The brains were removed, postfixed in the same fixative (4°C, 24 hours), rapidly frozen using CO₂, and stored at -80°C until sections were cut.

Coronal 10 μ m serial sections from the level of the central canal-fourth ventricle junction (13.6 mm posteriorly to bregma according to Paxinos²⁷) caudally to the inferior colliculus (located 9.6 mm posteriorly to bregma) were cut on a cryostat, mounted on gelatin-coated glass slides, and stored at -20°C until they were processed for (immune)histochemistry. To minimize staining-to-staining variations, staining experiments always included an equal amount of control and kindled samples.

Δ FosB immunohistochemistry

Sections were rinsed in Tris-buffered saline containing 0.3% Triton (TBS-T), and then incubated (4°C, 48 hours) with polyclonal rabbit-anti-mouse for B antibody (sc-48, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:250 in TBS-T containing 0.5% bovine serum albumin. Next, sections were rinsed in TBS-T, and incubated (room temperature, overnight) with donkey-anti-rabbit biotinylated antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1:400 in TBS-T containing 0.5% bovine serum albumin. Sections were then rinsed with TBS-T, incubated for two hours in avidin/biotin diluted 1:800 (Vectastain ABC-kit, Vector Laboratories Inc., Burlingame, CA, USA), and visualised by 3,3'-diaminobenzidine (DAB) containing NiCl₂. Finally, slides were dehydrated and coverslipped.

COX histology

COX was histochemically detected as described previously^{29, 30}. Briefly, sections were air dried and subsequently incubated (37°C, four hours) in the dark in a nickel enhanced 0.1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution (1% NiCl₂, pH 7.4) containing 0,0224% cytochrome C oxidase, 0,115% DAB, and 4,5% sucrose. The reaction was stopped by transferring the sections to an icecold pH neutral buffered 4% paraformaldehyde medium for ten minutes. Finally, slides were dehydrated and coverslipped.

Quantification of stainings

The dentate nucleus (DN), interpositus nucleus (IN) and fastigial nucleus (FN) were photographed in all sections at 4X magnification using an Olympus AX70 bright-field microscope with an Olympus DP70 camera (analysis Imaging System, Münster, Germany). A mean of 10 sections per animal for the Δ fosB staining and 16 sections per animals for the COX-staining were analysed. The deep cerebellar nuclei were easily identifiable due to the contrast between nuclei and surrounding white matter, allowing delineation using the freehand drawing function in ImageJ (version 1.43). Artifacts were excluded from analysis.

Cells showing Δ fosB immunoreactivity in the nucleus, cytoplasm, or both were counted by an observer that was blinded for the treatment and expressed as number of immunopositive cells per square millimeter. In total, 84 sections from 7 kindled animals, and 72 sections from 9 sham animals were analysed. Cytochrome oxidase staining was analysed by optical density (OD) using ImageJ. For each nucleus, the OD value was corrected for background activity by subtracting the OD of the adjacent white matter, resulting in negative values as ImageJ applies an inverted grey value scale. In total, 92 stained sections from 6 kindled animals and 101 sections from 6 sham animals were analysed.

Statistical analysis

Shapiro-Wilk test was performed as test for normal distribution of data. Group differences from normally distributed data were tested by a two-tailed independent samples t-test was used, while data that were not normally distributed were analysed using a Mann-Whitney test. Data were pooled when there was no significant difference between the left and right hemisphere. Within the dataset of one nucleus from one rat, an outlier was defined as a value that deviated more than 2 standard deviations from the mean. This was corrected for by adjusting the value to maximal 2 standard deviations. If the averaged values for one nucleus exceeded 2 standard deviations from the mean of this nucleus in the treatment group it was excluded from the analysis. The correlation between the expression of neuronal activity markers and the kindling parameters (pre-KADT, post-KADT, Δ ADT and kindling rate) was tested by bivariate analysis and presented as Pearson's correlation coefficient. Data are expressed as

mean \pm standard error of the mean (SEM). $p < 0.05$ was considered statistically significant.

Results

There were no significant differences in Δ FosB immunopositive cell densities or in COX grey values between left and right cerebellar nuclei in shams nor in kindled animals. Therefore, to increase the power of the analysis, values from both hemispheres were pooled.

Δ FosB

Density of Δ FosB immunoreactive (Δ FosB-ir) cells was lower in all three cerebellar nuclei of kindled animals than in shams (DN 108.5 ± 12.4 vs 152.1 ± 7.7 ; FN 87.2 ± 7.8 vs. 115.5 ± 2.2 and IN 91.3 ± 9.1 vs. 116.2 ± 3.5 ; $p < 0.05$ for DN and IN; $p < 0.01$ for FN), see Figure 6.1. In both sham and kindled animals, the DN contained a significantly ($p < 0.05$) higher Δ FosB-ir cell density (shams 152.1 ± 7.7 ; kindled 108.5 ± 12.4) than FN (shams 115.5 ± 2.2 ; kindled 87.2 ± 7.8) and IN (shams 116.2 ± 3.5 ; kindled 91.3 ± 9.1).

In kindled animals, Δ FosB-ir cell density was negatively correlated with pre-KADT in all three nuclei (DN: $r = -0.726$; IN: $r = -0.635$; FN: $r = -0.664$, $p < 0.05$). Similarly there was a negative correlation between Δ FosB-ir cell density and Δ ADT in the DN and IN of kindled animals (DN: $r = -0.664$; IN: $r = -0.625$, $p < 0.05$), meaning that a lower Δ FosB-ir cell density is associated with a higher pre-KADT and a larger Δ ADT (see Figure 6.2). No correlation was found between Δ FosB immunopositive cell density and post-KADT or kindling rate (data not shown).

Δ FosB immunohistochemical staining was not confined to the cell nucleus, as would be expected from a nuclear staining, but was seen in the cytoplasm as well (see Figure 6.3). This ' Δ FosB-like immunoreactivity' was almost exclusively found in the most lateral regions of the deep cerebellar nuclei, and was particularly observed in the DN.

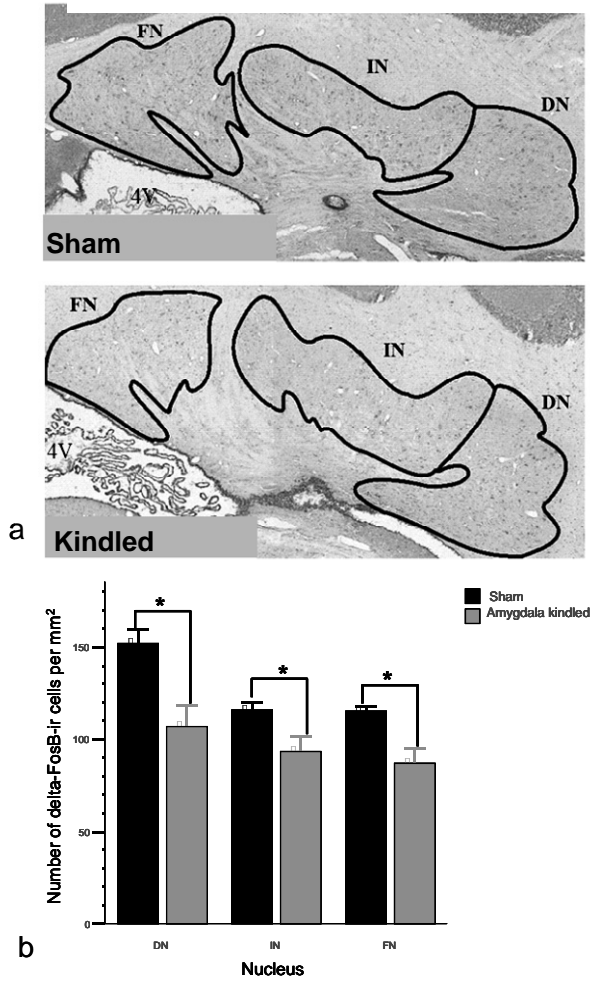


Figure 6.1 Amygdala kindling is associated with a reduced cerebellar Δ FosB expression. (a) Representative photomicrographs (4x magnification) showing Δ FosB immunoreactivity in the deep cerebellar nuclei of a sham and a fully amygdala kindled rat. Note the overall reduction in Δ FosB immunoreactivity in the kindled rat, suggesting a general decrease in neuronal activity. Coronal sections were taken at bregma level -11.30. 4V: fourth ventricle; DN: dentate nucleus; FN: fastigial nucleus; IN: interpositus nucleus. (b) Number of Δ FosB immunoreactive (ir) cells/mm² in the deep cerebellar nuclei. Data represent mean \pm SEM of 72 sections from 9 sham animals and 84 sections from 7 kindled animals. * $p < 0.05$.

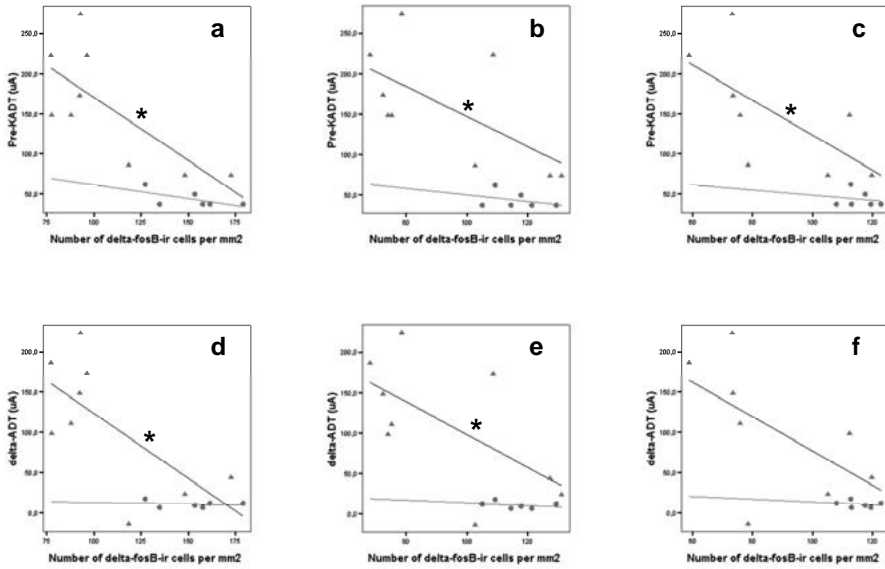


Figure 6.2 The neural activity of the deep cerebellar nuclei correlates negatively with seizure threshold. Correlations between Δ FosB immunoreactivity in the nuclei (a and d: dentate; b and e: interpositus; c and f: fastigial) and the pre-kindling afterdischarge threshold (a-c) or the change in afterdischarge threshold (d-f). ●: sham; ▲amygdala kindled rats; * $p < 0.05$.

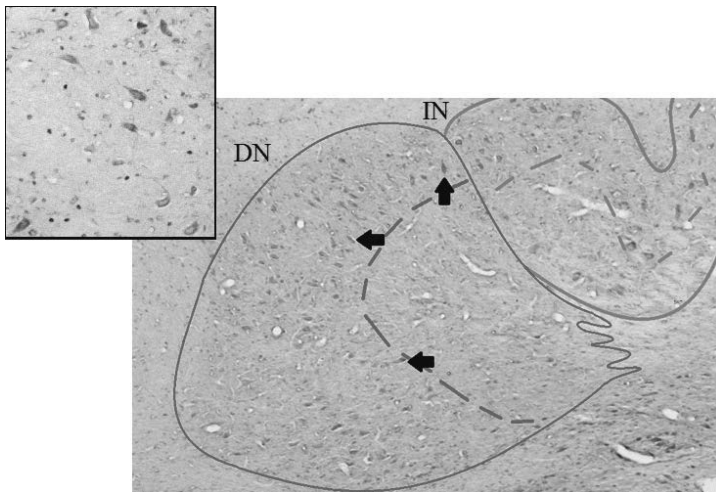


Figure 6.3 Photomicrograph (10x magnification) showing the distribution of Δ FosB immunoreactive cells in the lateral deep cerebellar nuclei. The cytoplasmic staining of Δ FosB is seen in relatively large cells (arrows) that are almost exclusively localized in the outer regions of the nuclei. These cells are seen in the deep cerebellar nuclei of both sham and amygdala kindled rats. The inset is a 40x magnification showing the cytoplasmic staining in more detail. DN: dentate nucleus; IN: interpositus nucleus.

COX

Kindled animals expressed significantly less COX than shams in both FN and IN (FN: -32.7 ± 3.4 vs. 21.9 ± 2.8 , $p < 0.05$; IN: -47.7 ± 4.2 vs. -32.4 ± 4.8 , $p < 0.05$, see Figure 6.4). In the DN, COX expression was also lower in the kindled animals than in the shams, but this difference was not statistically significant (-39.4 ± 5.5 vs. -55.5 ± 5.2 ; $p = 0.078$). COX-expression showed no significant correlation with any of the kindling parameters.

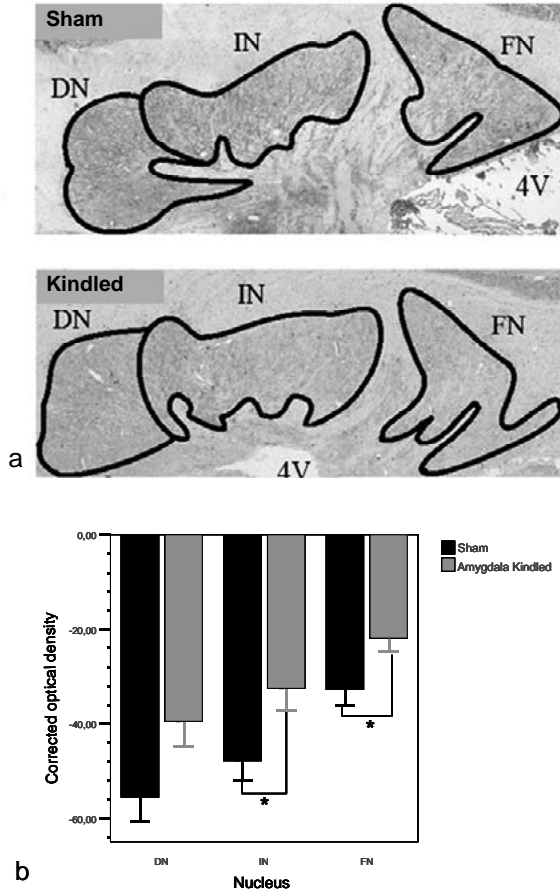


Figure 6.4 Amygdala kindling is associated with a reduced cerebellar cytochrome oxidase (COX) expression. (a) Representative photomicrographs (4x magnification) showing COX activity in the deep cerebellar nuclei of a sham and a fully amygdala kindled rat. Note the overall reduction in COX activity in the kindled rat, suggesting a general decrease in neuronal activity. Coronal sections were taken at bregma level -11.30 . 4V: fourth ventricle; DN: dentate nucleus; IN: interpositus nucleus; FN: fastigial nucleus. (b) Corrected optical density of COX activity in the deep cerebellar nuclei. The optical density was corrected for background staining by subtracting the optical density of the adjacent white matter, leading to a negative value. Data represent mean \pm SEM of 101 sections from 6 sham animals and 92 sections from 7 kindled animals. * $p < 0.05$.

Discussion

In this study, we have identified the pattern of interictal neuronal activity in the deep cerebellar nuclei of chronically epileptic rats. We were able to confirm our hypothesis that chronically epileptic rats show less neuronal activity in these nuclei than shams, reflected by a significantly lower density of Δ FosB immunopositive cells in the IN, FN and DN and by a significantly lower expression of COX in the FN and IN. We have furthermore shown that a lower level of neuronal activity is associated with a higher pre-KADT and a higher Δ ADT.

Methodological considerations

All experiments were carried out in fully kindled animals that were sacrificed 48 hours after their last kindled seizure. This means that we investigated interictal changes in a chronic epilepsy model. Data on interictal cerebellar activity in humans are scarce and are indicative of hypometabolism on PET³¹ and SPECT³². Epileptic dogs did not show any change in cerebellar metabolism interictally³³. These imaging studies mainly estimate cerebellar cortical activity, while the deep cerebellar nuclei are not evaluated this way. It is therefore not possible to compare our data with in vivo imaging data.

Some areas of the brain are more involved in generalized tonic-clonic seizures than others^{9,34-36}. The degree of involvement can be immunohistochemically determined by markers for immediately early genes, such as c-fos, FosB and Δ FosB. C-Fos and FosB peak at 2 and 6 hours after a stimulus, respectively. Δ FosB is a highly stable FosB isoform that persists in the brain for several weeks after an initial stimulus³⁷. Since the kindling protocol involves repetitive stimuli with interstimulus intervals of more than six hours, we chose to determine the Δ FosB immunopositive cell density as a marker of neuronal activation. To the best of our knowledge, no data are available in literature on basal cerebellar Δ FosB levels, nor on cerebellar Δ FosB expression in epilepsy. Our results in sham rats reveal that basal expression of Δ FosB is present in all deep cerebellar nuclei, and that among the deep nuclei, the DN contains the highest density of Δ FosB immunopositive cells. The DN receives input from the more lateral parts of the cerebellar cortex while IN and FN receive input from the vermal cortex (for a review see Voogd *et al.*³⁸). Our data suggest that in shams, the vermal cortex has a stronger inhibitory effect on its target nuclei than the cortex of the cerebellar hemispheres.

Furthermore, there are three types of Fos immunoreactivity: nuclear, cytoplasmic, or both³⁹. We found cells with nuclear Δ FosB immunoreactivity, but also several cells with cytoplasmic staining. This phenomenon has been described for the hippocampus, dentate gyrus and amygdala by others⁴⁰, and appears to be present in the deep cerebellar nuclei as well.

COX is a large transmembrane protein located in mitochondria. It transforms redox energy from the oxidative respiratory chain into a proton-motive force across

the mitochondrial membrane. In this process it receives an electron from four cytochrome c proteins and donates these electrons to free oxygen molecules, thereby converting them to water^{41,42}. Under physiological conditions, COX is involved in the cellular energy supply, while in the process of seizure-induced neurodegeneration it plays a role in the activation of pro-apoptotic pathways^{12,43-47}. Here, we used COX as a marker of oxidative metabolism. COX activity has been shown in the rat cerebellum previously (e.g. by using spectrophotometry and immunoblot⁴⁸), but cerebellar COX expression has not been evaluated before using COX histology. On the other hand, COX histology has been used before to quantify the degree of cerebral oxidative metabolism after electrical stimulation in the rat³⁰.

Epilepsy affects activity of the deep cerebellar nuclei

We have shown that kindled animals expressed a lower Δ FosB immunopositive cell density than shams in all three cerebellar nuclei. Neurons in the FN showed the largest decrease, followed by those in the IN. Our COX data indicate as well that FN and IN are more affected by kindling than the DN (see Figure 6.4).

Apparently, amygdala kindling decreases FN activity more than it reduces the activity of DN and IN. This may be the result of direct antidromic effects of amygdala kindling, bearing in mind the possible anatomical connection between the FN and the amygdala⁴⁹. These connections have been suggested to exist based on several findings. First, behavioural changes are observed following low frequency stimulation of the FN⁵⁰ or lesioning of the deep cerebellar nuclei⁵¹. Second, electrophysiological studies have shown that both low-⁵⁰ and higher frequency stimulation⁴⁹ of the FN evokes responses in hippocampus and amygdala bilaterally. Third, lesioning the FN leads to degeneration of synaptic terminals in the amygdala bilaterally⁴⁹.

Next to the possible antidromic effect of amygdala kindling on the cerebellum, activity of the deep cerebellar nuclei may have been affected by the generalized seizures that the animals in this model suffer from, since chronic epilepsy with generalized seizures in humans is also associated with cerebellar changes^{52,53}. Decreased activity of the deep cerebellar nuclei may therefore have been secondary to multiple seizures. Output of the FN is putatively glutamatergic⁵⁴, and decreased activity of the FN may lead to a decreased activation of its most important target structure, the thalamus. This may facilitate further spread of seizures, since the thalamus has been shown to play an important role in seizure control^{55,56}.

The FN is of particular interest with regard to the treatment of epilepsy since low frequency stimulation of this nucleus as well as stimulation of the vermal cortex (that projects to the FN) are both anticonvulsive^{16,26}, while GABA agonist injections into the FN significantly decrease seizure threshold⁵⁷. Furthermore, complete destruction of either the FN is proconvulsive, while partial destruction inhibits seizures⁵⁸. In addition, FN lesions abolish the anticonvulsive effects of vermis stimulation⁵⁹. On the other hand, low frequency stimulation of the DN does not affect seizure activity²² and GABA-ergic injections into the DN do not affect seizures⁵⁷.

Conclusion

In our study we confirmed our hypothesis that the deep cerebellar nuclei are hypoactive in chronic epilepsy. This decrease was most pronounced in the FN. Functionally located between limbic system, where seizures often arise, and thalamus, where seizures are propagated, the FN is an interesting target for epilepsy treatment; we hypothesize that low frequency stimulation of the FN restores its activity and thereby its control over thalamus function, explaining anticonvulsive effects of this treatment.

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Chapter 7

General discussion and conclusion

General discussion and conclusion

The role of the cerebellum in modulating behaviour is currently the subject of extensive investigation using a variety of techniques. Research aimed at the anatomical and functional connections of the cerebellum forms one way of addressing this topic. Deep brain stimulation (DBS) can affect distant structures and can thus be used to analyse the existence of functional connections between these structures. In **chapter 1** we presented the results of a literature search showing that this method has not been used frequently to analyze functional connections with cerebellar structures. In our studies we analysed cerebellar activity following DBS-induced behaviour. More specifically, we presented findings on the changes of neuronal activity of individual deep cerebellar nuclei (DCbN) following DBS in animal models. Here we summarize the main findings of these studies, discuss their relevance, and propose future research.

Cerebellum and behaviour

The cerebellar cognitive affective syndrome (CCAS) and the cerebellar mutism syndrome (CMS, a.k.a. posterior fossa syndrome, PFS) described in patients with cerebellar lesions include inappropriate or disinhibited behaviour or decreased initiation of activity^{1,2}. Studies on the relationship between impulsivity and the cerebellum are described in **chapters 2 and 3**. Here we demonstrated that DBS of the mediodorsal thalamic nucleus (MD) results in an increase of impulsive behaviour and decreased activity of the DCbN (**chapter 2**). In contrast, DBS of the ventrolateral thalamic nucleus (VL) had no influence on impulsivity and did not change neuronal activity of the DCbN. DBS of the subthalamic nucleus (STN) has previously been shown to improve motor parameters and decrease impulsivity in naïve rats³ as well as in a rat model of Parkinson's disease⁴. In **chapter 3** we showed that DBS of this nucleus resulted in increased neuronal activity of the DCbN.

The dorsolateral periaqueductal grey (dIPAG) and the ventromedial hypothalamus (VMH) are both constituents of the fear network. DBS of these areas in rats leads to panic-like behaviour and can therefore be used as a model for panic attacks^{5,6}. Using this model we showed that panic behaviour is accompanied by decreased activity of the DCbN (**chapter 4**). dIPAG stimulated animals that are returned to the experimental arena twelve hours after stimulation show a freezing response indicating conditioned fear⁷. In contrast to the findings following acute dIPAG stimulation, we found no altered activity of DCbN following this conditioned response (**chapter 5**).

The relationship between the cerebellum and epilepsy was first suggested by Hunt when he noted the coexistence of myoclonic epilepsy with cerebellar atrophy^{8,9}. Lesions of the cerebellum have been shown to induce or facilitate seizures and seizure spread. Cerebellar stimulation has been explored as possible treatment, with inconsistent results¹⁰. Amygdala kindling is a model in which repeated subthreshold

stimulation of the amygdala causes seizures of increasing severity, which is used as a model of chronic epilepsy¹¹. Fully kindled rats were characterized by a significantly decreased neuronal activity in the DCbN (**chapter 6**). This activity correlated negatively with the afterdischarge threshold.

These results lead us to conclude that abnormal or disruptive behaviour is accompanied by decreased activity in the DCbN, whereas favourable behaviour is associated with increased activity in the DCbN. These findings add to the evidence that the cerebellum plays a role in modulating behaviour. Several authors have suggested that the cerebellum in fact modulates all aspects of neurological functioning^{12,13}. Schmahmann has suggested that cerebellar modulation serves as a dampener of oscillation around a homeostatic baseline¹³. Cerebellar damage then results in dysmetria of thought as well as of movement, depending on the localisation of the lesion. The exact manner in which the cerebellum does this is still unclear. It has become clear that the cerebellum is involved in motor learning processes by long term depression (LTD) mechanisms of Purkinje cells (PC's)^{14,15}. Functional imaging studies have shown that the cerebellum is activated in the process of motor sequence learning¹⁴. The cerebellar cortex shows activation in the early phase of learning, which shifts towards the DCbN during the learning process and finally disappears with extended practice¹⁴. This may be the explanation for the lack of activation changes found in the DCbN in conditioned fear (**chapter 5**). It has been suggested that the cerebellum uses this learning process to form internal models, which are fine-tuned by error signals^{16,17}. Using these internal models the brain can direct movements precisely and swiftly without the need to wait for sensory feedback¹⁸. It is suggested that the cerebellum forms internal models of mental processes in the same manner¹⁸.

Cerebellar connections

The uniform microstructure of the cerebellar cortex and the fact that it contains about 50 billion neurons suggests that the cerebellum has powerful mechanisms for information processing. It is likely that all incoming information is processed in the same manner, as mentioned previously. To be able to influence cognitive and behavioural processes the cerebellum needs to have afferent and efferent connections with cerebral areas that are involved in these processes. The input to the cerebellum has been shown to come from diffuse cortical areas, including the prefrontal and parietal cortex. In contrast to what was previously believed the output of the deep cerebellar nuclei is not solely directed at the primary motor cortex. Efferents from the DCbN project to diffuse areas in the cortex through the thalamus. Efferents from the DCbN to the VL thalamic nucleus are well-known, while efferents to the MD nucleus of the thalamus have been shown to exist more recently¹⁹. It has been shown that these nuclei form part of anatomical closed loops between the cerebellum and the cerebral cortex²⁰. Each of these loops seems to subserve a different function. The cerebellar

recipient parts of the MD and VL nuclei of the thalamus project to the prefrontal cortex (PFC) and these cortical areas project back to the cerebellum, forming a loop that is thought to be involved in cognitive functioning. Our results support the existence of a cerebello-thalamo-cortical loop through the MD nucleus of the thalamus and the PFC and suggest that it is functionally involved in impulsive behaviour (**chapter 2**). In addition to closed loops with diverse cortical areas, recent tracing experiments in non-human primates have shown the existence of a closed loop between the cerebellum and the basal ganglia which seems to be independent of cortical input²¹. This loop is formed by a bisynaptic projection from the DN to the striatum²² and a similar projection from the STN to the cerebellar cortex via the pontine nuclei²³. We have shown that there is indeed a functional connection between the STN and the DCbN (**chapter 3**). The projections from the STN to the cerebellum arise in all subterritories of the STN, suggesting that the pathways between the basal ganglia and the cerebellum are involved in motor as well as non-motor processing.

The cerebellum also has connections with multiple brain stem nuclei and midbrain structures. In the rat, projections from the DCbN to the PAG have been shown to exist²⁴. Reciprocal connections between the cerebellum and widespread areas of the hypothalamus have been described using tracing experiments²⁵. These anatomical connections linking the cerebellum with the fear network are supported by our results (**chapter 4**). Connections between the fastigial nucleus (FN) and the amygdala have been suggested based on behavioural changes following cerebellar lesioning or stimulation^{26,27}. Classic electrophysiological studies also support a functional connection between the FN and the amygdala²⁸, however, an exact anatomical pathway has never been shown²⁹. The decreased metabolic activity in the DCbN following amygdala kindling which we found in **chapter 6** may be the result of antidromic effects of stimulation in the basolateral amygdala. However, the rats in this model suffered from many stimulation-induced generalized seizures. Chronic epilepsy has been associated with cerebellar changes in human imaging studies, possibly related to duration and severity of the disorder³⁰⁻³³. Therefore the altered activity we find in the DCbN may also be explained as secondary to repeated seizure activity rather than a direct effect of electrical stimulation.

All cerebellar connections described above are based on anatomical and functional studies in animal models. Reproduction of these anatomical connections in the human situation has been difficult. However, with the advent of functional imaging the possibility has risen to study functional connections in the human brain in healthy controls as well as in neurological patients. Functional imaging such as PET, SPECT and fMRI is increasingly used in patients undergoing DBS to elucidate the mechanism of DBS. In **chapter 1** we have summarized the functional imaging studies in patients with Parkinson's disease (PD) treated with DBS. Changes of activation are often seen in the cerebellum, suggesting functional connections between the stimulated structures and the cerebellum. Functional neuroimaging studies have also shown cerebellar activation during a variety of cognitive tasks³⁴. Functional connectivity between the cerebellum

and prefrontal and posterior parietal cortex was shown using resting-state functional connectivity MRI³⁵, suggesting an anatomical connection. Recent developments in diffusion tensor imaging (DTI) have made it possible to analyse the connections of white matter tracts of the human cerebellum. Using this technique the connection between the dentate nucleus (DN) and the prefrontal and posterior parietal cortex described previously in monkeys has been confirmed in humans³⁶. A connection between the STN and the cerebellum was also shown using DTI³⁷.

The exact localization of activation changes in functional imaging of the cerebellum and especially the DCbN is however hampered by the small size and the lack of a reliable MRI atlas³⁸. Recent improvement of neuroimaging techniques has resulted in an increased spatial resolution and now allows visualization of DCbN and cerebello-cortical sublayers. It has even been proven possible to visualize subunits of the DN³⁸. A meta-analysis of functional imaging studies and human cerebellar lesion studies shows that there is a functional topography in the cerebellum in which sensorimotor functions are localized to lobules I-VI in the anterior lobe, extending into lobule VI and posterior lobules VIIIA and VIIIB. Cognitive functions are localized to the lateral regions of lobules VI and VII of the posterior lobe³⁴. Functional connectivity studies support the mainly contralateral projection from cerebellum to cerebral cortex. Functional imaging during cognitive tasks show lateralization of language and spatial processing respectively to the right and left cerebellar hemispheres, which is in concordance with symptoms found in patients with lateralized cerebellar lesions³⁴. When the analysis is directed to the DCbN, the DN, and especially the ventrocaudal region is activated in cognitive tasks. This is in concordance with anatomical projections to the prefrontal and posterior parietal cortices which originate mainly in the DN. In contrast, we found activation changes in all DCbN following behavioural changes induced by DBS. However, functional imaging of the interpositus nucleus (IN) and the fastigial nucleus (FN) is still subject to methodological restrictions due to their limited volume. Further improvements of these imaging techniques are therefore necessary to be able to give reliable information on the activation of these DCbN on functional imaging. Future research using these imaging techniques may enable us to further analyse anatomical and functional connections between the cerebellum and the rest of the brain and within the cerebellum itself. This approach has recently proven its value, for imaging of connections and activation patterns in neuropsychiatric diseases have given insight in the involvement of the cerebellum (for example^{39,40}). It is to be expected that this knowledge will extend to other neurological and psychiatric disorders.

Cerebellar stimulation

Similar to DBS of cerebral targets, cerebellar stimulation has frequently been used in research settings as well as in clinical settings. Stimulation of cerebellar cortex or DCbN

has been performed to analyse connections of the cerebellum with the limbic system^{28,41} and to elucidate the role of the cerebellum in epilepsy^{42,43}. Cerebellar stimulation as a treatment option has been studied intensively in relation to epilepsy (for review see Fountas *et al.*¹⁰). The rationale was based on the concept of an inhibitory influence of the cerebellum on the thalamus, resulting in decreased activity of excitatory thalamocortical projections. This would then result in decreased cortical excitability, thereby inhibiting seizures or seizure spread. However, these studies showed inconsistent results, possibly due to variations in methodology, such as targets and stimulation parameters. Furthermore, clinical studies included patients with different types of epilepsy and seizures. Interestingly, the patients included in these studies often showed improvement of cognitive functioning, either subjectively or psychologically assessed, which is independent of the effect on seizures¹⁰. Cerebellar stimulation has also been studied as a possible treatment option for spasticity especially in cerebral palsy (CP), with positive effects⁴⁴. Most of the above mentioned studies applied cerebellar cortex stimulation. Only three animal studies performed stimulation of the DCbN. Two of these studies found an inhibition of seizure activity^{45,46}. One study observed no effect on seizures induced by electrical stimulation, but found a significant reduction of spasticity⁴⁷. In humans Sramka *et al.* performed bilateral stimulation of the DN leading to a transient improvement of seizure in all three patients⁴⁸. Dentate nucleus stimulation was shown to improve spasticity and speech quality in a patient with spasticity due to CP⁴⁹. This patient also showed improved status on psychological testing.

Cerebellar stimulation as a therapeutic option needs more research before it can be added to the standard treatment options in epilepsy and spasticity. With the recently improved stimulation hardware and software, it seems logical to go back to animal research to evaluate the effect of cerebellar stimulation and optimize target and stimulation settings. We have shown that generalized seizures elicited by amygdala kindling are associated with decreased activity in the DCbN (**chapter 6**). Activating the DCbN by low frequent stimulation may therefore prove beneficial. Since the FN seems to be the most important of the DCbN in epilepsy, it may be the best target to analyse.

With respect to behaviour, several options for further research present themselves. In the past cerebellar stimulation has been performed eliciting behavioural changes such as sham rage, predatory attack, grooming and eating^{50,51}. It would be interesting to repeat and elaborate on these experiments using modern stimulation technology in animal models. A possible setup would be to evaluate the effect of stimulation of the different DCbN with high and low frequency on behavioural aspects such as impulsivity, fear and depression. Further experiments might involve similar stimulation studies in animal models of neuropsychiatric diseases. The cerebellum has been implicated in diseases such as schizophrenia, autism and depression. It was suggested that these patients suffer from a lack of specific internal models related to mental processes¹⁸. Cerebellar stimulation may prove beneficial by improving the

function of the cerebellum leading to better internal model to direct behaviour. Results from cerebellar stimulation in animal models of these diseases may in the end lead to therapeutic implementation.

Conclusion

This thesis demonstrates that the cerebellum plays a role in the modulation of behaviour. Decreased activity of the deep cerebellar nuclei is associated with abnormal behaviour (impulsivity, panic attacks and seizures). This adds to the body of evidence that the cerebellum modulates all aspects of neurological functioning, possibly by forming internal models that allow motor and mental processes to be performed accurately and swiftly. Cerebellar lesions can lead to behavioural and cognitive problems, depending on the localisation of the lesion. This also explains cognitive and behavioural problems seen in congenital cerebellar abnormalities and in children following cerebellar tumour resection. Furthermore cerebellar abnormalities are found in neuropsychiatric diseases. These aspects of cerebellar function still receive too little attention in clinical practice. Future studies are necessary to increase our knowledge on the exact role of the cerebellum and the mechanisms underlying its influence on the brain. This will hopefully lead to more awareness of the presence of cognitive and behavioural problems in patients with cerebellar lesions. Finally, our results justify the evaluation of direct cerebellar stimulation in models of neurological and psychiatric disease.

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Summary

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The role of the cerebellum in modulating behaviour is currently a subject of extensive investigation using a variety of techniques. Research aimed at the anatomical and functional connections of the cerebellum forms one way of addressing this topic. Deep brain stimulation (DBS) can affect distant structures and can thus be used to analyse the existence of functional connections with cerebellar structures. In our study we analysed the involvement of the cerebellum in behaviour modulated by DBS. In this thesis, the results of several studies in which we examined the neuronal activity of the deep cerebellar nuclei (DCbN) following DBS in animal models have been presented.

Changes of impulsive behaviour have been described in patients with cerebellar lesions. In **chapters 2 and 3** we studied the relationship between impulsivity and the cerebellum. Here we demonstrated that DBS of the mediodorsal nucleus of the thalamus (MD) results in an increase of impulsive behaviour and decreased activity of the DCbN (**chapter 2**). In contrast, DBS of the ventrolateral thalamic nucleus (VL) had no influence on impulsivity and did not change neuronal activity of the DCbN. DBS of the subthalamic nucleus (STN) has previously been shown to improve motor parameters and decrease impulsivity in naïve rats as well as in a rat model of Parkinson's disease. In **chapter 3** we showed that DBS of this nucleus resulted in increased neuronal activity of the DCbN.

The dorsolateral periaqueductal grey (dIPAG) and the ventromedial hypothalamus (VMH) are both constituents of the fear network. DBS of these areas in rats leads to panic-like behaviour and can therefore be used as a model for panic attacks. Using this model we showed that panic behaviour is accompanied by decreased activity of the DCbN (**chapter 4**). dIPAG stimulated animals that are returned to the experimental arena twelve hours after stimulation show a freezing response indicating conditioned fear. In contrast to the findings following acute dIPAG stimulation, we found no altered activity of DCbN following this conditioned response (**chapter 5**).

The relationship between the cerebellum and epilepsy was first suggested by Hunt when he noted the coexistence of myoclonic epilepsy with cerebellar atrophy. Lesions of the cerebellum have been shown to induce or facilitate seizures and seizure spread. Cerebellar stimulation has been explored as possible treatment, with inconsistent results. Amygdala kindling is a model in which repeated subthreshold stimulation of the amygdala causes seizures of increasing severity, which is used as a model of chronic epilepsy. Fully kindled rats were characterized by a significantly decreased neuronal activity in the DCbN (**chapter 6**). This activity correlated negatively with the afterdischarge threshold.

These results lead us to conclude that abnormal or disruptive behaviour is accompanied by decreased activity in the DCbN, whereas favourable behaviour is associated with increased activity in the DCbN. This adds to the evidence that the cerebellum plays a role in modulating behaviour. It has been proposed that the cerebellum forms internal models of neurological processes which are constantly fine-

tuned by error signals. These internal models allow the brain to direct these processes, either motor or mental, without having to rely on direct sensory feedback. Damage to the cerebellum may hamper the fine-tuning and accessibility of these models, leading to motor, behavioural or cognitive problems depending on the localisation of the lesion.

The uniform manner in which the cerebellum seems to process information means that the neurological processes it has influence on are defined by its anatomical connections. Animal research has shown that there are several independent loops between the cerebellum and the cortex, passing through several thalamic nuclei, as well as between the cerebellum and the basal ganglia. Our results confirm the existence of functional connections between the cerebellum and the MD nucleus of the thalamus (**chapter 2**), the STN (**chapter 3**), the dIPAG and the VMH (**chapter 4**), and the amygdala (**chapter 6**). Improvement of neuroimaging techniques such as functional MRI and diffusion tensor imaging have already proven their value in analyzing these anatomical and functional connections in humans. Their role in this research is expected to grow in the coming years.

Cerebellar stimulation has been performed in the past in animal models to analyse connections of the cerebellum with the limbic system and to elucidate the role of the cerebellum in epilepsy. It has also been studied as a therapeutic option in patients with epilepsy and spasticity, with inconsistent results. We suggest future research using DBS of the cerebellar nuclei to further evaluate their role in behaviour and epilepsy and to analyse the possible therapeutic effect in animal models of epilepsy and neuropsychiatric diseases. The results of these studies may lead to therapeutic implementation of cerebellar stimulation.

Conclusion

This thesis demonstrates that the cerebellum plays a role in the modulation of behaviour. Decreased activity of the deep cerebellar nuclei is associated with abnormal behaviour (impulsivity, panic attacks and seizures). This adds to the body of evidence that the cerebellum modulates all aspects of neurological functioning, possibly by forming internal models that allow motor and mental processes to be performed accurately and swiftly. Cerebellar lesions can lead to behavioural and cognitive problems, depending on the localisation of the lesion. This also explains cognitive and behavioural problems seen in congenital cerebellar abnormalities and in children following cerebellar tumour resection. Furthermore cerebellar abnormalities are found in neuropsychiatric diseases. These aspects of cerebellar function still receive too little attention in clinical practice. Future studies are necessary to increase our knowledge on the exact role of the cerebellum and the mechanisms underlying its influence on the brain. This will hopefully lead to more awareness of the presence of cognitive and behavioural problems in patients with cerebellar lesions. Finally, our results justify the

evaluation of direct cerebellar stimulation in models of neurological and psychiatric disease.

Samenvatting

Samenvatting

De rol van het cerebellum in het moduleren van gedrag wordt momenteel met verschillende technieken onderzocht. Een van deze technieken betreft het onderzoeken van anatomische en functionele verbindingen van het cerebellum. Diepe hersenstimulatie (DBS; deep brain stimulation) kan gebieden op afstand beïnvloeden en kan dus worden gebruikt om de aanwezigheid van functionele verbindingen van de gestimuleerde gebieden met de kleine hersenen te onderzoeken. In onze studie onderzochten wij de betrokkenheid van het cerebellum bij gedrag dat gemoduleerd was door DBS. In dit proefschrift hebben wij de resultaten gepresenteerd van een aantal studies waarin we de neuronale activiteit van de diepe kernen van het cerebellum (DCbN; deep cerebellar nuclei) als gevolg van DBS in diermodellen hebben onderzocht.

Veranderingen van impulsief gedrag zijn beschreven bij patiënten met schade van het cerebellum. In **hoofdstuk 2 en 3** hebben wij het verband tussen het cerebellum en impulsiviteit onderzocht. We hebben aangetoond dat DBS van de mediodorsale (MD) thalamuskern leidt tot een toename van impulsiviteit en een afname van de neuronale activiteit in de DCbN (**hoofdstuk 2**). DBS van de ventrolaterale thalamuskern (VL) heeft daarentegen geen invloed op impulsiviteit of neuronale activiteit van de DCbN. Het is al eerder aangetoond dat DBS van de subthalamische kern (STN) in naïeve ratten en in een ratmodel van de ziekte van Parkinson leidt tot een verbetering van motore parameters en een afname van impulsiviteit. In **hoofdstuk 3** hebben we laten zien dat DBS van deze kern leidt tot een toename van de neuronale activiteit in de DCbN.

Het dorsolaterale periaqueductale grijs (dIPAG) en de ventromediale hypothalamus (VMH) maken beide onderdeel uit van het angst netwerk. DBS van deze gebieden leidt tot paniekachtig gedrag en kan worden gebruikt als een model voor paniekaanvallen. Gebruik makend van dit model hebben wij aangetoond dat paniekgedrag gepaard gaat met een afgenomen neuronale activiteit in de DCbN (**hoofdstuk 4**). Dieren die in het dIPAG zijn gestimuleerd en 12 uur later weer terug worden gezet in de experimentele arena vertonen angstgedrag hetgeen duidt op geconditioneerde angst. In tegenstelling tot de bevindingen na acute dIPAG-stimulatie vonden we geen verandering in de neuronale activiteit van de DCbN na dergelijke geconditioneerde angst (**hoofdstuk 5**).

De relatie tussen epilepsie en het cerebellum werd voor het eerst gelegd door Hunt toen hij het samen voorkomen van myoclonie epilepsie en cerebellaire atrofie beschreef. Letsels van het cerebellum kunnen leiden tot epileptische aanvallen of het opwekken en spreiden hiervan faciliteren. Cerebellaire stimulatie werd reeds onderzocht als mogelijke behandeling, met tegenstrijdige resultaten. Amygdala kindling is een model waarbij herhaalde elektrische stimulatie van de amygdala leidt tot epileptische aanvallen met toenemende ernst, hetgeen gebruikt wordt als model voor chronische epilepsie. Volledig gekindelde ratten toonden een significante afname

van de neuronale activiteit in de DCbN (**hoofdstuk 6**). Deze activiteit toonde een negatieve correlatie met de afterdischarge drempel.

Deze resultaten hebben geleid tot de conclusie dat afwijkend gedrag is geassocieerd met een afgenomen activiteit van de DCbN terwijl gunstig gedrag is geassocieerd met een toename van deze activiteit. Dit versterkt het bewijs dat het cerebellum is betrokken bij het moduleren van gedrag. Er is al voorgesteld dat het cerebellum interne modellen vormt van neurologische processen en dat deze modellen continu worden bijgeschaafd door feedback signalen. Deze interne modellen stellen de hersenen in staat deze motore en mentale processen vlot te laten verlopen zonder afhankelijk te zijn van directe feedback. Beschadiging van het cerebellum kan het bijwerken en de beschikbaarheid van deze modellen hinderen en leiden tot motorische, gedragsmatige of cognitieve problemen, afhankelijk van welk gebied van het cerebellum is aangedaan.

De uniforme manier waarop het cerebellum informatie lijkt te verwerken betekent dat de neurologische processen waar het invloed op uitoefent bepaald worden door de anatomische verbindingen. Dierexperimenteel onderzoek heeft aangetoond dat er meerdere onafhankelijke gesloten circuits bestaan tussen de cerebrale cortex en het cerebellum die door meerdere thalamuskernen lopen. Tevens bestaat er een dergelijk circuit tussen de basale kernen en het cerebellum. Onze resultaten bevestigen de aanwezigheid van functionele verbindingen tussen het cerebellum en de MD thalamuskern (**hoofdstuk 2**), STN (**hoofdstuk 3**), het dIPAG en de VMH (**hoofdstuk 4**) en de amygdala (**hoofdstuk 6**). Verbetering van beeldvormende technieken zoals functionele MRI en diffusion tensor imaging zijn al waardevol gebleken in het onderzoek van deze verbindingen in mensen. Hun rol in dit onderzoek zal naar verwachting alleen maar toenemen in de komende jaren.

Cerebellaire stimulatie werd in het verleden reeds toegepast om verbindingen van het cerebellum met het limbische systeem te onderzoeken en om de rol van het cerebellum in epilepsie op te helderen. Het werd tevens onderzocht als mogelijke therapeutische optie in patiënten met epilepsie en spasticiteit, met wisselend resultaat. Wij stellen verder onderzoek voor met DBS van de DCbN om hun rol in gedrag en epilepsie verder te verduidelijken en om het mogelijk therapeutische effect te onderzoeken in diermodellen van epilepsie en neuropsychiatrische ziektebeelden. De resultaten van deze studies zouden kunnen leiden tot de therapeutische implementatie van cerebellaire stimulatie.

Conclusie

Dit proefschrift toont aan dat het cerebellum een rol speelt in het moduleren van gedrag. Afgenomen activiteit in de diepe cerebellaire kernen is geassocieerd met afwijkend gedrag (impulsiviteit, angst en epilepsie). Dit voegt toe aan het bewijs dat het cerebellum alle neurologische processen beïnvloedt, mogelijk door interne

modellen te vormen die snelle en accurate uitvoering van motore en mentale processen mogelijk maken. Cerebellaire laesies kunnen leiden tot motore en gedragsproblemen, afhankelijk van de lokalisatie van de laesie. Dit verklaart ook de cognitieve en gedragsproblemen die worden gezien in kinderen met aangeboren afwijkingen van het cerebellum of na verwijdering van een cerebellaire tumor. Verder worden cerebellaire afwijkingen ook gevonden in neuropsychiatrische aandoeningen. Deze aspecten van de functie van het cerebellum krijgen nog veel te weinig aandacht in de klinische praktijk. Verder onderzoek is noodzakelijk om de precieze rol van het cerebellum en de manier waarop het de hersenen beïnvloedt te begrijpen. Dit leidt hopelijk tot meer aandacht voor de aanwezigheid van cognitieve en gedragsproblemen in patiënten met cerebellaire beschadigingen. Als laatste rechtvaardigen onze resultaten onderzoek naar directe cerebellaire stimulatie in modellen van neurologische en psychiatrische aandoeningen.

List of publications

List of publications

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Dankwoord

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Lieve Rob. Het is af. Ik hou van je. Dankjewel dat ik dit heb mogen doen.

Curriculum Vitae

Curriculum Vitae

Véronique Maria Peter Moers-Hornikx werd geboren op 5 Juli 1980 in Kigali, Rwanda als oudste dochter van Nederlandse ontwikkelingshulpmedewerkers. Ze groeide op in Rwanda en Nigeria en verhuisde in 1995 met haar ouders, zus en broers terug naar Helmond waar ze in 1998 eindexamen VWO deed aan het Carolus Borromeus College. Datzelfde jaar begon zij aan de opleiding geneeskunde aan de Universiteit Maastricht. Na het succesvol afleggen van het doctoraal examen in 2003 volgde zij haar coschappen in verschillende Limburgse en Brabantse ziekenhuizen. Het coschap interne geneeskunde volgde zij in het Centre Hospitalier Universitaire te Saint-Étienne, Frankrijk. In 2003 volgde zij haar wetenschapsstage onder begeleiding van prof. dr. Y. Temel, waar zij voor het eerst kennis maakte met het gebruik van diepe hersenstimulatie in diermodellen. Na afronding van het artsexamen in 2004 begon zij als arts-assistent niet in opleiding bij de vakgroep neurologie van het azM/MUMC⁺, waar zij in 2005 in opleiding kwam. In 2008 verrichte ze basaal onderzoek in het kader van dit proefschrift onder leiding van prof. dr. J.S.H. Vles waarvoor de opleiding neurologie tijdelijk werd onderbroken. In 2009 hervatte zij de opleiding neurologie en continueerde daarbij de wetenschappelijke activiteiten die geleid hebben tot dit proefschrift. In 2013 hoopt ze de opleiding neurologie af te ronden. In 2005 trouwde zij met Rob Moers. Zij hebben samen drie kinderen: Myrthe (5 jaar), Rianne (3 jaar) en Xander (1 jaar).

Véronique Maria Peter Moers-Hornikx was born in Kigali, Rwanda on the 5th of July 1980. She was the eldest daughter of Dutch developmental aid workers. She grew up in Rwanda and Nigeria. In 1995 she moved back to Helmond, the Netherlands, with her parents, sister and brothers. She finished her secondary school education at the Carolus Borromeus College in 1998. The same year she started her medical education at the Maastricht University. In 2003 she passed her doctoral exam, after which she followed her internships in several hospitals in the south of the Netherlands. She followed her internship internal medicine in the Centre Hospitalier Universitaire in Saint-Étienne, France. In 2003 she was first introduced to the use of deep brain stimulation in animal models by prof. dr. Y. Temel when he was her mentor during her research internship. In 2004 she finished her medical education and started as a neurology resident not in training in the azM/MUMC⁺, where she started her neurology training in 2005. In 2008 she performed basal research under supervision of prof. dr. J.S.H. Vles as part of this thesis for which she temporarily interrupted her neurology residency. In 2009 she again took up her residency, meanwhile continuing the research activities that have led to this thesis. She hopes to finish her neurology residency in 2013. In 2005 she married Rob Moers. They now have three children: Myrthe (5 years), Rianne (3 years) and Xander (1 year).

