THESE

PRESENTEE A

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BEHAVIOURAL CHARACTERIZATION OF HABENULA LESION IN RATS. INVOLVEMENT IN COGNITIVE PROCESSES AND POSSIBLE RELEVANCE FOR THE COGNITIVE SYMPTOMS OF SCHIZOPHRENIA

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"Froid est le vide de l'espace, froides sont les lumières du couloir et froids sont les yeux de la femme dans l'entrebâillement de la porte, froids sont les médicaments sur le plateau.

Les étoiles regardent fixement l'hopital et la solitude de la mer est profonde.

Peut-être que (Chagalle) verrait les vaches voler s'il était là, à la fenêtre où je suis maintenant, à regarder les vagues hérissées et la mer noire.

Parfois j'entends les violons du monde.

Parfois je vois les montagnes enneigées avancer d'un pas lourd.

Une fois j'ai vu des sorcières voltiger dans les airs sur leur manche à balai, mais c'était la Saint-Sylvestre et les gens tiraient des feux d'artifice.

Je grimpe au sommet et touche les étoiles du doigt. Je prends les nuages et les enroule comme un foulard autour de mon cou. Je vole avec les oiseaux et disparait dans les profondeurs comme une baleine.

J'essai de me figurer les coups de pinceau : comment on dessine le temps, comment on colore la vie. A quel maître attribuer cette oeuvre étrange.

Je contemple l'immensité, je vois la mer noire, les ténèbres, le froid et les lumières. Je suis maintenant à l'intérieur du château que (Bernstein) peignait quand le soleil brillait, à l'intérieur du cercle, derrière les fenètres noires, de l'autre côté des coups de pinceau."

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"Maintenant que la fin approche, que les murs s'ecroulent et que le rideau tombe, je le dis haut et clair: J'ai vécu sous une lune pleine, voyagé par la voute céleste et dans les grandes profondeurs.

J'ai aimé, j'ai ri, j'ai pleuré, et maintenant que les larmes coulent et qu'on s'amuse bien, je vous le dis: « I did it my way. »

Non cette tombe n'est pas assez profonde pour contenir nos sentiments à tous.

Vous, hommes et femmes qui avez sauté dans l'abîme.

Vous, jours pluvieux dont les pleurs ont ruisselé sur les vitres.

Dieu, quelle misère que ce chemin creux; comme il ya peu qui reste et comme ce qu'il y a est peu.

Eternelle est la nuit du silence."

Einar Mar Gudmundsson – "Les anges de l'univers"
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Chapter I - General introduction

I.1 About schizophrenia

I.1.1 The symptoms

In current terminology, schizophrenia is generally composed of 5 different classes of symptoms (from Stahl, 2000):

- **Positive symptoms**, which are considered to be the reflection of an over expression of the normal functions, and are composed of delusions, hallucinations, and disorganization of speech, communication and behaviour. Delusions are considered as being erroneous interpretations of the perceptions or experiences, the more common one being the delusion of persecution. Hallucinations can be of any sensory modality (hearing, vision, olfaction, taste or tact), but the more common ones and characteristic of schizophrenia are the auditory hallucinations.

- **Negative symptoms**, which are on the other hand considered to be due to an under expression of the normal functions, and express themselves as anergia, flattened affect, emotional blunting and indifference, lower productivity of thoughts, lack of drive, initiative and concern, as well as lack of sexual motivation and social withdrawal.

- **Cognitive symptoms**, which comprise impaired attention (Bleuler, 1950; McGhie & Chapman, 1961; Orzack & Kornetsky, 1966; Chen & Faraone, 2000; Barr, 2001), reduced verbal fluency (Bokat & Goldberg, 2003), and impairment of executive functions (Bersani *et al*, 2004) and certain forms of memory, especially working and declarative memory (Pilkonis, *et al*, 1980; Goldberg & Schmidt, 2001; Lieberman *et al*, 2001; Pillman *et al*, 2003; Braff *et al*, 1978, 1992, 1999; McKenna *et al*, 1990; Meltzer & McGurk, 1999).

- **Anxiety** and **depression** are often associated with schizophrenia, and are shown as tension, irritability, fear and guilt. Wetherell *et al* (2003), having studied 160

elderly inpatients suffering from schizophrenia or schizoaffective disorder, noticed that anxiety was particularly present in those who had a bad outcome and a poor quality of life. Pallanti *et al* (2004) found, among 80 schizophrenics, a greater propensity for suicide, as well as a more frequent history of consumption of drugs of abuse (especially alcohol), and less favourable social adaptation. Nonetheless, none of these different features was correlated with any of the schizophrenic symptoms. It is interesting to note that a meta-analysis, performed by Whitehead *et al* (2003), did not show any ability for the antidepressant drugs to relieve the depressive symptoms of schizophrenics.

- **Aggressiveness**, which can be physical or verbal and is more frequently directed at other people. However, it can be directed at the patient himself, the ultimate state being the suicide attempt. Arseneault *et al* (2003) showed a positive correlation between a violent personality during childhood (7-11 years old) and violence expressed during adulthood (≥ 26 years old) among subjects having developed schizophrenia in the meantime. In their review of the literature, Walsh *et al* (2002) found a more pronounced tendency for violence in schizophrenics than in healthy subjects, which was worsened by the consumption of drugs of abuse. Indeed, Arseneault *et al* (2004) established a positive correlation between the cannabis intake in young subjects and a higher risk (x 2) to later develop schizophrenia, and Veen *et al* (2004) correlated the intake of cannabis with a lower age of appearance of the first negative symptoms in males. The same profile is obtained with cocaine and alcohol, associated with treatment noncompliance, violence, housing instability and homelessness (Dixon, 1999).

*I.1.2 The diagnostic and Statistical Manual of Mental Disorders - Fourth Edition (**DSM-IV**)*

According to the DSM-IV, the diagnosis of schizophrenia requires that the patient has shown at least two of the following symptoms for a significant part of one month: delusions, hallucinations, disorganized speech (*e.g.* frequent derailment or incoherence), grossly disorganized or catatonic behaviour, negative symptoms (*i.e.* affective flattening, alogia, or avolition). In addition for at least 6 continuous months the patient

must have shown some evidence of the disorder. These manifestations must be accompanied, for a significant portion of time, by social/occupational dysfunction (*i.e.* work, interpersonal relations or self-care are markedly below the level achieved prior to the onset). Exclusion criteria are that the disturbance is not due to the direct physiological effects of a substance (*e.g.* a drug of abuse, a medication) or a general medical condition. Delusions are considered as being erroneous interpretations of the perceptions or experiences, the more common one being the delusion of persecution. Hallucinations can be of any sensory modality (hearing, vision, olfaction, taste or tact), but the more common ones and characteristic of schizophrenia are the auditory hallucinations.

The different subtypes of schizophrenia are as follow:

- *Paranoid Type*. Preoccupation with one or more delusions or frequent auditory hallucinations. None of the following is prominent: disorganized speech, disorganized or catatonic behaviour, or flat or inappropriate affect.

- *Catatonic Type*. The clinical picture is dominated by at least two of the following: motoric immobility as evidenced by catalepsy (including waxy flexibility) or stupor; excessive motor activity (that is apparently purposeless and not influenced by external stimuli); extreme negativism (an apparently motiveless resistance to all instructions or maintenance of a rigid posture against attempts to be moved) or mutism; peculiarities of voluntary movement as evidenced by posturing (voluntary assumption of inappropriate or bizarre postures); stereotyped movements, prominent mannerisms, or prominent grimacing; echolalia or echopraxia.

- *Disorganized Type*. All of the following are prominent: disorganized speech, disorganized behaviour, flat or inappropriate affect. The criteria are not met for Catatonic Type.

- *Undifferentiated Type*. A type of Schizophrenia in which symptoms meet the criterion of two of the following: delusions, hallucinations, disorganized speech (*e.g.* frequent derailment or incoherence), grossly disorganized or catatonic

behaviour, negative symptoms (*i.e.* affective flattening, alogia, or avolition), but the criteria are not met for the Paranoid, Disorganized, or Catatonic type.

- *Residual Type*. A type of Schizophrenia in which the following criteria are met: Absence of prominent delusions, hallucinations, disorganized speech, and grossly disorganized or catatonic behaviour; there is continuing evidence of the disturbance, as indicated by the presence of negative symptoms or two or more of the following symptoms for schizophrenia: delusions, hallucinations, disorganized speech (*e.g.* frequent derailment or incoherence), grossly disorganized or catatonic behaviour, negative symptoms (*i.e.* affective flattening, alogia, or avolition), present in an attenuated form (*e.g.* odd beliefs, unusual perceptual experiences).

Finally, associated features are: learning problems; hypoactivity; psychosis; euphoric mood; depressed mood; somatic or sexual dysfunction; hyperactivity; guilt or obsession; sexually deviant behaviour; odd/eccentric or suspicious personality; anxious or fearful or dependent personality; dramatic or erratic or antisocial personality.

Psychiatrists possess several tools to diagnose schizophrenia and assess its severity. The presence of the different symptoms is generally rated using the positive and negative syndrome scale (PANSS), which consists in collecting information about the patients, generally from clinical interview and reports of primary care staff, but also from their family. The interview of the patient, or future patient, allows the observation of physical symptoms (*e.g.* tension, mannerisms and posturing, excitement and blunting of affect), interpersonal behaviour (*e.g.* poor rapport, uncooperativeness, hostility and impaired attention), cognitive verbal processes (*e.g.* conceptual disorganization, stereotyped thinking and lack of spontaneity and flow of conversation), thought content (*e.g.* grandiosity, somatic concern, guilt feelings and delusions) and response to structured questioning (*e.g.* disorientation, anxiety, depression and difficulty in abstract thinking) (Kay *et al*, 1987). The brief psychiatric rating scale (BPRS) can also be used, and consists of 18 items rated on a 7 point scale from “not present” to “very severe”. The items consist of somatic concern, anxiety, emotional withdrawal, conceptual disorganization, guilt feelings, tension, mannerisms and posturing, grandiosity, depressive mood, hostility, suspiciousness, hallucinatory behaviour, motor retardation,

uncooperativeness, unusual thought content, blunted affect, excitement and disorientation (Overall & Gorham, 1962). Another tool is the quality of life scale (QLS), which is a 15-item instrument that measures five conceptual domains of quality of life (*i.e.* material and physical well-being, relationships with other people, social, community and civic activities, personal development and fulfillment and recreation) (Burckhardt & Anderson, 2003).

To assess the cognitive abilities of the patients, several tests are used in psychiatry, some of which have also been adapted to animal research. To rate higher cognitive functions, there are tests of motor planning that involves frontal lobe areas; those tests include the Tower of London task, and the Wisconsin Card Sorting Test, the latter being based on the acquisition of a rule and the ability of the subject to switch to a new rule and match the changing requirements of the task as it progresses. Tasks of working memory are also used, either matching to position tasks, or more verbal tasks. They have been adapted in animal research (*e.g.* radial mazes, T-maze, swim-test). Finally, attention is usually assessed by means of the continuous performance task (CPT), which gave rise to the 5-Choice serial reaction time task for rats.

I.1.3 Childhood-onset versus adult-onset schizophrenia

This disorder of the central nervous system affects about 1 % of the population worldwide. Despite a steep rise in incidence in the early twenties, the age of onset of the first symptoms varies between 15 and 35 years old, but they also can appear in children (8-9 years old) or in elderly (70-80 years old). Beitchman (1985), distinguishing childhood-onset schizophrenia from autism, drew a portrait of the young schizophrenics (less than 15 years old) quite similar to that observed in people developing the illness in adulthood: he noticed the presence of paranoid delusions, disorder of the thought as well as the appearance of auditory hallucinations. The main behavioural features of the young patients were a propensity for physical inactivity, loneliness and reverie, as well as irritability, and a decrease of interest in things and other people. On the other hand, they had little or no speech disturbance (echolalia or neologisms). While there was a more marked disturbance of identity below the age of 10, it was in subjects of more than 10 years old that delusions and hallucinations were the most prominent. Concerning the epidemiology, the childhood-onset schizophrenia was more frequent

between 10 and 15 years old, compared to between 5 and 9 years old. Rapoport & Inoff-Germain (2000), who followed young schizophrenics during several years, found that they had speech and motor disturbances, which probably were an indication of pronounced maturation deficits. Furthermore, those children had a greater family history of psychological disorders, which led the authors to hypothesize that this made them more vulnerable to environmental or genetic risk factors, compared to the schizophrenics that develop the illness in adulthood. Medication, especially the atypical class of neuroleptics, was more effective in childhood-onset schizophrenics, particularly regarding negative symptoms, and produced less secondary effects. From an anatomical point of view, they had the same pattern of ventricular enlargement and volume loss (essentially grey matter at the cortical level, where the white matter was quite well spared) than adult-onset schizophrenics. An interesting fact is that the study of Rapoport & Inoff-Germain (2000), conducted during a 4-year period, showed progressive changes concerning the neuronal loss (increase of ventricular volume and decrease of temporal lobe structures), leading the authors to consider schizophrenia as being a neurodevelopmental disease. Similarly, Sporn *et al* (2003), during a two-year MRI study, found that the speed of neuronal loss (cerebral gray matter) was greater in individuals that had developed the illness during their childhood compared to healthy subjects. Russell *et al* (1989), studying 35 young schizophrenics, did not find any lowering of the IQ compared to normal subjects of the same age, and noticed the presence of delusions in 63 % and auditory hallucinations in 80 % of the subjects. Finally, these young inpatients had a common premorbid history of attention deficits and conduct disturbance. A more complete neuropsychological study of 88 schizophrenics and 190 control subjects, from 9 to 13 years old, using the kiddie formal thought disorder rating scale (KFTDRS) and the Halliday and Hassan's analysis of cohesion, revealed more formal thought disorders and cohesive deficits in young schizophrenics compared to normal children; according to the authors, this state of mind represents a step, necessary but not sufficient, toward the diagnosis of schizophrenia (Caplan *et al*, 2000). Moreover, these disorders were materialized by difficulties in the organization of the thought, the frequent production of illogicalities, and less use of pronouns, articles and demonstratives to describe a previously heard text (referential cohesion). Finally, these subjects used fewer conjunctions to link their ideas, which were enunciated through contiguous sentences (Caplan *et al*, 2000).

I.1.4 The cost of schizophrenia

Schizophrenia is a disease that affects all populations and all social classes without distinction. One of the major problems is that, beyond the normal cost of the treatment itself, between 25 and 50 % of the schizophrenics make one or several suicide attempts. Indeed, Baldessarini (2003) noted that between 10 and 13 % of the schizophrenics die every year from suicide. Moreover, about 25 % of all hospital beds are occupied by schizophrenics, and schizophrenia accounts for 40 % of all long-term care days in the USA (Pickard, 1995). The annual direct cost of their care in the USA was estimated to be $ 19 billion in 1991, with an additional indirect cost of $ 46 billion due to lost productivity (Barondes *et al*, 1997).

I.1.5 Gender differences

Even though, from an anatomical point of view, Lauriello *et al* (1997), comparing men and women, did not notice any significant differences at the level of many regions involved in schizophrenia (see below), some authors did find gender differences in schizophrenia. One example is given by Pulver *et al* (1990), who studied 366 schizophrenics and 1851 of their first-degree relatives. These authors found that the sooner schizophrenia appeared in men, the higher was the risk for their relatives to develop the disease, which was not the case for women. Whereas men schizophrenics had a lesser percentage of mortality, in reaction to the disease, women with schizophrenia reached a higher social level, and their rate of hospital readmission, after they were treated with neuroleptics, was lower. In numerous studies, men show slightly earlier onset of schizophrenia than women, who show a second peak of onset after 40 years old (Hambrecht *et al*, 1992). In fact, the better socialization of women is attributed to the fact that they develop the illness relatively later than men, and thus have more time before onset of the illness to better integrate into society, to get married and to have children. Moreover, women have a lower rate of progression of the disease, and a better response to neuroleptics, which is generally attributed to the beneficial action of the estrogenic hormones. Indeed, the latter enhance the efficacy of neuroleptics by directly acting upon some monoaminergic systems, especially the dopaminergic system through the D_2 receptor, and the serotonergic system through the $5HT_{2A}$ receptor (Fink *et al*, 1996). In order to explain the greater degree of mortality encountered among the women population, these authors proposed that it is the

reflection of a more pronounced tendency to commit suicide, and also consider a possible antagonistic effect of neuroleptics upon the protective action of the estrogenic hormones, especially at the level of the cardiovascular system. Finally, tardive dyskinesia (*i.e.* the appearance of involuntary movements of the muscles of the face), a secondary effect due to neuroleptics, seems to be more pronounced in women after menopause (Seeman, 1986).

I.1.6 Evolution of theories of schizophrenia

I.1.6.1 Emil Kraepelin (1896)

One of the first psychiatrists to study the illness that would later become known as schizophrenia is Emil Kraepelin. He named this pathology dementia praecox, regarding its particular character and because he noted that it appeared earlier in life than other forms of dementia. According to him, it is a "*peculiar destruction of the internal connections of the psychic personality* (which) *predominate in the emotional and volitional spheres of mental life*" (p. 3). Raising a clinical picture of the disease, he defined two principal groups of disorders: "*a weakening of those emotional activities which permanently form the mainsprings of volition*" and "*the loss of the inner unity of the activities of intellect, emotion, and volition in themselves and among one another*" (p. 74-75). At this period, there was no distinct categories of symptoms, like nowadays, so Kraepelin defined this pathology by attributing a series of psychological and physical manifestations:

- **Psychological symptoms**. These include difficulties in sustaining attention, hallucinations (the most frequent being auditory), disturbances of the thought (patients think that they do not possess their senses any more), perturbation of taste and olfaction (patients define what they eat and drink as being diabolic), morbid tactile sensations (patients feel as if they are being touched), poverty of thought (patients' thoughts are limited and elaborated with difficulty, including erroneous associations; moreover, they are inefficient because they are too inattentive, bored and weary), stereotypies which can be psychological (flatness, persistence of simple ideas) or physical (maintenance of a posture or repetition of a movement), affected judgement faculties, delusions that can be transitory or permanent (*e.g.* hypochondria, ideas of sin or persecution), exalted ideas

(patients think that they are the messiah, or god), perturbation of the expression of emotions, attenuation of the will, impulsive deeds [someone asked them to do it (*e.g.* god)], manias (or obsessions), autistic withdrawal, incoherence in the way they think, talk or write (with the frequent appearance of neologisms), negativism (patients often show resistance to the dialogue, giving elusive answers). Finally, memory seemed to be rather unaffected, with however the appearance of pseudo-memories (confabulations).

- **Physical symptoms**. Headaches, disturbance of the pupillary behaviour (flattened reaction to the light), exacerbation of the tendon reflex, psychomotor disorders (especially disorder of equilibrium and stumbling), crisis (dizziness, fainting and sometimes epileptiform violent shaking), vasomotor disturbances (appearance of cyanosis at the level of the hands), fluctuation of the temperature (generally lower, but it can vary throughout the day, reaching as low as 34 or as high as 39ºC), disturbances of sleep, irregular food intake from total refusal to voracity, with a great fluctuation of the patient's weight.

According to Kraepelin, the clinical forms of the disease can be very diverse, by virtue of the presence of the different symptoms: *dementia simplex*, *simple depressive dementia*, *delusional depressive dementia*, *agitated dementias* or *paranoid dementias*.

I.1.6.2 Eugen Bleuler (1911)

Following the work of Kraepelin, fundamental progress was made by the Swiss psychiatrist Eugen Bleuler. In fact, he is the one who created the term "schizophrenia", derived from the greek words *schizein* (cleavage) and *phrên* (spirit), in a view of underlying splitting and disorganization of various psychological functions. This change of terminology showed his will of separating his work from Kraepelin and the dementia praecox, because he thought that numerous symptomatic manifestations such as catatonia which, according to him, belonged to dementia praecox but without being the reflection of a psychological deterioration, often appeared later. Then, dementia praecox became for him a particular form of dementia in young people.

Bleuler defined schizophrenia as being constituted of a group of psychoses whose progression is sometimes chronic, and sometimes gives rise to intermittent attacks, and

can stop at anytime without allowing the total recovery of the psychological abilities. According to him, this disorder is marked by a type of degradation which is specific for thought, feelings and links with the surrounding world. The expressions of the emotions are absent in the extreme cases while, in the moderate cases, one can notice that their intensities are not related to their real cause, which gives a large spectrum of emotions, from quiescence to the utmost agitation. Other symptoms are also present, like for example hallucinations, delusions, stupor, manias, melancholy and catatonia. The diverse presence or absence of these symptoms allows to precisely diagnose schizophrenia.

Nevertheless, Bleuler differentiated two main classes of symptoms:

- **Fundamental symptoms**, which characterized the disease: disturbance of association and of the expression of emotions, propensity for fantasies, tendency to detach from reality, attention deficits, disturbance of the will, of action and of the ability to reason.

- **Accessory symptoms**, which "*may be present throughout the whole course of the disease, or only in entirely arbitrary periods of the illness*" (p. 95): hallucinations (essentially auditory or tactile), delusions (being poisoned, persecution, grandeur), accessory memory disturbances, disruption of writing and speaking, and of the ego (there can be fragmentation of the personality, each of the fragments being able to govern the subject alternately).

He divided the pathology in four sub-classes, depending of the presence of the different symptoms: paranoid, catatonic, hebephrenic and simple schizophrenia.

Concerning the memory deficits, it is interesting to underline a few facts, without forgetting to note that, if nowadays it is recognized that memory deficits belong to the pathophysiology of schizophrenia (*cf* McKenna *et al*, 1990), at that time, scientists did not possess the experimental and theoretical tools that later allowed to substantial progress in this field. In fact, to assess memory functions, the doctors were questioning the patients about old facts of their life (which are now termed as "episodic memory"), and attributed the incorrect answers to a problem of association, to negativity and to a

lack of interest for this kind of test. Bleuler also talked about motor skills, which were not affected by such a disease. In fact, they are nowadays mentioned as "procedural memory", and the fact that they are not affected by schizophrenia seems now an evidence, as procedural memory has been extensively shown to be the kind of memory the better preserved in many diseases, psychiatric or not. A very interesting fact is that Bleuler referred to a memory test made with schizophrenics; it was designed to assess the visual memory of the subjects, who were asked to remember series of items with intervals, between presentation and recall/recognition, from 10 to 30 seconds. The results were that the subjects performed rather poorly. In fact, one can notice that this test requires what is now called "working memory", a form of memory which has been shown to be altered in schizophrenics.

Delusions

According to Bleuler, the delusions possess no common-sense, no unity, and their contents are consonant with the schizophrenic's mood. He defines them as: "*In the schizophrenic confusional states, there arises an apparently wild chaos of false notions which the patients believe in.*" (p. 130). Two contradictory delusions can be concomitant or can follow each other in a short time interval. The most common is the delusion of persecution, but one can also find delusions of magnificence or inferiority. They are prevalent during the acute manifestations of the illness, and can occur during the melancholic or maniac phases. They are not constantly present in the patient's life, and most of them, after many repetitions, can become secondary, lose their emotional character, and progressively stop affecting the patient's behaviour. The patient can stop focussing on a particular delusion, without correcting it, but it can re-emerge after a fortuitous association, either very clearly like it was before, or vaguely as if the delusion had been partly forgotten.

"*In delusions everything which one wishes and fears may find its level of expression*" (p. 117). It is interesting to note the terms used by Bleuler to define delusions, which seem to arise primordially, in their definitive shape, from the unconscious. To this purpose, he specified that certain delusions arose during an acute phase of the disease, and then fell into forgetting, often to re-emerge fortuitously, with sometimes a more elaborated structure. According to Bleuler, this means that these delusions are not only

dormant, but may also unconsciously mature. To his point of view, dreams and delusions are often linked, and he even related the case of patients whose dreams were suddenly projected in real life as delusions, *i.e.* as very strongly-held false beliefs.

I.1.6.3 Gabriel Langfeldt (1966)

Langfeldt characterized schizophrenia according to a very specific dichotomy. On one side, "true schizophrenia" which is linked to the kraepelinian dementia praecox and evolves towards dementia, and on the other side the "schizophreniform psychosis", which is reactive, toxic or organic, and has a better outcome. In fact he differentiates two types of schizophrenia, or schizotypal state, one being psychogenetic, and the other constitutional.

Langfeldt's conception of schizophrenia is well explained by Rousselot (1981). In fact, Langfeldt gives as main supporting evidence the notion of evolution of the patient's state, thinking that typical schizophrenia remains endemic and that its only issue is a catastrophic degradation. He describes this pathology following two constitutive poles, "process symptoms" and a group of "factors" which are susceptible to precise the diagnostic:

- **Process symptoms**. Specific emotional disturbances (a hypersensitivity that can provoke a complete withdrawal from the surrounding world), specific motor disturbances (psychomotor anomalies), hallucinations, primary delirious ideas (especially in the paranoid forms of the disease), specific disturbance of associations (incoherence, deviation from the main point in writing or speaking, neologisms, echolalia, stereotypies and perseverance of ideas), symptom of depersonalization. The presence of at least two of these symptoms is required to diagnose schizophrenia.

- **Factors**. Biotype, premorbid temper (especially the presence of introversion), accelerating factors [(varied organic injuries, intoxications, psychological trauma: they are rare and do not provoke the appearance of schizophrenia, but they can reveal it); heredity, intelligence (the deterioration is more prevalent in persons with low IQ), social and familial environment].

In the early 80s, two quite similar conceptions of schizophrenia arose, one elaborated by Andreasen and Olsen, and the other by Crow.

I.1.6.4 Nancy Andreasen and Scott Olsen (1982)

These authors divided schizophrenia into two sub-types: positive schizophrenia and negative schizophrenia. Positive schizophrenia is diagnosed when the patient has at least one of the following symptoms, in a preponderant way: hallucinations, delusions, thought disorders (incoherence, illogicality), disorganized behaviour and, at the same time, has none of the following symptoms: alogia (poverty of speech and of its content), emotional flattening, apathy, anhedonia, attention deficits. Negative schizophrenia is diagnosed when the patient has at least two of the following symptoms: alogia, emotional flattening, apathy, anhedonia, attention deficits, without any positive symptoms (hallucinations, delusions, thought disorders, strange behaviour).

According to these authors, the patients suffering from negative schizophrenia show a rather limited premorbid social adaptation, as well as an indication of cerebral damage, whereas patients suffering from positive schizophrenia have a better outcome, a better integration within society, and do not have cerebral atrophy.

I.1.6.5 Timothy Crow (1982, 1985)

Crow qualifies his approach under the terms: “The two-syndrome concept”. He divides schizophrenia according to two distinct types, taking into account the response to neuroleptics, and the anatomical encephalic disturbances.

- **Type I**. This has an organic origin, is reversible, and is associated with the positive symptoms, including delusions, hallucinations and thought disorder. It is present during the acute psychotic phases, and it exhibits quite a good response to neuroleptics (especially the dopaminergic antagonists, leading Crow to propose that this syndrome is linked to a defect of dopaminergic transmission).

- **Type II**. It has a functional origin, because it is associated to neuronal loss and the enlargement of cerebral ventricles, and its development is progressive. It is

present during the chronic psychotic episodes, and it is associated with negative symptoms, including flattened affect and poverty of speech. It is also characterized by a poor response to neuroleptics.

Differences between Crow's conception and that of Andreasen and Olsen is that the former integrates anhedonia in the depressive state, and attention deficits in the positive class of symptoms. Furthermore, Crow thinks that the patients can evolve, over time, from type I to type II, but only rarely in the opposite direction. Finally, according to him, these two syndromes have a common aetiology, as he considers that one can sometimes find both coexisting within a single person.

I.1.7 Toward an anatomy of schizophrenia: the contribution of histology and imaging techniques

During the last two decades, substantial progress has been made in the field of psychological and psychiatric diseases thanks to the improvement of histological, as well as imaging techniques: positron-emission tomography (PET) and magnetic-resonance imaging (MRI). Studies performed with such techniques are complementary to the behavioural and neurochemical investigations which have limitations. For example, post-mortem studies can reveal anomalies which we do not know if they are belonging to the pathophysiology of the disease, or if they are in fact due to the use of a certain type of medication. As an example, Silvestri *et al* (2000) found an enhancement of the specific binding on prefrontal dopaminergic D_2 receptors after a long treatment with neuroleptics. Imaging studies overcome this limitation since they can be performed at the onset of the disease. Despite the fact that certain discoveries made with imaging techniques have sometimes not been confirmed, they however have allowed the identification of several regions, altered more or less systematically in schizophrenia: **cerebral ventricles** (lateral, third) and **cerebrospinal fluid spaces** (sulci and fissures) at the cortical level (frontal, temporal and parietal), **temporal lobe** (hippocampal complex and amygdala), **thalamus** and certain cortical and sub-cortical areas (Fannon *et al*, 2000; Lieberman *et al*, 2001).

It is interesting to note that childhood-onset schizophrenia and adult-onset schizophrenia both lead to the same anatomo-pathological profile. Sowel *et al* (2000), in

a MRI volumetric study with 9 schizophrenics from 9 to 16 years old and 10 control subjects, showed, in the patients, enlarged ventricles, especially in the posterior horn of the lateral ventricles. They also had more subtle volume reductions of the corpus callosum, the cingulate gyrus, the caudate and the thalamus, also at the posterior level. Nevertheless, the authors do not know if these anatomical traits are primary or if they are due to the enlargement of the ventricles. While it seemed, in that study, that the volume of the temporal lobe of the childhood-onset schizophrenics was spared compared to the adult-onset schizophrenics, another MRI study by Levitt *et al* (2001), on 13 schizophrenic children and 20 control subjects, showed that the volume of the amygdala was slightly greater in the schizophrenics, this enhancement being more pronounced on the left side, while the hippocampal volume was unchanged.

I.1.7.1 The cerebral ventricles

One of the first PET studies that revealed a tendency for an enlargement of the ventricles in chronic schizophrenics was performed by Johnston *et al* (1976). Furthermore, they found that this enlargement was correlated with cognitive impairments. The authors therefore considered whether this enlargement was a consequence of the disease, or if it was particular to a specific form of the disease for which cognitive impairments were prevalent. Later, Weinberger *et al* (1980) showed that the schizophrenics with ventricular enlargement had a poorer response to neuroleptics (unspecified) than those without such anomaly. This correlation between ventricular enlargement and schizophrenia was confirmed by Raz & Raz (1990), in a meta-analysis, with a more pronounced change at the level of the third ventricle. This is logical if we consider that the structures that are commonly affected in schizophrenia are situated close to it (see below, hippocampus and thalamus for example). For example, Gaser *et al* (2004), in 39 schizophrenics, showed a positive correlation between ventricular enlargement and the reduction of thalamic volume, and more particularly the median nucleus, as well as the reduction of striatal volume (posterior putamen) and of the adjacent insular cortex. Finally, an MRI study with 20 schizophrenics (10 men, 10 women) by Buckley *et al* (1999) showed an enlargement at the level of the temporal horn of the lateral ventricle, as well as the inter-ventricular foramen of Monro, that was significant only in men.

A debate is still present concerning the correlation between the presence of either positive or negative symptoms and the tendency to ventricular enlargement. This thesis, asserted by Crow (see above), was investigated by numerous teams, with discrepant outcomes. A study by Andreasen *et al* (1982), on 32 schizophrenics comprising 16 with enlarged ventricles and 16 with normal ventricles, showed on the one hand a correlation between the prevalence of negative symptoms and the presence of enlarged ventricles, coupled with poor cognitive performance, and on the other hand a correlation between normal ventricles and the prevalence of positive symptoms. A CT scanner study on 19 schizophrenics (11 men and 8 women) and 46 control subjects, by Pearlson *et al* (1984), showed an increase of the ventricular volume in schizophrenics, with an increase of the difference in non-employed schizophrenics (since at least 6 months) compared to the employed (for at least 6 months); moreover, this enlargement was correlated with a prevalence of the negative symptoms. Similarly, Kemali *et al* (1985) confirmed the concomitance of lateral ventricle enlargement and the presence of negative symptoms, as well as the worsening of cognitive symptoms and social withdrawal.

In contrast to the preceding studies, Losonczy *et al* (1986) found enlarged ventricles in schizophrenics but without any correlation with the severity of negative or positive symptoms. An important study is that of Farmer *et al* (1987), who found, contrary to Crow's conception, a correlation between positive symptoms and ventricular enlargement. Finally, Keilp *et al* (1988), on 28 schizophrenics under medication, showed correlation between enlarged lateral ventricles in the anterior frontal horn area, and poor neuropsychological performance, and between an enlargement of the central part of the lateral ventricles and motor and immediate verbal memory deficits; nevertheless, this study did not show any correlation between ventricular size and prevalence of positive or negative symptoms.

I.1.7.2 The temporal lobe

The temporal lobe comprises the hippocampal formation (dentate gyrus, ammonic fields CA_1 to CA_4, subiculum and entorhinal cortex), and in certain studies, a wider area also including the amygdala. A MRI study by Suddath *et al* (1989) on 17 schizophrenics (10 men and 7 women) found a 15 % reduction of the temporal lobe volume; moreover, the grey substance showed a 18 % volume decrease on the right hemisphere, and a 21 % reduction on the left, the white substance remaining unchanged.

There have been very few imaging studies concerning the amygdala *per se*. One of them showed a volume reduction, in 18 schizophrenics compared to 22 control subjects (Joyal *et al*, 2003). Concerning the hippocampus, a meta-analysis by Nelson *et al* (1998) found a 4 % bilateral reduction of the volume, a reduction that was increased when the amygdala was included. An MRI study by Szeszko *et al* (2003) on 56 schizophrenics after a first episode, showed that the volume reduction was limited to the anterior part of the hippocampal formation, and that its posterior part, and the amygdala, were less or not affected. Later, performing post-mortem cell-counting, they showed a 40 % reduction of the number of non-pyramidal neurons, at the level of the CA_2, the pyramidal neurons being unaffected. Moreover, this difference was equally present in medicated and non-medicated patients (Benes *et al*, 1998). According to the authors, this is the reflection of a deficient neuronal modulation exerted by GABA interneurons. Finally, Highley *et al* (2003) did not find any difference in any of the hippocampal pyramidal cells fields investigated (CA_1, CA_2, CA_3, CA_4 and subiculum), which could also indicate that the changes in cell number only occur in the non-pyramidal neuron population, although Conrad *et al* (1991) showed abnormalities in the orientation of the pyramidal cells in the right hemisphere.

At the level of the entorhinal cortex, two studies with small group sizes have found differences, while a study with a more adequate group size did not. A post-mortem study by Arnold *et al* (1991) on the brains of 6 schizophrenics and 16 controls showed abnormalities of its rostral and intermediate portions that included aberrant invaginations of the surface, disruption of cortical layers and heterotopic displacement of neurons. Also, Jakob & Beckman (1994), on the brains of 5 schizophrenics (3 men and 2 women) revealed a defect in the orientation, as well as in the volume of the neurons that was decreased at the level of the pre-α second layer, and the pre-β third layer; the authors proposed that this was due to a fault in the migration of the neurons during the second trimester of pregnancy. This is very interesting concerning schizophrenia, because the entorhinal cortex is linked to many regions, as it is the link between primary and secondary sensory cortices (except the olfactory cortex), as well as somato-sensory cortical areas, and the hippocampus. Moreover, the pathway composed of the entorhinal cortex layers II and III – perforant path – hippocampal formation – entorhinal region is the centre of the limbic system. In fact, the entorhinal region filters the informations from the sensory areas toward the hippocampus, and

anomalies at this level could well explain the schizophrenics' deficits in sensory gating, as well as many cognitive disorders. However, a post-mortem morphometric study by Bernstein *et al* (1998) on 31 medicated schizophrenics (16 men and 15 women) and 45 control subjects (25 men and 20 women) did not show any difference at this level.

Some studies showed a correlation between the neuropsychological deficits encountered in schizophrenia and the temporal lobe anomalies. For example, a MRI study by Shenton *et al* (1992) in 15 right-handed schizophrenics and 15 control subjects showed, on the left hemisphere, a reduction of the volume of an area constituted by the anterior hippocampus and the amygdala (19 %), of the parahippocampal gyrus (13 %) and of the superior temporal gyrus (15 %), the latter being correlated with poor cognitive performance. One year later, a MRI study by Nestor *et al* (1993), coupled to the Wechsler memory scale, and the Wechsler adult intelligence scale (abstraction/categorisation), on 15 male right-handed schizophrenics with chronic positive symptoms, revealed two correlations: on one hand between categorisation and abstraction deficits and the reduction of the temporal lobe volume in both hemispheres (parahippocampal gyrus and posterior superior temporal gyrus), and on the other hand between a verbal memory deficit and the reduction of the posterior superior temporal lobe volume in the left hemisphere.

Recently, a pioneering MRI study has been performed by Milev *et al* (2003). During a 5-year follow-up study of 123 schizophrenics after their first episode of psychosis, the authors found that only the temporal lobe volume was predictive of the outcome; in fact, the patients with a smaller temporal lobe volume at the beginning of the illness showed a longer persistence of hallucinations, essentially auditory. Moreover, Tepest *et al* (2003) found that the relatives of schizophrenics showed temporal lobe volume reduction, leading them to state that hippocampal pathology in schizophrenia could be the consequence of a genetic vulnerability.

I.1.7.3 The thalamus

One of the first post-mortem studies of the thalamus in schizophrenia was performed by Pakkenberg (1990) who found, in a study of 12 schizophrenics' brains and 12 control brains, a general reduction of the total number of neurons in the thalamus in the schizophrenics, and especially in the mediodorsal nucleus, with 44 % reduction for the

astrocytes, and 45 % reduction for the oligodendroglia. Following this discovery, Andreasen *et al* (1994) used MRI to further explore the hypothesis according to which cognitive deficits in schizophrenia could be due to a disruption in neuronal circuitry mediating attention and information processing centred on the thalamus. Performed with 47 schizophrenics and 39 control subjects, all males, they found a decreased volume in schizophrenics, especially in the lateral and medial regions, with a prevalence in the right hemisphere. Staal *et al* (1998), using the same technique with 30 schizophrenics, and including 30 non-schizophrenic patients' relatives (brothers or sisters), found a thalamic volume reduction in 87.5 % of the schizophrenics, compared to control subjects. Moreover, 87.5 % of the relatives had a smaller thalamus than their normal comparison subjects and, according to these authors, the thalamic abnormalities in schizophrenics could have a genetic origin. More recently, Kemether *et al* (2003), in a MRI study with 41 schizophrenics (32 men and 9 women) and 60 control subjects (45 men and 15 women), noticed a thalamic volume reduction in the schizophrenics, concerning the mediodorsal, the pulvinar and the centromedian nucleus, the latter being a region particularly involved in arousal and attentional processes. Finally, Hazlett *et al* (2004), in 41 schizophrenics and 60 control subjects, showed a decrease in glucose metabolism in the mediodorsal and centromedian nuclei, and an increase in the pulvinar nucleus of the schizophrenics compared to control subjects. As these nuclei are closely linked with the cortex, according to the authors this study is consistent with the view that schizophrenia comprises a disruption of cortico-thalamic synaptic connections. Moreover, the authors showed a correlation between the pathology of the pulvinar nucleus and the prevalence of hallucinations, as well as positive symptoms in general on the one hand, and between the pathology of the mediodorsal nucleus and the prevalence of negative symptoms on the other hand. Contrariwise, a MRI study by Portas *et al* (1998), on 15 schizophrenics and 15 control subjects, did not reveal any difference in thalamic volume.

I.1.7.4 The basal ganglia

Concerning the striatum, a study by Bogerts *et al* (1985) did not show any difference in volume between 13 schizophrenics' brains (3 men and 10 women) and the brains of 9 control subjects (6 men and 3 women), at the level of the left hemisphere; but a very interesting fact is that those were the brains of people who died between 1928 and 1953, *i.e.* before the extensive introduction of the neuroleptics. In fact, nowadays it is quite

well accepted that the administration of typical neuroleptics leads to an increase of the striatal volume, and then to the appearance of secondary effects, such as motor disturbances (Corson *et al*, 1999a; Keshavan *et al*, 1994). Recently, a MRI study was conducted over a two-year period in 30 schizophrenics after their first episode, 12 schizophrenics who had received chronic treatment with atypical antipsychotics, and 13 control subjects. This study showed a lack of striatal volume difference between first-episode schizophrenics and control subjects, an increased striatal and pallidal volume in schizophrenics after chronic treatment with typical antipsychotics, and a lack of difference in patients after chronic treatment with risperidone (Lang *et al*, 2001). In contrast, Corson *et al* (1999b) found a volume reduction of caudate in 36 schizophrenics after a first episode compared to 43 control subjects. According to the authors, it could mean that striatal pathology is a common feature of schizophrenia not induced by medication.

I.1.7.5 The cerebral cortex

Weinberger *et al* (1979), in a computer tomography (CT) study, showed that 32 % of the schizophrenics observed had a cortical atrophy, especially at the level of the Sylvius fissure. Moreover, these authors noticed that some of the patients had this anomaly without any change in the ventricular volume. Finally, they found that this atrophy was not greater in old patients, leading them to state that this feature was not due to a progressive neurodegenerative process, as in Parkinson's or Alzheimer's diseases. More recently, important advances have been made for the comprehension of the cortical anomalies in schizophrenics, at the volumetric and cellular levels. A post-mortem study on 35 µm thick slices, performed by Pakkenberg (1993), on the brains of 8 schizophrenics and 16 control subjects, did not show any significant difference in terms of cell number in either hemisphere. This result favours the hypothesis that the volume reduction is more due to a decrease of the number of connections than a decrease of the number of neurons.

Study of neurons and the glia at the cortical level had produced interesting findings related to the behavioural consequences of frontal deficits in schizophrenics (attention, memory and categorisation), and reinforced the Selemon and Goldman-Rakic's hypothesis of the "reduced neuropil" (see below). An MRI study by Lim *et al* (1998), was devoted to exploring gray and white matter volumes, by assessing the level of N-

acetylaspartate (NAA), a neuronal marker selective for neurons, because it is most exclusively present in the cell bodies, axons and dendrites, and not in glia. They found in schizophrenics a decrease of the volume of the cortical gray matter, but with an identical NAA signal compared to control subjects, while the white matter was the same in both groups, but with a lower NAA signal in schizophrenics. The authors concluded that gray matter abnormality was due to a deficit of neurons and glia, while white matter abnormality was only due to axonal deficit. On the other hand Flynn *et al* (2003), using MRI in 30 schizophrenics and 27 control subjects, found a 12 % global reduction of the white matter volume, with a most prominent effect on the left genu of the corpus callosum. Moreover, post-mortem study showed a significant decrease of oligodendroglia-associated proteins detected by immunohistochemistry in schizophrenics. This was confirmed by Hof *et al* (2003) who found a 28 % decrease in the number and density of the cortical oligodendrocytes (layer III of the Brodmann's area 9), as well as a 27 % volume decrease of the adjacent white matter (frontal gyrus). Finally, Hakak *et al* (2003) noticed, during a post-mortem study of the brains of 12 medicated schizophrenics and 12 brains of control subjects, that 5 genes, mostly expressed in oligodendrocytes and involved in myelination as well as in synaptic plasticity, neuronal development and signal transduction, were under-expressed in schizophrenics in dorsolateral frontal cortex (Brodmann's area 46).

From a cognitive point of view, as schizophrenia has been associated with a "hypofrontality", Andreassen *et al* (1992) examined frontal activation during the Tower of London test. They found a lower activation of the frontal area in schizophrenics during this test. Moreover, in the same kind of experiment, using a mental arithmetic task, Hugdahl *et al* (2004) found a dissociation between frontal and parietal lobes. In fact, during the task, the frontal lobe was under-activated in schizophrenics compared to normal controls, while the parietal lobe was over-activated. According to the authors, this parietal over-activation could reflect an attempt to compensate the frontal under-activation.

Recently, Selemon and Goldman-Rakic (1999) formulated a new hypothesis, called "The reduced neuropil hypothesis". In fact, exploring the cortical neuronal density, they noticed an increase, in schizophrenics, in particular in the dorsolateral prefrontal area (Brodmann's area 9 and 46), but concomitant to a reduction of the cortical volume. As

schizophrenics had very little neuronal loss, this enhancement of the neuronal density could be due to a reduction of the distance between neurons; this could be the consequence of a reduction of the number of connections between neurons (less dendritic arborisations and less cortical afferent connections), and then less cortico-cortical connections. Thus, this anatomical disorganisation could explain some cognitive deficits affecting attention, working memory, sensory gating and goal-directed behaviours. Refering to this theory, Matsumoto *et al* (2003) studied the level of expression of the catechol-O-methyltransferase (COMT) mRNA, an enzyme that degrades catecholamines, by *in situ* hybridization in the brains of 14 schizophrenics and 15 control subjects. They did not find any global quantitative differences, but some anomalies in its laminar distribution, the enzyme being less present, in schizophrenics, in layers II and III, and more abundant in deeper layers IV and V. The authors concluded that, if COMT is more particularly present in dendrites, the fact that it is less expressed could mean that the interneuronal connections are affected in schizophrenia, leading to a frontal disturbance of monoaminergic transmission.

I.1.8 What may cause schizophrenia ? The different hypothesis stemming from fundamental research

Despite its high prevalence and well-known symptomatology, the anatomical and physiological causes of schizophrenia remain obscure. Initially, observations of the effects produced by drugs of abuse acting at the level of the central nervous system suggested the involvement of some neurotransmitter systems in the appearance of schizophrenic symptoms, the main candidates being *dopamine*, *glutamate*, *serotonin* (5-hydroxytryptamine, 5-HT), *acetylcholine* and *gamma-amino-butyric acid* (GABA). The investigations performed gave rise to numerous hypotheses, at first isolated from each-other, and then becoming more and more complementary, the extreme variety of symptoms suggesting the concomitant involvement of several cortical and sub-cortical areas of the brain, and the involvement of several neurotransmitter systems.

I.1.8.1 Schizophrenia and dopamine

To date, dopaminergic dysfunction remains the most robust hypothesis, which arose from several facts and observations: ***1*** - the compounds (*e.g.* amphetamine) that lead to an elevated release of dopamine can worsen some symptoms, and their prolonged use

can lead to a state resembling paranoid schizophrenia. ***2*** - L-DOPA, a dopamine precursor, used as medication that ameliorates the symptoms of Parkinson's disease by enhancing the dopamine level in the brain can cause or exacerbate positive symptoms (hallucinations and delusions), whereas in the early phase of the introduction of neuroleptic medication, schizophrenics showed Parkinson-like signs of tremor, rigidity and akinesia. ***3*** – typical neuroleptics, compounds that are potent and effective in improving schizophrenia, are antagonists of dopamine receptors. ***4*** - neuroleptics (*e.g.* chlorpromazine and haloperidol) provoke an increase of catecholamine metabolites in the mouse brain, which is attributed to their blocking effect at dopamine receptor leading to a compensatory enhanced catecholamine turnover (Carlsson & Lindqvist, 1963).

The first studies concerning the action of neuroleptic molecules led several investigators to consider schizophrenia as being the exclusive reflection of a dopaminergic hyperactivity, as compounds like chlorpromazine and haloperidol were found to be dopaminergic antagonists, acting on the D_2 receptor (Creese *et al*, 1976). More recently, it has been considered a dual dopaminergic dysfunction, namely a cortical hypoactivity and a sub-cortical hyperactivity (Davis *et al*, 1991). Indeed, a study in the rat showed that an induced cortical dopaminergic hypoactivity, provoked by the 6-hydroxydopamine lesion of prefrontal dopaminergic endings, leads to a subcortical dopaminergic hypersensitivity, especially at the level of the accumbens nucleus and the striatum (Pycock *et al*, 1980).

There are two main classes of dopamine receptors: the D_1 (and D_5) which is excitatory and stimulates adenylate cyclase, and the D_2 (and D_3 and D_4) which is inhibitory and inhibits the adenylate cyclase. In the rat, D_1 has been found in high concentrations in striatum, nucleus accumbens, islands of Calleja, olfactory tubercle and *zona reticulata* of the substantia nigra, cerebral cortex, amygdala, thalamus, suprachiasmatic nucleus, choroids plexus, claustrum, endopiriform nucleus, dorsal lateral geniculate and dentate gyrus (Wamsley *et al*, 1989). D_2 is the typical site of action of neuroleptics and, in the rat, is mostly expressed in striatum, nucleus accumbens, islands of Calleja, olfactory tubercle and the *zona compacta* of the substantia nigra (very few in the *zona reticulata*), bed nucleus of the stria terminalis, hypothalamus, habenula, lateral mammillary nucleus, periaqueductal gray, inferior colliculus, intermediate lobe of the pituitary,

stratum lacunosum moleculare of the hippocampus, dorsal horn of the spinal cord and glomerular layer of the olfactory bulb (Wamsley *et al*, 1989), while D_4 is expressed in frontal cortex and amygdala, two regions very implicated in the pathophysiology of schizophrenia. While most antipsychotics have lower affinity for D_2 than for D_4 receptors the affinity of clozapine for the D_4 site is 15 times higher than for D_2 (Reynolds, 1995).

It has been proposed that the different dopaminergic pathways may be involved in the manifestation of schizophrenia symptoms, in various ways (Kandel *et al*, 2000) (**Fig 1.1**)

- The *mesolimbic* pathway, from the ventral tegmental area (A10 cell group) towards the nucleus accumbens, the septum, the olfactory tubercles, the amygdala and the hippocampus, which is believed to be essentially involved in mood, reward and motivational functions, is associated with positive symptoms. Hyperactivity of this path is supposed to be the origin of hallucinations and delusions, as well as thought disorganisation and aggressive behaviour.

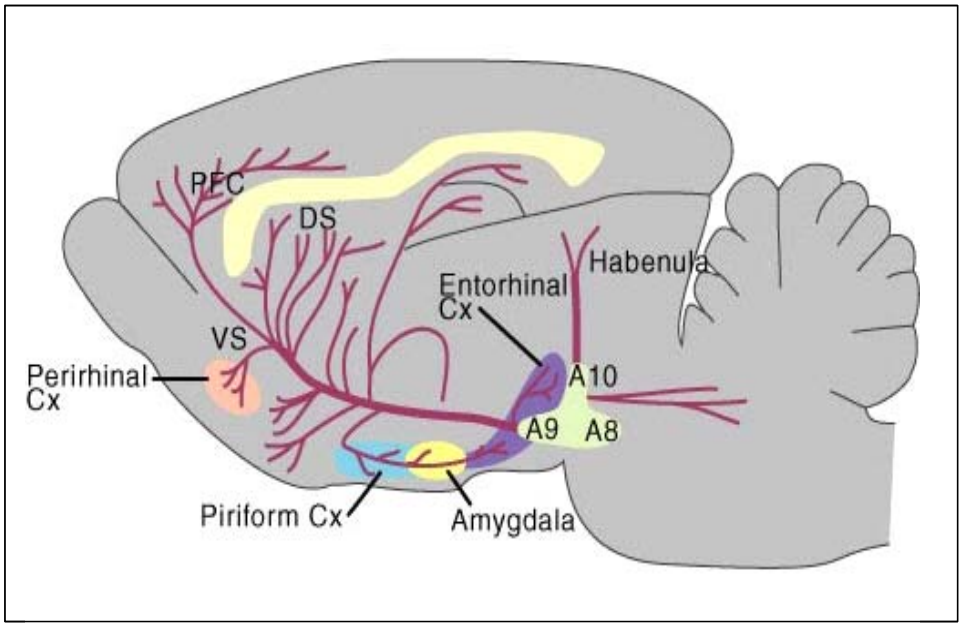
- The *mesocortical* pathway, from the ventral tegmental area (A10 cell group) towards the frontal and temporal cortices, involved in attentional processes and cognition, is associated with negative symptoms, as well as cognitive deficits, probably because of a dopaminergic deficit at the level of the limbic prefrontal cortex, and especially the dorsolateral prefrontal cortex.

- The *nigrostriatal* pathway, from the substantia nigra (A8 and A9 cell group) towards the striatum, which is part of the extrapyramidal system involved in the control of the movements, but is also involved in cognition.

- The *tuberoinfundibular* pathway, that controls the hormonal secretion from the pituitary. At this level, dopamine normally inhibits the release of prolactin. In fact, a hypoactivity of this pathway, provoked by neuroleptics that block the D_2 dopamine receptors, leads to an enhancement of prolactin release, and then may lead to the appearance of breast-swelling (gynecomastia in men), galactorrhea and amenorrhea in schizophrenic women. A role of dopamine in prolactin

release was proposed by Brown *et al* (1976) after they found haloperidol/dopamine receptors in the pituitary and not in the basal hypothalamus of the rat, leading them to postulate that in this region dopamine could serve as a prolactin release inhibiting factor.

Fig 1.1 Schematic representation of dopaminergic pathways in the rat



Abbreviations: **A10**, ventral tegmental area; **A8-A9**, substantia nigra; **DS**, dorsal striatum (*neostriatum*); **PFC**, prefrontal cortex; **VS**, ventral striatum (*accumbens nucleus*) (From Squire *et al*, 2003).

Several substances, that produce a psychotic-like state by acting on dopamine receptors, have been studied, and especially drugs of abuse like cocaine or amphetamine. Those compounds provoke a certain type of behaviour that resembles some positive symptoms (enhancement of locomotor activity in rats, hallucinations in man) by leading to the enhancement of catecholaminergic, and especially dopaminergic, activity. This could implicate the nucleus accumbens, the prefrontal cortex and the striatum, that receive a dopaminergic input from the ventral tegmental nucleus (the frontal cortex is well known to be cortical the structure that has the most important number of dopaminergic receptors). In fact it is suggested that dopamine could be involved in the deficit of information processing, linked to the prefrontal cortex, in schizophrenia (Walters,

2002). Kapur (2003) gives an interesting point of view about the role of dopamine, and the structures that are targets of dopaminergic pathways, in the occurrence of psychosis (*i.e.* delusions, hallucinations, and their secondarily related behaviours). According to Kapur, the dopaminergic system, and especially the mesolimbic system, is implicated in the attribution of motivational salience to a particular event (*i.e.* "A process whereby events and thoughts come to grab attention, drive action, and influence goal-directed behaviour because of their association with reward or punishment"); hence, a hyperactivity of the dopaminergic system would cause aberrant assignment of salience to external objects and internal representations. This process, that would persist in absence of sustaining stimuli, would create delusions and hallucinations.

Indeed, since the critical review of Robinson & Becker (1986), chronic amphetamine administration has been proposed to be a good animal model for psychosis, as it produces psychosis when intensively used in humans. Moreover, like the chronically-treated animals, the amphetamine abusers also become hypersensitive to amphetamine. In rats, if amphetamine is injected at low acute dose, it produces an increase of forward locomotion, head movement, sniffing and rearing, mediated by mesolimbic/mesocortical dopamine release (Kelly *et al*, 1975). If amphetamine is repeatedly and intermittently administered, it produces an exacerbation of these symptoms, with a reduction of their time of onset, which is called *sensitization*. This enhancement of dopaminergic transmission involving mesolimbic and mesocortical pathways is then considered as a good model for amphetamine psychosis (Robinson & Becker, 1986). Indeed, it has subsequently been shown, by Abi-Dargham *et al* (1998) for example during PET studies on 15 untreated schizophrenics and 15 control subjects, that a challenge by amphetamine produced a significantly enhanced dopamine release, observed in the striatum, in schizophrenics. Moreover, this was associated with a transient emergence or worsening of psychotic symptoms. Finally, Laruelle *et al* (1999), in a PET study on 34 schizophrenics and 34 control subjects, showed that dopamine dysregulation was present at time of onset and relapses but not during remissions.

An interesting hypothesis is stated by Gottesmann (2002) and aims at strengthening the link between dreams, dopamine and schizophrenia. During paradoxical sleep (rapid-eye movement sleep, REM-sleep), when dreams occur, the neurochemical profile of the brain is the following: the cerebral cortex is still activated by cholinergic inputs from

the brain stem, as during active waking, and the inhibitory influences exerted by histamine, noradrenaline and serotonin, that usually counterbalance the excitatory influences and thus rationalise the thought contents, become silent. At the same time, the inhibitory influence exerted by dopamine at the cortical level remains. According to Gottesmann, this could be a parallel between the dopaminergic pattern in schizophrenia and thus explain why schizophrenics have hallucinations and delusions, that resemble the content of dreams (*cf* the description of the psychological features of dreams content by Hobson (1998)).

However, the view concerning dopamine dysfunction as being the only important neurochemical feature of schizophrenia has been reconsidered following several observations. For example, the blockade of the D_2 receptors occurs immediately after the administration of neuroleptics, but the effects of the medication on the symptoms is clearly apparent only after several days or weeks; also, some schizophrenics are resistant to treatment by these drugs, and only experience side-effects (Hietala & Syvälahti, 1996). Moreover, it has never been clearly demonstrated that in schizophrenics the level of dopamine receptors was affected, and there are discrepant findings of an enhanced, or unchanged, number of D_2 receptors in the striatum of schizophrenics. For example, studies often show that there is no change in the amount of D_2 receptors in the brains of schizophrenics as revealed by PET scanning experiments (*e.g.* Zakzanis & Hansen, 1998). One of the major problems is that the findings from autoradiography and binding studies are made post-mortem, usually on brains of intensively medicated schizophrenics. In fact, it has been shown that a 6-week administration of haloperidol in rats leads to an increase of striatal and pallidal D_2-like receptors (65 % and 95 % respectively), similar to what is observed in drug-treated schizophrenics (Reynolds, 1995). Another possibility is that, as there exists for both types of dopamine receptors a state of low affinity and a state of high affinity, schizophrenia involves a failure of D_2 receptors to desensitize to a low-affinity state (Seeman, 1987). Nevertheless, an increased D_1 receptor concentration, in the dorsolateral prefrontal cortex, in never medicated schizophrenics has been found (Abi-Dargham *et al*, 2002). According to the authors, this could be due to an up-regulation of the number of receptors consecutive to a lack of stimulation of these receptors by mesocortical dopamine, which could lead to the working memory deficits encountered in schizophrenics.

However, as D_2 receptor medication failed to completely cure schizophrenia, new dopaminergic targets have been considered. D_3 and D_4 receptors are highly present in cortical and limbic areas, but only sparsely represented in the striatum, and could thus be good candidates in order to treat cognitive and negative symptoms with only little effect on motor functions (Jardemark *et al*, 2002). For example, the D_3 receptor, which is highly present in the shell of the nucleus accumbens and in the cerebral cortex, and is co-expressed with the D_1 receptor (Sokoloff *et al*, 1998), is postulated to be involved in the pathology of schizophrenia, with probably an action during early brain development (Schwartz *et al*, 2000). It is envisaged to be a target for future compounds, as its antagonism does not seem to induce tolerance and side effects (Joyce, 2001). Similarly, new advances have been made concerning the D_4 receptor, since it was found that the atypical antipsychotic clozapine, which produces much less side-effects than the typical ones, has a 10-fold higher affinity for D_4 compared to D_2 and D_3 receptors; moreover, it has been found to be upregulated in brains of schizophrenics (Sanyal & Van Tol, 1997). Unfortunately, clinical studies showed that a potent, selective D_4 antagonist was ineffective in a number of preclinical and clinical tests (Bristow *et al*, 1997; Kramer *et al*, 1997).

I.1.8.2 Schizophrenia and glutamate

The fact that antipsychotics that target dopamine receptors do not treat the broad set of symptoms of schizophrenia led many researchers to explore new avenues in order to explain schizophrenia. One of the potential candidates is glutamate (Moghaddam, 1999). Indeed, molecules acting as antagonists of ionotropic glutamatergic NMDA-type receptors provoke psychoses equivalent to what is currently observed in schizophrenics (paranoia, agitation, auditory hallucinations, stereotyped behaviour, social withdrawal, apathy, poverty of thoughts, as well as cognitive symptoms such as working memory deficits). Such substances are drugs of abuse like phencyclidine (PCP, or “angel dust”) and ketamine, or dizocilpine (MK-801), which are non-competitive antagonists of the NMDA receptor. Moreover, Mohn *et al* (1999) found that genetically-modified mice with 95% reduction of the NR_1 subunit, making the NMDA receptor inefficient, showed behavioural deficits similar to the symptoms observed in schizophrenia, including increased locomotor activity, stereotypies, and deficits in sexual behaviour as well as social withdrawal. Focussing on the ability of PCP to induce psychosis-like states in human, and using social behaviour in rats, Sams-Dodd (1996) created a model of

schizophrenia in rats, showing that PCP produces, in a dose-dependent manner, symptoms that are comparable to what is observed in schizophrenics: stereotyped behaviour and disturbances of the social behaviour. Moreover, these behavioural features were improved by haloperidol and clozapine, in doses equivalent that those administered in humans, the haloperidol improving negative-like symptoms, and clozapine improving positive-like, as well as negative-like symptoms. On the other hand, in order to test the NMDA antagonists model of schizophrenia, Adams & Moghaddam (2001) challenged a treatment with PCP in rats with a typical (haloperidol), and an atypical (clozapine) neuroleptic, as well as with a pure 5-HT_{2A} antagonist (M100907), in order to verify their potency in reversing the increased prefrontal cortical glutamate release. In fact, none of the compounds were able to block the PCP-induced enhancement of glutamatergic transmission, showing that this model was not useful in predicting efficacy for conventional antipsychotics.

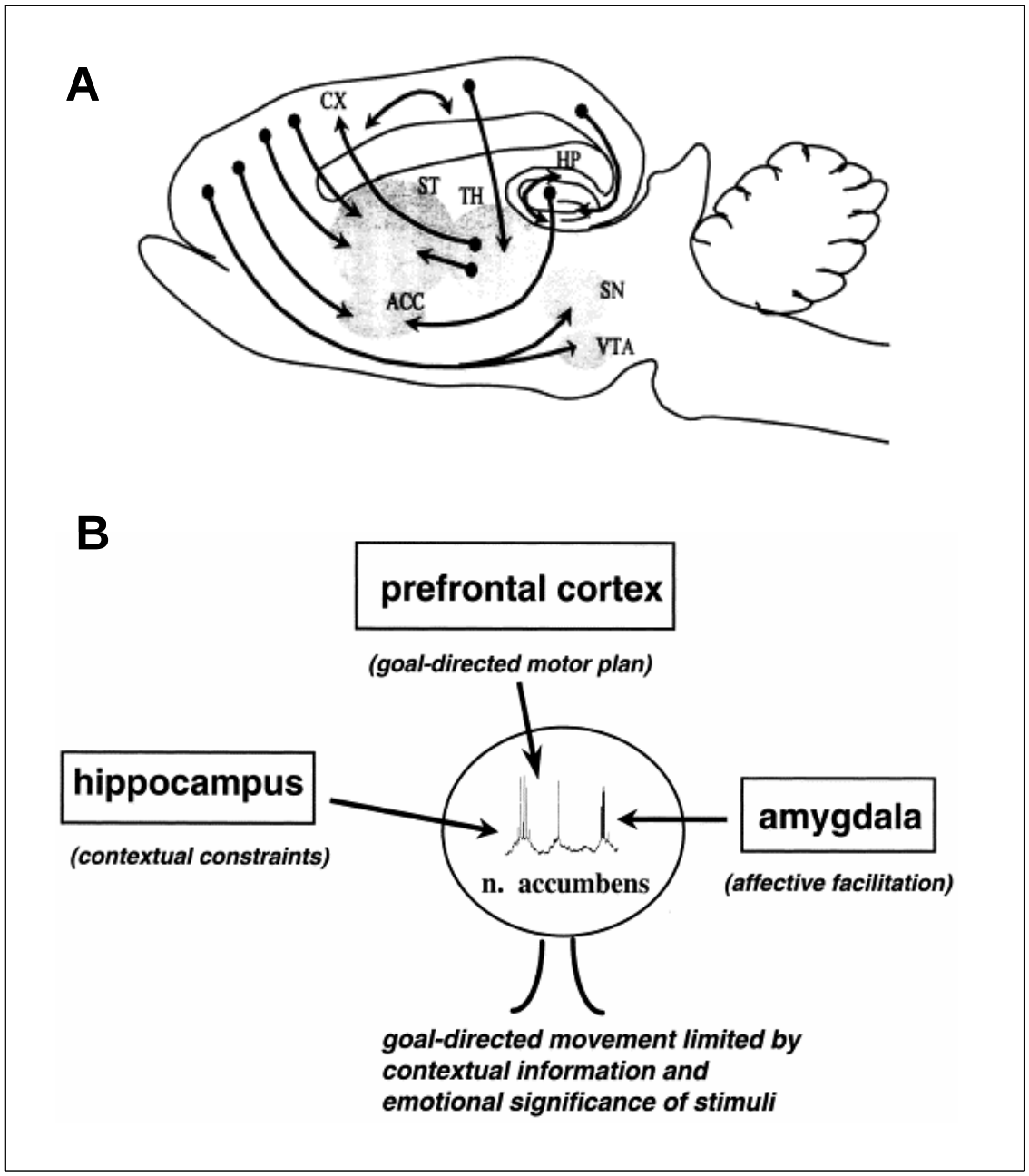
According to some investigators, the effects of NMDA antagonists on the brain is not only as originally supposed, *i.e.* the induction of schizophrenia-like symptoms by the suppression of postsynaptic glutamatergic neurotransmission. In fact, it has been shown in the rat, for example, that a subanesthetic dose of ketamine (30 mg/kg) produces an increase of the release of glutamate in the prefrontal cortex of rats. It seems that ketamine, by antagonizing NMDA receptors, disinhibits the release of glutamatergic neurons by blocking the action of GABAergic interneurons (Moghaddam *et al*, 1997). Then, a new aspect is that NMDA antagonists lead to the enhancement of the release of glutamate which in turn acts at non-NMDA receptors. Takahata & Moghaddam (2000), by administering AMPA/kainate antagonist in the ventral tegmental area in rats, produced an increase of the release of dopamine in the nucleus accumbens, and a decrease in the prefrontal cortex, showing that, under normal conditions, glutamate, through NMDA/kainate receptors, exerts a tonic inhibition upon dopaminergic neurons projecting to the nucleus accumbens, and a tonic excitation on those projecting to the prefrontal cortex. This has subsequently been confirmed by Jackson *et al* (2001), who induced a decrease of the release of dopamine in the nucleus accumbens by stimulating the prefrontal cortex with a frequency equivalent to that encountered during cognitive tasks. According to the authors, glutamatergic neurons of the prefrontal cortex act either directly by activating GABAergic interneurons in the ventral tegmental area, or they

stimulate GABA neurons in the nucleus accumbens that control the dopaminergic neurons of the ventral tegmental area.

Recent discoveries strengthen the view that some of the schizophrenic symptoms could be due to a concomitant alteration of the glutamatergic and the dopaminergic systems. For example, sub-chronic administration of PCP in rats induces a decrease of dopamine utilization in the prefrontal cortex, and induces cognitive deficits comparable to those observed in schizophrenia (Jentsch *et al*, 1997) (also see reviews by Goff & Coyle, 2001; Tsai & Coyle, 2002). It has been proposed that a circuit involving the mesocortical and mesolimbic dopaminergic pathways, as well as the glutamatergic pathways from the frontal cortex towards the dopaminergic areas of the midbrain could be the main system altered in schizophrenia; this could involve the midbrain-cortex-midbrain loop, in which altered dopaminergic regulation of prefrontal cortical neurons, especially glutamatergic (hypofrontality), could provoke schizophrenic symptoms by disturbing the descending glutamatergic efferent pathways towards the limbic system (dopaminergic ventral tegmental area, hippocampus, nucleus accumbens and amygdala) (Svensson, 2000). However, it has been shown by Kapur & Seeman (2002) that PCP and ketamine can act directly at the level of dopaminergic and serotonergic receptors as they have found equivalent affinities, for these compounds, at NMDA and D_2 and 5-HT_2 receptors. Finally, Kegeles *et al* (2000) using the amphetamine challenge model (see above) during an imaging study (SPECT) on 8 healthy volunteers, showed that the amphetamine-induced increase of striatal dopamine release was exacerbated (greater than two-fold) by the administration of ketamine. These authors concluded that this is consistent with the view that alteration of dopamine transmission in schizophrenia results from a disruption of glutamatergic neuronal regulation. Thus several investigators propose that contrary to what is stated by the dopamine theory of schizophrenia, the dopaminergic system itself is not deficient, and it is the regulatory system (*e.g.* the glutamatergic system) which is dysfunctional. This dysfunction could take place within the corticolimbic loops that connect prefrontal cortex, amygdala, nucleus accumbens and ventral tegmental area. It has been postulated that, as the prefrontal cortex is involved in higher cognitive functions (*i.e.* motor planning and learning that involves an emotional component) in collaboration with subcortical structures such as hippocampus, amygdala and nucleus accumbens, a dysregulation of the circuitry linking those structures would lead to the appearance of some of the

symptoms of schizophrenia. Indeed, the nucleus accumbens receives information from frontal cortex (linked to goal-directed motor planning), subiculum (linked to the context) and amygdala (linked to the emotional valence). According to Grace (2000), and considering that hippocampus and amygdala gate cortical information from the prefrontal cortex to the nucleus accumbens, a dysfunction of the glutamatergic inputs to the nucleus accumbens would lead to impaired regulation of output pathways exerted by dopamine, inducing inappropriate responses to particular situations (**Fig 1.2**). This would take place because of the subsequent disturbance of the transmission of cortical information, through the nucleus accumbens, towards the ventral pallidum and the thalamus. Indeed, it has been shown that glutamatergic inputs to the nucleus accumbens were modulated by D_1 dopaminergic receptors, strengthening the view that mesolimbic dopamine regulates information flow within the nucleus accumbens (Charara & Grace, 2003).

Fig 1.2 Schematic representation of relevant glutamatergic pathways in the rat



Top. Abbreviations: **Acc**, accumbens nucleus; **CX**, frontal cortex; **HP**, hippocampus; **SN**, substantia nigra; **ST**, striatum; **TH**, thalamus, **VTA**, tegmental ventral area. ***Bottom.*** Implication of glutamatergic inputs to the accumbens nucleus in schizophrenia, according to Grace (2000).

Several researchers suggest that agonists or antagonists of the glutamatergic system could be potent antipsychotics, at least for some classes of symptoms that are not improved by dopaminergic drugs. For example, the metabotropic mGluR5 receptor,

which belongs to the group I of glutamate receptors, is highly present in many structures involved in schizophrenia (*i.e.* hippocampus, striatum, neocortex, nucleus accumbens, thalamus and lateral septum), and the $mGluR_{2/3}$ autoreceptors, that belong to the group II of glutamate receptors, are highly present in cortex, hippocampus and cerebellar cortex. Moreover, $mGluR_5$ and $mGluR_2$ are abundant in the hippocampus and in the neurons of the nucleus accumbens that project to the ventral pallidum (Chavez-Noriega *et al*, 2002). Thus, metabotropic receptors are considered as good candidates, at least to ameliorate cognitive symptoms. Either agonists of the $mGluR_5$ subtype, which can enhance NMDA neurotransmission, or agonists of the $mGluR_{2/3}$ autoreceptors, which have been found to block PCP-induced increased locomotion and stereotypies in rats (Moghaddam & Adams, 1998). Finally, another possible treatment of the cognitive symptoms of schizophrenia would be to act upon the ionotropic NMDA receptor and enhance its function; indeed D-cycloserine a partial agonist at the glycine recognition site of the NMDA receptor, as D-serine is a modulator of the NMDA receptor at the glycine site, has been shown to enhance cognitive symptoms in schizophrenia (Coyle *et al*, 2002). Moreover, D-cycloserine (50 mg/day) improved negative symptoms when added to the atypical antipsychotic risperidone (Evins *et al*, 2002).

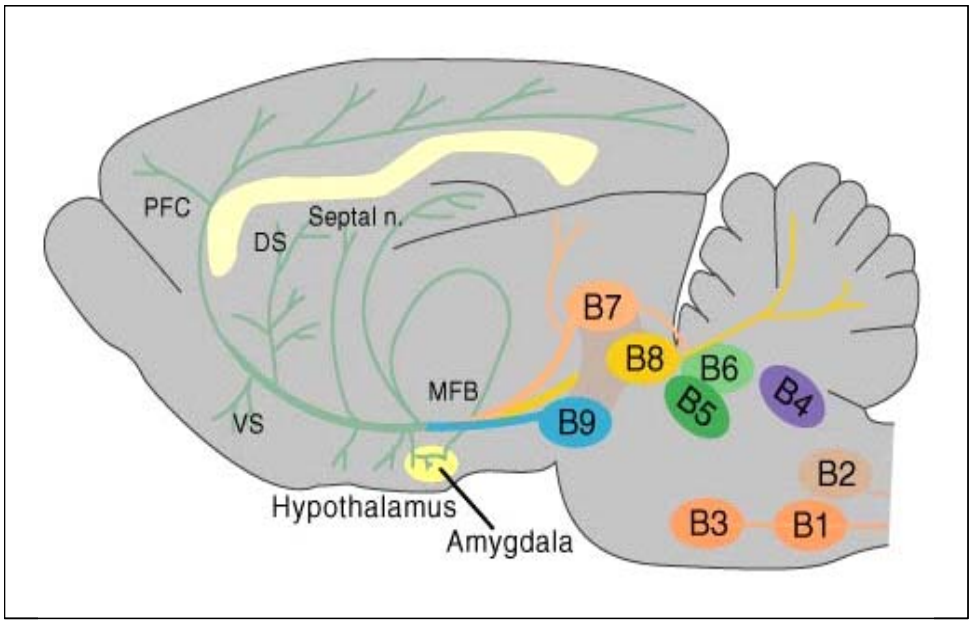
Even if altered glutamatergic neurotransmission, according to preclinical and clinical studies, seems to be a good candidate for the appearance of some of the symptoms of schizophrenia (*i.e.* negative and cognitive), deficiencies in the glutamatergic system still need to be strongly confirmed in schizophrenia itself, since for example genetic and receptor mapping studies present some discrepancies. Tsai *et al* (1995) found a lower amount of glutamate, as well as aspartate, in the prefrontal cortex and hippocampus in schizophrenics. They also found a higher concentration of N-acetylaspartylglutamate (NAAG), which is a precursor of glutamate and can also act post-synaptically as a NMDA antagonist, as well as a lower concentration of the enzyme that converts NAAG into glutamate. Concerning the ionotropic receptors, the $GluR_{5\text{-}6\text{-}7}$ subunits of the kainate receptors have been found to be down regulated at the level of the pyramidal cells in the CA_1, CA_2 and CA_3 regions of the hippocampus in schizophrenics (Benes *et al*, 2001). Also in the hippocampus, Porter *et al* (1997) found a lower expression of the mRNA of the $GluR_1$ and $GluR_2$ AMPA sub-units and of the $GluR_6$ and KA_2 kainate sub-units, and Gao *et al* (2000) found decreased concentration of the NR_1 and increased concentration of the NR_{2B} sub-units of NMDA receptor. However, a study by Martucci

et al (2003) did not show any difference in polymorphisms of the GRIN1 gene coding for the NR_1 subunit of the NMDA receptor in schizophrenics compared to controls. Crook *et al* (2002) found no difference in the post-mortem immunoreactivity for metabotropic receptors of the type II class in Brodmann's area 46 of the dorsolateral prefrontal cortex of medicated schizophrenics compared to controls. No polymorphisms were found in schizophrenics concerning the genes for the metabotropic receptors of the type III class ($mGluR_7$ and $mGluR_8$) which presynaptically inhibit glutamate (Bolonna *et al*, 2001; Bray *et al*, 2000). Also, in the German population, Marti *et al* (2002) did not find any variation in the expression of the GRM3 gene coding for the $mGluR_3$ receptor, and Ohtsuki *et al* (2001) did not find any mutation in the GRM4 gene coding for the mGluR4 receptor in a Japanese cohort. Nevertheless, an interesting finding suggests an alternative analysis of such results, as Ohnuma *et al* (2000), studying the expression of genes coding for the $mGluR_5$ receptor as well as for the excitatory amino acid transporter 2 (EAAT2) in the hippocampus, did not find any difference between schizophrenics and control, but when the ratio $mGluR_5$/EAAT2 was compared, they found an enhancement in the parahippocampal gyrus, that could be the reflection of a hypo-glutamatergic transmission in this structure.

I.1.8.3 Schizophrenia and serotonin

The findings that hallucinogenic substances such as the indoleamines (*e.g.* lysergic acid diethylamide, or LSD) or the phenethylamines (*e.g.* mescaline) bound to serotonergic receptors led some researchers to consider the involvement of a defective serotonergic system in the occurrence of schizophrenia (**Fig. 1.3**).

Fig 1.3. Schematic representation of the serotonergic pathways in the rat



Abbreviations: **B4-B9**, serotonergic cell groups in the raphé nuclei; **DS**, dorsal striatum (neostriatum); **MFB**, medial forebrain bundle; **PFC**, prefrontal cortex; **VS**, ventral striatum (accumbens nucleus) (From Squire *et al*, 2003).

For example, the hallucinogenic properties of the LSD have been attributed to its binding to the 5-HT_{2C} and 5-HT_{2A} receptors. This supposition was later strengthen by the finding that atypical antipsychotics (*e.g.* clozapine) have elevated affinity for the 5-HT_{2A} and 5-HT_{2C} serotonin receptors and a quite low *in vivo* occupancy for the D_2 dopamine receptor at therapeutic dose (Reynolds, 1995). These compounds cause less extrapyramidal side-effects than typical neuroleptics, and in the case of clozapine can alleviate symptoms in schizophrenics that do not respond to classical neuroleptics (Kane *et al*, 2001). Indeed, quetiapine, an atypical antipsychotic with minimal D_2 receptor occupancy, has been found, during a PET study on 12 schizophrenics, to bind to the 5-HT_{2A} receptor, binding which was correlated with the improvement of clinical symptoms and to the absence of motor side-effects (Kapur *et al*, 2000). Also, risperidone efficacy has been shown to be associated with the 5-HT_{2A} receptor polymorphism (Lane *et al*, 2002).

In their review, Iqbal & Van Praag (1995) describe interactions between serotonergic and dopaminergic systems, and consider as simplistic the view that schizophrenia could

simply occur because of an elevation, even marked, of the level of serotonin transmission. In fact, antagonizing the serotonergic system generally affects dopaminergic transmission, in both mesolimbic and mesostriatal systems, and is proposed to contribute to the beneficial effects of atypical antipsychotics concerning extrapyramidal symptoms (EPS) like tardive dyskinesia and even negative symptoms. For example, 5-HT_2 antagonists can act postsynaptically at the level of the substantia nigra, or presynaptically at the level of the striatum, and thus release dopamine from inhibition, leading to the decrease of EPS; the same effect can also be obtained by stimulating raphé 5-HT_{1A} autoreceptors and thus inhibiting serotonin transmission, which in turn leads to the enhancement of dopamine transmission in the striatum. Moreover, 5-HT_{2A} antagonists lead to the increase of dopamine release in the prefrontal cortex, which is proposed to be the mechanism by which they ameliorate negative symptoms (Lieberman *et al*, 1998). However, Ruiu *et al* (2000) have shown, on isolated frontal cortex synaptosomal fractions, that the 5-HT_2 antagonist ritanserin inhibited dopamine re-uptake, suggesting that this mechanism, leading to an increased extracellular dopamine concentration, may be of importance.

Aghajanian & Marek (2000) investigated the interactions between the serotonergic and glutamatergic systems, and proposed an alternative hypothesis to the serotonin hypothesis, based on the concomitant involvement of those two systems. These authors noted that hallucinogenic substances (*i.e.* indoleamines and phenethylamines) act through 5-HT_{2A} receptor that are located in the prefrontal cortex (pyramidal layer V) and the locus cœruleus. In the former, they induce enhancement of the glutamatergic transmission, and, in the latter, they facilitate activation of the glutamatergic transmission by sensory stimuli (since noradrenaline enhances the signal-to-noise ratio of sensory information), through the enhancement of the glutamatergic transmission. It could be through these mechanisms that these substances disturb thalamocortical loops and induce schizophrenia-like symptoms.

Abi-Dargham *et al* (1996), in a quite complete overview of the involvement of serotonin in schizophrenia, concluded that the studies did not give consistent results, whether serotonergic receptors or the serotonin transporter were investigated. For example, Serretti *et al* (2000) did not find any changes in the regional concentration of 5-HT_{2A} receptors in schizophrenics, and Okubo *et al* (1997), in a PET study, did not

find any difference in the binding for 5-HT_2 receptors in any brain regions in schizophrenics, including drug-free patients, compared to controls. Concerning the 5-HT_{2A} receptor, a review by Dean (2003) revealed how hard it is to associate this receptor to schizophrenia. First of all, there have been many discrepant post-mortem and imaging studies that showed either a decreased or an increased binding in the prefrontal cortex of schizophrenics. In fact, it seems that the 5-HT_{2A} receptor shows a down-regulation in reaction to both agonists and antagonists; thus, it is not clear if decreased binding is due to the administration of antipsychotics, some of which being antagonists of this receptor, or to the disease *per se*, which could be the reflection of an increased serotonergic tone. Van Oekelen *et al* (2003) postulated that an antagonistic action at both 5-HT_{2A} and 5-HT_{2C} receptors could lead to the phosphorylation of the receptors, their internalisation followed by their degradation in lisosomes. They propose a circuit in which such a mechanism could take place following the administration of drugs with a 5-HT_2 antagonistic action, and which involves GABA-ergic and glutamatergic neurons of the frontal cortex and dopaminergic neurons of the ventral tegmental area that project on the former. According to them, this down-regulation could maintain the therapeutic action of the antipsychotics.

Post-mortem studies showed that a property of the human serotonergic 5-HT_{2C} receptor, which has also been investigated with respect to schizophrenia, is its RNA editing. This phenomenon leads, after the substitution of some nucleotidic bases in the mRNA sequence (adenosine-to-inosine) at five positions within the second intracytoplasmic loop, to receptor variants with distinct pharmacological properties (Sodhi *et al*, 2001). Discrepant findings have been found about this phenomenon: Sodhi *et al* (2001) showed that editing in the frontal cortex of schizophrenics that led to the predominant formation of the more active receptor to the detriment of the less active, and was considered to lead to the frontal disturbances seen in schizophrenia, and to the different responses to the various neuroleptics. On the other hand, a study by Niswender *et al* (2001) did not show any differences in the same region. However, in the latter case, they found enhanced editing at one site in the patients that committed suicide, suggesting that abnormal editing could play a role in this feature of any psychiatric diseases. This was confirmed in a post-mortem study by Iwamoto & Kato (2003) who extended this view to major depression in addition to suicide. Interestingly, this demonstrates that anomalies may occur not in terms of number of receptors, but in terms of receptor

properties, and shows why it is so hard, using conventional techniques (imaging or histological), to demonstrate the involvement of any receptors in pathologies such as schizophrenia.

I.1.8.4 Schizophrenia and acetylcholine

It is well-known that a majority of schizophrenics are smokers (between 70 and 80 %, compared to 30% in the general population), and this percentage is also the highest among psychiatric illnesses (Hughes *et al*, 1986). Given the fact that smoking withdrawal exacerbates some of the symptoms, it has been proposed that smoking could be a self-medication attempt. In fact, studying the impact of nicotine on schizophrenia, Smith *et al* (2002) found that administration of high dose of nicotine through cigarettes tended to reduce the severity of negative symptoms, while it had no effect by nasal spray administration; nonetheless, this was only efficient in heavy smokers. Having noticed that nicotine withdrawal worsened these symptoms, the authors concluded that nicotine could maintain negative symptoms at a basal level of severity. On measures of cognitive performance, cigarettes had no effect, whereas the nasal spray administration tended to improve the deficits during various tests (spatial processing, visual and verbal memory). Zammit *et al* (2003), in a longitudinal study between 1970 and 1996, performed with 50,087 young Swedish conscripts, found that those that had started smoking around 18-20 years old had a reduced risk for the appearance of schizophrenia. According to these authors, this study confirms the neuroprotective role of nicotine.

Nicotinic acetylcholine receptors, which are ligand-gated ion channels, have been studied in schizophrenia and, because of their effects on cognition, nicotinic agonists are under development as possible treatment of cognitive deficits in schizophrenia. Nicotinic receptors are involved in higher cognitive functions such as attention, learning and memory, as shown extensively in rodents by the fact that nicotinic antagonists impair those functions, and that nicotinic agonists improve deficits-induced in attention or learning and memory (Levin & Simon, 1998). In the human brain these receptors are pentamers composed of a combination of 2 types of subunits [α(α2 to α7, α9) and β(β2 to β4)], and there are two main sub-populations of them, α7 and α4β2. The receptors of the α7 type exhibit a low affinity for nicotine and are mainly located in cerebellum, thalamus (reticular nucleus and less pronounced in the lateral geniculate), in subfields of the hippocampus, in layers I-II of temporal cortex (Brodmann's area 42), lateral

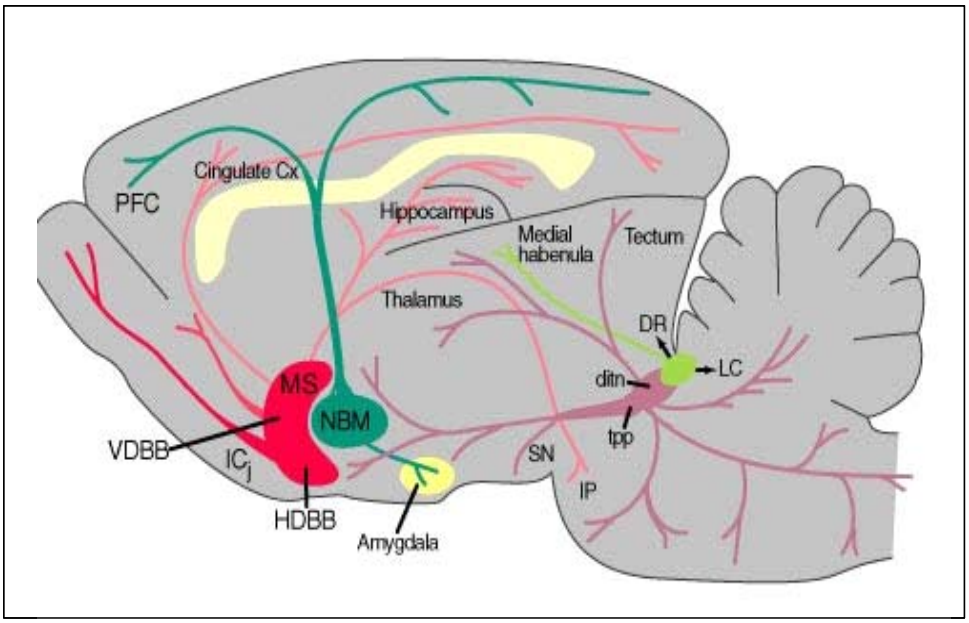
nucleus of the amygdala, nucleus basalis of Meynert, in the pons and in the deep cerebellar nucleus (Graham *et al*, 2002; Lena & Changeux, 1998). The receptors of the α4β2 type exhibit a high affinity for nicotine and are mainly located in the thalamus and the hippocampus, as well as in the striatum (Lena & Changeux, 1998; Graham *et al*, 2002). An important fact concerning schizophrenia is that the α7 are mainly located in the reticular thalamic nucleus, which is known to be entirely composed of GABAergic interneurons and is responsible for inhibitory feedback control of thalamo-cortical pathways (Freedman *et al*, 2000). Thus, deficits in sensory gating, a role played by the thalamus, and represented by a defect in diminishing the magnitude of the P50 response to the second of two identical stimuli (= cortical evoked potential after repeated auditory stimuli), have been proposed to be due to an abnormal expression (Leonard *et al*, 1998), or a desensitization (Griffith *et al*, 1998) of the α7 receptor in schizophrenia. Whereas treatment with conventional neuroleptic does not improve this impairment, nicotine administration has been shown to correct it, as well as clozapine, but only in patients that show a good therapeutic response concerning other types of symptoms (McEvoy & Allen, 2002). Indeed, two genetic studies of the chromosome 15q14 site have revealed in schizophrenics promoter variants of the α7 receptor that could account for its decreased expression and is postulated to be at the origin of the sensory gating deficits (Leonard *et al*, 2002). Moreover, Freedman *et al* (1997) found that this defect on the chromosome 15 was shared by schizophrenics and their non-schizophrenic relatives, showing that this feature of the disease was inherited genetically. An interesting fact is underlined by Freedman *et al* (2000), and supports the view conceived by Benes & Berretta (2001) (see below). These authors postulate that a dysregulation of the inhibitory action of GABAergic interneurons could be at the origin of deficits in schizophrenia, including sensory gating deficits. Freedman *et al* (2000) pointed out that post-mortem studies of four different groups of researchers revealed a decrease of the density of α7 receptors, which were mainly colocalized with GABAergic interneurons in the hippocampus and in the thalamus. They also summarized evidence that this decrease could lead to a failure of activation of inhibitory interneurons, and then to sensory gating deficits encountered in schizophrenia.

Concerning the α4β2 receptor, a post-mortem study by Durany *et al* (2000) has revealed a 30% decrease of its expression in the striatum of schizophrenics, and the authors

propose that nicotine consumption could then be a way to compensate this decreased number which leads to a decreased efficacy of cholinergic transmission.

In fact, many studies have been performed on cholinergic function and some contradictory results did not allow any general agreement concerning that question (see review by Hyde & Crook, 2001). Nevertheless, a very interesting fact is that the cholinergic pathway, linking the basal forebrain and the prefrontal cortex (**Fig. 1.4**), is under the influence, from the accumbens nucleus, of GABAergic neurons that are themselves regulated by a dopaminergic inhibitory influence (Moor *et al*, 1999).

Fig 1.4. Schematic representation of the cholinergic pathways in the rat



Abbreviations: **DR**, dorsal raphé nucleus; **HDBB**, horizontal limb of the diagonal band of Broca; **IP**, interpeduncular nucleus; **LC**, locus coeruleus; **MS**, medial septum; **NBM**, nucleus basalis magnocellularis (Meynert in primates); **PFC**, prefrontal cortex; **VDBB**, vertical limb of the diagonal band of Broca.

Thus, these authors propose that a dysregulation of the mesolimbic dopaminergic system could induce, because they are indirectly connected, a dysregulation of the cholinergic system, which could be the origin of the cognitive deficits encountered in schizophrenia (attention, learning and memory). This link between the cholinergic and

the dopaminergic system is very important and it has been stressed that, for example, nicotine, which stimulates dopamine release, could be used by schizophrenics, through smoking, to compensate the dysregulation of the dopaminergic system. George *et al* (2000), studying the effects of stress, which has been shown to exacerbate schizophrenic disorders, found that low, but not high, dose of repeated nicotine pre-treatment attenuated the increased dopamine utilization in the medial prefrontal cortex, and the associated acute stress-induced immobility responses in rats. Together with its effects on cognition, this provides a basis for the view that in schizophrenics nicotine has a real beneficial role.

Concerning the muscarinic receptors, it has recently been shown that the atypical antipsychotic clozapine acts as partial weak agonist of the post-synaptic excitatory muscarinic cholinergic receptors M_1 and M_4, the affinity being higher than for the D_1 and D_2 dopaminergic receptors (Pavel *et al*, 1999). Also, olanzapine, another atypical antipsychotic, has been shown in vivo to be an M_2 receptor antagonist (Raedler *et al*, 2000). According to these authors, this could account for the low extrapyramidal effects observed with such a compound. Moreover, Raedler *et al* (2003) showed that a treatment with clozapine induced a decreased binding to muscarinic receptors in 8 schizophrenics in cortex, striatum and thalamus. In fact, a post-mortem study has shown that the binding for M_1 and M_4 was reduced in the hippocampal formation of a cohort of schizophrenics (n=15 compared to 18 healthy control subjects), including dentate gyrus, subdivisions of Ammon's horn (CA_1 to CA_4), subiculum and parahippocampal gyrus (Crook *et al*, 2000). Another study has shown a decreased population of M_1 and M_4 receptor in the prefrontal cortex of 17 schizophrenics compared to 20 control subjects (Crook *et al*, 2001).

I.1.8.5 Schizophrenia and GABA

GABA is the main inhibitory neurotransmitter in the central nervous system, and the major transmitter of interneurons in cortical areas. There are two types of GABA receptors: the ionotropic $GABA_A$ receptor which also possesses a binding site for benzodiazepines, and the metabotropic $GABA_B$ receptor. Post-mortem studies have revealed changes in the GABA system of schizophrenics. Mizukami *et al* (2000) showed that the $GABA_B$ immunoreactivity was markedly reduced in the hippocampus of 5 schizophrenics compared to 3 control subjects, throughout all the CA fields, and

that it was also reduced in the pyramidal cells of the entorhinal cortex, throughout all layers, and in the inferior temporal cortex, in layer V. These authors consider that those results can be interpreted according to the view that some symptoms of schizophrenia, such as gating deficits, arise, at least partly, from disturbances within the hippocampus involving loss of inhibition upon the pyramidal cells by the GABA interneurons could easily contribute to cause hippocampal dysfunction. A highly replicated finding is up-regulation of the $GABA_A$ subtype in the prefrontal cortex (Brodmann's area 9 and 10), and hippocampus (CA_{2-4} region), in schizophrenia (Benes *et al*, 1996a,b; Dean *et al*, 1999; Ohnuma *et al*, 1999). This enhanced number of $GABA_A$ receptors is postulated to occur in reaction to a decrease of the GABAergic tone that could participate in the hypofrontality characteristic of schizophrenia. These receptor binding changes were confirmed by Ohnuma *et al* (1999) who also found in the same region a decrease of the GABA content and a decrease of the expression of the GABA transporter messenger RNA.

Benes & Berretta (2001), extending the above-mentioned post-mortem studies (cell counting, GABAergic endings labelling or specific labelling of the GABA receptors), concluded that some of the schizophrenic symptoms could occur because of dysregulation of the inhibitory/disinhibitory GABAergic system in the prefrontal cortex, as well as the dysregulation of the inhibitory role of GABA in the hippocampus. Indeed, they found a decreased number of non-pyramidal cells in prefrontal cortex (layer II), anterior cingulate cortex (layers II and IV) and hippocampus (CA_2 region). As these regions receive input from the amygdala, they suggested that a hyperactivity of this amygdalar afferent pathway could lead to the changes observed in the GABAergic system. In fact, these authors are some of the pioneers of the hypothesis that the disturbances of the GABAergic system could appear during the early development of the cortex, and could be due to a default in the cellular migration, and in the formation of cell layers. These features could also be linked to a pre- or/and post-natal stress, as the GABAergic system has been shown to be highly sensitive to stress-induced physiological variations, especially affecting the glucocorticoid hormones, that intensify the cellular response mediated by the $GABA_A$ receptor (Lambert *et al*, 1987). More precisely, the fact that the changes in $GABA_A$ receptor appear essentially in layer II of the prefrontal cortex, is in favour of the statement that there could be a defect during the

perinatal development of the brain, as this layer is the last to appear and to be differentiated in the early development.

Unfortunately, to date, no other *in vivo* study has brought solid data to confirm the changes in the GABA system in schizophrenics. Busatto *et al* (1997) and Abi-Dargham *et al* (1999) failed to find any difference at the cortical level using post-mortem benzodiazepine receptor binding, and if polymorphisms have been observed at the level of the $GABA_B$ gene in chromosome 6, there was no statistical confirmation that they could confer a particular vulnerability for schizophrenia (Imai *et al*, 2002). However, a study by Papadimitriou *et al* (2001) showed an association between schizophrenia and a polymorphism of the gene coding for the alpha 5 subunit of the $GABA_A$ receptor in a population of Greek late-onset schizophrenics.

I.1.8.6 Is there a genetic susceptibility for schizophrenia ?

Studies of mono- or dizygotic twins raised in the same or separate families, as well as studies of "high-risk" families, has shown a considerable genetic involvement in the appearance of schizophrenia. The probability to develop schizophrenia is 1 % in general population, and reaches 17 % in first-degree relatives and 50 % in monozygotic twins (Gottesman, 1991). Thus, there must be both genetic and environmental factors in the appearance of schizophrenia. Genetic studies have indicated many possible chromosomal effects (at chromosome 1, 2, 5, 6, 7, 8, 9, 10, 13, 15, 18, 22) (MacIntyre *et al*, 2003; Waterworth *et al*, 2002). According to Demirhan & Tastemir (2003), these chromosomic anomalies could give rise to a more pronounced risk to develop schizophrenia, in a non-specific manner, by disturbing embryogenesis of the nervous system. Most authors, in fact, believe that the appearance of schizophrenia requires the presence of several factors (multiple "hits"), including genetic predisposition, or "susceptibility genes", that may confer vulnerability. Nonetheless, they do not exclude the involvement of environmental stressors, well formulated for example by Gottesman (1991). Some of these risk factors are postulated to essentially act during pregnancy and birth of the child, and can lead to certain anatomical anomalies. Cannon *et al* (2002), who performed a meta-analysis, underlined three different categories of complications, correlated with the appearance of schizophrenia: ***1*** - pregnancy complications (bleeding, diabetes, rhesus incompatibility). Concerning that point, Mallard *et al* (1999), after having performed intrauterine growth-restriction in female guinea pig during gestation,

observed in pups some of the anatomical anomalies observed in schizophrenics (see above): ventricular enlargement and decrease of the hippocampal, striatal and cortical volumes. ***2*** - foetal abnormal development (low weight at the time of birth, congenital malformations, reduced head circumference); more particularly, the schizophrenics that had a low weight at the time of birth have a lower rate of premorbid social adaptation, and the prematures have a higher risk to present neurodevelopmental abnormalities (Kunugi *et al*, 2001). ***3*** - delivery complications (uterine atonia, asphyxia, caesarean operation in emergency).

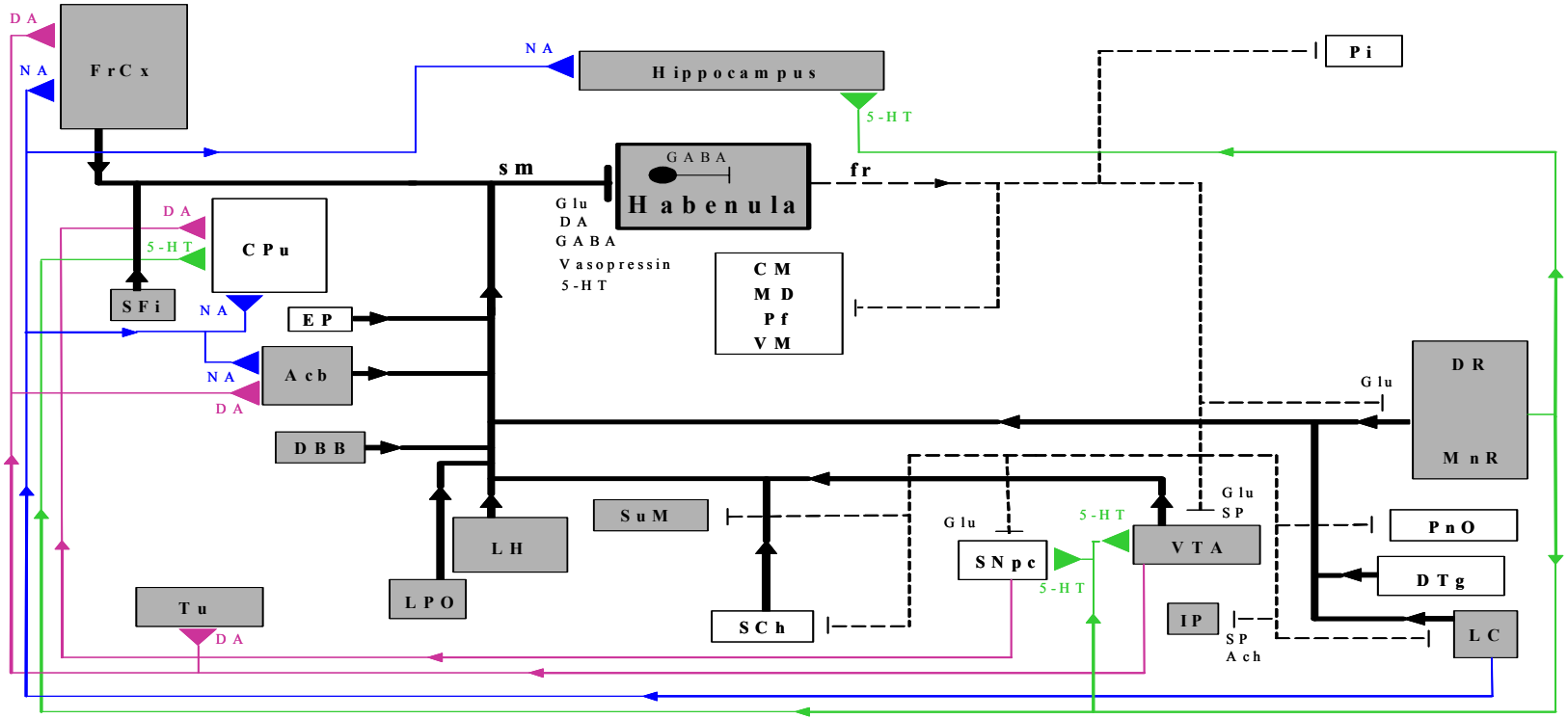
Recently, new genetic targets have emerged, such as neuregulin (NRG1) (Stefansson *et al*, 2002, 2003; Williams *et al*, 2003, Yang *et al*, 2003; Corfas *et al*, 2004), the Ca^{2+}-activated potassium channel, SK3 (Dror *et al*, 1999; Miller *et al*, 2001), but also dysbindin (DTNBP1), G72, D-amino-acid oxidase (DAAO), regulator of G protein-signaling-4 (RGS4), proline dehydrogenase (ProDH) and catecol-O-methyl transferase (COMT) (Tamminga & Holcomb, 2004).

I.2 The habenular complex

The habenular complex, or habenula (Hb), is a limbic structure within the dorsal diencephalon that forms part of the epithalamus. The neuroanatomy of the Hb has been best studied in the rat. It consists of two distinct nuclei, the lateral habenular nucleus (LHb), and the medial habenular nucleus (MHb) (Gurdjian, 1925), the former being composed of 10 sub-nuclei and the latter being composed of 5 sub-nuclei in the rat (Andres *et al*, 1999). The LHb and the MHb are very distinct from both an anatomical and physiological standpoint. Moreover, each nucleus has very distinct efferent and afferent connections and there are few, if any interactions between them (Cuello *et al*, 1978). From a neurochemical point of view, the MHb contains cholinergic (Eckenrode *et al*, 1987) as well as substance P-containing (Hökfelt *et al*, 1975) neurons, whereas the LHb contains almost exclusively glutamatergic neurons (Geisler *et al*, 2003) but also some GABAergic neurons (Contestabile *et al*, 1987). The major afferent pathways of both nuclei are contained in the stria medullaris, while the efferent connections leave the habenular complex through the fasciculus retroflexus (Pellegrino *et al*, 1979). The fibers stemming from the LHb form the periphery of the fasciculus retroflexus while those stemming from the MHb are situated at the core of the fasciculus retroflexus (Herkenham & Nauta, 1979). Those three entities (stria medullaris-Hb-fasciculus retroflexus) form the dorsal diencephalic conduction system (Sutherland, 1982).

An overview of the connections of the habenular complex is shown in **Fig 1.5**.

Fig 1.5. Connections of the habenular complex in the rat and in the cat



Abbreviations: **5-HT**, serotonin; **Acb**, accumbens nucleus; **ACh**, acetylcholine; **CM**, central medial thalamic nucleus; **CPu**, striatum; **DBB**, diagonal band of Broca; **DR**, dorsal raphé nucleus; **DTg**, dorsolateral tegmental nucleus; **EP**, entopeduncular nucleus; **fr**, fasciculus retroflexus; **FrCx**, frontal cortex; **Glu**, glutamate; **IP**, interpeduncular nucleus; **LC**, locus coeruleus; **LH**, lateral hypothalamic area; **LPO**, lateral preoptic area; **MD**, mediodorsal thalamic nucleus; **MnR**, median raphé nucleus; **Pf**, parafascicular thalamic nucleus; **Pi**, pineal gland; **PnO**, pontine reticular formation; **SCh**, suprachiasmatic nucleus; **SFi**, septofimbrialis nucleus; **sm**, stria medullaris; **SN**, substantia nigra pars compacta; **SP**, substance P; **SuM**, supramammillary nucleus; **Tu**, olfactory tubercle; **VM**, ventromedial thalamic nucleus; **VTA**, ventral tegmental area. Black bolded line: habenular afferent connections. Black dashed line: habenular efferent connections. White boxes: extrapyramidal system. Grey boxes: limbic system. Colored lines: *serotonergic*, *dopaminergic* and *noradrenergic* pathways altered by habenular manipulation in the rat or in the cat (stimulation or lesion).

I.2.1 Afferent connections to the habenular complex

In her classical paper, Gurdjian (1925) using multiple histological techniques (brain sections cut in three dimensions, transverse, saggital and horizontal, and prepared with either the Ranson pyridine-silver method, Cajal's silver method or stained with toluidine blue) described the dorsal diencephalon in the albino rat as follows. Firstly, the MHb, adjacent to the ependymal lining of the third ventricle, is divided into a ventromedial part, that shows compactly placed cells which form an irregular triangular mass, and a dorsomedial part, that was comprised of a group of more loosely packed cells. Secondly she described the LHb, which contains smaller, much more scattered cells than those seen in the MHb. Moreover, the Hb was shown to be the structure that receives the fibers included within the stria medullaris. These fibers arise from different regions of the forebrain and form different part of the stria medullaris: some fibers arise in the hippocampus, descend with the fornix columns in the region of the caudal one-third of the anterior commissure, are related to the MHb, and form the medial cortico-habenular tract; some fibers come from the ventromedial portion of the hemisphere, the pyriform lobe and the nucleus of the lateral olfactory tract to form the lateral cortico-habenular tract; fibers arise from the septum (= basal olfactory areas of the medial wall), and join the medial cortico-habenular tract to form the septo-habenular tract; some fibers come from the bed nuclei of the stria terminalis and the anterior commissure, from the caudal aspect of the tuberculum olfactorium and the rostral one-third of the preoptic area, to form the lateral olfacto-habenular tract; some fibers come from the medial portions of the preoptic area and the hypothalamic regions to form the medial olfacto-habenular tract. Later, Nauta (1958) emphasized that the stria medullaris-habenula-fasciculus retroflexus conduction system was one of the systems that serve to convey hippocampal, and also amygdalar, informations, via the septum and the lateral preoptic/hypothalamic area, towards the midbrain.

I.2.1.1 Afferent connections to the medial habenular nucleus

In the rat, the most prominent afferent connection of the MHb comes from two nuclei of the posterior septum, the *n. fimbrialis septi* and the *n. triangularis septi*, the former reaching the MHb at its rostral level, and the latter at its caudal level (Herkenham & Nauta, 1977; Parent *et al*, 1980). Interestingly, these septal nuclei receive their major input from the hippocampal formation (Nauta, 1958; Swanson & Cowan, 1979),

projections that were previously thought to reach the Hb (see above Gurdjian, 1925). Sperlágh *et al* (1998) have shown that this septal projection was the major source of ATP in the Hb that could be released by electrical stimulation. Kawaja *et al* (1990), who showed that this projection reaches the MHb to innervate the dendrites of substance P-containing and cholinergic neurons, have proposed that the septum, by this pathway, could then exert a control upon the habenulo-interpeduncular tract. The MHb also receives a dopaminergic input from the interfascicular nucleus of the ventral tegmental area (Phillipson & Pycock, 1982), and two noradrenergic afferent connections from the superior cervical ganglion and from the locus coeruleus (Gottesfeld, 1983). Two minor inputs have been found to be sent by the median raphé nucleus and by the nucleus of the diagonal band of Broca, the latter being cholinergic and GABAergic (Contestabile & Fonnum, 1983; Gottesfeld & Jacobowitz, 1979; Herkenham & Nauta, 1977). Finally, Sim & Joseph (1991) found a projection from the arcuate (infundibular) nucleus. In the cat, (Parent *et al*, 1980) found that the major inputs comes from the lateral hypothalamic and the lateral preoptic areas. They also found two connections coming from the substantia innominata, the postcommissural septum, the diagonal band of Broca and the entopeduncular nucleus which is the internal portion of the globus pallidus in non-primate mammals. Finally, a connection comes from the bed nucleus of the stria terminalis. Also, (Bobillier *et al*, 1976) found a projection from the median raphé nucleus in the cat. In the squirrel monkey, the projections to the MHb come from the anterior lateral hypothalamus, the internal portion of the globus pallidus, the diagonal band of Broca and the ventral tegmental area (Parent *et al*, 1980).

I.2.1.2 Afferent connections to the lateral habenular nucleus

In the rat, many afferent connections have been found to reach the LHb: GABAergic inputs come from the entopeduncular nucleus, the lateral hypothalamus and the lateral preoptic area (Araki *et al*, 1984; Garland & Mogenson 1983; Gottesfeld *et al*, 1977; Naguy *et al*, 1978; Vincent *et al*, 1982) and a cholinergic input comes from the entopeduncular nucleus (Moriizumi & Hattori, 1992). Some authors have also found afferent fibers that come from the medial frontal cortex (Greatrex & Phillipson, 1982), the ventrolateral septum, the diagonal band of Broca and the nucleus accumbens (Gottesfeld & Jacobowitz, 1979; Herkenham & Nauta, 1977; Mok & Mogenson, 1972). Sofroniew & Weindl (1978) found a projection which contains vasopressin/neurophysin from the suprachiasmatic nucleus to the lateral portion of the LHb, and several studies

have shown a dopaminergic projection from the ventral tegmental area (paranigral and interfascicular nuclei), towards the medial part of the LHb which has been shown to send fibers to the dorsal raphé nucleus and the substantia nigra (Phillipson & Pycock, 1982; Reisine *et al*, 1984; Skagerberg *et al*, 1984). Finally, the LHb receives inputs from the median and dorsal raphé nuclei (Vertes *et al*, 1999) which have also been found in the cat (Bobillier *et al*, 1976)

I.2.2 Efferent connections from the habenular complex

I.2.2.1 Efferent connections from the medial habenular nucleus

In the rat, substance P-containing as well as acetylcholine-containing projections have been found between the MHb and the interpeduncular nucleus (Artymyshyn & Murray, 1985; Contestabile *et al*, 1987; Eckenrode *et al*, 1987; Herkenham & Nauta, 1979; Villani *et al*, 1983). The cholinergic and substance P-containing components of the habenulo-interpeduncular path have different origins. While the cholinergic neurons are located preferentially in the ventral two-third of the MHb, the substance P neurons are located in the dorsal part of the nucleus. There has been divergent opinion on the nature of the habenular source to the IPN: studies using small injections of the retrograde tracer horseradish peroxidase (HRP) into the IPN revealed that the major projection was from the MHb, with a smaller component from the LHb (Contestabile & Flumerfelt, 1981). Based on loss of acetylcholinesterase (AChE) staining after kainic acid lesions of the Hb these authors inferred the existence of a AChE-rich projection from the LHb to IPN (Flumerfelt & Contestabile, 1982). Since AChE is also contained in non-cholinergic neurons this pathway may not be cholinergic. Studies using more selective markers, in fact, strongly indicate that the MHb is the major source of the cholinergic input to the IPN. Thus, using [^{3}H]choline as a neurotransmitter-specific retrograde transport label Villani *et al* (1983) found a high concentration of staining in the MHb after localized injections into the IPN. No label was found in the laterodorsal tegmental nucleus, nor in the triangular nucleus of the septum. Immunochemical studies of ChAT, which is specific for cholinergic neurons show that ChAT-stained neurons are present in the ventral portion of the MHb, whereas substance P-containing neurons were in the dorsal portion (Contestabile *et al*, 1987; Eckenrode *et al*, 1987). In the IPN substance P-staining was in the peripheral subnuclei whereas ChAT staining was in the central core (Contestabile *et al*, 1987). Lesions of the Hb or the fasciculus retroflexus resulted in loss

of both substance P- and ChAT-staining from the IPN, whereas lesions of the stria medullaris caused only a modest loss of ChAT from the IPN (Eckenrode *et al*, 1987). Based on AChE staining Woolf & Butcher (1985), concluded that the main cholinergic afferents to the interpeduncular nucleus comes from the diagonal band of Broca, the magnocellular preoptic area and the laterodorsal tegmental nucleus just as from the medial septal nucleus, substantia innominata, nucleus basalis and pedonculopontine tegmental nucleus in a smaller proportion. The fact that AChE is contained in non-cholinergic neurons may explain why this conclusion differs from the conclusions from studies in which more selective markers were used. Motohashi *et al* (1987) confirmed, by determining the reduction of [^{3}H]hemicholinium binding in the IPN after lesions, that the Hb and the posterior septum are the two main cholinergic projections to the interpeduncular nucleus. In contradiction with many studies, Cuello *et al* (1978) found a substance P pathway to the ventral tegmental area, rather than to the interpeduncular nucleus which, according to them seems to receive only a small number of substance P fibers. They also demonstrated an intra-habenular substance P connection between the MHb and the LHb. Rønnekleiv & Møller (1979) found a link between the MHb and the pineal gland (see also Korf *et al*, 1997), and Rønnekleiv *et al* (1980) found that the stimulation of the MHb produced a response in a type of cells called "silent cells", which are not influenced by light stimulation of the retina. Finally, Neckers *et al* (1979) found a decrease in substance P content in the dorsal raphé nucleus but not the median raphé nucleus after a bilateral lesion of the Hb (52% 24 hours after the lesion and 60% one week after), suggesting that the MHb, which contains the substance P nuclei, provides a substance P innervation to the dorsal raphé nucleus.

I.2.2.2 Efferent connections from the lateral habenular nucleus

One of the strongest efferent connections from the LHb is directed to the raphé nuclei (medial and dorsal), as first shown by Aghajanian & Wang (1977). Fibers have been demonstrated to reach the medial and dorsal raphé nuclei, the strongest connections being to the medial nucleus, as well as projections towards the hypothalamus (lateral, dorsomedial and posterior nuclei), the substantia innominata, the ventral tegmental area, the substantia nigra pars compacta and the oral part of the pontine reticular formation, and to various thalamic nuclei (mediodorsal, central medial, ventromedial), with only a few fibers projecting to the parafascicular nucleus (Araki *et al*, 1988; Herkenham & Nauta, 1979). Interestingly, other investigators did not find any efferent fibers from the

Hb to the lateral hypothalamus (Barone *et al*, 1981). Finally, sparse projections reach the pretectal area, the superior colliculus, the nucleus triangularis tegmenti pontis, the parabrachial nuclei and the locus coeruleus (Herkenham & Nauta, 1979). Phillipson & Griffiths (1985) found a link between the LHb and the nucleus accumbens, and Semba & Fibiger (1992) found a reciprocal link between LHb and the dorsolateral tegmental nucleus. Finally, Kiss *et al* (2002) found a strong projection, probably composed of glutamate or aspartate, between the LHb and the supramammillary nucleus. According to these authors, this pathway could participate in the generation of theta activity in the hippocampus, and in hippocampal activation.

I.2.3 Neurochemistry of the habenular complex

As we have seen above, the habenula receives many inputs, dopaminergic, serotonergic, GABAergic and glutamatergic. In this regard, one can find many receptors for all of these neurotransmitters.

I.2.3.1 Cholinergic receptors

The MHb, fasciculus retroflexus and the interpeduncular nucleus bind epibatidine, an agonist of the cholinergic receptor, with high affinity in the rat suggesting the presence of α4β2 receptors (Perry & Kellar, 1995). These structures also possess a strong immunostaining for the nicotinic receptor subunit β4 in the rat (Duvoisin *et al*, 1989) and mouse (Gahring *et al*, 2004). Moreover, the α3 subunit has been found in the Hb in the rat (Yeh *et al*, 2001), while the MHb lacks, or has very few, β2 subunits (Picciotto *et al*, 1995). Finally, the habenulo-interpeduncular path possesses a large population of α3β4 receptor (Zoli *et al*, 1998). These observations are consistent with findings that the habenulo-interpeduncular system contains α4β2, α3β2 and α3β4 receptors (Perry *et al*, 2002).

I.2.3.2 GABA receptors

In the rat central nervous system, autoradiographic studies have shown that the Hb, and especially the MHb, is one of the brain regions that possesses the largest population of $GABA_B$ receptors (Charles *et al*, 2001; Li *et al*, 2003; Princivalle *et al*, 2000). Moreover $GABA_{B(2)}$ protein mRNA has also been found to be highly expressed in the MHb in the

rat central nervous system (Durkin *et al*, 1999). Interestingly, systemic administration of a mixed GABA agonist, progabide, leads to a 20 % decrease of the glucose utilization in the LHb in the rat (Cudennec *et al*, 1987). Finally, $GABA_A$ receptors have been found on cholinergic neurons of the Hb in the rat (Rodriguez-Pallares *et al*, 2001).

I.2.3.3 Dopamine receptors

Consistent with dopaminergic afferents to the Hb from the ventral tegmental area, there is in the Hb a moderate number of D_1 (Savasta *et al*, 1986) and D_2 receptors (Wamsley *et al*, 1989) in the rat as assessed by autoradiography studies. Immunohistochemical studies have also shown that DARPP-32, a dopamine and adenosine 3′,5′-monophosphate-regulated phosphoprotein, which is present primarily in cells that receive a dopaminergic input and that express the dopamine D_1 subtype receptor and which is not synthesized in dopamine-containing cells, was present in the MHb in the primate (Ouimet *et al*, 1992), in the rat (Schalling *et al*, 1990) and in the mouse (Ouimet *et al*, 1984), while relatively high levels of its mRNA expression was found in the mouse MHb (Perez & Lewis, 1992).

I.2.3.4 Glutamate and ATP receptors

Glutamate AMPA receptors are found in the habenulo-interpeduncular system (Petralia & Wenthold, 1992), as well as mRNA of the kainate receptor which was demonstrated in the MHb (Gall *et al*, 1990). This region also contains a large proportion of the subunit NR_{2B} of the glutamate N-methyl-D-aspartate (NMDA) receptor in the rat (Khan *et al*, 2000). No marked expression of metabotropic glutamate receptors in the habenula has been reported. But glutamate is not the only fast activating neurotransmitter present in the Hb, as adenosine triphosphate (ATP) receptors have been found in the MHb by Edwards *et al* (1992). The function of these receptors is not clear, but their presence seems logical as the MHb receives inputs from the septum that have been shown to contain ATP (Sperlágh *et al*, 1998).

I.2.3.5 Serotonin receptors

Initially mRNA coding for the $5\text{-}HT_{2C}$ receptor was detected in the LHb in the rat, but no binding to this receptor was obtained in that nucleus (Mengod, 1990). A high level of

expression of the 5-HT_{2C} mRNA has been confirmed in the LHb in the rat (Pompeiano *et al*, 1994). 5-HT_2 receptors have also been detected in the MHb in the rat (Morilak *et al*, 1993), and a high level of expression of the 5-HT_4 receptor mRNA has been observed in the MHb in the rat (Ullmer *et al*, 1996; Vilaro *et al*, 1996). Finally, 5-HT_5 receptors have been found in high concentration in the Hb in the mouse (Waeber *et al*, 1998), while there has been found a large amount of 5-HT_{5B} receptor in the Hb in the rat (Grailhe *et al*, 1999; Kinsey *et al*, 2001).

I.2.4 Physiological implications of the position of the habenula within the central nervous system

The main physiological aspect of the Hb is that it represents a crucial link between the forebrain and the midbrain. It is a place of convergence of the extrapyramidal system (striatum, globus pallidus, thalamus, substantia nigra and pedonculopontine nucleus) and the limbic system (frontal cortex, septum, hypothalamus, tegmental ventral area, raphé nuclei and locus coeruleus). In fact, the LHb is the principal actor of this dialogue between forebrain and midbrain nuclei, while the MHb function is mainly to convey informations towards the interpeduncular nucleus. The LHb is the point by which forebrain's limbic and striatal structures modulate their monoaminergic afferents from the midbrain (Garland & Mogenson, 1983; Kalen *et al*, 1989; Nagy *et al*, 1978). Within the LHb, one can differentiate a medial subdivision, which receives afferent connections most exclusively from the limbic system and is called the limbic compartment, and a lateral subdivision, which receives afferent connections most exclusively from the globus pallidus and is called the pallidal compartment. The following sections describe the influences of the habenula on midbrain nuclei.

I.2.4.1 Dopamine

The Hb has a strong influence upon midbrain dopaminergic nuclei. Firstly, the Hb participates in the regulation of the neuronal activity within dopaminergic nuclei, as Christoph *et al* (1986) found after stimulation of the LHb in rats marked inhibition of the dopaminergic neurons in both the ventral tegmental area (84 % of the neurons tested) and the substantia nigra *pars compacta* (85-91 % of the neurons tested). The speculations concerning this pathway gave rise to different points of view, as some authors state that the influence upon the dopaminergic nuclei comes from the LHb

(Christoph *et al*, 1986), while others think that it arises in the MHb through substance P projection (Stinus *et al*, 1978; Nishikawa *et al*, 1986). The fact that Christoph *et al* (1986) recorded neurons showing inhibition followed by excitation after stimulation of the LHb has led to the proposition that the Hb could send inputs to the ventral tegmental area and the substantia nigra *pars compacta* which could be either directly projected onto dopamine neurons, or indirectly projected onto GABA interneurons. As both subnuclei of the Hb (LHb and MHb) have been shown to send projections to the ventral tegmental area, and considering that they have different neurotransmitter systems, we can suppose that they regulate differential effects upon the ventral tegmental area. In fact, this control is reciprocal and there is evidence of a regulation upon habenular activity by the dopamine system, injections of dopamine agonists producing a decreased (McCulloch *et al*, 1980) whereas dopamine antagonists produce an increased metabolism (Ramm *et al*, 1984) in the LHb. This is due to blockade of D_2 rather than D_1 receptors. Also, it is interesting to note that Herve *et al* (1987) found serotonergic terminals in the ventral tegmental area, on dopaminergic and probably also non-dopaminergic neurons. As the Hb is one of the strongest regulators of serotonergic activity, this leads to the possibility that the influence exerted by the Hb upon the ventral tegmental area could also be indirectly mediated by the raphé. Interestingly, Reisine *et al* (1984) found that a dopaminergic input to the LHb, coming from the ventral tegmental area, takes part in the control of serotonin release in the substantia nigra, but not in the striatum of cats. Finally, it has been found in rats and cats that the Hb participates in the control of dopamine release in the medial prefrontal cortex, the nucleus accumbens, the striatum and the olfactory tubercle, the overall effect of complete habenula lesion being to increase dopamine release (Lisoprawski *et al*, 1980; Nishikawa *et al*, 1986). Concerning this latter aspect, it is to be noted that while Lisopravski *et al* (1980) found an effect only on the mesocortical pathway, and not on the meso-accumbens and meso-striatal pathways, Nishikawa *et al* (1986) found also an effect on the meso-accumbens and meso-striatal pathways. These discrepant findings could be explained by the fact that, on the former case, the measurements were performed 6 days after bilateral electrical lesion, whereas on the latter case they were performed about 1 hour after infusion of tetrodotoxin.

I.2.4.2 Serotonin

Stimulation of the Hb has been shown to induce an inhibition of the serotonergic neurons of the raphé nuclei in the rat (Stern *et al*, 1979; Wang & Aghajanian, 1977). In fact, the projection from the Hb to the raphé is excitatory and synapses on GABA interneurons, inducing inhibition of the serotonergic neurons of the raphé, especially the dorsal nucleus, as Speciale *et al* (1980) observed increased serotonergic metabolism in dorsal but not median raphé nucleus 16 hours as well as 1 week after lesion of the Hb (Ferraro *et al*, 1997; Kalen *et al*, 1986; Nishikawa *et al*, 1985; Speciale *et al*, 1980; Zagami *et al*, 1995b). This projection serves to control ascending and descending serotonergic transmission, mainly towards the striatum, the hippocampus and the substantia nigra, as shown in the cat (Reisine *et al*, 1982; Sabatino *et al*, 1991; Soubrie *et al*, 1981), and in the rat (Kalen *et al*, 1989; Zagami *et al*, 1995a). The excitatory pathway from the Hb was originally thought to be made by substance P fibers (Neckers *et al*, 1979), but recently Geisler *et al* (2002) described an input from the LHb to the raphé lacking in substance P and GABA, and composed of glutamate. Others (Kalen *et al*, 1986) also provided evidence for a glutamatergic Hb-raphé projection, as shown by D-[^{3}H]aspartate tracing and loss of high-affinity glutamate uptake in the raphé after Hb lesion. Moreover, Sabatino *et al* (1991) revealed in the cat a synergistic action of the internal pallidum and the LHb upon serotonin release from the median raphé nucleus to the hippocampus, and Ferraro *et al* (1997) found that the habenular influence upon the hippocampus was mainly directed towards the CA_1 field. Zagami *et al* (1995b) found in the rat that when a low-frequency current (1-3 Hz) was applied in LHb, the pyramidal cells of the hippocampus responded in different ways, as some were activated while others were inhibited. Moreover, when stimulation frequency was raised to 5-10 Hz some of the neurons inhibited at low frequency showed activation, and some that were activated at low frequency showed inhibition. However, as for habenular control upon dopamine transmission, the different studies gave rise to contradictory data due to different experimental designs, and the effect of Hb upon serotonergic transmission is still unclear. For example, while increased serotonin release in the striatum has been obtained in rats after stimulation of the LHb (Kalen *et al*, 1989), decreased serotonin release in both the striatum and the substantia nigra has been obtained after stimulation of the LHb in cats (Reisine *et al*, 1982). It is to be noted that in the case of Kalen *et al* (1989) high frequency stimulation (15 Hz) was applied, whereas in the case of Reisine *et al* (1982), low frequency stimulation was applied.

I.2.4.3 Noradrenaline

There is limited information about the influence of the Hb on noradrenergic neurotransmission. In the rat, electrical stimulation of the LHb increases the release of noradrenaline in hippocampus (Kalen *et al*, 1989) and in medial prefrontal cortex, nucleus accumbens and striatum (Cenci *et al*, 1992). In the former study, as this increase was abolished by the transection of the dorsal noradrenaline bundle rostral to the locus coeruleus, we can conclude that the LHb acts directly on the locus coeruleus. This is supported by the fact that this effect was still present after complete lesion of both raphé nuclei, indicating that the action of the LHb upon the noradrenergic system does not require the serotonergic system.

I.2.4.4 Acetylcholine

Apart from the prominent efferent acetylcholine-containing projections between the Hb and the interpeduncular nucleus, the Hb plays further roles in cholinergic signaling. In the rat, Nilsson *et al* (1990) induced a 4-fold increase of the level of acetylcholine release in the hippocampus (dentate gyrus – CA_1 area) after stimulation of the LHb. This increase was totally blocked by the transection of the fasciculus retroflexus, and was reduced by about 95% by a fimbria-fornix lesion. According to them, this could take place through an action upon the diagonal band of Broca or the reticular formation. Interestingly, Girod & Role (2001) have found that low doses of acetylcholine can lead to a long-term facilitation of glutamate transmission in the interpeduncular nucleus, through nicotinic receptors, whereas a short-lasting inhibition is mediated via muscarinic receptors.

I.2.5 Habenula and behaviour

I.2.5.1 Pain

Various receptors involved in pain processes have been found in high concentration in the Hb in the rat. For example, the MHb has been shown to contain a high concentration of μ1 opiate (morphin/enkephaline) receptors (Goodman & Pasternak, 1985). Mezey *et al* (1999) have found in the Hb mRNA expression for vanilloid receptor subtype 1 (VR1) which is postulated to be a molecular integrator of painful stimuli, but a binding study did not show any immunolabeling in the Hb for this receptor. High expression of

the mRNA for a receptor named LC132, which is an orphan opioid-like receptor, has been found in the Hb in rats (Bunzow *et al*, 1994). The heptadecapeptide orphanin FQ has been shown to be an agonist of this receptor, and also to be a functional anti-opiate peptide, as it reverses opiate-mediated stress-induced antinociception (Mogil *et al*, 1996).

The response to painful stimuli, and thus the study of pathways involved in nociception, is generally studied in rodent by means of either the formalin test, which consists on moderate, continuous pain generated by injured tissue after the injection of formalin in a hindpaw of the rodent (Tjolsen *et al*, 1992), or by different types of noxious stimulation: electrical (current shock), mechanical (tail pinch), chemical (intragastric injection of HCl) or heat (hotplate and tail-flick). The involvement of the Hb in pain/analgesia processes has been shown by several authors in rats. Fabian & Ableitner (1995), by means of the 2-deoxyglucose method, found an increased activation in the LHb after the injection of a μ receptor agonist. Benabid & Jeaugey (1989) found that two-third of the neurons of the LHb respond to peripheral noxious stimulation, the Hb being strongly activated by either tail pinch (Smith *et al*, 1997) or intragastric injection of HCl (Michl *et al*, 2001). It has been found that analgesia could be produced during the formalin test either by Hb stimulation (Cohen & Melzack, 1986), or by injecting morphine into the Hb (Cohen & Melzack, 1985). On the other hand, Ma *et al* (1992) found that naloxone injected into the Hb could reverse the analgesic effect of morphine injected into the periaqueductal gray in rabbits. An interesting fact, is that lesion of the Hb had no effect in such a test (Cohen & Melzack, 1993). These authors suggest that the Hb is one area where morphine can produce analgesia, but that is not tonically active in modulating pain or necessary for the analgesic effects of systemically administered morphine (Cohen & Melzack, 1993). Finally, Gao *et al* (1990, 1996) demonstrated that dopaminergic neurons in the substantia nigra pars compacta that are electrophysiologically influenced by tail pinch (either inhibition, 78% or excitation, 15%) are also affected by stimulation or lesions of the LHb and postulate that the LHb is part of a nociceptive pathway involving the substantia nigra.

I.2.5.2 Sexual and maternal behaviours

A role of the Hb in sexual behaviour was demonstrated in rats by Modianos *et al* (1974), who showed that lesions of the Hb and the stria medullaris in females increased

the rate of rejection of males and decreased the rate of copulatory responses, but in males did not affect sexual behaviours. Interestingly, this contrasts with previous results concerning the role of the medial forebrain bundle in the same behaviours (Modianos *et al*, 1973), that indicated that lesions of the medial forebrain bundle affected the sexual behaviour of males, but not females. Thus, it seems that the dorsal diencephalic conduction system, to which the Hb belongs, is involved in female sexual behaviours, whereas the medial forebrain bundle is involved in male sexual behaviours. The reduction of female sexual behaviour after Hb lesion is not due to a disruption of the estrous cycle since lesions of the Hb induced a decrease in the mating behaviour of female rats, without affecting estrous cyclicity and spontaneous ovulation (Rodgers & Schneider, 1979). One factor facilitating sexual behaviour by acting on the Hb is progesterone, as Tennent *et al* (1982) showed that progesterone implants in the Hb, and especially the MHb and the medial portion of the LHb, facilitated sexual behaviours in ovariectomized female rats [*i.e.* receptivity (lordosis) and proceptivity (hopping, darting and ear wiggling)]. The Hb is also involved in a related behaviour, namely maternal behaviour. Matthews-Felton *et al* (1995) showed that a bilateral lesion of the LHb induced in female rats a severe disturbance of postpartum maternal behaviour: it induced a marked decrease of pup-retrieving after parturition, and a marked decrease of pup-cleaning, nursing and nest-building. Moreover, an experiment performed with sensitized female rats to pups showed that a lesion of the LHb induced severe deficits in pup-mediated retrieval and nest-building (Matthews Felton *et al*, 1998). Taken together, these findings show that the Hb, and more specifically the LHb, is essential in the hormonal onset of maternal behaviours and pup-mediated nonhormonal maintenance of maternal behaviours in the rat. Although Wagner *et al* (1998) found estrogen receptors in the LHb, estrogen implants alone in the LHb do not stimulate the onset of maternal behaviour in female rats (Matthews-Felton *et al*, 1999).

I.2.5.3 Sleep

As stated previously, the Hb is linked to regions such as interpeduncular nucleus, raphé nuclei, suprachiasmatic nucleus and pineal gland, that are involved in circadian rhythms and sleep (Aghajanian & Wang, 1977; Artymyshyn & Murray, 1985; Contestabile *et al*, 1987; Herkenham & Nauta, 1979; Rønnekleiv & Møller, 1979; Rønnekleiv *et al*, 1980; Sofroniew & Weindl, 1978). In particular, the link with the serotonergic system would be of particular interest for a possible involvement in the sleep and waking processes, as

serotonin has been shown to be involved in the appearance of sleep, as a permissive factor (Gottesmann, 1999). Moreover, Inagaki *et al* (1988) have found histaminergic fibers in both the LHb and the MHb in the rat, while histamine from the posterior hypothalamus is involved in arousal. A small number of melatonin receptors was found in the medial portion of the LHb in rats (Weaver *et al*, 1989; Williams *et al*, 1995), and Goldstein & Psatta (1984) postulated that the Hb is part of a system involved in the regulation of sleep, in which a peptide arginine-vasotocin plays a key role, including the pineal gland and the dorsal raphé nucleus. In fact, the Hb is involved in the control of the release of a vasotocin-like peptide (Goldstein, 1983, 1985), which is associated with paradoxical sleep (Pavel *et al*, 1979). Interestingly, Haun *et al* (1992) reinstated a substance P-dependent component of sleep (atonia during paradoxical sleep) and also a cholinergic-dependent non paradoxical sleep aspect (sleep duration) by transplanting embryonic habenular cells near the interpeduncular nucleus in rats with a lesion of the fasciculus retroflexus. As the interpeduncular nucleus receives substance P and cholinergic inputs from the MHb (Artymyshyn & Murray, 1985; Contestabile *et al*, 1987; Herkenham & Nauta, 1979) it is highly plausible that the disruption arose because of the interruption of such connections. Moreover, a reduction of the time spent in particular stages of sleep (*i.e.* intermediate stage and rapid-eye movement stage), while slow-wave sleep was spared, was observed following a lesion of the fasciculus retroflexus in rats (Valjakka *et al* 1998). If the influence of the Hb on sleep seems established, this influence may be reciprocal, as increased metabolism within the LHb has been found in rats after deprivation of paradoxical sleep or of total sleep (Landis *et al*, 1993; Peder *et al*, 1986).

I.2.5.4 Anxiety and depression

Lesions of the Hb in rats (Lee & Huang, 1988) or of the fasciculus retroflexus in female rats (Murphy *et al*, 1996) led to an elevated level of anxiety, as assessed by tests such as open-field or elevated plus-maze. Murphy *et al* (1996) found that the animals that were lesioned at 3 days of age were more anxious on the elevated plus-maze, while there was no difference in the open-field compared to the sham operated. On the other hand, rats that were lesioned at 70 days of age did not show more anxiety than controls in the elevated plus-maze, but showed more grooming and more locomotor activity in the open-field; moreover, both groups showed the same pattern of anxiety after an additional stress (24 h food deprivation and 5 days of social isolation). Thornton &

Davies (1991) using specific behavioural testing (a tank filled with water and separated into two different compartments, whose surrounding panels could be changed, and in which an escape platform could be placed and removed) showed that the Hb-lesioned animals were impaired in the acquisition of such a test, and that the Hb could be involved in the ability of animals to switch their response strategies under stress. Certainly stress affects the Hb. Stress-induced Fos-like immunoreactivity (FLI) has been found in the medial portion of the LHb in rats (Chastrette *et al*, 1991; Wirtshafter *et al*, 1994). Amat *et al* (2001) found, using inescapable and escapable shocks in the shuttle box on rats, that the lesion of the Hb suppressed in both cases the rise of serotonin that normally occurs in the dorsal raphé nucleus during a stressful situation. These authors postulate that the Hb is necessary for the stress-induced increase of the level of serotonin and that it plays a role in the induction of learned helplessness/behavioural depression. Finally, Shumak *et al* (2003) have found a marked elevation of the metabolism in the Hb (LHb + MHb) as well as within the interpeduncular nucleus in congenitally helpless rats, while there was a reduced metabolism in the ventral tegmental area, in the basal ganglia and in the basolateral and the central nuclei of the amygdala.

I.2.5.5 Reward

Sutherland & Nakajima (1981) showed that rats engaged in self-stimulation of the Hb, indicating that electrical stimulation of the Hb can induce a rewarding effect. They also showed that it requires the involvement of the median raphé nucleus. Thus the dorsal diencephalic conduction system represents a rewarding system, in addition to the medial forebrain bundle. In fact, stimulation of the medial forebrain bundle in rats leads to an increased metabolism in the LHb (Bielajew, 1991; Hunt & McGregor, 1998; Konkle *et al*, 1999). Ullsperger & von Cramon (2003), used magnetic resonance imaging (MRI) in humans during a task of error monitoring where they had to predict the appearance of an event, after which a rewarding (in case of correct answer = positive feedback) or a non-rewarding (in case of incorrect answer = negative feedback) stimulus occurred. During this task, the Hb was selectively activated in the case of the negative feedback, while the nucleus accumbens was activated during the positive feedback. This is in agreement with the view that positive feedback involves dopamine release in the nucleus accumbens, while negative feedback does not, and with the fact that the Hb has been

shown to induce the inhibition of dopaminergic neurons within midbrain areas (Christoph *et al*, 1986). This is also in accordance with the results obtained by Park & Carr (1998) who observed a decreased metabolism in both the MHb and the LHb following the ingestion of palatable food in rats, and with the results of Smith *et al* (2002) who showed that palatable food regimen-induced obesity in rats led to an increased μ-receptor expression (40%) in the MHb in the high-weight gain group of animals. According to these authors, this phenomenon could reflect a decreased β-endorphin production, in an attempt to reduce the effects of palatable food and counteract weight gain. Finally, ethanol consumption has been shown to produce a decreased metabolic activity in the Hb in rats with a history of ethanol consumption (Williams-Hemby *et al*, 1996).

The above discoveries suggest that the Hb is affected by rewards, more than it influences reward-mediated behaviors. Consistent with such a view are findings that chronic stimulation with certain drugs of abuse exerts toxic effects on the Hb. For example, chronic administration of cocaine induces a marked selective degeneration of the LHb and fasciculus retroflexus (Ellison, 1992; Ellison & Switzer, 1993) and results in a decrease of the number of GABA immunolabeled terminals within the LHb (Meshul *et al*, 1998). According to the results obtained by Meshul *et al* (1998), the degeneration seems to arise from a decreased inhibition. Finally, chronic administration of nicotine also results in degeneration of both the MHb, which contains cholinergic neurons, and the fasciculus retroflexus, which conveys the cholinergic tract towards the interpeduncular nucleus (Carlson *et al*, 2001).

I.2.5.6 Cognition

1.2.5.6.1 Learning and memory

Using a swim test with or without escape (a rope suspended vertically above the tank), Thornton & Evans (1982) showed that Hb-lesioned animals were impaired in escaping from the tank. These results reveal a deficit of attention and an inability to use new environmental elements in order to switch their strategy towards a more appropriate one. On the other hand, Vale-Martinez *et al* (1997) found no Hb lesion-induced differences in the acquisition of a two-way active avoidance test. Thornton *et al* (1990) found that rats lesioned with 6-hydroxydopamine in the Hb were impaired in the

acquisition of differential reinforcement of low rate of responding (DRL) operant behaviour, as they showed overesponsiveness and obtained less reinforcement. In general, and especially when we consider the experiments performed by Thornton *et al* (1990, 1994), it seems that Hb-lesioned animals show impairments during cognitive tests performed under stressful situations, and exhibit a lack of behavioural flexibility and plasticity. This is confirmed by an experiment by Thornton & Bradbury (1989), who found impaired acquisition on a one-way active avoidance test in Hb-lesioned rats only with a high level of stress. Finally, Tronel & Sara (2002) found an increased metabolism in the LHb of rats during the retrieval of an olfactory memory, and Villarreal *et al* (2002) found a decreased metabolism in the LHb of aged memory impaired rats in the Morris water-maze compared to young unimpaired rats. The results we obtained by studying the effects of Hb lesion on spatial memory, by means of the Morris water-maze, are presented in **Chapter II**.

I.2.5.6.2 Attention

As we have seen in the previous paragraph, Thornton & Evans (1982) postulated that lesion of the Hb could lead to deficits of attention. This suggestion has not been previously investigated with a well-accepted test of attention. The Hb certainly can influence brain regions involved in attention. For this reason, the effects of Hb lesion on attention were studied (see **Chapter IV**).

I.3 Introduction to the study

As seen in the above paragraphs, schizophrenia is one of the most common and devastating psychiatric diseases, with a prevalence of 0.5 – 1 % in most societies and a well-known symptomatology (Gottesman, 1991). However, despite numerous findings from experimental research, coming for example from physiological and imaging studies, it has not yet been possible to clearly discover its exact origins. This failure is reflected in the fact that since the introduction of the neuroleptics in the 1950s', several generations of treatments succeeded each other without being really able to cure or treat all the symptoms. Discovering more effective compounds requires several levels of investigation. Fundamental research is one of the most critical steps through this process because it may give the first clear indication about the pathology and aetiology of the disease. Experimental methodologies in preclinical research are numerous, ranging from animal behaviour after lesion or drug injection to imaging techniques and the elaboration of transgenic animals. One of the crucial points in the utilization of animal behaviour is the elaboration of a good animal model of the pathology studied, in order to have an adequate substrate on which the effect of potential new treatments can be tested. In the case of schizophrenia the elaboration of such model is particularly challenging when we consider the pathophysiology of schizophrenia, which is characterized by numerous symptoms sometimes almost diametrically opposed, as for example "positive" and "negative" symptoms (Kay *et al*, 1987). Despite this fact, undeniable progress has been made, and several animal models have been elaborated, either pharmacological such as those based on phencyclidine treatment (see Sams-Dodd, 1996), or based on lesions during development, such as the neonatal ventral hippocampal lesion model (see Lipska, 2000) (**Table 1.1**). However, some structures that could be involved in schizophrenia have not received the attention that they should have had. This is the case for the epithalamus, composed of the pineal gland and the habenular complex, whose dysfunction could generate, according to several authors, some of the symptoms of schizophrenia [see reviews by Ellison (1994), Sandyk (1991) and Sutherland (1982)].

Table 1.1 Different models of schizophrenia in rodents

Pharmacological models	Species	Behavioural deficits	Anatomical correlates	Reference
Glutamate NMDA receptor antagonist administration (PCP, ketamine, MK-801) - *neonatal* - *withdrawal* - *acute*	Rat	Working memory. Social interactions. Stereotypies, Prepulse inhibition, Latent inhibition, Hyperactivity.	Reduced prefrontal cortical dopamine release.	Bakshi *et al*, 1999; Becker *et al*, 2003; Kilts, 2001; Moghaddam & Jackson, 2003; Sams-Dodd, 1996.
Acute and chronic dopamine releaser (amphetamine) and receptor agonist (apomorphine) administration	Rat	Hyperactivity, Prepulse inhibition, Latent inhibition.		Sills, 1999; Tenn *et al*, 2003.
5-HT_2 agonists administration	Rat	Latent inhibition, Prepulse inhibition.		Geyer, 1998; Hitchcock *et al*, 1997.
Genetic models				
NMDA NR_1 subunit KO	Rat	Sexual and social interactions, Hyperactivity, Stereotypies.		Mohn *et al*, 1999.
Dopamine transporter (DAT) reduced expression	Mouse	Hyperactivity, Impaired response habituation in novel environment.		Zhuang *et al*, 2001.
Other models				
Maternal deprivation	Mouse	Prepulse inhibition.		Ellenbroek *et al*, 2004.
Neonatal excitotoxic ventral hippocampal lesion	Rat	Increased response to stress and to NMDA antagonists and dopamine agonists, Prepulse inhibition, Diminished sensitivity to rewarding stimuli, Social interactions, Working memory.	Reduced cortical N-acetylaspartate and glycogen synthase kinase-3ß; Reduced cortical glutamate transporter EAAC1; Reduced BDNF expression; Reduced GAD-67 expression.	Lipska, 2004.
Maternal influenza	Mouse	Increased anxiety, Spatial reference memory (Morris water-maze), Prepulse inhibition, Exploratory behaviour, Social interactions.	Loss of gene expression (RGS4 and calcium/calmodulin-dependent protein kinase IIα); Neurodegeneration in medial habenula and paraventricular thalamic nucleus.	Beraki *et al*, 2004; Mori *et al*, 1999; Shi *et al*, 2003.

I.3.1 A new hypothesis

Recently, Kelly (1998), linking several facts concerning the epithalamus, comprised of the habenular complex and the pineal gland, suggested that its dysfunction, and particularly that of the habenular complex, could lead to the appearance of primary delusions in schizophrenics. The principal arguments are the following:

- The events (dream-events) that occur in the dreams that we experience each night are not intended to enter long-term memory. In fact, the period of dreaming is around 90 minutes per night during paradoxical sleep (or rapid-eye movement sleep, REM-sleep), and if we sometimes remember about a dream, it is because we woke up during or just after it. According to this view dream-events are not meant to be remembered, and certain mechanisms are involved in preventing long-term memories of them from being made.

- The presence of vasotocin, or a molecule with vasotocin-like bioactivity, has been detected during paradoxical sleep in man. The experiment that demonstrated this was conducted by Pavel *et al* (1979), and consisted in sampling cerebrospinal fluid of people awakened during different stages of sleep. Only when the subjects were awakened during REM sleep or immediately after a REM sleep period was this activity detected.

- Vasotocin is reported to inhibit memory formation. De Wied *et al* (1991), using the passive avoidance paradigm in rats, have shown that, when applied intracerebroventricularly, low doses of vasotocin (0.03-0.3 ng) disrupted memory formation, like oxytocin, whereas at a high dose (10 ng) it lost this action, and even facilitated memory formation in the same way as vasopressin. Thus if the REM sleep-related release of vasotocin-like compound were too high or too low, then the inhibition of dream-event memory formation would fail.

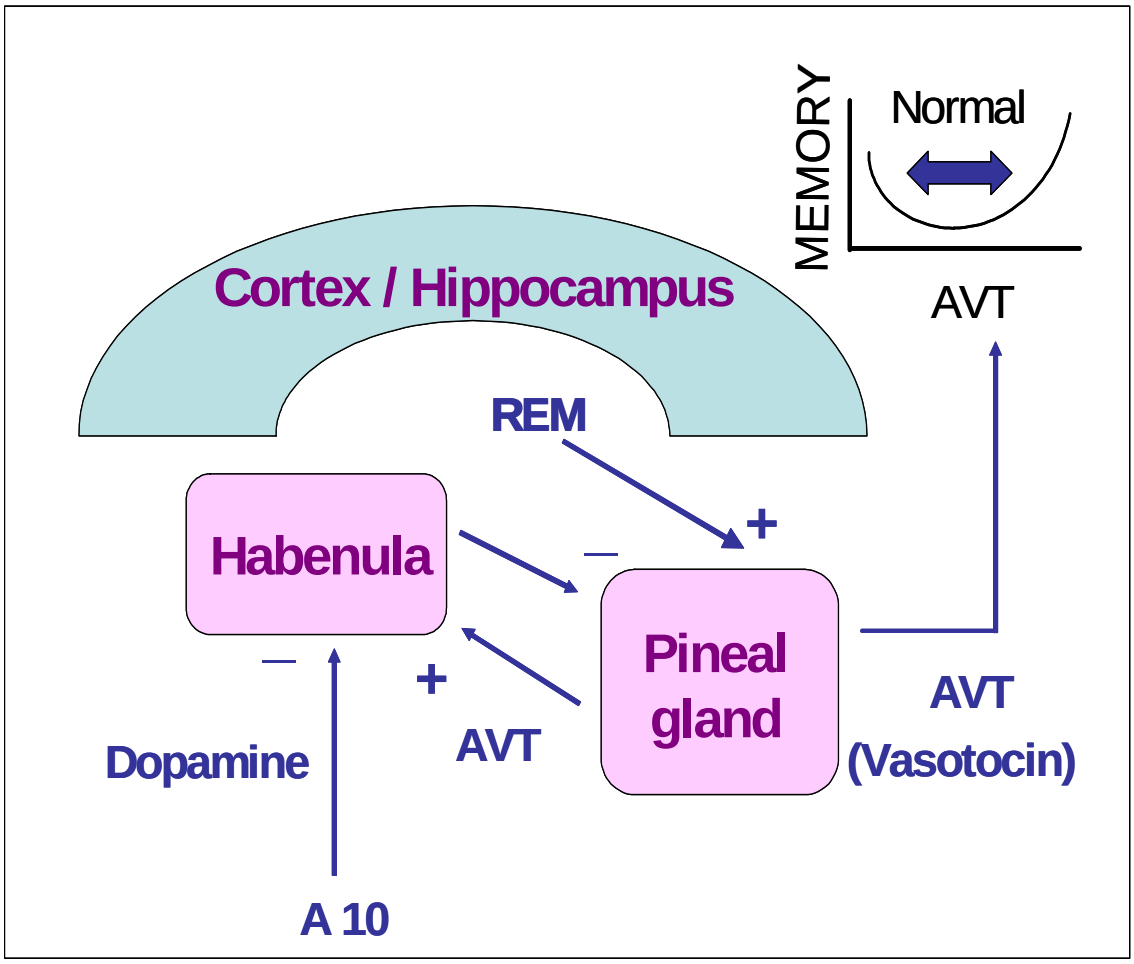
- The vasotocin-like bioactivity is believed to be released from ependymal cells of a pineal region under the influence of the habenula, and both of these regions are reported to be excessively calcified in patients with schizophrenia. Several studies indicate that vasotocin bioactivity is released from ependymal cells of a region that includes the pineal organ, the pineal stalk and the subcommissural

organ (Benson *et al*, 1976; Pavel, 1971; Pavel *et al*, 1977). Moreover after lesions of the habenula the concentration of vasotocin bioactivity in the cerebrospinal fluid increased (Goldstein, 1985). In this regard it is interesting that imaging studies indicate a possible link between habenular and pineal pathology and schizophrenia. For example in computer tomography (CT) studies, Sandyk (1992) reported greater calcification of these regions in schizophrenics. Caputo *et al* (1998) also in a CT study confirmed that there was a larger calcification of the epithalamus as a whole in schizophrenics, and also found that this calcification was correlated to other encephalic abnormalities, at the level of the cerebral cortex and the ventricles.

- As predicted if delusions arise from dream memories, there are marked similarities between the contents of dreams and the contents of delusions. Thus *threat to the person* or *persecution* is said to be the most common schizophrenic delusion (Sims, 1988) and is also frequent in dreams.. For example, in a sample of college students, the most common theme of recurrent dreams was of being threatened or pursued (Robbins & Houshi, 1983). Moreover, the occurrence of this theme is increased by stress, as shown by the fact that 86% of a sample of women undergoing a stressful event, divorce, reported in a single night at least one dream involving threat (Trenholme *et al*, 1984). Also, the *delusional misidentification syndromes*, which share the common theme that persons have been replaced by others or can change into others are common in schizophrenia (Ellis *et al*, 1994; Odom-White *et al*, 1995), corresponding to the fact that metamorphosis of one person into another occurs in about 1% of dreams (Domhoff, 1996; Hall *&* Van de Castle, 1966). Finally, the *delusion of being younger than one is*, a phenomenon that has been called "age disorientation". Crow (1990) has reviewed a number of studies indicating that this delusion is fairly common among hospitalised or chronic schizophrenics. At least qualitatively, it is possible that this age delusion stems from memories of dream events, since dreams of the past are quite common (Domhoff, 1996; Hall *&* Van de Castle, 1966). In one particularly long series of dream reports the proportion of dreams exhibiting regression was fairly constant at 15-40% over several decades with these dreams showing on average 8-21 years of regression (Smith & Hall, 1964).

Through these observations, Kelly (1998) developed an hypothesis devoted to explaining the occurrence of primary delusions in schizophrenics: following the dysfunction of epithalamic structures responsible for the control of its release, a molecule similar to vasotocin could be released in a too high or too low amount during REM-sleep, from the pineal body into the cerebrospinal fluid, leading to the placement of dream contents in the usual memory store. Moreover, this inclusion of dream contents would lead to the long-term strengthening of erroneous neuronal connections, probably at cortical level, leading to the mixing of these dream contents and the 'normal' memories, such that the schizophrenics would then not be able, during the waking state, to differentiate between dreamed facts and reality (**Fig 1.6**).

Fig 1.6. The dream-events hypothesis



I.3.2 The goals of the study

The above mentioned data concerning the epithalamus led us suppose that it could be involved in the disturbances that occur in schizophrenia. The goal of the present studies was to examine a prediction of the hypothesis that epithalamus dysfunction is involved in causing the symptoms of schizophrenia. The prediction is that lesions of epithalamus components should produce some behavioural deficits resembling those encountered in schizophrenia, a prediction which has the advantage of being accessible to testing by behavioural experiments.

To this purpose bilateral electrolytic lesions of the habenula were made in adult rats, who were then subjects in different experiments devoted to exploring the behavioural features, and also some neurochemical features, relevant to schizophrenia.

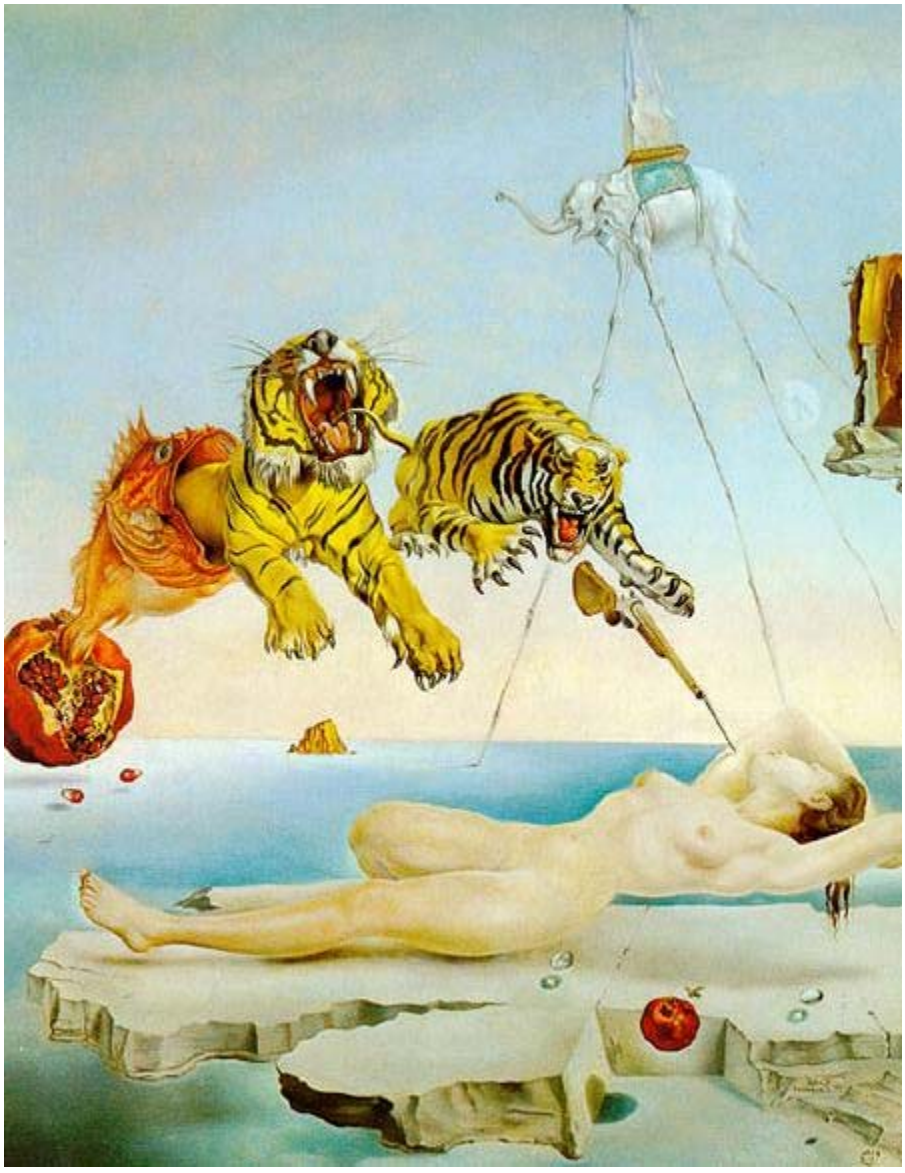
- **First**, after selective bilateral lesions of the habenula, or control lesions, rats performed a series of experiments that were destined to give a broad view of the behavioural effects of the lesion, focusing on tests generally employed in rodent to parallel deficits observed in schizophrenic patients. The prepulse inhibition test and the social interaction test were used: the former has an analogous version used in human studies and is devoted to analyzing sensory gating, which is disturbed in schizophrenia; the latter is a simple way to see if a lesion produces social isolation, which is among the negative symptoms of schizophrenia. Spatial learning in the Morris maze was used because memory impairment is now widely recognised as a feature of schizophrenia.

- **Second**, we performed the exact same series of experiments, but examining animals with either a lesion of the pineal gland, or with a complete lesion of the epithalamus (habenula + pineal gland), in order to investigate whether pineal damage contributes to behavioural deficits, since imaging studies and studies of melatonin levels strongly suggest dysfunction of the pineal component of the epithalamus in schizophrenia.

- **Third**, we performed a test of attention, the 5-choice serial reaction time task, in order to explore this other aspect of cognition that is impaired in schizophrenia.

- **Fourth**, to investigate in a direct way alterations in forebrain functioning after habenula lesions, regional cerebral blood flow was examined with magnetic resonance imaging (MRI) techniques.

- **Fifth**, we assessed if the lesion of the habenula would induce changes in certain populations of receptors which are postulated to be involved in schizophrenia, *i.e.* the serotonergic 5-HT_{1A} and 5-HT_{2A}, the dopaminergic D_1 and D_2, the cholinergic nicotinic alpha-7 and the glutamatergic NMDA, in structures that have been postulated in man to be affected by the schizophrenia syndrome (*e.g.* frontal cortex, striatum, basal ganglia and temporal lobe). To this purpose, we used the autoradiographic technique.



"One second before awakening from a dream caused by the flight of a bee around a pomegranate"

Dali – 1944

Chapter II - Effects of habenula lesion on social interactions, sensory gating and spatial memory

II.1 Introduction

The habenula, receiving inputs from forebrain structures and exerting influences on certain midbrain cell groups, is an evolutionarily conserved link between forebrain and midbrain (Sutherland, 1982). It is implicated in functions as diverse as maternal behaviour (Corodimas *et al*, 1993; Felton *et al*, 1998), pain (Cohen & Melzack, 1993), sleep (Haun *et al*, 1992; Valjakka *et al*, 1998), anxiety (Murphy *et al*, 1996), reward (Sutherland & Nakajima, 1981) and behavioural flexibility (Thornton & Evans, 1982), but its exact roles in these functions are not completely understood. One of its peculiarities is that it is the brain region most susceptible to damage by drugs of abuse, the lateral region by stimulants and the medial nucleus by nicotine (Carlson *et al*, 2000, 2001; Ellison, 1994, 2002).

In recent years several findings have suggested that pathology of the epithalamus, which is comprised of the habenula and the pineal organ, could be involved in the pathophysiology of schizophrenia. For example Sandyk (1992) reported that large calcifications of the pineal and habenula are more common in schizophrenics than normal controls. In a study where calcification of the epithalamus as a whole was evaluated Caputo *et al* (1998) also found a larger area of epithalamus calcification in schizophrenics compared to control subjects. That this may be mainly due to increased calcification of habenula, rather than of the pineal, is suggested by observations of no difference in size of pineal calcification in schizophrenics (Bersani *et al*, 1999). Based on several findings, particularly the fact that chronic treatment with cocaine or amphetamine severely damages the fasciculus retroflexus output pathway of the habenula in rats and results in a schizophrenia-like state in man, Ellison (1994) has proposed a role of habenula pathology in schizophrenia. Interestingly, drug abuse has been identified as a risk factor for schizophrenia (Kelly & Murray, 2000). Several mechanisms of how habenula dysfunction could result in the symptoms of schizophrenia have been proposed (Ellison, 1994; Kelly, 1998 Sandyk, 1991).

Despite these findings, there is a paucity of studies of the habenula in behaviours related to schizophrenia. If damage to the habenula is indeed involved in schizophrenia then lesions of the habenula are predicted to result in schizophrenia-like symptoms in experimental animals. This prediction is investigated in the studies presented here. We have made localized lesions of the habenula in rats, and examined their effects on several behaviours. As an important control for the possibility that the changes observed might be due to incidental damage to the overlying dorsal hippocampus, a control group with restricted lesions of this structure was included. The behaviours examined were social interaction, prepulse inhibition (PPI) and memory function since considerable evidence indicates that these functions are disturbed in schizophrenia (Braff *et al*, 1978, 1992, 1999; Goldberg & Schmidt, 2001; Lieberman *et al*, 2001; McKenna *et al*, 1990; Meltzer & McGurk, 1999; Pilkonis, *et al*, 1980; Pillman *et al*, 2003).

II.2 Materials and methods

II.2.1 Experimental series

Two series of experiments were performed. In the *first series* sham-operated and habenula-lesioned animals were compared. The social interaction test was performed 11-15 days, after lesioning, the PPI test 22-26 days post-lesion and the Morris maze starting on post-lesion day 37-41. This order of testing was chosen so that the tests judged to be more stressful for the animals were performed later. In animals of this series lesions were verified histologically, but not neurochemically. In the *second series* of experiments a hippocampal-lesioned group with restricted damage to the dorsal hippocampus lying above the habenula was included to address the question that the changes observed in habenula-lesioned animals might have been due to incidental damage to the hippocampus. Additionally, biochemical assay of choline acetyltransferase (ChAT) in homogenates of the interpeduncular nucleus (IPN) was performed to provide a biochemical index of damage to the habenulo-interpeduncular tract in addition to histological verification of lesions. In this series the social interaction test was performed 19-26 days, after lesioning, the PPI test 33-50 days post-lesion and the Morris maze starting on post-lesion day 39-56.

II.2.2 Animals

The experiments were carried out on male Sprague-Dawley rats (Iffa Credo, France) of body weight 250-300 g at the time of surgery. The animals were housed in individual cages (Macrolon, 42 × 26 × 15 cm) in a temperature-regulated (22 ± 2°C) animal room on a 12 h/12 h light/dark cycle (lights on at 06:00), with laboratory rat chow (Nafag AG, Switzerland) and water available *ad libitum*. The operations and behavioural tests were performed during the light period, at least one week after their arrival. All testing procedures were in accordance with the Swiss animal protection law for the care and use of animals and were approved by the Cantonal Veterinary Authority of the City of Basel.

II.2.3 Surgical procedures

Animals were anaesthetized with sodium pentobarbital (60 mg/kg, *i.p.*) and placed in a stereotaxic frame with the tooth bar 5 mm above the ear bars to correspond to the stereotaxic atlas of Pellegrino *et al* (1979). Bilateral electrolytic lesion of the habenula nuclei (lateral plus medial) was performed with stainless steel electrodes (00 gauge insect pins, Emil Arlt, Vienna, Austria, insulated except at the tip) by passage of a DC current of 1 mA for 15 s through the anode in the brain and a saline-soaked cotton swab on the tail as cathode. Constant current was provided by a Heinzinger LNG 350-03 power supply through a 100 kΩ series resistor, and was monitored by a current meter. The electrodes were inserted at a 10° angle in order to avoid the saggital sinus. Thus to position the electrode tip at the point AP -2.2 mm, ML 0.6 mm, 4.8 mm below dura (Pellegrino *et al*, 1979) the "lateral" displacement of the electrode carrier (still at an angle of 10° to the vertical) after positioning it at the midpoint of the saggital sinus at the desired AP coordinate was 1.5 mm, and the depth displacement of the electrode was 4.9 mm along the 10° angle track. For the sham-operated animals, the electrode was inserted at the same coordinates for the same amount of time, but no current was passed. The coordinates for the small hippocampal lesions were as for the habenula lesions, except that the electrode was inserted only 4.0 mm below the surface of the brain, where a current of 0.5 mA for 5 seconds was delivered. After the animals awoke, they were injected (1 ml/kg *s.c.*) with an analgesic (0.3 mg/ml Temgesic®, Essex Chemie AG, Luzern) and returned to their home cage where they were allowed to recover for at least 11 days.

II.2.4 Behavioural procedures

II.2.4.1 Social interaction test

This test was adapted from the social memory test of Thor & Holloway (1982) and the social interaction test of File (1980). As an index of social interaction the amount of time the test animal spent investigating a novel juvenile male of the same strain was quantified. The test rat was placed with the juvenile conspecific weighing 100 – 130 g in a Macrolon cage (42 × 26 × 15 cm), whose floor was covered in bedding to a depth of 1-2 cm, for a 5-min test. A different juvenile was used for each test rat, and before testing the first animal, two naïve animals were allowed to explore the cage for 5 min so

that the bedding was already tainted by rat odours. Any investigation by sniffing directly at any point of the juvenile's body was recorded and cumulated to obtain the total investigation time.

II.2.4.2 Prepulse inhibition of the acoustic startle response

Startle responses were measured with a commercially available startle system (Coulbourn Instruments, Allentown, PA, USA), modified such that the acoustic stimuli were presented to the animals via a single Visaton wide range tweeter (type DHT 9 AW-NG) in the center of the roof of a ventilated, sound-attenuated test chamber. Responses were recorded with a quartz force sensor for measuring dynamic and quasistatic forces (Type 9203, Kistler Instruments AG, Winterthur, Switzerland) mounted directly below the animal enclosure, consisting of a plastic box covered with a metal grid, (16 x 8 x 8 cm). This sensor was connected to a charge amplifier (Kistler, type 5011). The output signal of the charge amplifier was digitized (sample rate 1 kHz for 200 msec, 8 bit) and stored on a microcomputer.

The animals were placed in the startle apparatus one animal in each corner, each in an animal enclosure. From session to session, treatment groups were assigned to different startle-sensors (clockwise rotated), in order to rule out artifacts related to sensor and/or session differences.

Once in the startle apparatus, an adaptation period of three minutes was given, before delivery of various auditory stimuli. After this period rats were exposed to three startle-eliciting stimuli. These first stimuli were not included in the analysis, but presented in order to achieve a more stable level of startle reactivity for the remainder of the session. Following these first stimuli, subjects were exposed to pulse alone stimuli (PA: 105 dB, 40 msec) and to prepulse-pulse stimuli (PPP). The prepulse had a duration of 20 msec and was initiated 100 msec prior to the startle-eliciting pulse. Sound pressure levels of the prepulses were +8, +12 or +16 dB above the continuous background noise of 62 dB. Stimuli were presented in 3 blocks (PA1, PA2 and PA3), which each contained 10 PA-stimuli. In addition the second block contained PPP-stimuli (ten of each amplitude) to monitor PPI (prepulse inhibition). Within one block, stimuli were presented in a

randomized order and with a randomized interval between 9 and 21 sec. The whole session lasted 20 minutes.

Startle amplitudes (g) recorded for each animal were averaged over the ten stimuli of the same type, within one block. Prepulse inhibition was computed according the formula

$$\% \text{ PPI} = 100 \times (\text{Response}_{PA} - \text{Response}_{PPP}) / \text{Response}_{PA},$$

where Response_{PA} is the response to the PA-stimuli in the block PA2 which included the PPP stimuli, and Response_{PPP} is the response to PPP stimuli in the same block.

Immediately after the startle session was completed, animals were transported to an adjacent room and placed singly in activity monitors for a period of 60 min for the measurement of locomotor activity. The activity monitors consisted of cages (53 x 33 x 19 cm) in enclosures (60 x 40 x 50 cm) that each had a black-and-white video camera mounted centrally above the cage. Each second a single video frame was acquired with a monochrome frame grabber board (Data Translation Inc., Marlboro, MA; type DT3155). Using in-house developed software, digitized pixels of two successive frames were compared and the total number of pixels with altered intensity was counted (independently for pixels with increased and decreased intensity). This allowed the detection of the animal's position within the cage (the centre of pixels with increased intensity, because animals were light compared with background). Distance traveled (distance in cm between centers of activity when movement was > 10 % body size) was analyzed and stored every 5 min.

II.2.4.3 Morris water maze

The Morris water maze consisted of a circular black-plastic pool, 133 cm in diameter and 60 cm in depth, filled with water at room temperature (22 ± 1°C), standing on a 40 cm high pedestal. A black-plastic circular escape platform (11 cm in diameter, covered with wire mesh) was submerged 2 cm below the surface of the water and located at the centre of one of the four quadrants. The movements of the rats were followed by a

computer-based video tracking system (VP200 advanced tracker, Water for Windows software, HVS Image, Hampton, UK).

On the first day, the animals were given a practice swim, during which they were put in the pool without any platform, and allowed to swim for 60 seconds.

Starting on the following day, the acquisition phase took place over five consecutive days during which the hidden escape platform remained in the same quadrant (south-east; SE). On each test day the animals received a block of 4 trials: they were placed once at each starting position (north (N), south (S), east (E) and west (W) in a different randomized order each day), facing the wall of the pool, and were allowed 90 s to find the platform and climb onto it. After they successfully found the platform, they were allowed to stay on it for 30 s before the start of the next trial. In case of not finding the platform within 90 sec they were placed on it and left for 30 s before the start of the next trial. The time to reach the platform and the distance covered were recorded for every trial. On the sixth day the animals were given a retention test by administering a probe trial during which the platform was removed from the pool: the animals were placed in the pool, facing the wall at one of the four starting positions (which were randomized from rat to rat) and allowed to swim for 90 s during which the times spent in each of the quadrants were recorded [north-west (NW), south-west (SW), south-east (SE) or north-east (NE)].

On days 8-10 (experiment of *first series*) or day 8-9 (experiment of *second series*), to evaluate the performance of the animals in a visible platform condition, we administered on each day a four-trials session in which the position of the submerged platform was indicated by a white ball (3.7 cm in diameter), attached to it by a black wire, and positioned approximately 20 cm above the platform. The ball was capped by a piece of black plastic so that it was not tracked by the video system. On each of the four consecutive trials the starting position (N, S, E or W) and the platform position (NW, SW, NW or center) were randomly changed for each trial, and the distance swum and latency to find the platform were measured.

II.2.5 Histology

Animals were sacrificed by decapitation, the interpeduncular nucleus quickly dissected out for assay of choline acetyltransferase, and the remaining brain was freeze-sectioned in a cryostat. Twenty five-micron slices were taken through the entire habenula, mounted on slides and stained with Toluidine blue. Lesions of the habenula were considered acceptable when surrounding regions (*i.e.* dorsal hippocampus and paraventricular, dorsomedial, lateral and parafascicular thalamic nuclei) were spared.

II.2.6 Assay of choline acetyltransferase (ChAT)

In order to have a neurochemical index of the lesion, the choline acetyltransferase (ChAT) activity of the interpeduncular nucleus (IPN) was assayed by a slight modification (Kelly & Moore, 1978) of the method of Fonnum (1975), using [^{14}C]acetyl coenzyme A (Amersham, U.K.) as acetyl donor. This activity provides an index of the lesion of the medial habenula, which contributes the majority of the cholinergic innervation of the IPN (Contestabile *et al*, 1987; Eckenrode *et al*, 1987; Villani *et al*, 1983).

The IPN was dissected out and homogenized in ice-cold 10 mM EDTA, pH=7.4, containing 0.5 % Triton X-100. A final dilution of 1:400 was made from which 5 µl were added to 10 µl of an incubation mix and the assay performed as previously described (Kelly & Moore, 1978).

II.2.7 Statistics

The statistical significance of differences between treatment groups were analyzed by analysis of variance (ANOVA), followed by pairwise comparisons (Dunnett's test, two-tailed), using the SYSTAT software package (Version 10.2, SPSS Inc., Chicago, IL).

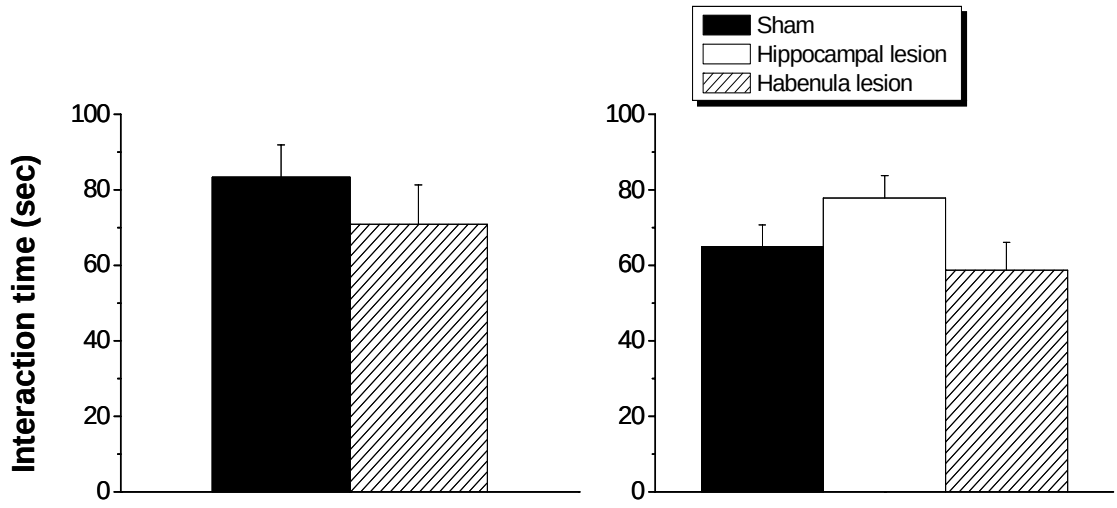
II.3 Results

After exclusion of animals with unsatisfactory lesions, there were in the *first series* of experiments 12 sham-operated animals and 8 habenula-lesioned animals, and in the *second series* 16 sham-operated animals, 16 animals with small dorsal hippocampus lesions and 10 habenula-lesioned animals.

II.3.1 Social interaction test

The results are shown in **Fig 2.1**. In the experiment from the *first series*, one-way ANOVA showed no significant effect of lesion group ($F_{2,29} = 0.52$, $p > 0.1$) on social interaction time. Similarly in the experiment from the *second series* of animals there was also no significant effect of lesion group (one-way ANOVA, $F_{2,39} = 2.287$, $p > 0.1$).

Fig 2.1. Time spent in social interaction



Results (mean ± SEM, sec) are shown for the sham group (n=12) and the habenula-lesioned group (n=8) in the first experimental series (**left panel**), and for the sham group (n=16), the habenula-lesioned group (n=10) and the group with restricted dorsal hippocampal lesions (n=16) in the second experimental series (**right panel**).

*Les resultats (moyenne ± ESM, sec) sont indiqués pour le groupe sham (n=12) et le groupe habenulo-lésé (n=8) lors de la première série d'expériences (**gauche**), et pourle groupe sham (n=16), le groupe habénulo-lésé (n=10) et le groupe ayant subi une lésion partielle de l'hippocampe dorsal (n=16) lors de la seconde série d'expériences (**droite**).*

II.3.2 Prepulse inhibition (PPI) of the auditory-evoked startle response

The average magnitude of the startle response to pulse alone stimuli during the different trial blocks is shown in **Table 2.1**. For the experiment of the *first series*, two-factor ANOVA (group, trial block as repeated factor) revealed no difference between groups ($F_{1,17} = 3.88$, $p > 0.05$) and no group x trial block interaction ($F_{3,51} = 0.92$, $p > 0.1$) but a significant effect of trial block ($F_{3,51} = 12.0$, $p < 0.0001$). Also in the experiment of the *second series* there was no significant effect of group ($F_{2,39} = 1.29$, $p > 0.1$), no significant group x trial block interaction ($F_{6,117} = 1.29$, $p > 0.1$) but a significant effect of trial block ($F_{3,117} = 23.0$, $p < 0.0001$).

Table 2.1. Startle amplitude

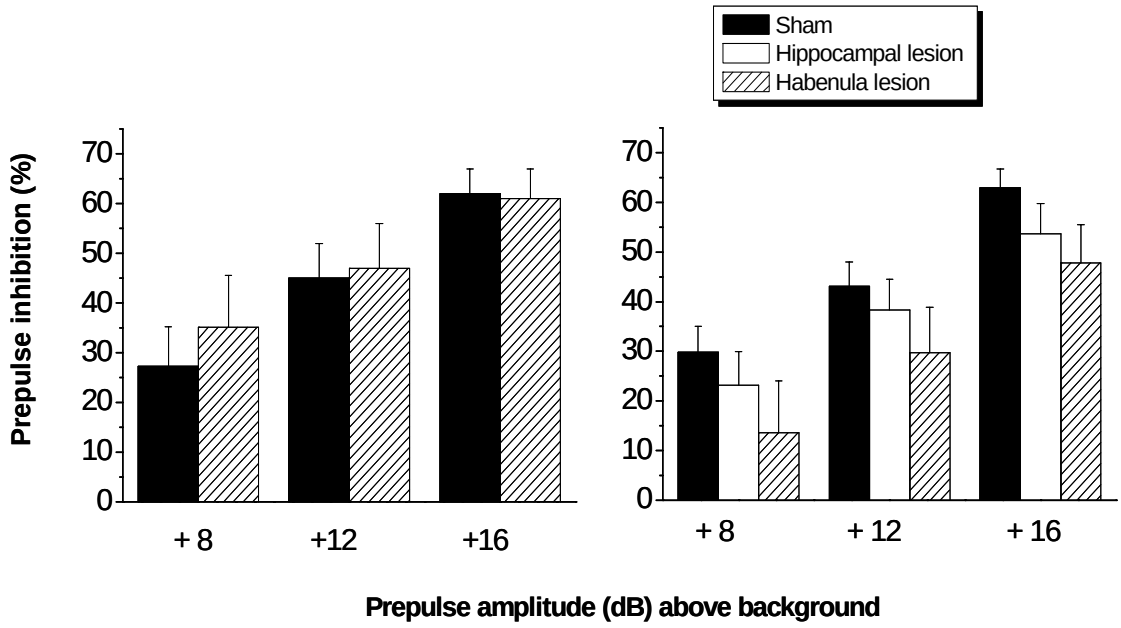
Group	PA0	PA1	PA2	PA3
First series				
Sham-operated	599 ± 75	371 ± 47	451 ± 60	486 ± 50
Habenula-lesioned	809 ± 79	583 ± 80	570 ± 67	596 ± 78
Second series				
Sham-operated	516 ± 47	389 ± 42	418 ± 46	439 ± 57
Habenula-lesioned	702 ± 117	477 ± 76	437 ± 66	516 ± 56
Dorsal hippocampus lesioned	705 ± 75	489 ± 81	516 ± 78	570 ± 64

Amplitudes (mean ± SEM; grams) to the first three pulse alone stimuli (PA0) and to pulse alone stimuli during blocks 1, 2 and 3 (PA1, PA2 and PA3).

Amplitudes (moyenne ± ESM; grammes) du sursaut en réponse au premier stimulus seul (PA0) ainsi qu'aux stimuli seuls durant les blocks 1, 2 et 3 (PA1, PA2 and PA3).

The percentage PPI results are shown in **Fig 2.2**. In the first experiment two-factor ANOVA (*factors*: lesion group, prepulse intensity as a repeated factor) showed no effect of lesion group ($F_{1,17} = 0.08$, $p > 0.1$) and no interaction of lesion group with prepulse intensity ($F_{2,34} = 0.55$, $p > 0.1$), but the expected highly significant effect of prepulse intensity ($F_{2,34} = 23.5$, $p < 0.0001$). Also in the experiment of the *second series* the two factor ANOVA showed a significant effect of prepulse intensity ($F_{2,78} = 136$, $p < 0.0001$) but no significant effect of group ($F_{2,39} = 1.29$, $p > 0.1$) and no group x prepulse intensity interaction ($F_{4,78} = 0.36$, $p > 0.1$).

Fig 2.2. PPI as percentage of startle amplitude in absence of a prepulse



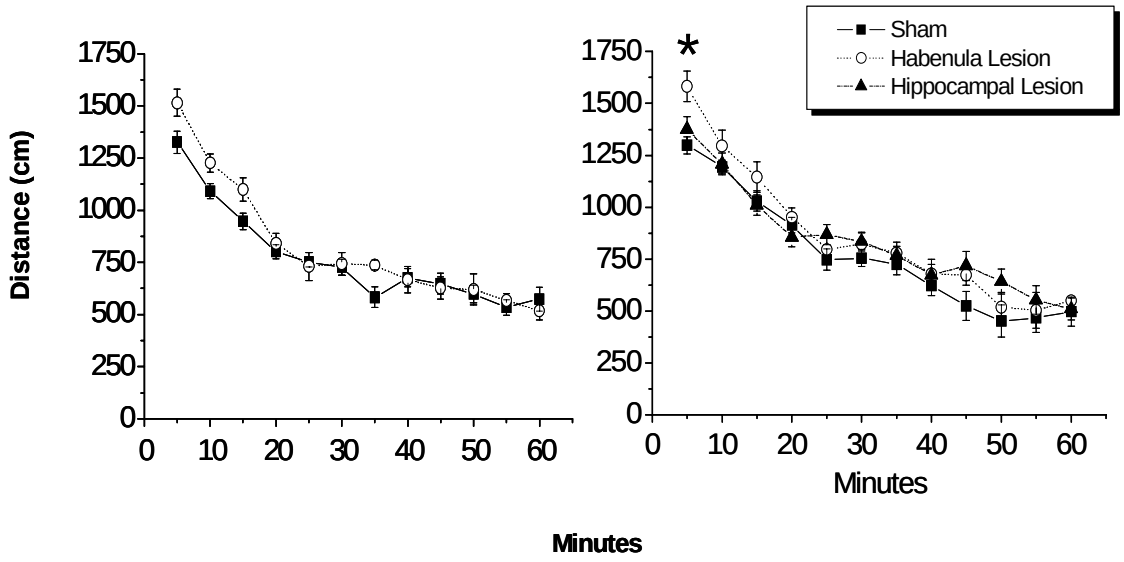
Results (mean ± SEM, %) are shown for the sham group (n=12) and the habenula-lesioned group (n=8) in the first experimental series (**left panel**), and for the sham group (n=16), the habenula-lesioned group (n=10) and the group with restricted dorsal hippocampal lesions (n=16) in the second experimental series (**right panel**).

*Les resultats (moyenne ± ESM, %) sont indiqués pour le groupe sham (n=12) et le groupe habenulo-lésé (n=8) lors de la première série d'expériences (**gauche**), et pourle groupe sham (n=16), le groupe habénulo-lésé (n=10) et le groupe ayant subi une lésion partielle de l'hippocampe dorsal (n=16) lors de la seconde série d'expériences (**droite**).*

II.3.3 Locomotor activity

The results are shown in **Fig 2.3**. In the experiment from the *first series* two-factor ANOVA revealed a highly significant effect of time ($F_{11,187} = 72.9$, $p < 0.0001$) but no significant effect of group ($F_{1,17} = 2.34$, $p > 0.1$) and no significant group x time interaction ($F_{11,187} = 1.71$, $p > 0.05$). Nevertheless there was a strong suggestion that habenula-lesioned animals showed greater activity during the early phase of the test. In the second experiment during the first 5 minutes of the session the habenula-lesioned rats again showed hyperactivity compared to the two other groups.

Fig 2.3. Locomotor activity per 5-min



Results (mean ± SEM, cm) are shown for the sham group (n=12) and the habenula-lesioned group (n=8) in the first experimental series (**left panel**), and for the sham group (n=16), the habenula-lesioned group (n=10) and the group with restricted dorsal hippocampal lesions (n=16) in the second experimental series (**right panel**). * $p < 0.05$ *vs* sham (2-tailed Dunnett's test).

*Les resultats (moyenne ± ESM, cm) sont indiqués pour le groupe sham (n=12) et le groupe habenulo-lésé (n=8) lors de la première série d'expériences (**gauche**), et pourle groupe sham (n=16), le groupe habénulo-lésé (n=10) et le groupe ayant subi une lésion partielle de l'hippocampe dorsal (n=16) lors de la seconde série d'expériences (**droite**). * p < 0.05 vs sham (test de Dunnett à deux bornes).*

Based on the results of the previous experiment a planned comparison of values during the first 5 minutes showed a significant effect of group ($F_{2,39} = 5.55$, $p < 0.01$) with the habenula-lesioned group differing significantly from the sham group (Dunnett's test, p = 0.004, 2-tailed). Over the whole recording period two-factor ANOVA showed that there was no significant effect of lesion group ($F_{2,39} = 1.61$, $p > 0.1$) and no significant group x time interaction ($F_{22,429} = 1.40$, $p > 0.1$) but the expected highly-significant effect of time ($F_{11,429} = 107$, $p < 0.0001$).

II.3.4 Morris water maze

II.3.4.1 Practice swim

One animal from the *second series* showed poor swimming and was withdrawn from the experiment. Otherwise one way ANOVAs revealed no differences between the

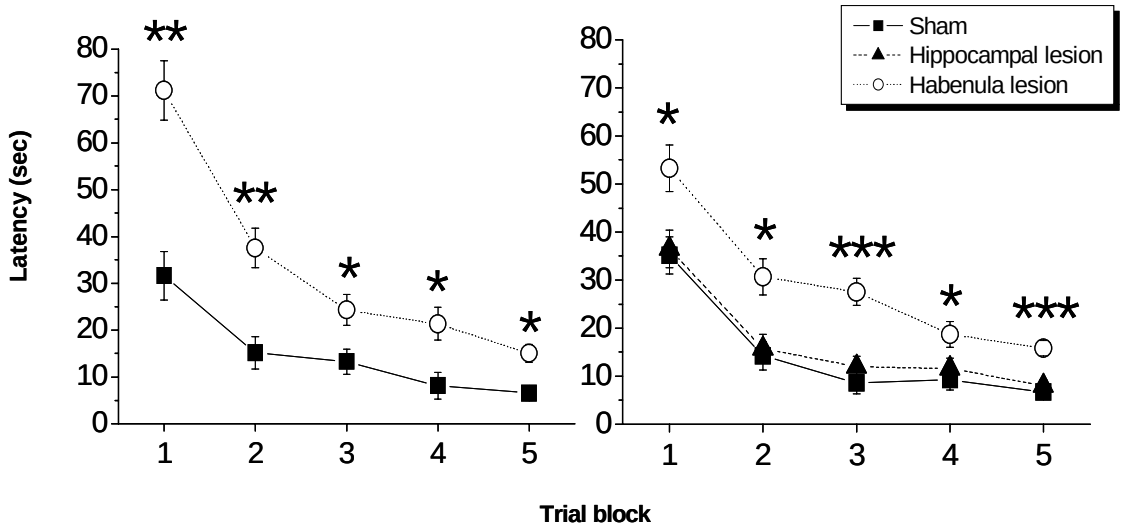
different groups in the distance swum during the practice swim (*first series*: $F_{1,18} < 1$, $p > 0.1$; *second series*: $F_{2,39} = 1.6$, $p > 0.1$) (results not shown).

II.3.4.2 Hidden platform trials

Escape latency. The results are shown in **Fig 2.4**. In the first experiment two-way ANOVA showed that there were significant effects of treatment group ($F_{1,18} = 40.83$, $p < 0.0001$) and trial block ($F_{4,72} = 42.96$, $p < 0.0001$), and a significant interaction of these factors ($F_{4,72} = 6.64$, $p < 0.001$). This interaction reflects the fact that the difference between groups declined over days. In the second experiment the two-way ANOVA revealed a significant effects of group ($F_{2,39} = 16.013$, $p < 0.0001$) and day ($F_{4,156} = 73.71$, $p < 0.0001$), but no significant interaction of group x trial block. In view of the significant effects of group, one-way ANOVAs at each time point were performed followed by pairwise comparisons with the 2-tailed Dunnett's test, to see at which time points the groups differed. The results of these comparisons are shown in **Fig 5**. At no time point was there any difference between the sham group and the group with restricted hippocampal lesions.

In summary, both experiments showed similar results in that the habenula-lesioned group took longer to find the platform at all time points.

Fig 2.4. Latency to find the hidden platform in the water maze as a function of trial block (4 trials per block)



Results (mean ± SEM, sec) are shown for the sham group (n=12) and the habenula-lesioned group (n=8) in the first experimental series (**left panel**), and for the sham group (n=16), the habenula-lesioned group (n=10) and the group with restricted dorsal hippocampal lesions (n=16) in the second experimental series (**right panel**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* sham (2-tailed Dunnett's test).

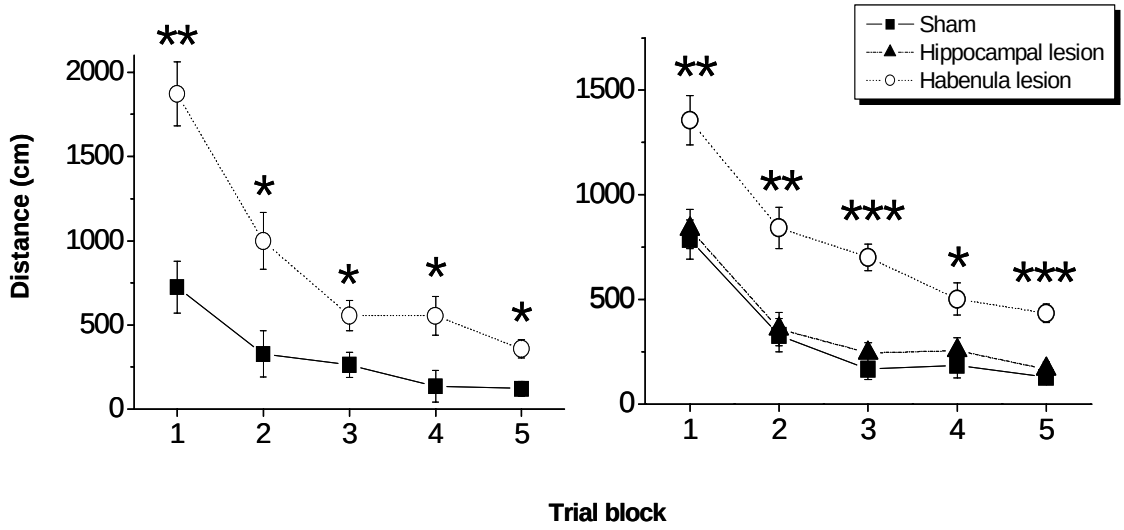
*Les resultats (moyenne ± ESM, sec) sont indiqués pour le groupe sham (n=12) et le groupe habenulo-lésé (n=8) lors de la première série d'expériences (**gauche**), et pourle groupe sham (n=16), le groupe habénulo-lésé (n=10) et le groupe ayant subi une lésion partielle de l'hippocampe dorsal (n=16) lors de la seconde série d'expériences (**droite**). * p < 0.05, ** p < 0.01, *** p < 0.001 vs sham (test de Dunnett à deux bornes).*

Distance swum before finding the platform. The results are shown in **Fig 2.5**, and in **Fig 2.7** examples of sham and lesioned animals patterns of swimming are shown. In the first experiment two-way ANOVA showed that there were significant effects of treatment ($F_{1,18}$ = 23.77, $p < 0.001$), trial block ($F_{4,72}$ = 34.8, $p < 0.0001$), and a significant interaction of these factors ($F_{4,72}$ = 6.66, $p < 0.001$). For the second experiment the analysis of the distance swum before finding the platform also revealed that the habenula lesioned rats swam a longer distance to find the platform than did rats from the other groups. Thus the two-way ANOVA showed a significant effect of group ($F_{2,39}$ = 22.9, $p < 0.0001$), and of trial block ($F_{4,156}$ = 8.96, $p < 0.0001$) but no significant interaction of group x trial block. One-way ANOVAs followed, in the case of a significant effect, by pairwise comparisons (Dunnett's test, two-tailed) showed significant differences between the habenula lesion group and the sham group at each

time point, but no significant differences between the sham group and the group with small dorsal hippocampal lesions.

Thus, both experiments showed similar results in that the habenula-lesioned group swam a longer distance before finding the platform on all days.

Fig 2.5. Distance swum before finding the hidden platform in the water maze as a function of trial block (4 trials per block)



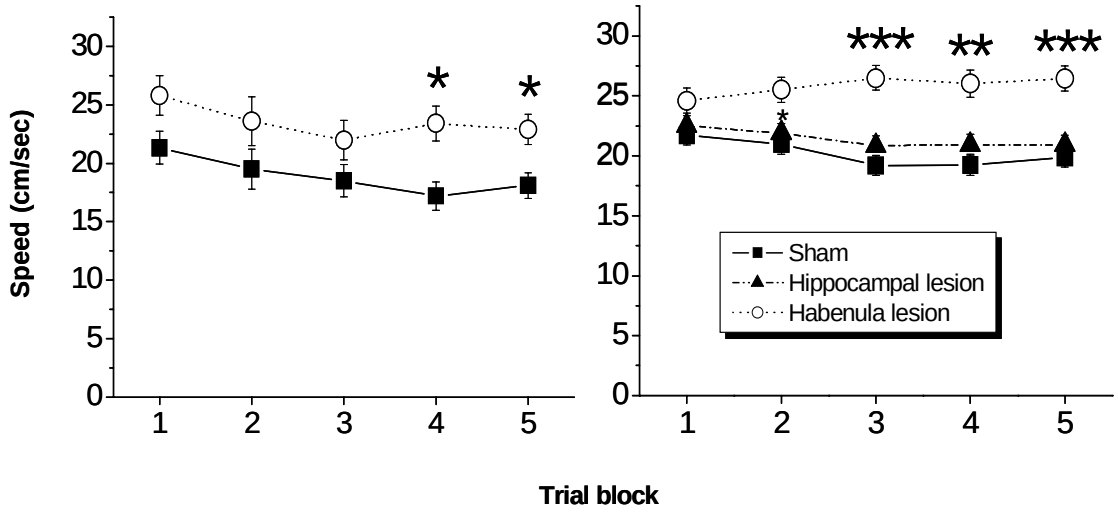
Results (mean ± SEM, cm) are shown for the sham group (n=12) and the habenula-lesioned group (n=8) in the first experimental series (**left panel**), and for the sham group (n=16), the habenula-lesioned group (n=10) and the group with restricted dorsal hippocampal lesions (n=16) in the second experimental series (**right panel**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* sham (2-tailed Dunnett's test).

*Les resultats (moyenne ± ESM, cm) sont indiqués pour le groupe sham (n=12) et le groupe habenulo-lésé (n=8) lors de la première série d'expériences (**gauche**), et pourle groupe sham (n=16), le groupe habénulo-lésé (n=10) et le groupe ayant subi une lésion partielle de l'hippocampe dorsal (n=16) lors de la seconde série d'expériences (**droite**). * p < 0.05, ** p < 0.01, *** p < 0.001 vs sham (test de Dunnett à deux bornes).*

Swimming speed. The results are shown in **Fig 2.6**. In the first experiment two-way ANOVA showed that the habenula-lesioned group swam faster during hidden platform trials ($F_{1,18} = 5.74$, $p < 0.05$). The effect of trial block was also significant ($F_{4,72} = 5.46$, $p < 0.05$), whereas the interaction of lesion group and trial block was not ($F_{4,72} = 0.7$, $p > 0.1$). In the second experiment there was a significant effect of group ($F_{2,39} = 13.818$,

$p < 0.0001$), no effect of trial block and a significant interaction of group x trial block ($F_{8,156} = 2.341$, $p < 0.05$). The consistent finding in both experiments, therefore, was that the habenula-lesioned animals swam faster than control animals.

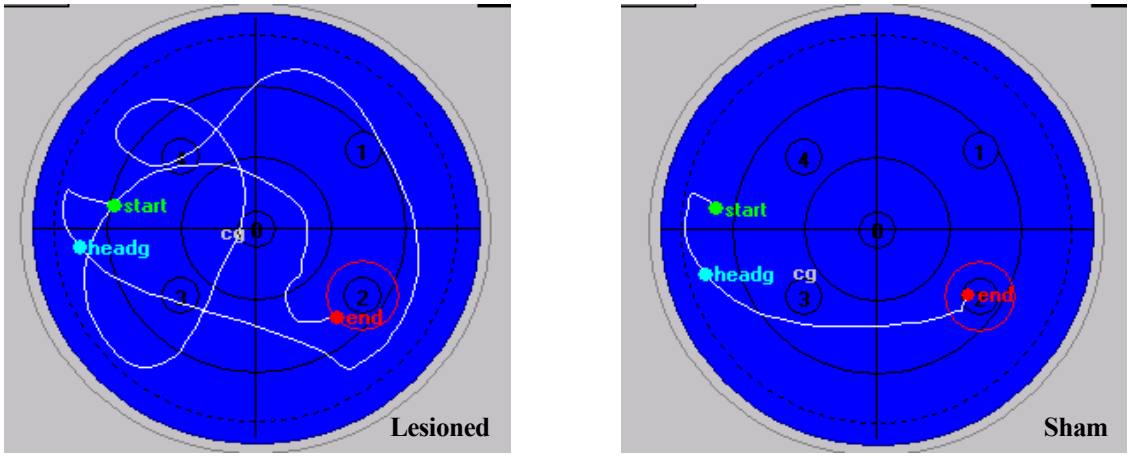
Fig 2.6. Swimming speed in the water maze as a function of trial block (4 trials per block)



Results (mean ± SEM, cm/sec) are shown for the sham group (n=12) and the habenula-lesioned group (n=8) in the first experimental series (**left panel**), and for the sham group (n=16), the habenula-lesioned group (n=10) and the group with restricted dorsal hippocampal lesions (n=16) in the second experimental series (**right panel**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* sham (2-tailed Dunnett's test).

*Les resultats (moyenne ± ESM, cm/sec) sont indiqués pour le groupe sham (n=12) et le groupe habenulo-lésé (n=8) lors de la première série d'expériences (**gauche**), et pourle groupe sham (n=16), le groupe habénulo-lésé (n=10) et le groupe ayant subi une lésion partielle de l'hippocampe dorsal (n=16) lors de la seconde série d'expériences (**droite**). * p < 0.05, ** p < 0.01, *** p < 0.001 vs sham (test de Dunnett à deux bornes).*

Fig 2.7. Swim patterns



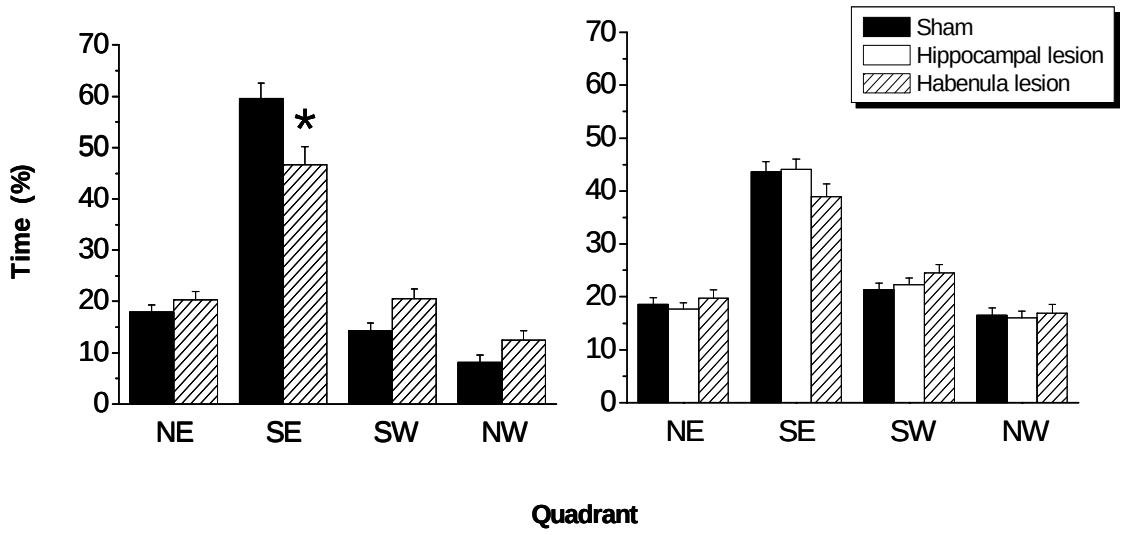
Typical examples of swim patterns, on day 5, for a habenula-lesioned rat (**left panel**) and a sham rat (**right panel**).

*Exemples typiques de navigation, au cinquième jour d'apprentissage, d'un rat avec lésion de l'habénula (**gauche**) et d'un rat sham (**droite**).*

II.3.4.3 Probe trial

The percentages of time spent in the quadrants of the pool during the probe trials are shown in **Fig 2.8**. Each group swam preferentially in the quadrant where was located the platform during the learning phase shown by a great effect of quadrant after two-way ANOVAs with quadrant as a repeated factor (*first series*: $F_{3,87} = 164$, $p < 0.0001$; *second series*: $F_{3,117} = 121$, $p < 0.0001$). One-way ANOVA of the percentage of time spent in the target (SE) quadrant showed a significant effect of lesion group in the *first series* ($F_{1,18} = 7.5$, $p < 0.05$). In the *second series* a similar tendency of the habenula-lesioned group to spend less time in this quadrant was not quite statistically significant ($F_{2,39} = 1.5$, $p > 0.1$).

Fig 2.8. Percentage of time spent in the four quadrants of the water maze during the probe trial. In the previous hidden platform trials the platform was located in the SE quadrant



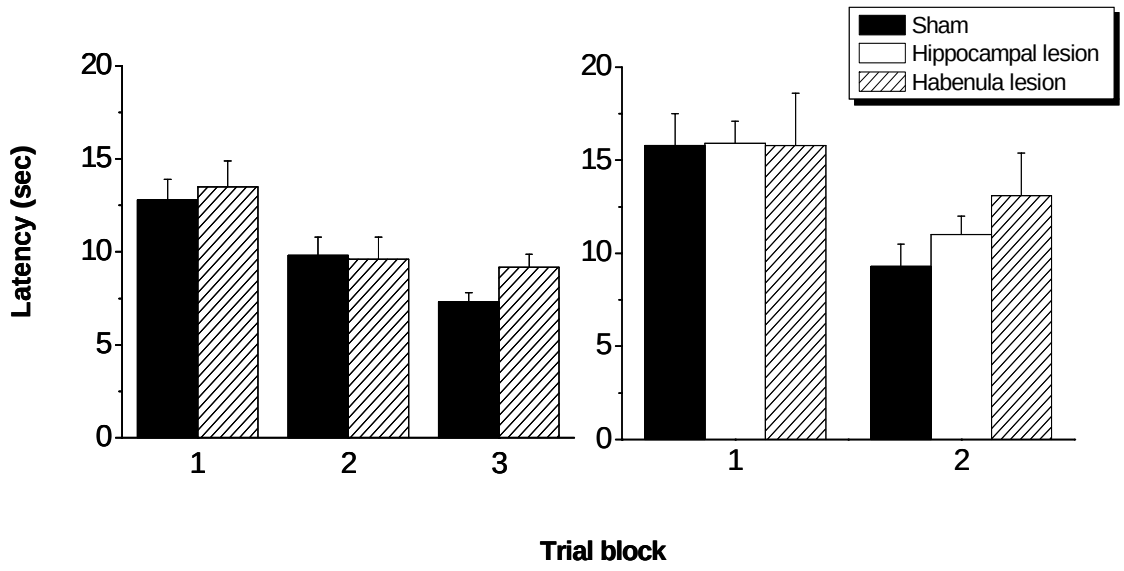
Results (mean ± SEM, %) are shown for the sham group (n=12) and the habenula-lesioned group (n=8) in the first experimental series (**left panel**), and for the sham group (n=16), the habenula-lesioned group (n=10) and the group with restricted dorsal hippocampal lesions (n=16) in the second experimental series (**right panel**). * $p < 0.05$ *vs* sham (2-tailed Dunnett's test after significant ANOVA).

*Les resultats (moyenne ± ESM, %) sont indiqués pour le groupe sham (n=12) et le groupe habenulo-lésé (n=8) lors de la première série d'expériences (**gauche**), et pourle groupe sham (n=16), le groupe habénulo-lésé (n=10) et le groupe ayant subi une lésion partielle de l'hippocampe dorsal (n=16) lors de la seconde série d'expériences (**droite**). * p < 0.05 vs sham (test de Dunnett à deux bornes suivant une ANOVA significative).*

II.3.4.4 Visible platform condition

Escape latency. Results are shown in **Fig 2.9**. A two-way ANOVA, with block as a repeated factor showed no significant difference between each group [*first series*: no effect of group ($F_{1,18} = 0.7$, $p > 0.1$) but a significant effect of block ($F_{2,36} = 11.5$, $p < 0.001$) and no group x block interaction ($F_{2,36} = 0.5$, $p > 0.1$); *second series*: no effect of group ($F_{2,39} = 0.46$, $p > 0.1$) but a significant effect of block ($F_{1,39} = 15.8$, $p < 0.001$) and no group x block interaction ($F_{2,39} = 0.8$, $p > 0.1$)].

Fig 2.9. Performance in the visible platform condition. Latency to find the platform in the water maze as a function of trial block (4 trials per block)



Results (mean ± SEM, sec) are shown for the sham group (n=12) and the habenula-lesioned group (n=8) in the first experimental series (**left panel**), and for the sham group (n=16), the habenula-lesioned group (n=10) and the group with restricted dorsal hippocampal lesions (n=16) in the second experimental series (**right panel**).

*Les resultats (moyenne ± ESM, sec) sont indiqués pour le groupe sham (n=12) et le groupe habenulo-lésé (n=8) lors de la première série d'expériences (**gauche**), et pourle groupe sham (n=16), le groupe habénulo-lésé (n=10) et le groupe ayant subi une lésion partielle de l'hippocampe dorsal (n=16) lors de la seconde série d'expériences (**droite**).*

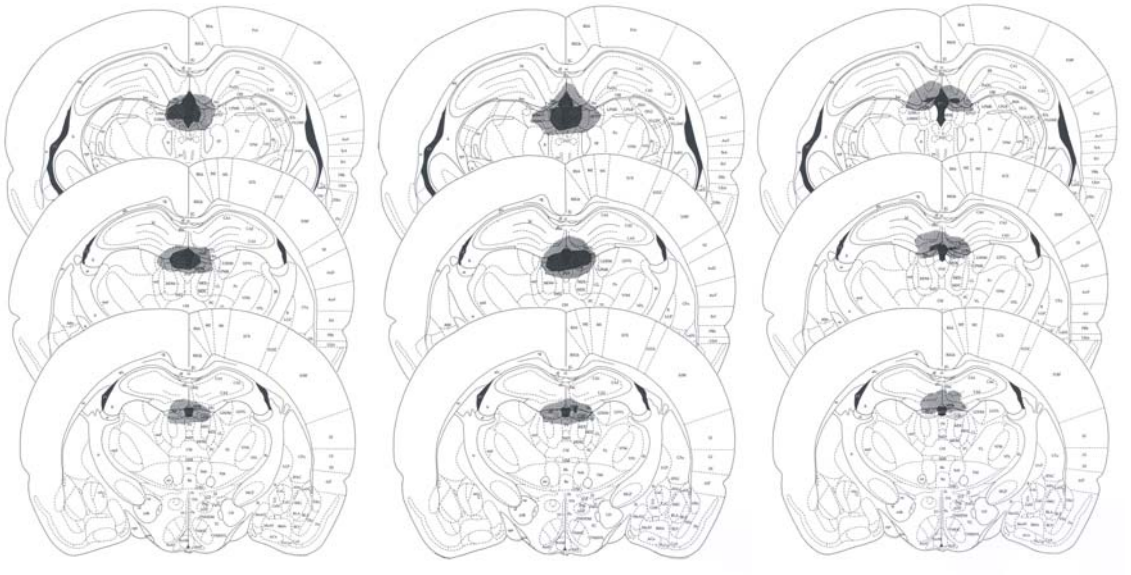
Distance swum before finding the platform. A two-way ANOVA, with block as a repeated factor showed no significant difference between each group [*first series*: no effect of group ($F_{1,18} = 3.7$, $p > 0.05$) but a significant effect of block ($F_{2,36} = 6.97$, $p < 0.01$) and no group x block interaction ($F_{2,36} = 2.13$, $p > 0.1$); *second series*: no effect of group ($F_{2,39} = 2.9$, $p > 0.05$) but a significant effect of block ($F_{1,39} = 12$, $p < 0.05$) and no group x block interaction ($F_{2,39} = 0.8$, $p > 0.1$)] (results not shown).

II.3.5 Histology

As shown in **Fig 2.10** habenula lesions destroyed a large proportion of both medial and lateral habenula without causing substantial damage to neighboring structures. In the group with small dorsal hippocampal lesions included in the *second series* of

experiments, the amount of hippocampal damage was greater than the incidental damage to the dorsal hippocampus in habenula-lesioned animals.

Fig 2.10. Representation of the brain lesions in the different lesion groups



Maximal (lightly-shaded areas) and minimal (heavily-shaded areas) extensions of the lesions. ***Left column***: habenula-lesions from the first series. ***Middle column***: habenula-lesions from the second series. ***Right column***: restricted dorsal hippocampus lesions from the second series. Plates are modified from the atlas of Paxinos & Watson (1998).

Etendues maximales (aires gris clair) et minimales (aires gris foncé) des différentes lesions. ***Gauche****: lésion de l'habénula lors de la première série d'expériences.* ***Centre****: lésion de l'habénula lors de la seconde série d'expériences.* ***Droite****: lésion partielle de l'hippocampe dorsal lors de la seconde série d'expériences. Coupes modifiées depuis l'atlas de Paxinos & Watson (1998).*

II.3.6 ChAT assay

ChAT assay was performed only for sham and habenula-lesioned animals of the *second series*. As shown in Table 2.2 animals with lesions of the habenula had a marked reduction, by 80 %, of ChAT in the interpeduncular nucleus. This result is consistent with those of previous neurochemical and histochemical studies (Contestabile *et al*, 1987; Eckenrode *et al*, 1987; Villani *et al*, 1983) and indicates marked degeneration of the habenulo-interpeduncular tract.

Table 2.2. Choline acetyltransferase in homogenates of interpeduncular nucleus

Group	ChAT (μmoles/g wet wt/hr)
Sham-operated	122.5 ± 6.5
Habenula-lesioned	23.9 ± 3.2 (19.5 ± 2.6 %) **

Results (mean ± SEM; μmoles/g wet wt/hr) are shown for the sham group and the habenula-lesioned group of the first series of experiments. Values in parentheses are expressed as percentage of the sham value. ** $p < 0.001$ *vs* sham-operated (2-tailed t-test).

*Les resultats (moyenne ± ESM; μmoles/g de matière sèche/hr) sont indiqués pour le groupe sham et le groupe habenulo-lésé lors de la première série d'expériences. Les valeurs entre parenthèses sont exprimées en pourcentage des valeurs correspondantes issues du groupe sham. ** $p < 0.001$ vs sham (test t à deux bornes).*

II.4 Discussion

The aim of the present studies was to challenge the hypothesis that habenula damage plays a role in the generation of the symptoms of schizophrenia. To this end we examined a prediction of this hypothesis, namely that lesion of the habenula would cause schizophrenia-like changes in experimental animals. The schizophrenia-like changes examined were reduction of social interaction and prepulse inhibition and impairment in performance of a spatial memory task. In both series of experiments the results were very similar: there were no significant effects of habenula lesions on social exploration time or on prepulse inhibition, whereas clear impairment was observed in the Morris water-maze test of spatial memory. As an important control it was shown that restricted damage to the dorsal hippocampus immediately above the habenula, comparable to the incidental damage after habenula lesions, did not impair water maze performance. The results therefore indicate a role of the habenula in memory, and are consistent with the view that habenula dysfunction contributes to memory deficits. Our results do not support a major role of habenula pathology in reductions of social interaction and PPI in schizophrenia.

Social interaction was examined since there is much evidence that individuals with schizophrenia are more socially withdrawn. Many schizophrenics feel anxious during social interactions and consider this a problem (Pilkonis *et al*, 1980). In self-report questionnaires schizophrenic patients receiving neuroleptics showed less extraversion than control patients with non-mental illness (Pillman *et al*, 2003), and more shyness and lower sociability than a control group of undergraduates (Goldberg & Schmidt, 2001). Longitudinal studies suggest that even in the pre-schizophrenia phase patients with schizophrenia had difficulty establishing social relationships and may have avoided their peers more than other children (Auerbach *et al*, 1993; Cannon *et al*, 1997; Davies *et al*, 1998; Done *et al*, 1994; Jones *et al*, 1994; Nuechterlein, 1986). Also, ethological studies indicate that schizophrenics show less non-verbal expressions of social interaction (Pitman *et al*, 1987; Troisi *et al*, 1998). Although the present results do not indicate a role of habenula damage in social withdrawal it is possible that the test we used does not correspond closely enough to situations in which social withdrawal is a feature of schizophrenia. For example in the present test social interaction time is believed to be determined by the balance of fear and a tendency to explore a novel animal (File, 1980), whereas in social interactions over a longer time-scale other

motivations such as the pleasure of social contact may be more relevant. Certainly in another form of social behaviour, namely maternal behaviour, there is strong evidence that the habenula plays a role and that there is disturbance of this behaviour in schizophrenia. Thus, numerous studies of maternal behaviour in the rat (Corodimas *et al*, 1992, 1993; Felton *et al*, 1998; Matthews-Felton *et al*, 1995, 1998) have shown that the lateral habenula is necessary for not only the hormone-dependent onset of maternal behaviours, but also for their non-hormone-dependent maintenance. In this respect the lesion effect resembles schizophrenia, in that several studies have indicated that, on average, parental care is impaired in mothers with schizophrenia (Goodman, 1987; Ragins *et al*, 1975; Sobel, 1961, 1964).

In neither series of our experiments was there a significant alteration of PPI of the acoustic startle response in habenula-lesioned animals. These results provide no evidence that habenula damage contributes to the often-described reduction of PPI in schizophrenics (Braff *et al*, 1978, 1992, 1999; Grillon *et al*, 1992). It should be noted, however, that in a recent study of PPI in never-medicated patients a deficit in PPI compared to controls was found with a 60 msec prepulse-pulse interval, but not with intervals of 30, 120 or 240 msec (Ludewig *et al*, 2003). Thus the impairment of PPI in never-medicated schizophrenics may not be as dramatic as previously observed in other groups of schizophrenics, and in further studies of habenula lesions it will be of interest to include the prepulse-pulse interval as a systematically varied parameter.

In the water maze task of spatial memory, deficits were observed in both experiments in the habenula-lesioned group. In both experiments there were deficits of latency to find the hidden platform and distance swum before finding it. In the first experiment there was also impairment according to the search pattern in the probe trial, with the lesioned animals spending less time in the quadrant that previously contained the escape platform. The lack of a significant change in this measure in the second experiment may be attributed to a certain amount of overtraining, such that by the end of hidden platform training both groups were performing well. In the water maze visual platform condition there was no impairment in the habenula-lesioned animals. This indicates that these animals have the necessary motivation and perceptual and motor abilities to perform the task. Their impairment in the hidden platform task can be more likely attributed to a deficit of spatial memory. Their impairment cannot be attributed to the small amount of

incidental damage to the overlying hippocampus, since the hippocampal lesion group, with comparable hippocampal damage, showed no impairment. This is in keeping with other studies showing that impairment in the Morris maze was observed only when more than 20 % of the dorsal hippocampus was damaged (Moser *et al*, 1993). The experiments thus strongly indicate that memory deficit, a well-documented feature of schizophrenia (Calev, 1984a, b; Cutting, 1985; Elliot & Sahakian, 1995; Gold *et al*, 1992; Goldberg *et al*, 1989; Meltzer & McGurk, 1999; McKay *et al*, 1996; McKenna *et al*, 1990; Sharma & Antonova, 2003; Tamlyn *et al*, 1992) can result from habenula damage. Spatial memory may be particularly pertinent to schizophrenia, since spatial learning tests in rodents are considered as model of declarative memory in humans (O'Keefe & Nadel, 1978), and declarative memory appears to be selectively impaired in schizophrenia (Perry *et al*, 2000).

A further alteration of behaviour observed in both Morris maze hidden platform experiments was that habenula-lesioned animals showed an increase of swimming speed. The fact that they were also hyperactive during the early minutes of the measurement of locomotor activity may suggest that lesioned animals are hyper-reactive to stress, be this due to the frustration of failing to find the platform in the Morris maze or to the novelty of the locomotor activity apparatus.

Although a relationship between the habenula and memory and cognition has not previously been emphasized, earlier studies are also consistent with this view. For example, Thornton & Evans (1982) reported that in a cylinder of water habenula-lesioned rats showed fewer categories of behaviour than controls, indicating a reduction of behavioural plasticity. Moreover when a rope was hung so that it just touched the water surface at the centre of the cylinder most control animals escaped by climbing the rope, compared to only very few habenula-lesioned animals. The lesioned animals therefore seemed impaired in selecting behavioural strategies in a stressful situation. Further, Thornton & Bradbury (1989) showed that habenula-lesioned rats were impaired in learning a one-way active avoidance response when the intertrial-interval was short and shock level was moderate, but were unimpaired if shock levels were low and the inter-trial interval was long. They suggested that differences in stress and effort could account for previous variable findings on this task. Thornton & Davies (1991) in a spatial two-choice water maze task showed that habenula-lesioned rats were impaired in

acquiring and reversing a spatial discrimination. These studies are thus also consistent with a deficit in cognition after habenula lesions.

A number of possible mechanisms could account for the memory impairment observed here. The habenula is a major link by which forebrain regions influence the activity of cell groups that project widely to the forebrain (Garland & Mogenson, 1983; Greatrex & Phillipson, 1982; Kalen *et al*, 1989; Nagy *et al*, 1978; Sutherland, 1982;). Through a habenulo-raphé pathway (Aghajanian & Wang, 1977) that is a major inhibitory influence on serotonergic cells of the dorsal raphé (Nishikawa & Scatton, 1985; Speciale *et al*, 1980; Wang & Aghajanian, 1977) it alters serotonergic activity in many structures including the striatum and substantia nigra (Reisine *et al*, 1982; Soubrié *et al*, 1981) and the hippocampus (Ferraro *et al*, 1997; Sabatino *et al*, 1991). Similarly, other workers have found that the lateral habenula acts via a pathway to the locus coeruleus to influence the noradrenergic activity in the hippocampus, prefrontal cortex, striatum and the nucleus accumbens (Cenci *et al*, 1992 ; Kalen *et al*, 1989). Other studies, have shown that the lateral habenula projects directly to the ventral tegmental area and the substantia nigra, to influence mesocortical, mesostriatal and mesolimbic dopaminergic pathways (Christoph *et al*, 1986; Lisoprawski *et al*, 1980; Matsuda & Fujimura, 1992). Moreover habenula stimulation results in an increase release of acetylcholine in hippocampus (Nilsson *et al*, 1990). Thus there are multiple mechanisms by which habenula damage could result in dysfunction of the hippocampus, a structure that is important in spatial learning (Morris *et al*, 1982, 1990; Moser *et al*, 1993; Sutherland *et al*, 1983). It has been recently reported that the induction of long-term depression (LTD) in the hippocampus is prevented by the presence of serotonin (Chakalova *et al*, 2001). Since activity of the lateral habenula inhibits the activity of dorsal raphé neurons (Wang & Aghajanian, 1977) it is possible that in habenula-lesioned animals the dorsal raphé is never adequately inhibited, such that LTD is impaired. Other possible mechanisms for memory disturbance after habenula lesions are suggested by findings that vasotocin-like bioactivity is elevated in cerebrospinal fluid (CSF) shortly after habenula lesions (Goldstein, 1985) and that intraventricular vasotocin inhibits memory formation with a U-shaped dose-response curve (De Wied *et al*, 1991). Thus an elevation of a vasotocin-like substance during waking could contribute to impairment of memory formation after habenula lesions. A further mechanism is suggested by the finding that vasotocin-like bioactivity is found in human CSF only during REM sleep (Pavel *et al*, 1979). Based

partly on the above observations it has been proposed that the habenula is part of a system that prevents REM-sleep neural activity ("dream-events") being placed in the memory store and being treated as reality (Kelly, 1998). Habenula lesions, by causing REM sleep levels of vasotocin-like activity to deviate from the range that inhibits memory formation, would therefore result in dream-events being stored as normal memories. Cognitive disturbance after habenula lesions would then result from an accumulation of erroneously-strengthened neural connections that are the basis of these delusional memories, such that input into and retrieval from such a disorganized memory store containing incorrect information, would be inefficient and error-prone.

In summary the present results provide evidence for a role of habenula damage in cognitive disturbances, and rule out incidental dorsal hippocampal damage as an explanation. Our results provide no support for a role of habenula damage in social withdrawal or deficits in PPI. It remains to be elucidated which of the several alternative mechanisms are involved in the memory disturbance observed. Moreover, since the habenula consists of at least fifteen subnuclei (Andres *et al*, 1999; Geisler *et al*, 2003), disturbance of multiple mechanisms may contribute to the pattern of behavioural disturbances, depending on the exact distribution of damage.

"Guernica"

Picasso - 1937

Chapter III - Effects of pineal lesion and complete epithalamic lesion on social interactions, sensory gating and spatial memory

III.1 Introduction

The epithalamus is a structure that belongs to the diencephalon and forms its dorsal posterior subdivision. It is composed of the pineal gland, the habenula and associated fiber bundles. The habenula has been shown to be involved in many behaviors such as olfactory guided behaviour, mating, control of behaviour by aversive stimuli, ingestion, anxiety, brain stimulation reward, sleep, behavioural flexibility as well as endocrine secretion (Cohen & Melzack, 1983; Corodimas *et al*, 1993; Felton *et al*, 1998; Haun *et al*, 1992; Murphy *et al*, 1996; Sutherland, 1982; Sutherland & Nakajima, 1981; Thornton & Evans, 1982; Valjakka *et al*, 1998), while the pineal gland is a major component of the photoneuroendocrine system, responsible for the release of the indoleamine melatonin, whose functions include regulation of circadian rhythms (Korf *et al*, 1998).

A number of recent studies have suggested that there is dysfunction of the epithalamus in patients with schizophrenia. For example computer tomography (CT) studies have suggested that large calcifications of the habenula and of the pineal are more frequent in patients with schizophrenia than in age-matched controls (Sandyk, 1992). Other studies have revealed greater calcification of the epithalamus as a whole in schizophrenia (Caputo *et al*, 1998). Although some studies found increased calcification of the pineal in schizophrenia only in a restricted age subgroup (Bersani *et al*, 1999), further support for the view that pineal function is disturbed in schizophrenia is provided by several reports of reduced plasma melatonin concentrations in patients with schizophrenia (Fanget *et al*, 1989; Ferrier *et al*, 1982; Monteleone *et al*, 1992; Vigano *et al*, 2001), both in drug-free patients (Ferrier *et al*, 1982; Monteleone *et al*, 1992; Vigano *et al*, 2001) as well as in patients receiving neuroleptics (Monteleone *et al*, 1992; Vigano *et al*, 2001). The fact that in these studies differences between patients and controls are most pronounced at the peak of melatonin secretion, near the middle of the dark phase, may explain why one study found no difference between schizophrenia patients and

controls in melatonin concentration in cerebrospinal fluid when collected in the morning (Beckmann *et al*, 1984).

Such correlational studies, however, cannot indicate whether the reported dysfunction is causal or not in the symptoms of the disease. In order to shed more light on this question we have begun to investigate whether lesions of discrete components of the epithalamus in rats result in any schizophrenia-like symptoms (see **Chapter II**). The behaviours initially examined were social interaction, prepulse inhibition (PPI) and memory function, since many reports indicate that these behaviours are disturbed in schizophrenia (Braff *et al*, 78, 92, 99; Goldberg & Schmidt, 2001; Lieberman *et al*, 2001; McKenna *et al*, 1990; Meltzer & McGurk, 1999; Pilkonis *et al*, 1980; Pillmann *et al*, 2003). The results showed that bilateral lesions of the habenula caused marked impairment of spatial memory in the Morris maze without alteration of social contact time in a brief social interaction test, and without alteration of PPI of an acoustic startle response (see **Chapter II**). To extend these studies to the other major component of the epithalamus, the pineal body, the present experiments examine social interaction, PPI and memory function in the Morris maze after pinealectomy alone, or after combined pinealectomy.

III.2 Materials and methods

III.2.1 Experimental series

Two series of experiments were performed; the *first series* comprised a sham-operated group and a group with a pinealectomy, whereas the *second series* was carried out on sham-operated animals and rats with an entire epithalamic lesion (habenula lesion plus pinealectomy). In both series the social interaction test was performed three weeks after completion of surgery, the PPI test was performed two weeks after the social interaction test, and the Morris maze started three weeks after the PPI test. This order of testing was chosen so that the tests judged to be more stressful for the animals were performed later. Additionally, biochemical assays of choline acetyltransferase (ChAT) in homogenates of the interpeduncular nucleus (IPN) were performed in the *second series* of experiments, to provide a biochemical index of damage to the habenulo-interpeduncular tract, and assay of melatonin in blood samples at day and night time-points was performed in the *first series* of experiments to verify the pinealectomy, in addition to histological verification of lesions.

III.2.2 Animals

The experiments were carried out on male Sprague-Dawley rats (Iffa Credo, France) of body weight 250-270 g at the time of surgery. The animals were housed in individual cages (Macrolon, 42 × 26 × 15 cm) in a temperature-regulated (22 ± 2°C) animal room on a 12 h/12 h light/dark cycle (lights on at 06:00), with laboratory rat chow (Kliba AG, Switzerland) and water available *ad libitum*. The operations and behavioural tests were performed during the light period, at least one week after their arrival. All testing procedures were in accordance with the Swiss animal protection law for the care and use of animals and were approved by the Cantonal Veterinary Authority of the City of Basel.

III.2.3 Surgical procedures

Pinealectomy. Animals were anaesthetized with sodium pentobarbital (60 mg/kg, *i.p.*) and placed in a stereotaxic frame. A circular hole, 5 mm in diameter, was drilled in the skull using a trephine, centered above the position of the pineal gland. After the circular piece of skull was removed, the pineal gland was extracted by means

of a curved forceps. Then, the piece of skull was put back in place and the skin was sutured. Animals who received the sham pinealectomy procedure underwent the identical procedure except that the forceps were not inserted and the pineal gland was not removed.

For the rats with a complete epithalamic lesion in the *second series* of experiments, pinealectomy and habenula lesions were performed with a three-week delay interval, the pinealectomy being performed first.

Habenula lesion. The procedure has been previously described in **Chapter II** (page 69).

III.2.4 Behavioural procedures

Behavioural procedures were identical in both experiments, and have been previously described in **Chapter II** (pages 69-71), to the following exceptions:

- concerning social interactions, in the *second series* of experiment the test lasted 10 minutes instead of 5.

- in the present study the water-maze used was 180 cm in diameter, instead of 133 cm. Moreover, in the *second series*, the hidden platform experiment was performed only for 4 days. Finally, in the visible platform condition, a single session of 4 trials was performed for both series.

III.2.5 Blood samples for melatonin radioimmunoassay

In order to have an index of the reduction of melatonin secretion associated with the pinealectomy, assay of melatonin in blood samples was performed for each animal at the end of the *first series* of experiments. Blood samples were taken, from sham and pinealectomized animals, by intracardiac puncture under light isoflurane anesthesia (Forene®, Abbott, USA). A first sample was taken during the light period (two hours before the transition light/dark, and two other samples during darkness under dim red light (six and nine hours after the beginning of the dark period, respectively). Blood

samples were placed into heparinized tubes and were kept on ice until centrifugation (4°C, 9000 rcf, 10 min). Plasma was divided into several aliquots and stored at –20°C until used in assay.

III.2.6 Melatonin radioimmunoassay

Melatonin was extracted from plasma samples using dichloromethane, according to the method of Brown *et al* (1985). Plasma melatonin concentrations were determined in duplicate by radioimmunoassay (RIA), using rabbit antiserum (R19540, INRA, Nouzilly, France) and labeled [^{125}I]-2-iodomelatonin (Amersham). Standards were extracted using the same procedure as for plasma samples. The RIA has been previously validated for rat plasma by parallelism and recovery studies (McKenna *et al*, 1990). The limit of sensitivity of the assay was 1 pg/tube. The inter-assay coefficients of variation were 4, 8, and 12% at the levels of 2, 10, and 20 pg/tube, respectively. The intra-assay coefficients of variation were 4, 3, and 3 % at the same levels, respectively.

III.2.7 Histology

Histological procedures for inspections of habenula lesions have been previously described in **Chapter II** (pages 72-73). Concerning pinealectomies, they were confirmed by visual inspection in both series of experiments.

III.2.8 Assay of choline acetyltransferase (ChAT)

The procedure has been previously described in **Chapter II** (page 73).

III.2.9 Statistics

The statistical significance of differences between treatment groups were analyzed by analysis of variance (ANOVA), followed by pairwise comparisons (Dunnett's test, two-tailed), using the SYSTAT software package (Version 10.2, SPSS Inc., Chicago, IL).

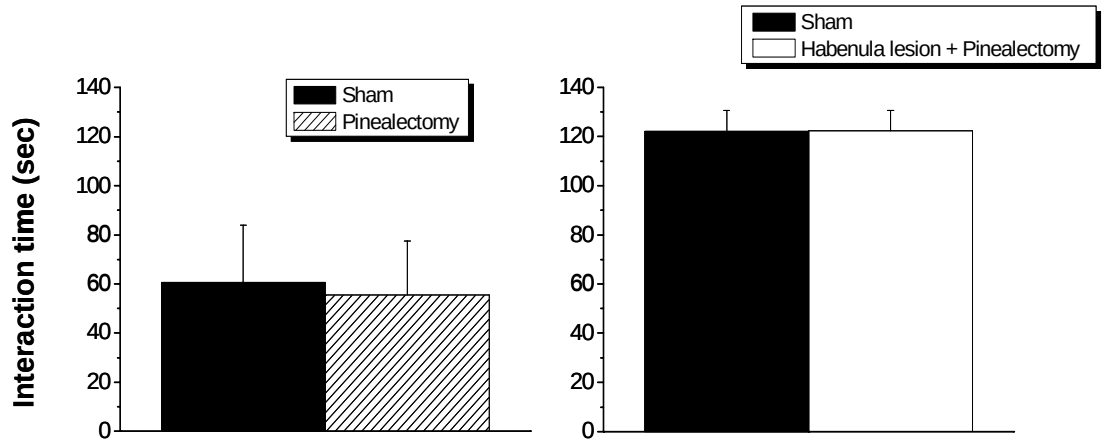
III.3 Results

After exclusion of animals with unsatisfactory lesions, there were 9 sham-operated animals and 15 pineal-lesioned animals in the *first series of experiments*, and 9 sham-operated animals and 11 animals with lesion of the epithalamus in the *second series of experiments*. In the *second series of experiments*, three rats were eliminated because of unsatisfactory habenular lesion, and two were removed because of cortical damages in the region of the pineal, made during the removal of the pineal gland.

III.3.1 Social interaction test

The results are shown in **Fig 3.1**. One-way ANOVA showed no significant effect of group on social interaction time in both series of experiments (*first series:* $F_{1,21} = 0.31$, $p > 0.1$; *second series*: $F_{1,18} = 1.8 \times 10^{-4}$, $p > 0.1$).

Fig 3.1. Time spent in social interaction



Results (mean ± SEM, sec) are shown for the sham group (n=9) and the pinealectomy group (n=15) in the first experimental series (**left panel**), and for the sham group (n=9) and the group with entire epithalamic lesion (n=11) in the second experimental series (**right panel**).

*Les resultats (moyenne ± ESM, sec) sont indiqués pour le groupe sham (n=9) et le groupe pinéalectomisé (n=15) lors de la première série d'expériences (**gauche**), et pour le groupe sham (n=9) et le groupe ayant subi une lésion épithalamique totale (n=11) lors de la seconde série d'expériences (**droite**).*

III.3.2 Prepulse inhibition (PPI) of the auditory-evoked startle response

Baseline startle response. The average magnitude of the startle response to pulse alone stimuli during the different trial blocks is shown in **Table 3.1**. For both experiments, two-factor ANOVA (group, trial block as repeated factor) revealed no difference between groups (*first series*: $F_{1,22} = 0.001$, $p > 0.1$; *second series*: $F_{1,18} = 3 \times 10^{-4}$, $p > 0.1$) and no group x trial block interaction (*first series*: $F_{3,66} = 0.7$, $p > 0.1$; *second series*: $F_{3,54} = 0.2$, $p > 0.1$). On the other hand, while there was a significant effect of trial block in the *first series* ($F_{3,66} = 9.0$, $p < 0.0001$), there was no effect of trial block in the *second series* ($F_{3,54} = 0.4$, $p > 0.1$). We have no explanation for this difference, except that it may be related to different batches of animals being tested at different times of the year, as well as the fact that all animals in the *second series* underwent two operations.

Table 3.1. Startle amplitude

Group	PA0	PA1	PA2	PA3
First series				
Sham-operated	569 ± 111	414 ± 106	371 ± 102	365 ± 106
Pinealectomized	545 ± 60	363 ± 48	370 ± 60	430 ± 88
Second series				
Sham-operated	512 ± 84	480 ± 94	550 ± 91	515 ± 87
Lesioned	500 ± 78	530 ± 56	570 ± 75	544 ± 88

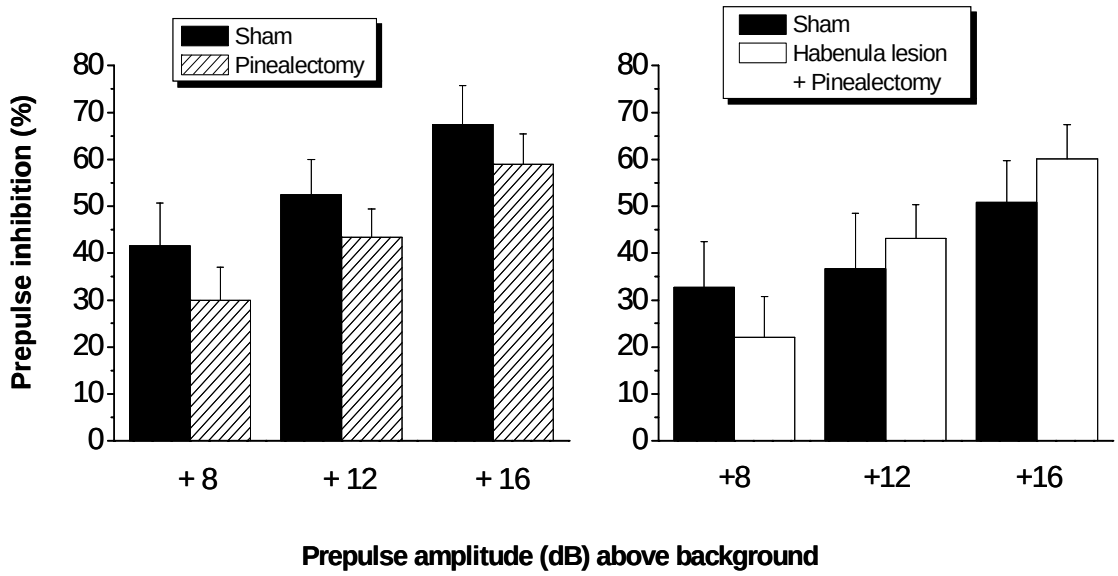
Amplitudes (mean ± SEM, grams) to the first three pulse alone stimuli (PA0) and to pulse alone stimuli during blocks 1, 2 and 3 (PA1, PA2 and PA3).

Amplitudes (moyenne ± ESM, grammes) du sursaut en réponse au premier stimulus seul (PA0) ainsi qu'aux stimuli seuls durant les blocks 1, 2 et 3 (PA1, PA2 and PA3).

Prepulse inhibition. The percentage PPI results are shown in **Fig 3.2**. In the experiment from the *first series* two-factor ANOVA (factors: group, prepulse intensity as a repeated factor) showed no effect of group ($F_{1,22} = 0.9$, $p > 0.1$), no interaction of group with prepulse intensity ($F_{2,44} = 0.14$, $p > 0.1$), but a significant effect of prepulse intensity ($F_{2,44} = 36.6$, $p < 0.0001$). In the experiment from the *second series* a two factor

ANOVA showed a significant effect of prepulse intensity ($F_{2,36}$ = 49, p < 0.0001), no significant effect of group ($F_{1,18}$ = 0.02, p > 0. 1) but a group x prepulse intensity interaction ($F_{2,36}$ = 7.2, p < 0.01). However at no individual prepulse intensity did the sham and epithalamus-lesioned groups differ significantly (2-tailed t-tests, p > 0.05).

Fig 3.2. PPI as percentage of startle amplitude in absence of a prepulse



Results (mean ± SEM, %) are shown for the sham group (n=9) and the pinealectomy group (n=15) in the first experimental series (**left panel**), and for the sham group (n=9) and the group with entire epithalamic lesion (n=11) in the second experimental series (**right panel**).

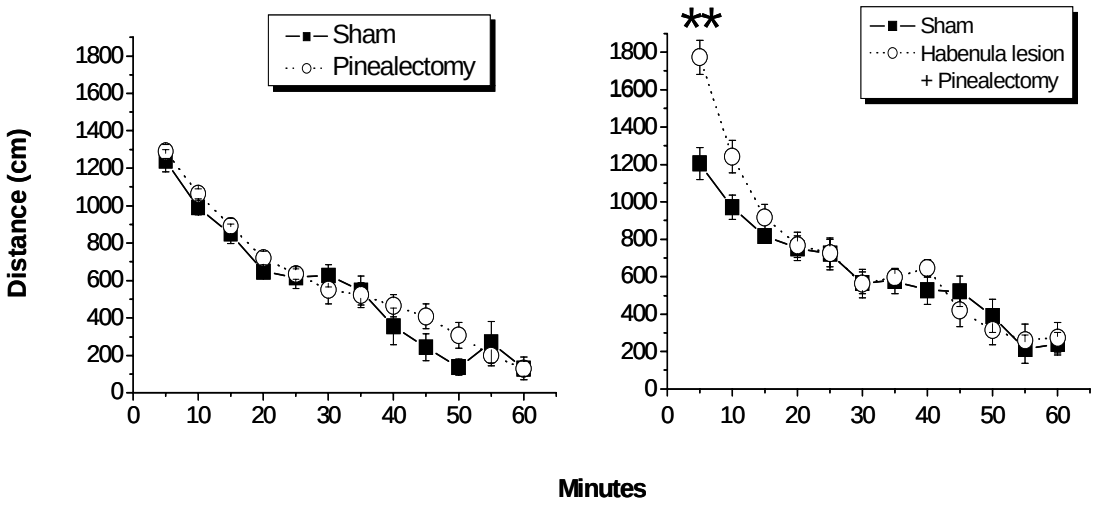
*Les resultats (moyenne ± ESM, %) sont indiqués pour le groupe sham (n=9) et le groupe pinéalectomisé (n=15) lors de la première série d'expériences (**gauche**), et pour le groupe sham (n=9) et le groupe ayant subi une lésion épithalamique totale (n=11) lors de la seconde série d'expériences (**droite**).*

III.3.3 Locomotor activity

The results are shown in **Fig 3.3**. In the experiment from the *first series* two-factor ANOVA revealed a significant effect of block ($F_{11,242}$ = 82.8, p < 0.0001) but no significant effect of group ($F_{1,22}$ = 0.77, p > 0.1) and no significant group x block interaction ($F_{11,242}$ = 1.12, p > 0.1). In the experiment from the *second series*, during the first 5 minutes of the session the lesioned rats showed hyperactivity compared to the sham animals, as previously shown (see Chapter II). Indeed, a planned comparison of values during the first 5 minutes showed a significant effect of group ($F_{1,18}$ = 20, p <

0.001). Over the whole recording period two-factor ANOVA showed that there was no significant effect of group ($F_{1,18}$ = 1.5, p > 0.1) but a significant group x block interaction ($F_{11,198}$ = 4.5, p < 0.001) and a significant effect of block ($F_{11,198}$ = 73, p < 0.0001).

Fig 3.3. Locomotor activity per 5-min



Results (mean ± SEM, cm) are shown for the sham group (n=9) and the pinealectomy group (n=15) in the first experimental series (**left panel**), and for the sham group (n=9) and the group with entire epithalamic lesion (n=11) in the second experimental series (**right panel**). * p < 0.05 *vs* sham (2-tailed Dunnett's test).

*Les resultats (moyenne ± ESM, cm) sont indiqués pour le groupe sham (n=9) et le groupe pinéalectomisé (n=15) lors de la première série d'expériences (**gauche**), et pour le groupe sham (n=9) et le groupe ayant subi une lésion épithalamique totale (n=11) lors de la seconde série d'expériences (**droite**). * p < 0.05 vs sham (test de Dunnett à deux bornes).*

III.3.4 Morris water maze

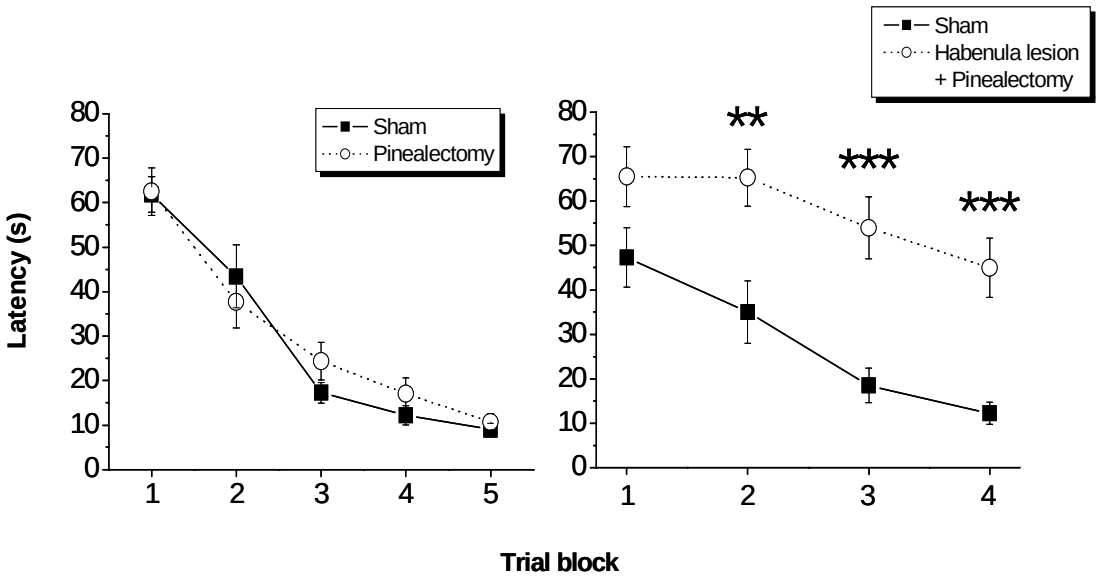
III.3.4.1 Practice swim

One way ANOVAs revealed no differences between the different groups in the distance swum during the practice swim (*first series*: $F_{1,22}$ = 2.7, p > 0.05; *second series*: $F_{1,17}$ = 0.7, p > 0.1) (results not shown).

III.3.4.2 Hidden platform trials

Escape latency. The results are shown in **Fig 3.4**. In the experiment from the *first series* two-way ANOVA showed that there were no significant effects of group ($F_{1,22} = 0.16$, $p > 0.1$), there was a significant effect of trial block ($F_{4,88} = 63.5$, $p < 0.0001$), and no significant interaction of these factors ($F_{4,88} = 0.8$, $p > 0.1$). In the experiment from the *second series* the two-way ANOVA revealed a significant effect of group ($F_{1,17} = 17.0$, $p < 0.01$) and trial block ($F_{3,51} = 14.0$, $p < 0.0001$), but no significant interaction of group x trial block ($F_{3,51} = 1.0$, $p > 0.1$).

Fig 3.4. Latency to find the hidden platform in the water maze as a function of trial block (4 trials per block)



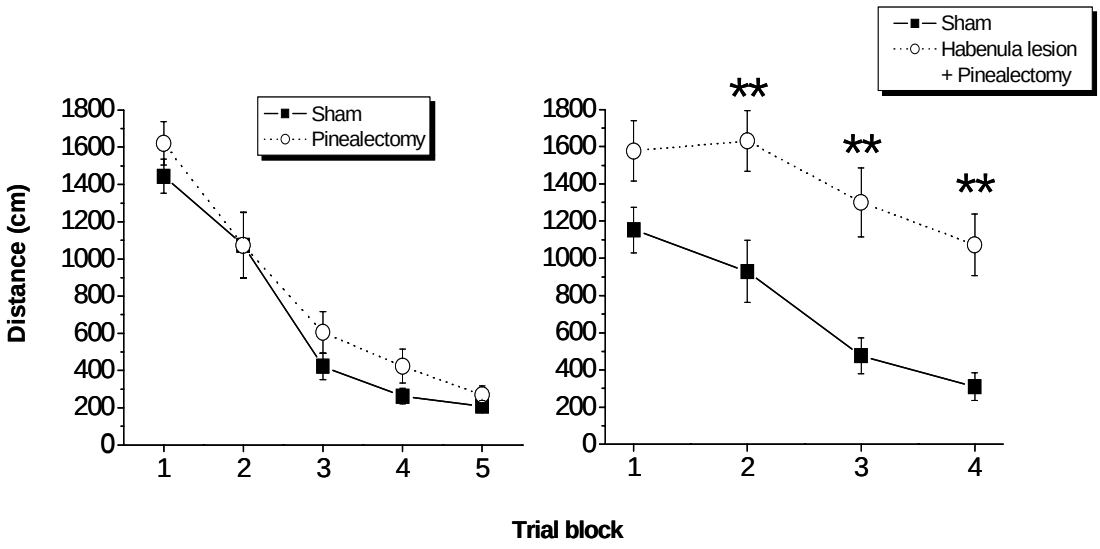
Results (mean ± SEM, sec) are shown for the sham group (n=9) and the pinealectomy group (n=15) in the first experimental series (**left panel**), and for the sham group (n=9) and the group with entire epithalamic lesion (n=11) in the second experimental series (**right panel**). ** $p < 0.01$, *** $p < 0.001$ *vs* sham (2-tailed Dunnett's test).

*Les resultats (moyenne ± ESM, sec) sont indiqués pour le groupe sham (n=9) et le groupe pinéalectomisé (n=15) lors de la première série d'expériences (**gauche**), et pour le groupe sham (n=9) et le groupe ayant subi une lésion épithalamique totale (n=11) lors de la seconde série d'expériences (**droite**). ** p < 0.01, *** p < 0.001 vs sham (test de Dunnett à deux bornes).*

Distance swum before finding the platform. The results are shown in **Fig 3.5**. In the experiment from the *first series* two-way ANOVA showed that there were no significant

effects of group ($F_{1,22}$ = 1.0, p > 0.1), a significant effect of trial block ($F_{4,88}$ = 65.5, p < 0.0001), but no significant interaction of these factors ($F_{4,88}$ = 0.4, p > 0.1). In the experiment from the *second series* the analysis revealed that the lesioned rats swam a longer distance to find the platform than did rats from the sham-operated group. Thus the two-way ANOVA showed a significant effect of group ($F_{1,17}$ = 13.0, p < 0.01), and of trial block ($F_{3,51}$ = 19.0, p < 0.0001) but no significant interaction of group x trial block ($F_{3,51}$ = 1.5, p > 0.1).

Fig 3.5. Distance swum before finding the hidden platform in the water maze as a function of trial block (4 trials per block)



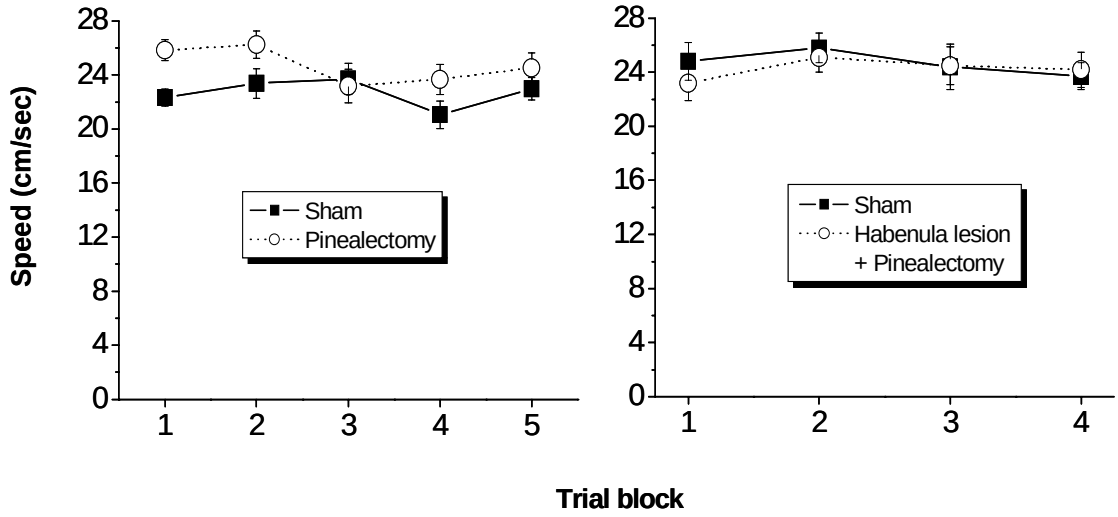
Results (mean ± SEM, cm) are shown for the sham group (n=9) and the pinealectomy group (n=15) in the first experimental series (**left panel**), and for the sham group (n=9) and the group with entire epithalamic lesion (n=11) in the second experimental series (**right panel**). ** p < 0.01, *** p < 0.001 *vs* sham (2-tailed Dunnett's test).

*Les resultats (moyenne ± ESM, cm) sont indiqués pour le groupe sham (n=9) et le groupe pinéalectomisé (n=15) lors de la première série d'expériences (**gauche**), et pour le groupe sham (n=9) et le groupe ayant subi une lésion épithalamique totale (n=11) lors de la seconde série d'expériences (**droite**). ** p < 0.01 vs sham (test de Dunnett à deux bornes).*

Swimming speed. The results are shown in **Fig 3.6**. In the experiments from both series two-way ANOVA revealed no significant effect of group (*first series*: $F_{1,22}$ = 3.3, p > 0.05; *second series*: $F_{1,17}$ = 0.1, p > 0.1). There was no effect of trial block (*first series*:

$F_{4,88}$ = 2.0, p > 0.05; *second series*: $F_{3,51}$ = 0.8, p > 0.1), and no interaction of these factors (*first series*: $F_{4,88}$ = 1.6, p > 0.1; *second series*: $F_{3,51}$ = 0.4, p > 0.1).

Fig 3.6. Swimming speed in the water maze as a function of trial block (4 trials per block)



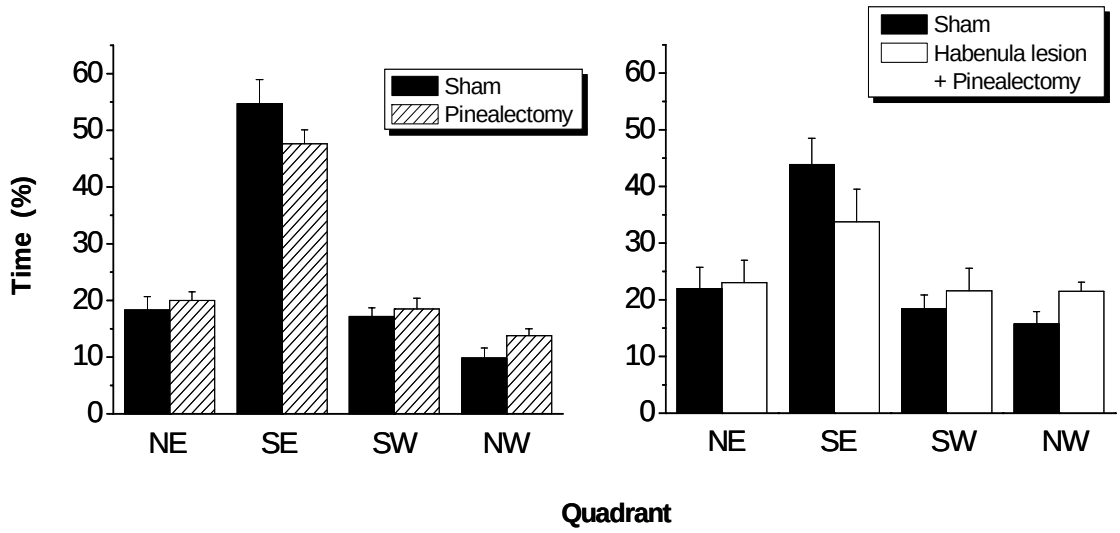
Results (mean ± SEM, cm/sec) are shown for the sham group (n=9) and the pinealectomy group (n=15) in the first experimental series (**left panel**), and for the sham group (n=9) and the group with entire epithalamic lesion (n=11) in the second experimental series (**right panel**).

*Les resultats (moyenne ± ESM, cm/sec) sont indiqués pour le groupe sham (n=9) et le groupe pinéalectomisé (n=15) lors de la première série d'expériences (**gauche**), et pour le groupe sham (n=9) et le groupe ayant subi une lésion épithalamique totale (n=11) lors de la seconde série d'expériences (**droite**).*

III.3.4.3 Probe trial

The percentages of time spent in the quadrants of the pool during the probe trials are shown in **Fig 3.7**. In both experiments, each group swam preferentially in the quadrant where the platform had been located during the learning phase, as shown by a highly significant effect of quadrant after two-way ANOVAs with quadrant as a repeated factor (*first series*: $F_{3,66}$ = 94.6, p < 0.0001; *second series*: $F_{3,51}$ = 8, p <0.001). One-way ANOVA of the percentage of time spent in the target (SE) quadrant showed no significant effect of group in either series (*first series*: $F_{1,22}$ = 2.37, p > 0.1; *second series*: $F_{1,17}$ = 1.6, p > 0.1).

Fig 3.7. Percentage of time spent in the four quadrants of the water maze during the probe trial. In the previous hidden platform trials the platform was located in the SE quadrant



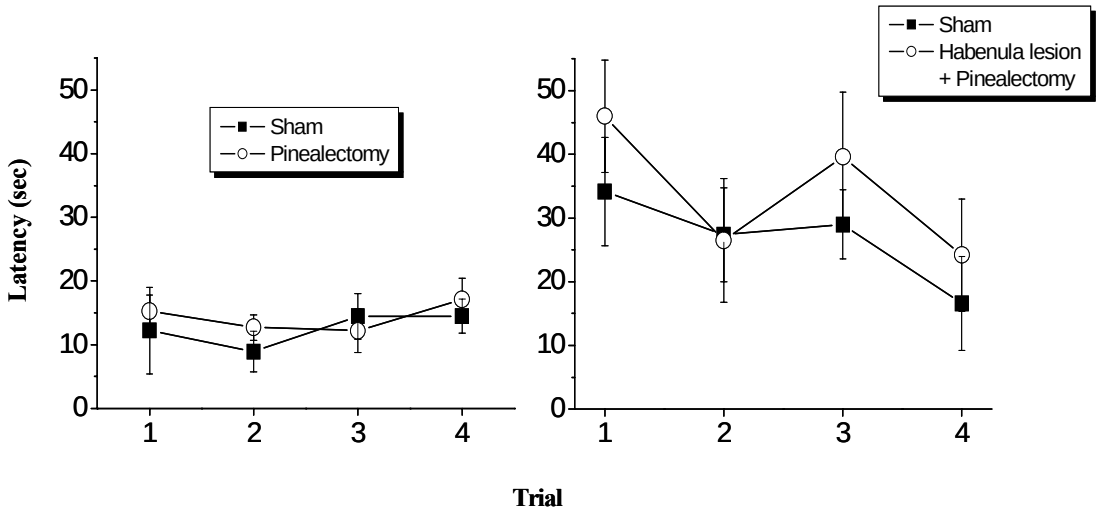
Results (mean ± SEM, %) are shown for the sham group (n=9) and the pinealectomy group (n=15) in the first experimental series (**left panel**), and for the sham group (n=9) and the group with entire epithalamic lesion (n=11) in the second experimental series (**right panel**).

*Les resultats (moyenne ± ESM, %) sont indiqués pour le groupe sham (n=9) et le groupe pinéalectomisé (n=15) lors de la première série d'expériences (**gauche**), et pour le groupe sham (n=9) et le groupe ayant subi une lésion épithalamique totale (n=11) lors de la seconde série d'expériences (**droite**).*

III.3.4.4 Visible platform condition

The results are shown in **Fig 3.8**. Since in both experiments animals were swimming directly to the platform by the end of the first block of trials, the experiment was ended after this first block. In the experiment of the *first series* two-factor ANOVA (group, trial as repeated factor) showed no significant effect of group on the escape latency ($F_{1,22} = 0.22$, $p > 0.1$), no effect of trial ($F_{3,66} = 1.2$, $p > 0.1$) and no interaction of these factors ($F_{3,66} = 0.5$, $p > 0.1$). Similarly, in the experiment of the *second series* there was no significant effect of group ($F_{1,17} = 0.83$, $p > 0.1$), no effect of trial ($F_{3,51} = 2.27$, $p > 0.05$) and no interaction of these factors ($F_{3,51} = 0.25$, $p > 0.1$).

Fig 3.8. Performance in the visible platform condition. Latency to find the platform in the water maze as a function of trial



Results (mean ± SEM, sec) are shown for the sham group (n=9) and the pinealectomy group (n=15) in the first experimental series (**left panel**), and for the sham group (n=9) and the group with entire epithalamic lesion (n=11) in the second experimental series (**right panel**).

*Les resultats (moyenne ± ESM, sec) sont indiqués pour le groupe sham (n=9) et le groupe pinéalectomisé (n=15) lors de la première série d'expériences (**gauche**), et pour le groupe sham (n=9) et le groupe ayant subi une lésion épithalamique totale (n=11) lors de la seconde série d'expériences (**droite**).*

III.3.5 Histology

As previously shown (see **Chapter II**) habenula lesion destroyed a large proportion of both medial and lateral habenula without causing substantial damage to neighbouring structures.

III.3.6 ChAT assay

ChAT assay was performed for sham and lesioned animals of the *second series*. As shown in **Table 3.2** animals with lesions of the habenula had a marked reduction, by 75 %, of ChAT in the interpeduncular nucleus. This result is consistent with those of previous neurochemical and histochemical studies (Contestabile *et al*, 1987; Eckenrode *et al*, 1987; Villani *et al*, 1983) and indicates marked degeneration of the habenulo-interpeduncular tract.

Table 3.2. Choline acetyltransferase in homogenates of interpeduncular nucleus

Group	ChAT (μmoles/g wet wt/hr)
Sham-operated	78.3 ± 3.6
Lesioned animals	19.9 ± 0.9 (25.4 ± 1.15)**

Results (mean ± SEM; μmoles/g wet wt/hr) are shown for the sham group and the habenula-lesioned group of the second series of experiments. Values in parentheses are expressed as percentage of the sham value. ** $p < 0.001$ *vs* sham-operated (2-tailed t-test).

*Les resultats (moyenne ± ESM; μmoles/g de matière sèche/hr) sont indiqués pour le groupe sham et le groupe habenulo-lésé lors de la deuxième série d'expériences. Les valeurs entre parenthèses sont exprimées en pourcentage des valeurs correspondantes issues du groupe sham. ** $p < 0.001$ vs sham (test t à deux bornes).*

III.3.7 Melatonin assay

Melatonin assay of plasma samples was performed for sham and pinealectomized animals of the *first series*. The results are shown in **Table 3.3**. Plasma melatonin concentrations in sham-operated animals were consistent with the typical light-dark rhythm, and in pinealectomized animals were markedly reduced. An ANOVA with sampling time as a repeated measure showed significant effects of group ($F_{1,22} = 110.8$, $p < 0.00001$), sampling time ($F_{2,44} = 120.0$, $p < 0.00001$) and a significant interaction of group x sampling time ($F_{2,44} = 113.6$, $p < 0.00001$). Comparisons between sham and pinealectomized animals at individual sampling times showed significant differences at midnight and 3 a.m. (2-tailed t-tests, $p< 0.00001$ in each case).

Table 3.3. Plasma melatonin concentration

	At 4 p.m.	At midnight	At 3 a.m.
Sham	4.8 ± 2.4	82.0 ± 8.4	80.3 ± 9.0
Pinealectomy	2.0 ± 1.4	3.0 ± 2.2***	3.1 ± 1.8***

Results (mean ± SEM; pg/ml) are shown for the sham group and pinealectomized group of the first series of experiments. Values in parentheses are expressed as percentage of the sham value. *** $p < 0.00001$ *vs* sham-operated (2-tailed t-test).

*Les resultats (moyenne ± ESM; pg/ml) sont indiqués pour le groupe sham et le groupe pinéalectomisé lors de la première série d'expériences. Les valeurs entre parenthèses sont exprimées en pourcentage des valeurs correspondantes issues du groupe sham. *** p < 0.00001 vs sham (test t à deux bornes).*

III.4 Discussion

The aim of the present experiments was to test the hypothesis that pineal damage contributes to the symptoms of schizophrenia. We tested whether loss of pineal function alone caused schizophrenia-like changes in social behaviour, memory function and prepulse inhibition. The results showed that none of these functions were altered by pinealectomy. To investigate whether pineal damage might interact with habenula damage such that more behavioural alterations would occur than after habenula damage alone, these behaviours were also examined in animals with combined habenula lesion plus pinealectomy. The results showed that pinealectomy superimposed on lesion of the habenula did not alter the qualitative pattern of behavioural alterations previously found after habenula lesions. The possibility of some quantitative alteration remains open however, since we did not directly compare habenula lesions and combined lesions in the same experiments. These points are discussed in more detail below.

First, it is of interest to compare the lack of effect of pinealectomy on memory function observed here, with the results of previous studies. Among the earliest studies, Relkin (1970) studied prepubertal or postpubertal pinealectomy on maze performance for food reward in male rats. Neither number of runs to criterion, number of errors, nor running time was altered by pinealectomy in either bright or dim light conditions. The present results are therefore in good agreement with those obtained by Relkin (1970). Also, similar to these results, are those of Catala *et al* (1985), who investigated the effects of pinealectomy on light-signaled two-way active avoidance in male rats. During the dark phase there was no difference between pinealectomized and sham-operated animals in speed of acquisition, whereas during the light phase pinealectomized rats actually conditioned more rapidly. Appenrodt & Schwarzberg (2003) also found no effect of pinealectomy on the acquisition of a conditioned active avoidance response, though pinealectomy prevented the prolongation of extinction produced by vasopressin. Moreover, in a test of social recognition memory, pinealectomy had no effect on performance, but affected the modulation of this memory by intraseptal vasopressin (Appenrodt *et al*, 2002). Similarly, in passive avoidance learning in rats pinealectomy alone had no effect on performance, but blocked the increase of retention latency caused when vasopressin was injected before the retention test (Juszczak *et al*, 1996). Thus our results are in agreement with most previous results from a variety of memory tests that

indicate, that baseline memory function is not impaired after pinealectomy. The effect of the combined habenula lesion and pinealectomy confirms the previously observed effects of habenula lesion alone (see **Chapter II**). Memory impairment was clearly shown by latency to find the platform and distance swum before finding the platform, whereas in the probe trial the difference between lesioned and sham animals in time spent in the target quadrant tended to show worse performance in the lesioned animals but was not statistically significant. Whereas in our previous study there was some reduction of latency in habenula-lesioned animals over the first 4 blocks of trials, there was almost no such change in the present studies. However direct comparison is complicated by the use of a larger maze in the present studies, so that further experiment would be necessary to determine if this represents a quantitatively greater impairment produced by pinealectomy in habenula-lesioned rats.

Neither pinealectomy alone, nor in combination with bilateral habenula lesions, had any effect on PPI of the acoustic startle response, and to our knowledge the effect of pinealectomy on PPI has not previously been studied. The results therefore provide no support for an effect of pineal dysfunction on PPI or in having any effect on PPI in animals with habenula damage.

In the case of social behaviour we found no effect of pinealectomy on the time spent in social contact during a brief exposure to a juvenile of the same strain. Neither did pinealectomy combined with habenula lesion result in any alteration of social exploration in this situation. There have been relatively few previous studies of social behaviour in pinealectomized animals. In examining aggressive encounters between mice McKinney *et al* (1975) found that during 15 min pairings, latency to initiation of fighting was increased twofold and duration of fighting was reduced approximately 40 percent if the pair contained at least one pinealectomized animal. Pinealectomized and sham-operated mice were equally likely to initiate aggression, but sham males were ranked as dominant in 75 percent of pairings of a sham and a pinealectomized animal. Also, melatonin is reported to facilitate aggression in mice (Paterson & Vickers, 1981). Thus, although pinealectomy may not affect the earliest aspects of social encounters it does appear to impair aggressive behaviour. Therefore positive evidence for a role of the pineal in social behaviour may be more likely to be obtained in longer social encounters than those examined here. Some further support for a role of the pineal in

aggressive behaviour under certain conditions is provided by a study of female hamsters showing that animals maintained under short photoperiod exhibited the highest level of offensive behavior and the lowest level of defensive behavior, and that pinealectomy eliminated these effects of short photoperiod (Fleming *et al*, 1988). Thus further study of the effect of pinealectomy on social behaviour in situations more refined than a simple short encounter could be of interest. In that patients with schizophrenia are generally less effective in competing with their peers, i.e. they are over-represented in the lower socioeconomic groups, it is a topic for further research whether this corresponds to reduced aggression due to pineal dysfunction.

Although the present experiments suggest no direct involvement of pineal dysfunction in spatial memory, social behaviour or PPI, further considerations must be taken into account in considering whether pineal dysfunction might play a role in the symptoms of schizophrenia in man. One consideration is that the rat has a superficial and a deep pineal, whereas humans have only a deep pineal (Vollrath, 1992). The location of the deep pineal of the rat is located in very close proximity to the habenula, considerably removed from the superficial portion, so that the pinealectomy performed here removed only the superficial pineal. Thus it remains possible that damage to the deep pineal might play a role in schizophrenia-like behavioral changes. This question could probably best be approached by studies in an animal with a more prominent deep pineal than the rat. A further consideration is that the pineal hormone melatonin is antioxidant and neuroprotective in a variety of situations (Iacovitti *et al*, 1997; Kondoh *et al*, 2002; Raghavendra & Kulkarni, 2001; Reiter, 1998), whereas pinealectomy can potentiate neurotoxicity (De Butte *et al*, 2002). Since patients with schizophrenia show indices of greater oxidant stress (Dakhale *et al*, 2004; Sirota *et al*, 2003) lack of melatonin might amplify the neurotoxic effects of this oxidant stress. Such an effect would not have been revealed in the present experiments because of the relatively short duration of the pinealectomy, and because the animals were not subjected to any enhanced oxidant stress.

In summary, in agreement with previous studies in different memory tests, pinealectomy had no effect on memory function in the Morris maze. It also did not affect social contact in a brief encounter with a juvenile conspecific, although evidence from previous studies of aggression indicates that pinealectomized animals are less

aggressive under certain conditions. Pinealectomy also did not affect PPI of an acoustic startle response. Thus there appear to be no direct effects of short-term pinealectomy on memory formation or PPI. By analogy with previously published effects on aggression, effects on other social behaviours deserve further attention. Also in human diseases, such as schizophrenia, involving oxidant stress or neurodegeneration, possible indirect effects due to reduction of the protective effects of melatonin must be considered.

Finally, considering the lack of effects of the pinealectomy, we decided after this series of experiments to focus on habenula lesions and not to perform pinealectomy anymore in the following studies.

"La cathédrale de Rouen"

Monet - 1894

Chapter IV - Attentional performances of habenula-lesioned rats assessed by the 5-choice serial reaction time task

IV.1 Introduction

The anatomical connections of the habenula indicate that it is a link between forebrain areas and midbrain cell groups such as the locus coeruleus, raphé nuclei, substantia nigra and ventral tegmentum that project widely to most brain regions (Sutherland, 1982). Consistent with such widespread influences it is implicated in diverse functions including anxiety (Kurumaji *et al*, 2003; Murphy *et al*, 1996), stress (Amat *et al*, 2001; Sica *et al*, 2000), analgesia (Cohen & Melzack, 1993), maternal behavior (Corodimas *et al*, 1993; Felton *et al*, 1998), sleep (Haun *et al*, 1992; Valjakka *et al*, 1998), behavioral flexibility (Thornton and Evans, 1982), reinforcement (Sutherland & Nakajima, 1981) and spatial memory (see **Chapter II**). The recent description of fifteen subnuclei within the habenula (Andres *et al*, 1999; Geisler *et al*, 2003) should eventually be of great assistance for future research aimed at elucidating the particular circuits that mediate these specific behaviors.

Recently a number of findings have begun to suggest that pathology of the habenula could be involved in some of the symptoms of schizophrenia. Thus Sandyk (1992) reported that large calcifications of the habenula are more frequent in schizophrenia patients. The epithalamus as a whole, comprising habenula plus the pineal organ, has also been shown to exhibit greater calcification in schizophrenia patients than controls (Caputo *et al*, 1998). A role of habenula pathology in schizophrenia has been proposed by Ellison (1994) based on several lines of evidence, particularly the findings that chronic administration of amphetamine or cocaine to rats causes degeneration in the fasciculus retroflexus output pathway of the habenula, and in man can elicit a schizophrenia-like state. Several hypotheses of how habenula pathology could generate symptoms of schizophrenia have been proposed (Sandyk, 1991; Ellison, 1994; Kelly, 1998). If as suggested, habenula pathology does contribute to the symptoms of schizophrenia then habenula lesions in experimental animals should produce behavioral changes resembling those in schizophrenia. Recently, we began to examine this prediction by investigating the effects of habenula lesions on functions that are impaired

in schizophrenia, namely memory performance, prepulse inhibition (PPI) and social interaction. Our results showed that habenula-lesioned rats exhibited a deficit in spatial learning in the Morris water maze, but no change of prepulse inhibition (PPI) or of social exploration, implicating habenula pathology particularly in memory impairment (see **Chapter II**).

Another frequent neurocognitive abnormality in schizophrenia, noted already by Kraepelin (1919/1971) and Bleuler (1911/1950) is disturbed attention. The concept of attention nowadays encompasses several aspects, such as sustained attention or the continuous allocation of sensory processing resources for the detection of rare events, divided attention, to monitor and respond to several different sensory channels, and selective attention, the ability to focus sensory processing resources on certain types of stimuli, while ignoring others (Robbins, 2002). Introspective reports from patients with schizophrenia (McGhie & Chapman, 1961), such as "I can't concentrate. It's diversion of attention that troubles me", indicated problems in selective and sustained attention. Quantitative studies of sustained attention have mostly used the continuous performance task (CPT), in which the subject must respond to a target stimulus whenever it appears in a rapid succession of non-target stimuli. In this task, and versions of it that either increase the working memory load by requiring a sequence of stimuli to be detected, or increase the sensory processing burden by degrading the stimuli, numerous studies have shown that patients with schizophrenia have, on average, impaired performance (Cadenhead & Braff, 2000; Nestor & O'Donnell, 1998; Orzack & Kornetsky, 1966). Such deficits are indicated not only by hit rate and errors, that are influenced by responses bias, but also by changes in the information processing sensitivity index, d', which is independent of responses bias. A recent meta-analysis has shown that worse performance by patients with schizophrenia in this task, assessed by d', correlates with negative symptoms (Nieuwenstein *et al*, 2001). Similar effects in a substantial proportion of non-affected siblings of patients with schizophrenia (Chen & Faraone, 2000; Finkelstein *et al*, 1997) suggest that attention deficit may be a marker of genetic susceptibility to schizophrenia.

To further test the hypothesis that habenula lesions in experimental animals should produce behavioral changes resembling those in schizophrenia the present studies examine if habenula lesions modify performance in a well-studied attention task, the 5-

choice serial reaction time task (5-CSRTT) in which a rat must attend to one wall of a Skinner box containing five recesses. The rat must quickly respond to a brief light stimulus that appears randomly in one of these recesses in order to obtain reinforcement (Carli *et al*, 1983; Robbins, 2002). This task allows changes in different aspects of attentional performance such as choice accuracy, premature responding and perseverative responding to be determined in the same test. Moreover a considerable amount has been discovered about the physiology and pharmacology of this task (Robbins, 2002), so that the results obtained may be integrated with this knowledge.

IV.2 Materials and methods

IV.2.1 Animals

The experiments were carried out with 24 male Lister-Hooded rats (Iffa Credo, France) housed in pairs in Macrolon cages (42 × 26 × 15 cm) in a temperature-regulated (22 ± 2°C) animal room on a 12 h light/dark cycle (lights on at 0600). Lister-Hooded rats were chosen because they are the best performers in such a test. Drinking water was available ad libitum. Rats performed the test daily, except that during the pre-lesion training they were not run at weekends. On testing days the animals received 15 - 16 g of food (Nafag 890 Rat Chow, Provimi Kliba, Switzerland) per day per rat, given immediately after the test (equally distributed in two opposite corners of the cage). When no tests were performed at the weekend lab chow was available ad libitum from Friday evening until 11 a.m. on Sunday morning. The experiments were approved by the Cantonal Veterinary Authority of the City of Basel. Animals were acclimatized to the animal quarters for at least a week before starting the experiments, which took place during the light phase.

IV.2.2 Surgical procedures

Lesions of the habenula or sham operations were performed as previously described (see **Chapter II**, page 69) except that the animals were anesthetized with isoflurane (Forene®, Abbott, USA)

IV.2.3 Apparatus

Six nine-choice serial reaction time task chambers (Med Associates, Vermont, USA) were used during this study. Each rat was run in the same chamber during the entire series of experiments. Five of the nine nose-poke recesses were used (numbers 1, 3, 5, 7 and 9), the others were blocked by screwed-on metal plates. The chambers were equipped with a dim house light, a stimulus light inside each recess and a pellet dispenser in the wall opposite to the stimulus recesses. Nose-pokes into the stimulus light recesses or into the food pellet well were registered by photocell beam assemblies. The chambers were housed in sound-insulated and ventilated enclosures.

IV.2.4 The 5-choice serial reaction time test

The test was as originally described by Carli *et al* (1983). All experimental contingencies and collection of data were controlled by a computer program written in the MED-PC language (Med Associates, Vermont, USA). The test began by the switching on of the house light and delivery of a food pellet into the feeder. When the rat retrieved a pellet from the feeder a 5-sec intertrial interval (ITI) was initiated, followed by switching on a stimulus light in one of the stimulus recesses. If the animal nose-poked into the correct recess during the stimulus or within an immediately-following limited hold period, a reward (45 mg Noyes pellet, Bilaney AG, Frankfurt, Germany) was delivered into the feeder (see **Fig 4.1**). Throughout the experiment the parameters analyzed were the following:

Correct responses. A correct response was recorded when the first nose-poke after the stimulus onset within the time allowed (stimulus duration + limited hold) was into the recess where the stimulus appeared.

Incorrect responses. An incorrect response was recorded when the first nose-poke after the stimulus onset within the time allowed (stimulus duration + limited hold) was into a recess where the stimulus did not appear.

Response omissions. An omission was recorded when no recess was nose-poked within the time allowed (stimulus duration + limited hold).

Premature responses. A premature response was recorded for every nose-poke response (i.e. into any stimulus recess) made in the intertrial interval (ITI) between the animal retrieving a pellet and the onset of a stimulus (*i.e.* responses made after initiating a trial but before the appearance of the stimulus).

Perseverative responses. Responses made after a correct or incorrect response in the same or any other recess before nose-poking the food-reward well.

Latency to collect the food pellet. The time from a correct response to nose-poking the food-reward well to collect the food pellet, averaged over the session.

Latency of correct responses. The time from onset of the stimulus light until a correct nose-poke response, averaged over the session.

Latency of incorrect responses. The time from onset of the stimulus light until an incorrect nose-poke response, averaged over the session.

Whereas correct responses were rewarded by delivery of a food pellet, incorrect responses were followed by a timeout (TO) when the house light was switched off until the rat nose-poked into the pellet feeder well, which ended the TO and started another 5-sec ITI before stimulus presentation. Premature responses during the ITI caused a 5-sec TO, during which any further premature responses reset the TO to the beginning. Moreover, if the animal made no nose-poke into any recess within the duration of the stimulus and limited hold then this "missed response" also initiated an unlimited TO, from which the only means of exit was a nose-poke into the food well.

Percent correct responses, the measure of choice accuracy, and percent omissions were calculated daily to determine if a rat should progress to a higher level of difficulty. *Percent correct responses* was calculated as correct responses / (correct responses + incorrect responses) x 100. *Percent omissions* was calculated as response omissions / (correct responses + incorrect responses + response omissions) x 100.

One test was given per day, lasting 30 minutes or 100 reinforcements, whichever came first. During each session, the stimulus light was presented approximately an equal number of times in each of the five holes, chosen randomly.

The animals were operated after at least 4 weeks at level 6, and their allocation into the two surgery groups was conducted in a counterbalanced manner based on their pre-operative baseline performance. Animals that did not reach level 6 were not included in the experimental analysis. However, so that each cage contained either two lesioned or two sham-operated animals that were treated identically, they were operated appropriately and performed the test daily, receiving the same drug treatment as their cage partners. In this way variability between cages, in terms of number of rats per cage and number of drug-treated rats per cage, was reduced.

IV.2.5 Pre-operative training

Before starting training rats were food-deprived (15 – 16 g of rat chow per day) and allowed to eat the food pellets in their home cages for 2 days, in order to habituate them to this new food. On the next day the rats were placed singly in the test chambers with 3 food pellets in the food well and each stimulus light recess, and allowed to explore the box and eat the pellets. The following day automatic training was initiated at the easiest level of difficulty, level 1. Whenever an animal met on two consecutive days criteria of >80 % correct responses and <20 % omissions, as defined below, it was progressed on the following day to the next level of difficulty (see **Table 4.1**).

Table 4.1. Stimulus and Limited hold durations across the 6 levels of difficulty

Levels	Stimulus duration (sec)	Limited hold duration (sec)
1	30	30
2	15	15
3	5	15
4	2	10
5	1	10
6	0.5	5

IV.2.6 Post-operative evaluation

The postoperative evaluation of the lesioned and sham operated control animals was comprised of the following phases:

Phase 1: Baseline performance 9-20 days post-surgery

Post-operative testing began 9 days after surgery. Of eleven animals that received lesions four were excluded because of not satisfying the criteria of substantial bilateral damage to the medial and lateral habenula with only minimal damage to neighboring structures. The sham group was composed of nine rats. To assess the effect of the lesion on baseline performance shortly after lesion the animals performed the task at level 6 once per day for twelve successive days.

Phase 2: Effect of d- amphetamine on task performance

To evaluate the response of the rats to an enhancement of dopaminergic transmission, a single dose of amphetamine (0.2 mg/kg *s.c.*, 30 minutes before the session), was administered to the animals in a crossover design that allowed sensitive within-subjects statistical comparisons. On the first day every rat received a vehicle injection (0.9% NaCl, 1 ml/kg *s.c.*, 30 min before the session) to accustom them to being injected. On the second day, half the animals (alternate animals) were given drug and half vehicle. On the third day, all rats received vehicle. On the fourth day, the animals that had previously received drug received vehicle, and those that had previously received vehicle received drug. The data collected in the tests of the second and the fourth day were used for statistical comparisons.

Phase 3: Baseline performance 41-52 days post-surgery

Following the amphetamine experiment, the animals were not tested for a week, after which they were returned to the baseline task and tested drug-free for a further twelve days.

Phase 4: Effect of haloperidol on task performance

On the day immediately following the previous phase the animals were injected with vehicle (1% lactic acid, pH 4.5, *i.p.*, 30 min before the session), and on the following day all of them received an acute injection of haloperidol (0.1 mg/kg in 1% lactic acid, pH 4.5, *i.p.*, 30 min before the session). It was clear that after 0.1 mg/kg haloperidol most animals responded very little in this task. Therefore, the rats were returned to baseline drug-free for four consecutive days, and then were tested with two lower doses of haloperidol or vehicle in a Latin square design. The treatments were vehicle (1% lactic acid, pH 4.5) or haloperidol (0.01 mg/kg and 0.03 mg/kg). All treatments were administered *i.p.* 30 minutes before the session. Between treatment days, to allow elimination of drug, the animals performed the task after vehicle treatment during three consecutive days.

Phase 5: Baseline performance 77-84 days post-surgery

Following the haloperidol experiment, the animals were not tested for a week, after which they were returned to the baseline task and tested drug-free for a further eight days.

Fig 4.1. The five-choice serial reaction time task

(**Left**) The rat must nose-poke in the correct recess where the stimulus light appears. (**Right**) Once the nose poke is made, the rat must collect the food pellet which is automatically delivered in the food well in the opposite wall of the Skinner box.

*(**Gauche**) Le rat doit placer son museau au niveau de l'ouverture dans laquelle est apparu le stimulus lumineux. (**Droite**) Ensuite, il doit récupérer la pastille de nourriture automatiquement délivrée dans la mangeoire se situant dans le mur opposé.*

IV.2.7 Drugs

All solutions were prepared fresh on the day of use. D-amphetamine sulfate (Siegfried, Zofingen, Switzerland) was dissolved in 0.9% NaCl and injected in a volume of 1 ml/kg. Haloperidol (Sigma, St Louis, MO, USA) was dissolved in 1% lactic acid, brought to pH 4.5 by addition of 1 M and 0.1 M NaOH and injected in a volume of 1 ml/kg. Vehicle-treated animals received the same volume of the corresponding vehicle.

IV.2.8 Assessment of the lesions

The procedures (histology and ChAT assay) have been previously described in **Chapter II** (pages 72-73).

IV.2.9 Statistics

Concerning the post-operative drug-free performances, average values of the various performance parameters were calculated per rat for each of the three drug-free blocks of trials that comprised the phases 1, 3 and 5 described in the Methods, and these were analyzed by two factor ANOVA with lesion group as one factor and trial block as a repeated factor. In the case of a significant effect of lesion group or a significant lesion group x session interaction, the data of the two groups in individual trial blocks were compared by one-way ANOVA to see when the differences occurred. Within the sham and lesion groups comparisons of the data from block 1 to that from later blocks were made by paired t-tests. Data from the amphetamine and haloperidol experiments were analyzed by ANOVA with drug treatment as a repeated factor to correspond to the use of crossover and Latin square designs in these experiments. In the case of a significant effect of lesion group or a significant lesion group x drug treatment interaction, the data of the two groups at individual dose levels were compared by one-way ANOVA. Within the sham and lesion groups comparisons of individual doses to the vehicle control condition were made by paired t-tests. All statistical analyses were performed by means of the SYSTAT software package (Version 10.2, SPSS Inc., Chicago, IL, USA).

IV.3 Results

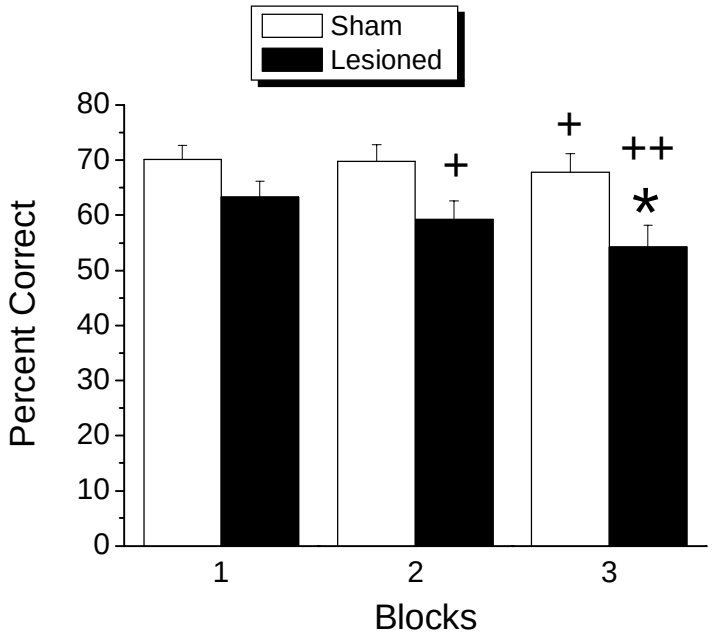
After exclusion of animals with unsatisfactory lesions there were 7 lesioned animals and 9 sham-operated animals. From the 24 rats at the beginning of the training session, 4 failed to reach the criterion and were not included in the statistical analysis. From the 11 lesioned animals, 4 did not have a satisfactory lesion and were also eliminated from the statistical analysis. Moreover, treatment with 0.1 mg/kg of haloperidol did not allow any analysis by ANOVA, as this dose markedly reduced responding, probably due to effects on the motor system, sham and lesioned animals making only 3.9 ± 1.3 and 3.4 ± 1.5 (mean ± SEM) total responses respectively. Otherwise no data was excluded from analysis.

IV.3.1 Choice accuracy

Effects of bilateral habenula lesions on drug-free performance

Two factor ANOVA showed a significant difference between groups ($F_{1,14} = 5.27$, $p < 0.05$), a significant effect of trial block ($F_{2,28} = 15.98$, $p < 0.0001$) and a significant interaction of these factors ($F_{2,28} = 5.42$, $p < 0.01$). These results confirm statistically the conclusion that can be drawn from inspection of **Fig 4.2**, that lesioned animals performed less accurately than control animals, and that the difference between control and lesioned animals was greater as training progressed. In fact in comparisons between the groups during the individual trial blocks, a significant difference between the control and lesion groups occurred only in the third block.

Fig 4.2. Percent correct across the 3 blocks of baseline performances



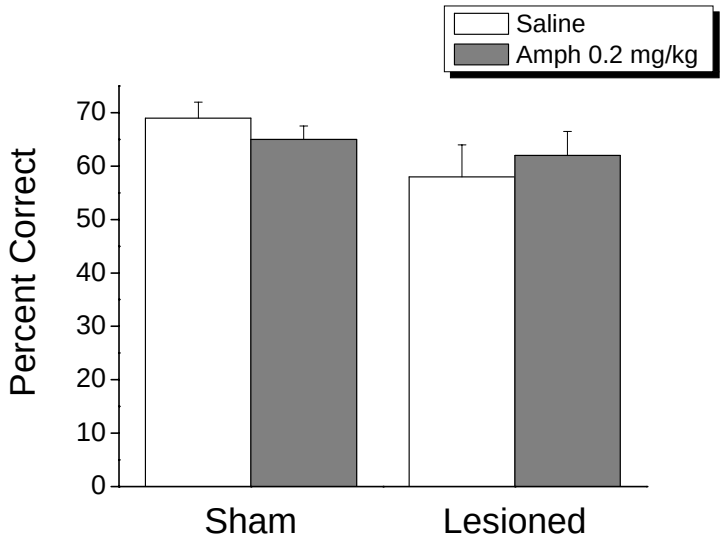
Choice accuracy (mean ± SEM) is shown during blocks of drug-free performance on post-operative days 9-20 (block 1), 41-52 (block 2) and 77-84 (block 3). Columns and bars show the means ± SEM of sham-operated (n=9) and bilateral habenula-lesioned subjects (n=7). * $p < 0.05$ compared to the sham group at the same time point (one-factor ANOVA); + $p < 0.05$, ++ $p < 0.01$ *vs* same group at block 1 (2-tailed paired t-test).

*Les performances de base (moyenne ± ESM) du groupe sham (n=9) et du groupe habénulo-lésé (n=7), concernant le pourcentage de réponses correctes, sont indiquées en fonction des trois sessions de mesures, soit du jour 9 au jour 20 après l'opération (session 1), du jour 41 au jour 52 après l'opération (session 2) et du jour 77 au jour 84 après l'opération (session 3). * p < 0.05 comparé aux performances du groupe sham au cours de la même session (ANOVA à un facteur); + p < 0.05, ++ p < 0.01 comparé aux performances du même groupe au cours de la session 1 (test t à deux bornes).*

Effects of d-amphetamine treatment

There was no effect of lesion group on the percentage of correct responses ($F_{1,14} = 2.4$, $p > 0.1$). Neither was there a significant effect of drug treatment ($F_{1,14} = 0.007$, $p > 0.1$) nor any significant interaction of lesion group x drug treatment ($F_{1,14} = 1.9$, $p > 0.1$) (**Fig 4.3**).

Fig 4.3. Effects of d-amphetamine treatment



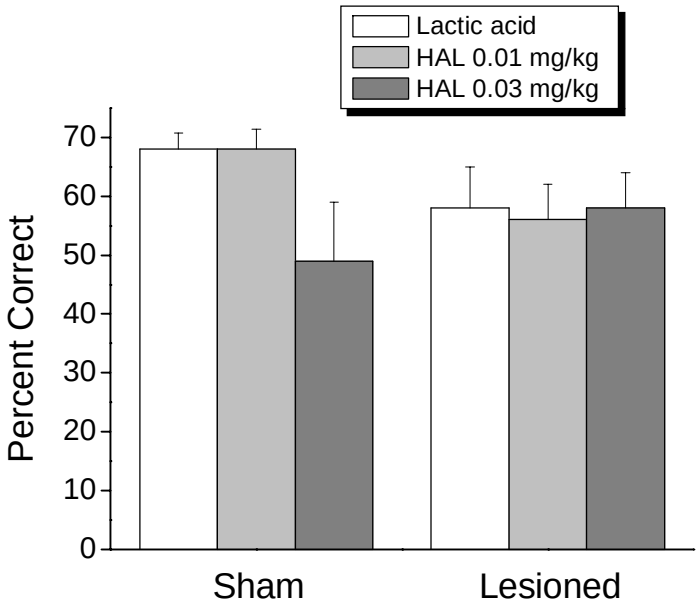
Choice accuracy (mean ± SEM) is shown during sessions where the rats received saline (NaCl 0,9 %, s.c.) or d-amphetamine (0.2 mg/kg, *s.c.*) 30 minutes before testing. Columns and bars show the means ± SEM of sham-operated (n=9) and bilateral habenula-lesioned subjects (n=7).

Les performances (moyenne ± ESM) du groupe sham (n=9) et du groupe habénulo-lésé (n=7), concernant le pourcentage de réponses correctes, sont indiquées suite à une injection de solution saline (NaCl 0,9 %, s.c.) ou de d-amphétamine (0,2 mg/kg, sc) 30 minutes avant le test.

Effects of haloperidol treatment

The results are shown in **Fig 4.4**. There was no significant effect of lesion group on the percentage of correct responses ($F_{1,14} = 0.3$, $p > 0.1$). Neither was there a significant effect of drug treatment ($F_{2,28} = 3.2$, $p > 0.1$), but the interaction of lesion group x drug treatment was significant ($F_{2,28} = 3.8$, $p < 0.05$). Inspection of the data showed that the 0.03 mg/kg dose of haloperidol impaired choice accuracy in sham, but not lesioned animals. Post-hoc comparison in the sham group between the 0.03 mg/kg dose of haloperidol and vehicle treatment showed a difference that was not quite significant (paired t-test, $p = 0.055$), so this effect is currently best considered to be a strong tendency.

Fig 4.4. Effects of haloperidol treatment



Choice accuracy (mean ± SEM) is shown during sessions where the rats received lactic acid (1% pH 4.5, *i.p.*) or haloperidol (0.01 or 0.03 mg/kg, *i.p.*) 30 minutes before testing. Columns and bars show the means ± SEM of sham-operated (n=9) and bilateral habenula-lesioned subjects (n=7).

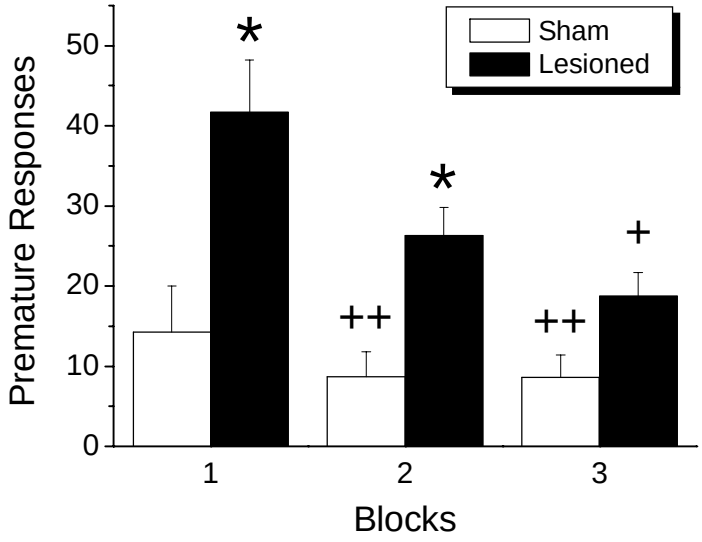
Les performances (moyenne ± ESM) du groupe sham (n=9) et du groupe habénulo-lésé (n=7), concernant le pourcentage de réponses correctes, sont indiquées suite à l'injection d'une solution d'acide lactique (1% pH 4.5, i.p.) ou d'halopéridol (0,01 et 0,03 mg/kg, i.p.) 30 minutes avant le test.

IV.3.2 Premature responses

Effects of bilateral habenula lesions on drug-free performance

Two-factor ANOVA showed significant effects of lesion group ($F_{1,14}$ = 12.28, p < 0.005), trial block ($F_{2,28}$ = 13.96, p < 0.001) and a significant interaction of these factors ($F_{2,28}$ = 4.73, p < 0.05). These results are consistent with the conclusion that premature responses were increased in habenula-lesioned animals and that, in contrast to the change in choice accuracy, this effect declined in magnitude over trial blocks (see **Fig 4.5**).

Fig 4.5. Premature responses across the 3 blocks of baseline performances



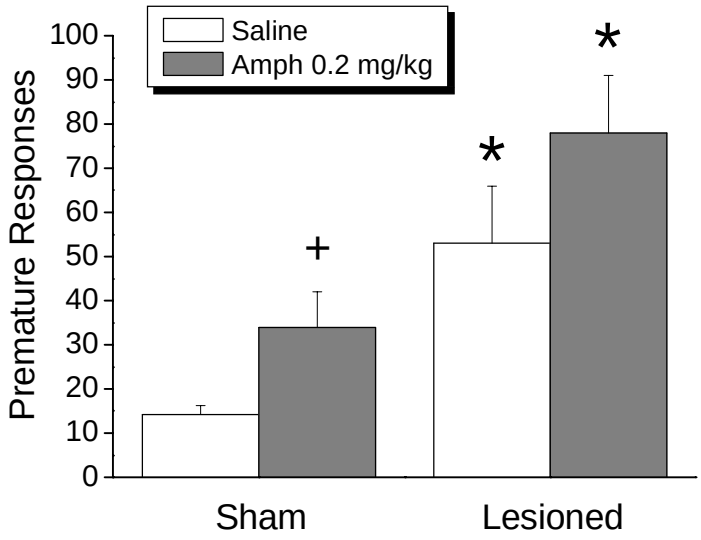
Premature responses are shown during blocks of drug-free performance on post-operative days 9-20 (block 1), 41-52 (block 2) and 77-84 (block 3). Columns and bars show the means ± SEM of sham-operated (n=9) and bilateral habenula-lesioned subjects (n=7). * $p < 0.05$ compared to the sham group at the same time point (one-factor ANOVA); + $p < 0.05$, ++ $p < 0.01$ *vs* same group at block 1 (2-tailed paired t-test).

*Les performances de base (moyenne ± ESM) du groupe sham (n=9) et du groupe habénulo-lésé (n=7), concernant le nombre de réponses prépaturées, sont indiquées en fonction des trois sessions de mesures, soit du jour 9 au jour 20 après l'opération (session 1), du jour 41 au jour 52 après l'opération (session 2) et du jour 77 au jour 84 après l'opération (session 3). * $p < 0.05$ comparé aux performances du groupe sham au cours de la même session (ANOVA à un facteur); + $p < 0.05$, ++ $p < 0.01$ comparé aux performances du même groupe au cours de la session 1 (test t à deux bornes).*

Effects of d-amphetamine treatment

Two-factor ANOVA showed significant effects of lesion group ($F_{1,14} = 11.69$, $p < 0.01$) and amphetamine treatment ($F_{1,14} = 10.34$, $p < 0.01$), but no significant interaction of lesion group x drug treatment ($F_{1,14} = 0.1$, $p > 0.6$). Thus premature responses were higher in lesioned animals, and were increased by amphetamine (see **Fig 4.6**).

Fig 4.6. Effects of d-amphetamine treatment



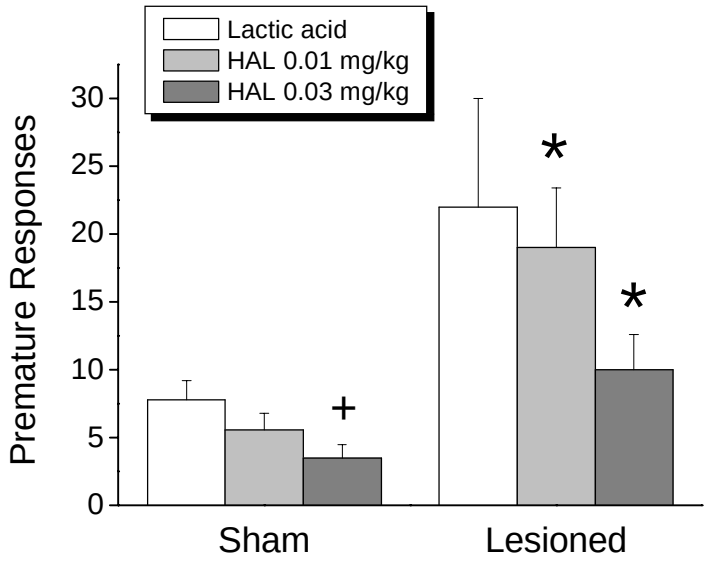
Premature responses are shown during sessions where the rats received saline or d-amphetamine (0.2 mg/kg *s.c.*) 30 minutes before testing. Columns and bars show the means ± SEM of sham-operated (n=9) and bilateral habenula-lesioned subjects (n=7). * $p < 0.05$ compared to corresponding sham group data (one-factor ANOVA); + $p < 0.05$ *vs* same group saline condition (2-tailed paired t-test).

*Les performances (moyenne ± ESM) du groupe sham (n=9) et du groupe habénulo-lésé (n=7), concernant le nombre de réponses prématurées, sont indiquées suite à une injection de solution saline (NaCl 0,9 %) ou de d-amphétamine (0,2 mg/kg, s.c.) 30 minutes avant le test. * $p < 0.05$ comparé aux performances du groupe sham dans les mêmes conditions (ANOVA à un facteur); + $p < 0.05$ comparé aux performances du même groupe après injection de la solution saline (test t à deux bornes).*

Effects of haloperidol treatment

There was a significant effect of lesion group on the mean number of premature responses ($F_{1,14} = 8.8$, $p < 0.05$), and a significant effect of drug treatment ($F_{2,28} = 4.4$, $p < 0.05$), but no significant interaction of lesion group x drug treatment ($F_{2,28} = 1.1$, $p > 0.1$). These results confirm the impression from inspection of the data that premature responses were elevated in habenula-lesioned animals, and that haloperidol reduced the number of premature responses (see **Fig 4.7**).

Fig 4.7. Effects of haloperidol treatment



Premature responses are shown during sessions where the rats received lactic acid (1% pH 4.5, *i.p.*) or haloperidol (0.01 or 0.03 mg/kg *i.p.*) 30 minutes before testing. Columns and bars show the means ± SEM of sham-operated (n=9) and bilateral habenula-lesioned subjects (n=7). * $p < 0.05$ compared to the sham group after the same drug treatment (one-factor ANOVA); + $p < 0.05$ *vs* same group saline condition (2-tailed paired t-test).

*Les performances (moyenne ± ESM) du groupe sham (n=9) et du groupe habénulo-lésé (n=7), concernant le nombre de réponses prématurées, sont indiquées suite à l'injection d'une solution d'acide lactique (1% pH 4.5, i.p.) ou d'halopéridol (0,01 et 0,03 mg/kg, i.p.) 30 minutes avant le test. * $p < 0.05$ comparé aux performances du groupe sham dans les mêmes conditions (ANOVA à un facteur); + $p < 0.05$ comparé aux performances du même groupe après injection de la solution saline (test t à deux bornes).*

IV.3.3 Response omissions

Effects of bilateral habenula lesions on drug-free performance

Two-factor ANOVA of the percentage omissions showed a significant effect of trial block ($F_{2,28} = 8.46$, $p < 0.005$) but no effect of lesion group ($F_{1,14} = 1.09$, $p > 0.1$) and no interaction of these factors ($F_{2,28} = 0.34$, $p > 0.1$) (see **Table 4.2**).

Effects of d-amphetamine treatment

The percentage of omitted responses was not affected by lesion group ($F_{1,14} = 0.2$, $p > 0.1$). Neither was there any significant effect of drug treatment ($F_{1,14} = 1.3$, $p > 0.1$) nor

any significant interaction of lesion group x drug treatment ($F_{1,14}$ = 1, p > 0.1) (see **Table 4.3**).

Effects of haloperidol treatment

There was a significant effect of lesion group on the percentage of omitted responses ($F_{1,14}$ = 4.63, p < 0.05), as well as a significant effect of drug treatment ($F_{2,28}$ = 11.6, p < 0.01), but there was no significant interaction of lesion group x drug treatment ($F_{2,28}$ = 2.03, p > 0.1) (see **Table 4.4**).

IV.3.4 Perseverative responses

Effects of bilateral habenula lesions on drug-free performance

As for response omissions there was no effect of lesion group and no lesion group x trial block interaction. Two-factor ANOVA showed a significant effect of trial block ($F_{2,28}$ = 3.74, p < 0.05) but no effect of lesion group ($F_{1,14}$ = 0.005, p > 0.1) and no interaction of these factors ($F_{2,28}$ = 0.005, p > 0.1) (see **Table 4.2**).

Effects of d-amphetamine treatment

Lesion group exerted no significant effect on the mean number of perseverative responses ($F_{1,14}$ = 2.2, p > 0.1). There was also no effect of drug treatment ($F_{1,14}$ = 1.64, p > 0.1) and no significant interaction of lesion group x drug treatment ($F_{1,14}$ = 1.3, p > 0.1) (see **Table 4.3**).

Effects of haloperidol treatment

There was no significant effect of lesion group on the mean number of perseverative responses ($F_{1,14}$ = 0.02, p > 0.1). However, there was a significant effect of drug treatment ($F_{2,28}$ = 14.3, p < 0.0001) reflecting a reduction of perseverative responses at the higher dose. There was no significant interaction of lesion group x drug treatment ($F_{2,28}$ = 1.56, p > 0.1) (see **Table 4.4**).

IV.3.5 Response latencies

Effects of bilateral habenula lesions on drug-free performance

Two-factor ANOVA on latencies of correct responses showed a significant effect of trial block ($F_{2,28}$ = 5.96, p < 0.01), no effect of lesion group ($F_{1,14}$ = 1.96, p > 0.1) and

no significant interaction of these factors ($F_{2,28} = 3.23$, $p > 0.05$). The significant effect of trial block reflects a trend for latencies to become slightly longer in the later trial blocks (see **Table 3.2**). Two-factor ANOVA on latencies of incorrect responses, in contrast, showed a significant effect of lesion group ($F_{1,14} = 10.3$, $p < 0.01$). There was no effects of trial block ($F_{2,28} = 2.76$, $p > 0.05$) and no interaction of the factors ($F_{2,28} = 0.25$, $p > 0.1$) (see **Table 4.2**).

Thus the main conclusion that can be drawn concerning response latencies is that habenula-lesioned animals had shorter latencies on incorrect trials, and that this difference was constant over trial blocks.

Effects of d-amphetamine treatment

Lesion group exerted no significant effect on the mean latency of correct responses ($F_{1,14} = 0.9$, $p > 0.1$). Neither was there a significant effect of drug treatment ($F_{1,14} = 2.35$, $p > 0.1$) nor a significant interaction of lesion group x drug treatment ($F_{1,14} = 3.65$, $p > 0.05$) (see **Table 4.3**). There was no significant effect of lesion on the mean latency of incorrect responses ($F_{1,14} = 1.22$; $p > 0.1$). However there was an effect of drug treatment ($F_{1,14} = 5$; $p < 0.05$) but no significant interaction of lesion group x drug treatment ($F_{1,14} = 0.05$, $p > 0.1$) (see **Table 4.3**).

Effects of haloperidol treatment

The analysis showed no significant effect of lesion group on the mean reaction time of correct responses ($F_{1,14} = 3.18$; $p > 0.05$). There was no effect of drug treatment ($F_{2,28} = 3.03$, $p > 0.05$), and no significant interaction of lesion group x drug treatment ($F_{2,28} = 0.74$, $p > 0.1$) (see **Table 4.4**). Lesion group exerted no significant effect on the mean latency of incorrect responses ($F_{1,14} = 0.004$, $p > 0.1$). There was no effect of drug treatment ($F_{2,28} = 0.5$, $p > 0.1$), and no significant interaction of lesion group x drug treatment ($F_{2,28} = 1.9$, $p > 0.1$) (see **Table 4.4**).

IV.3.6 Latency to collect the food pellet

Effects of bilateral habenula lesions on drug-free performance

According to two-factor ANOVA habenula-lesioned animals showed significantly shorter latencies to collect the food pellet ($F_{1,14} = 26.24$, $p < 0.001$). There was also a

significant effect of trial block ($F_{2,28} = 3.51$; $p < 0.05$), but no significant interaction of these factors ($F_{2,28} = 0.52$, $p > 0.5$) (see **Table 4.2**).

Thus habenula-lesioned animals collected the food pellet with a shorter latency than controls, an effect which was constant over trial blocks.

Effects of d-amphetamine treatment

Lesion group had a significant effect on the mean latency to collect the pellet ($F_{1,14} = 11.8$, $p < 0.01$), but there was no effect of drug treatment ($F_{1,14} = 1.98$, $p > 0.1$) and no significant interaction of lesion group x drug treatment ($F_{1,14} = 0.41$, $p > 0.1$) (see **Table 4.3**).

Effects of haloperidol treatment

There was no significant effect of lesion group ($F_{1,14} = 1.12$, $p > 0.1$), no significant effect of drug treatment ($F_{2,28} = 1.45$, $p > 0.1$), and no significant interaction of lesion group x drug treatment ($F_{2,28} = 0.03$, $p > 0.1$) on the mean latency to collect the food pellet (see **Table 4.4**).

Table 4.2. Performance parameters of sham control and habenula-lesioned animals during drug-free phases

Parameters	Block 1		Block 2		Block 3	
	Sham	**Lesioned**	**Sham**	**Lesioned**	**Sham**	**Lesioned**
% Omissions	17.0 ± 2.3	15.2 ± 1.7	20.1 ± 3.1	16.1 ± 1.4	24.1 ± 2.9	20.3 ± 2.1
Perseverative responses	22.6 ± 2.5	22.7 ± 2.8	19.7 ± 2.4	19.7 ± 3.4	16.8 ± 1.9	17.2 ± 2.7
Latency, correct responses	0.86 ± 0.04	0.88 ± 0.05	0.87 ± 0.04	0.97 ± 0.05	0.89 ± 0.03	1.03 ± 0.08
Latency, incorrect responses	2.09 ± 0.09	1.81 ± 0.10	2.26 ± 0.08	1.92 ± 0. 09	2.18 ± 0.07	1.92 ± 0.08
Latency, pellet	2.30 ± 0.13	1.59 ± 0.14	2.28 ± 0.13	1.62 ± 0. 15	1.96 ± 0.08	1.45 ± 0.09

The table shows % omissions, total perseverative responses per session, and latencies in seconds. All values are shown as mean ± SEM.

Tableau indiquant, pour chaque session, le pourcentage de réponses omises, le nombre total de réponses persévératives et les latences en secondes. Toutes les valeurs sont des moyennes (± ESM).

Table 4.3. Performance parameters of sham control and habenula-lesioned animals after a treatment with either saline or amphetamine (0.2 mg/kg)

Parameters	Sham		Lesioned	
	Saline	**Amph**	**Saline**	**Amph**
% Omissions	14.17 ± 3.59	14.36 ± 3.87	10.66 ± 1.48	14.13 ± 2.47
Perseverative responses	29.77 ± 4.55	29.22 ± 7.28	24.42 ± 4.13	15.00 ± 3.30
Latency, correct responses	0.77 ± 0.05	0.78 ± 0.04	0.91 ± 0.07	0.79 ± 0.07
Latency, incorrect responses	1.95 ± 0.18	1.67 ± 0.14	1.72 ± 0.10	1.49 ± 0.16
Latency, pellet	3.35 ± 0.63	2.28 ± 0.47	1.66 ± 0.20	1.26 ± 0.09

The table shows % omissions, total perseverative responses per session, and latencies in seconds. All values are shown as mean ± SEM.

Tableau indiquant pour chaque groupe, en fonction du traitement, le pourcentage de réponses omises, le nombre total de réponses persévératives et les différentes latences en secondes. Toutes les valeurs sont des moyennes (± ESM)

Table 4.4. Performance parameters of sham control and habenula-lesioned animals after treatment with either vehicle or haloperidol (Hal) (0.01 or 0.03 mg/kg)

Parameters	Sham			Lesioned		
	Vehicle	Hal, 0.01 mg/kg	Hal, 0.03 mg/kg	Vehicle	Hal, 0.01 mg/kg	Hal, 0.03 mg/kg
% Omissions	33.20 ± 10.69	22.63 ± 4.32	50.06 ± 10.36	14.05 ± 2.14	13.13 ± 2.58	26.09 ± 4.17
Perseverative responses	15.77 ± 1.80	23.00 ± 4.67	6.22 ± 2.98	19.71 ± 3.96	17.28 ± 2.30	6.42 ± 1.64
Latency, correct responses	1.12 ± 0.20	0.82 ± 0.03	0.67 ± 0.15	1.24 ± 0.16	1.04 ± 0.10	1.10 ± 0.16
Latency, incorrect responses	2.14 ± 0.14	2.21 ± 0.08	1.97 ± 0.40	1.85 ± 0.16	1.99 ± 0.13	2.52 ± 0.27
Latency, pellet	2.03 ± 0.53	2.40 ± 0.40	1.76 ± 0.58	1.39 ± 0.24	1.88 ± 0.58	1.29 ± 0.04

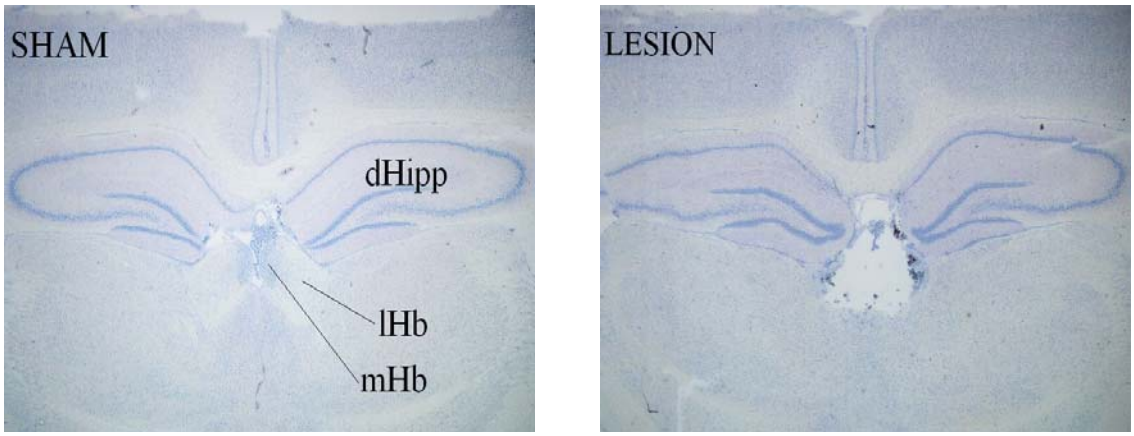
The table shows % omissions, total perseverative responses per session, and latencies in seconds. All values are shown as mean ± SEM.

Tableau indiquant pour chaque groupe, en fonction du traitement, le pourcentage de réponses omises, le nombre total de réponses persévératives et les différentes latences en secondes. Toutes les valeurs sont des moyennes (± ESM).

IV.3.7 Histology

As illustrated in **Fig 4.8**, habenula lesions destroyed a large proportion of both medial and lateral habenula without causing significant damage to neighboring structures. Only animals with such lesions were included in the analysis of behavioral effects.

Fig 4.8: Typical sections through the habenula region of a sham-operated rat and a bilateral habenula-lesioned rat



Only animals with similar lesions, showing large destruction of the habenula without significant damage to neighboring structures, were included in the analysis of behavioral effects. Abbreviations: **mHb**, medial habenula; **lHb**, lateral habenula; **dHipp**, dorsal hippocampus.

Seuls les animaux ayant des lesions circonscrites à l'habénula et n'affectant pas ou très peu les structures avoisinantes furent incluses dans les analyses statistiques. Abréviations: mHb, noyau médian de l'habénula; lHb, noyau latéral de l'habénula; dHipp, hippocampe dorsal.

IV.3.8 Assay of choline acetyltransferase (ChAT)

As shown in **Table 4.5**, rats with lesions of the habenula had a marked reduction, by 78 %, of ChAT activity of the interpeduncular nucleus, compared to the sham-operated animals. This result is consistent with those of previous neurochemical studies (Villani *et al*, 1983; Contestabile *et al*, 1987; Eckenrode *et al*, 1987) and indicates marked degeneration of the habenulo-interpeduncular cholinergic tract.

Table 4.5. Choline acetyltransferase in homogenates of interpeduncular nucleus from sham and habenula-lesioned animals.

Group	ChAT (µmoles/g wet wt/hr)
Sham-operated	83.5 ± 6.6
Lesioned	18.5 ± 9.6 (22.1 ± 11.5 %) **

Results (mean ± SEM; µmoles/g wet wt/hr) are shown for the sham group and the habenula-lesioned group. Values in parentheses are expressed as percentage of the sham value. ***p* < 0.001 *vs* sham-operated (2-tailed t-test).

*Les resultats (moyenne ± ESM; µmoles/g de matière sèche/hr) sont indiqués pour le groupe sham et le groupe habenulo-lésé. Les valeurs entre parenthèses sont exprimées en pourcentage des valeurs correspondantes issues du groupe sham. **p < 0.001 vs sham (test t à deux bornes).*

IV.4 Discussion

The aim of the present studies was to examine whether bilateral lesions of the habenula would cause deficits in performing the 5-CSRTT, a task in which attention plays an important role. The results clearly showed that multiple deficits resulted from such lesions, and to our knowledge are the first data to demonstrate a role for the habenula in the performance of a test of attention.

The major behavioral consequences after the habenula lesion in the 5-CSRTT are a marked increase of the number of premature responses and alterations in accuracy. Interestingly, the deficits observed during the task can be distinguished in terms of their time-course. At one extreme increased premature responses appeared immediately upon reinstating testing after the lesion, and thereafter declined in magnitude. At the other extreme choice accuracy was not significantly altered in the first test sessions upon reinstating testing, and became progressively worse in the habenula-lesioned animals as testing progressed. Falling between these two extremes were shortened latencies for incorrect responses and for retrieving the food pellet in lesioned animals, that appeared already in the first block of post-operative test sessions and then remained stable. Given that the habenula consists of fifteen subnuclei (Andres *et al*, 1999; Geisler *et al*, 2003) it is understandable that alterations with different properties exist. Elucidating the circuits involved in these various alterations will clearly require further experiments. Here we restrict ourselves to considering the possible role of elevated dopamine in the changes of premature responses and choice accuracy.

Concerning first the increase of premature responding occurring shortly after the lesion, one possible behavioral explanation is that this increase reflects an impaired ability to focus attention only on the most relevant aspect of the task, namely the occurrence of a stimulus light. Instead, the stimulus recesses themselves, that have become associated with reward during training, elicit an inappropriate level of responding. As only a slight, short-lasting increase of locomotor activity is present in habenula-lesioned animals (see Chapter II) it is unlikely that the increase of premature responding is merely a reflection of generalized hyperactivity. Moreover it seems unlikely that the increased premature responding could be explained by a motor effect of the lesion to non-specifically facilitate responding in the stimulus alcoves, since there was no increase at all of

perseverative responding caused by the lesion. It is currently difficult to conclude whether the increase in premature responses in habenula-lesioned rats corresponds to a particular aspect of the behavior of patients with schizophrenia. Premature responding has been considered to represent some aspect of impulsivity (Winstanley *et al*, 2004), which is a troubling symptom of a proportion of schizophrenics (Hoptman *et al*, 2002; Spivak *et al*, 1997, 2003). Future pharmacological characterization of this behavior in rats and of the various measures of impulsivity in man will be invaluable in deciding how well these behaviors correspond to each other.

It has been emphasized (Chudasama *et al*, 2003; Robbins, 1998) that the ability to inhibit inappropriate responses in a complex situation such as the 5-CSRTT is an important aspect of executive control, probably involving frontal cortical regions (Duncan & Owen, 2000). One candidate as a likely neural mechanism responsible for this effect, that is consistent with previous findings and the present data, is increased release of dopamine in mesolimbic or mesocortical dopaminergic pathways. The habenula exerts an inhibitory influence on dopaminergic cells of the ventral tegmentum (Christoph *et al*, 1986). Correspondingly, acute interruption of impulse flow in the habenula increases the turnover of dopamine in mesolimbic and mesocortical regions (Nishikawa *et al*, 1986) while chronic lesions of habenula produce a long-lasting increase of dopamine turnover selectively in frontal cortex (Lisoprawski *et al*, 1980). Cole & Robbins (1989) showed that the marked increase of premature responding evoked by amphetamine was greatly attenuated by 6-hydroxydopamine-induced lesions of the nucleus accumbens that damage mesolimbic dopamine terminals and mesocortical dopamine axons running through that region. This evidence is consistent with the view that lesions of the habenula result in increased mesolimbic/mesocortical dopamine release, which then causes the observed increase in premature responding. The effects of the dopamine antagonist, haloperidol, observed here are consistent with this view, as the effect of haloperidol on premature responding was to reduce it. The fact that haloperidol clearly reduced premature responding in non-lesioned animals suggests that there is some dopamine release in control animals that contributes to premature responding. For comparison, one previous study reported no significant effect of haloperidol on premature responding (Carli & Samanin, 1992) without showing the absolute data. Conceivably the possibility of obtaining a reduction was limited by a "floor effect". A further study showed a tendency to reduced premature responses after

the dopamine D_2 antagonist, sulpiride, and a significant reduction after the dopamine D_1 antagonist SCH23390 (Harrison *et al*, 1997). It is possible that both a reduction in the secondary-reinforcing properties of the stimulus alcoves and some motor disturbance contribute to these effects of haloperidol. However, arguing against any pronounced motor disturbance, response latencies and latencies to collect food pellets were not lengthened. In agreement with previous studies in intact animals (Cole & Robbins, 1987, 1989) amphetamine, an enhancer of dopamine release, increased premature responses in sham-operated animals. The effect of amphetamine does not seem to be due to any motor effect that generally increases nose-poking into the stimulus alcoves since perseverative responding was completely unaltered.

Since global depletion of brain serotonin by 5,7-dihydroxytryptamine increases premature responding in this task (Harrison *et al*, 1997; Winstanley *et al*, 2004) it should be considered whether increased premature responding after amphetamine or habenula lesions might be due to reduced serotonin release. Concerning amphetamine, this seems unlikely since the effect of amphetamine on serotonin release is facilitation , albeit at doses a hundred-fold higher than those that affect catecholamine release (Rothman *et al*, 2001). Moreover no effect of serotonin depletion on increased premature responses provoked by low amphetamine doses was observed (Harrison *et al*, 1997). Concerning the habenula lesion effect, with the exception of Nishikawa & Scatton (1985), the majority of studies indicate an inhibitory influences of habenula activity on serotonergic neurons (Reisine *et al*, 1982; Speciale *et al*, 1980; Wang & Aghajanian, 1977) such that the effect of lesions would be elevated serotonin release which would be predicted to decrease premature responding.

In contrast to premature responding, choice accuracy was not significantly altered shortly after habenula lesion, but was impaired at later times. Whereas a degree of behavioral recovery often occurs after other brain lesions, we are not aware of another example of a progressive deficit after a restricted brain lesion in an adult animal. This different time-course compared to that of the elevation of premature responding suggests that different neural mechanisms are involved. Also, unlike premature responding, this lesion-induced deficit was not ameliorated at all by haloperidol. Neither was choice accuracy worsened by *d*-amphetamine, in agreement with the effects of comparable doses in several previous studies (Cole & Robbins, 1987, 1989; Harrison *et*

al, 1997; Muir *et al*, 1995). Theoretically, sensitization of dopaminergic transmission by prolonged hyperactivity (Vanderschuren *et al*, 1999, 2000), until it exceeded a threshold level could be a possible explanation of the time course of impairment of choice accuracy. However the lack of effects of haloperidol and *d*-amphetamine in doses that exerted effects on premature responding in the same experiment indicates that elevated dopaminergic transmission does not appear to play a role in this impairment. Alternative explanations include the possibility of some progressive neural degeneration after habenula lesion. While we have not observed any obvious neural degeneration, further studies are necessary to examine this possibility in detail. Finally, there remains the possibility of a mechanism according to which habenula dysfunction results in a progressive accumulation of delusional memories, so that cognitive functions are progressively disturbed by the accumulation of wrongly-strengthened synaptic connections that are the neural substrate of these delusional memories (Kelly, 1998). The progressive deficit in choice accuracy is consistent with such a mechanism, but of course does not prove it. Further, we can reasonably eliminate the possibility that this late-occurring impairment of choice accuracy arises from an impairment of visual ability, since in a different cognitive task, the Morris water-maze, rats with a lesion of the habenula performed the visible platform condition of the test as well as sham-operated controls, although they were impaired in finding the hidden platform (see **Chapter II**).

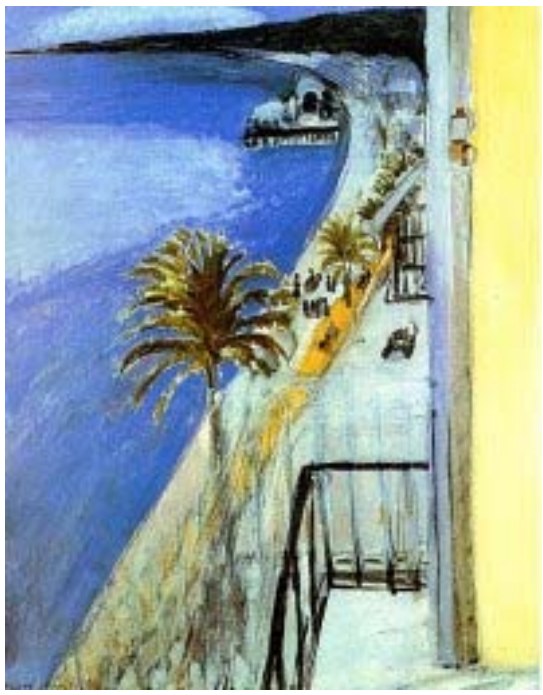
The effects of habenula lesions observed here in the 5-CSRTT do not appear to be caused by a lesion-induced increase in food motivation. A consistent finding from several laboratories is that when food motivation is reduced by pre-feeding then there is an increase of "percentage omissions", the percentage of stimuli that elicit no response (Bizarro & Stolerman, 2003; Carli & Samanin, 1992; Grottick & Higgins, 2000, 2002; Harrison *et al*, 1997). In none of these studies was choice accuracy altered by manipulating food motivation. In the present studies the lesion resulted in a delayed reduction in choice accuracy and no alteration of percentage omissions with the exception of an overall lesion effect in the haloperidol experiment. This latter result is difficult to explain currently. Thus, the lesion-induced changes cannot readily be accounted for by an alteration of motivation. Moreover, previous lesion evidence suggests that the habenula is not involved in food intake (Mok *et al*, 1973).

Even if attention deficits in schizophrenia are due to habenula dysfunction it is not necessarily the case that animals with habenula lesions are a model of attentional deficit in schizophrenia in the pharmacological sense that chronic treatment with neuroleptics is expected to improve measures such as choice accuracy. It may be, for example, that the marked effect of neuroleptics on activity of the habenula (McCulloch, 1982; Palacios & Wiederhold, 1985; Room *et al*, 1991) is necessary for their improvement in attentional performance when administered chronically (Orzack *et al*, 1967; Spohn & Strauss, 1989). In this case animals with complete habenula lesions would be a model of patients who are non-responders to neuroleptics concerning their attentional choice accuracy deficit. Moreover, although in many studies of patients with schizophrenia attentional performance was improved by chronic neuroleptic treatment, there are other studies in which such treatment had no significant beneficial effect (Allen *et al*, 1997; Liu *et al*, 2000; review: Blyler & Gold, 2000). Factors contributing to such different outcomes may include the exact nature of the test, concomitant medication with the anticholinergic agent benztropine, sample size and duration of the medication-free period before baseline assessment. It is beyond the scope of this discussion to consider all the possible factors that may contribute to such different outcomes. However, consistent with a progressive loss of the substrate for beneficial effects of neuroleptic treatment on attention, it is interesting that better effects were found in younger patients compared to older patients (Harvey *et al*, 2003a, b) and no positive effects were found in a patient population that comprised a high proportion of patients that were neuroleptic-non-responders with respect to other symptoms (Epstein *et al*, 1996).

The present results are relevant to the hypothesis that habenula dysfunction is involved in the cognitive symptoms of schizophrenia. This hypothesis is based on several lines of evidence. For example, excessive calcification of the habenula or of the epithalamus, comprising the habenular nuclei plus the pineal organ, is observed in schizophrenia patients (Caputo *et al*, 1998; Sandyk, 1992). Moreover, chronic stimulant exposure selectively damages the lateral habenula and its fasciculus retroflexus output pathway in experimental animals (Ellison, 1992), and in man can lead to a schizophrenia-like state (Satel & Edell, 1991; Sato *et al*, 1983). In examining the prediction that habenula lesions would therefore cause schizophrenia-like symptoms in rats we recently found that such lesions produced cognitive disturbance in the Morris water maze spatial reference memory task (see **Chapter II**) that is thought to be analogous to declarative

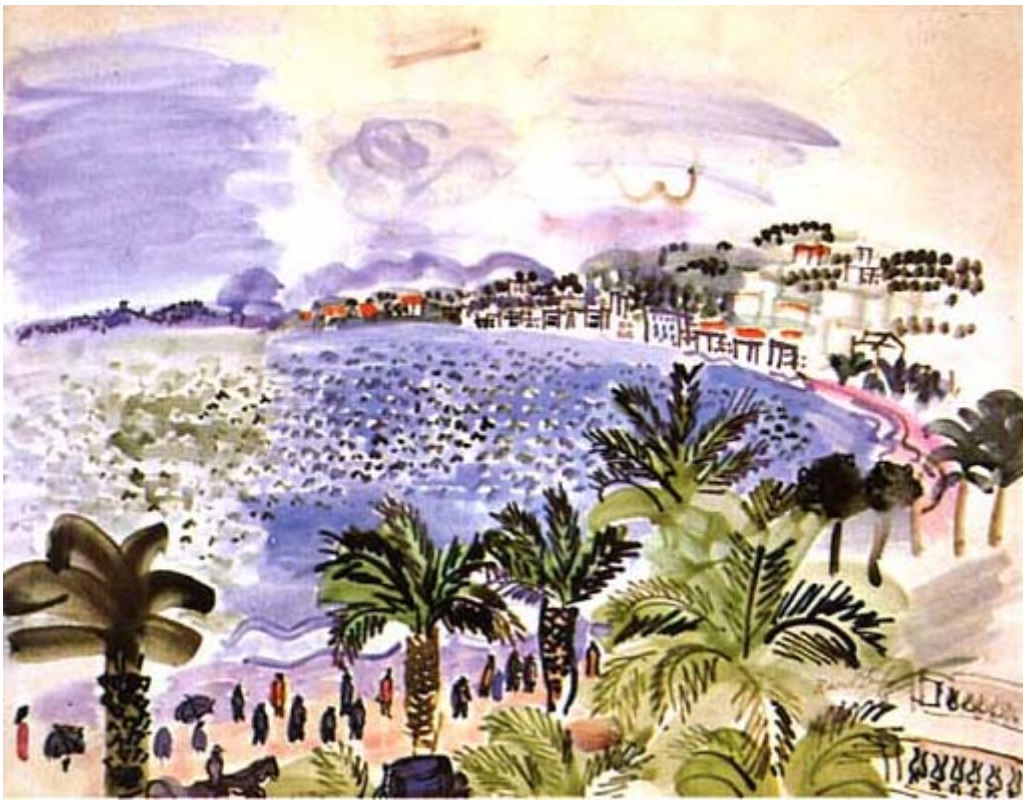
memory (O'Keefe & Nadel, 1978) which is impaired in schizophrenia (Cirillo & Seidman, 2003; Perry *et al*, 2000). In the same series of studies we found no deficit of prepulse inhibition (PPI) of a startle response, a phenomenon that is impaired in schizophrenia patients (Braff *et al*, 1978, 1992, 1999). Interestingly however a deficit in PPI in habenula-lesioned mice has recently been described which was not initially present, but which appeared after the experience of fear-conditioning (Heldt & Ressler, 2004). Similar to the deficit in choice accuracy observed here, this deficit resulting from habenula lesion is therefore experience-dependent.

In the present experiments the prediction that habenula lesions would cause schizophrenia-like symptoms was further tested by examining performance in a test of attentional mechanisms, since numerous studies have emphasized that disturbances of attention are common in schizophrenia (Barr, 2001; Bleuler, 1950; Chen & Faraone, 2000; McGhie & Chapman, 1961). Our results showed that habenula lesions caused multiple deficits in an attention task that is modeled after the continuous performance test of attention (Chudasama & Robbins, 2004), that schizophrenia patients are impaired on. Thus a prediction of the hypothesis that habenula lesions contribute to cognitive impairments in schizophrenia was confirmed, so that the hypothesis withstood this challenge. The results add to accumulating evidence that the hypothesis that habenula dysfunction contributes to cognitive impairment in schizophrenia deserves further consideration.



"La baie de Nice"

Henri Matisse - 1918



"La baie des anges"

Raoul Dufy - 1928

Chapter V - Influence of habenula lesion on brain structures activity, with or without stimulation of monoamine systems: A regional brain blood flow magnetic resonance imaging study

V.1 Introduction

The results from the studies in the previous chapters indicate that in some respects the behavioural changes, particularly cognitive aspects, produced by bilateral lesions of the habenula resemble those in schizophrenia. Thus two major cognitive dysfunctions of schizophrenia, dysfunction of memory (see **Chapter II**) and dysfunction of attention (see **Chapter IV**), were clearly produced in rats by bilateral habenula lesions.

As part of the basis for these alterations, because the habenula is a major influence on ascending serotonergic pathways (Nishikawa & Scatton, 1985; Speciale *et al*, 1980; Wang & Aghajanian, 1977), noradrenergic pathways (Cenci *et al*, 1992; Kalen *et al*, 1989) and nigrostriatal, mesolimbic and mesocortical dopaminergic pathways (Christoph *et al*, 1986; Lisoprawski *et al*, 1980; Matsuda & Fujimura, 1992; Sasaki *et al*, 1988, 1990) that project widely throughout the brain, it was argued that lesions of the habenula may cause marked alteration of forebrain function. Particularly of interest for at least two reasons is the possibility that such lesions might alter the functioning of the frontal cortex, first because frontal cortical regions are important in performance of attentional tasks (Chudasama *et al*, 2003; Muir *et al*, 1996) and spatial learning in the Morris maze (Dallison & Kolb, 2003; Vafaei & Rashidy-Pour, 2004; Wright *et al*, 2003) and, second, there is extensive evidence of impaired functioning of frontal cortical regions in schizophrenia (Davidson & Heinrichs, 2003; Hill *et al*, 2004; Weinberger & Berman, 1988).

Therefore the purpose of the present experiments was to investigate using a direct method, the measurement of cerebral blood flow by magnetic resonance imaging (MRI, see **Fig 5.1**), whether there were any marked alterations of cerebral functioning, particularly of the frontal cortex, after habenula lesions.

In addition to lesions, subchronic amphetamine treatment was included as a factor in the experimental design. The rationale for this is that some evidence suggests that continuously-elevated dopamine release might contribute to schizophrenia-like functional brain changes in man (Laruelle *et al*, 1999) and animals (Joseph *et al*, 2000; Tenn *et al*, 2003). Moreover, chronic amphetamine or cocaine intake has been found to produce schizophrenia-like state in healthy people, and to worsen the state of schizophrenic patients (). Therefore it was investigated whether there could be an interaction between habenula damage and subchronic amphetamine.

Finally, in addition to baseline blood flow, amphetamine-stimulated cerebral blood flow was also measured, since differences between schizophrenics and controls in frontal cortex blood flow may be seen if the frontal cortex is activated, for example by a cognitive task, rather than under baseline conditions (Berman *et al*, 1986; Cohen *et al*, 1987; Volkow *et al*, 1987; Weinberger *et al*, 1986a, 1988).

V.2 Materials and methods

V.2.1 Animals

The experiments were carried out on male Sprague-Dawley rats (Iffa Credo, France). The animals arrived with a body weight of 120-140 g and were housed in individual cages (Macrolon, 42 × 26 × 15 cm) in a temperature-regulated (22 ± 2°C) animal room on a 12 h/12 h light/dark cycle (lights on at 06:00), with laboratory rat chow (Nafag AG, Switzerland) and water available *ad libitum*. The operations and behavioural tests were performed during the light period, and were in accordance with the Swiss animal protection law for the care and use of animals and were approved by the Cantonal Veterinary Authority of the City of Basel.

V.2.2 Surgical procedures

One week after their arrival, the rats were operated, and divided in two groups: habenula-lesioned (n = 12) or sham-operated (n = 12). Lesions of the habenula or sham operations have been performed as previously described (see **Chapter II**, page 69). However, a difference was that, at the time of operation, the animals weighed between 150 and 160 g, so that the atlas of König & Klippel (1963) for small rats was used. Thus to position the electrode tip, from interaural line, at the point AP 4.2 mm, ML 0.5 mm, 3.7 mm below dura (König & Klippel, 1963) the "lateral" displacement of the electrode carrier (still at an angle of 10° to the vertical) after positioning it at the midpoint of the saggital sinus at the desired AP coordinate was 1.3 mm, and the depth displacement of the electrode was 4.0 mm along the 10° angle track.

V.2.3 Food deprivation

Because of the fixed size of the animal holder for the MRI apparatus the growth of the animals had to be slowed so that they would fit into the apparatus for a second MRI, 12 weeks after the first. Therefore, starting on the seventh day following the operation, a modest food deprivation procedure began. To be maintained at constant body weight the rats received 17 g of food each day throughout the experiment, with water available *ad libitum*.

V.2.5 Drug preparation

All solutions were freshly prepared on the day of use. D-amphetamine sulphate (Siegfried, Zofingen, Switzerland) was dissolved in 0.9% NaCl and injected in a volume of 1 ml/kg. Saline-treated animals received the same volume of saline (0.9% NaCl).

V.2.4 Drug administration

Three days after the beginning of the food deprivation, the rats were placed in the MRI apparatus for the first measurement (*MRI 1*). Three days later, the subchronic drug pretreatment began. The rats were divided in four groups: sham-operated animals pretreated with either saline (NaCl 0.9%, 1 ml/kg/day *s.c.*, n = 6) or amphetamine (2.5 mg/kg/day, *s.c.*, n = 6), and habenula-lesioned animals pretreated with either saline (NaCl 0.9%, 1 ml/kg/day *s.c.*, n = 6) or amphetamine (2.5 mg/kg/day, *s.c.*, n = 6). During the pretreatment procedure, all animals received a daily injection of the corresponding treatment (saline or amphetamine) during four consecutive days. On the following day, *i.e.* one week after *MRI 1*, the second measurement (*MRI 2*) was performed. During both MRI measurement, 1 mg/kg amphetamine was injected *i.v.* through an indwelling catheter after completion of baseline measurements.

V.2.6 MRI

Rats were anaesthetized with isoflurane (2%) in a mixture of oxygen and nitrous oxide (1:2). Cannulae were inserted into the tail vein (for injection of contrast agent). The rectal temperature was monitored with a thermistor and was maintained at 37.5 °C by ventilation with warm air (**Fig 5.2**). The first MRI experiment (*MRI 1*) was made 3 days after the beginning of food deprivation, *i.e.* 10 days after surgery, and the second MRI experiment (*MRI 2*) was made on the fifth day of subchronic drug (or saline) treatment.

Each MRI experiment comprised two measurements: measurement of relative cerebral blood flow (rCBV) and measurement of rCBV-change induced by intravenous injection of 1mg/kg amphetamine.

V.2.6.1 MRI-protocol and data analysis to measure rCBV

rCBV maps were derived from MRI data sets acquired using a T_2-weighted 3D fast-spin-echo sequence (3D-FSE) with the following imaging parameters: repetition delay T_R = 900 ms, echo delay T_E = 60 ms, RARE-factor = 16, field-of-view FOV = 3.28×3.00×2.5cm, data matrix = 96 × 80 × 42, number of acquisitions NEX = 2. Readout direction was anterior-posterior. An oversampling-factor of 2 was used in readout direction to avoid aliasing. Total acquisition time was 6min 28sec. Slices were oriented horizontally and reconstructed to a matrix of the dimension 128 × 128 × 64.

After acquisition of a pre-contrast scan, 0.7 ml of Endorem (AMI-25, Guerbet, Paris) were injected into the tail vein of the animal. Acquisition of a post-contrast data-set was started approximately 20 seconds after injection thereby avoiding effects due to the first pass of the contrast agent CA[16].

Image processing and analysis was carried out using BioMAP software (M. Rausch, Novartis). In brief, data sets from all animals were co-registered with a rat brain template, smoothed and analyzed using the general linear model. Modeling was carried out on a pixel-by-pixel basis. The atlas of Paxinos & Watson (1998) was used to identify anatomical structures. Regions of interest (ROI) analysis was used in addition to assess rCBV changes on a more global level, while the calculation of parametric maps allowed identifying smaller foci of CBV change, including sub-areas of larger brain structures. A height threshold of $p < 0.05$ and an extent threshold of 27 pixels was applied to the probability maps, which were overlaid onto the brain template for further analysis. Significance of rCBV change was determined by using the two-tailed t-test.

V.2.6.2 MRI-protocol and data analysis to measure rCBV changes after pharmacological stimulation with amphetamine

Dynamic T2-weighted images were acquired using a 3D- FSE sequence with the following imaging parameters: repetition delay T_R = 736 ms, echo delay T_E = 55 ms, RARE-factor = 16, field-of-view FOV = 3.28 × 3.00 × 2.5cm, data matrix = 96×64×42, number of acquisitions NEX = 1. Readout direction was anterior-posterior. An oversampling-factor of 2 was used in readout direction to avoid aliasing. Total

acquisition time was 2 min 31 sec. Slices were oriented horizontally and reconstructed to a matrix of the dimension 128 × 64 × 64. Sixteen of these volume data sets were acquired consecutively. Amphetamine was injected after acquisition of 5 baseline volumes. To be able to calculate fractional changes in rCBV, one additional volume was acquired before administration of Endorem.

Data analysis comprised the following steps:

Calculation of the relative change in blood volume on a pixel-by-pixel basis using

$$\frac{\Delta CBV(t)}{CBV(t)} = \frac{\ln\left(S(t)/S(0)\right)}{\ln\left(S(0)/S_{pre}\right)}$$

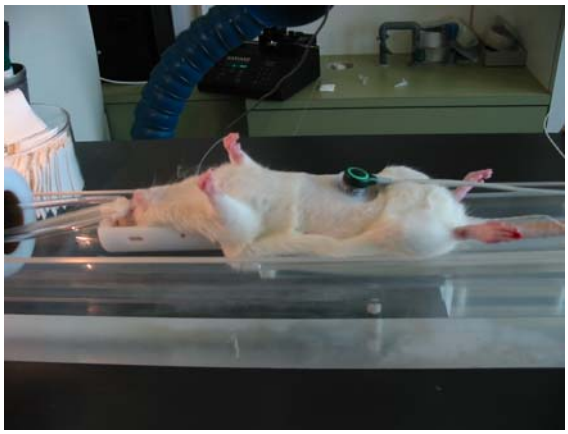
Here, **S(t)** is the signal intensity of a voxel at time point t, **S0** the mean baseline intensity derived from the first 4 volumes and **Spre** the signal intensity of the dataset, which was acquired before administration of contrast agent.

The responsiveness to amphetamine was defined as the area-under-the-curve (AUC). Volumes 5 to 10 were included into this calculation.

The response-map was resliced to the reference brain using the coordinates determined from the high-resolution 3D-FSE maps.

Fig 5.1. The MRI scanner

Fig 5.2. Rat placed in the coil of the scanner



One can see the rectal electrode for temperature monitoring, as well as the electrode placed on the abdomen of the rat which monitors pCO2 and the mouth catheter for the artificial ventilation.

On peut apercevoir l'electrode rectale servant à mesurer la temperature du rat, l'electrode de mesure de la pCO2 placée sur l'abdomen ainsi que le cathéter placé dans le museau et servant à la ventilation artificielle.

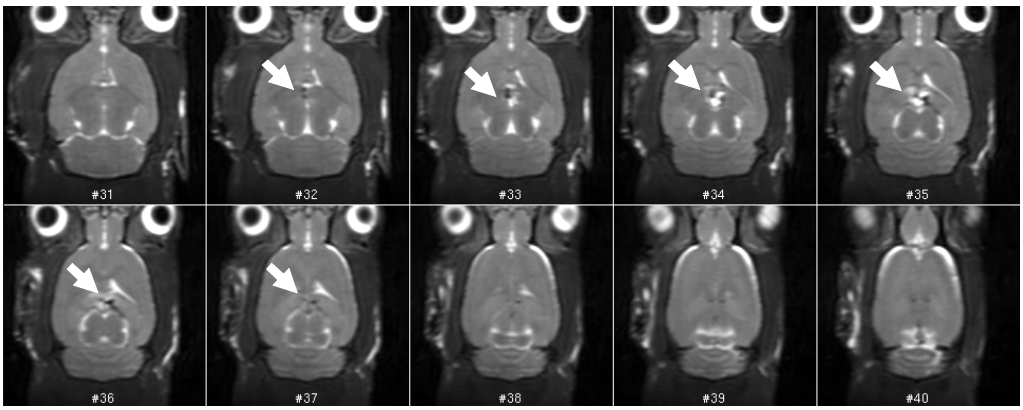
V.3 Results

Because the MRI procedure is highly constraining for the rats (deep anaesthesia + paralysis + intubation), several of them died, either during the first or the second MRI. Thus, statistical analysis were performed with 7 lesioned and 6 sham for the first MRI (MRI 1), which were divided as follow for the pretreatment procedure and the second MRI (MRI 2): 4 sham-operated (Sh/Cont) and 4 lesioned (Les/Cont) rats which were subchronically treated with saline during the pretreatment procedure, and 2 sham-operated (Sh/Sens) and 3 lesioned (Les/Sens) rats which were subchronically treated with amphetamine during the pretreatment procedure.

V.3.1 Habenula lesion

The habenula lesion was clearly detectable on T2-weighted FSE scans: it was characterized by heterogeneous structure consisting of hyperintense edema and dark structures representing hemorrhagic transformations (**Fig 5.3**). No signal abnormalities were visible in this area in sham-operated animals.

Fig 5.3. Localisation of the lesion



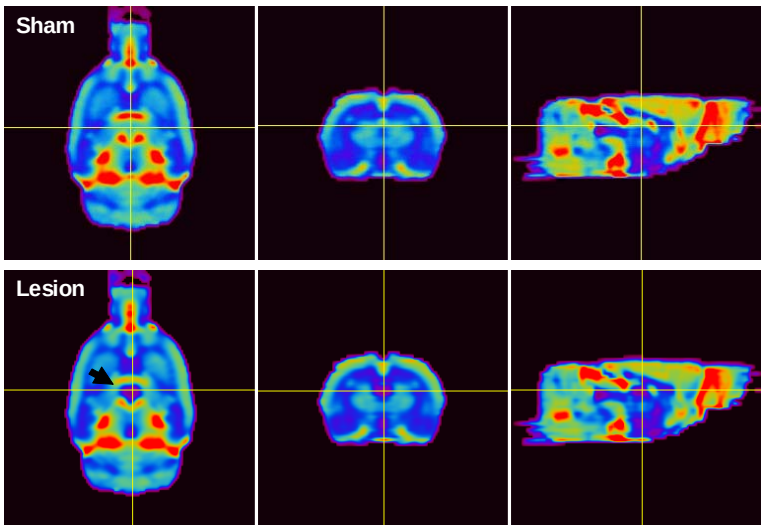
The figure shows a data set from an individual animal. The white arrow shows the localization of the habenula where one can distinguish oedema small hemorrhagic transformations around and within the lesion.

La figure montre un exemple de lesion. La flèche blanche indique la zone dans laquelle est située l'habénula, où l'on peut distinguer des traces d'oedéme ainsi que de légères traces d'hémorragie.

V.3.2 Comparison of blood volume in the habenula region during MRI 1 baseline measurement between sham-operated and lesioned animals

Measurement of baseline blood volume in sham and lesioned animals during MRI 1 revealed clear differences between the two groups. The baseline blood volume was reduced in the area covering the lesion by around 44%. Visual inspection of the rCBV maps revealed no abnormalities in rCBV for the sham operated animals (**Fig 5.4**).

Fig 5.4. Baseline rCBV during the first measurement (MRI 1)



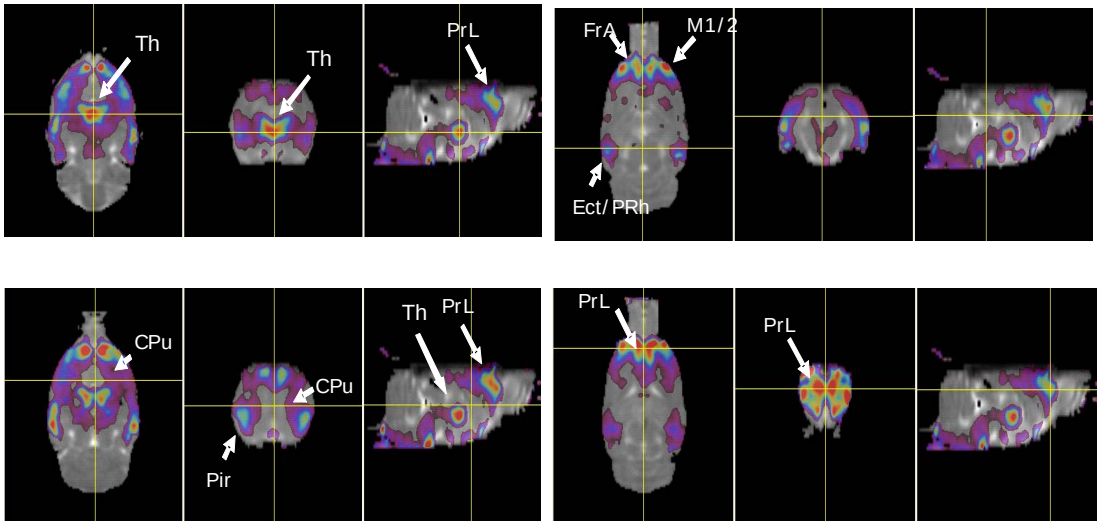
Comparison of rCBV maps from sham and lesioned animals. The rCBV maps including the habenula represent the average baseline over all animals belonging to one of the two groups, which were recorded before treatment onset during MRI 1. The black arrow indicates the position of the habenula. The signal intensity, which reflects the blood flow, increases according to the following set of colors: purple < blue < green < yellow < orange < red.

Sur cette figure est représentée la moyenne, pour chacun des deux groupes (sham et lésés), du flux sanguine de base avant le traitement à l'amphétamine durant le premier IRM (IRM 1) sur des coupes incluant l'habénula. La flèche noire indique la position de cette dernière, et l'on peut observer une baisse marquée de l'intensité du signal chez les animaux lésés par rapport aux animaux shams. L'intensité du signal, qui reflète le flux sanguin, croît selon le code de couleur suivant: violet < bleu < vert < jaune < orange < rouge).

V.3.3 Activation by amphetamine during MRI 1 in sham animals

Regions activated by acute amphetamine treatment (1 mg/kg) in sham animals are represented in **Fig 5.5**.

Fig 5.5. Regions activated by acute amphetamine (1 mg/kg) in sham animals



Structures activated by amphetamine in sham animals during MRI 1. Structures indicated in the figure are: Th, Thalamu; PrL, Prelimbic cortex; FrA, Frontal association cortex; PRh/Ect, Ectorhinal/perirhinal cortex; M1/M2, Primary/secondary motor area; Pir, Piriform cortex; CPu, Caudate Putamen.

Figure représentant les aires du cerveau actives par le traitement à l'amphétamine chez les animaux sham Durant le premier IRM (IRM 1). Les structures indiquées sont: Th, Thalamu; PrL, cortex prélimbique; FrA, cortex associatif frontal; PRh/Ect, cortex ectorhinal/perirhinal; M1/M2, aires motrices primaire/secondaire; Pir, cortex piriform; CPu, néo-striatum (noyau caudé/putamen).

V.3.4 rCBV results

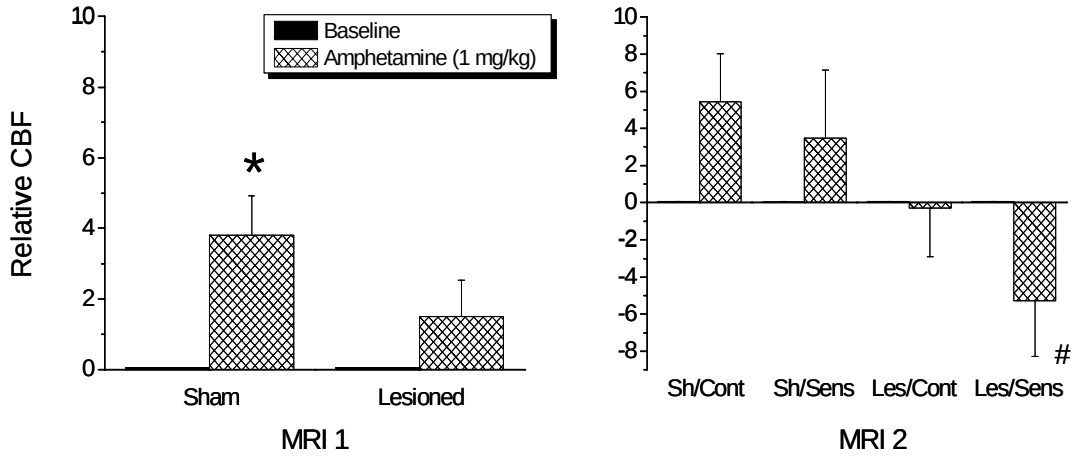
V.3.4.1 Frontal cortex

The results are shown in **Fig 5.6**

MRI 1. Two factor ANOVA (lesion as between subjects factor, acute drug treatment as a repeated factor) showed a significant effect only of drug ($F_{1,11} = 11.9$, $p < 0.005$).

MRI 2. Three factor ANOVA (lesion and subchronic treatment as between subjects factors, acute drug treatment as a repeated factor) showed a significant effect of lesion ($F_{1,9} = 5.80$, $p < 0.05$) and a significant lesion x drug interaction ($F_{1,9} = 5.81$, $p < 0.05$). Inspection of the results shows that the activation by amphetamine was markedly blunted in the lesioned animals, and even reversed. The fact that there was a lesion effect at the second, but not the first MRI, suggests that the effect of lesion was increasing with time. However this could not be shown with by 4-factor ANOVA (between subjects factors: lesion, subchronic treatment; nested within subjects factors: MRI number and acute drug treatment). This analysis showed significant effects only of lesion ($F_{1,9} = 8.27$, $p < 0.05$) and a lesion x drug interaction ($F_{1,9} = 8.28$, $p < 0.05$).

Fig 5.6. rCBV measurements in the frontal cortex



Relative cerebral blood flow (arbitrary units) before and after amphetamine (1 mg/kg, *i.v.*) during the first MRI (**Left**) and the second MRI (**Right**). Animals, either sham (Sh) or with habenula lesion (Les), were treated subchronically (4 days) with saline control (Sh/Cont, n=4; Les/cont, n=4), or with amphetamine 2.5 mg/kg/day to induce sensitization (Sh/Sens, n=2; Les/Sens, n=3) between the first and the second MRI. *p<0.05 *vs* baseline blood flow of same group (2-tailed paired t-test following significant ANOVA); # p<0.05 *vs* same acute treatment in corresponding sham group (2-tailed t-test following significant ANOVA).

*Flux sanguin cerebral relatif (unite arbitraire) avant et après amphétamine (1 mg/kg, i.v.) durant le premier IRM (**Gauche**) et le second IRM (**Droite**). Les animaux, soit sham (Sh) soit avec lésion de l'habénula (Les), reçurent un traitement subchronique (4 jours) de liquide physiologique (Sh/Cont, n=4; Les/cont, n=4), ou d'amphétamine 2.5 mg/kg afin d'induire une sensibilisation (Sh/Sens, n=2; Les/Sens, n=3) entre le premier et le second IRM. *p<0.05 comparé au flux sanguine de base du même groupe (test t à deux borned=s suivant une ANOVA significative); #p<0.05 comparé au groupe sham dans les mêmes conditions (test t à deux borned suivant une ANOVA significative).*

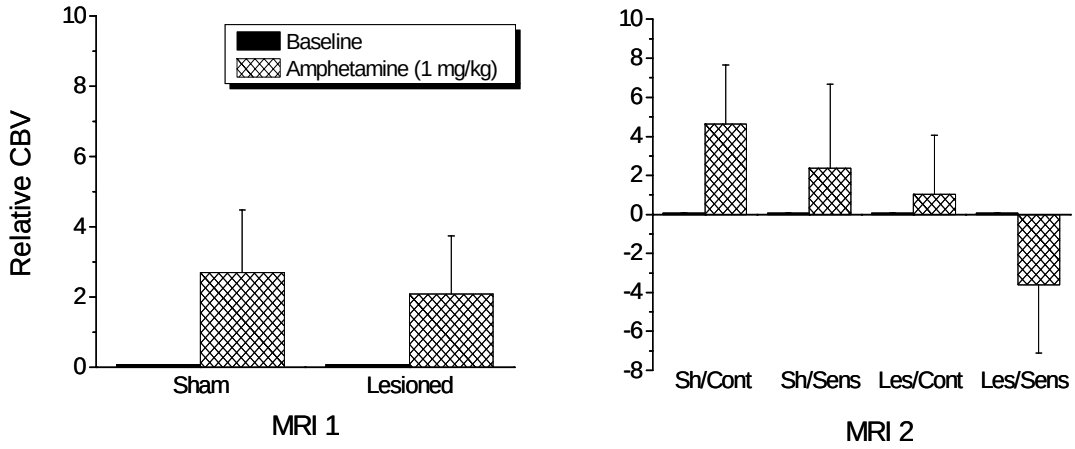
V.3.4.2 Cingulate cortex

The results are shown in **Fig 5.7**

MRI 1. Two factor ANOVA (lesion as between subjects factor, acute drug treatment as a repeated factor) showed no significant effect of group ($F_{1,11}$ = 0.063, p > 0.8), no effect of drug treatment ($F_{1,11}$ = 3.612, p = 0.084) and no interaction between these two factors ($F_{1,11}$ = 0.062, p > 0.8).

MRI 2. Three factor ANOVA (lesion and subchronic treatment as between subjects factors, acute drug treatment as a repeated factor) showed no significant effect of any of the parameters, nor of any of the interactions, analyzed.

Fig 5.7. rCBV measurements in the cingulate cortex



Relative cerebral blood flow (arbitrary units) before and after amphetamine (1 mg/kg, *i.v.*) during the first MRI (**Left**) and the second MRI (**Right**). Animals, either sham (Sh) or with habenula lesion (Les), were treated subchronically (4 days) with saline control (Sh/Cont, n=4; Les/cont, n=4), or with amphetamine 2.5 mg/kg/day to induce sensitization (Sh/Sens, n=2; Les/Sens, n=3) between the first and the second MRI.

*Flux sanguin cerebral relatif (unite arbitraire) avant et après amphétamine (1 mg/kg, i.v.) durant le premier IRM (**Gauche**) et le second IRM (**Droite**). Les animaux, soit sham (Sh) soit avec lésion de l'habénula (Les), reçurent un traitement subchronique (4 jours) de liquide physiologique (Sh/Cont, n=4; Les/cont, n=4), ou d'amphétamine 2.5 mg/kg afin d'induire une sensibilisation (Sh/Sens, n=2; Les/Sens, n=3) entre le premier et le second IRM.*

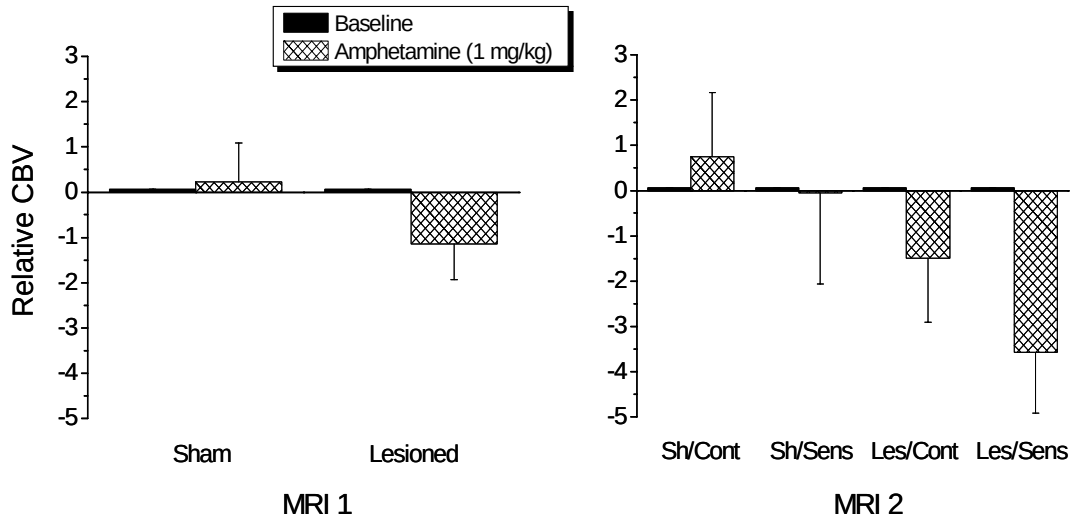
V.3.4.3 Parietal cortex

The results are shown in **Fig 5.8**

MRI 1. Two factor ANOVA (lesion as between subjects factor, acute drug treatment as a repeated factor) showed no significant effect of group ($F_{1,11}$ = 1.334, p = 0.273), no effect of drug treatment ($F_{1,11}$ = 0.782, p = 0.396) and no interaction between these two factors ($F_{1,11}$ = 1.343, p = 0.271).

MRI 2. Three factor ANOVA (lesion and subchronic treatment as between subjects factors, acute drug treatment as a repeated factor) showed no significant effect of any of the parameters, nor of any of the interactions analyzed.

Fig 5.8. rCBV measurements in the parietal cortex



Relative cerebral blood flow (arbitrary units) before and after amphetamine (1 mg/kg, *i.v.*) during the first MRI (**Left**) and the second MRI (**Right**). Animals, either sham (Sh) or with habenula lesion (Les), were treated subchronically (4 days) with saline control (Sh/Cont, n=4; Les/cont, n=4), or with amphetamine 2.5 mg/kg/day to induce sensitization (Sh/Sens, n=2; Les/Sens, n=3) between the first and the second MRI.

*Flux sanguin cerebral relatif (unite arbitraire) avant et après amphétamine (1 mg/kg, i.v.) durant le premier IRM (**Gauche**) et le second IRM (**Droite**). Les animaux, soit sham (Sh) soit avec lésion de l'habénula (Les), reçurent un traitement subchronique (4 jours) de liquide physiologique (Sh/Cont, n=4; Les/cont, n=4), ou d'amphétamine 2.5 mg/kg afin d'induire une sensibilisation (Sh/Sens, n=2; Les/Sens, n=3) entre le premier et le second IRM.*

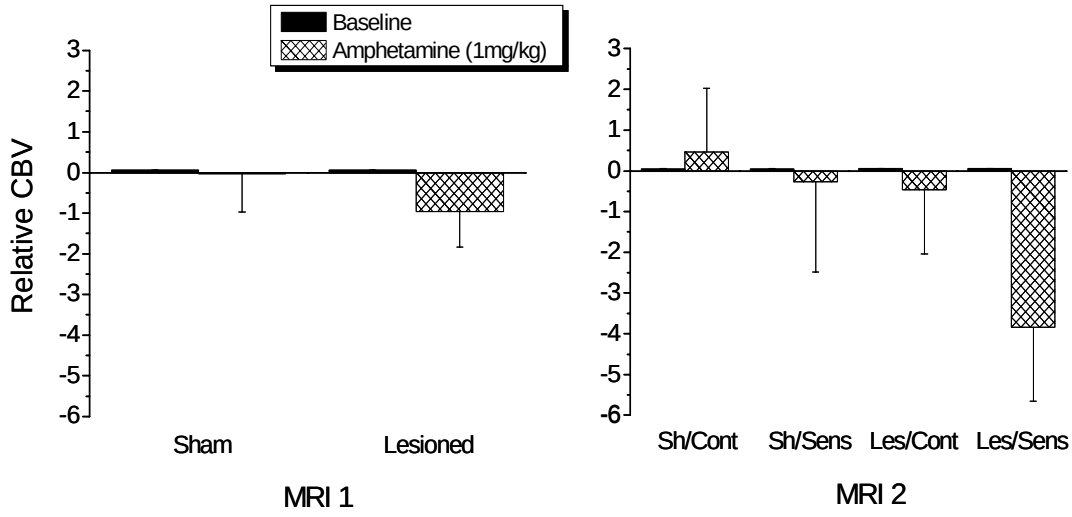
V.3.4.4 Caudate-Putamen

The results are shown in **Fig 5.9**

MRI 1. Two factor ANOVA (lesion as between subjects factor, acute drug treatment as a repeated factor) showed no significant effect of group ($F_{1,11}$ = 0.52, p = 0.486), no effect of drug treatment ($F_{1,11}$ = 0.716, p = 0.416) and no interaction between these two factors ($F_{1,11}$ = 0.519, p = 0.486).

MRI 2. Three factor ANOVA (lesion and subchronic treatment as between subjects factors, acute drug treatment as a repeated factor) showed no significant effect of any of the parameters, nor of any of the interactions analyzed.

Fig 5.9. rCBV measurements in the caudate-putamen



Relative cerebral blood flow (arbitrary units) before and after amphetamine (1 mg/kg, *i.v.*) during the first MRI (**Left**) and the second MRI (**Right**). Animals, either sham (Sh) or with habenula lesion (Les), were treated subchronically (4 days) with saline control (Sh/Cont, n=4; Les/cont, n=4), or with amphetamine 2.5 mg/kg/day to induce sensitization (Sh/Sens, n=2; Les/Sens, n=3) between the first and the second MRI.

*Flux sanguin cerebral relatif (unite arbitraire) avant et après amphétamine (1 mg/kg, i.v.) durant le premier IRM (**Gauche**) et le second IRM (**Droite**). Les animaux, soit sham (Sh) soit avec lésion de l'habénula (Les), reçurent un traitement subchronique (4 jours) de liquide physiologique (Sh/Cont, n=4; Les/cont, n=4), ou d'amphétamine 2.5 mg/kg afin d'induire une sensibilisation (Sh/Sens, n=2; Les/Sens, n=3) entre le premier et le second IRM.*

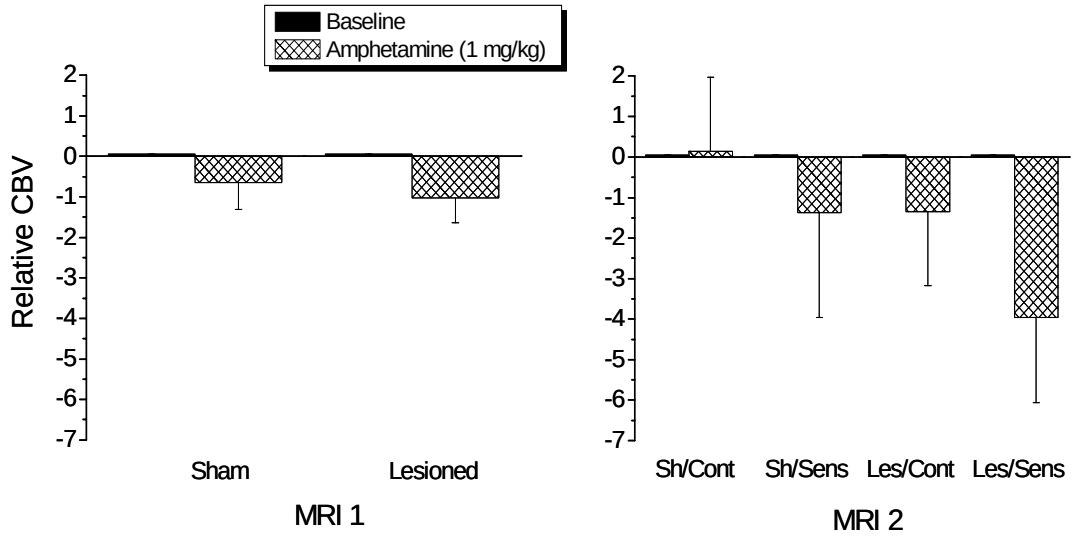
V.3.4.5 Hippocampus

The results are shown in **Fig 5.10**

MRI 1. Two factor ANOVA (lesion as between subjects factor, acute drug treatment as a repeated factor) showed no significant effect of group ($F_{1,11}$ = 0.176, p = 0.683), no effect of drug treatment ($F_{1,11}$ = 3.799, p = 0.077) and no interaction between these two factors ($F_{1,11}$ = 0.175, p = 0.684).

MRI 2. Three factor ANOVA (lesion and subchronic treatment as between subjects factors, acute drug treatment as a repeated factor) showed no significant effect of any of the parameters, nor of any of the interactions analyzed.

Fig 5.10. rCBV measurements in the hippocampus



Relative cerebral blood flow (arbitrary units) before and after amphetamine (1 mg/kg, *i.v.*) during the first MRI (**Left**) and the second MRI (**Right**). Animals, either sham (Sh) or with habenula lesion (Les), were treated subchronically (4 days) with saline control (Sh/Cont, n=4; Les/cont, n=4), or with amphetamine 2.5 mg/kg/day to induce sensitization (Sh/Sens, n=2; Les/Sens, n=3) between the first and the second MRI.

*Flux sanguin cerebral relatif (unite arbitraire) avant et après amphétamine (1 mg/kg, i.v.) durant le premier IRM (**Gauche**) et le second IRM (**Droite**). Les animaux, soit sham (Sh) soit avec lésion de l'habénula (Les), reçurent un traitement subchronique (4 jours) de liquide physiologique (Sh/Cont, n=4; Les/cont, n=4), ou d'amphétamine 2.5 mg/kg afin d'induire une sensibilisation (Sh/Sens, n=2; Les/Sens, n=3) entre le premier et le second IRM.*

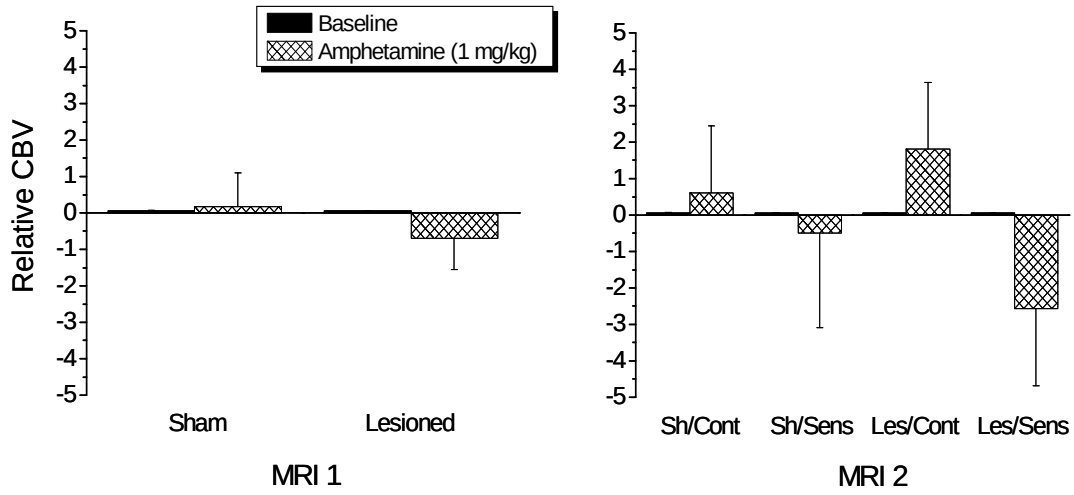
V.3.4.6 Medulla

The results are shown in **Fig 5.11**

MRI 1. Two factor ANOVA (lesion as between subjects factor, acute drug treatment as a repeated factor) showed no significant effect of group ($F_{1,11}$ = 0.474, p = 0.505), no effect of drug treatment ($F_{1,11}$ = 0.259, p = 0.621) and no interaction between these two factors ($F_{1,11}$ = 0.466, p = 0.509).

MRI 2. Three factor ANOVA (lesion and subchronic treatment as between subjects factors, acute drug treatment as a repeated factor) showed no significant effect of any of the parameters, nor of any of the interactions analyzed.

Fig 5.11. rCBV measurements in the medulla



Relative cerebral blood flow (arbitrary units) before and after amphetamine (1 mg/kg, *i.v.*) during the first MRI (**Left**) and the second MRI (**Right**). Animals, either sham (Sh) or with habenula lesion (Les), were treated subchronically (4 days) with saline control (Sh/Cont, n=4; Les/cont, n=4), or with amphetamine 2.5 mg/kg/day to induce sensitization (Sh/Sens, n=2; Les/Sens, n=3) between the first and the second MRI.

*Flux sanguin cerebral relatif (unite arbitraire) avant et après amphétamine (1 mg/kg, i.v.) durant le premier IRM (**Gauche**) et le second IRM (**Droite**). Les animaux, soit sham (Sh) soit avec lésion de l'habénula (Les), reçurent un traitement subchronique (4 jours) de liquide physiologique (Sh/Cont, n=4; Les/cont, n=4), ou d'amphétamine 2.5 mg/kg afin d'induire une sensibilisation (Sh/Sens, n=2; Les/Sens, n=3) entre le premier et le second IRM.*

V.4 Discussion

The results of the present study can be considered preliminary, since the group sizes were quite small, because of unexpected deaths of animals between the first and second MRI experiment. Nevertheless certain statistically significant results were obtained.

The main finding was that bilateral habenula lesion caused a type of "hypofrontality" in the sense that the frontal cortex was less markedly activated by amphetamine in lesioned animals than in sham-operated animals. In fact, at least qualitatively, amphetamine caused a decrease in relative cerebral blood flow in lesioned animals, in contrast to the increase that it caused in sham-operated animals. This result bears strong resemblance to the results from several studies of regional brain metabolism and blood flow in schizophrenia, that indicate physiological hypoactivity of the prefrontal cortex (see review by Weinberger & Berman, 1988) particularly during the performance of behavioural tasks that activate the prefrontal cortex of controls (Berman *et al*, 1986; Cohen *et al*, 1987; Volkow *et al*, 1987; Weinberger *et al*, 1986a, 1988).

In other cortical areas similar results were obtained, but were not statistically significant. The amphetamine-induced reduction of blood flow in cerebellum and superior colliculus are new findings, and were unaltered by habenula lesion.

In comparing the effects of amphetamine in the present study to previous findings it is most relevant to compare them to imaging studies, in which anesthesia was used, rather than to deoxyglucose experiments in the freely-moving animal. Such studies are relatively few, but in general the present results are similar to them. For example, using a much higher dose of amphetamine (20 mg/kg, *i.p.*) in rats Silva *et al* (1995) observed the greatest increase in blood flow in frontal cortical regions, and a reduction in the hippocampus, which was also a statistically non-significant tendency in the present studies. An increase in the caudate-putamen was also obtained in the study of Silva *et al* (1995). In the present study this effect was not observed. One possible reason for this discrepancy is the marked difference in amphetamine doses used. Chen *et al* (1997) also used an amphetamine dose (3 mg/kg, *i.v.*) considerably higher than that used here. Activation of cortical regions was highly similar to that found in the present study in that frontal cortex was activated more than cingulated cortex, and parietal cortex was activated the least. At this dose the striatum was also activated. Apart from the

difference in amphetamine dose, another factor that may partly account for the apparent difference in effects on striatal blood flow, is regional variation within the striatum. For example in one deoxyglucose study in mice (Miyamoto *et al*, 2000), regions within the striatum activated by amphetamine can clearly be seen, whereas in the region as a whole no overall effect was obtained. Such an explanation may also apply to the finding here of activated regions in the medulla, but not of activation of the medulla as a whole.

It is possible that the hypoactivation of the frontal cortex could contribute to elevated subcortical dopamine release, resulting in the increase of premature responding in the 5-choice serial reaction time task shown by rats with such lesions (see **Chapter IV**). Supporting such a suggestion are, for example, studies that show that lesion of the frontal cortex potentiates amphetamine-induced behaviours (Iversen, 1971) such as locomotor activity and stereotypy that are mediated by mesolimbic and nigrostriatal dopamine release (Kelly, 1977). In agreement with such a view other studies show that activation of the frontal cortex decreases dopamine release in nucleus accumbens probably via projections to the dopaminergic cell bodies of the ventral tegmental area (Jackson *et al*, 2001). Moreover the frontal cortex is involved in acquisition of the Morris water-maze spatial memory task, as shown by lesion and functional inactivation studies (Dallison & Kolb, 2003; Vafaei & Rashidy-Pour, 2004) as well as by the fact that indices of neuronal plasticity are increased in frontal cortical regions after Morris maze training (Wright *et al*, 2003). Thus the frontal cortex changes observed here may also contribute to the impairment of Morris water-maze learning caused by habenula lesion.

Future mechanistic studies are clearly required to determine the intermediate steps leading from habenula lesion to diminished frontal cortex response to amphetamine. The numerous habenula-brainstem nucleus-frontal cortex pathways are obvious candidates for further study. Since there was a tendency in lesioned/sensitized animals for amphetamine to decrease blood flow in areas receiving little or no dopaminergic innervation such as the occipital cortex and hippocampus, or no noradrenergic innervation, such as the caudate-putamen, serotonergic mechanisms, which innervate all of these regions, seem to deserve investigation.

Finally it is not presently possible to decide whether in schizophrenia there is a blunting of the amphetamine-induced activation of frontal cortex, which would be a similarity to the effects of habenula lesion observed here. The reason for this is that previous studies of the effects of amphetamine on cerebral metabolism or cerebral blood flow in schizophrenia have used doses that have an inhibitory effect in controls (Wolkin *et al*, 1987, 1994). Interestingly there was a tendency for the inhibitory response in frontal cortex to be blunted in schizophrenia patients. Only recently has it been shown that slightly higher doses of amphetamine cause more pronounced stimulatory effects on glucose utilization in several brain regions including anterior cingulate cortex (Vollenweider *et al*, 1998). Hopefully in the future there will be a study in schizophrenics compared to controls where similar doses are used, and where blood flow is measured rather than glucose utilization, which would be more comparable to the study reported here. It would also be more comparable to perform the study in anaesthetized subjects, if this is acceptable from safety and ethical viewpoints, since anesthesia markedly activates the habenula (Herkenham, 1981), such that these conditions may be optimal to observe lesion-induced effects.

Because of the preliminary nature of the present results it will be important to confirm them, and to extend them by finer regional analysis, as well as following the time-course for longer times after lesioning of the changes observed.

In summary, the present results demonstrate that activation of the frontal cortex by amphetamine is blunted in rats with bilateral lesions of the habenula. Since this cortical region is involved in attention and spatial memory this “hypofrontality” might contribute to the deficits in these behaviours caused by habenula lesion, if a similar reduction of activation by a behavioural task, rather than amphetamine, occurs. The similarity of these results to mechanisms in schizophrenia cannot be directly compared, since there have not been directly similar studies of amphetamine performed in schizophrenics compared to controls. However in a general way they are similar to extensive evidence of hypofrontality in schizophrenia.

6 Janvier 1969

Chapter VI - Does habenula lesion alter populations of receptors linked to schizophrenia ? An autoradiographic study

VI.1 Introduction

It has been shown in rodents and cats that habenular manipulation, by either lesion or stimulation, leads to marked variations of the release of several neurotransmitters (Garland & Mogenson, 1983; Greatrex & Phillipson, 1982; Kalen *et al*, 1989; Nagy *et al*, 1978; Sutherland, 1982). More precisely, through a habenulo-raphé pathway (Aghajanian & Wang, 1977), which is a major inhibitory influence on serotonergic cells of dorsal raphé (Nishikawa & Scatton, 1985; Speciale *et al*, 1980; Wang & Aghajanian, 1977), habenula activity alters serotonergic activity in striatum and substantia nigra (Reisine *et al*, 1982; Soubrié *et al*, 1981), and the hippocampus (Ferraro *et al*, 1997; Sabatino *et al,* 1991). Similarly, it has been found that the lateral habenula acts directly on locus coeruleus to positively influence the noradrenergic activity in hippocampus, prefrontal cortex, striatum and nucleus accumbens (Cenci *et al*, 1992 ; Kalen *et al*, 1989). Other studies have shown that the lateral habenula projects directly to the ventral tegmental area and substantia nigra to negatively influence mesocortical, mesostriatal and mesolimbic dopaminergic pathways (Christoph *et al*, 1986; Lisoprawski *et al*, 1980; Matsuda & Fujimura, 1992). Moreover habenula stimulation results in an increased release of acetylcholine in the hippocampus (Nilsson *et al*, 1990).

In previous chapters it was shown that complete bilateral lesion of the habenula resulted in marked cognitive deficits. First of all, using the Morris water maze, we have found that the lesion impaired spatial memory, as attested by greater latency and distance to find the hidden platform (see **Chapter II**). Second the lesion induced marked attention deficits that were twofold: first, the lesioned rats showed marked immediate enhancement of the number of premature responses, an index of impulsivity, which decreased with time and was antagonized by haloperidol treatment; second, the lesioned rats showed a late-appearing impairment of choice accuracy in this attention test, that worsened with time and was not improved by haloperidol treatment (see **Chapter IV**). The latter results led us to consider that lesion of the habenula provokes two distinct deficits: a short-term deficit that seems to involve dopaminergic and possibly other

monoaminergic systems, and a long-term deficit, that seems to be independent of those monoaminergic systems, but may be due to cumulative erroneous changes in synaptic connectivity that are postulated to account for the appearance of delusions in schizophrenics (Kelly, 1998). Finally, in an MRI study, we observed blunted response to amphetamine, represented by blunted increase of relative cerebral blood flow, in rats with lesion of the habenula (see **Chapter V**).

In an attempt to correlate our behavioural and imaging findings with possible changes at the synaptic level, we examined here the impact of bilateral habenula lesion on several receptors by means of the autoradiography technique. This technique, using labelled ligand binding of either agonists or antagonists, is a very useful tool for quantifying a population of receptors in any region of the brain. We therefore investigated the consequence of the lesion upon the serotonergic 5-HT_{1A} and 5-HT_{2A}, the dopaminergic D_1 and D_2, the glutamatergic NMDA and the cholinergic nicotinic alpha-7 receptors, as those receptors have been intensively shown to be involved in schizophrenia. For example, potent antipsychotics bind to the dopaminergic D_2, for the typical, and the serotonergic 5-HT_{2A}, for the atypicals, receptors. The cholinergic nicotinic alpha-7 and the glutamatergic NMDA receptors have been associated with the cognitive deficits of schizophrenia. Several cortical and sub-cortical regions of the brain have been investigated, as they have been intensively shown to be implicated in the aetiology of schizophrenia (see **Chapter I**), as well as structures directly influenced by habenular efferent pathways. Autoradiography was performed at two time-points on two different groups of rats, either one week or twelve weeks after the lesion, in order to investigate the possible short- and long-term effects of the lesion, as we have noted during our attention test that the lesion induced both immediate and delayed deficits (see **Chapter IV**).

VI.2 Materials and methods

VI.2.1 Animals

The experiments were carried out with 24 male Sprague-Dawley rats (Iffa Credo, France) housed by groups of four in Macrolon cages (60 x 37 x 20 cm) in a temperature-regulated (22 ± 2°C) animal room on a 12 h light/dark cycle (lights on at 06:00). Food and water were available *ad libitum*. The experiments were approved by the Cantonal Veterinary Authority of the City of Basel. Animals were acclimatized to the animal quarters for at least a week before starting the experiments, which took place during the light phase.

VI.2.2 Surgical procedures

Lesion of the habenula or sham procedure were performed as previously described (see **Chapter II**). After completion of the operation, rats were housed four to a cage, two lesioned and two sham-operated rats.

VI.2.3 Receptor autoradiography

VI.2.3.1 Serotonergic 5-HT_{1A} receptor

After 30 min of preincubation in buffer containing 170 mM Tris-HCl pH 7.6, 4 mM $CaCl_2$, 0.01% ascorbic acid, 1 µM pargyline and 1 µM fluoxetine at room temperature, the sections were incubated for 60 min at room temperature in the same buffer supplemented with 2 nM [^{3}H]-8-OH-DPAT (229 Ci/mmol; Amersham). Non-specific binding was determined in a set of adjacent slides by incubation in the presence of 10 µM 5-HT. The washing of labelled sections was carried out as follows: two 5 min washes in ice-cold incubation buffer and a brief dipping in ice-cold water to remove salts. Finally the sections were dried under a stream of cold air. Autoradiograms were generated by apposing the labelled tissues to BioMax MR Films (Eastman Kodak Company, Rochester, New York 14650) during 3 weeks.

VI.2.3.2 Serotonergic 5-HT_{2A} receptor

After 1 min of preincubation in a buffer (50 mM Tris-HCl pH 7.4, 4 mM $CaCl_2$, 0.1% ascorbic acid, 0.1 % BSA and 10 nM mesulergine) at room temperature, the sections were incubated at room temperature for 90 min in the same medium supplemented with 0.2 nM [^{125}I]-(±)DOI ((±)-1-(2,5,-dimethoxy-4-[^{125}I]iodophenyl)-2-aminopropane; 2200 Ci/mmol; Perkin-Elmer, NEN). Non-specific binding was determined in a set of adjacent slides by incubation in the presence of 10 µM 5-HT. The labelled sections were washed as follows: two 10 min washes in the former buffer (without ligand) was followed by a brief dipping in ice-cold distilled water to remove salts. The sections were then dried under a stream of cold air. The autoradiograms were generated by apposing the [^{125}I]-(±)DOI labelled tissues at 4°C to BioMax MR Films (Eastman Kodak Company, Rochester, New York 14650) during 2 weeks.

VI.2.3.3 Nicotinic acetylcholine receptor alpha-7

Receptor autoradiography was performed according to the procedure of Verbois *et al* (2000). After 30 min of preincubation at room temperature in KRH buffer: Krebs-Ringer HEPES buffer containing 20 mM HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid) pH 7.4, 118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 10 mM NaOH, the sections were incubated for 2 hours at room temperature in KRH buffer, supplemented with 0.05 mg/ml BSA (bovine serum albumin) and 2.5 nM [^{125}I]α-Bungarotoxin (136 Ci/mmol, Perkin Elmer). Non-specific binding was determined in a set of adjacent slides by incubation in the presence of 10 mM (-)-Nicotine. The washing of labelled sections was carried out as follows: three 20 min washes in the ice-cold KRH buffer (without ligand), 20 sec in ice-cold 10 times diluted KRH buffer and a brief dipping in ice-cold distilled water to remove salts. Finally the sections were dried under a stream of cold air. Autoradiograms were generated by apposing the labelled tissues to BioMax MR Films (Eastman Kodak Company, Rochester, New York 14650) at 4^{o}C for 1 week.

VI.2.3.4 Glutamatergic NMDA receptor

Receptor autoradiography was performed according to the procedure of Giraldez & Girardi (1998): after 1 hour of preincubation at 4^{o}C in buffer containing 50 mM Tris-

HCl pH 7.5, the sections were incubated for 45 min at room temperature in 50 mM Tris-HCl pH 7.5, 10 µM glutamate, 10 µM glycine and 1 mM spermidine, supplemented with 10 nM [^{3}H]-MK801 (17.1 Ci/mmol, Perkin Elmer). Non-specific binding was determined in a set of adjacent slides by incubation in the presence of 0.1 mM MK801. The washing of labelled sections was carried out as follows : three 1 min washes in the preincubation buffer (without ligand) and two brief dippings in ice-cold distilled water to remove salts. Finally the sections were dried under a stream of cold air. Autoradiograms were generated by apposing the labelled tissues to BioMax MR Films (Eastman Kodak Company, Rochester, New York 14650) at 4^{o}C for 5 weeks.

VI.2.3.5 Dopaminergic D_1 receptor

Receptor autoradiography was performed according to the procedure of Lillrank *et al* (1999). After 20 min of preincubation at room temperature in buffer containing 50 mM Tris-HCl pH 7.4, the sections were incubated for 90 min at room temperature in 50 mM Tris-HCl pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 µM mianserin, supplemented with 5 nM [^{3}H]-SCH23390 (75.5 Ci/mmol, NEN). Non-specific binding was determined in a set of adjacent slides by incubation in the presence of 10 µM dopamine. The washing of labelled sections was carried out as follows: a brief dipping in ice-cold distilled water followed by two 10 min washes in the ice-cold former buffer (without ligand) and a brief dipping in ice-cold distilled water to remove salts. Finally the sections were dried under a stream of cold air. Autoradiograms were generated by apposing the labelled tissues to BioMax MR Films (Eastman Kodak Company, Rochester, New York 14650) at 4^{o}C for 2 weeks.

VI.2.3.6 Dopaminergic D_2 receptor

Receptor autoradiography was performed according to the procedure of Coronas *et al* (1997). After 15 min of preincubation at room temperature in buffer containing 50 mM Tris-HCl pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ the sections were incubated for 30 min at room temperature in the same buffer supplemented with 0.1 nM [^{125}I]-Iodosulpride (2000 Ci/mmol, Amersham). Non-specific binding was determined in a set of adjacent slides by incubation in the presence of 1 µM spiperone. The washing of labelled sections was carried out as follows: a brief dipping in ice-cold distilled water followed by two 2 min washes in the ice-cold former

buffer (without ligand) and a brief dipping in ice-cold distilled water to remove salts. Finally the sections were dried under a stream of cold air. Autoradiograms were generated by apposing the labelled tissues to BioMax MR Films (Eastman Kodak Company, Rochester, New York 14650) at 4°C for 2 days.

VI.2.4 Histology

Twenty five-micron slices were taken through the entire habenula, mounted on slides and stained with Toluidine blue. Lesions of the habenula were considered acceptable when a large proportion of the habenula was destroyed and surrounding regions (*i.e.* dorsal hippocampus and paraventricular, dorsomedial, lateral and parafascicular thalamic nuclei) were spared.

VI.2.5 Data analysis

Data from binding were analyzed by optic densitometry of BioMax MR Films using a computerized image analysis system (MCID, Imaging Research, St Catherines, Ontario, Canada). For a given labelled region, the optic density (O.D.) corresponding to the total binding and non specific binding was measured. For each brain region, two sections measuring total binding and two sections measuring nonspecific binding were analyzed for each animal; means of the values from the two "total binding" sections were used for total binding for that animal and similarly for nonspecific binding. Specific binding was calculated by subtracting the value for nonspecific binding for each animal from the value for total binding for that animal. Both right and left hemispheres were analyzed and the value used for that brain region was the mean of values for the two hemispheres.

The statistical significance of differences between treatment groups at the two different time-points were analyzed by analysis of variance (ANOVA), using the SYSTAT software package (Version 10.2, SPSS Inc., Chicago, IL).

VI.3 Results

The statistical analysis was performed with 3 sham-operated and 4 lesioned animals. As some slides were damaged by the optic densitometry film technique, there have been animals that lacked some regions for some receptors. In case of too few animals for one of the two groups, the regions were not analyzed in each groups.

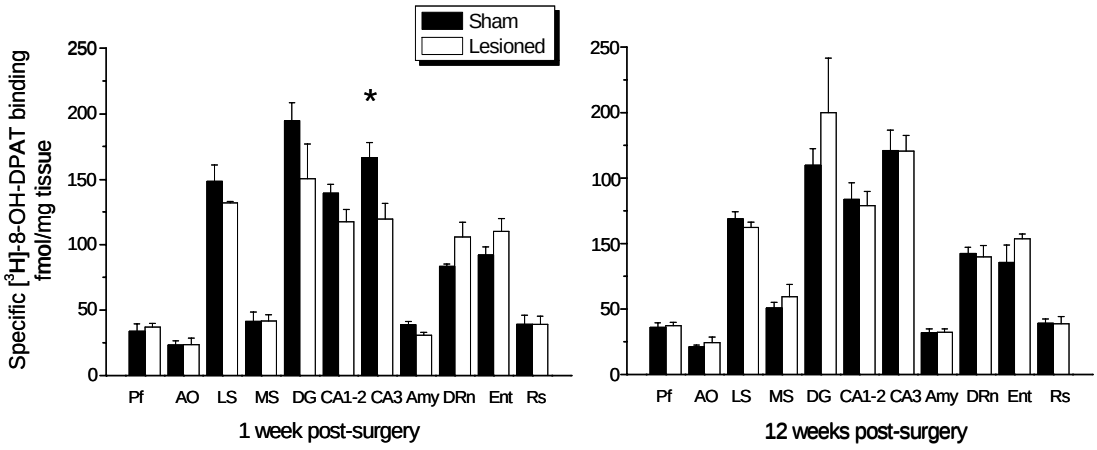
VI.3.1 Serotonergic 5-HT_{1A} receptor

The results are presented in **Fig 6.1**.

One week. One-way ANOVA showed a significant effect of the lesion in the CA_3 field of the hippocampus, the receptor binding being lower in the lesioned animals ($F_{1,5}$ = 7.05, $p < 0.05$). One-way ANOVA also showed a tendency for the lesioned animal to have a decreased binding in the $CA_{1\text{-}2}$ field of the hippocampus ($F_{1,5}$ = 2.97, $p > 0.1$), and in the amygdala ($F_{1,5}$ = 4.95, $p > 0.05$), and a tendency for an increased binding in the dorsal raphé nucleus ($F_{1,5}$ = 2.75, $p > 0.1$). On the other hand, one-way ANOVA showed no effect of lesion in any of the other regions studied.

Twelve weeks. The results are presented in **Fig 6.2**. One-way ANOVA showed no significant effect of the lesion for any of the regions studied.

Fig 6.1. Specific binding on the serotonergic 5-HT$_{1A}$ receptors



Results (mean ± SEM; fmol/mg tissue) are shown for the sham and the lesioned groups. * $p < 0.05$. Abbreviations: Pf, prefrontal cortex; AO, anterior olfactory nucleus; LS, lateral septum; MS, medial septum; DG, dentate gyrus; CA1-2, CA_{1-2} fields of the hippocampus; CA3, CA_3 field of the hippocampus; Amy, amygdala; DRn, dorsal raphé nucleus; Ent, entorhinal cortex; Rs, retrosplenial cortex.

VI.3.2 Serotonergic 5-HT$_{2A}$ receptor

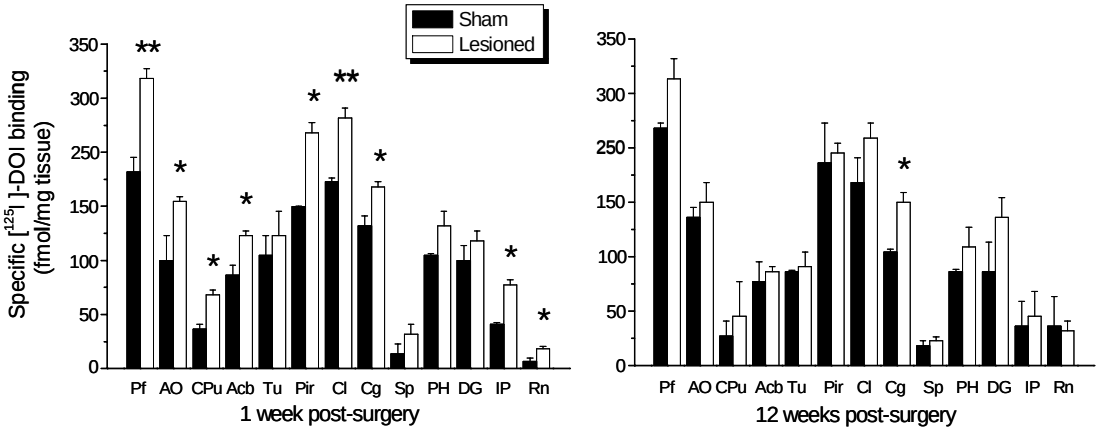
The results are presented in **Fig 6.2**, and in **Fig 6.3** are shown examples of autoradiograms at the level of the frontal cortex one week post-surgery.

One week post-surgery. One-way ANOVA revealed a marked effect of the lesion, as lesioned animals showed increased specific binding in prefrontal cortex ($F_{1,4} = 24.73$, $p < 0.05$), anterior olfactory nucleus ($F_{1,4} = 12.50$, $p < 0.05$), neostriatum (caudate-putamen) ($F_{1,4} = 16.74$, $p < 0.05$), ventral striatum (accumbens nucleus) ($F_{1,4} = 14.22$, $p < 0.05$), piriform cortex ($F_{1,4} = 15.46$, $p < 0.05$), claustrum ($F_{1,4} = 49.7$, $p < 0.01$), cingulate cortex ($F_{1,4} = 12.06$, $p < 0.05$), interpeduncular nucleus ($F_{1,4} = 17.94$, $p < 0.05$) and raphé nuclei ($F_{1,4} = 12.46$, $p < 0.05$). On the other hand, one-way ANOVA showed no effect of lesion in olfactory tubercle ($F_{1,4} = 0.42$, $p > 0.1$), septum ($F_{1,4} = 2.27$, $p > 0.1$), posterior hypothalamus ($F_{1,4} = 1.43$, $p > 0.1$) and dentate gyrus ($F_{1,4} = 1.63$, $p > 0.1$).

Twelve weeks post-surgery. One-way ANOVA showed that the significantly increased binding in lesioned animals remained in only one region, *i.e.* cingulate cortex ($F_{1,3} =$

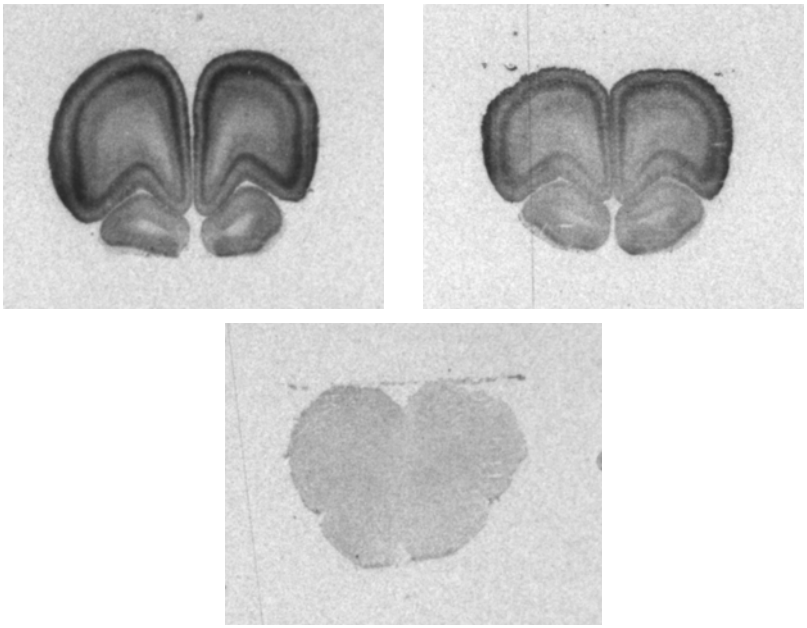
21.49, $p < 0.05$). One-way ANOVA revealed no significant difference between the two groups in any of the other regions studied.

Fig 6.2. Specific binding on the serotonergic 5-HT_{2A} receptors



Results (mean ± SEM; fmol/mg tissue) are shown for the sham and the lesioned groups. Abbreviations: Pf, prefrontal cortex; AO, anterior olfactory nucleus; CPu, caudate-Putamen, Acb, accumbens nucleus; Tu, olfactory tubercle; Pir, pyriform cortex; Cl, claustrum; Cg, cingulate cortex; Sp, septum; PH, posterior hypothalamus; DG, dentate gyrus; IP, interpeduncular nucleus; Rn, raphé nuclei.

Fig 6.3 Autoradiograms of the serotonergic 5-HT_{2A} receptors one week post-surgery



Autoradiograms are shown of a lesioned rat (**top left**) and a sham rat (**top right**). At the **bottom** is represented the non-specific binding at the same level.

*Autoradiogrammes à partird'un rat lesé (**partie supérieure gauche**) et d'un rat sham (**partie supérieure droite**). La **partie inférieur** représente le marquage non-spécifique au même niveau.*

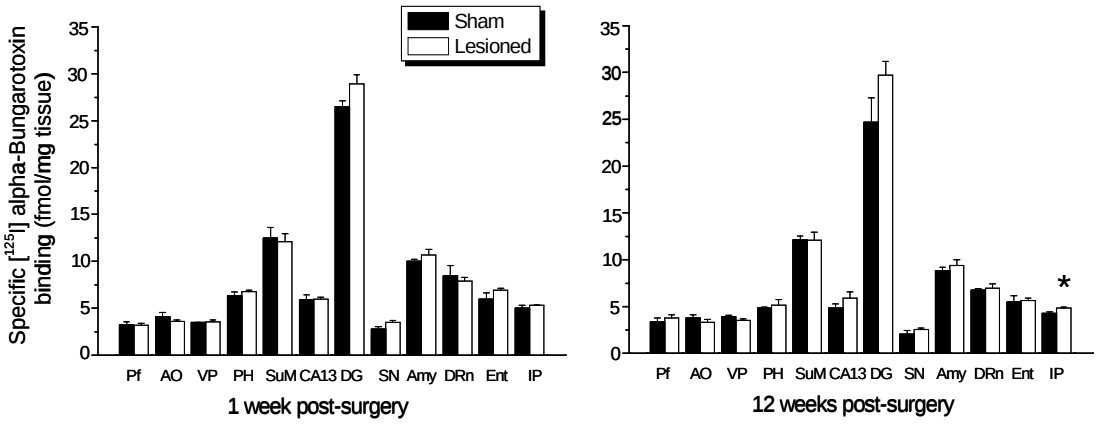
VI.3.3 Nicotinic acetylcholine receptor alpha-7

The results are presented in **Fig 6.4**.

One week post-surgery. One-way ANOVA showed no significant effect of the lesion in any of the regions studied.

Twelve weeks post-surgery. One-way ANOVA revealed no significant effect of the lesion in any of the regions considered at both tome-points.

Fig 6.4. Specific binding on the cholinergic nicotinic alpha-7 receptors



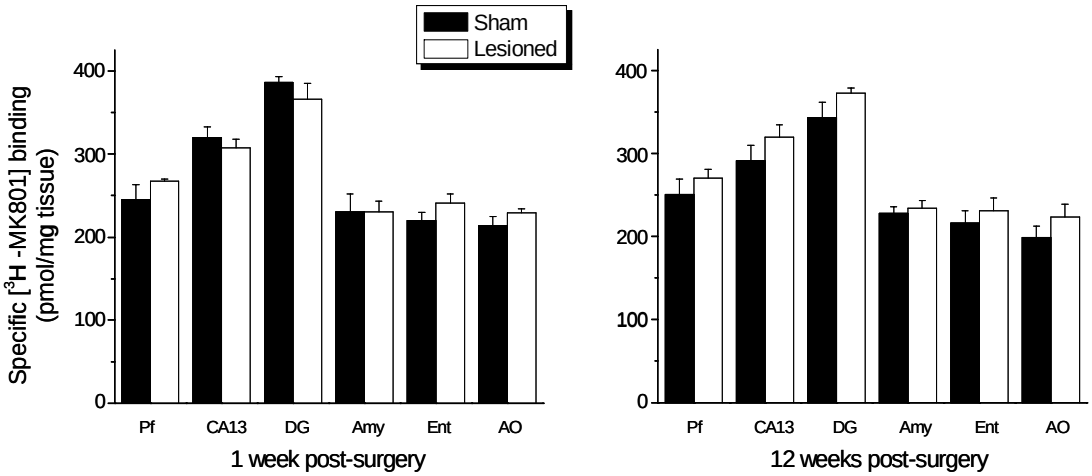
Results (mean ± SEM; fmol/mg tissue) are shown for the sham and the lesioned groups. Abbreviations: Pf, prefrontal cortex; AO, anterior olfactory nucleus; VP, ventral pallidum; PH, posterior hypothalamus; SuM, supramammillary nucleus; CA13, $CA_{1\text{-}3}$ fields of the hippocampus; DG, dentate gyrus; SN, substantia nigra; Amy, amygdala; DRn, dorsal raphé nucleus; Ent, entorhinal cortex, IP, interpeduncular nucleus

VI.3.4 Glutamatergic NMDA receptor

The results are presented in **Fig 6.5**.

One-way ANOVA showed no significant effect of the lesion in any of the regions studied at both time points, **one week** and **twelve weeks** post-surgery.

Fig 6.5. Specific binding on the glutamatergic NMDA receptors



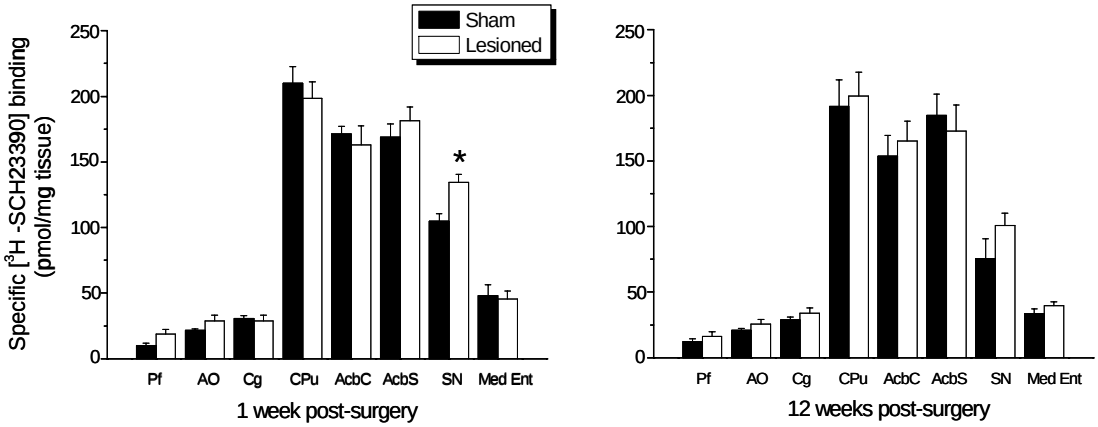
Results (mean ± SEM; pmol/mg tissue) are shown for the sham and the lesioned groups. Abbreviations: Pf, prefrontal cortex; CA13, CA_{1-3} fields of the hippocampus; DG, dentate gyrus; Amy, amygdala; Ent, entorhinal cortex; AO, anterior olfactory nucleus.

VI.3.5 Dopaminergic D_1 receptor

One week. The results are presented in **Fig 6.6**. One-way ANOVA revealed a significant effect of the lesion in the substantia nigra, where the lesioned animals showed an increased binding ($F_{1,5} = 10.99$, $p < 0.05$). On the other hand, no significant effect of the lesion was detected in any of the other regions studied.

Twelve weeks. One-way ANOVA showed no effect of the lesion in any of the regions studied.

Fig 6.6. Specific binding on the dopaminergic D_1 receptors



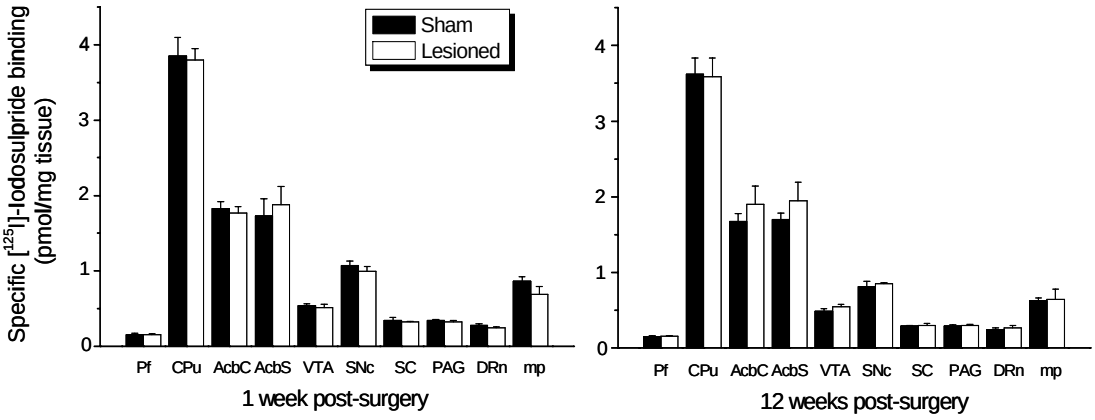
Results (mean ± SEM; pmol/mg tissue) are shown for the sham and the lesioned groups. Abbreviations: Pf, prefrontal cortex; AO, anterior olfactory nucleus; Cg, cingulate cortex; CPu, caudate-Putamen, AcbC, core region of accumbens nucleus; AcbS, shell region of accumbens nucleus; SN, substantia nigra; Med Ent, medial part of the entorhinal cortex.

VI.3.6 Dopaminergic D_2 receptor

The results are presented in **Fig 6.7**.

One-way ANOVA revealed no significant effect of the lesion in any of the regions studied at both time-points, ***one week*** and ***twelve weeks*** post-surgery.

Fig 6.7. Specific binding on dopaminergic D_2 receptors one week after the lesion



Results (mean ± SEM; pmol/mg tissue) are shown for the sham and the lesioned groups. Abbreviations: Pf, prefrontal cortex; CPu, caudate-Putamen, AcbC, core region of accumbens nucleus; AcbS, shell region of accumbens nucleus; VTA, ventral tegmental area; SNc, substantia nigra *pars compacta*; SC, superior colliculus; PAG, periaqueductal gray; DRn, dorsal raphé nucleus; mp, mammillary peduncle.

VI.4 Discussion

The aim of the present study was to investigate the effects of bilateral lesion of the habenula on receptors that have been postulated to play a role in the pathophysiology of schizophrenia, namely the dopaminergic D_1 and D_2, the serotonergic 5-HT_{1A} and 5-HT_{2A}, the glutamatergic NMDA and the cholinergic nicotinic alpha-7 receptors. To this aim, we have used the autoradiography technique in which labeled ligands that bind specifically to a receptor are incubated with brain slices to allow the detection and subsequent quantification of receptor binding. Since we have previously shown that lesion of the habenula induced distinct immediate and delayed deficits in a cognitive task of attention (see **Chapter IV**) which resembled the cognitive deficits encountered in schizophrenic patients, we investigated the effects of the lesion at two different time points, a short-term evaluation one week after the lesion, and a second long-term evaluation twelve weeks after the lesion. Moreover, as during any behavioural testing animals are strongly stimulated, we grouped our rats by cages of four from the time they were lesioned until the autoradiography was performed, in order they were in a stimulating environment which could partly mirror behavioural testing conditions that may affect receptor populations in the brain.

The results obtained were the following. Although there was no significant differences between the two groups at both time points for the dopaminergic, glutamatergic, cholinergic and serotonergic 5-HT_{1A} receptors, we observed a marked effect of the lesion on the serotonergic 5-HT_{2A} receptor at the first time point, *i.e.* one week after the lesion was performed. Indeed, the habenula lesion induced a marked increase of the concentration of the 5-HT_{2A} receptor in frontal cortical areas, namely prefrontal cortex, pyriform cortex, cingulate cortex and claustrum, but also in sub-cortical areas such as the striatum (caudate-putamen and accumbens nucleus), the interpeduncular nucleus and the raphé nucleus. On the other hand, at the second time point there were no significant differences with the exception of the cingulate cortex where the lesioned animals still showed an increased binding on 5-HT_{2A} receptors. At this time there were still marked tendencies for an increased binding in lesioned animals compared to sham, in frontal cortex and claustrum.

The reasons we particularly investigated these receptors reside in the fact that many researchers have postulated their implication in the pathophysiology of schizophrenia (see **Chapter I** for more details). In summary, for example, serotonin receptors have been first postulated to be involved in the pathology of schizophrenia when it was discovered that fact that hallucinogenic substances, such as lysergic acid diethylamide (LSD) or mescaline, bind to 5-HT_{2A} and 5-HT_{2C} receptors. It is now known that they are agonists at these receptors. Moreover, clozapine, which belongs to the atypical class of antipsychotics, is an antagonist of the 5-HT_{2A} receptor (see **Chapter I**). Dopamine was also hypothesized to be involved in schizophrenia according to similar reasons, as amphetamine, which provokes the release of dopamine, or cocaine, which inhibits its reuptake, provoke a schizophrenia-like psychosis including hallucinations, when chronically used. Moreover, the typical neuroleptics haloperidol or chlorpromazine are potent antagonists of dopaminergic D_2 receptor. Also, glutamate is increasingly investigated in schizophrenia because NMDA receptor antagonists, such as PCP or ketamine, produce paranoia and hallucinations. On the other hand, in the case of acetylcholine, this may be linked to cognitive deficits of schizophrenia, since in animal models cognitive deficits are provoked by antagonizing muscarinic or nicotinic cholinergic transmission. Also, the fact that the majority of schizophrenics are heavy smokers strengthens the idea of some cholinergic alterations, nicotine being thought to relieve some of the symptoms. Moreover, rivastigmine, a reversible acetylcholinesterase inhibitor, has been shown to improve quality of life of schizophrenics, through an action on cognitive but also negative symptoms (Lenzi *et al*, 2003), and has also been shown to improve cognitive deficits of elderly patients suffering of comorbid schizophrenia and dementia (Mendelsohn *et al*, 2004).

However, although animal experimentation gave interesting results in trying to replicate schizophrenia symptomatology by acting on the above-mentioned receptors, human studies, either post-mortem histological studies, imaging investigations or genotyping, have given mostly discrepant findings and did not allow, to date, a clear demonstration of alteration of any of those receptors in schizophrenics. While imaging studies revealed striatal alteration of dopaminergic transmission, after amphetamine challenge but not under baseline conditions, in schizophrenics or patients with schizotypal personality disorder (Abi-Dargham *et al*, 1998, 2004), attempts to demonstrate changes in dopamine receptors in schizophrenics have given discrepant findings of an enhanced, or

unchanged, number of D_2 receptors in the striatum of schizophrenics. For example, studies often show that there is no change in the amount of D_2 receptors in the brains of schizophrenics as revealed by PET scanning experiments (*e.g.* Zakzanis & Hansen, 1998), while Abi-Dargham *et al* (2002) found an increased D_1 receptor concentration in the dorsolateral prefrontal cortex in never medicated schizophrenics. Concerning glutamate receptors, subunits of kainate receptors have been found to be down regulated in the hippocampus in schizophrenics (Benes *et al*, 2001), while Gao *et al* (2000) found decreased concentration of the NR_1 and increased concentration of the NR_{2B} subunits of NMDA receptor, also in the hippocampus. Concerning serotonin receptors, a review by Abi-Dargham *et al* (1996) revealed that none of the studies gave consistent results. Serretti *et al* (2000) did not find any changes in the regional concentration of 5-HT_{2A} receptors in schizophrenics, and Okubo *et al* (1997), in a PET study, did not find any difference in the binding for 5-HT_2 receptors in any brain regions in schizophrenics. On the other hand, studies of cholinergic receptors in schizophrenia gave the most consistent findings, as decreased binding for the alpha-7 receptor has been found in schizophrenics, as well as in frontal cortex and reticular nucleus of the thalamus (Freedman *et al*, 2000).

It has been shown in the rat and in the cat that habenula lesion induced an increased release of dopamine in frontal cortex, accumbens nucleus and neo-striatum, but there seems to be a difference in the time-course of the effects regarding the different regions. Indeed, while Nishikawa *et al* (1986) found an enhanced release of dopamine in frontal cortex, nucleus accumbens and neostriatum 6 hours after injection of tetrodotoxin in the habenula, Lisopravski *et al* (1980) found an enhanced release of dopamine only in frontal cortex 6 days after lesion of the habenula. It is then somewhat surprising that no changes occurred in dopaminergic receptors after habenula lesion, because even if effects in striatum seem to be short-lasting, at least effects in frontal cortex are much longer. An interesting aspect of the effects of habenular manipulation on dopamine activity which may be relevant, is the fact that lateral habenula stimulation in the rat activates some neurons of the ventral tegmental area while inhibiting others (Christoph *et al*, 1986).

Concerning serotonin, it seems that the influences of habenula manipulation are as complicated as those exerted upon the dopamine systems. Indeed, Speciale *et al* (1980)

observed increased serotonergic metabolism in dorsal but not median raphé nucleus 16 hours as well as 1 week after the lesion of the habenula. Soubrié *et al* (1981) found increased serotonin release in substantia nigra and striatum after infusion of KCl in the lateral habenula. According to these authors, these results are consistent with the view that lateral habenula normally inhibits raphé cells, and they consider that the infusion of KCl produced a blockade of lateral habenula neurons through an excessive depolarizing action. Nishikawa & Scatton (1985) found decreased serotonin release in striatum, substantia nigra, hypothalamus and hippocampus after electrolytic habenula lesion in the rat. In the hippocampus, Zagami *et al* (1995a,b) found in the rat that when a low-frequency current (1-3 Hz) was applied in lateral habenula, the pyramidal cells responded in different ways, as some were activated while others were inhibited. Thus it seems that habenular influence upon serotonergic neurons of the raphé is exerted in both ways. According to these authors, and considering that the serotonergic pathway from the raphé to the hippocampus is inhibitory, low frequency stimulation of the habenula, by releasing only a small amount of neurotransmitter, might excite only the small GABA interneurons of the raphé and thus cause an inhibition of the serotonergic neurons resulting in excitation of hippocampal pyramidal cells by subsequent disinhibition; on the other hand, high frequency stimulation, releasing more neurotransmitters, might directly activate the serotonergic raphé neurons and thus induce inhibition of hippocampal pyramidal cells. Concerning the striatum and the substantia nigra, when lateral habenula was stimulated, serotonin release was significantly lowered in both structures, whereas when picrotoxin was applied in the lateral habenula, serotonin was unchanged in the substantia nigra with picrotoxin concomitantly applied in the dorsal raphé while serotonin release was still decreased in the striatum. It seems thus that regulation of serotonin release in both structures by the lateral habenula is exerted in different manners (Nishikawa *et al*, 1986).

Indeed, even if, according to current views, an increased receptor binding is a compensatory response to a decreased neurotransmission, the discrepant findings obtained by the different studies, as well as the bimodal action of habenula upon serotonin raphé cells, make our results very difficult to explain. Moreover, regulation of 5-HT_{2A} receptors is paradoxical since down-regulation of 5-HT_{2A} receptors with chronic treatment of either agonists or antagonists is observed (Gray & Roth, 2001). As the influence of habenula upon serotonin release in frontal cortical areas has not been

studied to date, we can hypothesize that the short-term effect of habenula lesion, *i.e.* increased number of 5-HT_{2A} receptors at frontal cortical level, is a consequence of a decreased serotonergic flow from the raphé, in accordance with the results obtained by Nishikawa & Scatton (1985). The discrepancies of the different findings concerning the habenulo-raphé pathway could be explained by several factors. The size of the lesion may differ from one study to the other, and it is also important to consider both nuclei that can be more or less affected. The impact of the lesion on the substance P/acetylcholine ratio could be crucial, as the interpeduncular nucleus, which receives its major influence from the medial habenula, sends one of its most important efferent pathways to the raphé nuclei (Groenewegen *et al*, 1986; Montone *et al*, 1988). Moreover, it has been shown that the projection from the interpeduncular nucleus to the median raphé nucleus is excitatory (Maciewicz *et al*, 1981). We should then consider the effects of habenula lesion on raphé activity not only through the disruption of direct connections from the lateral habenula, but also through the disruption of the habenulo-interpeduncular pathway that originates in the medial habenula. Another explanation for the discrepant findings would be that, when stimulation studies were performed (Ferraro *et al*, 1997; Kalen *et al*, 1989; Sabatino *et al*, 1991; Wang & Aghajanian, 1979), the electrodes were implanted in the lateral habenula without any regard to their specific localization, while we know now that it is composed of 10 subnuclei (Andres *et al*, 1999), which may have different mode of action upon their target areas.

In human post-mortem studies the results are the opposite of what we have obtained. Indeed, Burnet *et al* (1996b) found, during a post-mortem study, decreased 5-HT_{2A} binding in prefrontal areas in schizophrenics. The fact that chronic treatment with clozapine in the rat leads to a decreased binding as well as gene expression (mRNA) of the 5-HT_{2A} receptor, which is paradoxical given post-mortem results, while haloperidol has no effect, suggests that human data are difficult to interpret, and should take into account the effects of medication (Burnet *et al*, 1996a). Indeed, Gurevitch & Joyce (1997) found decreased binding on 5-HT_{2A} receptors in frontal cortical areas in schizophrenics that were medicated at the time of death, compared to healthy subjects, whereas there was no significant difference with healthy subjects when the schizophrenics were off drug at the time of death. According to Burnet *et al* (1996b), 5-HT_{2A} decreased binding seen in schizophrenic patients may reveal an attempted

compensatory response to primary pathophysiological events, *i.e.* enhanced serotonin transmission, furthered by clozapine.

From a behavioural point of view, if the increased binding on 5-HT_{2A} receptors reflects a decreased serotonin release consecutive to lesion of the habenula, this is consistent with the increase of premature responding, induced by habenula lesion, that we observed during the 5-choice serial reaction time task (see **Chapter IV**). In fact, while Harrison *et al* (1997) obtained an increased number of premature responses in rats with global serotonin depletion, Winstanley *et al* (2004) decreased premature responding with a 5-HT_{2A} antagonist treatment. According to the latter results, and considering that the lesion seems to decrease serotonin neurotransmission, the subsequent increased impulsivity could therefore be partly due to a loss of a serotonergic mechanism that reduces impulsivity. Interestingly, one possibility is an action through 5-HT_{2C} receptors (Winstanley *et al*, 2004), but there might be others.

The fact that 12 weeks after the lesion levels of receptor were no longer different between sham and lesioned animals could explain that at this stage, the long-term effects of the lesion on attention are no more due to effects at the level of monoamine receptors, but shifted to another mechanism that could be changes in synaptic connectivity, inducing another type of deficit. This could explain why, during the attention test, the long term effects of the lesion were to decrease choice accuracy, which was not present shortly after the lesion, while increased premature responding was present immediately after the lesion and then progressively normalized.

Finally, concerning our MRI data (see **Chapter V**), *i.e.* a decreased excitatory response to amphetamine at frontal cortical level, it is difficult to conclude whether changes in serotonin receptors and neurotransmission are involved in this, given that there have been paucity of studies investigating the effects of selective serotonin ligands on frontal cortical blood flow. However, opposite effects of serotonergic agonist compounds on impulsivity in the attention test when applied to frontal cortex suggest that there are multiple serotonin mechanisms in this region. One can site a study by Underwood *et al* (1992) who investigated cortical blood flow in rats following dorsal raphé stimulation. They found that brief stimulation of the dorsal raphé elicited an increase of the blood flow at cortical level. Moreover, stimulation of the rostral dorsal raphé elicited a

decreased blood flow, while sustained intermittent stimulation of the caudal dorsal raphé elicited an increased blood flow at cortical level.

"La vie de chaque homme est un chemin vers soi-même, l'essai d'un chemin, l'esquisse d'un sentier. Personne n'est jamais parvenu à être entièrement lui-même; chacun, cependant, tend à le devenir, l'un dans l'obscurité, l'autre dans la lumière, chacun comme il le peut. Chacun porte en soi, jusqu'à sa fin, les restes de sa naissance, les dépouilles, les membranes d'un monde primitif. Beaucoup ne deviennent jamais des hommes, mais demeurent grenouilles, lezards ou fourmis. Tel n'est humain que dans sa partie supérieur, et poisson en bas. Mais chacun de nous est un essai de la nature, dont le but est l'homme. Tous nous sortons du même sein, mais chacun de nous tend à émerger des ténèbres et aspire au but qui lui est propre. Nous pouvons nous comprendre les uns les autres, mais personne n'est expliqué que par soi-même."

Hemann Hess – "Demian"

Chapter VII – General discussion

The role in behaviour of the habenula, an epithalamic structure, has been sparsely investigated. Influences of the habenula on electrophysiological and neurochemical aspects of brain functioning are now quite well known, but very few studies have been performed in animals to investigate the behavioural consequences of its dysfunction. While the role of the habenula has been clearly established concerning the reaction to stressful events, pain, mating and maternal behaviour, very little was previously known about its role in higher brain functions, such as cognition. For over a decade, since the findings by Sandyk (1992) and Caputo *et al* (1998) of a higher degree of habenula calcification in schizophrenics compared to healthy subjects, the habenula has been postulated to be involved in the pathophysiology of schizophrenia (Ellison, 1994; Kelly, 1998; Sandyk, 1991). Indeed, dysfunction of the habenula in schizophrenics has been postulated to lead to the memorization of "dream-events" which would lead to the introduction of wrong connections at cortical level and the mixing of those wrong connections, related to the dreams, with the normal memory store. This would be the basis of the appearance of primary delusions in schizophrenics. Moreover, this phenomenon is further hypothesized to worsen with time, by accumulation of those connections (Kelly, 1998). To date, no studies in rodents had been performed in order to investigate the consequences of a defective habenula on behaviors linked to this schizophrenia. Traditionally in science hypotheses are challenged by testing whether predictions of the hypothesis are supported by experiment. If a prediction is not confirmed by experiment then it is wrong and the hypothesis must be discarded or modified. If the prediction of the hypothesis is confirmed then it becomes more likely that the hypothesis is correct. Many predictions can be made from the above-mentioned hypothesis. By analogy with the progress that was made in treating Parkinson's disease once it became clear that this involved a deficit of dopaminergic neurons, or with the progress in Alzheimer's disease after a dysfunction of cholinergic neurons was demonstrated, we felt that determining the neural system involved in schizophrenia would be very valuable for future research. Therefore the prediction was made that, according to the hypothesis, lesions of the epithalamus, *i.e.* habenula and pineal body, should result in schizophrenia-like symptoms in experimental animals. The present study was therefore devoted to exploring the behavioural consequences of an

electrolytic lesion of the habenula in rats, and to further investigating its possible role in the appearance of some of the symptoms of this disorder.

To study the effects of habenula lesions on behaviour linked to schizophrenia, we have used tools allowing parallels between animal behaviour and human pathology, as several tests utilized in man to assess the severity of schizophrenia symptoms have been adapted to animal research. The *prepulse inhibition of the startle reflex* (PPI), is a test used in man to explore sensory gating deficits. The *social interaction* test, derived from the social memory test of Thor & Holloway (1982), is a rather simple way to evaluate the degree of interactions of a rat with a conspecific, which can be related to a parameter of socialization and compared to the often observed lack of communication and social involvement in schizophrenic patients. To assess cognitive functioning we have analyzed spatial reference memory ability, which is postulated to be a good model of human declarative memory (O'Keefe & Nadel, 1978), with the *Morris water-maze*, and we have explored attention abilities by means of the *5-choice serial reaction time task*, which is mirrored by the continuous performance test used in man, and allows the separate analysis of choice accuracy and impulsive behaviour (Robbins, 2002).

The goal of the first study was to characterize habenula lesion effects using tests related to schizophrenia symptoms: prepulse inhibition, social interaction and Morris water-maze. While prepulse inhibition and social interaction time were unaffected by the lesion, the Morris water-maze experiment revealed marked deficits in the lesioned group. Indeed, the habenula-lesioned animals showed greater latency, as well as greater distance swum, before finding the hidden platform. Moreover, as electrolytic lesion of the habenula can damage a small portion of the dorsal hippocampus, which has been shown to be involved in spatial reference memory a control group with small dorsal hippocampal lesion, identical to that resulting from habenula lesion, was included in a second series of experiments using the same tests. Rats with restricted dorsal hippocampal lesion showed no impairment during the Morris water-maze task, leading us to conclude that memory deficits were only due to habenula lesion. Finally, in visible platform conditions the habenula lesioned rats performed at the same level as sham-operated controls, which confirms the fact that the deficits are due to memory alteration. The conclusion of the first study was that the habenula appeared to be involved in

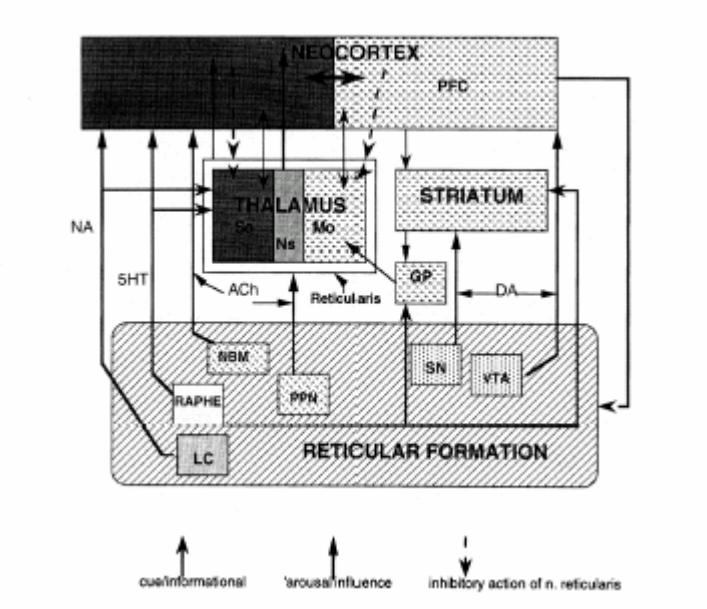
memory impairments in schizophrenia. Under the conditions used no role in social behaviour or prepulse inhibition was demonstrated.

As human imaging studies have shown concomitant alterations of habenular and pineal structures, the two components of the epithalamus, it has been postulated that the epithalamus as a whole could be involved in the manifestation of schizophrenia symptomatology (Sandyk, 1992). Therefore, the goal of the second study was first to determine whether pineal damage could lead to the same deficits encountered with habenula lesion, and second to assess the consequences of a complete epithalamic lesion (electrolytic habenula lesion + pinealectomy), in order to assess if combined lesions would worsen the deficits encountered with habenula lesion. To this purpose we performed the same experiments as during our first study, except that the lesion was either a pinealectomy or a habenula lesion plus pinealectomy. The results were that pinealectomy alone did not produce deficits in any of the tests, while complete epithalamic lesion led to the same pattern of deficits as habenula lesion alone, *i.e.* spared prepulse inhibition and social interaction, and marked visual reference memory impairments. The conclusion of the second study was that, while the epithalamus as a whole may show pathological changes in schizophrenia, habenula dysfunction alone accounts for the appearance of cognitive deficits. Therefore, in the subsequent studies, we focussed on the effects of habenula lesion alone.

The third study was designed to test the hypothesis that habenula lesion would produce attention deficits, as postulated by Thornton & Evans (1982), using the 5-choice serial reaction time task. We obtained two kinds of habenula lesion-induced impairments, distinct by their nature and also by their time-course. One week after receiving a habenula lesion, the rats showed a marked increase of the number of premature responses, which is an index of impulsivity, while response accuracy, a different aspect of attentional performances, was unaffected. Subsequently, over two months of testing, lesioned animals showed a progressive decrease of the number of premature responses, which tended to normalize, while at the same time choice accuracy progressively decreased. Moreover, treatment with haloperidol, a dopamine antagonist, significantly reduced the number of premature responses, while it was ineffective concerning choice accuracy. If we refer to the diagram proposed by Robbins *et al* (1998) (**Fig 7.1**), we can notice that many areas involved in the arousal processes, *i.e.* frontal cortex, thalamus,

striatum, globus pallidus, raphé nuclei, tegmental ventral area, substantia nigra and locus coeruleus, share connections with the habenula.

Fig 7.1: Neuronal substrates of arousal



Abbreviations: PFC = prefrontal cortex; Se = sensory thalamus; Ns = nonspecific thalamic nuclei; Mo = motor thalamus; GP = globus pallidus; NBM = nucleus basalis magnocellularis; PPN = pedonculopontine nuclei; SN = substantia nigra, *pars compacta*; VTA = ventral tegmental area; ACh = acetylcholine; DA = dopamine; NA = noradrenaline; 5-HT = 5-hydroxytryptamine; LC = locus coeruleus. (From Robbins *et al*, 1998).

In particular, the habenula is a regulator of the dopaminergic activity from the ventral tegmental ventral area to the frontal cortex and from the substantia nigra to the striatum, the serotonergic activity from the raphé nuclei to the striatum and the noradrenergic activity from the locus coeruleus to the striatum and the frontal cortex. Thus, these different results led us to conclude that if the elevated premature responding may at least partly due to increased dopamine activity (Robbins, 2002), the results obtained here probably reflecting an increased mesolimbic and/or mesostriatal dopamine transmission, which has been previously shown in habenula-lesioned animals (Lisoprawski *et al*, 1980; Nishikawa *et al*, 1986). Moreover, the autoradiographic study revealed that the lesion induced an enhancement of the number of serotonergic 5-HT_{2A} receptors, which could probably be the reflection of a decreased serotonergic transmission. Thus, increased premature responding may also be produced by this decreased activity, as shown by Harrison *et al* (1997) in serotonin depleted animals. The

concomitant action of those two processes, increased dopamine activity and decreased serotonergic activity could explain the strength of this phenomenon and its duration, as premature responses are still high in lesioned animals when serotonin receptor levels are back to normal. On the other hand, as haloperidol had no effect on choice accuracy, the impairment of this latter parameter seems to be the consequence of dysfunction of mechanisms independent of direct alteration of monoamine transmission. Indeed, this progressive impairment of choice accuracy is, to our knowledge, the first example of a progressive cognitive deficit after a restricted brain lesion. While there is generally compensation for lesion effects, a cumulative mechanism such as the accumulation of delusional memories (Kelly, 1998) could account for such a progressive impairment.

In order to investigate the possible changes of brain activity and neurotransmitter receptors that may be the consequence of habenula lesion throughout the brain, we performed two experiments. First we explored regional brain activation by means of functional MRI. Moreover, amphetamine was administered to the rats, both acutely and chronically, in order to determine if the lesion of the habenula would induce a particular sensitivity to alteration of the dopamine system, as is the case for schizophrenics. Second, we investigated possible changes in populations of receptors that belong to systems involving the habenula, and which have been postulated to have a role in the pathophysiology of schizophrenia, using the autoradiographic technique. These two experiments allow us to isolate a structure that may be involved in the behavioural deficits observed in our study, namely the frontal cortex. Indeed, amphetamine treatment induced particular changes in lesioned animals, *i.e.* a poorer response in frontal cortical regions, which could be a parallel to frontal hypofunctioning observed in schizophrenics during cognitive tasks requiring this area. Also the autoradiographic study revealed a short-term enhancement of the number of 5-HT_{2A} receptors in the frontal cortex, that may reflect a decreased serotonergic transmission subsequent to habenula lesion. Whether these phenomena are the basis of the cognitive impairments observed during our study is very difficult to know. Interestingly, 5-7-dihydroxytryptamine-induced lesion of the serotonin system has recently been shown to induce deficits in the Morris water-maze in a test were rats were pre-trained before the lesion and retested two days after the lesion during five consecutive days (Mogensen *et al*, 2003).

The conclusion that can be drawn from these experiments is, therefore, that the prediction of the hypothesis was partly confirmed. Lesions of the habenula caused schizophrenia-like changes in memory and attention, but did not cause changes in social interaction time or PPI. Thus, the hypothesis was not proven wrong, but was limited to the cognitive changes in schizophrenia. From the different results obtained, we can hypothesize that primary effects of the lesion could be due to alterations of ascending monoamine systems, and that long-term effects could occur through secondary processes that may take place in cortical areas. Moreover, the effects of the lesion seem to be "experience dependent" as we observed a progressive deterioration of the performances in the attention task.

As we have seen above (see **Chapter I**), the habenula is the node of a reciprocal route of communication between limbic and extrapyramidal structures of the midbrain and the forebrain. Moreover, the habenula has been shown to take part in the regulation of the ascending monoaminergic pathways, comprising the dopaminergic, serotonergic and noradrenergic systems. Even if, as found by Mok & Mogenson (1974), stimulation of the habenula influences 15% of the neurons recorded in the upper brain stem (midbrain reticular formation, midbrain central gray area, ventral tegmental nucleus of Gudden, ventral tegmental area of Tsai, interpeduncular nucleus), while stimulation of the lateral hypothalamus influences 41% of the neurons recorded, thus demonstrating a weaker influence of the former, it is not to be neglected. Moreover, the habenula is composed of 15 sub-nuclei (Andres *et al*, 1999), which renders the evaluation of its functions difficult, and suggests a tight compartmentalization of its functions. For example, Corodimas *et al* (1993) found disruption of mating behaviour in rats with an almost complete lesion of the lateral habenula (95-98 %), whereas in rats with an incomplete lesion (74 %) this behaviour was preserved. It would then be very interesting in future investigations to perform studies with restricted lesion of either the medial habenula, or the lateral habenula, in order to investigate which route is essential in specific functions of the habenula and which could be particularly relevant for the pathophysiology of schizophrenia. Thus, because of its action on monoaminergic systems, its link with structures involved in various illnesses, and its averred involvement in behaviours such as maternal and mating behaviour, sleep, reward and cognition, studying the habenula may help us better understand the genesis of psychiatric diseases, such as schizophrenia or mood disorders. Indeed, several lines of evidence strengthen this statement, and we

can envisage many ways, that need to be confirmed, by which the habenula could influence brain functioning. For example, as serotonin has recently been shown to be essential at the level of the prefrontal cortex for cognitive flexibility in monkeys (Clarke *et al*, 2004), it would be interesting to clearly establish the effect of habenular manipulation on serotonergic flow in this region. Through its indirect influence upon the activity of hippocampal pyramidal cells, the habenula could be implicated in the genesis of cellular plasticity by influencing the generation of long term potentiation (LTP) and long-term depression (LTD). We have seen during this study that habenula lesion does not modify sensory gating, nor social interaction. Maybe it would be relevant for the study of schizophrenia, to elaborate a model that would take into account several different aspects of such pathology. For example, coupling ventral hippocampal lesion (*cf* Lipska, 2000) and lesion of the habenula could make a stronger model for schizophrenia. As certain genetic risk factors for schizophrenia can also be expected to preferentially affect habenula function, recently discovered targets seem interesting to investigate as they have been shown to be strongly linked to schizophrenia. For example, in several populations, genetic polymorphisms of neuregulin 1 have been linked to schizophrenia (Stefansson *et al*, 2002, 2003; Williams *et al*, 2003, Yang *et al*, 2003; Corfas *et al*, 2004). Neuregulin plays an important role in growth and cell differentiation (Carraway & Burden, 1995; Burden & Yarden, 1997) and, interestingly, erbB4, a major receptor for neuregulin, has been shown to be highly expressed in the medial habenula, in addition to dopaminergic cell body areas, hippocampus and cortex (Steiner *et al*, 1999). Another genetic link to schizophrenia concerns the Ca^{2+}-activated potassium channel, SK3. An association of longer CAG repeats in the gene for SK3 was found in Israeli Ashkenazi Jews (Dror *et al*, 1999). This has been further confirmed in some populations, but not others (see Miller *et al*, 2001 for review). The localization of this channel is highest in medial habenula, as well as the ventral tegmental area, raphé nuclei and hippocampus (Stocker & Pedarzani, 2000). Finally, another gene that has been linked to schizophrenia (Millar *et al*, 2000; Ekelund *et al*, 2001) is DISC1 (disrupted in schizophrenia-1). The highest level of expression of this gene in the primate brain is the dentate gyrus, but one of the few brain regions showing moderately high expression is the interpeduncular nucleus (Austin *et al*, 2003), so that alteration of this gene can be expected to alter habenulo-interpeduncular function. We have seen that effects of habenula lesion may be different in their time-course, and Murphy *et al* (1996) who showed different effects on anxiety if the lesion

was performed neonatally or in adult rats. It would then be interesting, in view of a neurodevelopmental model, to study the effects of neonatal lesion on the tests used during this study. It would be interesting to explore the changes that occur in habenula-lesioned animals within the progression of a test, to determine if changes are not dependent of the task and its time-course. Also, as the receptor number is not the only parameter that varies when a neurotransmitter pathway is affected, studies of sensitivity of the receptors could be done, and secondary messengers could be targeted, GTP gamma-S for example, in order to have an idea of the consequences of the lesion at the level of the receptors functionality. Finally, it would be interesting to study the effects of typical and atypical neuroleptics in the Morris maze experiment, and, as serotonin may be involved in the deficit of attention, at least in the enhancement of premature responses, to study the effects of neuroleptics that target the serotonergic system, *e.g.* clozapine, in such a test.

- "Vous avez beau dire, y'a pas seul'ment que d'la pomme, y'a aut'chose. Ca s'rait pas des fois d'la betterave ? Hein ?"

- "Si ! Y'en a aussi."

~~~~~~

- "Non, mais t'a déjà vu ça ? En pleine paix. Y chante et puis crac, un bourre pif !
Mais il est complètement fou ce mec ! Mais moi, les dingues, j'les soigne. J'vais lui faire une ordonnance, et une sévère ... J'vais lui montrer qui c'est Raoul. Aux quat' coins d'Paris qu'on va l'retrouver éparpillé par petits bouts, façon puzzle. Moi, quand on m'en fait trop j'correctionne plus: j'dynamite, j'disperse, j'ventile..."
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