# BEHAVIOURAL, NEUROCHEMICAL AND NEUROENDOCRINE EFFECTS OF

# PREDATOR STRESS

## IN MICE

**DISSERTATION**

**ZUR ERLANGUNG DES DOKTORGRADES**
**DER FAKULTÄT FÜR BIOLOGIE**
**DER LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN**

**VORGELEGT VON**
**MARJOLEIN BEEKMAN**
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**SUBMITTED BY**
**MARJOLEIN BEEKMAN**
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**Betreuerin:** Dr. Astrid C.E. Linthorst
**1. Gutachter:** Prof. Dr. Rainer Landgraf
**2. Gutachter:** Prof. Dr. Peter Schlegel

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# SUMMARY

Stress plays a role in the etiology of anxiety and mood disorders. To investigate these disorders, animal models are used, many of which incorporate a stressful stimulus. Recently, the interest in animal models that use a psychological stressor has grown, as the brain regions that are activated by this kind of stress might differ from those activated by more physical forms of stress, for example those based on nociception. It is thought that psychological stressors may more closely resemble stressful situations that in humans can lead to pathology. The study, described in this thesis, was undertaken to elucidate the effects predator exposure has on behaviour, on neurochemical parameters in various brain regions and on neuroendocrine parameters. Also the effects of repeated predator exposure were assessed.

For this purpose C57bl/6N and Balb/c mice were exposed to a rat for thirty minutes. The rat was introduced in a rat compartment of the mouse home cage, separated from the mouse compartment by a Plexiglas separation wall equipped with small holes. Animals could smell, see and hear each other, but not touch. The effect of such stimuli on behaviour was described comprehensively. Simultaneously, using a high time resolution microdialysis method, several neurochemical parameters were measured. In various strains of mice the effect of predator exposure on plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone were also determined.

Rat exposure caused marked changes in the behaviour of the mice. They became alert, started risk-assessment activities, which were followed by coping behaviour. Upon re-exposure the behavioural profile had slightly changed, showing less risk-assessment and more self-directed behaviour, interpreted as less arousing properties of the paradigm on a second trial. It was also found that the levels of free corticosterone were lower on a second day of exposure, confirming the less stressful nature of the paradigm when repeated. During rat exposure extracellular levels of serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were higher than baseline levels in the hippocampus, the prefrontal cortex and the lateral septum, but not in the caudate putamen of C57bl/6N mice. In Balb/c mice microdialysis was performed in the hippocampus, paraventricular nucleus (PVN) and anterior hypothalamus (AHP). Elevations in 5-HT and 5-HIAA were seen in the hippocampus, and to a lesser extent in the PVN and the AHP. The finding that extracellular levels of 5-HT were not ubiquitously increased under stressful conditions, but in selected brain regions only, underlines the role that 5-HT plays in emotion during stress.

Sharp increases were seen at the beginning of rat exposure in the extracellular levels of noradrenaline (NA) in the hippocampus of both strains of mice, indicating the arousing properties of the paradigm. In the PVN and AHP the levels of NA were not measurable, but increases in its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) were seen. Levels of the dopaminergic metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were either slightly or not increased respectively, in the hippocampus, PVN and AHP, which fits with findings in literature that these anatomical structures do not play an important role in dopaminergic neurotransmission during mild stress. Unlike with the behavioural parameters or with the levels of free corticosterone, no clear effects of re-exposure were seen on the neurochemical parameters. Also it was not possible to correlate certain behaviours, indicative of anxiety or coping, to the observed changes in neurotransmitter levels.

Comparison of plasma levels of ACTH and corticosterone in various strains of mice revealed strain differences, with C57bl/6N, Balb/c and B6C3F1 mice showing elevated levels of these hormones after rat exposure, which was not the case for C57bl/6J and DBA/2 mice. The strains exhibiting more pronounced neuroendocrine responses also had a different behavioural profile, with displaying increased rearing, sniffing in the air or at the separation wall or spending more time with food-related behaviour. On the contrary, in mice genetically altered to overexpress corticotropin-releasing hormone (CRH), which are thought to be more anxious by nature, rat exposure did not yield behavioural patterns that were clearly different from those seen in the CRH-wildtypes.

Taken together, behavioural, neurochemical and neuroendocrine parameters form a complimentary picture indicating that rat exposure in its current form had mild arousing properties. It would be worthwhile to increase the stressful properties of the paradigm in order to have a functional model to study the mechanisms, from both behavioural, neurochemical and hormonal points of view, underlying the way an organism copes with stress. Furthermore, it is interesting to see in which way these mechanisms are disturbed in pathological cases. Nevertheless, in this mild form predator exposure elicited a selective activation of brain regions and neurochemicals. This highly differentiated response may be of utmost importance to coordinate and to fine-tune the specific neuroendocrine, behavioural and autonomic responses to this form of stress.

# ZUSAMMENFASSUNG

Stress spielt eine wichtige Rolle in der Ätiologie von Angsterkrankungen wie auch von affektiven Störungen. Um diese Erkrankungen zu erforschen werden Tierverhaltensmodelle benutzt, von denen viele einen stressvollen Stimulus verwenden.

In der letzten Zeit bedient man sich immer häufiger Modellen, die auf der Verwendung von psychologisch stressvollen Reizen basieren, da in dieser Situation offensichtlich andere Gehirnareale aktiviert werden als bei anderen, mehr physischen Stressoren, wie etwa schmerzvollen Stimuli. Daher könnten derartige psychologische Stressoren möglicherweise ein besseres Model für Situationen darstellen, die für Menschen pathologische Folgen haben könnten.

Die vorliegende Arbeit beabsichtigt, die Effekte der Fressfeind-Exposition auf das Verhalten, neurochemische Parameter in unterschiedlichen Gehirnregionen und neuroendokrine Parameter zu beschreiben. Auch wurden die Effekte von wiederholter Exposition betrachtet.

Als Stressstimulus wurden C57bl/6N und Balb/c Mäuse für 30 Minuten einer Ratte ausgesetzt. Hierzu wurde die Ratte in ein durch eine Plexiglastrennwand abgegrenztes Kompartiment im Heimkäfig der Maus plaziert. In der Trennwand befanden sich kleine Löcher, so dass die Tiere einander riechen, sehen und hören, sich aber nicht berühren konnten. Die Effekte dieses Vorgangs auf das Verhalten der Maus wurden ausführlich beschrieben. Gleichzeitig wurden mittels einer Mikrodialysemethode mit hoher zeitlicher Auflösung unterschiedliche neurochemische Parameter gemessen. In unterschiedlichen Mäusestämmen wurden schließlich die Effekte von Fressfeind-Exposition auf die Plasmawerte von adrenocorticotropem Hormon (ACTH) und Corticosteron ermittelt.

Die Rattenexposition führte zu erheblichen Änderungen im Verhalten der Mäuse. Sie wurden aufmerksam, fingen an mit Risikoabschätzungsverhalten, gefolgt von Stressbewältigungsstrategien. Bei wiederholter Exposition hatte sich dieses Verhaltensprofil leicht geändert. Es gab weniger Risikoabschätzungsverhalten, dafür aber mehr selbstgerichtetes Verhalten, woraus geschlossen wurde, dass die Exposition beim zweiten Mal als weniger aufregend interpretiert wurde. Auch wurde nachgewiesen, dass die Werte des freien Corticosterons bei der Zweitexposition niedriger waren, was bestätigte, dass dieses Modell bei einer Reexposition weniger stressvoll war.

In Anwesenheit der Ratte waren die extrazellulären Werte von Serotonin (5-HT) und dessen Metabolit 5-Hydroxyindolessigsäure (5-HIAA) höher als unter basalen Bedingungen im

Hippocampus, präfrontalen Cortex und im lateralen Septum, aber nicht im caudaten Putamen der C57bl/6N Mäuse. In Balb/c Mäusen wurde Mikrodialyse durchgeführt im Hippocampus, paraventrikulären Nucleus (PVN) und im anterioren Hypothalamus (AHP). Anstiege in 5-HT wurden gesehen im Hippocampus und in geringerem Ausmaß im PVN und AHP. Das Ergebnis, dass 5-HT unter stressvollen Bedingungen nicht überall erhöht war, sondern ausschließlich in bestimmten Gehirnregionen, unterstreicht die Funktion von 5-HT in emotionaler Verarbeitung von Stressstimuli.

Am Anfang der Rattenexposition stieg der extrazelluläre Gehalt von Noradrenaline (NA) im Hippocampus der beiden Mäusestämme deutlich an, was darauf hinweist, dass dieses Verfahren erregende Eigenschaften hat. Im PVN und AHP konnte NA nicht bestimmt werden, jedoch wurde eine Erhöhung in dessen Metaboliten 3-Methoxy-4-hydroxyphenylglycol (MHPG) gemessen. Im Hippocampus, PVN und AHP waren die Mengen des dopaminergen Metaboliten Homovanillinsäure (HVA) leicht, die des Dihydroxyphenylessigsäures (DOPAC) nicht angestiegen. Diese Ergebnisse stimmen mit der Literatur überein. Hier wird beschrieben, dass diese anatomischen Strukturen keine ausgeprägte Rolle in der dopaminerge Neurotransmission während milder Stressexposition spielen. Im Gegensatz zu den beobachteten Verhaltensparametern oder den Mengen von freiem Corticosteron waren bei den neurochemischen Parametern keine Einflüsse von wiederholter Rattenexposition nachweisbar. Auch war es nicht möglich, bestimmte Verhaltensparameter, die Angst oder Stressbewältigung wiederspiegeln, mit den Neurotransmitterlevels zu korrelieren.

Ein Vergleich der Plasmakonzentrationen von ACTH und Corticosteron in unterschiedlichen Mäusestämmen zeigte, dass Unterschiede zwischen den Stämmen existierten. C57bl/6N, Balb/c und B6C3F1 Mäuse zeigten erhöhte Levels dieser Hormone, was für C57bl/6J und DBA/2 Mäuse nicht der Fall war. Die Stämme mit der ausgeprägteren Hormonantwort zeigten auch ein anderes Verhaltensprofil mit mehr „Männchen machen“, mehr schnuppern in Luft oder an der Trennwand und mehr Fressverhalten. Im Gegensatz dazu konnte in Mäusen, die genetisch manipuliert wurden, Corticotropin-releasing Hormone (CRH) zu überexprimieren und denen eine ängstlichere Natur unterstellt wird, kein Verhaltensprofil während Rattenexposition festgestellt werden, das sich von dem von den CRH-Wildtypen unterschied.

Zusammengenommen formen die Verhaltens-, neurochemischen und neuroendokrinen Parameter ein sich ergänzendes Bild, woraus hervor geht, dass Rattenexposition in seiner jetzigen Form leicht erregende Eigenschaften hatte. Es würde sich lohnen, um die stressvollen Eigenschaften des Modells zu verstärken, um ein funktionelles Modell zu

kreieren, mit dem die Mechanismen (sowohl aus verhaltensbezogenem, neurochemischem und neuroendokrinem Blickwinkel) untersucht werden können, auf denen die Stressbewältigungsstrategien eines Organismus beruhen. Anschließend wäre es interessant zu erforschen, wie diese Mechanismen pathologisch verändert sein können. Nichtsdestotrotz verursachte Rattenexposition auch in dieser milden Form eine ausgeprägte und selektive Aktivierung von Gehirnregionen und Neurotransmittern. Diese hochdifferenzierte Antwort könnte von großem Belang für die Koordination und Abstimmung der spezifischen neuroendokrinen, verhaltensbezogenen und autonomen Antworten auf diese Art Stress sein.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-HIAA | 5-hydroxyindole-3-acetic acid, the metabolite of serotonin |
| 5-HT | 5-hydroxytryptamine, serotonin |
| ACTH | adrenocorticotropic hormone |
| AHP | anterior hypothalamus |
| ANOVA | analysis of variance |
| AUC | area under curve |
| AVP | arginine vasopressin |
| BNST | bed nucleus of the stria terminalis |
| CBG | corticosterone binding globulin |
| CNS | central nervous system |
| COMT | cathechol-O-methyl-transferase, an enzyme degrading NA and DA |
| CRH | corticotropin-releasing hormone |
| DA | dopamine |
| Dex | dexamethasone, a synthetic glucocorticoid |
| DOPAC | 3,4-dihydroxyphenylacetic acid, a metabolite of dopamine |
| DRN | dorsal raphe nucleus |
| ECT | electroconvulsive therapy |
| EPM | elevated plus maze, a behavioural anxiety model |
| GR | glucocorticoid receptor |
| HPA axis | hypothalamic-pituitary-adrenocortical axis |
| HPLC | high performance (pressure) liquid chromatography |
| HVA | homovanillic acid, a metabolite of dopamine |
| LC | locus coeruleus, a cluster of noradrenergic cell bodies |
| LHPA axis | limbic-hypothalamic-pituitary-adrenocortical axis |
| LS | lateral septum |
| MAO | monoamine oxidase, an enzyme degrading 5-HT, NA, DA |
| MHPG | 3-methoxy-4-hydroxyphenylglycol, a metabolite of noradrenaline |
| mPFC | medial prefrontal cortex |
| MR | mineralocorticoid receptor |
| MRN | median raphe nucleus |
| NA | noradrenaline |

| | |
|---|---|
| NAc | nucleus accumbens |
| OCD | obsessive compulsive disorder |
| PFC | prefrontal cortex |
| PTSD | post-traumatic stress disorder |
| PVN | paraventricular nucleus of the hypothalamus |
| RIA | radioimmunoassay |
| S.E.M. | standard error of mean |
| SAP | stretched attend posture, a behavioural parameter |
| SSRI | selective serotonin reuptake inhibitor, an antidepressant drug |
| Tg | transgenic, genetically manipulated animal, overexpressing a protein |
| VTA | ventral tegmental area, the origin of a dopaminergic pathway |
| Wt | wildtype, the not manipulated counterpart of Tg |

# I. INTRODUCTION

The introduction will start off with a description of depression and the global involvement of monoaminergic neurotransmitters and the hypothalamic-pituitary-adrenocortical-axis (HPA axis) in mood and anxiety disorders to set the background against which the project described in this thesis was carried out.

Aspects relevant to the research at hand will then be described more elaborately.

## 1. SETTING THE SCENE

### *1.1. Depression in society*

Major depressive disorder, commonly referred to as depression, is a psychiatric disease that is becoming an increasingly important problem in modern society. The diagnosis of a depressive episode is made based on the criteria in the ICD-10 [348] or in the DSM-IV [5] (also listed in Table 1). The DSM-IV states that either an abnormal depressed mood, or an abnormal loss of interest and pleasure or, in persons 18 years of age or younger, an abnormal irritable mood must be present for most of the day, almost every day, for at least two weeks. In addition to at least one of those symptoms, at least five of the symptoms listed in Table 1 must be present.

**Table 1. Symptoms occurring in a depressive episode [5, 348].**

| ICD-10 based symptoms (F32) | DSM-IV based symptoms |
|---|---|
| • Depressed mood | • Abnormal depressed mood |
| • Loss of interest and enjoyment | • Abnormal loss of all interest and pleasure |
| • Bleak and pessimistic views of the future | • If 18 yrs or younger, abnormal irritable mood |
| • Reduced energy and increased fatigability | • Activity disturbance |
| • Reduced concentration and attention | • Abnormal poor concentration or indecisiveness |
| • Reduced self-esteem and self-confidence | • Abnormal self-reproach or inappropriate guilt |
| • Ideas of guilt and unworthiness | • Abnormal fatigue or loss of energy |
| • Disturbed sleep | • Sleep disturbance |
| • Diminished appetite | • Appetite or weight disturbance |
| • Ideas or acts of self-harm or suicide | • Abnormal morbid thoughts of death or suicide |

Not every depressive episode therefore needs to consist of the same symptoms. To distinguish between several depressive syndromes, depression can be classified based on

several factors, like severity (mild, moderate, severe), onset (early, postpartum, late), clinical course (single episode, recurrent, chronic) etc. This classification however remains based on subjective criteria; so far no biological markers have been identified that could facilitate the diagnosis, classification or the prognosis of the disease [347].

Depression has a lifetime prevalence of 10% to 25% for women and of 5% to 12% for men. Indeed, women are 2 – 3 times as vulnerable to suffer from a depressive episode as men. At any point in time, 5% to 9% of women and 2% to 3% of men suffer from this disorder. Its prevalence is unrelated to factors like ethnicity, education, income, or marital status. Yet, depression is more common in certain areas. For example in Italy 6-month prevalence rates of 8% are reported, whereas they are as low as 1.4% in rural Bavaria, Germany [347]. A depressive episode lasts 9 months on average, but in 20% of the persons it turns into a chronic disease.

Depression forms a risk factor for other disorders. In 80 to 90% of depressed patients, anxiety symptoms are also present, and about 30% have a full-blown anxiety disorder. In fact the symptomatology of depression and anxiety partly overlap. The outlook of patients with both disorders is gloomier than for depressed individuals that do not suffer from anxiety symptoms. Patients with depression also suffer more frequently from cardiovascular disease, and it has been associated with a decreased bone mineral density [213]. Depressive syndromes often occur in the context of other medical conditions as well, like Cushing's disease (hyperadrenalism), Addison's disease (hypoadrenalism) [149], Parkinson's disease, certain cancers, asthma, diabetes and stroke [224].

The death rate among depressed persons is high: 15% of the patients commit suicide [111]. It is a disease that seriously impairs the quality of life of both the patient and his social environment, and that is costly to society. For example: in the USA more than 19 million adults are affected, making it the most common serious brain disease in the United States. An estimated $43 billion was lost to the direct and indirect costs of the illness in 1990 [117], and the costs are estimated to be an annual $70 billion nowadays [116].

### *1.2. Etiology*

For obvious reasons, the etiology of depression and the related anxiety disorders are of considerable interest to many research institutes and pharmaceutical companies. However, there is no one cause for depression. The situation is much more complex. Preclinical studies as well as modern brain imaging technologies are revealing that, in depression, neural circuits responsible for the regulation of mood, thought, sleep, appetite, and behaviour fail to function

properly, and the balance of critical neurotransmitters are dysregulated. Also the involvement of growth hormone, the thyroid axis, opioid receptors, substance P and brain cytokines have been described [110, 112, 224, 341]. Reports even exist suggesting that an infection with the Borna-disease virus might cause some forms of depression [35].

Epidemiologic studies have shown that around 40 – 50 % of the risk for depression is genetic [93, 306]. Therefore genes involved in depression are sought. But as it is such a complex phenomenon, many genes might be involved, with each of them possibly responsible for a relatively small effect. Because vulnerability is only partly genetic, it is a combination of genetic and environmental factors that precipitates a depressive episode (see also Figure 1).

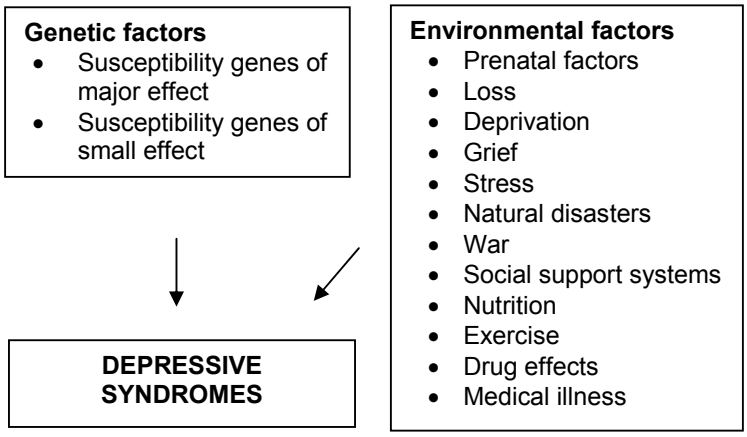



**Figure 1. A combination of genetic and environmental factors can cause depression. Taken from [347]**

Because of all the (multidisciplinary) effort that is put into the elucidation of the background of depression, much more is known now than 10 years ago. Still, furher knowledge and better treatments are necessary, as 24% of patients do not respond to pharmacological or psychotherapeutic treatment, and persons have a more than 80% chance to suffer from additional major depressive episodes at a 8-year follow-up. Additional drawbacks so far are the slow onset of effect of therapy and adverse effects of the drugs in use. Three kinds of treatments have been shown to be effective in fighting depression. These are antidepressant drugs, certain forms of psychotherapy (in particular cognitive and behavioural therapies) and electroconvulsive therapy (ECT) [223]. One of the ways to learn about depression is to study the working mechanisms of these therapies. Doing so with some agents that have antidepressant capacities, lead to the recognition of the role of monoamines in the disease.

# 2. ROLE OF MONOAMINES AND STRESS IN DEPRESSION

## *2.1. Monoamines*

Three serendipitous findings around the mid 20th century gave rise to the monoamine hypothesis of depression (also named the biogenic amine hypothesis). First, Bloch *et al.* [34] described in 1954 that iproniazid, an agent used to treat tuberculosis, improved the mood of some patients suffering from this disease. Later it was found that iproniazid inhibits the function of the enzyme monoamine oxidase (MAO), one of the enzymes that breaks down monoaminergic neurotransmitters and that iproniazid thus leads to an increase in central dopamine (DA), serotonin (5-HT) and noradrenaline (NA). Around the same time a derivate of chlorpromazine, which was very effective in the treatment of psychosis itself, called imipramine, was found by Kuhn [161] to have antidepressant effects. Towards the end of the 1950's, the mechanism of action was elucidated: imipramine inhibits the reuptake of monoamines, another way of increasing the availability of monoamines. Tricyclic antidepressants, based on the prototype imipramine, are still in use to treat depression.

Finally the reverse was found to be true when it became clear that reserpine, used to lower high blood pressure, lowers the concentration of monoamines in the brain by blocking the storage of monoamines in the vesicles, and can cause depression [106].

Based on this, it was hypothesised that depression is caused by a lack of noradrenaline and/or serotonin. The monoamine hypothesis has proven itself to be a very good working hypothesis. Indeed, all pharmacotherapeutic agents used nowadays in the treatment of depression interfere with the central availability of monoamines. All drugs, be it tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRI) or selective noradrenaline reuptake inhibitors (SNRI) work in one of three ways, all increasing synaptic levels of monoamines:

1. blockade of presynaptic transporter proteins that remove monoamines from the extracellular space,
2. inhibition of monoamine oxidase,
3. inhibition or excitation of pre- or postsynaptic receptors that regulate the firing rate of neurons or regulate the release of monoamines [223].

Although it is commonly accepted that an imbalance in 5-HT and NA contributes to the etiology of depression, there is more to the disease than just the monoamine hypothesis, which can also be conferred from the host of systems that are involved (see above).

The monoamine hypothesis for example does not explain why antidepressants need to be taken over several weeks, before clinical effects become apparent. It is now thought that

influencing the balance of 5-HT and NA also influences the transcription regulation of certain genes, and that it is through these genes that symptoms of depression are alleviated. Such mechanism of action requires a longer treatment time than is necessary for influencing neurotransmitter levels per se [224, 258, 347].

## 2.2. Stress and the HPA axis

### 2.2.1. Stress

Stress is known to be a factor in the cause of depression (for reviews see [10, 138, 139]). Interestingly, it seems to play a role in the onset of the first two episodes, but not in consecutive episodes. Stress per se however is probably not sufficient to cause a depression. Typically, horrendous stress does usually not cause a depression, but a post-traumatic stress disorder (PTSD). In vulnerable patients however, stressors that other individuals would consider mild, can play a major role in the manifestation of a depressive episode. This again underlines the interplay of multiple factors in the etiology of depression.

'Stress' is a very general concept, and relates to the responses of the body to external events that bring the physiological equilibrium out of balance. These external events can be of physical, chemical or psychological nature [107]. The term stress and stressors as those events that cause stress was introduced by Hans Selye in 1936 as part of his generalised adaptation syndrome [284]. The stress response comprises those behavioural, neuroendocrine and neurochemical changes that are evoked as a kind of alarm system that is initiated when there is a discrepancy between what an organism is expecting and what really exists [168]. In other words, the stress response is meant to cope with stressors and to find a new equilibrated state. If the coping process is not sufficient, and the stress response is therefore prolonged and sustained, the body and brain homeostasis can be threatened and health can be endangered [107]. In humans for example the incapacity to deal with (most often psychological) stressors can result in anxiety and mood disorders.

The stress response is mediated by activity of the autonomic nervous system and the HPA axis and these systems interact. In addition to these two components, behavioural changes occur.

The activation of the autonomic nervous system results in the release of noradrenaline from various autonomic nerve cell endings in practically every organ in the body, as well as the excretion of adrenaline from the adrenal medulla into the blood. These events result in an increase in pulse and blood pressure, digestion is slowed down, and nociceptors become less sensitive. This enables an organism to directly respond to the stressor with a flight-or-flight

reaction. The activation of the HPA axis will be discussed more elaborately in the next paragraph.

### *2.2.2. The hypothalamus and the HPA axis*

One component of the stress response is HPA axis activation. A pivotal structure in the HPA axis is the hypothalamus. Its role is to maintain homeostasis of the body. Input to the hypothalamus comes from various sensors and structures. These include among others thermoreceptors; osmoreceptors; projections from the nucleus of the solitary tract, informing about blood pressure and other visceral sensory information; projections from the nucleus suprachiasmaticus, enabling the hypothalamus to couple its activity to dark/light rhythms; and projections from limbic and olfactory structures, that play a role in the regulation of feeding behaviour, reproductive behaviour, but also in stress-induced responses (see paragraph 6.1 of the introduction). The output from the hypothalamus follows two major projections. One of them is the autonomic nervous system that through its sympathetic and parasympathetic branches controls heart rate, vasoconstriction, digestion etc. The second output involves endocrine signals. The hypothalamus consists of a number of specialised nuclei secreting various releasing factors. These factors induce the pituitary to excrete specific hormones, among which are adrenocorticotropic hormone, thyroid stimulating hormone, follicle-stimulating hormone, prolactin, somatotropic hormone. These hormones in their turn can affect peripheral organs. Thus the hypothalamus and pituitary form a part of various regulatory endocrine axes, of which the HPA axis is one.

Activity of the HPA axis results in the excretion of glucocorticoids (like corticosterone in rodents or cortisol in humans) from the adrenal cortex, that increase the availability of glucose in the blood by increasing gluconeogenesis, lipolysis, proteolysis and increasing insulin resistance. In addition, glucocorticoids have an anti-inflammatory function. Under non-stressful conditions the HPA axis is important for regulating osmotic and nutritional balances. The activity of this system shows a circadian rhythm, with the peak activity at the onset of the waking cycle [72, 74].

Also under stressful conditions the HPA axis is activated, as glucose is needed to supply enough energy to organs that are active during the stress response (see also Figure 2).

In this context the paraventricular nucleus (PVN) is the most important nucleus in the hypothalamus, as the focal point in the complex of interacting systems regulating the stress responses [232]. The PVN again consists of magnocellular cells, that produce and release arginine vasopressin (AVP) and oxytocin, and of parvocellular cells, that produce and release

corticotropin-releasing hormone (CRH) from their medial part [136]. From the median eminence, CRH is released into the portal system. CRH in turn elicits the release of adrenocorticotropic hormone (ACTH) from the pituitary into the circulation. AVP synergises with CRH and can enhance the ACTH response [7, 186, 340]. When ACTH reaches the adrenal cortex, corticoids are released.

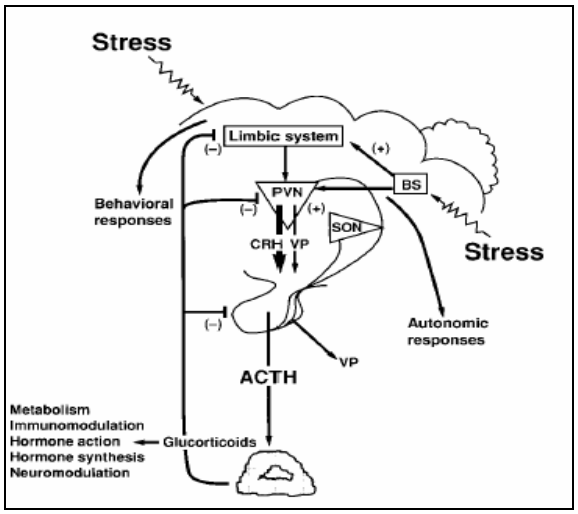



**Figure 2. Schematic representation of the HPA axis. BS: brainstem, SON: supraoptic nucleus (another source for VP), VP: vasopressin. For other abbreviations and explanation see the text.**

Various feedback loops inhibit these stress-induced processes. First, in a short feedback loop, ACTH inhibits the release of more ACTH from the pituitary. In a larger feedback loop (see also Figure 2), the excreted glucocorticoids are necessary to inhibit the responsiveness of the HPA axis over glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), which are mainly located in the hippocampus [73, 259, 260, 262].

In healthy individuals the HPA axis responds with increases in glucocorticoids to stressful events, which occupy GR and over this feedback mechanism induce a return to the equilibrated state of the system. Normal levels of glucocorticoids are thought to have beneficial effects on the hippocampal function and certain cognitive abilities [224].

In depressed patients on the other hand it has been shown that feedback mechanisms are not sufficiently functional anymore. Patients show a sustained activation of the sympathetic nervous system, higher levels of circulating cortisol (although this is not a universal finding in patients), an increased cortisol-response to ACTH, and also pituitary and adrenal glands are often found to be enlarged [22, 114, 258, 290]. Exemplifying of the deleterious effects of sustained high levels of cortisol is the fact that 40 – 90% of patients suffering from Cushing's

disease, which is characterised by high levels of cortisol, also suffer from depression [149]. Tests to illustrate the malfunctioning feedback mechanism are the dexamethasone (Dex) suppression test or the Dex/CRH test. In these, the synthetic glucocorticoid dexamethasone is administered. Dexamethasone can not cross the blood brain barrier and in healthy individuals suppresses, through occupation of the GR in the pituitary, the ACTH-release. In depressed patients, this feedback mechanism is disturbed, and ACTH release is suppressed less. In the Dex/CRH test, CRH is administered after a low dose of dexamethasone. In depressed patients, the ACTH and glucocorticoid response to this exogenous CRH is stronger than in healthy individuals. Reversely, the exaggerated response to CRH is often less pronounced in patients after clinical remission [214, 215]. This is another indication that the feedback mechanisms, that are meant to keep the system as stable as possible are out of balance during depression.

#### *2.2.3. Extrahypothalamic effects of CRH*

CRH is not only produced and released from the PVN, but also functions as a neurotransmitter in the central nervous system (CNS). CRH-containing neurons can be found in limbic and cortical areas. CRH and related peptides like urocortin I, II and III are also found in other areas, including hypothalamic nuclei, the locus coeruleus and the lateral septum. The peptide is released from the central amygdala in response to stressors [210, 211, 263]. Central administration elicits endocrine, autonomic and behavioural effects that resemble the effects caused by stressors [90, 111, 231, 316]. The effects of these neuropeptides are exerted through $CRH_1$ and $CRH_2$ receptors, the former mainly associated with anxiety and the latter with anxiolysis. CRH is thus able to elicit anxiety-like properties, that are unrelated to HPA axis activity [26, 43, 67, 169, 261, 296, 299, 313, 319, 342].

## 3. ANXIETY DISORDERS

### *3.1. Forms of anxiety disorders*

A variety of anxiety disorders is described in the ICD-10 and DSM-IV. These include panic disorder (with and without a history of agoraphobia), agoraphobia (with and without a history of panic disorder), specific phobia, social phobia, generalised anxiety disorder, obsessive-compulsive disorder (OCD), acute stress disorder, and PTSD. In addition, there are adjustment disorders with anxious features, anxiety disorders due to general medical conditions, substance-induced anxiety disorders, and the residual category of anxiety disorder not otherwise specified [5, 348].

A panic attack is a period of acute terror, with physical symptoms like shortness of breath, clammy sweat, irregular heartbeat, dizziness and feelings of irreality and wanting to flee the place where the attack began. In between the attacks, many people suffer from anticipatory anxiety, which may culminate in agoraphobia. The incidence of panic disorder is about 1 to 2%, with a two-fold chance for women to develop a panic disorder. Panic attacks commonly occur with social phobia, generalised anxiety disorder and major depression as well. Agoraphobia, severe anxiety in places from where escape is difficult, or avoidance of such places, has a 1-year prevalence of 5% and is also twice as common in women. Specific phobias are characterised by irrational fear, sometimes also taking the form of panic as described above, in situations that very often involve certain animals, heights, flying, storms, blood, needles etc. These phobias occur with a prevalence of 8% per year. Social phobia occurs in social situations, in which one could be embarrassed or ridiculed, for example when giving a presentation. It has a prevalence rate of 7%. Generalised anxiety is characterised as a long period of anxiety and worry, with accompanying symptoms like muscle tension, fatigue, poor concentration, insomnia and irritability, like in the forms of anxiety disorder described above, but without a focus on a special event or situation. The prevalence is 3%, again with a two-fold number of female patients suffering from it. The most common therapy with these disorders consists of behavioural-cognitive therapy, and in case of medication, administration of benzodiazepines [149].

Acute stress disorder and PTSD refer to the anxiety and the behavioural disturbances after being involved in an extreme trauma, like rape, combat, severe accidents, witnessing murder etc. A critical feature is dissociation, in which the world is perceived as unreal. Often memories to the traumatic event are impaired, although flashbacks can occur. The difference between acute stress disorder and PTSD lies in the duration of the symptoms. When they exceed a month, one speaks of PTSD. Because of the duration of symptoms, PTSD is often accompanied by decreased self-esteem, hopelessness, and difficulties in maintaining relationships. Pharmacologically, PTSD is often treated with SSRIs, also used as antidepressants.

The incidence for obsessive compulsive disorder is about 2%. Patients suffer from thoughts that will not leave them and behaviour that they can not help performing. Mostly these behaviours are counting, checking, cleaning or avoiding. Effective drug therapy involves the administration of SSRIs, whereas benzodiapines are not effective [48, 149].

Clearly the group of anxiety disorders is very heterogeneous, but all forms of anxiety disorder share a state of exaggerated arousal. Some have a strong genetic element (e.g. panic disorder), whereas others are rooted in stressful events [5, 348].

### *3.2. Anxiety and depression*

Although mood and anxiety disorders are treated as separate syndromes, the distinction is very often based on whether the patient subjectively has a primarily depressed or anxious mood. Many symptoms are common to both syndromes, like fatigue, impaired concentration, irritability, sleep disturbance and worry. The comorbidity of the two disorders is high. Additionally, 68% of the individuals with comorbid depression and anxiety were anxious for over 10 years before a depression developed, which might be an indication that the syndromes share some mechanisms [258]. In both disorders, the HPA axis and the limbic system play a major role. The involvement of stress, apart from its obvious role in the etiology of the acute stress disorder and PTSD, can be derived from the fact that CRH induces a blunted ACTH response in patients with panic disorder, when compared to healthy individuals [276], implying that in panic disorder as well the HPA axis is overactive. In addition, the administration of CRH causes increased fear-responses [42, 310]. Also in anxiety disorders, a role is reserved for the monoamines. For example, buspirone, a high-affinity 5-$HT_{1A}$ receptor agonist is an anxiolytic drug and several compounds that antagonise the action of 5-HT at the 5-$HT_2$ and 5-$HT_3$ receptor are also potential anxiolytics [281]. Decreases in the activity of the locus coeruleus, where many noradrenergic neurons have their origin, heightened anxious behaviour [305, 338]. In addition, many of the symptoms during anxiety are reminiscent of the effects of sympathetic activation.

The similarities between the involved brain circuits and symptoms of stress, anxiety and depression caused some scientists to state that the three form a vicious circle with stress leading to anxiety, leading to depression, leading to more stress etc. [247].

## 4. WHY THIS THESIS?

As outlined above, clinical evidence exists that among other factors, monoamines and stress are involved in depression, and in the tightly bound anxiety disorders. To get a better understanding of the interplay between serotonin and noradrenaline on one side and stress on the other side, the study presented here used the behavioural model of predator exposure to characterise the neurochemical changes under this paradigm. Another focus of the study was

formed by behavioural observations. Attempts to link those to neurochemical and neuroendocrine findings also formed part of the project. In the following sections, more detailed information will be given on animal models in general and predator exposure in specific, on the role of brain regions involved in depression and emotion and on the role of the neurotransmitters that were measured in this work. The introduction will conclude with the more detailed aims of this thesis.

## 5. BEHAVIOURAL ANIMAL MODELS

### *5.1. The optimal animal model*

The search for novel behavioural models for depression and anxiety continues [267, 281], as it is highly unlikely that a depressed mouse will ever be found that forms an optimal model. In general, animal models for psychiatric ailments should fulfil the criteria of predictive validity, face validity and construct validity [122, 344, 345]. Predictive validity means that the model must distinguish compounds that might influence the disease from compounds that do not, preferably in a dose dependent manner [321]. Face validity refers to phenomenological similarity between the model and the disorder, i.e. that 'symptoms' or behavioural parameters in the model resemble symptoms of the disease in humans. Finally, construct validity implies that the cause for the behavioural change in the animal is similar to the cause of the disorder in man. However in the case of depression, too little is known about the etiology or its pathophysiology to be able to base a model on construct validity [225]. Therefore a different set of criteria has been formulated [205] saying that the minimum requirements for an animal model of depression are:

- it is 'reasonably analogous' to the human disorder or its manifestations or symptomatology (refers to face validity),
- there is a behavioural change that can be monitored objectively,
- the observed behavioural changes should be reversed by the same treatments that are effective in humans (predictive validity),
- it should be reproducible between investigators.

In practise it means that certain symptoms of depression (endophenotypes, rather than the complete set of symptoms, the phenotype) are reproduced in animals, and mechanisms underlying these symptoms are elucidated and novel treatments are tested in these models [225]. Still it must be kept in mind that depression influences higher cognitive human processes such as motivation and self-esteem, and that it will remain unclear whether

mechanisms that are elucidated based on animal models truly represent the pathophysiologic background of depression or merely the effects of stress, pain or deprivation [347]. After all, the biological basis of the symptoms in animals could be different from those in humans [225].

### *5.2. Animal models used in depression research*

Most of the following models, which are in use to investigate depression, use stressors to induce behaviours that are sensitive to antidepressant treatment:

- Learned helplessness is a model developed in 1968 by Seligman that models some features of depression [283]. Animals that are exposed to an inescapable electric shock subsequently fail to escape from a situation in which escape is possible. These animals show some concomitant neurovegetative symptoms that are similar in depressed persons, such as alterations in REM (rapid eye movement) sleep, reduction in body weight and in sexual behaviour, as well as elevated CRH and corticosterone levels. Repeated dosing of antidepressants and ECT reduce the features of learned helplessness as well as the neurovegetative symptoms. Unfortunately, this model uses extreme stress and is very animal-unfriendly. It is also unclear whether it may model PTSD more than it does depression and finally, findings are less reliable and reproductive than in the forced swim test [225, 247].
- The (modified) forced swim test, also called the Porsolt test, has been very helpful in predicting the antidepressant properties of compounds [224, 236]. It measures the latency of a rat or mouse to become immobile when placed in a container filled with water and the time it swims, climbs and passively floats. Acute administration of antidepressants increases the coping responses to the swim stress. A variation to this is the tail suspension test, in which mice are hanged by their tails and the time is measured until mice stop struggling. The benefits of these tests are that they are fast and easy [225]. The question is however, whether these tests are capable of identifying anti-depressant compounds that are not monoamine-based medications. Also, antidepressants are already active after acute administration, whereas their clinical effect in patients takes more time to become apparent, which again raises the questions about the similarity in biological background of the parameters measured in the test and the symptoms in patients.

- In olfactory bulbectomy both olfactory bulbs of rats are removed. This causes hyperactive responses in novel, brightly lit open fields, which are normalised by chronic treatment of antidepressants. The effects of antidepressants are not secondary to the loss of smell in these animals [64, 197]. It is supposed to be a model for geriatric depression [294].
- In chronic stress paradigms animals undergo a variety of mild stresses such as social isolation, short food or water deprivation, disruption of the light/dark cycle etc. A variation is repeated social defeat. Animals that are stressed like this show anhedonia, and cardiovascular and neuroendocrine effects, that are sensitive to antidepressant treatment. These paradigms have face validity, but are poorly reproducible [225].
- Early life stress can take the form of prenatal stress, early postnatal handling or maternal separation. Each of these cause persistent neuroendocrine and behavioural changes in the pups that last until adulthood. Their HPA axis is hyperactive, they show elevated locomotor responses to novelty and greater vulnerability for learned helplessness. These models are gaining interest [225].
- Relatively new in the antidepressant field of research are models that use intracranial self-stimulation, a model known from research on drugs of abuse and reward mechanisms. Withdrawal symptoms, e.g. after amphetamine use, can represent an animal model of anhedonia. Antidepressants can reduce some of the withdrawal symptoms [64].

### *5.3. Animal models used in anxiety research*

As described before (in paragraph 3.1) many forms of anxiety disorders exist, in which different pharmacological agents are effective. This multitude should be reflected in the models of anxiety. However, no useful model exists to model phobia or PTSD, and apart from some commonalities between animals with certain stereotypies and OCD, no model exists that reflects OCD. Most models in use therefore are related to generalised anxiety disorder [180]. As a consequence these tests are especially sensitive towards the anxiolytic properties of benzodiazepines, but less to these of antidepressants [37]. The most common tests are listed below.

Many of the used paradigms are ‘conflict’ tests that use both an aversive stimulus (like open space or a brightly lit environment) and a rewarding stimulus (like a familiar or non-threatening environment, or food) [322]. The less animals avoid the aversive stimulus, the

less anxious they are. It does seem that the different tests tap into different aspects of anxiety [21, 271]. The tests can therefore be considered to be complimentary. An example of different aspects in anxiety is the distinction between state and trait anxiety [180]. State anxiety is the anxiety experienced at a particular moment and that is increased by an anxiogenic stimulus. Trait anxiety does not vary over time and is more like a baseline level of anxiety.

- A commonly used paradigm is the elevated plus maze (EPM), in which an animal is placed on a + -shaped maze with two opposite arms that are closed off at the sides, and the remaining two arms having no walls. The apparatus is elevated above the floor. The test uses the preference of animals to stay in shielded areas and measures among others the time on the open and closed arms as well as the number of entries in both kinds of arms. Anxiolytics cause an increase of time spent or entries onto the scarier open arms, driven by the urge to explore. Interestingly this is mainly seen on a first trial. When an animal is tested repeatedly, the measures become insensitive to benzodiazepines. It appears that on a first trial the animal's behaviour is mainly influenced by the openness of the arms, whereas on subsequent tests elevation is the more important aspect [100, 102]. An ethological version of the EPM has been developed in which more parameters, like aspects of defensive behaviour, are measured. Factor analysis has been performed by several research groups to identify the relationship between the test indices and different factors like anxiety and locomotion. Although results of the analysis are slightly variable among the groups, the general picture seems to be that for example the time in open arms, the number of entries into the open arms and the number of stretched-attend postures are reflective of fear or reactivity, whereas the total number of entries, and number of rearings is an index of locomotion and the time spent in the middle of the maze as well as grooming may have similarities with a decision-making process [97, 267, 270, 271, 322].
- The dark/light box consists of two separated rooms, one brightly lit, the other dark. Night-active animals like mice and rats prefer to be in the dark compartment. Animals are placed in the dark compartment and it is measured how long it takes until they enter the light compartment, how many entries they have into this compartment and the time spent on the light side. Anxiolytics increase the time spent in the light compartment. It has been suggested that the light/dark paradigm models state anxiety [24].

- In open-field tests the subject is placed in a wide arena and the time spent along the walls and in the center area is measured. The more anxious the animal is, the more time it spends close to the walls.
- Under bright light and in a novel environment, social interaction between animals is decreased. Anxiolytics increase social behaviour again. This test works well with rats, but because they are more aggressive to conspecifics, it is less useful with mice [180].

In all these tests it is important to control for effects of test compounds on locomotion, to exclude false positive or false negative predictions.

### *5.4. Rats vs. mice*

Traditionally, the rat has been the rodent of first choice in many areas of research and most behavioural tests have been optimised for rats. However, with the rise of genetics, the mouse has taken the place of rats. They are more easily to house, breed more quickly, recombination techniques have been standardised for mice, and their genome is more completely characterised. Behavioural models are now adapted from rats to the mouse situation. But this is not always successful. Part of the reason is that more knowledge is needed about the naturalistic behaviour of mice to develop better mouse behavioural tests [225]. Indeed it has been argued by many ethologists, like Lorenz, Tinbergen and Thorpe that a more comprehensive survey of the behavioural repertoire of animals is more likely to produce success when using behavioural models in research [267].

### *5.5. Genetic components*

What the models mentioned above lack so far is that they do not incorporate the fact that depression requires a certain genetic vulnerability. The paradigms are conducted with 'normal' mice, which might not have the genotype to develop analogues of depressive syndromes.

To a certain extent this can be overcome by using a proper strain of mice. Commercially available inbred strains of mice (and rats) already show a high variation in their responses to stress and in for example HPA axis reactivity and responses to antidepressants [62]. In a survey of 11 strains of mice in the forced swim test, tenfold differences in immobility scores were found. Also antidepressants had dissimilar effects in the strains [190]. Large differences were also reported in comparing the behaviour of different strains of mice in the tail suspension test [182]. Strains also differ in their anxiousness. However, in models of anxiety,

the ranking order for various strains is task-dependent [272, 329]. This is caused by the fact that the different models measure different aspects of anxiety, and also because the influence of the genetic background (from where the strain differences primarily arise) is not equally large among paradigms. For example, the time mice spent on the open arms of the EPM was found to be determined by their genetic background for about 78% [322]. The activity in the open field however was only for 26% dependent on solid strain differences [322].

Another strategy is to selectively breed animals that show for example high or low levels of swimming in the forced swim test, to generate animals with larger differences in depression-like (endo)phenotypes. It would be interesting to compare the brain systems and genes of these animals to 'normal' animals. In depression research, lines are developed with animals susceptible to learned helplessness, high/low responses in the forced swim test and high/low immobility scores in the tail suspension test. Recently, in the field of anxiety, animals exhibiting high- and low anxiety on the elevated plus maze have successfully been bred and several SNPs (single nucleotide polymorphisms) have been identified separating these animals from the normal phenotypes (personal communication with S. Krömer).

Mutational techniques are also employed to improve models of depression. Two approaches are currently used. Forward genetics use chemical mutagens such as ethylnitrosurea (ENU) to randomly induce mutations. Interesting phenotypes are investigated to find the responsible gene. In reverse genetics, a candidate gene is disrupted or overexpressed to investigate what the effects are on phenotype [225]. An example of this, also used in the experiments reported here, are mice that overexpress CRH. Two caveats with genetically altered mice are that the background strain needs to be carefully selected and that compensatory adaptive changes may occur. The latter happens especially in animals that were born with overexpressing or knocked-out genes, rather than animals in which the gene transcription can be switched on or off with conditional mutation techniques.

### *5.6. Example: CRH-transgenic mice*

The reason to develop mice with a mutated CRH gene is that abnormalities are found in CRH levels in depressed people. Some depressed patients show a hypersecretion of CRH, as well as a downregulation of CRH receptors and blunted ACTH responses to administered CRH [224]. Reversely, in healthy individuals, the effects of centrally administered CRH mimic symptoms of depression, like increased arousal, decreased appetite and increased blood pressure [224]. In rodents, the central administration of CRH reduces the exploration of novel surroundings, decreases sleep, enhances fear responses and decreases food and sexual

behaviour. These changes parallel those found in major depressive disorder, panic disorder and anorexia nervosa [303]. Mice that overexpress CRH have elevated levels of ACTH and glucocorticoids and develop a Cushing's syndrome phenotype [302]. They also show characteristics that fit enhanced stress-responsiveness and anxiety-like behaviour. For example, the transgenic animals spent less time on the open arms than controls and were less active in a novel environment [303].

### *5.7. Predator exposure*

As outlined above, most animal models in depression and anxiety research incorporate a stressful situation. Apart from the models that are mentioned above there is a host of other models that attempt to research the various mechanisms underlying stress per se. Many of them contain a physical component, such as pain (avoidance paradigms), stimulation of the immune system (haemorrhage, lipopolysaccharides), or a decrease of body temperature (cold exposure). In recent years, behavioural models that use predator exposure, of which rat exposure is a form, have gained increasing interest. Predator exposure provides a stress model that relates to the innate fear for a predator, whereas other models, like the ones used in avoidance paradigms, relate to learned fear. Another difference with some classical paradigms lies in the way in which the stressful stimuli are processed (see also paragraph 6.2). In models based on pain, or on, for example, ether stress or hypoxia (where animals are presented with a situation that is immediately threatening for survival) stimuli reach the paraventricular nucleus of the hypothalamus directly from the brainstem. Stimuli such as encountered in predator exposure first have to be cognitively processed by the limbic areas before they are dealt with [135]. Indeed, c-fos expression after predator exposure was increased in numerous brain regions, indicating the activation of different regions than those involved in hypoxia or restraint stress [83, 99]. The impact of psychological stressors depends on how they have been perceived, in which previous experience and the ability to cope with stressful situations are important factors [135, 159]. Thus, models dealing with innate fear and with processive stimuli may more closely resemble psychologically stressful situations in humans. Various forms of predator exposure are employed. Some involve the presentation of dogs to cats; fox odour, cat droppings or cats to rats [1, 30, 99, 246] and anaesthetised or awake rats to mice [28, 174]. Models may even be as exotic as exposing sea trout [167] or goldfish to blue-gills [147].

As the method of performing predator exposure differs among laboratories, in some cases not only the effect of the predator but also that of novel environment or handling mix in. In the

case of rat exposure, mice are very often placed in a small cage within a larger cage, containing the rat, enabling the rat to climb on top of the mouse compartment and sometimes to push the mouse compartment around (e.g. [6, 132]). A special form of predator exposure is used by the group of Caroline Blanchard and Robert Blanchard in their Mouse Defense Test Battery. They use an oval or closed-off runway and follow a mouse with a handheld anaesthetised rat. Carefully studying the effects of drugs on defensive behaviours displayed by the mice makes it possible to differentiate between anxiolytics and panicolytics [29, 32, 120, 121, 123].

The form of predator exposure in the experiments described in this thesis also used rats as a predator of mice. Rats are known to kill mice [195, 279], and mice show stressful reactions when presented with a rat. In the current setup, the rat was introduced into a compartment directly next to the mouse compartment in the mouse's home cage, thereby avoiding novel environment, transportation stress, or physical contact with the experimenter, and enabling simultaneous microdialysis (see also Materials and Methods, paragraph II.1.3.4 and [174]).

## 6. NEUROANATOMY

### *6.1. The LHPA axis*

Previously the HPA axis has been described as consisting of the hypothalamus, pituitary and adrenal medulla, which cooperate in the responses to stress. However, in some cases it would be more accurate to speak of an LHPA system, with the L standing for limbic. The limbic lobe was described by Paul Broca (1824 – 1880) as a ring-formed structure around the brainstem, consisting of the cingulate cortex, the temporal lobe cortex and the hippocampus. Later it was found that the limbic lobe was important for the generation of emotion. Papez showed that the limbic lobe, together with the anterior nuclei of the thalamus, formed a functional connection between the cortex and the hypothalamus [240]. This circuit was later extended to include the amygdala [156]. Now the limbic system, a term introduced by Paul MacLean in 1952 [191], is used to represent the brain regions that generate and regulate emotions (see also Figure 3 and Figure 4) [107]. This system is important in the modulation of the HPA axis activity, and hence the term LHPA axis is found more and more often.

It is well-described that the way an organism reacts to a stressor is related to characteristics of the stressor (severity, chronicity, predictability, controllability), previous experience and on

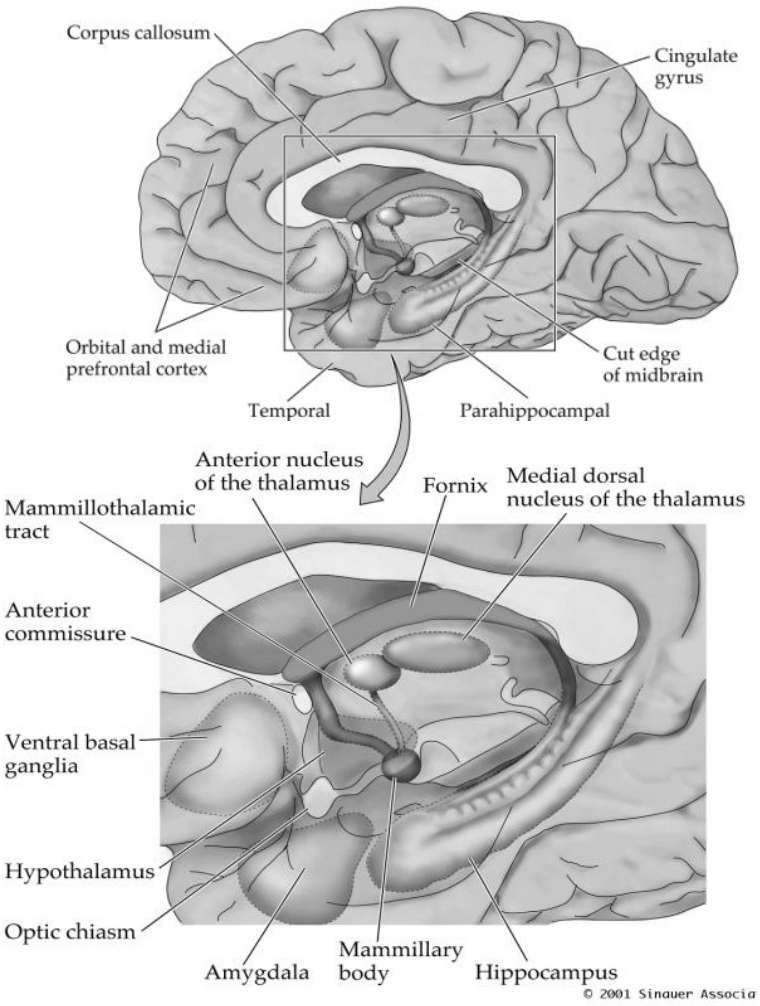



**Figure 3. Overview of the human brain with structures that form a part of the limbic system [36].**

the subject (age, strain, species) (see e.g. [6, 187, 209, 222, 338]). It is hypothesised by Herman *et al.* [135, 137] that the activation of different neuronal circuits may partly underlie these differences. In case of a genuine homeostatic challenge, in which a stressor is present that is recognised by sensors like nociceptors, chemo-, baro- and osmoreceptors, sensors that respond to glucose, leptin, insulin, renin-angiotensin, atrial natriuretic peptide or sensors reacting to inflammatory agents, a so-called 'systemic' pathway is thought to be activated.

This 'systemic' pathway relays information that enters the brain and needs little sensory processing directly from the brainstem to the hypothalamus, so that responses are reflexive and fast [137]. But also in absence of such a physiological stressor, HPA axis activation can occur in the anticipation of a challenge and its concomitant homeostatic disruption. These anticipatory responses can either be acquired by learning (conditioning paradigms), or can be

innate (reactions to predators, unfamiliar situations, social challenges, or e.g. brightly lit places in case of rodents). To avoid an unnecessary allostatic load (the 'cost' of prolonged stimulation or overstimulation of adaptive processes [202, 203]) the HPA responses to anticipated stressors are thought to be under the control of limbic structures, such as the hippocampus, amygdala and the prefrontal cortex (PFC), that first need to appraise the emotional significance of the stimulus before the HPA axis is activated [137]. These higher brain structures receive polysensorial and associational input, rather than primary sensory information [137]. Further indications exist that even within the group of processive or psychological stressors, different brain areas may be activated. A predator odour for example activated dopamine metabolism in the amygdala, but did not activate the nucleus accumbens, whereas the opposite was true for a conditioned fear situation [222]. Also purely psychological (psychogenic) stressors and those that also have a physical component (neurogenic stressor) differ in their impact [6, 187].

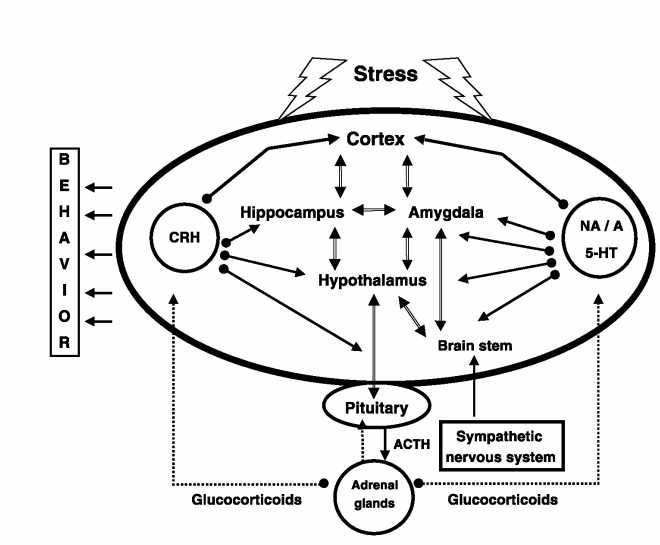



**Figure 4. A schematic representation of the LHPA axis [107].**

Of the higher brain structures that are involved in the responses to these stressors, the hippocampus exerts the strongest inhibitory influence on the hypothalamus (somewhat depended on subfield [92]), via a polysynaptic pathway, in which acetylcholine, gamma-aminobutyric acid (GABA) and glutamate are important mediators [137]. Also the prefrontal cortex, especially its dorsal subregions, can inhibit the HPA axis responses to anticipatory stressors. The neurotransmitters NA and dopamine play a role in this [137, 308]. The lateral septum is implied in stress regulation as well. Neurons in this area are robustly activated by various processive stressors, that then exert an inhibitory influence on the PVN via

GABAergic neurons [135, 137]. In contrast, the amygdala offers an excitatory influence [135, 137, 224]. The amygdala again consists of different nuclei. The central nucleus is involved in some systemic stress responses, whereas the basolateral nucleus is activated by anticipatory stressors. The medial nucleus takes an intermediate position [137]. The structures mentioned here can exert their influences also via other brain regions, like the nucleus of the solitary tract, the preoptic area and the PVN surrounding areas. These latter regions may also mediate 'systemic' responses [137].

### *6.2. Relevant brain areas in emotion and depression*

Before listing brain regions that are relevant for emotion, it is important to realise that there are several phases that are part of the perception of emotion. First, a stimulus must be identified and appraised as having emotional significance. Second, an affective state has to be produced in response to the stimulus, which includes autonomic, neuroendocrine and behavioural components. Third, the affective state and emotional behaviour must be regulated, influencing the first and second step, so that it stays appropriate to the situation [248]. Both animal and human studies have shown that the amygdala is the most important structure in the first step of the process. Also the insula and the ventral striatum and thalamus have been mentioned as important to recognise displays of disgust especially.

For the production of an affective state again the amygdala is mentioned as a key player. From here, projections to many brain regions mediate physiological and behavioural reactions. Septal nuclei and the bed nucleus of the stria terminalis (BNST, also called part of the 'extended amygdala' [68]) are functionally and anatomically related to the amygdala, and also play a role in fear and anxiety-like responses [224]. Also important in the production of an affective state are the reward pathways, the anterior cingulate cortex, the orbital and the medial prefrontal cortex (mPFC).

To regulate affective states, the prefrontal cortex is important as well as the hippocampus. The latter structure has been hypothesised to play an important role in the inhibition and facilitation of defensive behaviour and anxiety in response to (potentially) threatening situations, as well as in spatial cognition and episodic memory. The hippocampus also has been named a 'general purpose comparator' [115], with a central role in determining the extent of conflict between behaviours that serve different goals, in facilitating exploratory patterns over defensive patterns of behaviour and allowing resolution of the goal conflict.

The role of the prefrontal cortex is dependent upon subregion. The dorsal regions for example are implicated in cognitively demanding tasks, which need to take attention away from

emotion and for planning-based regulation of emotional behaviour. Activity of the dorsal PFC is found to be decreased in depressed patients [27, 86, 87]. The ventral regions mainly have a role in regulation at an unconscious or automatic level.
The anterior cingulate gyrus is involved in early stages of learning, and disruptions in this region causes errors and attentional deficits. The anterior cingulate is also important in the anticipatory reactions to arousal or in the performance of relaxation tasks [248].

In depression research the hippocampus has been the brain region most extensively investigated. But to be expected from the above, it becomes more and more clear that many brain areas mediate the diverse symptoms of depression [225]. Brain imaging studies have shown volume reduction or altered activity in and thus implied the involvement of not only the hippocampus, but also of areas in the prefrontal and cingulate cortex, striatum, amygdala and thalamus, although some findings are contradictory [224].
The neocortex and hippocampus might mediate the cognitive symptoms of depression (feelings of worthlessness, guilt, suicidality). The reward pathways in the brain, which encompass the dopaminergic neurons from the ventral tegmental area (VTA) in the ventral midbrain to the forebrain (i.e. the nucleus accumbens and the ventral striatum) regulate the animal's response to natural reinforcers such as food, sex and social interaction. Interest in all these activities is diminished during a depression, suggesting a role of the reward centres in the disease [225]. The striatum and the amygdala, also important for emotional memory, are therefore thought to be involved in anhedonia, anxiety and reduced motivation. Volume reduction of the amygdala may result in a restricted range of emotions to be recognised and experienced, with a bias towards the identification of negative emotions [249].
The hypothalamus finally might mediate the neurovegetative symptoms of depression, like sleep disturbance, appetite and energy loss, and disinterest in sex and pleasure [224].

The brain structure that is focused on in anxiety is the amygdala (see Figure 5). It processes sensory information via two pathways. Through one rapid pathway, sensory information is received directly from the thalamus, the structure where all sensory information from the body is received and filtered. The second pathway is more complex and involves input from nuclei in the brainstem, from the hippocampus, the medial prefrontal cortex and cortico-striato-thalamo circuits. All sensory information, gated through the thalamus, reaches the amygdala in the lateral nucleus. From here it is passed on to other nuclei. The central nucleus coordinates the information from multiple nuclei and generates a behavioural response.

Connections from the central nucleus to PVN, striatum and other brain regions make sure that the response is executed [153].

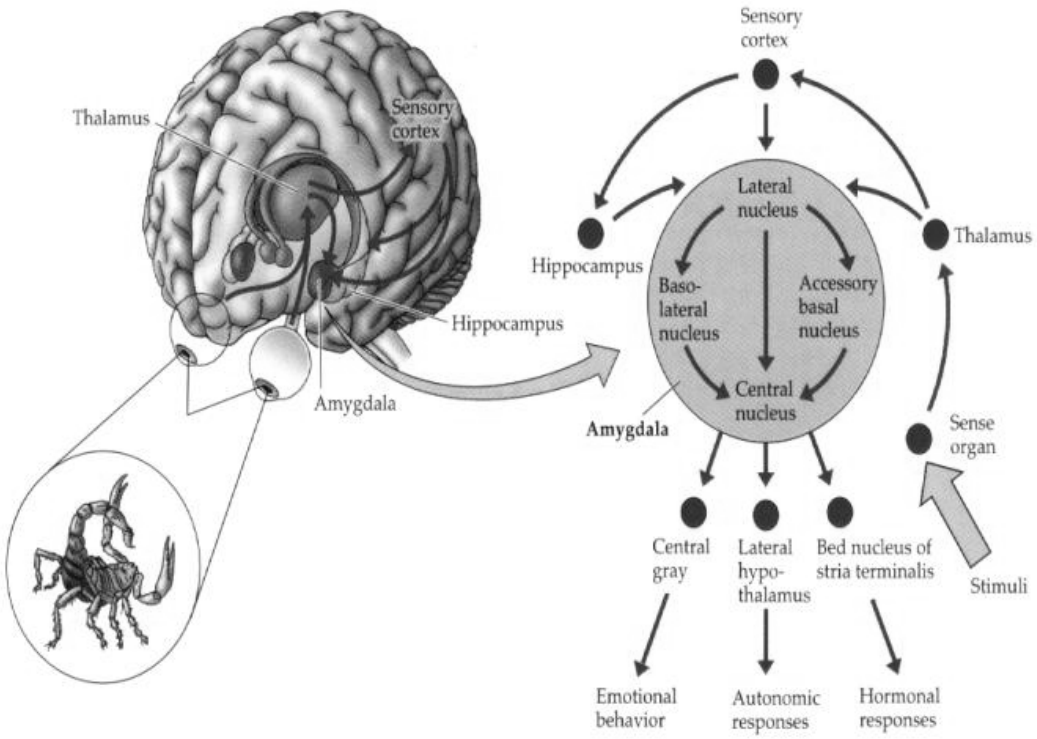



**Figure 5. Schematic representation of brain regions, including the amygdala, that are involved in formulating a response upon presentation of an aversive stimulus [36].**

Under normal conditions the amygdala continuously monitors the sensory information that is redirected from the thalamus and cortical regions. These are compared to known aversive or appetitive stimuli. Recognition of such associations activates pathways involving the central nucleus or the BNST in case of anxiety or fear related stimuli or of the nucleus accumbens in case of pleasant associations.

Under basal conditions the hippocampus attenuates the responses of the amygdala. Under stressful conditions the hippocampus gives information on the context of the threat and makes it possible to retrieve information from explicit memory. It activates the amygdala and potentiates memory formation in case of aversive incidents. When the hippocampus is not properly functioning this often leads to a fail estimation of the context of a potential threat and an overgeneralised fear response, a hallmark of anxiety disorders [153].

The prefrontal cortex provides information on changes in the threatening stimulus or whether the danger has passed [153]. To extinct aversive memories, input from the cortex is needed. It

is proposed that the PFC provides cognitive control over stress and fear responses and mediates tolerance toward anger, anxiety and frustration [74, 163, 165, 166]. Psychotherapy might function through increasing the cortical control over the limbic pathways [165]. Reciprocally, under stressful circumstances the amygdala inhibits the functioning of the prefrontal cortex, to not have a delaying cognitive control when rapid instinctual responding is needed [111].

## 7. NEUROTRANSMITTERS

The role of monoamines in depression has been mentioned before. In the following characteristics will be given of the monoamines serotonin, noradrenaline and dopamine, as well as some indications of their involvement in stress, depression and anxiety.

### *7.1. Serotonin*

#### *7.1.1. General*

Serotonin owes its name to its discovery in serum, as a compound that makes muscles contract. Serotonin is also present in blood platelets, chromaffin cells of the intestinal mucosa and in the central nervous system.

Serotonin is synthesised (see Figure 6) from the essential amino acid tryptophan. Tryptophan is converted to 5-hydroxytryptophan by the enzyme tryptophan-5-hydroxylase, that is converted by l-aromatic amino acid decarboxylase to form serotonin. The synthesis is limited by the availability of tryptophan.

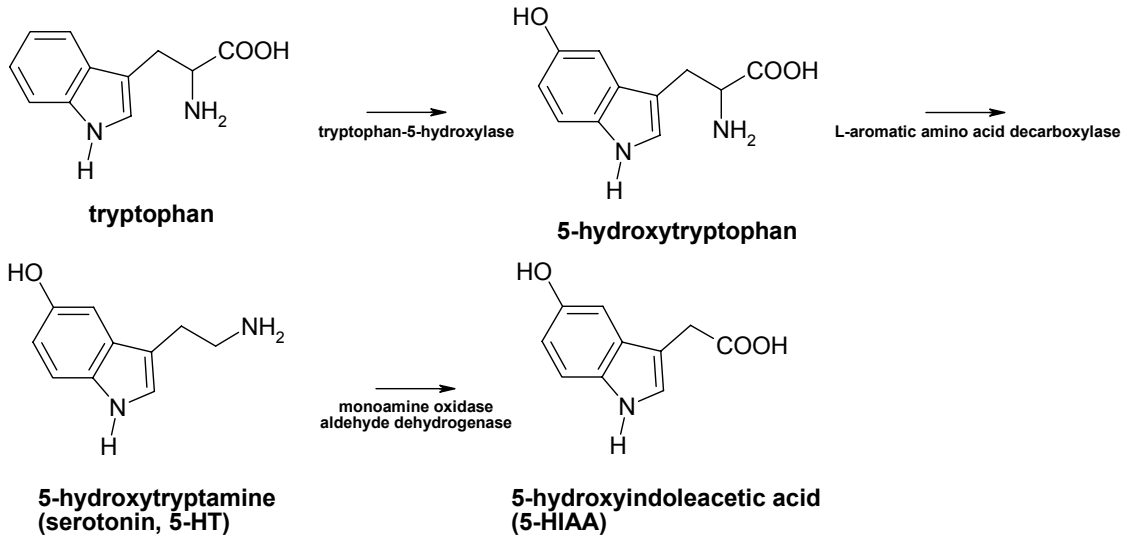



**Figure 6. The synthesis and metabolism of serotonin.**

After release of 5-HT, its action is terminated by the reuptake of serotonin through 5-HT transporters in the presynaptic membrane. Serotonin is also metabolised by monoamine oxidase and aldehyde dehydrogenase to yield 5-hydroxyindole-3-acetic acid (5-HIAA).

When serotonin is released in the synaptic cleft after an action potential, it exerts its effects via a multitude of 5-HT receptors. So far 7 families of receptors are known ($5\text{-HT}_{1\text{-}7}$) with 15 subtypes. All of these receptors are G-protein coupled, except the $5\text{-HT}_3$ receptor that is an ion-channel receptor.

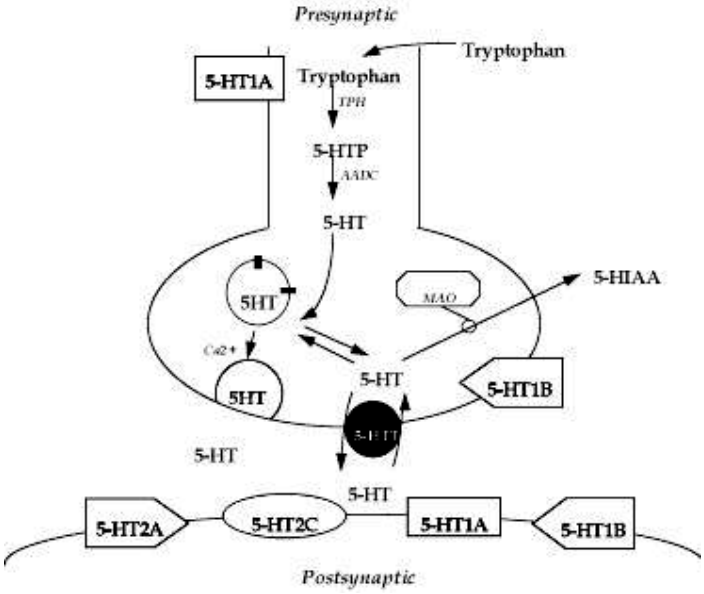



**Figure 7. Schematic representation of a serotonergic neurone with pre- and postsynaptic serotonergic receptors.**

The $5\text{-HT}_{1A}$ receptor exists both as postsynaptic receptor (in the hippocampus, septum, cortex, amygdala and other limbic structures) and as a presynaptic receptor that occurs on the cell body (mainly in the raphe nuclei). The postsynaptic receptor is a heteroreceptor, and can also be found on neurones that use other neurotransmitters, like NA. The presence of such heteroreceptors means that an alteration in serotonergic neurotransmission may modulate non-serotonergic systems as well. As presynaptic receptor it suppresses the activity of the serotonergic neurons.

Also $5\text{-HT}_{1B/D}$ receptors are found presynaptically, on axon terminals, where they inhibit the release of more 5-HT [71]. The highest density is found in the substantia nigra, but also in the hippocampus, caudate and the putamen. The $5\text{-HT}_{1B}$ receptor can also exist on non-

serotonergic neurons, and there may modulate the release of NA, dopamine, glutamate and GABA. Not only does 5-HT influence its own release, but GABA and histamine also have inhibitory effects on serotonergic neurotransmission [145]. Conversely, noradrenaline and glutamate have an excitatory influence [145].

$5\text{-HT}_2$ receptors are found postsynaptically, as are $5\text{-HT}_3$ receptors [145, 153].

The cell bodies of serotonergic neurons form clusters. The five caudally located clusters project to the spinal cord, whereas the four most rostral clusters in the dorsal and median raphe nuclei (DRN and MRN) project to the mid- and forebrain [145]. The effect of serotonin in the brainstem and spinal cord is mostly that of excitation, whereas it is usually inhibitory in the forebrain [145].

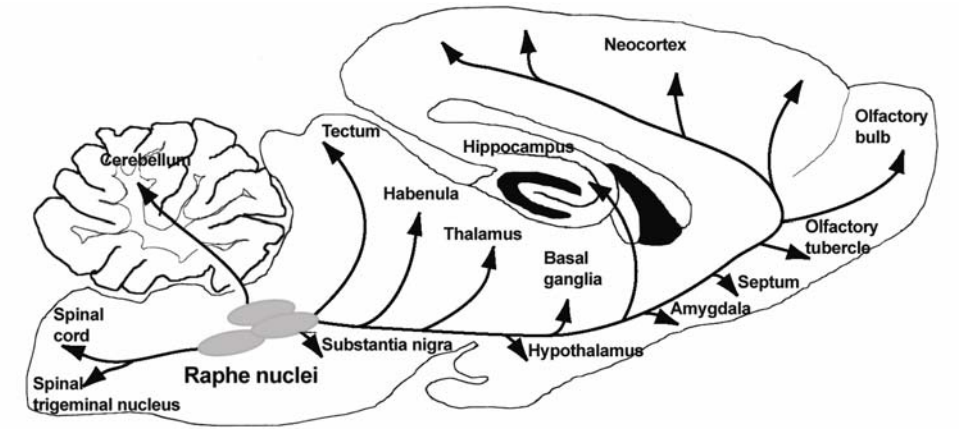



**Figure 8. Serotonergic pathways in the rodent brain. Adapted from [48].**

As can be taken from Figure 8, the serotonergic pathways project to a multitude of brain regions, that are involved in a host of functions. Serotonin is therefore involved in sleep and arousal, feeding behaviour, sexual behaviour, pain perception, the control of body temperature, memory formation, the regulation of mood and of motor behaviour, to name a few [48, 145, 189, 280]. The neurons originating from the dorsal and the median raphe nucleus project to different brain structures but with considerable overlap. Limbic structures seem to be mainly innervated from the median raphe nucleus, whereas structures in the basal ganglia, like the striatum, are primarily innervated from the dorsal raphe nucleus [258]. Inputs to the raphe nuclei arise from brainstem nuclei such as the ventral tegmental area (via dopaminergic neurons) and the locus coeruleus (LC, via noradrenergic neurons). Interestingly, the prefrontal cortex, hyperactive in some anxiety and depressive states, can inhibit raphe activity via $5\text{-HT}_{1A}$- and $GABA_A$ receptors [50, 87, 173], although, after depletion of 5-HT,

activation of the mPFC resulted in activation of the raphe nuclei, mediated by glutamate receptors [50].

### *7.1.2. Involvement in stress, anxiety and depression*

Reciprocal interactions between the serotonergic system and the HPA axis have been described. Neurons from the raphe nuclei project to the PVN, where 5-HT influences the levels of CRH, ACTH and other stress hormones through 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors [57, 124, 238, 327]. Most information from the raphe nuclei however reaches the PVN via GABA-ergic interneurons. 5-HT thus probably influences the PVN indirect via heavy innervation of limbic structures [237]. 5-HT also modulates the negative-feedback of glucocorticoids on the HPA axis [141, 194, 285].

Conversely, stress is associated with increased activity of the dorsal and median raphe nuclei [54, 129, 185, 250, 318], and most researchers find increases in the extracellular levels of 5-HT or in the activity of serotonergic neurons in several brain areas under stressful conditions (reviews: [54, 193, 277]). The 5-HT synthesis rate is reduced in adrenalectomised animals [331], which effect is probably mediated over GR receptors in the hippocampus that are projecting to the raphe nuclei. In the hippocampus, MR and GR are often colocalised with the 5-$HT_{1A}$ receptor, the expression of which is suppressed by corticosterone [146]. The expression of the 5-$HT_{1A}$ receptor can be influenced by corticosterone [52].

Serotonin, the serotonin transporter and practically all receptor subtypes also play a role in anxiety and depression [193, 207]. Indications for this are that the chance of a depression increases if one interferes with the availability of tryptophan, or when the synthesis of serotonin is blocked by PCPA (parachlorophenylalanine). Suicide victims also show reduced levels of serotonergic markers [78, 192].

A reduction in the serotonin transporter availability was found in the brains of depressed patients [196] and of patients with a generalised anxiety disorder [143]. Also the allele for the short form of the serotonin transporter promoter is associated with a poor response to SSRI [225].

Knockout mouse models of the 5-$HT_{1A}$ receptor induced an increase in anxiety like behaviour [133, 241]. The selective 5-$HT_{1A}$ agonist buspirone is effective in the treatment of anxiety and depression [331]. A 5-$HT_{1B}$ knockout had a less anxious behavioural profile [201, 353]. 5-$HT_{2A}$ receptor antagonists decreases the behavioural and physiological responses to stress, and are anxiolytic in some animal models. 5-$HT_{2B}$ agonists, 5-$HT_3$ antagonists and 5-$HT_{2C}$

antagonists appear to be anxiolytic, but the latter unfortunately have an increased food-intake and weight gain as a side effect.

Chronic treatment with antidepressants cause levels of 5-HT in the forebrain structures and in the raphe nuclei to increase [18, 33], along with a reduction in the density of 5-$HT_2$ receptors [274] and an increase in the density of 5-$HT_{1A}$ receptors [75, 131].

In preclinical models of anxiety an increase of 5-HT function is associated with aversive behaviours. Conversely, drugs that reduce serotonergic function reverse fearful behaviours. Acute administration of SSRIs increase serotonergic concentrations by blocking the serotonin reuptake transporter. Indeed, the acute administration of SSRIs is known to first increase anxiety symptoms in patients. This elevation in 5-HT however is tempered by activation of autoreceptors that inhibit further release of 5-HT. With time, the autoreceptors become less responsive. Simultaneously, postsynaptic 5-HT receptors become downregulated, and also other systems may be influenced, resulting in the antidepressant and anxiolytic properties of SSRI [153].

## *7.2. Noradrenaline*

### *7.2.1. General*

Noradrenaline is a catecholamine that is synthesised in central noradrenergic neurons, as well as in the periphery.

Noradrenaline is synthesised from the amino acid tyrosine (see Figure 9). The enzyme tyrosine hydroxylase catalyses the transition to DOPA, which is decarboxylated by DOPA-decarboxylase to dopamine. This in turn is transformed to noradrenaline by dopamine-β-hydroxylase. The step from tyrosine to DOPA is rate-limiting.

After release, the action of noradrenaline is mainly terminated by an efficient reuptake mechanism, but NA is also metabolised by MAO-A and COMT (cathechol-O-methyltransferase). The most significant metabolic product of NA is MHPG (3-methoxy-4-hydroxyphenylglycol) [149]. Noradrenaline can interact with three families of adrenergic receptors, all of which are G-protein coupled. These are the β-family, divided into $\beta_1$, $\beta_2$ and $\beta_3$ receptors, and the $\alpha_{1(a,b)}$ and $\alpha_{2(a,b,c)}$ families. The β receptors are the most important adrenergic postsynaptic receptors and all have an excitatory effect after activation. So are the $\alpha_1$ receptors. The $\alpha_2$ receptor in contrast is located on the cell body, as well as postsynaptically. Pre- and postsynaptically it decreases the release of NA [258].

The cell bodies of the noradrenergic neurons are organised in six clusters, of which the locus coeruleus is best-known. Here, extensive projections to numerous areas originate. The locus

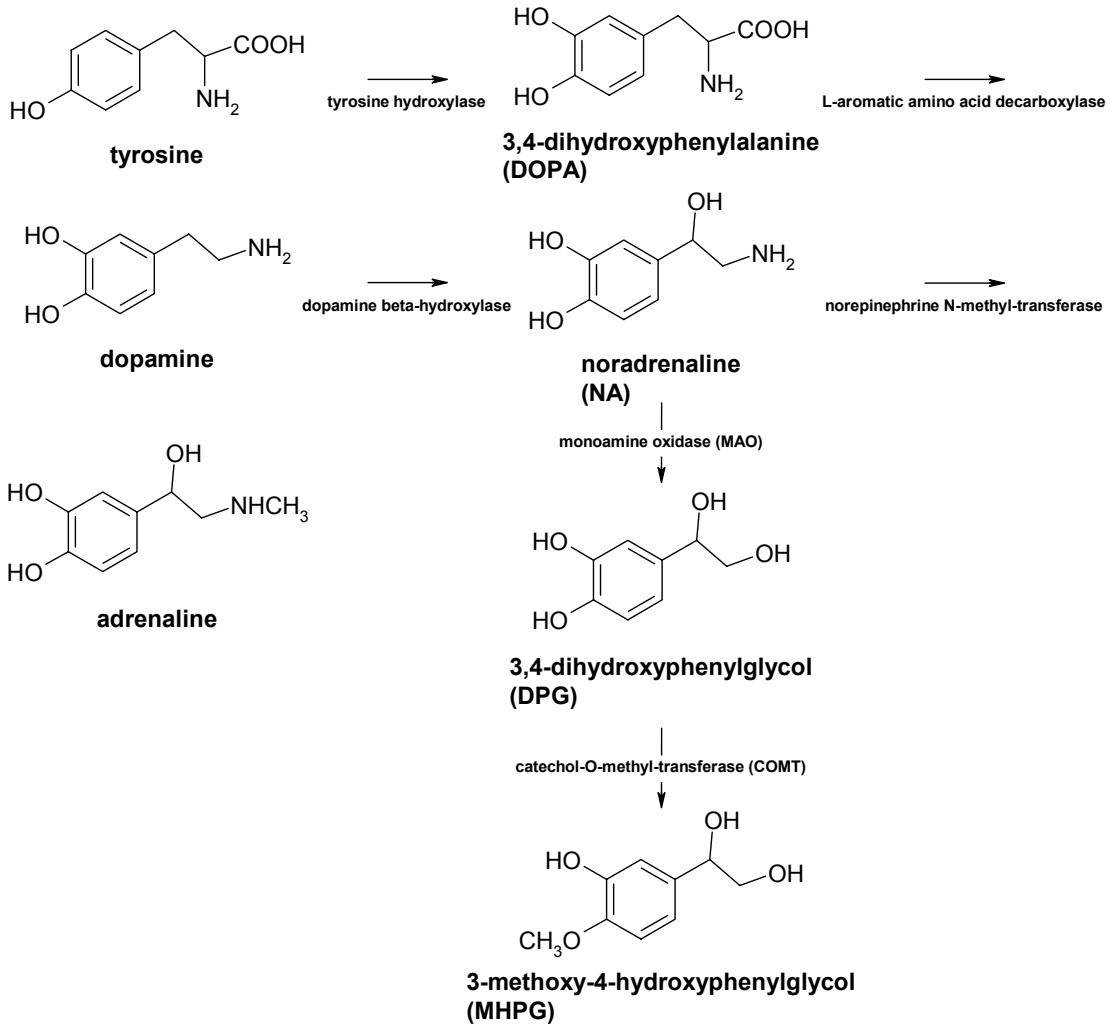



**Figure 9. Synthesis of dopamine and noradrenaline and the metabolism of NA.**

coeruleus projects to, among other structures, the prefrontal cortex, the amygdala, the bed nucleus of the stria terminalis and the hippocampus, all involved in the processing of stress as described before. More than 70% of the noradrenergic forebrain innervation stems from the LC [173]. The LC also receives innervation from various structures, among which a projection from the amygdala that uses CRH to stimulate the LC during stress [326].

### *7.2.2. Role in stress, depression and anxiety*

Exposure to stressful stimuli or novelty increases NA release and turnover in the LC, the hypothalamus, the amygdala and the hippocampus. Interestingly, this effect is stronger with uncontrollable or less predictable stress [153].

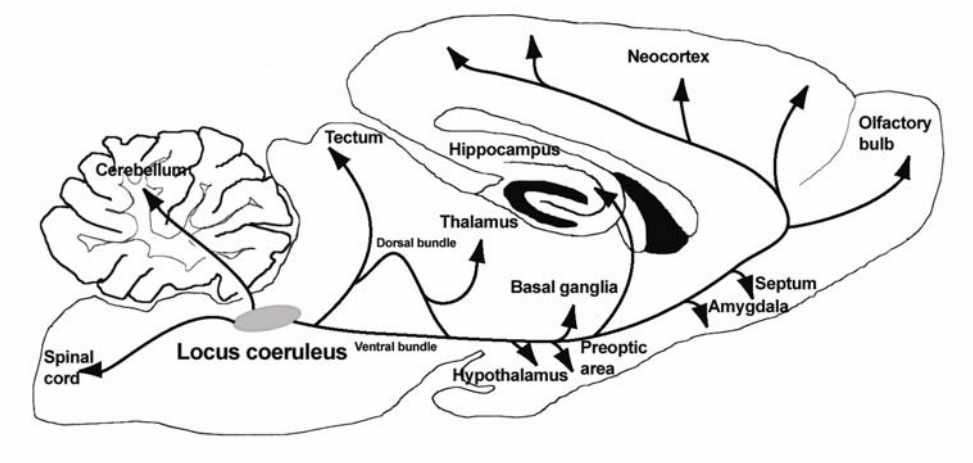



**Figure 10. Noradrenergic pathways in the mouse brain. The pathways originate from the medulla oblongata, pons (localised left beneath the locus coeruleus in the picture) and the locus coeruleus. Adapted from [48].**

One important structure that is noradrenergically innervated is the PVN. NA is a potent stimulator of the release of CRH [153]. For stressors like immobilisation, formalin injections, haemorrhage and insulin injections, the amount of noradrenaline that can be measured in the PVN of rats with microdialysis correlates with mean plasma ACTH levels [232]. During stress levels of CRH in the locus coeruleus are also increased, and when CRH is administered into the LC the response to anxiety is enhanced [153], as are the levels of NA in the cortex. High levels of glucocorticoids do not only decrease the synthesis of CRH, but also the levels of NA in the PVN and additionally decrease the effect of NA on the release of CRH [153].
In the hippocampus, NA elevations switch its function from a memory formation state when it is not behaviourally activated to a state of enhanced stimuli detection when aroused with novelty or stressed with aversion [115]. NA also modulates the amygdala to regulate stress or fear related memory [44].

It is thought that in patients the levels of NA are relatively low under the basal conditions, and that due to this decreased tone the receptors are supersensitive. During stress-induced increases in NA levels the system reacts sensitised.
Indications for this are a decreased neuronal density in the LC in depression [9], increases in the enzyme tyrosine hydroxylase to be able to synthesise more NA, and increases in the presynaptic $\alpha_2$ receptor densities and lower levels of the NA transporter, all regulatory mechanisms to increase the synaptic levels of NA when this is lowered over a longer period of time [155, 228, 229].

All noradrenergic receptors have been implicated in anxiety and depressed states. The prefrontal cortex for example receives a strong noradrenergic input from the locus coeruleus, implied in the responses to anticipatory stressors [137]. Stress-enhanced NA-release affects the function of the prefrontal cortex through $\alpha_1$ receptors and causes impaired performance on memory tasks [25]. These receptors are therefore a potential target to treat poor concentration and performance as seen during excessive anxiety.

The presynaptic $\alpha_2$ receptor antagonist yohimbine can induce panic, whereas the agonist for the same receptor, clonidine, has anxiolytic properties [39, 323].

The β receptors may play a role in the consolidation of traumatic memory, and β receptor antagonists as propranolol may have a role in the treatment of PTSD [45, 256].

Chronic treatment with antidepressants causes a decrease in LC firing, a downregulation of β receptors and a decrease in the activity of tyrosine hydroxylase.

### *7.2.3. Interactions between 5-HT and NA*

It has been suggested that the role of NA is mostly one of behavioural activation, vigilance and fight or flight. In contrast 5-HT seems to promote homeostasis via behavioural inhibition and tolerance to aversive stimuli, and via control of sleep, appetite and sexual behaviours [258]. Obviously, numerous interactions between the serotonergic and noradrenergic system must exist to coordinate these processes.

Serotonergic projections from the dorsal raphe indirectly inhibit firing of the locus coeruleus via 5-$HT_{1A}$ and 5-$HT_2$ receptors, whereas noradrenergic projections from the LC have an inhibitory effect on the median raphe via $\alpha_2$ receptors and both excitatory and inhibitory effects on the dorsal raphe via $\alpha_1$ and $\alpha_2$ receptors respectively. Apart from direct influences of the two neurotransmitter systems through heteroreceptors (see also Figure 11), numerous interactions exist mediated by interneurons, that usually use GABA or glutamate as neurotransmitter [337].

In depression and anxiety the serotonergic system seems to be underactive and the NA system seems to be overactive (see above). All this might cause an overactivation of amygdala, hippocampus and cortical pathways that activate the stress and fear responses and an underactivation of cortical areas that inhibit these responses. Chronic treatment with antidepressants normalises the balance. Again, it is not necessarily true that normalising the balance *per se* heals, it can also be the beginning of, or a part of, a cascade of adaptive processes involving many neurobiological systems.

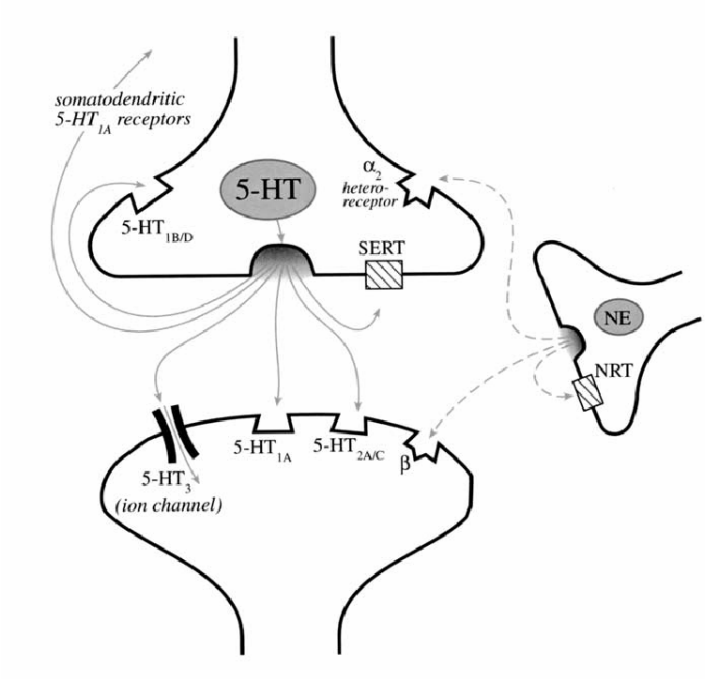



**Figure 11. Targets within the serotonin synaps, showing heteroreceptors and autoreceptors. SERT: serotonin reuptake transporter, NRT: noradrenaline reuptake transporter, heteroreceptors and autoreceptors [153].**

## *7.3. Dopamine*

### *7.3.1. General*

Dopamine is not only an intermediary product of the NA-synthesis (see Figure 9), but as Arvid Carlsson found out around 50 years ago, functions itself as well as a monoaminergic neurotransmitter in the CNS. The effects of dopamine, like noradrenaline, in the synaptic cleft are primarily terminated by reuptake and by metabolism. MAO metabolism of dopamine produces 3,4-dihydroxyphenylacetic acid (DOPAC), that is further metabolised by COMT to homovanillic acid (HVA) (see Figure 12).

Two families of dopamine receptors have been described, consisting of five receptor types in total. $D_1$ and $D_5$ receptors are related, as are $D_2$, $D_3$ and $D_4$ receptors. $D_1$ receptors can be found in the striatum, the substantia nigra and the cortex. These receptors have stimulating properties. $D_2$ receptors are located in the striatum, the cortex and the pituitary. The $D_2$ receptor also exists presynaptically and on the cell soma. Contrary to $D_1$ receptors they are inhibitory.

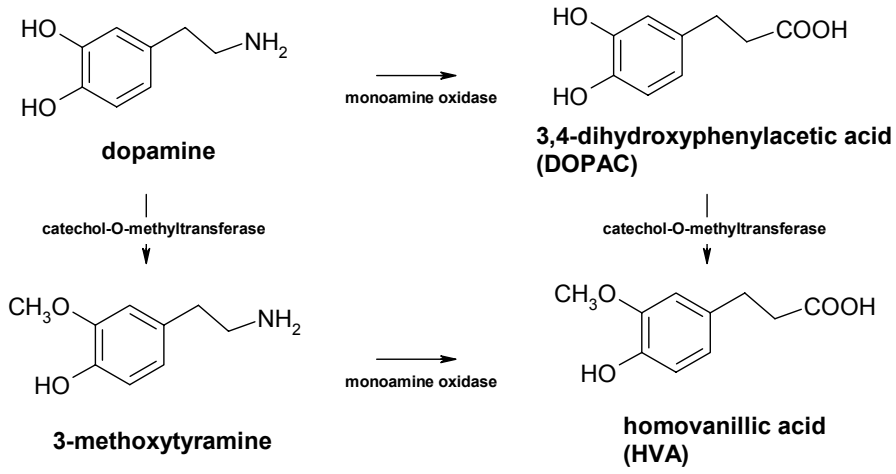



**Figure 12. Metabolism of dopamine.**

There are three dopaminergic tracts. Dopaminergic neurons that originate in the substantia nigra project to the striatum (caudate nucleus and putamen) and are involved in the initiation and coordination of movement (see Figure 13). This is the tract that degenerates in Parkinson's disease [149].

Then there are neurons from the periventricular area of the hypothalamus projecting to the

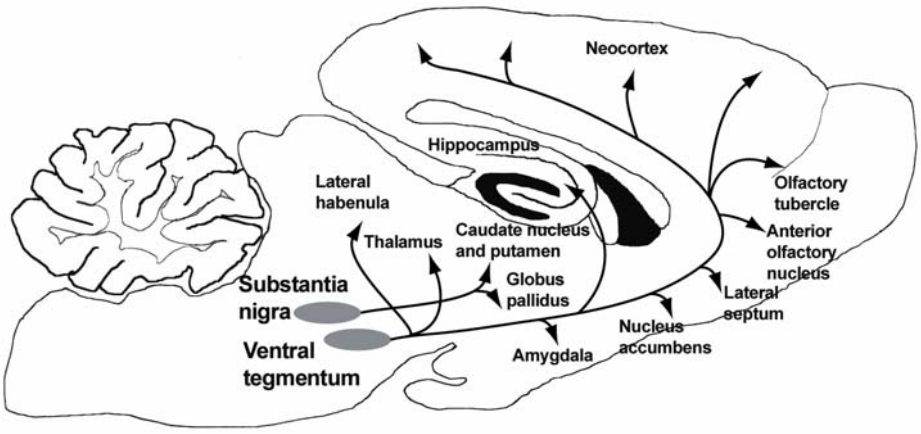



**Figure 13. Dopaminergic pathways. Adapted from [48].**

infundibulum and anterior pituitary, where DA inhibits the release of prolactin. A third dopaminergic pathway originates in the ventral tegmental area and projects to the limbic system and neocortex, and has a role in emotional expression [149]. One of the targets of dopaminergic neurons from the VTA is the nucleus accumbens (NAc). This structure and dopamine play a critical role in reward and are mostly studied in the context of drugs of

abuse. However, some reports hint that the VTA-NAc pathway is also associated with depression [224].

### *7.3.2. Role in stress and depression*

The PVN is innervated by neurons from the zona incerta, that are mainly dopaminergic [56]. Dopamine is known to activate the HPA axis [38], but it is unknown whether this happens directly or via interneurons. Also dopaminergic input to the prefrontal cortex is stress-sensitive and may affect the output of the HPA axis [137].

Acute stress, like restraint or footshock, increased the release of dopamine in the mesolimbic system [254]. A reduction in dopamine transporter binding sites was found in the caudate nucleus and putamen of stressed treeshrews [2]. Also in the learned helplessness model, significant alterations in $D_1$ and $D_2$ receptors were found [160].

Although dopamine is mainly implicated in Parkinson's disease (hypoactivity of the dopaminergic system) and schizophrenia (hyperactivity), dopaminergic hypoactivity has also been implicated in depression [212]. One indication is the higher incidence of depression in patients with Parkinson's disease. It also has been found for example that dopaminergic metabolites are decreased in some depressed patients, and it has been shown that l-DOPA, an antiparkinsonian drug, can be useful in the treatment of retarded depression [149]. Amphetamine and methylphenidate, dopamine reuptake inhibitors, cause euphoria in healthy individuals and depressed patients [181, 227] an effect that is blocked by dopamine antagonists. $D_2/D_3$ receptor agonists decrease the immobility time in the Porsolt swim test [36]. In clinical situations the dopaminergic system is manipulated by using for example sertraline, one of the SSRIs, which does not only inhibit the serotonin transporter, but also that of dopamine [224]. When antidepressant monotherapy is unsuccessful, sometimes a dopamine agonist (like methylphenidate) is added to potentiate the effect of the antidepressant.

## 8. AIMS

On the previous pages it has become clear that in the field of the depression and anxiety research the search for suitable behavioural models continues and that predator exposure has interesting features that make it a promising behavioural model. On an anatomical level brain areas have been mentioned that play an important role in emotional and behavioural responses and finally, on a neurochemical level, the major neurotransmitters have been

named, that all seem to play a role in the etiology of depression. All these levels were combined in experiments measuring various neurotransmitters and metabolites with microdialysis in various brain regions of freely moving mice, exposed to a rat exposure paradigm.

Prior experiments indicated that half an hour of rat exposure caused freezing behaviour and increases in free corticosterone, as well as in extracellular levels of 5-HT and 5-HIAA in the hippocampus of B6C3F1 mice [174]. It was decided to dedicate more time to a project concerning rat exposure, to learn more about the neurochemical aspects of this relatively new paradigm, evolving in the project at hand.

This time a more current strain was chosen as subjects, C57bl/6N mice. The first question that arose was how levels of 5-HT and 5-HIAA varied over the phases of rat exposure, i.e. before, during and after the paradigm. The analysis method was optimised to enable the measurement of samples that were collected during intervals as short as 5 minutes. In addition, behaviour of the mice was carefully observed. The idea was that this might enable a correlation of higher or lower neurotransmitter levels to certain behaviours that might indicate either anxiety or coping behaviour. Also it was thought that behavioural analysis might make it possible to pinpoint behaviours that could be focussed on as a novel model for stress or for depression/anxiety research. As a final step it was planned to test the effect of antidepressants, to see how behaviour and levels of 5-HT and 5-HIAA would be different under predator exposure. As the project progressed however, aims needed to be adapted. Gradually the following questions were formulated, that this thesis aims to answer.

- How do C57bl/6N mice behave before, during and after rat exposure?
- Which changes in behaviour during rat exposure indicate anxiety or coping?
- When animals are subjected to rat exposure again, does their behaviour differ from the first experience?
- How do levels of 5-HT and 5-HIAA change over time under a predator exposure paradigm in C57bl/6N mice in brain areas that are primarily involved in emotion, such as the hippocampus, prefrontal cortex and lateral septum (LS)?
- How do levels of 5-HT and 5-HIAA change in the caudate putamen, a region that was chosen as a control structure, because it is not primarily associated with emotion but with motor activity?
- Can levels of neurotransmission be related to specific behaviours?
- Is there an effect of the repetition of rat exposure on the levels of neurochemicals?

- The same applied to the measurement of NA, its metabolite MHPG and the metabolites of dopamine DOPAC and HVA in the hippocampus of C57bl/6N.

When it was found that the neuroendocrine effects of rat exposure on C57bl/6N mice differed from those on B6C3F1 mice the following questions became interesting:

- Do various strains of mice respond to predator exposure with differences in plasma levels of ACTH and corticosterone (CORT)?
- Do various strains of mice respond with a different behavioural sequelae to predator exposure?
- Do levels of corticosterone correlate with behaviour?
- Do CRH-Tg (CRH-transgenic) mice, genetically engineered to have more anxious traits, respond differently to rat exposure than the wild type mice?

As it was found that the profile of responses in Balb/c mice differed from that in C57bl/6N mice, microdialysis studies were performed in Balb/c mice as well, aimed to answer the following questions:

- Are differences in behaviour between C57bl/6N and Balb/c mice reflected in differences in extracellular levels of 5-HT, 5-HIAA, NA, MHPG, DOPAC and HVA in the hippocampus?
- Are behaviour and levels of neurochemicals differentially affected by re-exposure in these two strains?

Finally, brain structures known to be involved in HPA axis regulation were studied. Levels of 5-HT, 5-HIAA, MHPG, DOPAC and HVA were measured in the paraventricular nucleus and anterior hypothalamus (AHP) of Balb/c mice (NA remained below the detection limit). Questions related to this were:

- What are the differences between neurotransmitter and metabolite levels in the AHP, PVN and the hippocampus?
- What indications of stress can be conferred from these levels?

# II. MATERIALS AND METHODS

The experiments that were conducted in this study can roughly be divided into two groups: studies involving microdialysis and studies that involved the measurement of the plasma levels of the stress hormones ACTH and corticosterone. As the setup and methods for these experiments were different, the setups will be described separately.

## 1. EXPERIMENTS USING MICRODIALYSIS

The technique of microdialysis is introduced, followed by the specifications of the used setup. Then a general time frame of the experiments is given, followed by details about each step in this time frame.

### *1.1. Microdialysis - Theoretical background*

Microdialysis is a technique that enables the monitoring of the levels of neurotransmitters and other compounds in the extracellular space of specified brain regions. The levels that can be measured mirror the total of release, reuptake and metabolism of the substance of interest during a certain time period.

Microdialysis is a very refined technique that has not only the advantage that it can be performed in alive, freely moving animals, but also that it enables to get insight in the temporal course of neurotransmission [339]. This provides major advantages over for example brain punctures, where brain tissue is homogenised *post mortem* and analysed for

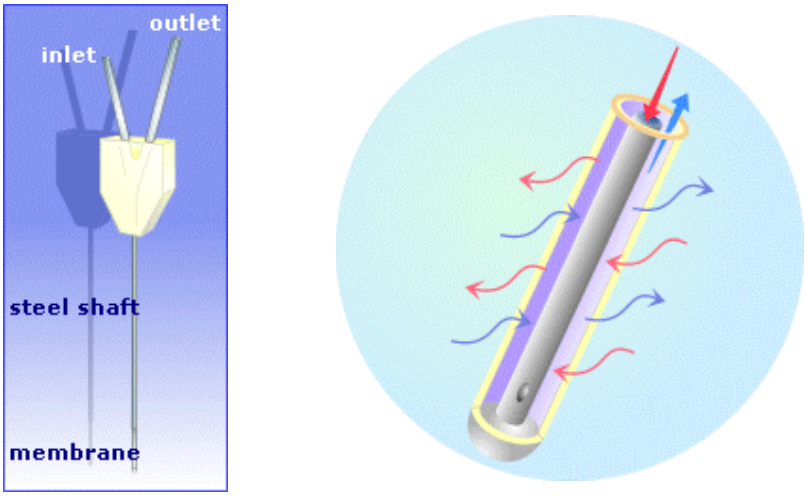



**Figure 14. Schematic representation of the microdialysis cannula and its tip, showing the inlet, outlet and liquid flow through the tip of the cannula. Taken from the website of CMA/microdialysis.**

the contents of neurotransmitters. Microdialysis is also more sensitive than voltammetry, the one other *in vivo* technique used to monitor the chemistry in the brain.

In microdialysis a cannula is implanted in the brain area of interest. The cannula has a tip, consisting of a semi permeable membrane of various lengths (for mice typically 1, 2 or 3 mm) (see Figure 14).

An inlet and outlet to the cannula make liquid flow possible through the cannula. When the liquid flows along the membrane, due to diffusion, an equilibrium will be approximated for molecules in the extracellular brain tissue surrounding the probe and the liquid flowing through the probe. As the membrane used in these studies has a cut off value of 6000 Da (i.e. the molecular weight at which 80% of the molecules are prevented from passing the membrane is 6000 Da), molecules like serotonin, noradrenaline and their metabolites can flow through the membrane, but proteins can not.

The recovery rate, i.e. the amount of neurotransmitter in the surrounding medium that can be detected in the dialysate, depends on the flow rate of the liquid through the probe (usually 2.0 µl/min), on the membrane length and diameter (the surface area over which exchange takes place), the weight, shape and charge of the molecule of interest and the temperature. Before endogenous substances are measured, the recovery of these should be established, to make sure that microdialysis is a suitable technique. Because a larger membrane can be chosen for larger brain regions, the recovery can easily be increased.

### *1.2. Microdialysis set-up*

In the setup that was used, the inlet of the microdialysis probe (CMA/11, CMA/Microdialysis AB, Stockholm, Sweden, cuprophane membrane) was attached to a dual channel low-torque liquid swivel (Instech Laboratories, Plymouth, PA, USA) with a 30-cm piece of fluorethylenepolymere tubing (Microbiotech, Stockholm, Sweden; dead volume of 1.2 µl/100 mm length), and from there to a microinfusion pump (TSE Technical and Scientific Equipment GmbH, Bad Homburg, Germany) with tubing of 50 cm long. Syringes with a volume of 2.5 ml, filled with sterile and pyrogen free Ringer (Delta Pharma, Pfullingen, Germany; composition 147 mM NaCl, 4 mM KCl, 2.25 mM $CaCl_2$), were placed in the pump and served as the source of liquid, perfusing the system at 2.0 µl/min. The outlet side of the probe was also connected to the swivel with 30 cm of tubing, and from there to an automated refrigerated fraction collector (Microsampler 820, Univentor, Malta), with 100 cm of tubing.

The swivel is a helpful tool to avoid that the inlet and outlet tubing to and from the microdialysis probe get tangled up. The swivel hanged from the side of the cage top on a counterbalancing arm, so that it did not pull or push on the head of the mouse once the microdialysis cannula was implanted. Use of a swivel did not interfere with the animals motions and was not stressful for them.

Dialysate was collected in cooled vials in the autosampler. The autosampler rotated an empty vial under the outlet after a programmable amount of time, so that the series of vials made it possible to follow neurotransmitter levels over time.

### *1.3. Overview of the experiments*

The general timeframe of the microdialysis experiments can be found in Table 2.

**Table 2. General timeframe of microdialysis experiments.**

| Time in days | Action |
|---|---|
| - 7 or earlier | • Arrival of animals, individually housed in standard cages |
| 0 | • Operation: implantation of guide cannula, housed in rat exposure cages |
| 8 | • Implantation of microdialysis cannula, start of perfusion |
| 10 | • Experiment: rat exposure |
| 11 | • Experiment: re exposure and killing |
| later | • Histological verification of probe location<br>• Analysis of microdialysate using HPLC with electrochemical detection<br>• Behavioural analysis<br>• Statistical analysis |

The following sets of experiments were conducted, all using the rat exposure and re-exposure paradigm as described above:

- Measurement of 5-HT and 5-HIAA in the hippocampus, prefrontal cortex, lateral septum and caudate putamen of C57bl/6N mice.
- Measurement of NA, MHPG, DOPAC, HVA in the hippocampus of C57bl/6N mice.
- Measurement of 5-HT, 5-HIAA and of NA, MHPG, DOPAC, HVA in the hippocampus of Balb/c mice.
- Measurement of 5-HT, 5-HIAA, MHPG, DOPAC and HVA in the anterior hypothalamus and the paraventricular nucleus of Balb/c mice.

Apart from these experiments, related or slightly different experiments were conducted, using the same time schedule or techniques. These were:

- In-vitro experiment

  To determine the recovery for the various compounds that were measured, an *in vitro* experiment was carried out. Two 1-mm membrane length and two 3-mm membrane length microdialysis probes were hung in Eppendorf vials filled with Ringer with known concentrations of 5-HT (5 fmol/15 µl), 5-HIAA (7000 fmol/15 µl), NA (5 fmol/15 µl), DOPAC (1500 fmol/15 µl, MHPG (500 fmol/15 µl) and HVA (2500 fmol/15 µl), kept at 37 °C with a water bath. Per probe four 15-min fractions of dialysate were collected and stored at -80 °C until analysis. In addition, before and after the in-vitro experiment, samples were taken directly from the Eppendorf vials. The samples were analysed later using HPLC with electrochemical detection. Recovery was calculated per compound as the average amount present in 15 µl of the dialysates, expressed as a percentage of the amount in 15 µl of the solutions taken directly from the vials.

- Free corticosterone measurements

  Using the time frame described in Table 2 the dialysate of some C57bl/6N mice was used to determine the content of free corticosterone, rather than of neurotransmitter levels. With 14 animals, all samples obtained during the experiments were analysed for their free corticosterone content to get an indication of the time course of this.

The separate steps of the general time schedule, as presented in Table 2 will be described in the following paragraphs.

### *1.3.1. Animals and arrival*

Adult male, experimentally naïve C57bl/6N and Balb/c mice were bought at Charles River (Charles River WIGA GmbH, Sulzfeld, Germany) at the age of 12-16 weeks. Upon arrival they were housed individually in standard Macrolon II cages with food and water available *ad libitum*. Animals were kept in a temperature (22 ± 1 ºC) and humidity (60 ± 5%) controlled room, with a 12 hr light-dark cycle (lights on at 6.00 a.m.).

Animals were allowed to adapt to their surroundings at least 7 days before any action took place.

As predator, adult male Wistar rats were used, that weighed 200-220 g upon arrival. Rats were housed four to a cage, with food and water available *ad libitum*. Rats were kept strictly

separated from the mice, and were not used as predator until a week after their arrival. They were used a maximum of three times, each a week apart.

The experimental protocols were approved by the 'Ethical Committee on Animal Care and Use' of the Government of Bavaria, Germany and all efforts were made to keep their number and suffering limited to the minimum.

### *1.3.2. Operation*

Animals were transported to the operation room, where they were anaesthetised in a bowl with isoflurane (CuraMED Pharma GmbH, Karlsruhe, Germany). During this narcosis, they were positioned and immobilised in a stereotactic frame (Stoelting Co., Wood Dale, IL, USA), equipped with rat ear bars, a teeth bar, nose rod and a mouse inhalation anaesthesia mask (designed in the institute), through which narcosis was continued with isoflurane.

The fur on the skull was removed with a clipper, after which the skin on the skull was cut open. Using the stereotactic instrument, the coordinates of bregma were determined, and the location where the guide cannula (length 14 mm, outside diameter 0.7 mm, inside diameter 0.4 mm, see also paragraph 1.3.12 ) should be implanted was measured off.

The area around the implantation site was roughened up with a dental drill with saw blade. Then, using a drill with conic head, a hole was made in the skull at the precise location of implantation.

The site of implantation was determined based on the mouse brain atlas [243] and on a series of dummy operations. In the following table, the used coordinates are listed for the structures that were dialysed.

**Table 3: Implantation coordinates for the different dialysed brain regions.**

| Brain region | Lateral (mm from bregma) | Posterior (mm from bregma) | Ventral (mm from bregma) |
|---|---|---|---|
| Hippocampus | - 3.2 | - 2.9 | -1.7 |
| Prefrontal cortex | - 0.3 | + 2.3 | -1.0 |
| Lateral septum | - 0.4 | + 1.2 | -2.2 |
| Caudate putamen | + 1.6 | + 0.8 | -2.2 |
| Anterior hypothalamus | - 0.3 | - 0.7 | -4.2 |
| Paraventricular nucleus | - 0.1 | - 0.7 | -3.7 |

The guide cannula, attached in the stereotactic instrument, was lowered very slowly through the hole in the skull. After reaching the required depth, the cannula was fixed on the skull using cyanacrylat glue and dental cement (Paladur, Heraeus Kulzer, Wehrheim, Germany). Not until the cement was hardened out, the guide cannula was released from the stereotactic instrument. Subsequently, a small metal peg with a tiny hole in the middle was fixed on the

skull using the same fixatives. This peg served to attach a metal wire (a tether) to during probe implantation, to connect the animal to a swivel and balancing arm.

The skin was sowed together and a small cap of silicon tubing, closed off at one side with silicon glue, was pushed onto the guide cannula, to prevent dirt to settle in the cannula.

Animals were weighed and returned to their room, where they were placed in the rat exposure cages, again with water and food available *ad libitum*.

The rat exposure cages were made of Plexiglas and consisted of a compartment of 15x25x30 cm (wxdxh), separated from a compartment of 10x25x30 cm by a Plexiglas separation wall. The mice resided in the larger part of the cage, whereas rats were placed in the smaller compartment during rat exposure. The separation wall was equipped with two rows of air holes (6 mm in diameter, 2 x 6 holes, 6 and 3.5 cm above the bottom of the wall respectively), so that mice and rats could see, smell and hear each other during rat exposure.

**Figure 15: A C57bl/6N mouse in rat exposure cage. The mouse has been implanted with a microdialysis cannula.**

### *1.3.3. Implantation of microdialysis cannula*

A day before the implantation, the microdialysis systems (swivel and connecting tubing) were flushed with Virkon S (Arovet, Zollikon, Switzerland), a disinfecting agent, and rinsed overnight with sterile water (both at a rate of 2.0 µl/min).

Implantation of the cannula occurred in the "home" room of the animals, on the eighth day after operation. Prior to the procedure, animals were weighed, and a counterbalancing arm with a swivel was mounted on the cage. The inlet side of the tubing was flushed with 100% ethanol during 30 minutes at 2.0 µl/min. The inlet of the microdialysis cannula was then attached to the tubing, and the membrane was hung in a sterile Eppendorf vial, filled with 100% ethanol. The inlet side of the tubing and the cannula were then perfused with sterile, pyrogen-free Ringer during 10 minutes at 2.0 µl/min. The ethanol served to remove glycerol from the probes. Consequently, the vial with 100% ethanol was exchanged with one containing sterile Ringer, and the probes, now hanging in Ringer, were perfused for an additional 10 min. Finally, the outlet of the probe was attached to the tubing, and perfusion continued for the duration of the experiment. Extreme care was taken not to introduce air into the system – any air bubbles visible with a magnifying glass in the probe were removed before the actual implantation took place.

During the implantation, animals were again briefly sedated using a bowl in which air containing isoflurane was guided, and then immobilised using earplugs, teeth- and nose bar, equipped with inhalation mask.

The silicon cap on the guide cannula was removed, and the microdialysis probe was slowly and carefully lowered into the guide cannula. When the probe was into place, it was fixed using a layer of cyanacrylat glue, dental cement, and glue again.

Probes had different membrane lengths, depending on region to be dialysed. For the hippocampus, prefrontal cortex and caudate putamen, a membrane length of 3 mm was used, for the lateral septum one of 2 mm, and for the anterior hypothalamic area and paraventricular nucleus a membrane of 1 mm. With the probe correctly located, the membrane protruded from under the guide cannula into the tissue.

A metal wire, attached to the swivel, was attached to the metal peg on the mouse's skull, using a tiny metal cylinder. The animal was then placed into its cage, now connected to the microdialysis system. Every morning and evening, the syringes containing Ringer were filled, so that the probe was continuously perfused at a rate of 2.0 µl/min.

### *1.3.4. Predator exposure and killing*

Rat exposure experiments were conducted on the second day after insertion of the microdialysis probe (i.e. the $10^{th}$ day after surgery). In the case that the mice were re-exposed to a rat, this happened on the following day, using the same time schedule. Between 8.30 and

**Figure 16. Rat exposure. In this situation no microdialysis was performed.**

10.30 hr baseline microdialysis samples of 15 min were collected. At 10.30 hr rats were brought into the experimental room and placed in the smaller compartment of the mouse home cage (see Figure 16). At 11.00 hr the rats were removed and brought back to their facilities. During the half hour of rat exposure and the consecutive 15 minutes, microdialysis samples were collected in 5-min intervals. Then, until 14.00 hr, samples were again collected every 15 minutes. To the 5-min samples 20 µl Ringer was added, so that the end volume in these vials was the same as the one in those with the 15 min samples. The samples were collected in cooled vials in an autosampler. Vials with dialysate were taken out of the autosampler at least once an hour and stored on dry ice until the end of the experimental day, when they were transferred to the – 80 °C freezer, were they were stored until analysis. Freezing did not affect the levels of the compounds to be measured.

On the days of experiments the animals were also videotaped from 8.30 hr till 14.00 hr using security cameras (black/white CCD cameras, Conrad, Munich, Germany). Using a quad-unit, four images of cages could be recorded on one tape simultaneously.

At the end of the second experimental day, mice were killed using an overdose of pentobarbital. Microdialysis probes were carefully removed and checked for integrity of the membrane and the absence of air bubbles. Brains were collected in 4% formaldehyde, and a global section was done to check for any anatomical or pathological anomalies of the subjects. Microdialysis systems were flushed with sterile water overnight.

### *1.3.5. Histological verification*

After the brain tissue was fixed with formaldehyde during at least 24 hours, brains were cut in 25 µm slices on a microtome at a temperature of about –20 °C. Every 3$^{rd}$ slice was

collected on a gelatinised slide, using the free floating method. In case of hippocampal dialysis, brains were cut horizontally, in all other cases transversally.

To prevent the two hemispheres to fall apart in the transversal cuts, brains were embedded in a medium (see paragraph 1.3.12) before freezing them onto the microtome.

Brain sections were coloured using cresyl violet (or Nissl-staining, see paragraph 1.3.12 for procedure). Under a microscope was examined where the guide cannula and probe location had been. Only the data obtained with microdialysis probes in the correct location were used.

### *1.3.6. Video analysis*

The video recordings enabled a detailed behavioural analysis. The behaviour of the mice was scored using an instantaneous scoring method, meaning that every 30 sec (pre- and post-rat exposure) or every 10 sec (during rat exposure and the following 5 min) it was recorded what the mouse was doing at that time point. Data were stored in Microsoft Excel spreadsheets. Table 4 lists the behaviours that were scored.

**Table 4. Description of the behaviours that were scored from video. Using a decision flow diagram, behaviours were assessed and scores could be given in the presented order. Only one score was given per time point. In this way, an animal that was rearing but making small head movements as well, received only the score 'rearing', not 'sniffing air'. * In case of freezing ('frozen' posture, with no movement but breathing, tensed tail, and head slightly protruding), which rarely happened, a note was made, but no separate score was given. ** Animals that were rearing whilst engaged in food-related behaviour were not given the separate score 'rearing' as this position was seen as necessary to reach the food.**

| Scored behaviour | Description |
|---|---|
| Inactive | Lying or sitting quietly* |
| Food-related behaviour | Drinking from bottle, eating from food-trough or in other parts of cage, moving food-pellets in trough |
| Rearing | Full vertical extension of hind limbs and body ** |
| Sniffing wall | Nose in one of the holes of the separation wall, or very close to the surface of the separation wall |
| Stretching, retracting | Full extension of the body, with arched back, or the opposite movement |
| Nesting, digging | Gathering bedding with front paws or moving bedding with head movements respectively |
| Sniffing bedding | Nose in or close above bedding, but without moving it around with nose. Especially during rat exposure, bedding close to head was simultaneously pushed backwards with paws. |
| Walking | Locomotion not including 'sniff air' |
| Grooming, scratching | Movement of head or paws over parts of the body, also included licking |
| Sniffing air | Small vertical movements of the head or nose, whilst sitting or lying |

Also the rat behaviour was scored, albeit that due to spatial limitations in the cage, the number of possible behaviours for the rat was less extensive. Scores were given every 10th second for: inactivity, rearing, grooming, sniffing separation wall, bedding, or air.

### *1.3.7. Analysis of dialysate – HPLC with electrochemical detection*

HPLC is the abbreviation of High Performance (or Pressure) Liquid Chromatography, and is a separation method as well as the name of the apparatus used for this separation. A liquid mixture of compounds (as for example neurotransmitters in Ringer) is injected onto a column. In reversed phase chromatography, this column is tightly packed with silica particles, to which aliphatic chains are attached. This makes up the stationary phase of the system. A pump presses a liquid hydrophilic, mobile phase through this column, which also carries the sample. Because the various compounds in the sample differ in hydrophobicity, and thus in affinity for the stationary and mobile phase, the time a compound spends on the column differs (and will be equal to the time it takes for the mobile phase to traverse the column for compounds that have no affinity at all for the stationary phase, or longer for those that can form hydrophobic interactions to the column material as well). Thus the compounds elute from the column with different retention times. When a detector is connected to the outlet of the column, the compounds can be identified.

An electrochemical detector exists of a cell with two electrodes with a specified voltage between them. When compounds flow through this cell and are oxidised, they give rise to an electrical current. These currents are represented as peaks in a chromatogram. The intensity of the current is a measure for the amount of oxidised compound, whereas the time it takes for the peak to appear after injection of the sample makes identification possible. Qualification and quantification of the peaks usually follows relative to a calibration curve, generated by the measurements reference standards with defined quantities of the compounds under investigation.

The chromatographical parameters such as retention time and peak height can be influenced by changing the speed of the mobile phase, the composition of the mobile phase (lipophility, pH etc.), the temperature of the column, the type of column used (packing material, size) and the detection potential.

Various HPLCs were used to analyse the samples in these experiments. They either consisted of a Sunflow 100 isocratic pump (Sunchrom, Friedrichsdorf, Germany), a Mistral column thermostat (Spark Holland Instruments, Emmen, The Netherlands), a Rheodyne 7125 injection valve (Rheodyne, Rhonert Park, CA, USA), and an Antec Electrochemical Detector (Antec Leyden, Zoeterwoude, The Neterlands) or of a Gynkotec M480 high-precision Pump (Gynkotec, Germering, Germany), a Rheodyne 7725 injection valve, and an electrochemical detector in combination with an oven (Decade, Antec Leyden). The detectors used an

Ag/AgCl reference electrode. The chromatography data system was Gynkosoft from Gynkotec. Columns (see Table 5) came from YMC (Schermbeck, Germany). Compounds were quantified relative to a standardcurve of four reference standards by measuring the height of the peaks. Other details are listed in Table 5.

**Table 5. Details to the HPLC parameters. SOS: sodium 1-octane sulfonate. EDTA: ethylenediaminetetra acetate.**

| | 5-HT and 5-HIAA | NA, MHPG, DOPAC, HVA |
|---|---|---|
| **Eluent** | 22% methanol<br>50 mM $NaH_2PO_4$<br>40 mg/l EDTA<br>0.29 mM SOS | 10% methanol<br>45 mM $NaH_2PO_4$<br>40 mg/l EDTA<br>0.40 mM SOS, pH 4.55 |
| **Alternative eluent** | 20% methanol<br>13.2 mM citric acid<br>40 mM sodium acetate<br>40 mg/l EDTA<br>0.15 mM SOS, pH 4.9 | 2% methanol<br>40 mM citric acid<br>60 mM NaOH<br>100 mM sodium acetate<br>40 mg/l EDTA<br>0.22 mM SOS |
| **Alternative eluent** | | 6.2% methanol<br>3.8 mM citric acid<br>16.5 mM sodium acetate<br>40 mg/l EDTA<br>0.21 mM SOS. pH 5.00 |
| **Flow rate of mobile phase** | 400 or 500 µl/min | 400 µl/min |
| **Oven temperature** | 30 °C | 40 °C |
| **Detector voltage** | 550 or 600 mV | 650 mV |
| **Column** | Pro C18, 150 x 3.0 mm | Pro C18 or Hydrosphere C18, 150 x 3.0 mm |

Two representative chromatograms are shown in the following figures.

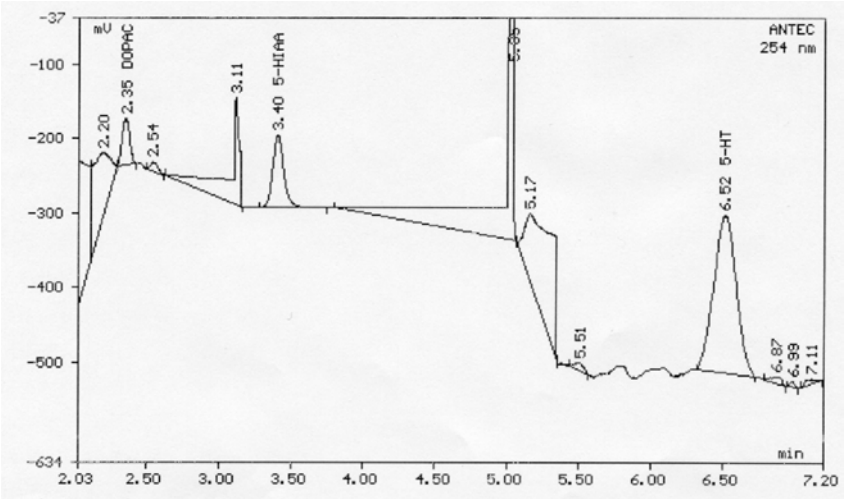



**Figure 17. Example of a chromatogram from the 5-HT analysis of a hippocampal sample from a Balb/c mouse. The y-axis shows the cell potential in mV, the x-axis time in min. 5-HIAA eluted with a retention time of 3.40 min, 5-HT had a retention time of 6.52 min.**

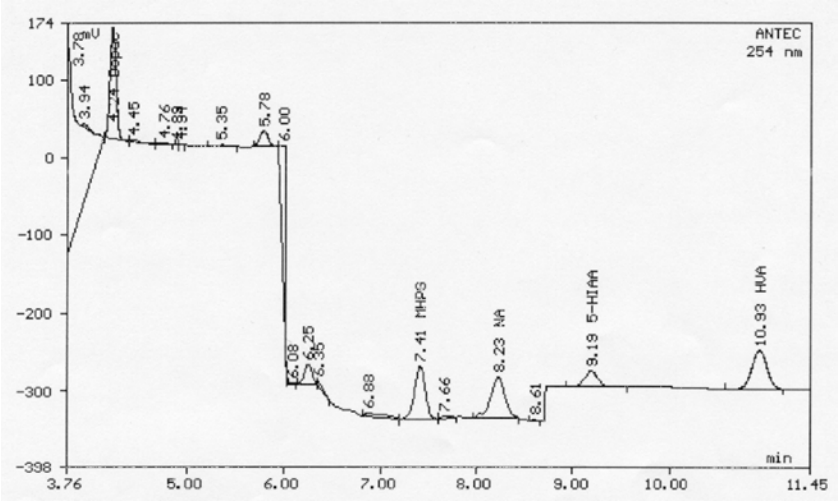



**Figure 18. Example of a chromatogram from the NA-analysis of a hippocampal sample of a C57bl/6 mouse. The y-axis indicates the cell potential in mV, the x-axis time in min. DOPAC had a retention time of 4.24 min, MHPG of 7.41 min, NA of 8.23 min, HVA of 10.93 min.**

Detection limits in the 5-HT analysis were: 0.4 – 0.8 fmol for 5-HT and 4.5 fmol for 5-HIAA per injection. In the NA-analysis the following detection limits were achieved: 1 – 2 fmol for NA, 6.5 fmol for MHPG, 6.5 fmol for DOPAC and 4.5 fmol for HVA per injection. All detection limits had a signal to noise ratio of 3.

### *1.3.8. Measurement of free corticosterone – radioimmunoassay (RIA)*

In a RIA, the sample containing the compound to measure, e.g. corticosterone or ACTH, is mixed with a known amount of that same compound that is radioactively labelled with $^{125}$I. A fixed amount of antibody against corticosterone or ACTH is added, and the non-labelled unknown amount of corticosterone or ACTH and the labelled form compete for binding to the antibody. After a fixed time period, a second antibody against the first antibody is added, that makes the complex precipitate. After centrifugation a pellet is formed, and the supernatant can be removed. The amount of radioactivity in the pellet is counted with a γ-counter. The more activity is present, the more labelled corticosterone (or ACTH) could bind to the primary antibody, which means that less of the compound was present in the sample. A standard curve makes it possible to calculate the amount that was present in the original samples.

As corticosterone passes the blood brain barrier, and evenly distributes over the extracellular fluid, it can also be detected in dialysate. To measure free corticosterone in the dialysate, a corticosterone RIA kit (MP Biomedicals (before: ICN Biochemicals), Costa Mesa, CA, USA) was used according to the instructions of the manufacturer. Samples however were not

diluted, and an extra standard curve was generated to reliably measure the low amounts of corticosterone in the dialysate.

The detection limit of the assay was 0.001 µg/100 ml dialysate.

### *1.3.9. Statistical analysis of behavioural data*

The behavioural scores were counted per 15-min (pre and post rat exposure) or per 5-min interval (during rat exposure and the 15-min following it) and counts for specific behaviours were expressed as percentage of total counts in that period. Similarly, all active behaviours (i.e. all scores except the score 'inactive') were collapsed under the term 'activity'. Thus, the time intervals, 28 per experimental day, of the behavioural scores matched those of the microdialysis, yielding information about the behavioural changes over time and enabling comparisons between behaviours and microdialysis data.

To reveal effects of 'time' and 'strain' in the specific behaviours or in 'activity', data were analysed using Analysis of Variance (ANOVA) with repeated measurements design. Behavioural data were arcsine transformed to approach the criteria of homogeneity and normality prior to ANOVA. Data were then summarised to six levels of 'time' as within-subject factor, to avoid type 1 errors due to too many levels. These levels were as follows: one level pre-exposure, consisting of the time periods corresponding with the eight basal microdialysis samples; two levels during rat exposure, both consisting of three consecutive samples; and three levels post-exposure, with the behaviour during three 5-min samples directly after rat-exposure as one level, and of the consecutive five, and last six 15-min samples as the other two levels. An additional between-subject factor of 'strain' (with two levels: C57bl/6N and Balb/c) was added.

When a significant effect of 'time' was found, post-hoc simple contrasts compared the different time levels to the pre-exposure values. Also the values during the second half of rat exposure were compared with those in the first half. The results of the post-hoc tests were Bonferroni corrected for multiple comparisons.

In case of a significant effect of 'strain' or of the interaction 'time by strain', the ANOVA with 6 levels of time was repeated within each strain, as were the post-hoc contrasts when an effect of 'time' was still present. Then, the data for C57bl/6N and Balb/c mice were compared per level of time as well, using post-hoc simple contrasts. Also these results were Bonferroni corrected.

Behavioural scores were also analysed by expressing the counts for specific behaviours before, during and after rat exposure as percentage of the total number of *activity* counts in

those periods (rather than as a percentage of *total* counts in those periods). Because inactivity was not, and time information was only limited taken into account in this analysis, this made clear how the animals behaved during their active periods, enabling comparisons in behavioural profile during activity periods before, during and after rat exposure. These comparisons were again done using ANOVA with repeated measures design, this time with four levels of 'time' (one level pre exposure, one level during exposure, and two levels post exposure, of which the first existed of scores obtained in the first 15 min following the rat exposure), and again two levels of 'strain'. When a significant effect of 'time' was found, simple contrasts within ANOVA, Bonferroni corrected, compared all time levels to the 'during rat exposure' level. In case of significance of 'strain' or of the interaction, this procedure was repeated within the strains, after which also the strains were compared per time level, using post-hoc simple contrasts. These results were Bonferroni corrected.

Similarly, an ANOVA was done for behavioural data that were collected on two consecutive days of rat exposure, to see whether the behaviour was different on a second day of rat exposure. Apart from two levels for 'strain' and four levels of 'time', a second within-subject factor of 'day' (with two levels) was added. In case of an effect of 'strain', or of an interaction involving 'strain' and 'day', the ANOVA was repeated within the two strains. When an interaction involving 'time' and 'day' was found, the data for the two days were compared per time level using post-hoc simple contrasts within ANOVA, to see which time period contributed to the effect of 'day'. Results of these post-hoc contrasts were Bonferroni corrected.

Finally, rat behavioural data, averaged over the thirty minutes of rat exposure, were analysed with ANOVA with repeated measures. Three levels of 'times used' examined whether there was an effect of the number of times a rat was used. As no significant effect of 'times used' was present for any of the scored behaviours, no further tests were done.

### *1.3.10. Statistical analysis of microdialysis data*

The amounts that were found in the microdialysis samples were calculated as fmol per 1-min dialysate collection (to account for differences in sample time), and then expressed as a percentage of baseline, defined as the average neurotransmitter level in the pre-rat exposure samples during which the behavioural activity of the animal was 10% or less.

To reveal time effects in the levels of 5-HT and 5-HIAA (for C57bl/6N mice), or in 5-HT, 5-HIAA, MHPG, DOPAC and HVA (for Balb/c mice) the data were analysed using ANOVA with 6 levels of 'time' (as described under the analysis of behavioural data), and a between-

subject factor ‘region’ (4 levels for C57bl/6N mice: hippocampus, prefrontal cortex, lateral septum and caudate putamen, 3 levels for Balb/c mice: hippocampus, anterior hypothalamus, paraventricular nucleus) to be able to determine effects of dialysed brain region. Levels of NA, MHPG, DOPAC and HVA in the hippocampus of C57bl/6N mice were analysed using ANOVA with repeated measures design as well, with six levels of ‘time’ as within-subject factors, as above. As these parameters were not measured in other brain areas in C57bl/6N mice, no ‘region’ factor was present. All data were ln-transformed before the ANOVA.

When a significant effect of ‘time’ or of the interaction ‘time by region’ was found, simple contrasts within ANOVA, Bonferroni corrected for multiple comparisons, were performed, to compare all time levels relative to baseline, as well as between the two time levels during rat exposure. Following a significant effect of ‘region’ or of ‘time by region’, the ANOVA was repeated for each brain region, and so were the post-hoc tests when an effect of ‘time’ was found again. Additional post-hoc Scheffé tests served to determine which brain regions differed from each other for the time periods.

To compare the effects of rat exposure on neurotransmission on day 1 and day 2 of rat exposure, the area under curve (AUC) of the full time vs. neurotransmitter or metabolite levels plots were calculated after ln-transformation. ANOVA with ‘day’ as within-subject factor and ‘region’ as between-subject factor was performed. In case of a significant effect of ‘region’ or of the interaction ‘day by region’ the ANOVA was repeated for the brain regions separately. When a significant effect of ‘day’ was found, simple contrasts within ANOVA were done, Bonferroni corrected, to identify the brain regions in which an effect of ‘day’ was apparent. A similar procedure was followed for the parts of the plots during rat exposure and the subsequent 15 minutes only. In the latter case, the AUC was calculated starting from a level of 80% upwards, as a result of which the values for the AUC did not need to be ln-transformed before the ANOVA.

### *1.3.11. Statistical analysis of free corticosterone data*

The free corticosterone data were analysed using ANOVA with repeated measurements design, after the data had been ln-transformed. As within-subject factors, seven levels of ‘time’ and two levels of ‘day’ were chosen. The levels of ‘time’ were as follows: one level pre exposure (consisting of four samples), two levels during rat exposure (each consisting of one sample), four levels after rat exposure (the first consisting of one, the following two levels consisting of two and the last one of three data points). In case of a significant effect of ‘time’ all levels were compared to baseline level using post-hoc simple contrasts, Bonferroni

corrected, to reveal which time levels were significantly higher than the baseline level. In case of an effect of 'day' each of the time levels of day 1 was compared to the corresponding level on day 2, using post-hoc simple contrasts, Bonferroni corrected.
Although large interindividual differences were found in the corticosterone responses, a group of animals with relatively large increases in free corticosterone following rat exposure was considered too small (n=3) to justify statistical analysis of those data separately. However, figures showing the data of this group, as well as of the remaining animals are shown in the results section for illustrative purposes.

Results with $p < 0.05$ were considered significant, unless Bonferroni corrections were applied, in which case the significance level depended on the number of comparisons. AUC were calculated with GraphPad Prism (GraphPad Software Inc., San Diego, CA, U.S.A.), whereas all other statistics were done with SPSS v10.0.7 (SPSS Inc., Chicago, IL, U.S.A.).

### *1.3.12. Other procedures and preparations*

- Gelatinised slides:
  3.5 g pulverised gelatine, 0.25 g $KCr(SO_4)_2$ x $12H_2O$ and 500 ml distilled water were mixed during half a day and filtered over filter paper. Slides were briefly dipped in the solution and dried during at least two days.
- Embedding medium:
  One egg yolk was undone from egg white using absorbing paper, sliced open and the inside of the yolk mixed with 0.8 ml 25% glutardialdehyde.
  This was briefly stirred by hand and immediately poured over the brain and solidified in about 5 minutes.
- Cresyl violet solution:
  230 ml distilled water, 50 ml 10% acetic acid, 10 ml 1 M sodium acetate and 1.5 g cresyl violet were mixed for an hour at 50 °C and filtered over paper.
- Nissl-staining procedure:
  Slides were kept in 70% alcohol at least 2 hours to dehydrate them and to fix the tissue to the glass. Then they were in the following liquids: 5 min in 96% ethanol, 5 min in 70% ethanol, 30 sec in tap water, 30 sec in distilled water, 3 min in cresyl violet, 30 sec in distilled water, 1 min in 70% ethanol, 2 min in 90% ethanol, 1 min in 96% ethanol with 0.5% acetic acid, twice 1 min in 96% ethanol, twice 1 min in 100%

ethanol and three times 5 min in Rotihistol. Slides were covered with Roti-Histokitt, coverslipped, and left to dry.

- Dummy probes:

  Pieces of about 15 mm were cut off from electrodes formerly used for monkeys, that had a diameter of 0.25 mm. The Teflon coat was removed with a scalpel and the rough end scoured with sand paper.

- Guide cannula:

  The sharp end of a needle, sized 0.70 mm by 30 mm, was grinded off, and the steel shaft was grinded until it had a length of 14.0 mm. The inside of the cannula was cleaned with sewing silk and the sharp edges were smoothed with sandpaper.

- Chemicals:

  All chemicals used (analytical or HPLC-grade) were obtained from Merck (Darmstadt, Germany) or Sigma-Aldrich Chemie (Taufkirchen, Germany) unless otherwise marked.

## 2. EXPERIMENTS MEASURING PLASMA LEVELS OF STRESS HORMONES

### *2.1. Overview of the experiments*

The general time frame of the experiments was as described in Table 6.

**Table 6. General timeframe of the experiments involving plasma hormone measurements.**

| Time (in days) | Action |
|---|---|
| - 7 or more | • Mice arrived and were housed singly |
| - 3 | • Mice were put in rat exposure cages |
| - 1 | • Mice were brought to experimental room |
| 0 | • Rat exposure or control experiment, killing, processing blood |
| Later | • Analysis of plasma |
| | • Video analysis |

The following experiments were conducted according to the schedule depicted above:

- Rat exposure and control experiments with C57bl/6N, C57bl/6J, Balb/c, B6C3F1, DBA/2 mice.
- Rat exposure and control experiments with C57bl/6N mice, bred at the institute.
- Rat exposure and control experiments with CRH-Tg (transgenics) and CRH-Wt (wildtype) mice.

The different steps are described in more detail in the following paragraphs.

### 2.1.1. Animals, arrival and housing

Male mice, 12 weeks old, were ordered from Charles River (all animals, except the transgenic animals and the corresponding wild types, or the C57bl/6N bred in the institute's facilities) and were housed in standard Macrolon II cages upon arrival, with food and water available *ad libitum*. They were kept in a housing room under the same conditions as in the experimental room (details as with the microdialysis experiments). Three days prior to the rat exposure experiment, they were singly housed in a rat exposure cage and brought to the experimental room on the day before the experiment.

As only four animals could participate in an experiment per day because only four animals could be recorded simultaneously on video, animals from different strains were used in a pseudo- randomised fashion.

CRH-Tg and CRH-Wt mice were already available in the animal facilities of the institute. These animals were transported to the housing room and singly housed at least 7 days prior to the experiment. At this time those mice were between 12 and 16 weeks old. The CRH-mice originally came from the group of Mary Stenzel-Poore, Portland, OH, USA. The mice that were used had been born in the institute facilities.

The institute bred C57bl/6N mice were the F1 generation of mice that came from Charles River and had been paired using the 'harem' method.

Other details were as with the microdialysis experiments, described in paragraph 1.3.1.

### 2.1.2. Rat exposure, killing, blood preparation

On the experimental day at 10.30 hr, a rat was put into the smaller compartment of the cage. The rats had been housed absolutely separated from the mice. Rats were used maximally three times, three days apart. After half an hour, at 11.00 hr rats were removed and placed outside the room. During control experiments no rat was placed in the Plexiglas cage but the experimenter did enter the room at 10.30 hr to remove the lid from the cage and to briefly extend a hand into the cage, to control for the vicinity of the experimenter to the mouse. During the entire experiment, from 8.30 – 11.00 hr, the behaviour of the mice and rats was recorded using security cameras.

At 11.00 hr mice were sedated in a glass with isoflurane, decapitated and trunk blood was collected in ice-cooled tubes coated with EDTA (ethylenediaminetetraacetate, KABE Labortechnik GmbH, Nürnbrecht, Germany) containing 6 µl Trasylol (Bayer, Leverkusen, Germany). The animals were decapitated within 30 – 45 seconds after being disturbed. Samples were kept on ice until they were centrifuged for 20 minutes at 2500 rpm in a

refrigerated centrifuge. Plasma was then aliquoted in 100 µl and 25 µl portions and stored at – 80 ºC until concentrations of ACTH and total corticosterone were measured. Storage did not affect the hormone levels.

### *2.1.3. ACTH and corticosterone measurement.*

The concentrations of ACTH and corticosterone were assessed using ACTH and corticosterone RIA kits respectively. The kits were used according to the manufacturer's manual. See also paragraph 1.3.8. The inter- and intra assay variation coefficient were 7% and 5% for ACTH and 7% and 4% for corticosterone. The detection limits of the assays were 2 pg/ml for ACTH and 0.4 ng/ml for CORT.

### *2.1.4. Behavioural analysis*

The behaviour of the mice during the half hour of rat exposure was scored using either the instantaneous method with Excel as described with the microdialysis experiments (C57bl/6N, C57bl6/J, Balb/c, B6C3F1, DBA), or using a continuous method (Noldus Observer Video Pro, v 4.0, Noldus Information Technology, Wageningen, the Netherlands) (C57bl/6N, Balb/c, B6C3F1; CRH-Wt and Tg; C57bl/6 bred at the institute).

**Table 7. Behavioural parameters that were scored using a continuous method with Noldus Observer Video Pro. *Latency* of an event: time in seconds from the beginning of rat exposure until the first event took place; *frequency* of an event: number of times an event took place during the half hour of rat exposure; *duration* of a behaviour: time in seconds that an animal was engaged in the behaviour during rat exposure**

| Behavioural parameter | | Definition |
|---|---|---|
| contact | latency | First physical contact with separation wall, while facing the rat compartment |
| rear | frequency, duration | Posture with front-paws away from the bottom, with or without supporting at the walls |
| backup | frequency | Transition of an imaginary line separating the mouse compartment in a half close to the rat and a half away from the rat |
| stretched attend posture | frequency | Full extension of the body, with arched back |
| defensive bury | frequency, duration | Heaping up bedding with front paws against separation wall |
| nosepoke | latency, frequency, duration | Putting nose in one of the holes in the separation wall |
| groom | duration | Movement of head or paws over parts of the body, also included licking |
| food-related behaviour | duration | Drinking from bottle, eating from food-trough or in other parts of cage, moving food-pellets in trough around |
| inactivity | duration | Lying or sitting quietly for more than 5 seconds |
| time at separation wall | duration | Body located in the area bordered by the separation wall, approx. 7 cm wide. |

In the latter method every change of behaviour was recorded at the moment it occurred. The time resolution was 0.1 seconds. Parameters scored with the Noldus system are listed in Table 7.

### *2.1.5. Statistical analysis*

Plasma values of ACTH and corticosterone were ln-transformed and analysed using ANOVA, with 'strain' or 'genotype' (5 levels for C57bl/6N, C57bl/6J, Balb/c, B6C3F1 and DBA; 2 levels for CRH-Tg and CRH-Wt) and two levels of 'exposure' (control and exposed) as between-subject factors. In case of an effect of 'strain' or of the interaction 'strain by exposure', post-hoc simple contrasts within ANOVA compared the control and exposed levels per strain. These results were Bonferroni corrected for multiple comparisons. Occassional additional comparisons with the CRH-Wt and CRH-Tg were done with Student's *t*-tests, Bonferroni corrected. Control and exposed levels of the C57bl/6N mice bred at the institute were compared using one-way ANOVA. Additional comparisons between exposed levels of C57bl/6N mice bred at the institute and those bought from Charles River were done with unpaired Student's *t*-tests.

Behavioural data obtained with the Excel method, were counted per behaviour for every 5-minutes interval during rat exposure and expressed as the total number of behavioural counts in that period. The AUC of the time vs. behavioural intensity curves were calculated, and normalised so that a maximum AUC-value of 100 could be reached, in case that behaviour was observed throughout the rat exposure period. In case of freezing, not the AUC was used, but the number of 'freezing' counts. These AUC's were compared for each behaviour using one-way ANOVA, with 5 levels of 'strain' as the between-subject factor. When a significant effect of 'strain' was found, post-hoc Scheffé tests determined the homogeneous subsets of strains.

Behavioural data obtained with the Noldus-method were expressed in seconds (for latency and duration scores) for the 30 minutes of rat exposure, or as the number of times (for frequency scores) during the 30 minutes of rat exposure that a certain behaviour occurred. Per parameter one-way ANOVA was used to compare the strains C57bl/6N, Balb/c and B6C3F1. This ANOVA was repeated with a between-subject factor 'height of the hormone level'. For this purpose ACTH and corticosterone levels were divided into three groups. One group contained animals with levels lower than 50 pg/ml ACTH or 25 ng/ml CORT, one group consisted of animals with levels higher than 100 pg/ml ACTH or 50 ng/ml CORT and a group was formed by animals with an intermediate level.

Behavioural data for CRH-Wt and CRH-Tg mice were subjected to ANOVA with two levels of ‘exposure’ and two levels of ‘genotype’ as between-subject factors. After significant differences in the groups were found, post-hoc Duncan tests determined the homogeneous subsets. Student’s *t*-tests compared the control and exposure behavioural levels for C57bl/6N mice bred at the institute. Students *t*-tests were also used to compare the behavioural levels after exposure to those seen with animals from Charles River.

All results with $p < 0.050$ were considered to be significant, except for cases in which Bonferroni corrections were applied, in which the number of comparisons determined the level of significance. Calculations were all done with GraphPad and SPSS.

# III. RESULTS

## 1. BEHAVIOUR

### *1.1. Behaviour before rat exposure*

Figure 19 shows the variations in behaviour of the C57bl/6N mice (red curves) and of Balb/c mice (in blue) over the time course of the experiment.

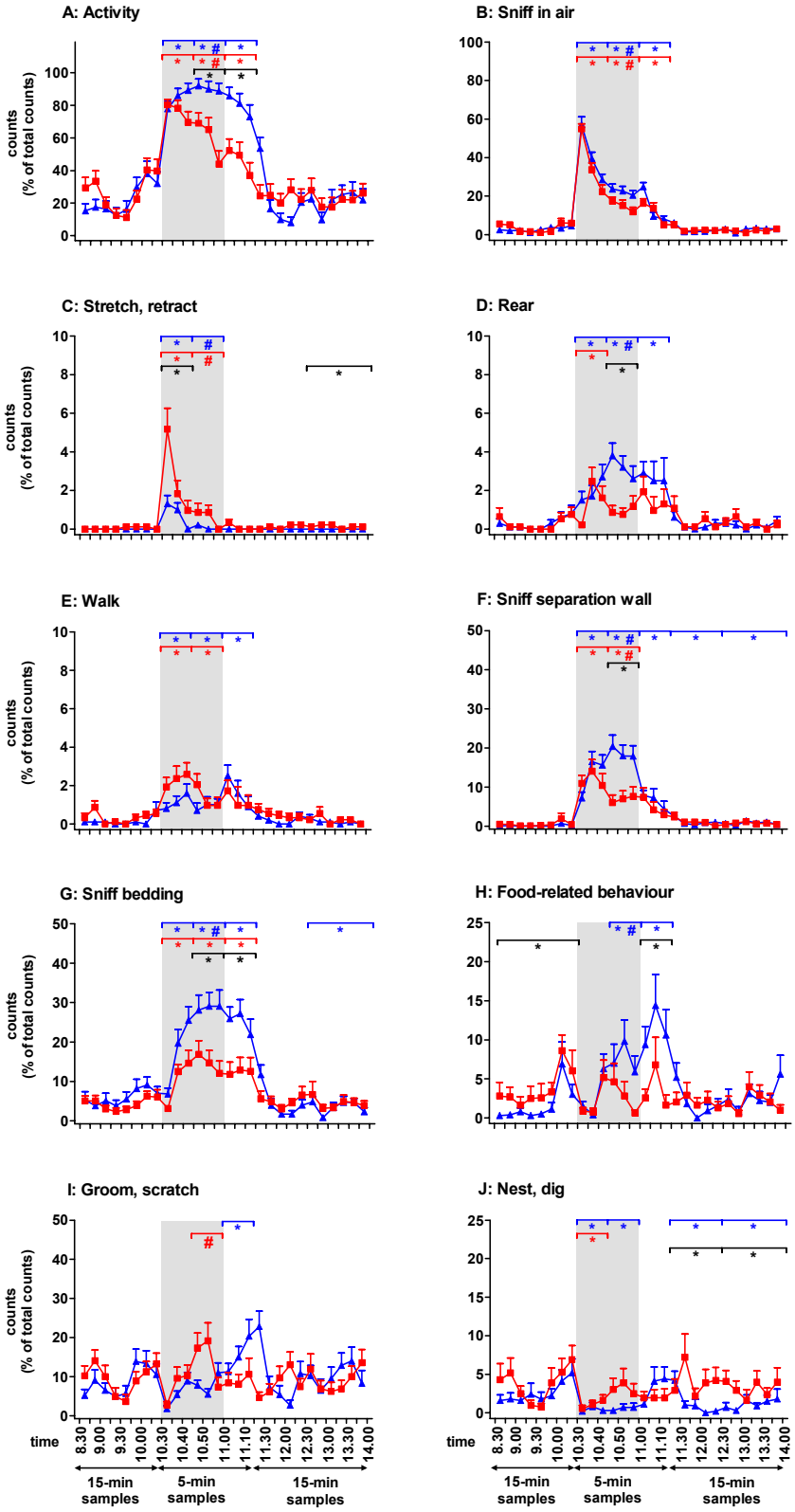
As the experiments were conducted during the light phase, animals were typically inactive before and after the rat exposure. However, this state was periodically interrupted by active periods that could last up to 30 minutes. Because these activity phases were not synchronised among animals, it resulted in an average activity level before and after rat exposure that was varying, but continuously more than zero (see panel A).

Figure 20 depicts the behavioural profile of animals during those active phases. During the phases before rat exposure (open bars), activity of C57bl/6N mice existed mostly of self-directed behaviour like grooming, food-related behaviour, nesting, and also of considerable amounts of calmly sitting while sniffing air and sniffing in the bedding of the cage which sometimes was followed by finding parts of food pellets in the bedding and eating. Balb/c mice exhibited the same gamut of behaviours before rat exposure, but the level of sniffing in the bedding was higher, whereas levels of food-related behaviour and nesting were lower than with C57bl/6N mice.

### *1.2. Behaviour during rat exposure*

At the beginning of rat exposure animals woke up and became alert and active, resulting in a significantly higher level of average activity (see Figure 19A). A first reaction was to move to a corner as far away from the rat compartment as possible. Freezing was hardly observed.

**Figure 19 (next page). Levels of activity (panel A) and specific behaviours (panels B-J) of C57bl/6N mice (red squares, n=31) and Balb/c mice (blue triangles, n=32) before, during (shaded area) and after their first exposure to a rat. Data are represented as the number of counts during which animals were active or showed a particular behaviour, expressed as a percentage (+ S.E.M.) of the total number of counts during a period in which one microdialysis sample was collected. 'Activity' (panel A) during a certain period is the sum of the percentages of the particular behaviours (panels B-J) in that same time interval. Please note differences in scaling of the y-axis, and the non-linearity of the time-axis. In all cases, ANOVA indicated an effect of strain or of the interaction time by strain. Black asterisks indicate significant difference in levels for certain time periods between C57bl/6N and Balb/c mice (post-hoc Student's *t*-test, Bonferroni corrected for multiple comparisons, $p < 0.010$). ANOVA also indicated an effect of time for both strains in all behaviours, except for food-related behaviour with C57bl/6N mice (see text). Red and blue asterisks indicate significant differences from baseline for C57bl/6N and Balb/c mice respectively, whereas coloured # indicate significant difference between the second half and first half of rat exposure (post-hoc simple contrasts, Bonferroni corrected for multiple comparisons, $p < 0.0083$).**


A: Activity
B: Sniff in air
C: Stretch, retract
D: Rear
E: Walk
F: Sniff separation wall
G: Sniff bedding
H: Food-related behaviour
I: Groom, scratch
J: Nest, dig
counts (% of total counts)
time
8.30
9.00
9.30
10.00
10.30
10.40
10.50
11.00
11.10
11.30
12.00
12.30
13.00
13.30
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15-min samples
5-min samples
15-min samples

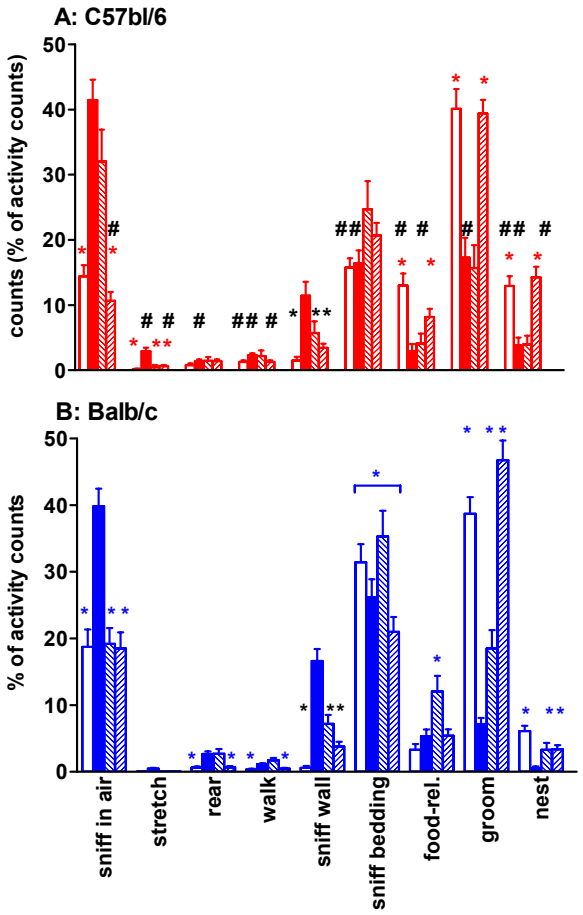



**Figure 20. Comparison of the behavioural make-up of active periods of C57bl/6N mice (panel A, n=31) and Balb/c mice (panel B, n = 32) before (clear bars), during (solid bars) and after (downwardly hatched bars: first 15 min afterwards; bars hatched upwards: rest of time) the first exposure to a rat. Data are represented as the counts (+ S.E.M.) of a specific behaviour, expressed as the percentage of total activity. Strain effects were found for all behaviours except sniffing separation wall. Black # in panel A show those bars that are significantly different between the two strains (Student's *t*-test, Bonferroni corrected, $p < 0.0125$). Except for rearing, walking and sniffing bedding for C57bl/6N mice and stretching for Balb/c mice, ANOVA indicated significant time effects for all behaviours in both strains. * depicts the results of post-hoc simple contrasts (Bonferroni corrected for multiple comparisons, $p < 0.017$) and indicates significant difference from 'during rat exposure'. Although a time-effect was present with 'sniffing bedding' in Balb/c mice, no single time period was significantly different from 'during rat exposure'. Food-rel. is food-related behaviour.**

Figure 19 shows that in the first minutes of rat exposure the activity no longer was made up of self-directed behaviours, but consisted of investigative behaviours like sniffing air, stretching towards the rat compartment, rearing, walking and sniffing at the separation wall. These behaviours were significantly more present in the first half of rat exposure, than under pre-exposure conditions (the following effects of 'time' were found for C57bl/6N and Balb/c mice respectively, activity: $F(5,305) = 33.98$, $p < 0.0005$; $F(5,305) = 104.16$, $p < 0.0005$; sniffing air: $F(5,305) = 85.04$, $p < 0.0005$; $F(5,305) = 122.27$, $p < 0.0005$; stretching:

$F(5,305) = 30.83$, $p < 0.0005$; $F(5,305) = 2.87$, $p < 0.02$; rearing $F(5,305) = 2.87$, $p < 0.02$; $F(5,305) = 17.84$, $p < 0.0005$, walking $F(5,305) = 11.13$, $p < 0.0005$; $F(5,305) = 7.65$, $p < 0.0005$; sniffing at wall: $F(5,305) = 13.26$, $p < 0.0005$; $F(5,305) = 39.92$, $p < 0.0005$; asterisks in Figure 19 indicate those time periods that were significantly different from pre-exposure levels). Also sniffing bedding ($F(5,305) = 6.91$, $p < 0.0005$; $F(5,305) = 43.87$, $p < 0.0005$) was increased, but a self-directed behaviour like nesting ($F(5,305) = 3.22$, $p < 0.01$; $F(5,305) = 3.33$, $p < 0.01$) was decreased. Time effects were also found for grooming ($F(5,305) = 2.76$, $p < 0.02$; $F(5,305) = 4.87$, $p < 0.0005$) and food-related behaviour in Balb/c mice ($F(5,305) = 13.21$, $p < 0.0005$), but these were not apparent from comparing the first half of rat exposure to baseline conditions. Remarkably, mice and rats occasionally had nose-to-nose contact when they simultaneously poked their noses through a hole in the separation wall. Over the course of rat exposure, activity, sniffing air, stretching and sniffing at separation wall decreased significantly in C57bl/6N mice when compared to the first half of rat exposure. In contrast, these mice were still intensively sniffing around in bedding, usually close to the separation wall. Small, but not significant increases in grooming and nest-building were seen as well in this phase. Occasionally, when mice had had their nest close to the separation wall, they would form a new nest at a larger distance from the rat compartment. Balb/c mice differed from this, in that their activity did not become less as rat exposure progressed. Decreases in investigative behaviours as sniffing air and stretching were compensated by higher levels of rearing, sniffing at the separation wall, and sniffing in the bedding, whereas also the self-directed food-related behaviour was more present.

Figure 20 does not reveal the temporal changes within the half hour of rat exposure, but does show that during rat exposure the levels of sniffing air, stretching (only for C57bl/6N), rearing and walking (only for Balb/c) and sniffing the separation wall, which all constitute investigative behaviours, are significantly higher than levels found during active periods before rat exposure or towards the end of the experiment. In contrast, self-directed behaviours like food-related activities (only for C57bl/6N mice), grooming and nest-building were significantly lower during rat exposure (effects of 'time' were found for sniffing air $F(3,183) = 27.30$, $p < 0.0005$ and $F(3,183) = 13.98$, $p < 0.0005$ for C57bl/6N and Balb/c respectively, stretching $F(3,183) = 37.22$, $p < 0.0005$ for C57bl/6N but not for Balb/c with $F(3,183) = 1.45$, $p > 0.05$; rearing $F(3,183) = 10.18$, $p < 0.0005$ for Balb/c but not for C57bl/6N with $F(3,183) = 0.70$, $p > 0.05$; walking $F(3,183) = 2.86$, $p < 0.05$ for Balb/c, but not for C57bl/6N with $F(3,183) = 2.26$, $p > 0.05$, sniffing wall $F(3,183) = 46.21$, $p < 0.0005$ (no effect or interaction of 'strain'), sniffing bedding $F(3,183) = 4.83$, $p < 0.005$ for Balb/c, not significant for

C57bl/6N $F(3,183) = 2.38$, $p > 0.05$, food-related behaviour $F(3,183) = 9.93$, $p < 0.0005$ for C57bl/6N and $F(3,183) = 6.65$, $p < 0.0005$; grooming $F(3,183) = 25.62$, $p < 0.0005$ for C57bl/6N and $F(3,183) = 50.23$, $p < 0.0005$ for Balb/c and nesting $F(3,183) = 27.89$, $p < 0.0005$, $F(3,183) = 4.61$, $p < 0.005$ for C57bl/6N and Balb/c respectively. Asterisks in Figure 20 represent those time periods that were different from during rat exposure).

### *1.3. Behaviour after rat exposure*

When the rats were removed from the rat exposure cages, mice often became more alert again and often moved to an opposite corner. In the first fifteen minutes after rat-exposure, C57bl/6N mice showed increased levels of activity, sniffing air and sniffing bedding when compared to baseline, but later all activity levels where no longer different from baseline level (see Figure 19). Balb/c mice also showed higher activity levels in the 15 min after rat exposure than during baseline. Here behavioural scores for sniffing in air, rearing, walking, sniffing at the separation wall, sniffing bedding, and for the self-directed behaviours grooming and food-related activity were still higher than during baseline. All of these, except for sniffing at the separation wall returned to levels as found before rat exposure. Sniffing bedding and nesting however showed levels even less than found before rat exposure.

Figure 20 underlines that the activity profile of mice during and briefly after rat exposure showed great resemblance, in which only stretching and sniffing at the separation wall occurred significantly more during rat exposure than shortly afterwards in C57bl/6N mice. Balb/c were sniffing air more during than briefly after rat exposure, but participated less in food-related behaviour, grooming or nesting. Behaviour during and shortly after rat exposure greatly differed from the behavioural profile before and longer after rat exposure.

### *1.4. Differences in behaviour between C57bl/6N and Balb/c mice*

For all behaviours in Figure 19 the ANOVA indicated significant effects of ‘strain’ (activity $F(1,61) = 12.61$, $p < 0.001$; stretching $F(1,61) = 13.95$, $p < 0.0005$; rearing $F(1,61) = 5.86$, $p < 0.02$; sniffing bedding $F(1,61) = 11.02$, $p < 0.005$; food-related behaviour $F(1,61) = 6.51$, $p < 0.02$; nesting $F(1,61) = 11.30$, $p < 0.01$) and/or of the interaction ‘strain by time’ (activity $F(5,305) = 14.73$, $p < 0.0005$; sniffing air $F(5,305) = 2.53$, $p < 0.05$; stretching $F(5,305) = 7.71$, $p < 0.0005$; rearing $F(5,305) = 4.38$, $p < 0.001$; walking $F(5,305) = 2.25$, $p < 0.05$; sniffing separation wall $F(5,305) = 7.46$, $p < 0.0005$; sniffing bedding $F(5,305) = 7.78$, $p < 0.0005$; food-related behaviour $F(5,305) = 5.50$, $p < 0.0005$; grooming $F(5,305) = 4.00$, $p < 0.005$; nesting $F(5,305) = 3.03$, $p < 0.02$).

Also in Figure 20 effects of 'strain' (stretching $F(1,61) = 17.19$, $p < 0.0005$, walking $F(1,61) = 8.49$, $p < 0.005$, sniffing bedding $F(1,61) = 10.51$, $p < 0.005$; nesting $F(1,61) = 35.55$, $p < 0.0005$) and/or of the interaction 'strain by time' (sniffing air $F(3,183) = 6.56$, $p < 0.0005$; grooming $F(3,183) = 4.29$, $p < 0.01$; stretching $F(3,183) = 12.35$, $p < 0.0005$; rearing $F(3,183) = 3.78$, $p < 0.02$; sniffing food $F(3,183) = 13.27$, $p < 0.0005$; nesting $F(3,183) = 8.71$, $p < 0.0005$) were found for all behaviours, except sniffing at the separation wall.

When comparing behaviour for the two strains in Figure 19 and in Figure 20, the most striking difference prior to rat exposure was the level of food-related behaviour that was lower in Balb/c than in C57bl/6N mice. Interestingly it was the same behaviour that was higher for Balb/c mice in the 15 minutes after rat exposure. During rat exposure, slight differences were found in the investigative behaviours stretching and walking, which were seen more in C57bl/6N mice and in rearing and sniffing bedding, which were scored more often with the Balb/c mice. Maximal levels of rearing, sniffing separation wall and sniffing bedding occurred later in Balb/c mice, and this strain stayed active for longer as well. Focussing on the self-directed behaviours it can be recognised that grooming was seen more often in C57bl/6N mice, especially towards the end of the exposure. Briefly after rat exposure it was the self-directed food-related behaviour that was seen preferentially in Balb/c mice briefly after rat exposure. In general however both strains showed mostly self-directed behaviours before and towards the end of the experiment, and mainly investigative behaviours during rat exposure.

### *1.5. Differences in behaviour on day 1 and 2*

The temporal course of behavioural changes over the phases of rat exposure as described above was highly the same when the paradigm was repeated on a second day. Interestingly, small changes in behavioural levels were found between the two days that were mostly independent of strain or of time period. Figure 21 illustrates this for the time during rat exposure. On day two the levels of most of the investigative behaviours (sniffing air, rearing, walking, and sniffing wall (the latter in case of C57bl/6N only, effect of strain by day $F(1,51) = 5.66$, $p < 0.05$) were less than on day 1. In contrast, levels of food-related behaviour, grooming and nesting were higher. The corresponding effects of 'day' were as follows: sniffing air $F(1,51) = 3.45$, $p < 0.001$; rearing $F(1,51) = 13.38$, $p < 0.001$; walking $F(1,51) = 4.71$, $p < 0.05$; sniffing wall $F(1,51) = 10.52$, $p < 0.005$; food-related behaviour $F(1,51) =$

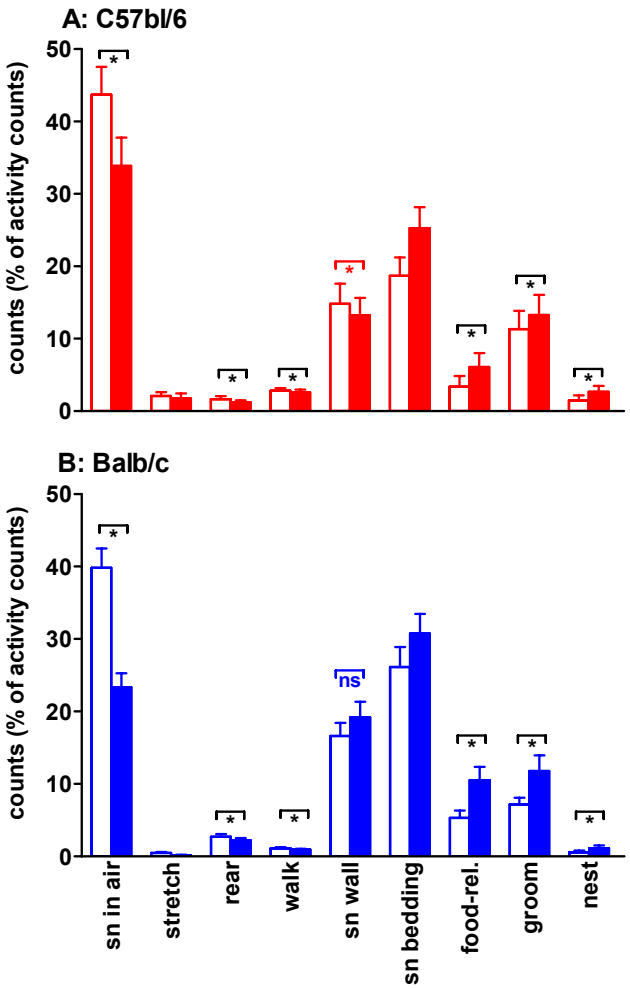



**Figure 21. Behavioural profile of C57bl/6N mice (panel A, n=21) and Balb/c mice (panel B, n=32) on two consecutive days (open bars: day 1; closed bars: day 2) during rat exposure. Data are represented as the duration (+ S.E.M.) of a specific behaviour, expressed as the percentage of time during which the animal was *active*. As an interaction of 'time by day' was only found for sniffing air and sniffing separation wall, the results before and after rat exposure have been omitted from this figure. Black asterisks indicate that post-hoc simple contrasts revealed that day 2 differed from day 1 ($p < 0.0125$), but that ANOVA had not indicated an effect of 'strain'. In case of sniffing separation wall a significant interaction of 'strain by day' was found and the coloured asterisk indicates that levels on day 1 and 2 differed for C57bl/6N mice, whereas the 'ns' signifies the absence of such a difference for Balb/c mice. No effects of 'day' were found for stretching and sniffing bedding.**

5.29, $p < 0.05$; grooming $F(1,51) = 14.58$, $p < 0.0005$; nesting $F(1,51) = 16.06$, $p < 0.0005$. In addition, significant interactions of 'time by day' were found for sniffing air $F(3,153) = 3.45$, $p < 0.02$, sniffing wall $F(3,153) = 4.17$, $p < 0.02$ and sniffing bedding $F(3,153) = 3.82$, $p < 0.02$.

Although not many, some day-differences existed that were not the same for each time period. In case of sniffing air, levels were lower on day 2 for each of the phases, but were not as

clearly lower in the phases after rat exposure. Balb/c mice were sniffing less at the separation wall after rat exposure on day 2 than on day 1, whereas there was no difference during rat exposure. For sniffing at the bedding, differences between the two days were variable for the phases, and in the end did not result in an effect of day. Other than the mentioned interaction of 'strain by day' in case of sniffing at the separation wall, no further significant interactions of 'strain by time' or of 'strain by time by day' were found.

### *1.6. Behaviour of rats*

When rats (data not shown) were introduced in the rat exposure cages, they first started with turning around a lot in their small compartment, with frequent sniffing at the separation wall, the floor and with rearing. This was typically followed by a period of intensive grooming, and by the end of rat exposure, animals were calmly sitting while sniffing in the air. Occasionally they had fallen asleep. ANOVA did not show an effect of 'times used' for the time animals were active ($F(2,12) = 0.31$, $p > 0.05$) or for the time they spent with any of the specific behaviours (grooming $F(2,12) = 2.78$, $p > 0.05$; sniffing air $F(2,12) = 0.46$, $p > 0.05$; rearing $F(2,12) = 1.21$, $p > 0.05$; sniffing separation wall $F(2,12) = 1.76$, $p > 0.05$; sniffing floor $F(2,12) = 0.11$, $p > 0.05$), indicating that this pattern was similar across the maximum of three times the rats were used.

## 2. MICRODIALYSIS

### *2.1. In-vitro experiment*

The in-vitro experiment was performed to determine whether the system set-up was functional and whether the compounds of interest could be measured using microdialysis.
Table 8 shows the recovery rates of the various compounds using a 1-mm and 3-mm probe. All compounds were measurable.

**Table 8. Recovery data for various compounds with 1-mm and 3-mm probes.**

| | Recovery 1 mm probes, % | Recovery 3 mm probes, % |
|---|---|---|
| **5-HT** | 4.5 | 15.4 |
| **5-HIAA** | 4.7 | 16.0 |
| **NA** | 4.0 | 11.3 |
| **MHPG** | 6.1 | 17.5 |
| **DOPAC** | 5.0 | 15.3 |
| **HVA** | 5.6 | 14.8 |

The values showed that when a membrane was used with three times the surface of another membrane, recovery was also three times as high, as predicted by theory. Based on the

recovery rates, it would be possible to convert the measured amounts in the dialysates back to what the extracellular concentrations surrounding the microdialysis probe in the brain must have been that were present during experiments. This was not done, so all figures in this thesis are based on the amounts in the samples.

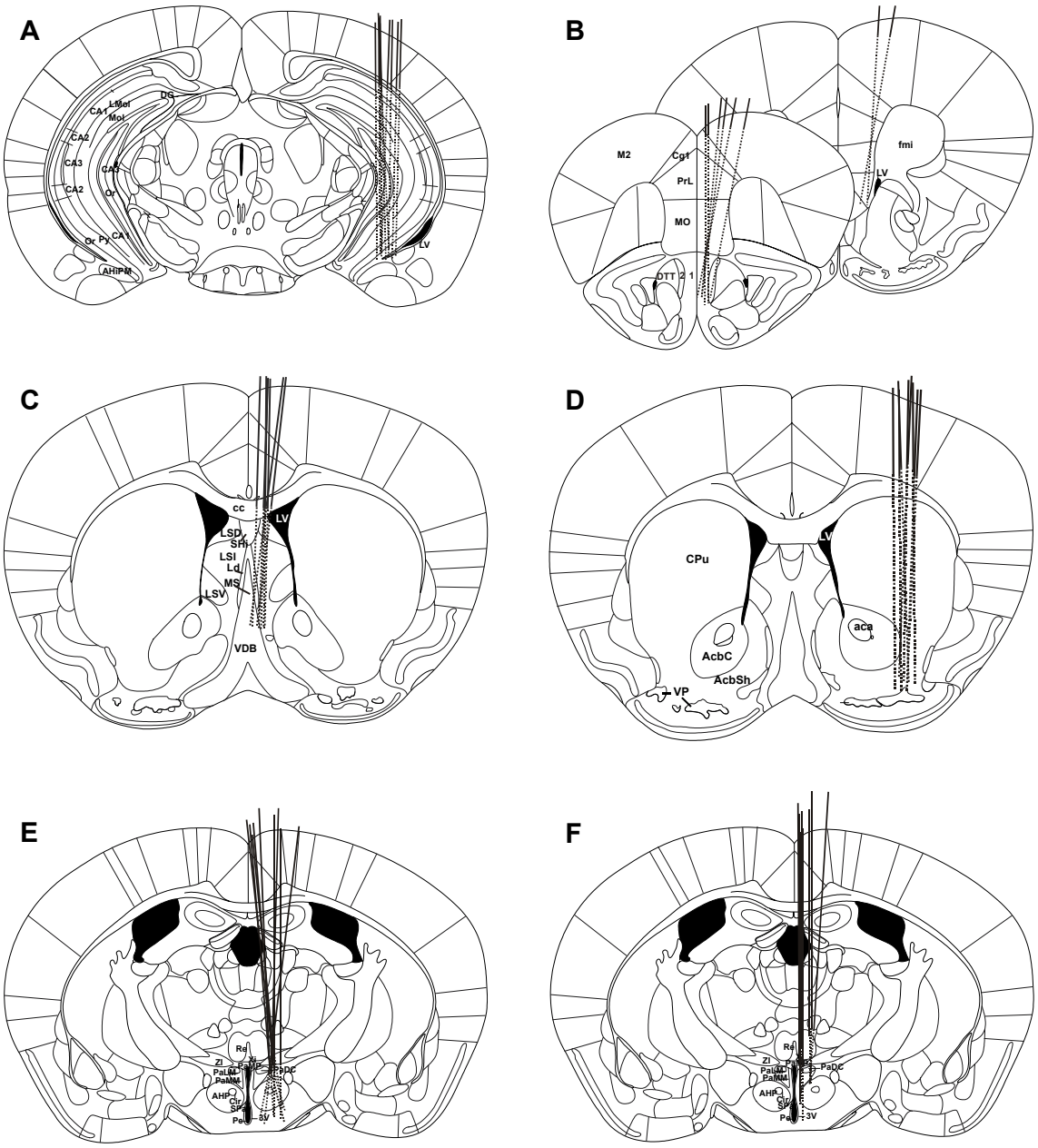


**Figure 22 (previous page). Schematic representation of probe locations in the hippocampus (panel A, coronal slice at -2.92 mm from bregma), prefrontal cortex (panel B, coronal slices at +2.34 mm and +1.94 mm from bregma), lateral septum (panel C, coronal slice at +0.74 mm from bregma) and caudate putamen (panel D, coronal slice at +1.10 mm from bregma) of C57bl/6N mice, as well as the anterior hypothalamus (panel E, coronal slice at -0.94 mm from bregma) and paraventricular nucleus (panel F, coronal slice at -0.94 mm from bregma) of Balb/c mice. Dotted lines indicate position of the semi-permeable membrane, continuous lines the guide cannula. Drawings reproduced from [243]. *1* layer 1 (tenia tecta); *2* layer 2 (tenia tecta); *aca* anterior commisure, anterior part; *3V* third ventricle; *AcbC* accumbens nucleus, core; *AcbSh* accumbens nucleus, shell; *AhipM* amygdalohippocampal area; *AHP* anterior hypothalamic area, posterior part; *CA1* field CA (cornu amonis) 1 of the hippocampus; *CA2* field CA2 of the hippocampus; *CA3* field CA3 of the hippocampus; *Cg1* cingulate cortex, area 1; *Cir* circular nucleus; *CPu* caudate putamen (striatum); *DG* dentate gyrus; *DTT* dorsal tenia tecta; *fmi* forceps minor of the corpus callosum; *Ld* lambdoid septal zone; *Lmol* lacunosum molecular layer of the hippocampus; *LSD* lateral septal nucleus, dorsal part; *LSI* lateral septal nucleus, intermediate part; *LSV* lateral septal nucleus, ventral part; *LV* lateral ventricle; *M2* secondary motor cortex; *MO* medial orbital cortex; *Mol* molecular layer of the dentate gyrus; *MS* medial septal nucleus; *Or* oriens layer of the hippocampus; *Pa* paraventricular nucleus, *-DC* dorsal cap, *-LM* lateral magnocellular part, *-MM* medial magnocellular part, *-MP* medial parvicellular part; *Pe* periventricular hypothalamic nucleus; *PrL* prelimbic cortex; *Py* pyrimidal cell layer of the hippocampus; *Re* reunions thalamic nucleus; *SHI* septohippocampal nucleus; *Spa* subparaventricular zone of the hypothalamus*; VDB* nucleus of the vertical limb of the diagonal band; *VP* ventral pallidum; *Xi* xiphoid thalamic nucleus; *ZI* zona incerta.**

### *2.2. Histological verification of probe location*

Figure 23 shows a schematic representation of the probe locations in the various brain regions. In case of the hippocampus, where brains were cut horizontally, the location in the frontal projection plane was approximated. All results that are shown in the microdialysis experiments are based on measurements obtained with these probes.

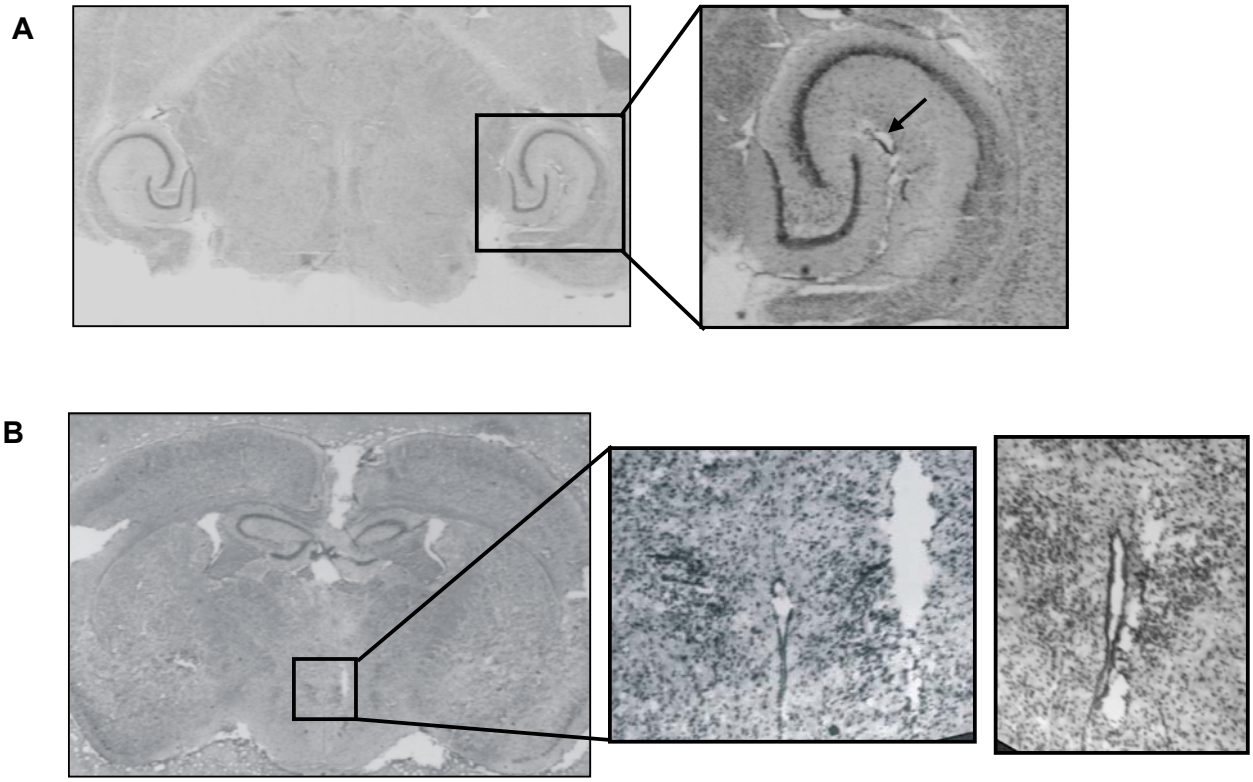


**Figure 23 (previous page). Pictures of probe locations in coronally cut slides of hippocampus (panel A, C57bl/6N mouse) and paraventricular nucleus (panel B, Balb/c mouse). Arrow in A points towards hole made by probe. In B both a more lateral (middle) and more medial (right) PVN-implantation locus are shown. The slices were Nissl-stained.**

To give a more detailed view of this, photos made from a horizontal hippocampal section and a frontal section showing the probe location around the paraventricular nucleus have been included in Figure 23.

## *2.3. Serotonergic neurotransmission in C57bl/6N mice*

### *2.3.1. Effect of rat exposure on 5-HT on day 1*

Figure 24 shows the time course of levels of 5-HT, 5-HIAA and behavioural activity, in animals that were dialysed in one of four different brain structures.

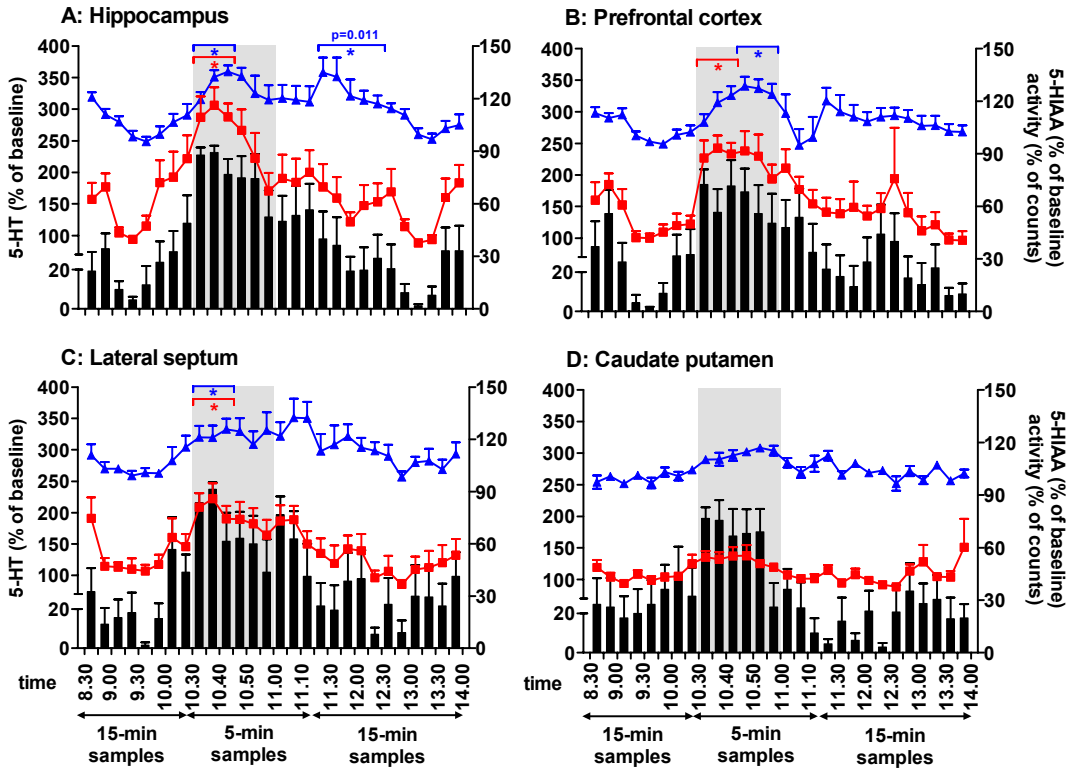



**Figure 24. Extracellular levels of 5-HT (red squares, left y-axis), 5-HIAA (blue triangles, right y-axis) in % of baseline (+ S.E.M.), and time behaviourally active (black bars, right y-axis) in % of sample time (+ S.E.M.), over the course of the experiment in hippocampus (n=9, panel A), prefrontal cortex (n=8, panel B), lateral septum (n=7, panel C) and caudate putamen (n=7, panel D) of C57bl/6N mice. Shaded area indicates the period of exposure to a rat. When the ANOVA indicated a significant effect of 'time', post-hoc simple contrasts were performed comparing all time levels to baseline and comparing the two levels during rat exposure. Results from the post-hoc tests are denoted by * in red or blue, which indicates significant difference from the baseline levels of the compound in the same colour (Bonferroni corrected for multiple comparisons, $p < 0.0083$). 5-HT baseline values were 0.40 ± 0.09 fmol/min in the hippocampus, 0.25 ± 0.08 fmol/min in the prefrontal cortex, 0.25 ± 0.06 fmol/min in the lateral septum and 0.66 ± 0.09 fmol/min in the caudate putamen. 5-HIAA baseline values were respectively 151.2 ± 13.3, 90.3 ± 4.9, 115.2 ± 8.7 and 212.5 ± 9.3 fmol/min.**

The behavioural activity pattern did not depend on the location of implantation. The overall ANOVA did not indicate an effect of 'region' ($F(3,27) = 0.69$, $p > 0.05$) or of 'region by time' ($F(15,135) = 0.81$, $p > 0.05$) for activity. As was the case in Figure 19, activity levels did vary with 'time' ($F(5,135) = 30.07$, $p < 0.0005$), with higher levels during rat exposure (results of corresponding post-hoc simple contrasts are not depicted in Figure 24).

Different was the situation for 5-HT, in which case the overall ANOVA not only indicated a significant effect of 'time' ($F(5,135) = 18.40$, $p < 0.0005$), but also of 'region' ($F(3,27) = 3.57$, $p < 0.05$). After further analysis, 'time within region' effects were present in the hippocampus ($F(5,135)= 9.53$, $p < 0.0005$), the prefrontal cortex ($F(5,135) = 7.69$, $p < 0.0005$) and lateral septum ($F(5,135) = 5.42$, $p < 0.0005$), but not in the caudate putamen ($F(5,135) = 1.11$, $p > 0.05$), meaning that the variations in 5-HT levels over the course of the experiment were not significant in the caudate putamen, as the only one out of four brain regions tested.

In the period before rat exposure, hippocampal levels varied between 94.0 ± 2.5% and 221.6 ± 37.1% of baseline, with an average of 155.7 ± 14.2%. This variation in pre-exposure levels was less in cortical 5-HT (ranging from 100.0 ± 7.3% to 184.8 ± 17.9% with an average of 131.2 ± 7.3%), septal 5-HT (ranging from 107.0 ± 10.0% to 191.0 ± 33.4%, with an average of 132.4 ± 8.1%), and a similar variation was hardly noticeable in the caudate putamen (93.6 ± 3.3% to 124.9 ± 14.3%, with an average of 107.2 ± 3.0%).

During rat exposure, extracellular levels of 5-HT increased rapidly, and again reached highest levels in the hippocampus, with a maximum of 306.2 ± 28.4%. In the prefrontal cortex, a maximum was reached of 242.3 ± 20.0% and in the lateral septum of 222.2 ± 24.5%. Post-hoc contrasts revealed that extracellular levels of 5-HT in the hippocampus, the prefrontal cortex and the lateral septum were significantly higher during the first half of rat exposure than under basal conditions (see Figure 24). In the caudate putamen, levels did not exceed 137.8 ± 14.4% during rat exposure, which was not significantly higher than pre-exposure level. According to post-hoc Scheffé test, hippocampal levels during this period were significantly higher than those in lateral septum and in caudate putamen, whereas also cortical levels were higher than in the caudate putamen. In the second half of rat exposure, extracellular levels decreased, and were no longer different from pre-exposure levels, the same of which holds true for the levels after rat exposure.

Interesting is that the pattern of increases and decreases in 5-HT in three out of the four regions resembled the pattern of increases and decreases in activity.

### 2.3.2. *Effect of rat exposure on 5-HIAA on day 1*

The overall ANOVA for 'time' and 'region' effects of 5-HIAA indicated a significant effect of 'time' ($F(5,135) = 12.01$, $p < 0.0005$). Similar to the results obtained with 5-HT, significant time effects were present in the hippocampus ($F(5,135) = 6.14$, $p < 0.0005$), prefrontal cortex ($F(5,135) = 5.54$, $p < 0.0005$) and lateral septum ($F(5,135) = 3.93$, $p < 0.005$), but not in the caudate putamen ($F(5,135) = 1.93$, $p > 0.05$). Variations in levels of 5-HIAA were more modest than the variations in 5-HT. Pre-exposure levels had an average of 106.0 ± 1.8% in the hippocampus, 104.0 ± 0.7% in the prefrontal cortex, 105.1 ± 2.0% in the lateral septum and 100.1 ± 0.9% in the caudate putamen. In the first three regions, extracellular levels of 5-HIAA increased significantly during rat exposure, but the increase was slighter than with 5-HT. Also the maximum levels were reached at a later time point than in case of 5-HT. Levels in hippocampal 5-HIAA peaked one sample later than in case of 5-HT and were 135.3 ± 3.7%. In the cortical region, only during the second half of rat exposure significantly higher 5-HIAA concentrations were found than during the pre-exposure period (peak level: 128.6 ± 5.4%). The septal maximum concentration of 5-HIAA was 132.4 ± 11.0%. In the putamen, 5-HIAA showed a non-significant increase to maximal 112.5 ± 2.7%.

### 2.3.3. *Effect of rat exposure on 5-HT and 5-HIAA on day 2*

The curves for 5-HT and 5-HIAA on a second day of predator exposure greatly resembled those shown in Figure 24. The curves of day 2 (shown in Figure 25 for the hippocampus) for 5-HT practically overlapped the ones of day 1, but the curves of 5-HIAA lay lower on the second day in the hippocampus and caudate putamen. To compare the effects of rat exposure

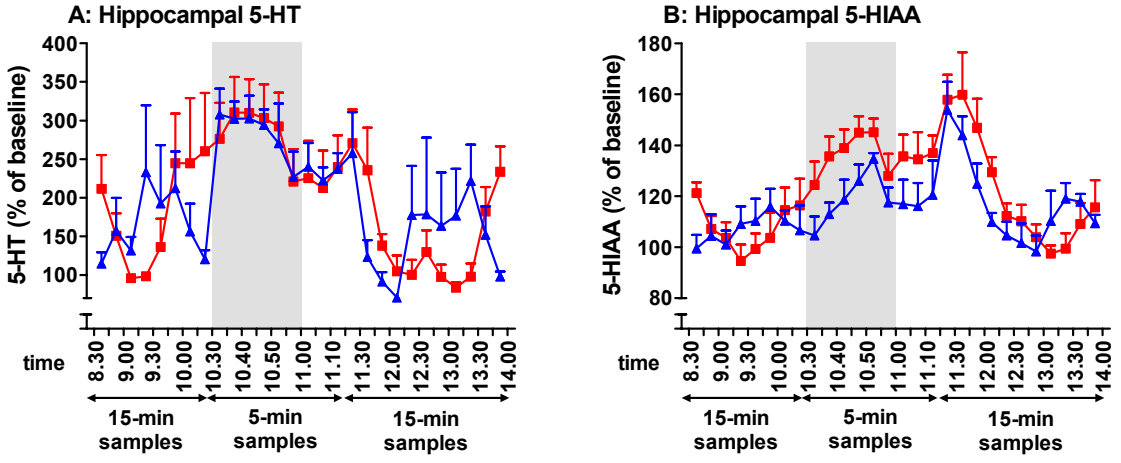



**Figure 25. Extracellular levels (average + S.E.M.) of 5-HT (panel A) and 5-HIAA (panel B) in the hippocampus of C57bl/6N mice (n=4) on day 1 (red squares) and day 2 (blue triangles). Figure serves illustrative purposes. Statistical analysis to compare day 1 to day 2 followed over analysis of the AUC, see Table 9.**

on day 1 and 2, the AUC of these curves were calculated both for the complete time curve, and for the part during rat exposure and the 15 minutes following it. These 15 minutes were added to the AUC, to account for effects on neurotransmission that might become visible with a temporal delay. The AUC are listed in Table 9. For all AUC effects of 'region' were found, which was not different from examining the curves themselves, as was done in Figure 24 for day 1. An effect of 'day' however was only found when considering the AUC of 5-HIAA during rat exposure. This indicated that on a second day of rat exposure, hippocampal 5-HIAA increased later or slower than on the first day.

**Table 9. AUC (in arbitrary units, average ± S.E.M.) for 5-HT and 5-HIAA in C57bl/6N mice, on two consecutive days of rat exposure. Also the significant *p*-values are listed for the effects of 'day' in the ANOVAs within region, when this was allowed based on an effect of 'region' in the overall ANOVA. In case the simple contrasts that were done afterwards also yielded a significant difference between day 1 and 2, the *p*-value is in italic. When the ANOVA did not indicate a significant effect of 'day' n.s. for non significant is listed. AUC for the hippocampus were based on n=4 because not all C57bl/6N mice with probes in the hippocampus had been subjected to the exposure twice, for the PFC on n=5 for a similar reason, for LS on n=7 and for the caudate putamen on n=5. Baseline levels were comparable to those measured on day 1.**

| Brain region | 5-HT: 8.30 – 14.00 hr | | | 5-HT: 10.30 – 11.15 hr | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | | Day 1 | Day 2 | |
| **Hippocampus** | 5286 ± 655 | 5327 ± 462 | n.s. | 1347 ± 297 | 1342 ± 151 | n.s. |
| **Prefrontal cortex** | 4076 ± 485 | 4202 ± 436 | n.s. | 839 ± 171 | 974 ± 235 | n.s. |
| **Lateral septum** | 3877 ± 367 | 4033 ± 231 | n.s. | 776 ± 142 | 837 ± 91 | n.s. |
| **Caudate putamen** | 2930 ± 131 | 2639 ± 150 | n.s. | 284 ± 46 | 177 ± 55 | n.s. |

| Brain region | 5-HIAA: 8.30 – 14.00 hr | | | 5-HIAA: 10.30 – 11.15 hr | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | | Day 1 | Day 2 | |
| **Hippocampus** | 3308 ± 46 | 3114 ± 34 | n.s. | 398 ± 46 | 277 ± 35 | *0.017* |
| **Prefrontal cortex** | 3014 ± 92 | 2984 ± 63 | n.s. | 304 ± 43 | 260 ± 38 | n.s. |
| **Lateral septum** | 3068 ± 121 | 3039 ± 114 | n.s. | 302 ± 49 | 285 ± 41 | n.s. |
| **Caudate putamen** | 2814 ± 29 | 2728 ± 63 | n.s. | 211 ± 16 | 147 ± 23 | n.s. |

## *2.4. Noradrenergic and dopaminergic neurotransmission in C57bl/6N mice*

### *2.4.1. Effect of rat exposure on day 1*

Whereas 5-HT and 5-HIAA could be measured simultaneously in the dialysate, a new set of experiments was performed to be able to measure NA with its metabolite MHPG, and the metabolites of dopamine, DOPAC and HVA. These compounds were only measured in the hippocampus.

Effects of time were present for NA, MHPG and HVA, but not for DOPAC, see Figure 26 (NA: $F(5,30) = 7.63$, $p < 0.0005$; MHPG: $F(5,30) = 4.85$, $p < 0.005$; HVA: $F(5,35) = 5.75$, $p < 0.001$ DOPAC $F(5,35) = 0.97$, $p > 0.05$).

Noradrenaline showed a steep increase at the beginning of rat exposure, peaking in the first sample during rat exposure with a level of 238 ± 11%. Levels were also still higher during the second half of rat exposure but then returned to baseline level. Its metabolite MHPG did not show such a steep increase, and during no time period the level was higher than under pre-exposure conditions. During the second half of rat exposure however, levels were slightly higher than during the first half.

Dopamine itself could not be measured (under detection limit), but its metabolites DOPAC and HVA could be detected in the dialysate. Rat exposure did not significantly influence the average levels of DOPAC, but HVA was increased during and briefly after rat exposure.

Not shown in Figure 26 are the results of the analysis of behavioural activity – for this effects of time were found and post-hoc contrasts indicated that the levels during rat exposure were higher than before or afterwards.

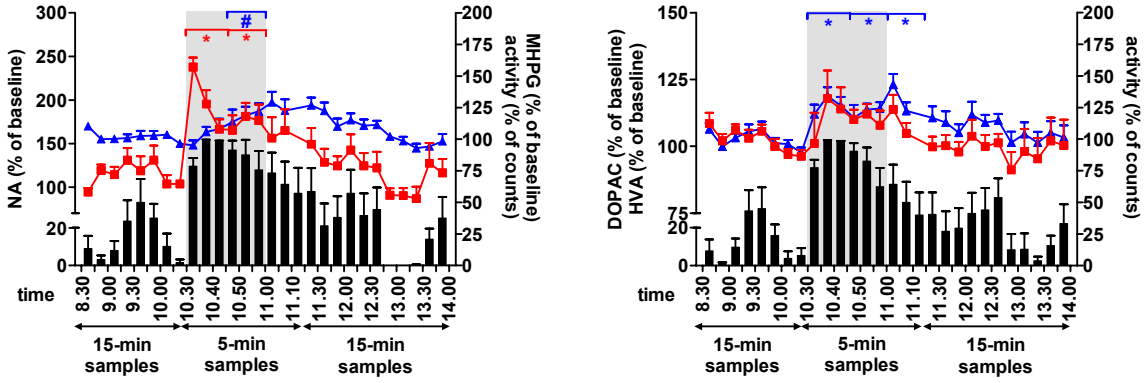



**Figure 26. Extracellular levels of noradrenaline (left panel, left y-axis, red squares, n=7), MHPG (left panel, right y-axis, blue triangles, n=7), DOPAC (right panel, red squares, n=8) and HVA (right panel, blue triangles, n=8) in the hippocampus of C57bl/6N mice. Baseline values were 0.48 ± 0.06 fmol/min for NA, 26.3 ± 1.8 for MHPG, 8.6 ± 0.8 for DOPAC and 20.8 ± 1.4 fmol/min for HVA. # indicates a significant difference between the first and second half of rat exposure (post-hoc simple contrasts, $p < 0.0083$). Other details as in Figure 24.**

### *2.4.2. Effect of rat exposure on day 2*

Table 10 shows that the AUC of the curves on repetition of the paradigm did not differ from the first exposure. The curves of day 2 (shown in Figure 27 for NA) practically overlapped those from day 1. Figure 27 also illustrates that the remarkable peak seen on day 1 with noradrenaline was also present on day 2, with a level of 212 ± 29%.

**Table 10 (next page). AUC (in arbitrary units, average ± S.E.M.) for hippocampal NA; MHPG, DOPAC and HVA on two consecutive days. None of the paired Student's *t*-tests comparing the values for day 1 with those of day 2 were significant. AUC were based on n=6 for NA, n=7 for MHPG, and on n=8 for DOPAC and HVA. Baseline values were similar to those encountered on day 1.**

| Compound | 8.30 – 14.00 hr | | | 10.30 – 11.15 hr | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | | Day 1 | Day 2 | |
| **Noradrenaline** | 3643 ± 213 | 3933 ± 244 | n.s. | 668 ± 112 | 810 ± 117 | n.s. |
| **MHPG** | 2921 ± 83 | 2869 ± 116 | n.s. | 246 ± 47 | 221 ± 61 | n.s. |
| **DOPAC** | 2781 ± 78 | 2782 ± 61 | n.s. | 234 ± 27 | 192 ± 36 | n.s. |
| **HVA** | 2914 ± 41 | 2849 ± 35 | n.s. | 249 ± 15 | 204 ± 23 | n.s. |

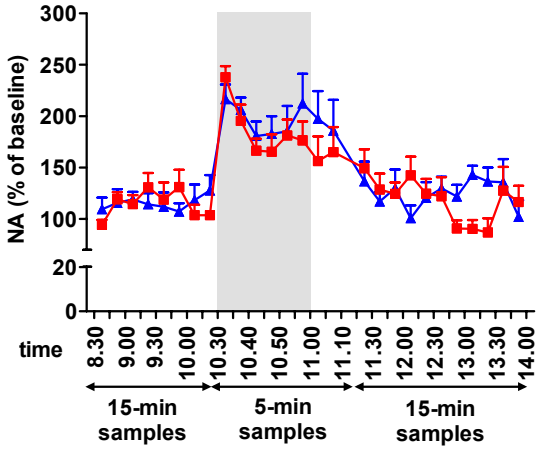


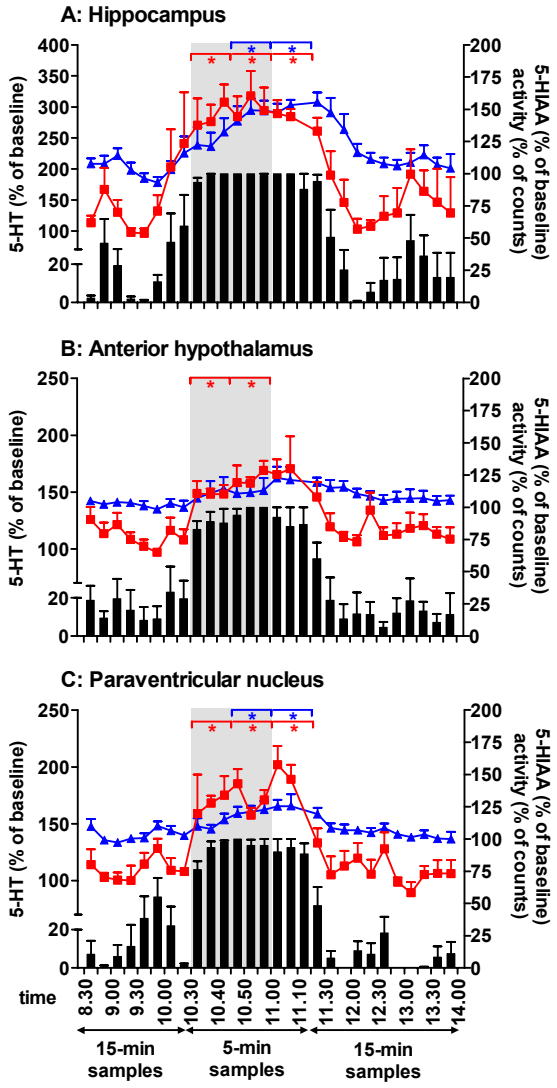

**Figure 27. Extracellular levels (average + S.E.M.) of NA in the hippocampus of C57bl/6N mice (n=6) on day 1 (red squares) and day 2 (blue triangles). Figure serves illustrative purposes. Statistical analysis to compare day 1 to day 2 followed over analysis of the AUC, see Table 10.**

## *2.5. Serotonergic neurotransmission in Balb/c mice*

### *2.5.1. Effect of rat exposure on day 1*

In Balb/c mice three brain structures were dialysed: the hippocampus, the anterior hypothalamus and the paraventricular nucleus. In the dialysate, levels of 5-HT, 5-HIAA, MHPG, DOPAC and HVA were measured. In the hippocampus, different animals needed to be used to measure 5-HT and 5-HIAA on the one side and the other compounds on the other side. In the AHP and PVN all could be measured in the same animals. Due to analytical difficulties however, it was not possible to measure 5-HT in all animals that were dialysed in the AHP or PVN. Therefore the n-number is lower for 5-HT in these cases than for MHPG, DOPAC and HVA. The results for 5-HIAA were chosen to match the individuals with successful analysis of 5-HT, and therefore also have a lower n-number. Levels of NA could not be measured in the AHP and PVN due to chromatographic problems. Also, from the placement of the microdialysis probes in Figure 22F and Figure 23B, it follows that some probes were located more in the medial, and others more in the lateral PVN. As no differences were found in the neurochemical results, these data have been pooled.

Figure 28 depicts the levels of 5-HT and 5-HIAA in the three structures, as well the behavioural activity. ANOVA did not indicate an effect of 'region' for the activity. Activity levels did change with time, but as these results were not different from those described above and depicted in Figure 19, no further details are given here.




Effects of 'region' however were found for 5-HT and 5-HIAA ($F(2,14) = 17.14$, $p < 0.0005$ and $F(2,14) = 5.43$, $p < 0.02$ respectively) as well as interactions of 'region by time' ($F(10,70) = 2.74$, $p < 0.01$ and $F(10,70) = 2.05$, $p < 0.05$ respectively), so the time course of these compounds was analysed separately for each region.

In all three regions effects of 'time' were present for 5-HT ($F(5,70) = 31.62$, $p < 0.0005$ in the hippocampus; $F(5,70) = 8.84$, $p < 0.0005$ in the AHP and $F(5,70) = 21.87$, $p < 0.0005$ in the PVN). Levels of 5-HT increased at the beginning of rat exposure to levels that were significantly higher than during pre exposure conditions. This increase was largest in the hippocampus

**Figure 28. Extracellular levels of 5-HT, 5-HIAA and behavioural activity in the hippocampus (panel A, n=5), anterior hypothalamus (panel B, n=6) and paraventricular nucleus (panel C, n=6) of Balb/c mice. Please note the difference in scaling of the left y-axis. Baseline values for 5-HT were 0.58 ± 0.10 in the hippocampus; 0.23 ± 0.05 in the AHP and 0.16 ± 0.03 fmol/min in the PVN. For 5-HIAA these were 156.5 ± 13.3; 147.0 ± 26.9 and 108.0 ± 10.9 fmol/min. Other details are as described in the caption to Figure 24.**

with a maximum level of 298.8 ± 35.2%, followed by the PVN with a maximum level of 171.3 ± 7.1%. In the first 15 minutes after rat exposure, levels stayed elevated in the hippocampus and PVN, before returning to levels that were no longer different from baseline. In the PVN it seemed as if the elevation in 5-HT fell off during the presence of the rat, to increase again after removal of the predator. In the AHP 5-HT was only higher during rat exposure. Post-hoc Scheffé tests (not represented in the figure) indicated that levels in the

hippocampus were different from AHP and PVN before rat exposure, during rat exposure and the 15 minutes after. Hippocampal levels were also higher than those in PVN in the last time period. Scheffé tests however did not indicate differences between the levels in AHP and PVN for any of the six time levels. An effect of 'time' was found for 5-HIAA in the hippocampus ($F(5,70) = 13.58$, $p < 0.0005$) and the PVN ($F(5,70) = 4.69$, $p < 0.001$), but just did not reach significance in the AHP ($F(5,70) = 2.32$, $p > 0.05$). In both former brain regions, levels of 5-HIAA were mildly increased during the second half of rat exposure, as well as in the 15 minutes following it. Again, the amplitude of the effect was largest in the hippocampus with a maximum of 150.8 ± 4.2% compared to 122.0 ± 9.2% in the AHP and 125.5 ± 5.9% in PVN. Here, post-hoc Scheffé tests showed that levels of hippocampal 5-HIAA were different from those in AHP during the second half of rat exposure, and from those in PVN in the second post exposure period.

### *2.5.2. Effect of rat exposure on day 2*

Whereas most curves looked the same on day 2 as on day 1, the levels of 5-HT in the hippocampus were higher during rat exposure on a second day of rat exposure, resulting in a significant effect of 'day' in the ANOVA (see Table 11 and Figure 29). Yet, a direct comparison of these AUC with paired *t*-tests, without taking data of the AHP and PVN into account did not yield significance. These higher levels were not seen in all animals, but in two out of the five that made up the average. In the AHP and PVN, curves did not look differently on day 2, as can be seen in Figure 29 as well.

**Table 11. AUC (in arbitrary units, average ± S.E.M.) for 5-HT and 5-HIAA in Balb/c mice on two consecutive days of rat exposure. The overall ANOVA indicated a significant effect of 'region', so ANOVA was repeated for each region separately. When the ANOVA within each region indicated a significant effect of 'day', the *p*-values for this effect are listed. When the ANOVA did not indicate a significant effect of 'day' n.s. for non significant is listed. The post-hoc test for 5-HT in the hippocampus was not significant. AUC for the hippocampus were based on n=5, in the AHP on n=9 and in the PVN on n=10. Baseline levels were comparable to those measured on day 1.**

| Brain region | 5-HT: 8.30 – 14.00 hr | | | 5-HT: 10.30 – 11.15 hr | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | | Day 1 | Day 2 | |
| **Hippocampus** | 5343 ± 430 | 6896 ± 538 | 0.024 | 1487 ± 186 | 2268 ± 248 | 0.038 |
| **AHP** | 3501 ± 152 | 3390 ± 125 | n.s. | 547 ± 47 | 540 ± 72 | n.s. |
| **PVN** | 3529 ± 131 | 4218 ± 586 | n.s. | 674 ± 45 | 1131 ± 462 | n.s. |

| Brain region | 5-HIAA: 8.30 – 14.00 hr | | | 5-HIAA: 10.30 – 11.15 hr | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | | Day 1 | Day 2 | |
| **Hippocampus** | 3316 ± 82 | 3371 ± 114 | n.s. | 418 ± 45 | 440 ± 50 | n.s. |
| **AHP** | 2947 ± 86 | 2813 ± 31 | n.s. | 239 ± 50 | 205 ± 23 | n.s. |
| **PVN** | 2955 ± 61 | 2968 ± 83 | n.s. | 269 ± 25 | 280 ± 35 | n.s. |

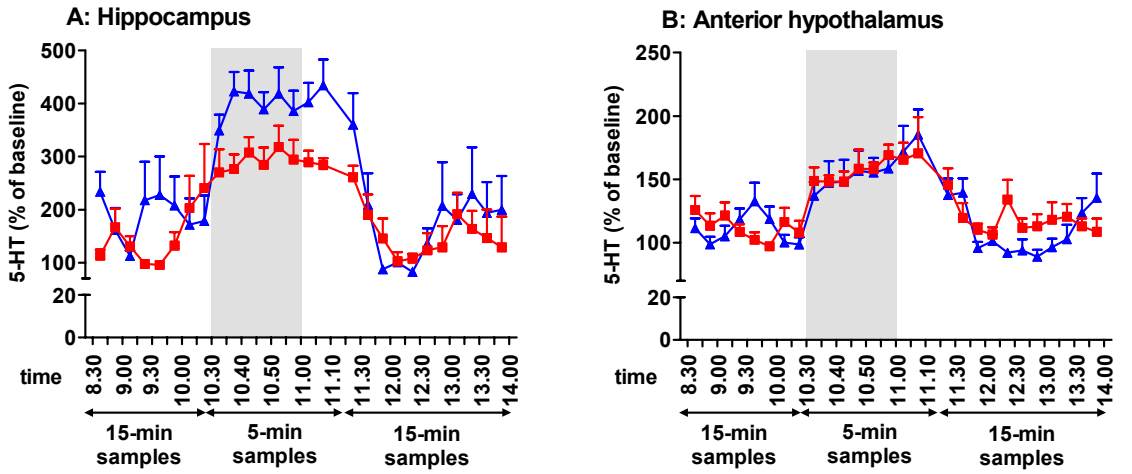



**Figure 29. Extracellular levels (average + S.E.M.) of 5-HT in the hippocampus (panel A, n=5) and the anterior hypothalamus (panel B, n=9) of Balb/c mice on day 1 (red squares) and day 2 (blue triangles). Figure serves illustrative purposes. Statistical analysis to compare day 1 to day 2 followed over analysis of the AUC, see Table 11.**

## *2.6. Noradrenergic and dopaminergic neurotransmission in Balb/c mice*

### *2.6.1. Effect of rat exposure on day 1*

The changes in the level of the metabolite of noradrenaline were similar among the dialysed regions, as depicted in Figure 30, and no effect of 'region' or of 'region by time' was indicated by the ANOVA ($F(2,21) = 0.01$, $p > 0.05$; $F(10, 105) = 1.49$, $p > 0.05$). Yet, an effect of 'time' was present ($F(5,105) = 33.35$, $p < 0.0005$). Levels of MHPG were higher during and in the two periods after rat exposure than prior to rat exposure. The levels in the second half of rat exposure were significantly higher than in the first. Although it was not allowed based on the results of the ANOVA to investigate this temporal effect for the regions separately, it is clear from Figure 30 that in the hippocampus MHPG was only clearly elevated above baseline level in the second half of rat exposure, where it reached a level of 128.8 ± 10.8%. It is also visible that the highest levels in the AHP and PVN are found in this period and the successive one, with respective maxima of 129.6 ± 6.0% and 129.0 ± 3.3. Unlike with 5-HT and 5-HIAA these amplitudes were of comparable size in the hippocampus and the two other structures.

Figure 31 shows the results for the metabolites of dopamine. For DOPAC and HVA as well, no effect of 'region' ($F(2,21) = 0.73$, $p > 0.05$; $F(2,21) = 2.33$, $p > 0.05$) or of the interaction 'region by time' ($F(10,105) = 1.06$, $p > 0.05$; $F(10,105) = 1.63$, $p > 0.05$) were found. ANOVA did not indicate an effect of time either for DOPAC ($F(5,105) = 1.00$, $p > 0.05$), but

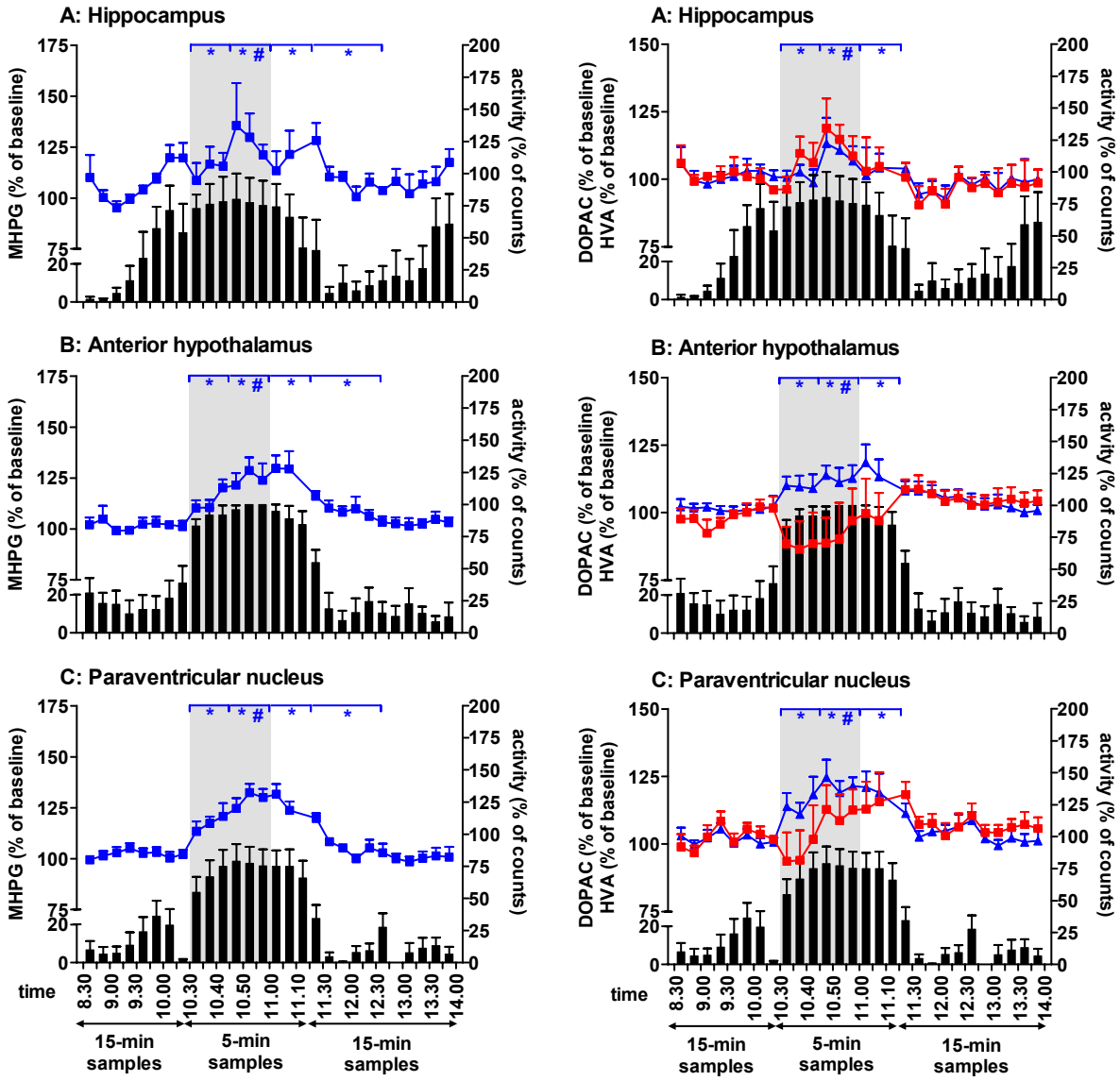



**Figure 30 (left). Extracellular levels of MHPG in the hippocampus (n=5), AHP (n=9) and PVN (n=10) of Balb/c mice. Baseline values were 14.9 ± 2.2; 13.9 ± 2.3 and 13.7 ± 1.0 fmol/min respectively. Other details as in Figure 24 and Figure 28.**

**Figure 31 (right). Extracellular levels of DOPAC and HVA in the hippocampus (n=5), AHP (n=9) and PVN (n=10) of Balb/c mice. Baseline values for DOPAC were 11.3 ± 1.7; 26.0 ± 4.6 and 27.5 ± 3.7 fmol/min for the hippocampus, AHP and PVN respectively. For HVA these were 25.8 ± 1.1; 59.1 ± 8.8 and 56.8 ± 5.7 fmol/min. Details as in Figure 24 and Figure 28.**

did so for HVA ($F(5,105) = 13.58$, $p < 0.0005$. HVA was slightly elevated during and shortly after rat exposure, especially in the latter half of the exposure. The maximal levels were comparable among the three regions. They were 110.2 ± 2.8% in the hippocampus, 115.9 ± 6.3% in the AHP and a bit higher in the PVN with 121.7 ± 3.7%. Although not significantly,

there seemed to be a tendency for a decrease in DOPAC at the beginning of rat exposure in the AHP, when a level was measured of 87.9 ± 8.8% and to a lesser extent in the PVN (96.4 ± 10.9%), but not in the hippocampus. After the paradigm, levels were not differing from those under baseline conditions.

### *2.6.2. Effect of rat exposure on day 2*

Curves for MHPG on the second day of rat exposure showed a tendency to increase less in the PVN, and to not increase until just after rat exposure in the AHP, resulting in an effect of 'day' for MHPG (no effect of 'region' $F(2,21) = 1.04$, $p > 0.05$). An effect of 'region by day' was found for HVA ($F(2,21) = 3.65$, $p < 0.05$ for the AUC of the full curve and $F(2,21) = 4.48$, $p < 0.05$ for the part during rat exposure). In the hippocampus, the AUC was less on day 2 for HVA according to the ANOVA, but a post-hoc direct comparison with paired *t*-tests was not significant (see also Figure 32). For DOPAC no differences between the two days were found. On day 2 a seeming decrease in DOPAC in the AHP was present as well.

**Table 12. AUC (in arbitrary units, average ± S.E.M.) for MHPG, DOPAC, HVA in Balb/c mice, on two consecutive days of rat exposure. Significant *p*-values are listed at the top of the column for the effects of 'day' for MHPG and 'day by region' for HVA in the ANOVA that compared all data combined. In case of HVA, ANOVAs within regions were allowed, and effects of 'day' per region are listed as well. The post-hoc test for HVA in the hippocampus was not significant. When the ANOVA did not indicate a significant effect of 'day' n.s. for non significant is listed. AUC for the hippocampus were based on n=5, in the AHP on n=9 and in the PVN on n=10. Baseline levels were comparable to those measured on day 1.**

| Brain region | MHPG: 8.30 – 14.00 hr | | | MHPG: 10.30 – 11.15 hr | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | 0.034 | Day 1 | Day 2 | 0.007 |
| **Hippocampus** | 3035 ± 104 | 3041 ± 125 | | 287 ± 51 | 260 ± 50 | |
| **AHP** | 3000 ± 56 | 2830 ± 30 | | 294 ± 26 | 218 ± 10 | |
| **PVN** | 2974 ± 44 | 2904 ± 52 | | 315 ± 22 | 267 ± 26 | |

| Brain region | DOPAC: 8.30 – 14.00 hr | | | DOPAC: 10.30 – 11.15 hr | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | n.s. | Day 1 | Day 2 | n.s. |
| **Hippocampus** | 2733 ± 84 | 2872 ± 71 | | 205 ± 33 | 253 ± 38 | |
| **AHP** | 2682 ± 80 | 2806 ± 52 | | 206 ± 31 | 178 ± 25 | |
| **PVN** | 2863 ± 89 | 2875 ± 82 | | 250 ± 35 | 232 ± 42 | |

| Brain region | HVA: 8.30 – 14.00 hr | | | HVA: 10.30 – 11.15 hr | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | 0.044 | Day 1 | Day 2 | 0.024 |
| **Hippocampus** | 2733 ± 37 | 2913 ± 70 | 0.027 | 180 ± 13 | 262 ± 40 | 0.012 |
| **AHP** | 2866 ± 60 | 2834 ± 48 | n.s. | 226 ± 29 | 215 ± 18 | n.s. |
| **PVN** | 2920 ± 60 | 2858 ± 62 | n.s. | 272 ± 28 | 248 ± 24 | n.s. |

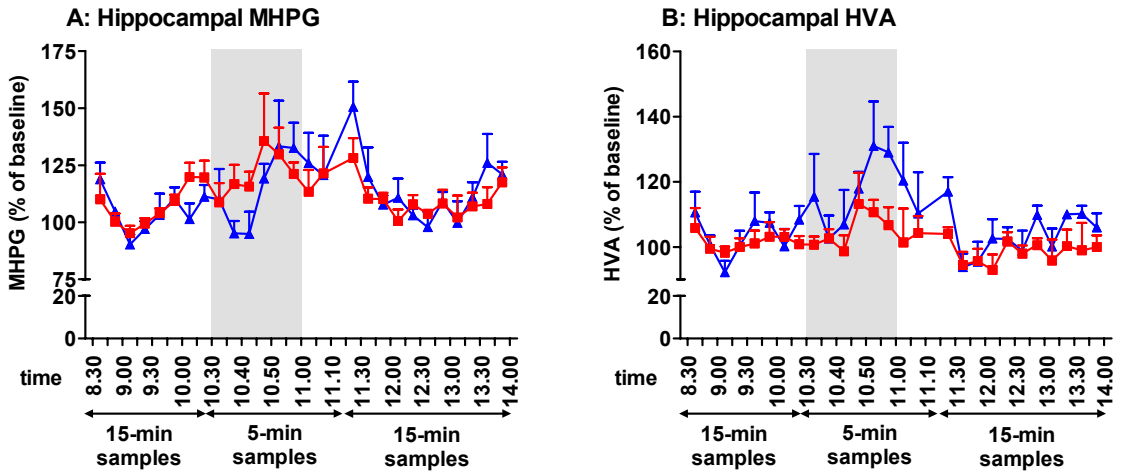



**Figure 32. Extracellular levels (average + S.E.M.) of MHPG (panel A) and HVA (panel B) in the hippocampus of C57bl/6N mice (n=5) on day 1 (red squares) and day 2 (blue triangles). Figure serves illustrative purposes. Statistical analysis to compare day 1 to day 2 followed over analysis of the AUC, see Table 12.**

### *2.7. Comparison of neurotransmission in C57bl/6N and Balb/c mice*

Because the microdialysis experiments were not primarily designed to compare the differential neurochemical effects of predator exposure on C57bl/6N and Balb/c mice, and the experiments with the Balb/c mice were performed after those with C57bl/6N mice, the neurochemical results for the two strains can not be compared statistically. Still, Figure 33 brings the results for the strains, that except for NA in Balb/c mice have been shown in previous figures, together to illustrate that only few differences in the neurochemical parameters existed between the two strains. This contrasts the differences that were seen when comparing the effect of rat exposure on behavioural parameters.

A strain difference only appears to be present in the extracellular levels of HVA that were higher in C57bl/6N mice. Differences were also seen in the absolute baseline values (see caption to Figure 33), with extracellular levels of NA being higher in Balb/c but levels of MHPG being lower.

Figure 33 also underlines the rapid increase in 5-HT and NA in both strains at the beginning of rat exposure, the latter having a clear peak in the first sample collected in presence of the rat. The metabolites of these neurotransmitters also increased, but slower and to a lesser extent. Where the levels of 5-HT and 5-HIAA in Balb/c stayed elevated for longer than in C57bl/6N mice, the same was true for the behavioural activity during that time.

Also the metabolites of dopamine increased somewhat during rat exposure in both strains, but very mildly.

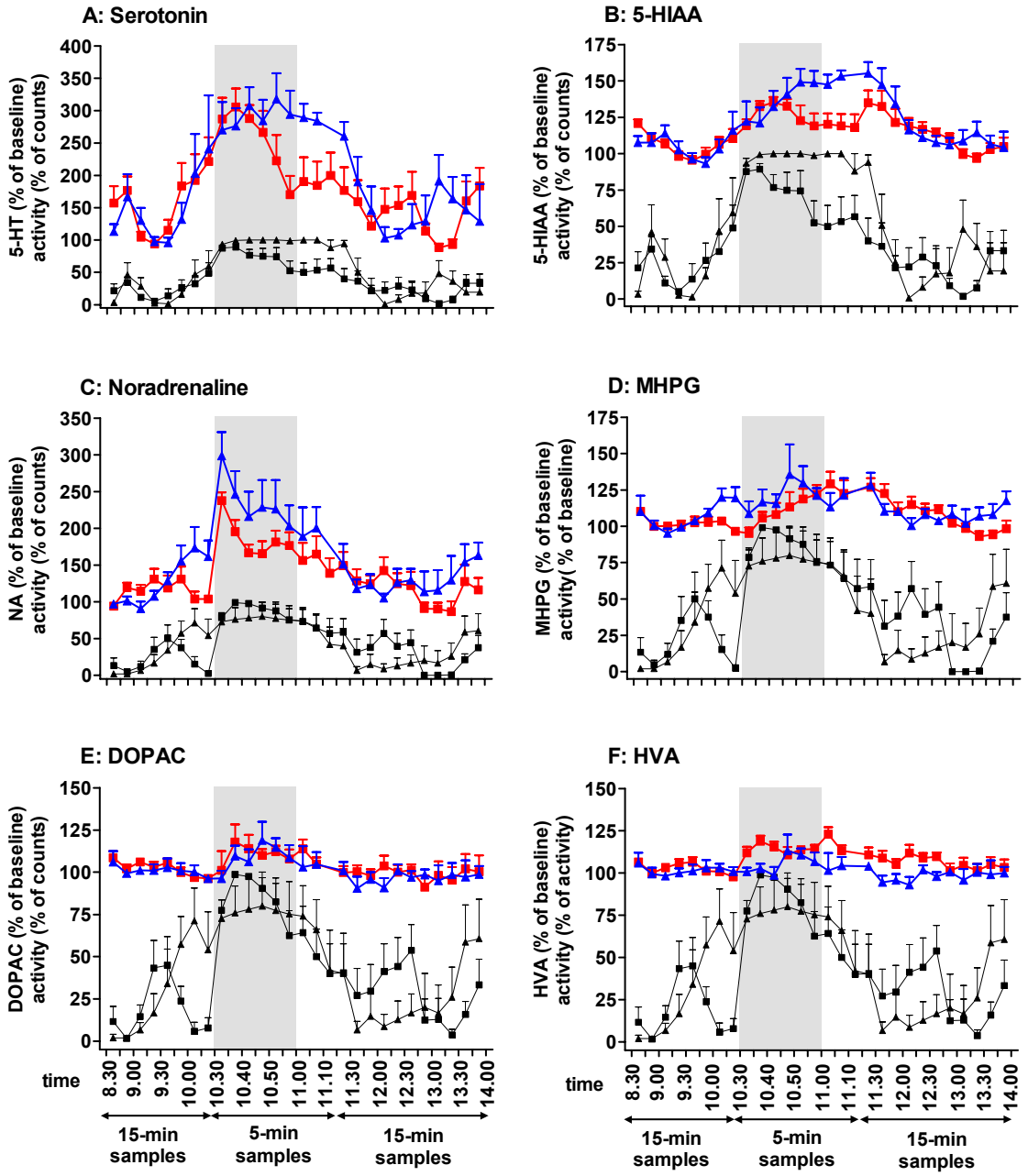



**Figure 33. Comparison of C57bl/6N (red curves, squares) and Balb/c (blue curves, triangles). Results for six compounds (coloured lines) and for behavioural activity (black lines) are depicted (average values + S.E.M.). Baseline values for C57bl/6N and Balb/c were 0.40 ± 0.09 (n=9) and 0.58 ± 0.10 (n=5) fmol/min for 5-HT; 151.2 ± 13.3 (n=9) and 156.5 ± 13.1 (n=5) fmol/min for 5-HIAA; 0.48 ± 0.06 (n=7) and 0.93 ± 0.15 (n=5) fmol/min for NA; 26.3 ± 1.8 (n=7) and 14.9 ± 2.2 (n=5) fmol/min for MHPG; 8.6 ± 0.8 (n=8) and 11.3 ± 1.7 (n=5) fmol/min for DOPAC; 20.8 ± 1.4 (n=8) and 25.8 ± 1.1 (n=5) fmol/min for HVA. Other details as described in the caption to Figure 24 and Figure 26.**

# 3. NEUROENDOCRINE DATA

## *3.1. Free corticosterone*

The dialysate of 14 C57bl/6N mice was used to determine the time curve of free corticosterone over the course of the experiment on the two days. The results are depicted in panel A of Figure 34.

Significant effects were found for 'time' ($F(6,78) = 3.80$, $p < 0.005$) and 'day' ($F(1,13) = 5.03$, $p < 0.05$), but not for the interaction 'time by day' ($F(6,78) = 1.75$, $p > 0.05$). Post-hoc tests showed that the corticosterone levels towards the end of the experimental day were higher than the levels during baseline. Also a modest increase could be seen in the levels of free corticosterone during and briefly after rat exposure on day 1. Overall, levels of corticosterone were lower on day 2 than on day 1, but post-hoc contrasts did not indicate a certain time period in which this difference was significant.

In the group of 14 animals interindividual differences were found, with some animals responding to rat exposure with larger increases in free

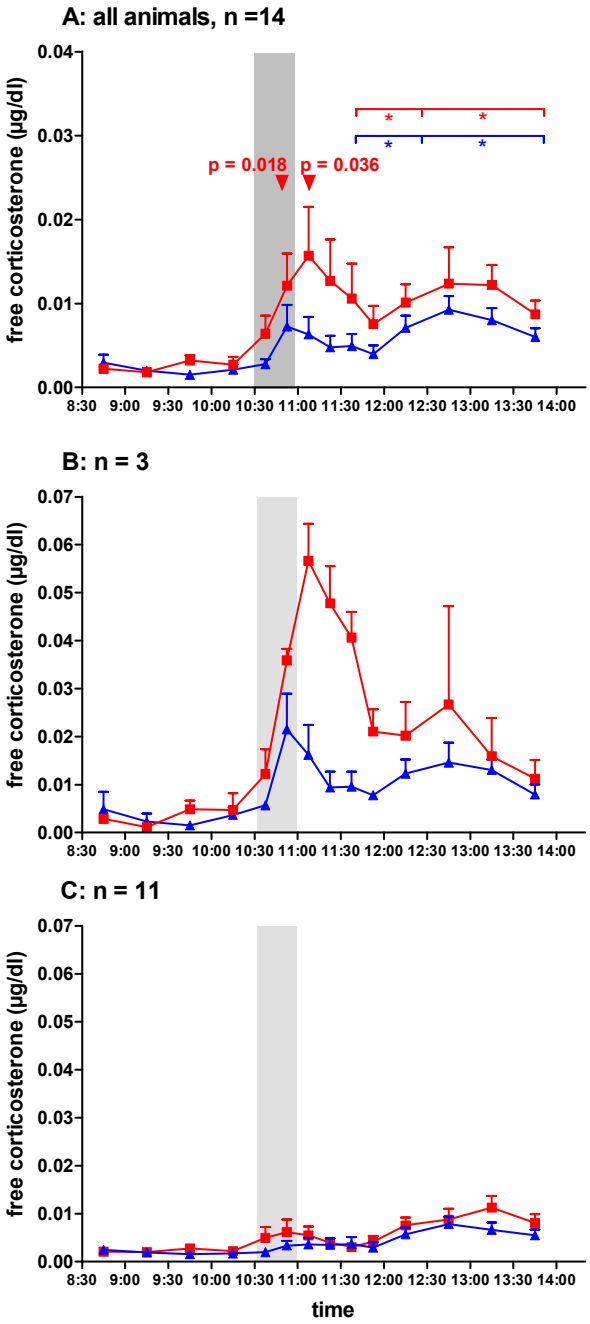



**Figure 34. Average (+ S.E.M.) free corticosterone – time curves for all C57bl/6N mice of which the dialysate was analysed (panel A, n=14) on day 1 (red squares) and day 2 (blue triangles) of rat exposure, as well as for 3 animals (panel B) of which the levels of free corticosterone clearly deviated from the other 11 animals (panel C). Please note differences in Y-axis scaling. Shaded area signifies the time frame during which the rat was present. Only the figure in panel A was analysed statistically. Asterisks indicate a significant deviation from baseline level (post-hoc simple contrasts, Bonferroni corrected for multiple comparisons, $p < 0.0083$).**

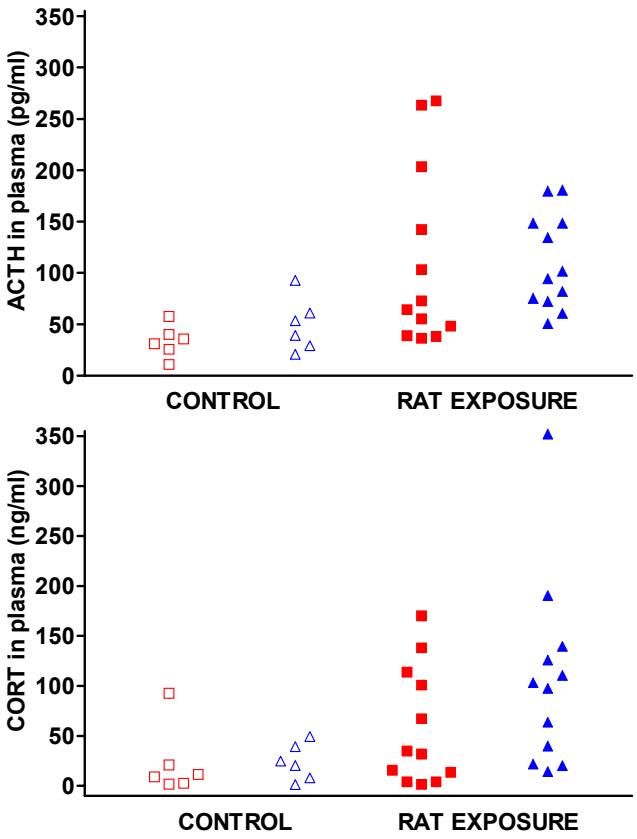



**Figure 35. Plasma levels of ACTH (top panel) and corticosterone (bottom panel) in C57bl/6N (red squares) and Balb/c (blue triangles) under control (open symbols, n=6) and rat exposure (closed symbols, n=12) conditions. See text for significant effects.**

corticosterone than others. The levels of free corticosterone in these two groups are illustrated in panels B and C of Figure 34. However, due to the low number of animals in the group with higher corticosterone levels, no statistics were done on this result. Nevertheless it can be recognised that in these animals the effect of rat exposure had less impact on free corticosterone levels when the paradigm was repeated on a second day.

A different situation was found when not the levels of free corticosterone, but the plasma levels of total corticosterone and of ACTH were measured in C57bl/6N and Balb/c mice (see Figure 35). Although interindividual differences could be noticed, on average, the levels of both corticosterone and ACTH were clearly higher after rat exposure than under control conditions. ANOVA showed a significant effect of 'exposure' on both ACTH ($F(1,32) = 20.00$, $p < 0.0005$) and corticosterone ($F(1,32) = 8.14$, $p < 0.01$). No effect of 'strain' or of the interaction 'exposure by strain' existed.

### 3.2. More strain differences – plasma values

After the finding that exposure leads to only mild increases in free corticosterone in C57bl/6N mice, whereas previous experiments with B6C3F1 mice had indicated larger effects, different strains of mice, known to differ in emotionality, were subjected to predator exposure or a control experiment and plasma ACTH and plasma corticosterone were measured. The results are presented in Figure 36. Control levels of ACTH and CORT were roughly the same for all strains, but not all strains showed an elevation in these hormones when they were subjected to rat exposure. C57bl/6J and DBA/2 mice did not respond with an increase in plasma concentrations for example. In the overall ANOVA an effect of 'exposure' was present ($F(1,50) = 15.43$, $p < 0.0005$ for ACTH and $F(1,50) = 7.05$, $p < 0.010$ for CORT). An

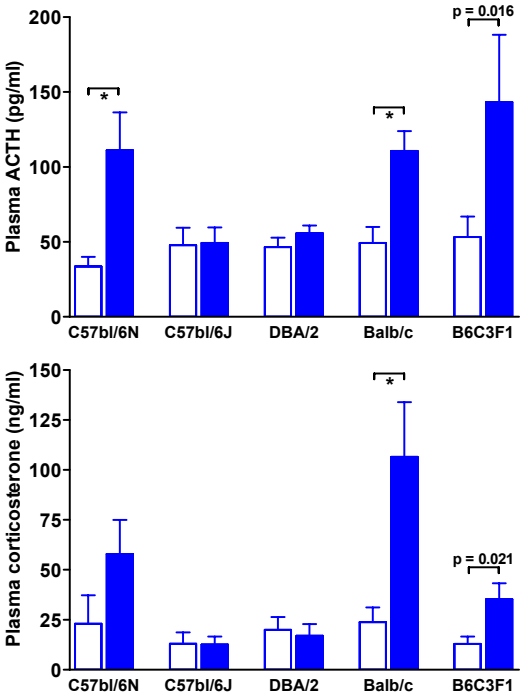



**Figure 36. Average (+ S.E.M.) plasma levels of ACTH (top panel) and corticosterone (bottom panel) for 5 strains of mice under control conditions (open bars, n=6) or after half an hour of rat exposure (closed bars, n=12 for C57bl/6N and Balb/c mice, n=6 for C57bl/6J, DBA/2 and B6C3F1 mice). Asterisks indicate a significant difference between control and exposed conditions (post-hoc simple contrasts, Bonferroni corrected for multiple comparisons, $p < 0.010$).**

effect of 'strain' was found for CORT with $F(4,50) = 3.22$, $p < 0.05$. In the case of ACTH this was $F(4,50) = 2.36$, $p > 0.05$. From Figure 36, however, is clear that both for ACTH and CORT, Balb/c were the most responsive mice. Also C57bl/6N and B6C3F1 mice seemed to show increased hormonal levels after rat exposure.

To investigate whether differences in hormone responses were reflected in behaviour, the behaviour during exposure was examined in the five strains. Results are shown in Table 13.

**Table 13 (next page). AUC (± S.E.M.) for all different behavioural parameters that were scored during rat exposure for five strains of mice (n=6 per strain). Behaviour was scored using the instantaneous Excel method and plotted in figures like in Figure 19. AUC were calculated and normalised so that an AUC value could be maximally 100, in case that behaviour had been exhibited throughout rat exposure. For freezing not the AUC was determined, but the total number of behavioural scores that indicated 'freezing'. Descriptions of behaviours can be found in Table 4 and Table 7. N.s. or * in the second column means that the ANOVA indicated a non-significant or significant effect of 'strain', in the latter case the results of homogeneous subsets according to Scheffé post-hoc tests are indicated in superscript behind the values of each strain.**

| | | C57bl/6N | C57bl/6J | DBA/2 | Balb/c | B6C3F1 |
|---|---|---|---|---|---|---|
| **GENERAL** | | | | | | |
| active | n.s. | 90.0 ± 5.0 | 86.6 ± 2.3 | 81.8 ± 9.2 | 97.5 ± 1.0 | 92.8 ± 2.6 |
| time close to wall | n.s. | 53.2 ± 10.6 | 25.1 ± 6.4 | 51.6 ± 17.2 | 55.6 ± 3.4 | 28.5 ± 6.2 |
| **INVESTIGATIVE** | | | | | | |
| freezing | * | $0.0 \pm 0.0^{1}$ | $0.7 \pm 0.7^{1}$ | $0.0 \pm 0.0^{1}$ | $0.0 \pm 0.0^{1}$ | $3.7 \pm 1.9^{1}$ |
| sniffing air | * | $30.1 \pm 3.1^{1,2}$ | $31.5 \pm 2.3^{2}$ | $14.9 \pm 2.3^{1}$ | $27.3 \pm 4.8^{1,2}$ | $32.0 \pm 3.4^{2}$ |
| stretching | n.s. | 0.3 ± 0.2 | 1.1 ± 0.7 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.4 ± 0.3 |
| rearing | * | $2.3 \pm 1.0^{1,2}$ | $1.3 \pm 0.5^{1,2}$ | $0.3 \pm 0.1^{1}$ | $4.2 \pm 0.4^{2}$ | $3.1 \pm 0.9^{1,2}$ |
| walking | n.s. | 1.9 ± 0.7 | 1.6 ± 0.4 | 0.6 ± 0.2 | 2.4 ± 0.7 | 1.4 ± 0.4 |
| sniffing wall | * | $23.2 \pm 5.9^{2}$ | $8.4 \pm 1.4^{1,2}$ | $5.5 \pm 1.7^{1}$ | $24.0 \pm 7.9^{2}$ | $9.3 \pm 3.4^{1,2}$ |
| sniffing bedding | n.s. | 15.6 ± 1.8 | 21.3 ± 2.7 | 16.8 ± 2.9 | 12.4 ± 3.6 | 13.7 ± 2.9 |
| defensive burying | n.s. | 0.0 ± 0.0 | 0.0 ± 0.0 | 5.8 ± 4.1 | 0.2 ± 0.1 | 3.0 ± 1.5 |
| **OTHER** | | | | | | |
| grooming | * | $7.2 \pm 1.9^{1}$ | $10.7 \pm 3.2^{1,2}$ | $28.6 \pm 8.2^{2}$ | $6.1 \pm 0.9^{1}$ | $7.2 \pm 2.0^{1}$ |
| food-related | * | $7.9 \pm 2.3^{1,2}$ | $7.6 \pm 3.2^{1,2}$ | $0.6 \pm 0.1^{1}$ | $20.6 \pm 4.0^{2}$ | $19.8 \pm 5.7^{2}$ |
| nesting, digging | n.s. | 1.6 ± 0.7 | 2.8 ± 0.9 | 8.7 ± 5.3 | 0.2 ± 0.1 | 1.7 ± 1.0 |

Strain differences were found for the following behaviours: freezing $F(4,25) = 3.00$, $p < 0.05$; sniffing air $F(4,29) = 4.57$, $p < 0.01$; rearing $F(4,29) = 5.25$, $p < 0.005$; sniffing separation wall $F(4,29) = 6.30$, $p < 0.001$; grooming $F(4,29) = 5.22$, $p < 0.005$ and food-related behaviour $F(4,29) = 5.95$, $p < 0.005$. For rearing, the distribution of the values matched those of hormone increases: strains with higher hormone levels also tended to show more rearing. They also seemed to show more sniffing at the separation wall and less grooming. Balb/c and B6C3F1 mice, both with higher corticosterone values during rat exposure than the other strains, also exhibited more food-related behaviour than the other strains. When the levels of behavioural intensities were compared to the levels of CORT in the same individuals, regardless of strain, no additional indications were found that certain behaviours were more intense with higher corticosterone levels, except for the aforementioned grooming, rearing and food-related behaviour.

For C57bl/6N, Balb/c and B6C3F1 it was decided to use a continuous scoring model as well to take another look at behavioural differences between the strains during rat exposure. Behavioural parameters as described in Table 7 were scored and are shown in Figure 37. Effects of 'strain' were not found for any of the parameters. This was partly due to the inclusion of the B6C3F1 strain in the analysis, because rearing was seen more often, and food-related behaviours were seen longer in Balb/c than in C57bl/6N mice when only these strains were compared. These results did not contradict those found in the behavioural scoring during the microdialysis experiments (see Figure 20 for example).

Also when the individual behavioural data were not sorted by strain but by height of the ACTH or CORT levels in the animals, hardly any correlations were found between hormone

levels and behaviour. An exception was the nosepoke frequency, for which an effect of the height of the CORT response was found ($F(2,17) = 3.72$, $p < 0.05$), with the lowest group of CORT values (less than 25 ng/ml) being associated with poking less often than the other two groups of CORT values.

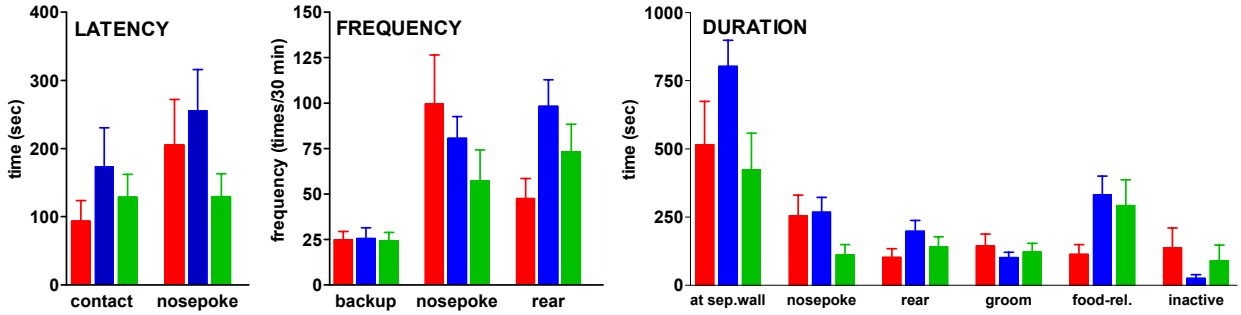



**Figure 37. Behavioural profile (averages + S.E.M.) of C57bl/6N (red bars), Balb/c (blue bars) and B6C3F1 (green bars) mice (n=6 per strain) during rat exposure.**

## 4. ADDITIONAL EXPERIMENTS

### *4.1. C57bl/6N mice bred at the institute*

Due to a preliminary experiment using transgenic animals that were bred in the institute, in which the wildtype animals showed very high corticosterone values (data not shown; experiment could not be repeated because too few animals were available), it seemed to be a good idea to examine whether the results for the experiments would be different when animals were bred in the institute. The first generation of C57bl/N mice that were born in the institute's animal facilities were subjected to a control experiment or to rat exposure.

Figure 38 shows that the hormone levels were higher in exposed than in non-exposed controls (effect of 'exposure' on ACTH: $F(1,15) = 13.34$, $p < 0.005$; on CORT: $F(1,15) = 6.93$, $p < 0.02$) .

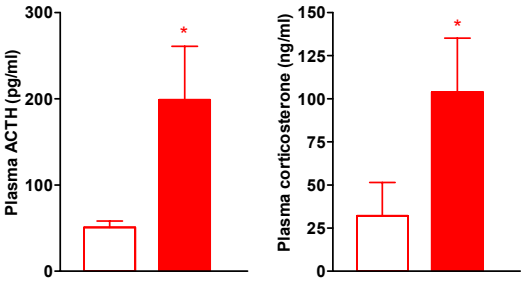



**Figure 38. Average (+S.E.M.) plasma levels of ACTH (left) and corticosterone (right) for C57bl/6N mice, born in the institute, with a control experiment (n=8) and after rat exposure (n=8). Asterisks indicates a significant difference between control (One-way ANOVA, $p < 0.05$)**

A comparison with Figure 36 indicates that both ACTH and CORT values after rat exposure lay higher (198.9 ± 62.0 and 103.9 ± 31.3) with the institute-bred animals, but still within the same range found with animals delivered from Charles River (111.2 ± 13.2 and 58.0 ± 17.0), as Student's *t*-test were not significant. Control levels were not different either.

Interestingly, differences in behaviour between the control and the exposed group were hardly seen. Only stretched-attend posture was observed with the exposed group but not with the control group. Due to the setup of this control experiment, in which an empty hand was inserted in the rat compartment, animals were alerted, and did not stay sleeping. Compared to the values of the C57bl/6N mice from Figure 37, these exposed mice were much more inactive (unpaired Student's *t*-test, $t = -2.20$, $p < 0.05$), and consequently, showed less often backups ($t = 3.21$, $p < 0.01$), nosepoking ($t = 3.89$, $p < 0.005$), and spent less time nosepoking ($t = 3.76$, $p < 0.005$) or at the separation wall ($t = 3.59$, $p < 0.005$).

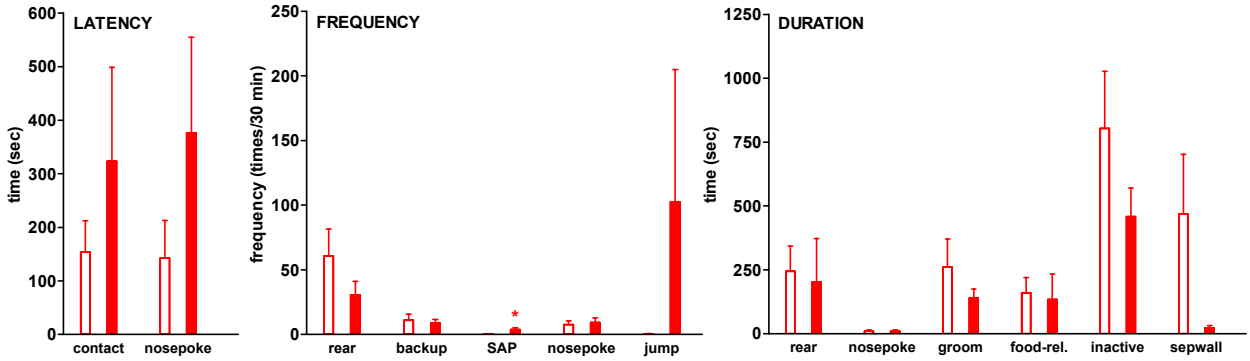



**Figure 39. Behavioural profile (average + S.E.M.) for C57bl/6N mice, born in the institute, under control (open bars, n=8) and exposure (closed bars, n=8) conditions. Asterisk indicates a significant difference between the two conditions (unpaired Student's *t*-test, $p < 0.05$).**

### *4.2. CRH-transgenic animals*

One experiment was performed with mice overexpressing CRH, which are thought to be more anxious than their wildtype littermates. Plasma ACTH was elevated in both Wt and Tg after exposure (effect of 'exposure' $F(1,20) = 8.17$, $p < 0.01$, no effect of 'genotype' or interaction). For corticosterone however, an effect of 'genotype' ($F(1,20) = 28.27$, $p < 0.0005$), 'exposure' ($F(1,20) = 4.69$, $p < 0.05$) and a significant 'genotype by exposure' interaction ($F(1,20) = 14.46$, $p < 0.001$) taught that Wt had elevated corticosterone levels

after exposure, but that Tg did not. Also, the control levels of Tg were higher than those of Wt, and even comparable to the exposed corticosterone levels of Wt (see Figure 40).

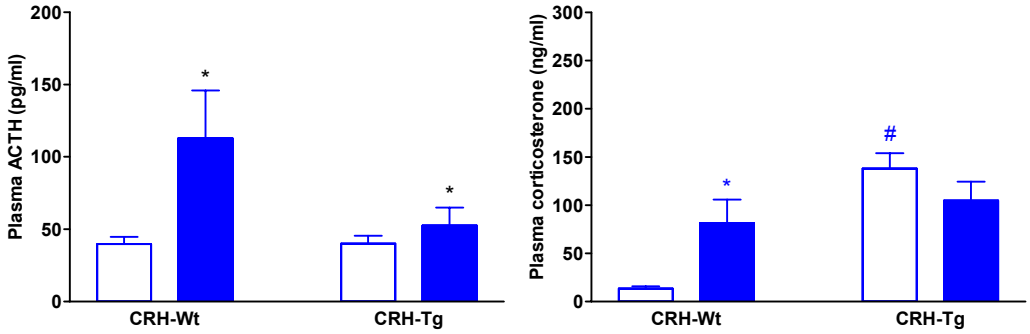



**Figure 40. Average ACTH (left panel) and CORT (right panel) levels in CRH-Wt (n=6) and CRH-Tg (n=6) after a control experiment (open bars) and after rat exposure (closed bars). Black * indicate that no effect of 'strain' was present and that exposed levels were different from control. Blue * indicates a difference with the control level of the same genotype (post-hoc simple contrasts, $p < 0.025$). # indicates a difference with the control level of the other genotype (Student's *t*-test, $p < 0.025$).**

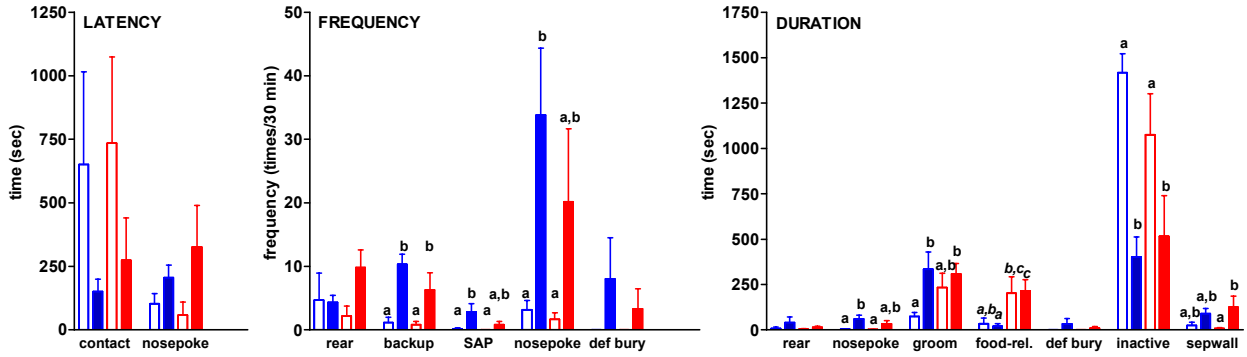



**Figure 41. Behavioural profile of CRH-Wt (blue bars) and CRH-Tg (red bars) under control (open bars) or exposure (closed bars) conditions. In all groups n=6. When ANOVA indicated a significant effect of 'exposure', the homogeneous subgroups according to Duncan post-hoc tests have been indicated in the figure with letters. In case of a significant effect of 'genotype', these letters are in italic (which is the case for food-related behaviour).**

Behavioural analysis of these animals revealed (see Figure 41) differences between the control and the exposed animals for backup ($F(1,3) = 20.40$, $p < 0.0005$), SAP ($F(1,3) = 6.28$, $p < 0.05$) and nosepoke ($F(1,3) = 9.83$, $p < 0.005$) frequencies, which were all seen more in exposed animals, as well as in the duration of nosepoking ($F(1,3) = 11.30$, $p < 0.005$), grooming ($F(1,3) = 6.00$, $p < 0.05$) and time spent at the separation wall ($F(1,3) = 7.20$, $p < 0.02$). Animals that were exposed to a rat were also more active than controls ($F(1,3) = 20.17$, $p < 0.0005$). Differences in this behavioural profile after rat exposure between the two

genotypes were small, but CRH-Tg tended to show less or less frequent SAP and nosepokes. Interestingly, a genotype difference was also found in the behaviour: transgenics spent more time engaged in food-related behaviours than their wild types, both under control and under exposure conditions ($F(1,3) = 9.80$, $p < 0.005$).

# IV. DISCUSSION

## 1. BEHAVIOURAL ASPECTS OF RAT EXPOSURE

### *1.1. Behaviour of C57bl/6N and Balb/c mice on day 1*

Introduction of a rat into the home cage changed the behavioural profile of C57bl/6N and Balb/c mice. The animals were asleep or mainly exhibited self-directed behaviours, such as grooming, food-related behaviour and nest-building, before and after rat exposure. During rat exposure and the 15 min afterwards however, the activity levels increased, and rather than self-directed behaviour, investigative behaviours were seen, such as sniffing in the air, stretching and sniffing at the separation wall. As a first reaction to the introduction of the rat, animals jumped to an opposite corner of the cage, in an attempt to flee from the rat, and oriented towards the rat compartment. From there, they started their risk-assessment activities, while keeping a close eye on the rat. This behavioural concatenation shows great resemblance with those seen in behavioural models of anxiety, for example in the Mouse Defense Test Battery, in which a mouse in an oval or closed-off runway is approached by a hand-held, anaesthetised rat. If there is an escape possibility, the mouse will flee, when not, it orients towards the threat, and starts risk-assessment behaviour [28]. Risk assessment serves to gain information about the anticipated threat. Especially when the nature and location of the threat are uncertain, approach and investigation of the possible danger is part of the behavioural repertoire of mammals [28]. In addition, risk assessment behaviour, especially stretching, is sensitive to anxiolytic drugs [19, 23, 28, 118, 267], and is therefore an indication of arousal, stress or even anxiety. Another clear sign of anxiety would have been freezing. However, unlike with previous experiments using B6C3F1 mice [174], jumping away from the rat compartment, a sign of anxiety by itself, was not followed by freezing in the C57bl/6N and Balb/c mice. An explanation for this could be in the distinction that Koolhaas *et al.* [159] make between two coping styles that species can exhibit under stressful conditions, a proactive and a reactive form. These differ in behavioural characteristics, e.g. freezing is found with the reactive coping style, whereas defensive burying is a characteristic of proactive coping. Possibly, C57bl/6N and Balb/c mice tend to more proactive coping styles than B6C3F1 mice. The absence of freezing would be in line with that assumption. On the other side, defensive burying, which under the present rat exposure paradigm would take the form of heaping up bedding against the separation wall, was not observed. A behaviour that might be considered to be related to defensive burying however, frantically digging in the

bedding close to the separation wall, has been seen extensively. The attribution of behavioural phenomena such as freezing to either proactive or reactive coping styles is not rigid. Also proactive animals can manifest freezing, for example in the absence of saw dust [159].

After a first intensive phase of risk-assessment, these behaviours became less intensive. This happened earlier in the C57bl/6N mice than in the Balb/c mice. Most animals exhibited a brief period of intense grooming, and sometimes of nest-building before they returned to a behavioural state that was no longer indicative of stress (i.e. sleeping, or mostly involving self-directed behaviour). The transitional state might be characterised as a state in which the animals were coping with the novel situation at hand. Grooming is considered a sign of de-arousal and coping after stress [62, 298, 316, 328]. Also nest-building has been mentioned as a behaviour related to coping [159, 295]. Based on the observation that food-related behaviour was seen more intensively in Balb/c just after the rat exposure than during any other time of the experiment, it is tempting to hypothesise that in this strain food-related behaviour is also a sign of de-arousal, and as such comparable in function to grooming and nesting. The behaviour may have an analogy with binge-eating in humans.

The brief revival in overall activity and sniffing the air seen in C57bl/6N mice when the rat was removed from the cage was probably due to the close presence of the experimentor, and to the necessity to now adapt to a situation without a rat. Taken together, the observed behavioural changes, some of which are reminiscent of those seen in anxious situations, indicate a slightly anxious, clearly arousing effect of rat exposure.

### *1.2. Behaviour of C57bl/6N and Balb/c mice on day 2*

Mice were re-exposed to a rat on the consecutive day to find out whether the mouse had learned from the previous experience and was less aroused or had become sensitised and would be more anxious. On the second day, a behavioural pattern unfolded that was highly similar to the one of the first day. Still, differences could be recognised. Overall, sniffing in the air, rearing, walking and sniffing at the separation wall (for C57bl/6N) were seen less, behaviours that are part of the risk assessment stage of the behaviour sequelae during and briefly after rat exposure. Groom and nest behaviour, indicative of coping, were more present on day two, especially briefly after rat exposure, as well as food-related behaviour. Although these differences were small, they may show a better coping with the situation after the second exposure. Also cat exposure only very slowly, after multiple sessions, changed the exhibited behaviour in rats [30].

Data in the literature show very diverse effects of a previous stressor on behaviour during a subsequent stressor. However, decreases in risk assessment and increases in self-directed behaviours are generally interpreted as decreased anxiety. Concerning risk assessment, it was found that DBA/2 mice after social isolation showed reduced risk assessment on the elevated plus maze. This was carefully interpreted as a reduction of anxiety [97, 269]. Also in T1 mice, a novel environment experience decreased anxiety [269]. These reports of decreased risk assessment after a prior stressful experience are contrasted by reports that DBA/2 mice on the other hand were more anxious on the elevated plus maze after a novel environment stress [269], social defeat or prior experience with the plus maze [268]. Also Blanchard *et al*. report that for example in situations in which a predator has been encountered before, risk assessment is elicited maximally [28, 31]. Differences in grooming were seen in Wistar rats, where repeated restraint stress increased, but mild chronic stress reduced grooming, of which both effects were prevented with the antidepressant desipramine [65]. Taken together, the effect of previous stress, is very much conditional upon strain, but also on the nature of the previous stressor, and on the frequency of prior exposition to stress. One report was found on repeated rat exposure, by Grootendorst *et al*., who exposed mice repeatedly to a rat over a period of two weeks, after which several tests were performed. These stressed C57bl/6 mice showed increased locomotor activity levels and defecated less when submitted to the circular hole board or the light/dark box [125]. These results seem to indicate a decrease in emotionality after repeated rat-exposure. In our case, the small differences in coping and risk assessment behaviour might also indicate a decrease in emotionality. The increase in food-related behaviour as well can be interpreted as an indication that the animals were less anxious. Although not often done nowadays, it used to be common to investigate the effect of potential anxiolytics on consummatory behaviour. Anxiolytics were found to increase food intake, regardless whether a fearful stimulus was present as well [321]. The same principle is found in the Vogel's punished drinking test, in which anxiolytics restore the amount animals drink after having associated drinking with an electric shock.

Concluding, re-exposure of mice to a rat resulted in small shifts in behaviour that seem to indicate a reduction in anxiety and arousal.

### *1.3. Behavioural strain differences*

The differences observed between the two strains could reflect genetic differences relating to emotionality or learning. Balb/c mice spent more time with risk assessment activities. The increased levels of grooming and nesting were seen later in this strain than in C57bl/6 mice.

This could indicate that Balb/c experienced more anxiety than C57bl/6N mice. Using a light/dark test, Balb/c mice were found to be highly reactionary, spending by far the least time in the lit compartment whereas C57bl/6 were found to be intermediate reactionary [119]. Other tests however found that Balb/c mice are less anxious than C57bl/6 mice. Balb/c mice showed high levels of open arm entries on the EPM, indicating low levels of anxiety, in comparison to C57bl/6 [272]. Another study, examining 16 strains of mice even found that C57bl/6 mice were among the most anxious on the EPM [322]. On the other hand, the EPM data were not confirmed by results in the open field test, where both strains spent a similar period of time in the central arena, an index for similar levels in emotionality [272]. From test batteries it also has become apparent that C57bl/6 mice are good learners, whereas Balb/c mice are impaired learners when it comes to spatial learning [60, 272], although the performance in contextual fear conditioning does not indicate a large difference [60, 286]. Still, it could be that better general cognitive capabilities of C57bl/6 mice enable them to understand faster that they have little to fear from the rat in the rat exposure paradigm. This might also contribute to the explanation why C57bl/6 mice showed a faster onset of coping behaviour when compared to Balb/c mice.

That strains differ behaviourally is also apparent from the results obtained from subjecting five different strains to rat exposure. Differences were seen with freezing, sniffing the air or at the separation wall, rearing, grooming and food-related behaviour. B6C3F1 mice spent little time sniffing at the separation wall, and they also exhibited freezing, as has been described before [174]. These observations indicate an anxious reaction to the presence of the rat and could be indicative of a reactive coping style as described before. Low levels of sniffing behaviour and rearing were seen with DBA/2 mice, whereas they exhibited high levels of grooming. Contrasting the results with B6C3F1 mice, this could mean low levels of arousal in DBA/2 mice. This would fit with the findings that they are known as impaired learners [60, 286], show low levels of acoustic or tactile startle [60], but are intermediate responders in a light/dark paradigm [119]. On the other hand, DBA/2 mice exhibited defensive burying behaviour, which is seen in response to aversive stimuli [321]. This strain has also been reported to be irritable, vocalising during handling, and to be anxious on the EPM [272].

In addition, it should be noted that within a strain, sublines may not respond similarly. This is reflected in different levels of sniffing at the separation wall between C57bl/6N (a subline bred by Charles River) and C57bl/6J (a subline bred by Jackson) mice. In general, the variance in behavioural parameters may be larger in the Charles River line (personal

communication with I. Sillaber). The Jackson line was reported to spend more time burying in the home cage (a spontaneous behaviour) and made more entries in the illuminated part of a light/dark box [329], whereas also differences in exploratory behaviour have been reported [63]. Subline differences were reported for behaviour in the open field with the 129 strain too [217]. As an additional consideration, even when the same (sub)strain and the exact same procedure would have been followed, results may still depend on the testing site, as was found by Crabbe *et al.* [61], who subjected several mouse strains to standardised tests in three different laboratories.

From the above it becomes clear that first, it is not possible to give definite statements on an animals emotionality based on one behavioural test, as the rank orders for strains too often differ among paradigms. This has to do with the fact that different drives are determining the behaviour of mice for parameters that are measured in the various anxiety paradigms. Factor analysis revealed for example that on the EPM the urge to explore is important, whereas neophobia and locomotion are more important in light/dark paradigms [21].

Secondly, various strains, known to cover a wide scala of emotionality and cognitive abilities, exhibited only small differences in the various behavioural parameters. Unless extensive further factor analysis would reveal that the combination of small differences in several parameters suffices to discriminate the typical properties of the strains, it appears that rat exposure, as applied here, may not be a powerful enough model to screen for such inherited differences.

## 2. NEUROCHEMICAL ASPECTS OF RAT EXPOSURE

### *2.1. Serotonergic neurotransmission*

#### *2.1.1. Effects of stress on 5-HT and 5-HIAA*

During rat exposure, levels of 5-HT and its metabolite 5-HIAA increased in three out of four brain areas dialysed in C57bl/6N mice, i.e. the hippocampus, the prefrontal cortex and the lateral septum. In a fourth region, the caudate putamen, significant increases were absent. In Balb/c mice as well increases in 5-HT were found in the hippocampus. Also in the two other dialysed structures of Balb/c mice 5-HT levels increased during rat exposure, but these were smaller in the PVN than in the hippocampus, and even less in the AHP. In this mouse strain increases in 5-HIAA were found in the hippocampus and the PVN, but not in the AHP. The largest effect of rat exposure on 5-HT and 5-HIAA in both strains was observed in the hippocampus. In both C57bl/6N and Balb/c mice the hippocampus was dialysed and 5-HT

and its metabolite had been measured. Similar baseline levels and rat exposure-induced increases in 5-HT and 5-HIAA were found in the hippocampus of the two strains of mice.

Previous results from our laboratory are in line with these findings. In that study [174] extracellular levels of 5-HT and 5-HIAA in the hippocampus of B6C3F1 mice were also found to increase as a consequence of rat exposure. In this case, the results of Linthorst *et al.* could be compared to the current findings, because microdialysis was used, and the setup of the experiments was similar to the one reported in this thesis. However, not many other studies combined predator exposure with measuring neurochemical parameters. Those that did used post-mortem tissue, or in case of microdialysis, did not have the same time-resolution as was used in the current study. Two other factors also confound the comparison of the present serotonergic results to literature findings. First, it has often been reported that 5-HT levels in various brain regions as well as the firing rate of raphe 5-HT neurons are highly correlated with behavioural activity [174, 178, 277, 277, 278, 292, 312], which is also described in this thesis. Nevertheless, remarks on the activity state of the animal at the time of microdialysis sampling are often omitted in the description of results, thus making it hard to attribute increases in levels of 5-HT to behavioural activity, stress, or a combination of both. Another confounding factor is that in some microdialysis studies an SSRI is added to the perfusion liquid to artificially increase levels of serotonin in the dialysate (eg. in [278], see also [277]). The way in which such an addition interferes with the physiological neurotransmission processes is unclear. In the present studies, no SSRI needed to be used, because the highly sensitive analysis method that was used, was able to measure in the physiological femtomol range of extracellular levels of serotonin.

Taking the above precautions into account, most other predator exposure studies measuring serotonin in various brain regions were in line with our observations in C57bl/6N and Balb/c mice and with the findings in B6C3F1 mice [174]. Hayley *et al*. [132] for example, measuring neurochemical parameters in brain micropunches, also found increases of 5-HIAA in the hippocampus and prefrontal cortex of both C57bl6/J and Balb/c mice when animals were killed 20 minutes after the end of rat exposure. On the other hand, no increases in 5-HT were found. In Swiss-Webster mice, killed directly after a 5-min exposure to a cat, 5-HT and 5-HIAA levels were not significantly increased in hippocampus, hypothalamus or striatum [19]. Although in these studies 5-HT and 5-HIAA increases were not or not always seen, it must be noted that also in the paradigm in the present study, 15 minutes after the rat exposure, neurotransmitter levels were no longer higher than under pre-exposure conditions.

One microdialysis study exists in which rats were exposed to a cat. During cat exposure, rats showed significant increases in alert waking, and 5-HT was significantly, around 30%, higher than baseline levels in hippocampus, prefrontal cortex, amygdala and also in the striatum, a structure where we found no significant changes in 5-HT levels. The reported increase was somewhat, but not significantly, larger in prefrontal cortex, followed by hippocampus and striatum. For 5-HIAA only significant increases of about 8% were seen in hippocampus and amygdala [278]. That the percent increases in that study are less than found in our experiments, can be explained by a different definition of 'baseline'. In our study baseline levels were calculated by only taking into account those samples, during the collection of which the activity was equal to or lower than 10% of the sample duration. When baseline neurotransmission is based upon a fixed number of samples, regardless of behavioural activation, the average level may be higher, and increases relative to that therefore smaller.

From the above follows that after predator exposure, often increases are found in 5-HT and 5-HIAA in the hippocampus and prefrontal cortex, whereas results are less unambiguous for structures like the striatum and hypothalamus.

Also other stressors have been described to lead to changes in 5-HT and 5-HIAA. In the hippocampus, stress induces increases in extracellular levels of 5-HT and 5-HIAA. This has been shown for example repeatedly in mice and rats after swim stress [108, 179, 230, 245, 278], although this was not always the case [4, 154]. In both of these latter studies however, baseline levels of 5-HT were calculated without correcting for the behavioural activity at the time of sampling. It is therefore unclear in how far the deviating results can be attributed to that. Apart from swim stress, stressors that involve the activation of pain pathways like with tail pinching and electrical shocks caused increases in 5-HT and its metabolite [108, 127, 278]. In addition, purely physical stress, such as stimulation of the immune system by lipopolysaccharide or cytokines had 5-HT neurotransmission stimulating effects [175, 178, 242] in the hippocampus. The magnitude of the increases that are found vary, but are mostly in the range of 130 to 300% of baseline 5-HT. These variations are hard to contribute to the severity of the stressor and of course depend greatly on the definition of baseline. Therefore it is not possible to draw conclusions about the severity of rat exposure based on the elevations that were seen in 5-HT and 5-HIAA.

Not only does literature confirm that stress leads to elevations in serotonergic indices in the hippocampus, the same holds true for the prefrontal cortex. In this structure as well, almost all studies report an increase after swim stress ([58, 108, 278], again with the exception of [4, 154]), tail pinch [108, 244, 278], shocks [130] and immune stress [91, 164].

Contrasting the abundance of studies investigating the effects of stress in the hippocampus and cortical areas, is the limited amount of studies involving microdialysis of the lateral septum. The group of Lucki *et al.* found a decrease in 5-HT and 5-HIAA in rats that were forced to swim for 30 minutes [154, 251], whereas we saw a mild increase during predator stress. As mentioned above, differences in baseline definition make it hard to compare the results.

Also for the striatum, where we saw no increases in 5-HT or 5-HIAA, different findings have been published for other stress paradigms. Forced swimming lead to increases in 5-HT [154, 278], but in the latter study also to a concomitant decrease in 5-HIAA. Tail pinch increased extracellular levels of 5-HT [278], but was not found to have affected the turnover of 5-HT when analysed in post-mortem tissue [244]. A decrease in striatal levels of 5-HT was seen after restraint stress in mice, but not in rats [158].

The hypothalamic region as well is a region in which conflicting effects of stress on serotonergic parameters are reported. Tail pinch did not affect the turnover of 5-HT [244], and after foot shock increases in the turnover have been reported [89] that sometimes occurred only in case of a strong shock, but not after a mild shock [142]. Also immune stress did not reliably lead to increases in 5-HT or its turnover (increase in [164], but not in [59, 91]).

All in all, the finding in the present study that predator stress increases extracellular levels of 5-HT and 5-HIAA in a brain-region specific manner is underlined by brain-region specificity of the effects of predator stress by other groups and of other stress paradigms. A differential role of 5-HT in these brain regions presents itself as an explanation for this observation. Also differences in the innervation of these brain regions could explain such findings.

### *2.1.2. The role of 5-HT in various brain regions*

One hypothesis concerning the role of 5-HT was put forward by Barry Jacobs and colleagues [277]. They hypothesised that, as behavioural activity correlates to such a high degree with 5-HT levels, the main function of 5-HT resides in the ubiquitous facilitation of motor output and the coordination of concurrent autonomic and endocrine responses [145, 277]. Some findings in the present study are not in line with this hypothesis. For one thing, in the caudate putamen, a region highly involved in the coordination of movement [149, 352], levels of 5-HT did not vary with behavioural activity. Also in the PVN and AHP, such a correlation was largely absent. In addition, other studies have shown brain region dependent increases in 5-HT that were not paralleled by behavioural activation. Immune stress for example increased

hippocampal but not preoptic levels of 5-HT in absence of behavioural activation [176, 178]. Linthorst *et al.* also described a dramatic increase in hippocampal 5-HT in diving rats during a swim stress paradigm. These levels of up to 1500% of baseline were much higher than found during other periods of behavioural activity, and could be related to a panic-like state [179]. Thus it seems that 5-HT is indeed increased during states of higher behavioural activity, but that this is especially true for limbic areas, like the hippocampus, prefrontal cortex and lateral septum, and not necessarily for a structure like the caudate putamen. The first three structures have in common that they are all part of the extended Papez-circuit [107, 205] and as such play a role in the generation and regulation of emotions [107, 150, 346]. The hippocampus and prefrontal cortex seem to be specifically involved in the more mnemonic or cognitive aspects of regulating emotion. Although the ventral striatum as well plays a role in emotion, it is part of the reward pathway and therefore especially important in the response to reinforcers [47, 225, 248]. It is feasible that this pathway is not activated during rat exposure, whereas the pathways involving the hippocampus and prefrontal cortex are. In this respect it would also be interesting to investigate the effects of predator exposure on neurotransmission in the amygdala, which plays a role in the appraisal of a stimulus and in anxiety [224, 248].

The PVN is a region that is involved in coordinating and executing the stress response. It is tempting to speculate that the increases in 5-HT in this region are related to the mild HPA axis stimulating properties of predator exposure. Also it appeared as if an elevation of extracellular 5-HT in the PVN was seen twice during the experiments: once at the beginning of rat exposure and once at the beginning of the post stress period. As in both cases a manipulation took place (i.e. placing and removing the rat from its compartment respectively), this would be time points at which an HPA axis response could be initiated. However, these observations are too preliminary to make definite statements.

The anterior hypothalamus is a region that is implied in thermoregulation. Internal body temperature is monitored by temperature-sensitive cells in this region and changes in autonomic nervous system activity, endocrine secretions, and behaviour can be initiated by the AHP to aid in thermoregulation. Serotonin appears to play a role in this and can cause hyperthermia [170, 171]. Apart from this, the AHP also has a role in rat offence behaviour, such as biting and kicking [3]. As there were no signs of offence behaviour, and an effect of rat exposure on body temperature is not likely, it may not be surprising that only very mild increases were found in this area.

The above explains how the results as they were found for serotonin could be explained in the light of the brain areas in which it was measured. However, differences in the innervation of

these structures can not be excluded to play a role as well in the region-specific increases in 5-HT and 5-HIAA. For example, two of the areas in which clear elevations in 5-HT were seen, the hippocampus and septum, are (partly) innervated by the median raphe nucleus. The dorsal part of the hippocampus even receives almost exclusively neurons from the MRN, whereas the ventral part is innervated by both raphe nuclei [145, 206, 226, 301]. The septum as well appears to receive a mixed [206] innervation of both the DRN [76, 333] and the MRN [16, 334]. The AHP is not densily innervated by serotonergic neurons [41] and only a mild increase in 5-HT was found in this area during rat exposure. Also for this structure indications exist that both raphe nuclei play a role in the innervation [144, 265]. In contrast, the striatum, where we did not find increases in 5-HT, seems to be a terminal area of dorsal raphe pathways [145, 206, 333].

Indeed, it could be that the MRN and DRN are differentially activated during stress. For example, it was shown that the expression of tryptophan hydroxylase mRNA was increased in the MRN after one session with immobilisation stress, but only after three sessions in the dorsal raphe [53]. Also, the MRN responded to lower concentrations of CRH than does the DRN [309]. Sound stress activated the median raphe nucleus but not the DRN [84], and correspondingly, elevations in 5-HT were found in the median but not dorsal raphe nucleus [66].

Still, the prefrontal cortex too is mainly innervated by the dorsal raphe, yet an increase in extracellular 5-HT was found during rat exposure. To a certain extent this also goes for the mild elevations found in the PVN, which serotonergic innervation, although not dense [41], comes from the dorsal raphe [85, 333, 351]. Possibly, differences in the effect rat exposure has on levels of 5-HT in regions innervated by the DRN could be attributable to a subdivision within the organisation of this nucleus. Neurons to the striatum for example originate from more lateral parts of the caudate DRN than those to the frontal cortex [325]. Adding to a dual role that the DRN may have under stressful conditions is the finding that activation of $CRH_1$ receptors in the DRN seems to mediate inhibition of serotonergic neurons, whereas stimulation of $CRH_2$ receptors can result in the opposite effect [128, 129]. Indeed it was found that low doses of CRH, administered into the DRN, mainly inhibited its activity and that this was followed by a facilitatory rebound effect after higher doses of CRH [318].

Summarising the above, it seems that the background for the brain region-specific increases in 5-HT and 5-HIAA is formed by a combination of the functionality and innervation patterns of the structures under investigation. Concluding it can be said that the picture is emerging that the neuroanatomically highly differentiated response of the 5-HT system to different

forms of stress subserves its various roles in the coordination of not only motor activity, neuroendocrine and autonomic responses, but also emotional behaviour.

#### *2.1.3. 5-HT and specific behaviours*

The collection of samples of short duration during rat exposure, combined with detailed behavioural observations, enabled us to try to relate increases in 5-HT to certain specific behaviours. It has been hypothesised that increased activity of the 5-HT system takes place especially during self-directed behaviours such as grooming and feeding and other oral buccal movements [15, 105, 126, 188, 264, 264]. Under these circumstances, 5-HT is thought to reduce the signal to noise ratio, making an organism less attentive to external stimuli. In contrast, noradrenaline signalling would be highest during situations in which animals are orienting toward their environment, thereby actually increasing the signal to noise ratio [258]. Indeed, fluctuations during baseline and post rat exposure recordings in the limbic forebrain (but not the caudate putamen) were often, but not exclusively, correlated with self-directed behaviours such as grooming. However, a sharp and immediate increase in extracellular levels of 5-HT, especially in the hippocampus, was observed during the first 5 min of rat exposure, when animals are almost only sniffing in the air, hence orienting to their environment. One explanation for this elevation could of course be that stress can also cause not-behaviourally related increases in serotonin. Another explanation could be that 5-HT is correlated with different behaviours during the various phases of the experiment. For example, before and after rat exposure, when no stressor is present, it could be correlated with grooming, the main constituent of activity during those phases, whereas 5-HT could be more related with sniffing air during the rat exposure. Hence, increased serotonergic neurotransmission in regions such as the hippocampus, septum and prefrontal cortex during psychological challenges may play an important role in the assessment of information on (changes in) the environment. Such information will eventually be used to modulate the emotional response of the organism. Concluding, whereas elevations in 5-HT can not be interpreted as an indication of stress, without taking the behavioural activation into account, elevations in 5-HT can also not be taken as a sign for the occurrence of specific behaviours, without taking the state of arousal into account.

### *2.2. Noradrenergic neurotransmission*

#### *2.2.1. Effects of stress on NA and MHPG*

In the hippocampus of both C57bl/6N and Balb/c mice, the extracellular levels of NA showed

a steep and immediate increase in the first 5 min of rat exposure. This peak value was seen in both strains and was similar among the two days of rat exposure. Contrasting the serotonergic results, the levels of NA appeared to be less correlated with behavioural activity. Also the peak at the beginning of rat exposure was not paralleled by a maximum in behavioural activation. Levels of the metabolite MHPG showed a mild increase, starting in the second half of rat exposure. This was seen in all three areas in which MHPG was measured, i.e. the hippocampus, PVN and AHP of Balb/c mice. The begin peak that was seen with NA in the hippocampus was not mirrored in hippocampal MHPG levels. Therefore it is not possible to derive from the MHPG results in the AHP and the PVN whether NA showed a maximum at the beginning of rat exposure in these areas as well. Still, it seems allowed to assume a general elevation of extracellular NA levels in these regions based on the elevation seen in MHPG.

The present findings of increased noradrenergic and metabolite levels are supported by results of other predator stress studies found in the literature. Elevations in MHPG were found in the post-mortem analysis of the hippocampus of C57bl/6 and Balb/c mice in a rat exposure study by Hayley *et al.* [132]. They also reported increases in the metabolite level in the locus coeruleus and prefrontal cortex of these mice [132]. In cat odour-exposed Swiss Webster the NMN level, another metabolite of NA, was elevated in the hippocampus but not in the hypothalamus, the latter contrasting our observation that MHPG was increased in the PVN and AHP. NA itself was not increased in the cat-odour exposed rats [19]. However, in the study brain micropunches were examined post-mortem, so it is very well possible that an increase in NA was missed due to the fact that it could not be determined at the beginning of exposure. Finally, ferret exposure was found to increase levels of noradrenaline in the PVN of both slow and fast seizing rats [204].

Also other types of stressors have been described to increase levels of NA and MHPG in the hippocampus. Measurements in rats sacrificed directly after immobilisation stress indicated elevated levels of MHPG in the hippocampus [134]. Tail pinch increased levels of noradrenaline in the hippocampus of rats [235, 273]. Rosario *et al.* [273] also showed that rats that were more anxious in a novel open field also had larger increases in hippocampal NA in response to tail pinching, suggesting a relation between emotionality and the height of the NA-response. Immune stress too, in the form of a challenge with endotoxin, caused increases in hippocampal levels of NA and MHPG [177].

Stress has also been shown to affect hypothalamic levels of NA and MHPG. Immobilisation stress caused increases in NA in the PVN of rats, as well as in the central amygdala and the

BNST [8, 218]. Tail pinch [134] caused increases in MHPG, but a decrease in the level of NA in the rat hypothalamus, measured post-mortem. Also inescapable footshock resulted in increased levels of MHPG in hypothalamic and cortical brain slices of Balb/c and C57bl/6J mice killed directly after the shock [287]. Interestingly, footshock intensity was found to correlate with increases of NA in the rat amygdala [255]. Assuming that the emotional response also increases with stress intensity, this would be another indication that emotionality and levels of NA are related.

Taken together, both the present study and findings in literature show that stress in mice and rats leads to increases in levels of NA and MHPG in a variety of brain structures.

### *2.2.2. Function of noradrenaline*

More than with serotonin, there is a concensus concerning the role of NA. Increases in the levels of this neurotransmitter are thought to be related to arousal. This arousal can be novelty, like in primates presented with new faces, but also arousal due to more classical stressors such as restraint, footshock etc. [12, 221, 253, 291, 314, 317, 324]. Psychogenic stressors such as predator exposure also fall into the arousing category. Under circumstances that ask for externally directed attention, as is often the case in behavioural stress paradigms, firing of the locus coeruleus, the nucleus where most noradrenergic fibers originate, increases [258]. Especially when an automatic behaviour is suddenly interrupted, and orienting responses become prevalent, the firing rates of the LC are highest [13, 14]. As the locus coeruleus also projects to the hippocampus, this would fit with the observation that peaks in noradrenaline were seen here in the beginning of rat exposure, in the time mice needed to orient towards the rat. The increases found in the hippocampus therefore can be seen as an index of arousal. This increase might have been necessary to switch the hippocampus to a state of enhanced stimuli detection [115].

Another area that is innervated by NA is the PVN. It receives its input mainly from the A1 and A2 regions in the medulla oblongata. These noradrenergic pathways are activated by systemic stressors, but also play a role in the response to stressors that have anticipatory aspects [137]. For systemic stressors, such as hypoglycaemia, insulin injections, formalin injections, and also for the neurogenic stressor immobilisation, the amount of noradrenaline in the PVN of rats correlates with mean plasma ACTH levels [232, 234]. It is tempting to speculate that ACTH levels in case of psychogenic stressors also correlate with paraventricular levels of NA. In that case, the observed elevations in MHPG during rat exposure, and the supposed increases in NA, would be mirroring the mild increases seen in

plasma ACTH levels. On the other hand, a correlation between NA and ACTH was not seen with stressors like cold exposure and hemorrage [234] and also in a study with rats involving repeated restraint stress, discrepancies were found between NA and ACTH values [317].

The function of an increase in noradrenergic neurotransmission in the anterior hypothalamus under the presented paradigm is unclear. In this region, noradrenaline is known to cause hypothermia [170], but possibly it is also released here to prepare an organism for attack [3]. Again, such an increase in noradrenergic levels is only assumed based on the observed elevations of MHPG.

Based on our current results, in which noradrenaline was only measurable in the hippocampus, it is not possible to draw conclusion about differences in the effect of predator exposure on structures that are differentially innervated by noradrenergic neurons. As indications exist that the levels of noradrenaline might correlate with stressor intensity, it would be interesting to investigate this under more challenging conditions as well. Concerning the assumed differential activation of pathways by systemic and more psychological stressors (see also paragraph 6.1 in the introduction), it would be interesting to see how noradrenaline reacts to different kinds of stress in not only the paraventricular nucleus, but also in the medial prefrontal cortex. This latter structure receives a strong noradrenergic input from the locus coeruleus. It has been proposed that NA plays an inhibitory role in the PFC under life threatening circumstances, to favour rapid instinctual responses over more complicated ones [12, 111]. On the other hand, the prefrontal cortex is also involved in the evaluation of anticipatory stressors [137]. Possibly the two categories have differential effects, for example over time, or when simultaneously measuring the effect in the PVN. The effect on the prefrontal cortex of rats has been investigated for a host of stressors, like tail pinch [104, 304], foot shock [70, 95], handling [151, 199], restraint [297], novelty [96, 253], exposure to fox odour [304] and administration of anxiogenic compounds [70]. These paradigms covered the scala of systemic to psychological stressors, and an increase in noradrenaline was seen in all cases. Nevertheless, a direct comparison of the effects of systemic and psychological stressors has hardly been performed, except for Kawahara *et al.* [152], who found that both hypotension and handling increased prefrontal cortical levels of noradrenaline to the same extent. However, handling is a neurogenic stressor as it still involves a strong physical component. Possibly, differences are found when comparing systemic with psychogenic stressors, such as predator exposure.

### *2.3. Dopaminergic neurotransmission*

#### *2.3.1. Effects of stress on dopaminergic metabolites and dopamine*

Dopamine itself could not be measured, but its metabolites DOPAC and HVA were assessable. In the hippocampus of C57bl/6N and Balb/c mice and in the AHP and PVN of Balb/c mice levels of HVA were higher during rat exposure, but DOPAC was not. This was due to the fact that some animals had actually shown a decrease in DOPAC at the beginning of rat exposure, thus affecting the average. No large effects of re-exposure were seen for DOPAC or HVA.

Different DOPAC results were obtained in a study by Belzung *et al.* [20] who exposed Swiss Webster mice to cat odour and found elevated levels of DOPAC in brain micropunches of the hypothalamic region and striatum, but not of dopamine. No effects of exposure were found in the hippocampus, also contrasting the present results. Brain region dependent effects were also seen in Sprague-Dawley rats exposed to fox odour. The DOPAC/DA ratio, measured in brain homogenates was increased in the PFC and the amygdala, indicating the activation of dopaminergic metabolism in these limbic areas after the aversive stimulus. This activation was absent in other areas, including striatum and nucleus accumbens. On a second trial with fox-odour exposure the activation was not seen at all [222]. Another study in rats confirmed with microdialysis increased levels of dopamine in the prefrontal cortex after exposure to the smell of red fox urine [17]. Additionally, increases were seen in the nucleus accumbens core but not in the nucleus accumbens shell [17].

In general the effects of stress on the levels of dopamine and its metabolites are complex. Also studies employing other paradigms show differential effects of stress on dopaminergic neurotransmission. The effects of stress that were found, appear to be brain region, stressor, species [158, 289] and hemisphere [307] dependent, and can also differ between a first or subsequent confrontation with the stimulus. Brain region dependency has partly been illustrated already in the mentioned studies involving predator exposure. Other examples are that for example chronic variate stress induced increases in hippocampal DOPAC levels, but a decrease in the hypothalamic levels of both HVA and DOPAC [109]. Intracerebroventricular injection of CRH or intraperitoneal administration of corticosterone only increased DA levels in the dorsomedial hypothalamus [184]. Also immobilisation did not increase DA in other hypothalamic regions except the dorsomedial one [162], but DOPAC on the other hand was found to be higher in the PVN after immobilisation stress in another study [233]. An example of stressor dependent effects is that the forced swim test

made dopamine and DOPAC increase in the whole brain of Swiss mice, but the tail suspension test did not [257].

In none of the studies it was found that the direction of change in the levels of HVA and DOPAC were opposite. It has been described that stress can result in decreased activity of monoamine oxidase, the enzyme that degrades dopamine [82]. However, this as well would not explain why HVA is not too decreased, as MAO is also a necessary enzyme for the metabolism of dopamine to HVA. The observed decrease in DOPAC levels in some animals can therefore not be explained.

Also it becomes clear from the literature that measuring the metabolites of dopamine do not substitute for the assessment of dopamine itself, and that no effects on extracellular levels of dopamine in the hippocampus or hypothalamic areas can be inferred from the levels of HVA or DOPAC. This notwithstanding, based on the role of dopamine (see paragraph 2.3.2 below), an increase in its levels would be expected after a psychological stimulus such as rat exposure is. As stress-induced increases in dopamine levels have been described before in structures like the hippocampus and hypothalamus, they could also have occurred under the present predator exposure conditions. The increases in HVA, and partly in DOPAC, might be reflective of this.

### *2.3.2. Function of dopamine*

Apart from its role in movement, dopamine is considered the principal neurotransmitter in motivated action, and plays a role in approaching appetitive and evading aversive stimuli, that can be both of a physical or psychological nature [239]. However, the role of dopamine in brain regions during appetitive stimuli and also addiction is beyond the scope of this section.

Emotional arousal is accompanied by increases in extracellular dopamine in the medial prefrontal cortex especially, and to a lesser extent, in other limbic areas and the striatum [51, 94, 96, 140, 208]. Increases in the prefrontal cortex are often associated with anxiety or with coping [69, 140, 148], and are thought to take the prefrontal cortex ‘offline’ to allow for the regulation of fast and more primitive forms of behaviour [350], which is similar to the alleged role of noradrenaline in this structure. This would make it interesting to also investigate the effects of predator exposure on dopaminergic transmission in the PFC. Aversive and appetitive stimuli also affect the nucleus accumbens shell and core. It is the latter region, as well as the PFC, that is also associated with the effect of novelty on dopaminergic stimulation. In these regions a reduction in the response to a stimulus is seen when novelty wears off [17,

343]. Possibly, re-exposure would yield interesting data obtained when these regions would be dialysed.

The effects of stress on dopamine levels are independent of locomotor effects [257]. However, dopaminergic neurotransmission is associated with specific (peri) oral movements like grooming and with spontaneous exploratory activity [98, 103, 252, 335]. Several brain structures, including the nucleus accumbens, the olfactory tubercle and the BNST, but not the hippocampus, could be identified to play a role in this [103]. Indeed no correlation was found between the occurrence of grooming and the changes in the levels of HVA and DOPAC. As the hippocampus and hypothalamic structures do not appear to be the structures that play a major role in the regulation of stress responses by dopamine, this could explain why also only small variations in the metabolites were found.

### *2.4. Effect of re exposure on neurotransmission*

In general, there were hardly differences observable in neurotransmission patterns on day 1 and 2 of rat exposure. The few observations that were made will be discussed in this section.

One finding was that the hippocampal levels of 5-HIAA in C57bl/6N but not in Balb/c mice, were slightly lower on day 2 than on day 1. Interestingly, such a difference was not present in the AUC of 5-HT. The background of this effect remains to be elucidated. However, it has been shown before that there is no consistent relationship between 5-HT release and its metabolism or synthesis, as reflected in 5-HIAA levels, and that these might be differentially regulated (reviewed in [277]). It has been suggested that CRH plays a role in this [179].

Also an interesting finding is that the beginning peak during rat exposure in extracellular levels of NA seen in the hippocampus was not attenuated upon re-exposure. If this noradrenaline has the function to switch the animal's attention to external events, it apparently makes no difference whether this event is novel, or has been experienced before. In this respect it would have been especially interesting to have measured noradrenaline in the hypothalamic areas, where a relationship between levels of NA and HPA axis activity has been described for certain stressors. As corticosterone levels were lower during re-exposure, it would have been worthwhile to measure whether levels of NA were correspondingly lower in the PVN. Although in our studies an overall effect of 'day' was found for MHPG, with the levels of the noradrenergic metabolite being lower on a second day, this is not enough to conclude that also levels of noradrenaline were lower on day 2 than day 1. On the other hand, it is also imaginable that levels of NA in the PVN were similar to the ones on the first day, despite declined HPA axis activation. Such deviation has been described before [317]. A

study by Shibasaki *et al.* [291] suggests that such an attenuation of extracellular NA-levels in the PVN of rats after repeated stress might be stressor dependent. A decreased NA-response was observed after restraint, but not after pain inflicted by tail pinch. This finding adds to the discussion in paragraph 2.2.2 above, that a difference may be found in the way systemic and psychological stressors may affect noradrenergic neurotransmission, for example over time.

It is interesting that 'day' differences were found for the behavioural parameters that were scored, as well as for the free corticosterone data. This is contrasting the lack of neurochemical alterations after prior experience with predator exposure. An absence between behavioural and neurochemical parameters has been mentioned in literature as well [204]. This indicates that behavioural and neuroendocrine responses are not under the influence of only 5-HT or NA, but under a multitude of modulating neurochemicals. The view that neurotransmitters do not play a solitary role is commonly accepted, and for example the interplay of serotonergic and noradrenergic neurotransmission is often described (e.g. [58, 183, 216, 258, 311]). Interestingly, strain differences do not seem to play such a large role in neurochemical studies as they do in behavioural or neuroendocrine studies. This also underlines the rudimentary function of elevations in neurochemicals, that only in interplay with other messengers result in differences in behaviour or neuroendocrine differences downstream.

## 3. NEUROENDOCRINE ASPECTS

### *3.1. Effects of stress on HPA axis activation*

Measurement of plasma levels of ACTH and corticosterone revealed that predator exposure stimulated the activity of the HPA axis in C57bl/6N, Balb/c and B6C3F1 mice, but not in C57bl/6J or in DBA/2 mice. Noteworthy is that the increases were mild, and that strain differences were present.

Increased levels of stress hormone levels have been reported more than once after predator exposure. The group of Anisman reported elevated corticosterone and ACTH levels in C57bl/6 and Balb/c mice after rat exposure [6, 187]. Also Sprague-Dawley rats exposed to fox odour had elevated plasma corticosterone levels, when compared to no or to control odour exposure [101, 222]. So-called fast and slow seizing rats both responded with increases in plasma ACTH and corticosterone to ferret exposure too [209]. A study by Figueiredo [99]

showed that when rats were exposed to cats, increases in ACTH and CORT were seen, but that these seemed to be differentially regulated when the rats were familiar with the testing apparatus already. Temporal dynamics of the hormone increases could underlie this observation. It is known that maximal values for ACTH are reached within a few minutes, but that it takes 20 to 30 min for corticosterone to reach a maximum. The same phenomenon could explain why we, in our study, saw that the strain in which ACTH was elevated most significantly, C57bl/6N mice, was not the strain with the largest increases in CORT.

The increases in plasma ACTH and corticosterone found in our study were only mild, when compared to values in the literature found with predator exposure or other stressors (e.g. [6, 88, 187]). This can be explained by the influence that the chosen method of predator exposure has on the outcome of hormonal levels. Anisman *et al*. [6] for example placed the mice in a clear plastic case in a rat arena, adding an element of novel environment to the paradigm, which is likely to increase the corticosterone responses of the animals by itself, and also to sensitise the subsequent response to the rat [99]. In addition, the rat could roam over the compartment in which the mouse was placed, which could lend the situation a more threatening aspect than when the mice and rat confrontation can only take place at one side of the mouse's home cage, as was the case in this study and in the one of Linthorst *et al*. [174]. Apart from such large differences in the procedures, it has even been described that even a factor like the diet of the predator can influence the reaction of the predated animal to its presence [23].

As predator exposure with the used paradigm was experienced as a mild psychological stressor, this could also explain why some strains and individuals did not respond with a more pronounced increase in stress hormone levels.

Such interindividual differences also became apparent with the free corticosterone measurements, in which 3 out of 14 animals only responded with a clear stress-induced increase in corticosterone levels. When analysing the data of all 14 animals simultaneously a significant increase in free corticosterone was seen towards the end of the experimental day, caused by the diurnal activity of the HPA axis (as described by [174, 230, 245]). At the time of rat exposure an increase was seen as well, but due to the Bonferroni correction this remained statistically non-significant. Such a difference between plasma and central levels of corticosterone can be explained by the fact that the two values may not directly be related. Free corticosterone is the fraction of corticosterone that is not bound to corticosterone binding globulin (CBG), and that is available for binding to glucocorticoid and mineralocorticoid receptors. When measuring the plasma value, the amount of free

corticosterone and the fraction bound to CBG can not be differentiated. The plasma value of corticosterone therefore does not necessarily reflect the biologically active amount of hormone, and might give a distorted image of the actual HPA axis activation. This is even more so because the amount of CBG also varies with time and stress-situation (levels of CBG decrease under stressful conditions). It is therefore not possible to predict the free amount of corticosterone based on the total levels [77, 79, 300, 315, 320].

Still, measuring free corticosterone levels and using the same rat exposure method, Linthorst *et al.* [174] found larger increases in B6C3F1 mice than we did with C57bl/6N mice. Indeed, the choice of strain is an important factor in determining the neuroendocrine and behavioural outcome of experiments (see also paragraph 1.3 for more discussion on strain differences and [6, 11, 40, 55, 81, 113, 200, 282, 287, 288, 322]). Apparently the observed differences between the free corticosterone values of C57bl/6N and B6C3F1 mice, as well as those in the plasma hormone levels are related to genetic differences of vulnerability to stress and HPA axis activity. Adding to this, changes in CBG are strain dependent [80]. Another contributing factor to strain differences when it comes to hormonal levels is the rate of steroid degradation, which is different among strains. C57bl/6N have a high rate of catabolism and Balb/c a lower rate [172, 198, 293].

The differentiation by Koolhaas *et al*. between reactive and proactive coping styles, of which the behavioural consequences have been discussed above in paragraph 1.1, also has neuroendocrine implications. With the reactive form, the reactivity of the HPA axis is high, whereas the proactive form is more associated with low levels of corticosterone [159]. In mild stressing circumstances, such as predator exposure here, reactive strains might not necessarily yield higher average levels of corticosterone, but a higher number of responders, than proactive styles. This would be consistent with the fact that from a behavioural point of view, B6C3F1 in this predator exposure paradigm seem to have a reactive coping style, whereas the C57bl/6 and Balb/c strains seem to be proactive.

A final observation from the neuroendocrine data is related to the effect of re-exposure. The free corticosterone data showed that the HPA axis is activated less during a second trial than with the first one. This has also been described with plasma values of corticosterone in rats repeatedly exposed to cat odour [101, 222]. This might indicate that the animals had learned from the previous experience that the rat did not oppose an acute threat. Based on the information from this prior exposure, it is possible for the hippocampus to exert an inhibitory influence on the HPA axis. This attenuated HPA-response also fits with the observed behavioural differences that were less indicative of stress on the second day of rat exposure.

### *3.2. Behavioural and hormonal correlates*

One of the questions to be answered in this thesis is whether specific behaviours correlate with hormonal levels. Therefore the behaviour of the 5 strains of which plasma hormone levels were assessed was analysed, which was repeated using another method for the three strains with the most pronounced activation of the HPA axis. A relation between hormonal levels and behaviour could be expected, based on the discrimination of reactive and proactive coping styles, which indeed classifies certain behaviours that are more likely to be related to higher or lower levels of corticosterone. The latter category for example has high levels of defensive burying and nest-building [159, 332]. The present results carefully indicated that mice that spent more time rearing or sniffing at the separation wall, and/or exhibited more food-related behaviour had higher stress hormone levels. This however was only a general tendency that did not apply to every individual with this profile. Contrasting this, mice that were freezing or jumping, did always show increased hormonal levels.

From literature becomes clear that it is more often so that specific behavioural parameters can not or hardly be related to stress hormone levels. In a study exposing rats to odours, among which a predator odour, no significant alterations in immobility, grooming, rearing or horizontal locomotion were found, although corticosterone levels between groups differed [222]. Balb/c mice exposed to a rat showed more stretch attend postures than controls and had higher corticosterone levels, whereas the reverse was true for C57bl/6J mice. In both strains, the level of freezing was positively correlated with corticosterone levels, and the levels of rearing negatively [6, 275]. On the other hand the amount of freezing was not related with corticosterone levels in rats receiving footshock stress [127]. No relation was found between the time various strains of mice spent in the open arms of the EPM or the exploratory locomotor activity in a novel environment and levels of corticosterone [40, 322]. Also levels of locomotion did not correlate with ACTH or corticosterone levels in rats exposed to ferret odour [246], nor did levels of struggling or immobility in the rat forced swim test [11].

Taken together, except maybe for some extremes in behaviour, like jumping, most behaviour is not reflecting the activity of the HPA axis. It can be that in individual cases, or occasionally on strain level, such correlations exist, but the large interindividual variance of both behavioural and neuroendocrine parameters makes it hard to extent such observations to whole species. This also reflects that differential brain circuits underlie the control of behaviour and neuroendocrine responses.

# 4. DISCUSSION OF ADDITIONAL EXPERIMENTS

## *4.1. C57bl/6N mice bred at the institute*

Many researchers use animals that are bred in their own facilities. This has the advantage that all events during the animal's life are known that might influence results. In addition it is known that animals that come from commercial vendors may vary in their behavioural responses, depending on where they come from [60, 63, 329]. Possibly even subtle differences in diet, housing and caretakers may underlie these different responses [46, 336, 349].

Hormonal responses to rat exposure were not different in animals that were born in the institute or that were delivered from Charles River. Behavioural differences however were seen. Institute-bred mice were less active in general and showed less investigative behaviours than animals from Charles River. In general an attenuated activity response to a novel situation is interpreted as a sign of anxiety. Also the occurrence of panic like jumping behaviour of one of the animals born at the institute would point into this direction. It is feasible that animals from Charles River have been confronted with the smell of rats before, for example through the cloths of caretakers, and therefore were not absolutely rat exposure naïve before used in the rat exposure experiments described here. Prior exposure to odours does indeed affect the response to consecutive odour exposures [266]. Also, animals coming from a vendor have been confronted with novelty and changing conditions more often than animals that were not confronted with transportation, different housing conditions, changing care takers etc. Taken together this could lead to predominating interest in the presence of a rat, rather than fear for it, and thus in higher levels of investigative behaviours in the bought animals than in the animals from the institute. Still, it remains debatable whether a different emotionality between the two groups truly exists. Hormonal levels did not confirm this and the overall impression of the animals when observing the behavioural videos also did not indicate a clear difference in anxiety. In addition, behavioural data of exposed institute-bred animals were not much different from those of unexposed ones. It would be necessary to conduct additional experiments, like the elevated plus maze, to clarify whether animals from the institute are more, less, or equally anxious as animals from somewhere else. Nevertheless, the results do confirm that differences in behaviour exist between groups of animals that differ in origin. Also the results show that behavioural differences are not necessarily reflected in hormonal differences.

### *4.2. CRH-transgenic animals*

Rat exposure exposure experiments were also conducted with CRH-transgenic and wildtype mice. As also mentioned in paragraphs 2.2.3 and 5.6 of the introduction, CRH plays a role in depression and anxiety. Consequently, CRH-transgenic mice were found to be more anxious than wildtypes on the EPM, in a novel environment and in the light-dark box [303, 330]. Contrasting these findings that relate to innate fear, CRH-Tg did not appear to be more anxious in conditioning paradigms [330].

In our study, only small differences were found between the two genotypes on a behavioural level. Under control conditions, CRH-Tg tended to groom more than Wt did. This observation was also made by Van Gaalen *et al.* [330] when observing spontaneous home cage behaviour of the mice after a 1-min confinement. This was interpreted as an indication of higher levels of anxiety that had been present in the CRH-Tg.

Rat exposure gave rise to investigative behaviours in both kinds of mice. Transgenics however tended to engage less frequently in these than wildtypes did. As with the C57bl/6 mice from the institute, discussed in the previous section, this might be signalling the more anxious nature of CRH-Tg mice. Similarly, in a novel environment study by Stenzel-Poore [303] CRH-Tg were less active than Wt in the first 5 min of the paradigm, also interpreted as a sign of increased anxiety. Throughout the rest of the novel environment paradigm however, differences between CRH-Wt and Tg were absent. This might also explain why no larger behavioural differences between the two genotypes were found in our study: possibly the time frame was too long to still see significant quantitative differences that might only occur in the first minutes. On the other hand, it is also possible to interpret the slightly lower levels of investigative behaviour in CRH-Tg as an indication that they were less aroused than wildtypes by the presence of the rat. As CRH-Tg react more dramatically to superimposed stressors than wildtypes do [303], an experiment pre-stressing the animals with social defeat for example would help elucidate how to interpret the behavioural differences between the genotypes. In case the difference between the levels of investigative behaviour increases, this would indicate that these are a measure of anxiety in the CRH-Tg.

The higher amount of food-related behaviour that was seen in CRH-Tg is more likely to be a result of genotype, than to be an expression of experienced stress, as it was seen both under unexposed and exposed conditions. Although increased food-intake has not been described with mice overexpressing CRH, various neuropeptides, including CRH, are known to play a role in consummatory behaviour. However, central administration of CRH induces a decrease in food consumption [219, 220]. It can be envisioned though, that in mice with lifelong

increased levels of CRH and corticosterone, these effects are different. Obesity is often seen in patients with Cushing's disease [157], and in obese rats it was shown that reduction of corticosterone levels reduced levels of food intake [49].

Whereas the behavioural differences between the genotypes were not very evident, differences in stress hormones were. Control levels of corticosterone were higher in mice overexpressing CRH than in the wildtypes, but were not elevated in response to rat exposure, where the wildtypes did have higher CORT-levels after stress. Levels of ACTH though were similar among the genotypes under control conditions and elevated after stress in both kinds of mice. However, this elevation was less pronounced in the CRH-transgenics.

The observation that the basal levels of CORT were higher in the CRH-Tg is in line with findings by Stenzel-Poore *et al.* [302]. However, they found higher basal levels for ACTH in the transgenics as well. Based on the ACTH-release stimulating function of CRH, we had also expected to find an elevated basal level of ACTH in the transgenics. The reason for this discrepancy is unclear, but may have to do with interindividual differences. Also in the study of Stenzel-Poore not every single transgenic animal had an ACTH level that was higher than the average level of wildtypes. The effects of rat exposure that we described on plasma ACTH and corticosterone are similar to those found after restraint stress in these animals [60]. Because the CRH-Tg by nature suffer from chronical HPA activation (reflected in the higher basal levels of CORT), the HPA axis gets desensitised to further stimulation. This results in attenuated stress hormone responses after a stressful experience [60, 302].

Concluding, the neuroendocrine findings with CRH-Tg and CRH-Wt mice were in correspondence with what is described in literature. The differences in behaviour between the genotypes during rat exposure did not convincingly indicate a more anxious nature of the CRH-Tg. This is might be an indication that rat exposure in this form was too mild to elicit a discriminative response.

## 5. CONCLUSIONS AND CONCLUDING REMARKS

This section will present the answers to the questions, posed in the aims section of the introduction (paragraph 8), in a condensed form. Following are concluding remarks on the value of rat exposure as a behavioural paradigm and on its use for neurochemical studies.

### *5.1. Answers*

Rat exposure in the form it was used in the described experiments is a model that is mildly

stressing. It resulted in changes in the behaviour of C57/bl/6N and Balb/c mice, as well as changes in diverse neurochemical parameters and measures of neuroendocrine functioning.

On a behavioural level, predator exposure caused mice of various strains to become alert, start risk-assessment activities like rearing and sniffing, followed by behaviour that indicates coping, such as grooming and nesting. In case of Balb/c possibly also the engagement in food-related behaviour forms an index of coping behaviour. Upon re-exposure mice spent less time with risk-assessment and more time with coping behaviour, hinting that they had learned from the previous experience and adapted their behavioural strategy accordingly.

These behavioural changes were paralleled by increases in 5-HT during the active phases, as well as in its metabolite 5-HIAA. These increases were found in the hippocampus, PFC and LS, all part of the limbic system, but were absent in the caudate putamen, a brain area that is not part of the limbic system. 5-HT was also found to be increased in the PVN, and to a lesser extent in the AHP. These findings indicate that brain-region specific alterations in 5-HT subserve the role of 5-HT in emotion.

Increases in NA were seen in the hippocampus, especially during the first 5 minutes of rat exposure, indicative of the arousing properties of the paradigm. Similar findings on a second day of rat exposure could mean that hippocampal NA-elevations are needed to switch attention to external events, and that this might not easily be subject to desensitisation. Increases in the metabolite MHPG were found in the hippocampus, but also in the anterior hypothalamus and paraventricular nucleus, potentially pointing towards an elevation of NA in those regions as well. Unfortunately it was not possible to make statements on the relation between HPA axis activation and hypothalamic levels of NA based on the MHPG-measurements.

Levels of the dopaminergic metabolite HVA, but not of DOPAC, were increased in the hippocampus, in the paraventricular nucleus, and in the AHP. Although it is not possible to conclude from this that also dopamine itself had been increased, it is likely that these increases too were caused by arousing properties of rat exposure.

It was not possible to correlate certain behaviours to the increases in neurotransmitters. But it does seem that 5-HT is not only elevated during oral-buccal movements, as a current hypothesis states. 5-HT and especially NA are mainly elevated during the beginning of rat exposure, in the alert and risk assessment phase. Whereas the behaviour was different during re-exposure, neurochemical patterns remained the same.

Predator exposure also affected the HPA axis activity, in which strain differences became apparent. Levels of total corticosterone and ACTH were higher after rat exposure in

C57bl/6N, Balb/c and B6C3F1 mice, but not in C57bl/6J and DBA/2 mice. Levels of free corticosterone were also elevated in subsets of C57bl/6N, and were lower on a subsequent trial. This underlines the behavioural data that indicated an attenuated stress effect of repetition of rat exposure and contrasts the neurochemical data that were not different on a second day. Differences in hormonal responses were partly reflected in behavioural differences as well. Higher levels of stress hormones seemed to be found in mice that were rearing more, sniffing more in the air or at the separation wall, or spent more time with food-related behaviour.

The arousing properties of rat exposure were confirmed by neuroendocrine findings with institute bred C57bl/6N mice or CRH-Tg mice. The behaviour of C57bl/6N mice from the institute was different from that of the mice coming directly from the vendour. This forms a good argument to breed animals for behavioural research as much as possible in house. Interestingly, behavioural observations with CRH-Tg could not unequivocally confirm the more anxious nature of these mice comparing to wildtypes. Possibly this was the case because rat exposure in its current form was a very mild stressor, and maybe not powerful enough to reveal differences in anxiety between the two genotypes.

Concluding, although rat exposure confronts mice with a mild stress, it has marked, strain dependent effects on behaviour and HPA axis activation. It also affects neurotransmission in a brain region-selective manner. Behaviour and neuroendocrine data were different with re-exposure, the neurochemical parameters were not. This underlines that behavioural, neurochemical and neuroendocrine effects are not mediated similarly, but are different, complimentary, pieces of the total picture of effects stress can have on an organism.

### *5.2. The value of predator exposure as a behavioural model*

Although predator exposure has repeatedly been named as a model for psychological stress, and in some cases serves as a model of panic [1, 28, 30, 122, 174, 222, 246], this view needs to be differentiated based on the present results. In order to function as a proper stress or panic model, it is important to make careful considerations about the strain of mice and about the exact procedure to employ.

Concerning the method used in this study, it may be worthwhile to couple predator exposure to a novel environment. After all, under naturalistic conditions as well, mice are not very likely to engage predators in their home dens, but when they are outside in the open. This would also enhance face validity of the model. A practical downside of such an adaptation could be that it complicates the performance of simultaneous microdialysis.

Once the rat exposure paradigm is potentiated by adding an element of novel environment for example, the behavioural profile of various of strains of mice, like C57bl/6N, Balb/c or B6C3F1 mice, should be re-analysed to find the strain with a preferably large HPA axis response, behaviours indicative of anxiety (like jumping or freezing), and preferably large differences in risk assessment and coping behaviour between the first and second rat exposure trial. In this case the neuroendocrine and behavioural window would be optimised to discriminate in anxiolytic, anxiogenic and possibly antidepressant effects of compounds. The predictive validity of the model could then be put to the test.

The rat exposure model certainly has potential as a behavioural model. Even in its mild form it was possible to discern an effect of 'day' on behaviour, which was not possible based on the neurochemical indices, and only clear in a subset of animals based on the hormonal data. This behavioural strength is due to the multitude of behavioural parameters that were examined. At the same time this also forms a practical drawback of predator exposure: the behavioural observations of the animals are very time consuming and need training. However, it may be very well possible that the future of behavioural models lies in ethological approaches. Ever finer differences in drug profiles should be discriminated. Closely observing animals makes the researcher sensitive to such subtle changes in the animals. The value of observing more than one behavioural parameter is already clear in the elevated plus maze and the forced swimming test. The difference between catecholaminergic drugs and the serotonergic SSRI only becomes visible in the forced swim test, when behaviour is more closely observed than just measuring the immobility time. Catecholaminergic antidepressants namely selectively increase the amount of struggling or climbing behaviour, whereas SSRI selectively increase the amount of swimming [64].

### *5.3. Rat exposure and neurochemistry*

If the rat exposure paradigm as it was used in the current experiments would be adapted so that it poses a more threatening situation to the mouse, it could very well be used to investigate neurochemical processes that underlie the various phases of the stress response. These phases would first be a baseline situation, then realising that a threatening situation is present, assessing the extent of danger, possibly take protective action and returning to an equilibrated state again. A technique like microdialysis offers itself to study the levels of certain neurotransmitters over time, although the temporal resolution may not be high enough to study all phases of the response. It would be interesting to more extensively study the effects rat exposure has on the prefrontal cortex and on the paraventricular nucleus. A

question could be whether the levels of noradrenaline in the prefrontal cortex, like those in the hippocampus, do not differ with repeated exposure and how this is in the PVN. The latter structure also presents itself to further look into the correlation between neurochemicals and neuroendocrine activity. Also the amygdala offers itself as an interesting, and technically challenging structure to explore. When certain behaviours seem to correlate with neurochemical changes in specific brain structures, it would be interesting to investigate whether pharmacological manipulation of the extracellular level of that compound affects behaviour. The list of possibilities is sheer endless to investigate the function of neurochemicals in certain brain structures in their relevance for behaviour and HPA axis activation.

### *5.4. A personal note*

It is my conviction that depression is a disease that can exclusively be found in humans (possibly in primates too), but not in rodents. It appears to me that depression is a state that develops from a situation in which cognitive control over emotional impulses is so strong that the balance between ratio and emotion is lost and gets pathological. Indeed, a characteristic of depression is emotional flattening and attenuated responses to emotional events.

Translated to a neuroanatomical level this could mean that cortical areas are too active in suppressing the activity of the limbic system. Indeed the cortex is larger in humans than in any other species. The prefrontal cortex takes up a third of human brain volume and plays a key role in complex planning, problem solving and provides a perspective on whether a task is proceeding satisfactorily [111]. Also the evolutionary role of the cortical areas seems to be to modulate behavioural responses originating in other limbic areas in such a way that future purposes or additional knowledge can be incorporated in the final behavioural action that is taken after presentation of a stimulus. Still, emotions generated by activity of the limbic system are the most effective warning systems that have been developed in evolution, and the behaviour that is a consequence of certain emotions has a function in the survival of an individual and a species. When emotions that are generated in humans and that function as an alert to take action, are chronically not followed by certain behavioural events, it is not surprising that this leads to increasing activity of the limbic system and stress system, necessitating more cognitive control. An intuitive approach says that such a strained system can only collapse in a state with predominantly suppressed emotions, as could be the case in depressive syndromes, or in a state controlled by exaggerated emotion, as could be the case in anxiety disorders.

What follows from the above is that anxiety is easier to model in animal models than depression is. After all, rodents have the neuroanatomical structures that enable the induction of a state of exaggerated emotion, but not so much the ability to create a situation in which emotions are suppressed the way humans can. What can be seen however in functional animal models of depression is that they rely on a learned element that is contra-natural. In the learned helplessness paradigm, animals no longer try to avoid an electric shock. In the forced swim test animals stop trying to get out of the water. Antidepressants effects are characterised by returning the animal's behaviour to a more intuitive, emotionally driven state.

Other implications of the above are that the future of psychiatric research may not be in the focus of a primary imbalance in neurotransmitters, but in the imbalance between complete networks. Such an imbalance will certainly be measurable in the form of changed neurotransmission in the brain, after all this is the means by which neuronal structures communicate. But not less important is to simultaneously investigate the resulting effects on the output of the brain: behaviour, endocrinology and autonomic nervous activation. To understand the processes that occur in the etiology of depression and anxiety it is therefore imperative to investigate how a disruption of an equilibriated state by a stressor leads to effects on all those different levels and how these effects are correlated. It appears especially interesting to find out in how far systemic and psychological stressors indeed are two of a kind.

In the end however it must be realised that as animals are not humans, it will not be possible to get the full picture based on animal studies. But *in vivo* animal studies will enable us to take a few steps towards the elucidation of neurobiological background of psychiatric disorders.

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Munich, March 2004

Marjolein Beekman

# CURRICULUM VITAE

| | |
|---|---|
| August 6, 1974 | Born in Amersfoort, The Netherlands |
| August 1980 – July 1986 | Primary education at School Op De Berg, Amersfoort, The Netherlands |
| August 1986 – June 1992 | Secondary education at Johan van Oldenbarnevelt gymnasium (grammar school), Amersfoort, The Netherlands |
| August 1992 – June 2000 | Pharmacy study, University of Groningen, The Netherlands. |
| October 1997 | Obtained M.Sc. degree<br>Thesis: The neuroactive steroid ganaxolone – a comparative behavioral study with antiepileptics and in conjunction with psychodisrupting drugs.<br>Research done at National Institute on Drug Abuse, Baltimore, USA, in the Drug Development Group of Dr. J. M. Witkin.<br>Awards: Organon Young Research Talent Prize; KNMP Award for (bio)pharmaceutists. |
| June 2000 | Obtained public pharmacist degree |
| September 1998 – August 1999 | One-year postgraduate course Japanese Language and Business Studies, Leiden University, the Netherlands and Japan Netherlands Institute, Tokyo, Japan.<br>Internship at Yamanouchi Pharmaceutical Co., Tsukuba, Japan, in the Pharmacology I (CNS) department of Dr. T. Yamaguchi, dementia section. |
| September 2000 – March 2004 | Ph.D.-student at the Max Planck Institute of Psychiatry, Munich, Germany, in the section of Neurochemistry of Dr. A. C. E. Linthorst. |

*Publications:*

Beekman, M., Flachskamm, C., Linthorst, A.C.E. Behavioural, neurochemical and neuroendocrine aspects of the rat exposure stress paradigm in C57bl/6N mice. *In preparation.*

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