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Review

Stress and the brain: Solving the puzzle using microdialysis

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Abstract

Aberrant functioning of the hypothalamic–pituitary–adrenocortical (HPA) axis seems to be involved in depression and anxiety. However, the mechanisms underlying the relationship between stress and mental illness are not completely resolved yet. The therapeutical efficacy of selective serotonin re-uptake inhibitors and benzodiazepines points to a key role of serotonin and gamma-aminobutyric acid (GABA) in depression and anxiety. Thus, it can be hypothesised that stress-induced changes in serotonin and GABA contribute to a dysregulation of the HPA axis and to the development of psychiatric disorders in susceptible subjects. It will, therefore, be crucial to increase our understanding of the effects of stress on serotonin and GABA. Various refinements have made *in vivo* microdialysis an extremely powerful method to study the highly dynamic neurotransmitter responses in stress physiology and behaviour. Furthermore, microdialysis can also be used to measure free corticosterone levels in the brain and, thus, HPA axis activity and neurotransmission can be monitored concomitantly. Here we review the effects of acute and chronic stress on serotonin and GABA, as assessed by microdialysis, in the hippocampus; a brain structure critically involved in the behavioural and neuroendocrine responses to stress. From the microdialysis data discussed, it can be concluded that both serotonin and GABA in the hippocampus are highly responsive to stress, but also that these responses are shaped by the exact nature of the stressor, i.e. the balance between the psychological and physical aspects of the stressful challenge.

Keywords: Hippocampus; Serotonin; 5-hydroxyindoleacetic acid; GABA; Corticosterone; Stress; Depression; Anxiety

Contents

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; ANOVA, analysis of variance; CRF, corticotropin-releasing factor; CRF1, corticotropin-releasing factor receptor type 1; GABA, gamma-aminobutyric acid; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenocortical; MR, mineralocorticoid receptor; SSRI, selective serotonin re-uptake inhibitor.

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1. Introduction

1.1. Stress and psychiatric disease

The link between stressful life events or the experience of chronic stress and developing a mental illness has long been recognised but it was only during the past two decades that the role of stress in psychiatric disease has received a firm neurobiological underpinning. It is now well-recognised that mood and anxiety disorders are often accompanied by alterations in the functioning of the hypothalamic–pituitary–adrenocortical (HPA) axis. Although not fully delineated, changes in HPA axis activity and stress responsivity may be apparent in different forms of psychiatric disease (for references see De Kloet et al., 2005). Elevated plasma levels of the stress hormone cortisol together with a flattening of its normal diurnal rhythm are found in many (but not all) severely depressed patients. In contrast, hypocortisolaemia (at least during the diurnal rhythm) may be a feature in post-traumatic stress disorder (for references see de Kloet et al., 2006). Apart from aberrations at the level of the 'end product', changes have also been found in the major driving force of the HPA axis, i.e. the corticotropin-releasing factor (CRF) system, and in the functioning of mineralocorticoid (MR) and glucocorticoid (GR) receptors. Elevated levels of CRF have been measured in the CSF of depressed patients (Nemeroff et al., 1984), which, together with post-mortem findings of increased CRF mRNA expression (Raadsheer et al., 1995) and a higher number of CRF/vasopressin-expressing neurons (Raadsheer et al., 1994) in the hypothalamic paraventricular nucleus, suggest CRF hyperactivity in depression. Whereas such hyperactivity of CRF may well result in elevated levels of cortisol, neuroendocrine challenge tests in depressed patients (e.g. the dexamethasone suppression and the dexamethasone/CRF test) suggest that reduced negative feedback of cortisol via GRs is also an important contributor in HPA axis dysregulation. Furthermore, as proposed by De Kloet and colleagues, an imbalanced interplay between MR and GR may increase the vulnerability of predisposed persons to develop a stress-related psychiatric illness (De Kloet et al., 2007; see also Reul et al., 2000). In this respect it is of interest to note that recently single nucleotide polymorphisms (SNPs) in the GR (e.g. ER22/23EK; van Rossum et al., 2006) and an associated protein (i.e. FKBP5; Binder et al., 2004) have been found which seem to predict the response of depressed patients to antidepressant treatment and to stress. Furthermore, a specific gene variant of MR (MRI180V) seems to be associated with higher cortisol and heart rate responses in a psychosocial stress test (DeRijk et al., 2006).

1.2. Stress and the brain: the questions we have

Although it is clear from the first section of this review that stress and depression are intricately linked, the precise central nervous system mechanisms involved in this relationship are far from resolved. The growing number of people feeling 'stressed' and the increased incidence of stress-related psychiatric disease in today's modern society demonstrate the need for a better understanding of the regulation and function of the stress response and, importantly, stress coping mechanisms. Virtually all current antidepressant treatments target monoaminergic neurotransmitters such as serotonin and noradrenaline, whereas the anxiolytic benzodiazepine class of drugs is aimed at the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Interestingly, the antidepressant selective serotonin re-uptake inhibitors (SSRI's) and the 5-HT_{1A} receptor partial agonist buspirone are also widely used in the treatment of anxiety disorders. It is therefore very well possible that stress-induced changes in the functioning of these neurotransmitter systems precipitate (or contribute to) a dysregulation of the HPA axis in (genetically) susceptible subjects culminating in the development of a mood or anxiety disorder. Thus, to understand the neurobiology of psychiatric disease it is essential to obtain full insight into the role of monoamines and GABA in the stress response. For this purpose, first the effects of acute stress on neurotransmitter circuits will need to be established. Second, stress-related psychiatric disease may be a result of malcoping strategies during a period of chronic stress, and thus it will be important to determine the effects of chronic stress on brain neurotransmitter circuits. Finally, given that stress may result in disease in vulnerable individuals, how stress impinges on neurotransmission in subjects with specific genetic or other susceptibilities, should be examined. A variety of research strategies and methods can be applied to address these questions, but in this review we will demonstrate that *in vivo* microdialysis is an extremely powerful strategy for this purpose.

1.3. The use of in vivo microdialysis in stress research

Since its introduction in the 1980's, *in vivo* microdialysis has been an important research tool in neuropharmacology both in academia and in industry. Recently, microdialysis has become an important method also in research on stress physiology and behaviour. Several refinements have contributed to the suitability of microdialysis for these research fields.

First, the use of gas anaesthesia and of guide cannulas (allowing longer recovery periods after surgery) reduce stress levels and permit animals to express normal behavioural patterns; normal behaviour is further facilitated by the use of low-torque swivels and counterbalance arm systems giving the animals freedom to move in a three-dimensional way in the home cage and also during various behavioural tests. In our experiments, corticosterone levels in rats and mice show a normal, diurnal rhythm with low levels during the morning and increasing levels towards the night, the active period of rodents (see Section 4 and Figs. 2 and 3).

Second, with the introduction of thinner microdialysis probes (e.g. membrane diameter of 0.24 mm) small brain regions important in stress physiology, such as the paraventricular nucleus, the amygdala and the raphe nuclei, can be microdialysed. Furthermore, with thin, short probes and guide cannulas, microdialysis could also be established in freely-behaving mice, giving researchers the unique possibility to implement genetic mouse models in their studies.

Third, analytical methods have improved significantly during the past years. Neurotransmitter levels in dialysates are normally assessed by HPLC methods. Improvement of detection limits,

among others by microbore and capillary HPLC strategies and by miniaturisation of detector cells (e.g. for electrochemical detection), has made the use of re-uptake inhibitors in the perfusion fluid superfluous. Thus, physiological and behavioural studies can be performed in a pharmacologically clean situation. Most importantly, because of the increased sensitivity of the analytical methods, presently the sampling time can be reduced profoundly, resulting in more detailed time profiles which are critical when studying the highly dynamic responses to stress.

Finally, *in vivo* microdialysis is now regularly combined with *in vivo* biotelemetry and/or EEG recording. Using these combinations it is possible to demonstrate changes in physiological and behavioural parameters in parallel with neurotransmitter changes in the brain which is an extremely powerful strategy when studying the neurobiology of stress. Taken together, the developments in microdialysis and associated technologies during the last 10–15 years have made the method an important tool in stress physiology and behavioural research. In the future, microdialysis may become an even more powerful method if the sensitivity of analytical assays can be further improved, resulting in a further increase in time resolution and in the number of substances that can be (concomitantly) measured in dialysates. In the remainder of this review we will outline how microdialysis has contributed to understanding the effects of stress on serotonergic (Section 2) and GABAergic neurotransmission (Section 3). Moreover, we will highlight how microdialysis can be used to monitor the activity of the HPA axis by measuring corticosterone levels directly in the extracellular fluid of the brain (Section 4).

2. Effects of stress on serotonin in the hippocampus

Serotonin is an important modulator of a wide spectrum of physiological functions and behaviours, as diverse as food intake, mood and fear, sleep, reproduction and HPA axis activity (Jacobs and Azmitia, 1992); all functions known to be disturbed in psychiatric disease. It is therefore not surprising that changes in serotonergic neurotransmission have been described in depression and anxiety (for review see Mann, 1998; Maes and Meltzer, 1995; Ressler and Nemeroff, 2000). Decreased levels of 5-hydroxyindoleacetic acid (5-HIAA, the metabolite of serotonin) have been found in (subsets of) depressed patients. Furthermore, post-mortem tissue studies and specific neuroendocrine challenge tests (e.g. fenfluramine-induced release of cortisol and prolactin) suggest changes in serotonin receptor expression and function (Maes and Meltzer, 1995; Cleare et al., 1998). However, the most striking indication for a role of aberrant serotonergic neurotransmission in the aetiology of depression (and anxiety) is the therapeutical efficacy of SSRIs (e.g. paroxetine, fluoxetine, citalopram, and others). Given the intricate involvement of both stress and serotonin in psychiatric disease, it is essential to characterise the effect of stressful challenges on brain serotonergic neurotransmission and to elucidate the mediators involved. Below we will describe how stress affects extracellular levels of serotonin and 5-HIAA as measured by microdialysis. We will focus this review on serotonergic neurotransmission in the hippocampus, because, the hippocampus presents, in addition to its prominent role in learning and memory, a key structure in the coordination of the neuroendocrine and behavioural responses to stress. Furthermore, the hippocampus receives a dense serotonergic innervation from the median raphe nucleus (dorsal and ventral hippocampus) and the dorsal raphe nucleus (ventral hippocampus).

2.1. Acute stress

Hippocampal extracellular levels of serotonin and 5-HIAA have been found to respond to a wide variety of stressful challenges largely in a stimulatory fashion. Thus, exposure to a predator (e.g. a rat when testing a mouse or a cat when testing a rat), which is a relatively pure psychological stressor, results in a rise in hippocampal serotonin and 5-HIAA levels (Rueter and Jacobs, 1996; Linthorst et al., 2000). These earlier studies have used relatively long sample durations (30 min). Because of our interest in the finer dynamics of the stress response, we have used a rapid sampling paradigm, i.e. 5-min samples during a 30-min rat exposure, to study the serotonin response to predator stress in C57BL6/N mice (Beekman et al., 2005). Male C57BL6/N mice were exposed to a male Wistar rat placed in a separate compartment of the mouse home cage. The animals were separated by a Plexiglas wall with small holes (for experimental details see Beekman et al., 2005). Using this paradigm, we demonstrated that hippocampal serotonin levels in C57BL6/N mice show a fast increase already within the first 5 min of exposure (Fig. 1). The maximum levels attained were around 300% of baseline. Serotonin levels remained elevated during the next 10 min, but decreased towards baseline (i.e. pre-stress) levels during the second 15-min period of the exposure to the predator (Fig. 1). It is of interest that also the behaviour of the mouse changes during the 30-min exposure to the predator. At the start of the exposure to the rat, mice become immediately alert and usually move to the corner of the cage. After this initial response, they start risk assessment behaviours, including sniffing in the air, stretching towards the separation wall and sniffing at the separation wall. These risk assessment behaviours take part especially during the first 5–15 min of the exposure, whereas during the second 15-min period mice are extensively sniffing the bedding of the cage (Beekman et al., 2005). These results indicate that during psychological stress hippocampal serotonin may play an essential role especially during the first phase of the response, i.e. during the period when the subject has to assess a new, potentially life-threatening, situation and to respond to it adequately.

Exposing animals to stressors which are mainly psychological but also have a clear nociceptive component, such as tail pinch and electric footshock, also increases extracellular levels of serotonin and 5-HIAA in the hippocampus. With regard to tail pinch (performed by putting a paper clip to the tail of a rodent), it is of interest to note that several groups (Kalén et al., 1989; Pei et al., 1990; Vahabzadeh and Fillenz, 1994) have found that tail pinch increases extracellular hippocampal serotonin maximally after the paper clip has been removed (using sample intervals between 10–20 min). After removal of the paper clip animals show mainly consummatory behaviours and grooming. It is at present not clear why serotonin levels show a delayed increase during this phase of the stress response,

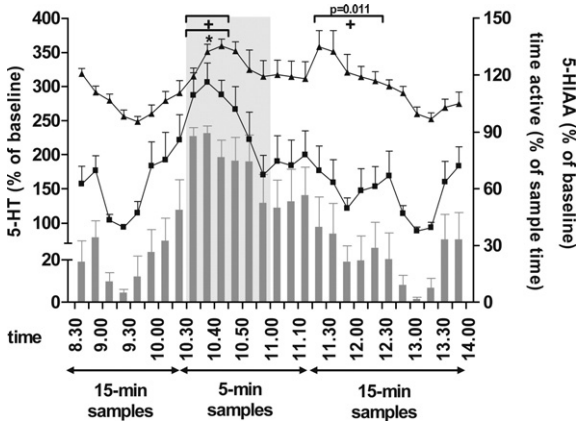

Fig. 1. Effect of predator stress (30-min exposure to a male Wistar rat; shaded area) on behavioural activity (dark grey bars; right *y*-axis), and on extracellular serotonin (squares; left *y*-axis) and 5-HIAA (triangles; right *y*-axis) in the hippocampus of male C57BL6/N mice as assessed by *in vivo* microdialysis (mean+SEM ($n=9$)). Behavioural activity is expressed as percentage of sample time. Concentrations of serotonin and 5-HIAA are expressed as percentage of baseline. Basal values of serotonin and 5-HIAA were calculated by averaging values of the pre-rat exposure period during which the animal was largely inactive (≤10% behavioural activity during collection of the sample). After collection of 8 baseline samples between 08:30–10:30 h, mice were exposed to a rat placed into a separate compartment of their home cage for 30 min (10:30–11:00 h). Post-stress samples were collected between 11:00-14:00 h. The sample duration was 15 min except for the rat exposure period and the first 15 min thereafter during which 5-min samples were collected. ANOVA showed a significant effect of the within-subject factor 'time' for all three parameters. Please note that the variation in serotonin levels in the period before the rat exposure is related to increases in spontaneous activity in the home cage (e.g. grooming). For a detailed discussion on the relationship between hippocampal extracellular serotonin and behavioural activity in the home cage the reader is referred to Linthorst, 2007. *, Significantly different from baseline for serotonin; +, significantly different from baseline for 5-HIAA (post-hoc contrasts with Bonferroni correction, $P<0.008$). From Beekman et al. (2005) with the permission of Wiley-Blackwell Publishing Ltd. (please see this original paper for further experimental details).

but it may be of relevance that in the dorsal raphe nucleus of the cat a subset of serotonin neurons is highly active during oral-buccal movements. In contrast, studies by Rueter and Jacobs (Rueter and Jacobs, 1996) and by Fujino and colleagues (Fujino et al., 2002) show an immediate increase due to tail pinch (using 30-min and 3-min sampling times, respectively).

The effects of footshock or tailshock and fear conditioning on hippocampal serotonin have hardly been studied using *in vivo* microdialysis. Hajós-Korcsok and colleagues described that giving rats a 0.2 s, 1 mA footshock increases ventral hippocampal extracellular serotonin profoundly to 650% of baseline during the first 10 min after the shock, but this effect disappeared when a second shock was given 1 h later (Hajós-Korcsok et al., 2003). Interestingly, using a learned helplessness paradigm, it was found that the effect of electric shocks on hippocampal serotonin levels depends on the coping possibilities provided to the animals. Thus, increases in ventral hippocampal serotonin were only found in rats that received an inescapable tailshock, but not in rats that could escape from the electric shock (Amat et al., 1998). Furthermore, using a fear conditioning paradigm, Wilkinson et al. demonstrated that hippocampal extracellular levels of serotonin only increase (to about 200% of baseline) when rats are exposed to the context in which they had received the aversive stimulus but not when they are exposed for a second time to the unconditioned and conditioned stimulus (Wilkinson et al., 1996). Unfortunately, in this study the effects of the first footshock, and the subsequent shocks during the conditioning phase, on serotonin have not been studied. However, given that during the contextual fear conditioning test session no physical stressors are present, the observed increase in hippocampal extracellular serotonin clearly demonstrates that psychological stress is able to stimulate hippocampal serotonergic neurotransmission possibly to support the animal's risk assessment of the precise nature and the environment of the stressful experience.

Although forced swim stress was originally developed as a rapid and simple test to detect putative antidepressant characteristics of new drug compounds (Porsolt forced swim test), this test has now gained popularity in studies on stress physiology. Forced swim stress activates the HPA axis (Wotjak et al., 1998; Abel, 1993; Bilang-Bleuel et al., 2005) and causes profound behavioural changes. During the swim session itself, rats and mice initially try to escape the container by climbing against the wall. In the case of rats, search attempts under the water surface and at the bottom of the container (diving) are often observed during the first phase of the exposure. These behaviours are followed by swimming in the container and by floating behaviour. Floating behaviour (immobility), although being used as an index for 'depressed behaviour' in the Porsolt test, is more likely to represent a behavioural coping strategy aimed at conserving energy (De Pablo et al., 1989; Korte, 2001; Bilang-Bleuel et al., 2005; Chandramohan et al., 2006). During the past years, we have performed an extensive series of microdialysis studies in rats to characterise the hippocampal serotonin response to a 15-min forced swim stress session. We demonstrated that forced swim stress changes hippocampal extracellular serotonin and 5-HIAA levels in a context- and water temperature-dependent manner (Linthorst et al., 2002; Linthorst et al., in press). Thus, whereas swimming in water at 35 °C causes a rapid and sustained increase in hippocampal serotonin and 5-HIAA during and after the stress, biphasic responses are observed at lower water temperatures (Linthorst et al., in press). Swimming in water of 25 °C results in an immediate rise in extracellular serotonin followed by a decrease to baseline levels after return to the home cage and a subsequent second rise. In contrast, when rats had to swim in cold, 19 °C, water, no effects on serotonin levels were observed during the swim stress. Serotonin levels only started to increase 15–30 min after the end of the stressful challenge. Interestingly, hippocampal extracellular levels of 5-HIAA also show water temperature-dependent effects. Most strikingly, hippocampal 5-HIAA levels decrease significantly below baseline levels during and immediately after swimming at 19 °C, which is followed by a gradual rise to maximum levels of about 140% of baseline. Maximum levels of 5-HIAA are only reached 105–120 min after completion of the swim stress session at 19 °C. Interestingly, in both C57BL/6N (Oshima et al., 2003) and 129/OLA-CD1 (Peñalva et al., 2002) mice, 10-min swim stress at 25 °C causes a monophasic increase in hippocampal serotonin, whereas a

significant transient decrease in hippocampal 5-HIAA was found in the latter strain. Moreover, also the behaviour of rats during and after the swim stress highly depends on the water temperature (Stone, 1970; Abel, 1993; Sandi et al., 1997; Bilang-Bleuel et al., 2005; Linthorst et al., in press). To find a possible explanation for the effects of water temperature on the outcome of the forced swim test, both with respect to hippocampal serotonin levels and behaviour, we performed an *in vivo* biotelemetry experiment to measure core body temperature during and after the swim stress period. 15-min forced swimming at 35 °C has only minimal effects on body temperature. In contrast, swimming in water of 19 and 25 °C decreased core body temperature by about 12 and 8 °C, respectively (Linthorst et al., in press). Thus, although forced swim stress is often considered to represent a psychological stressor, it is clear that this form of stress also causes profound changes in the physiology of the animal; changes that will contribute to the final outcome of the test with respect to diverse parameters such as hippocampal serotonin, and forced swimming and post-forced swimming behaviour. The finding that the hippocampal serotonin response is water temperature-dependent not only bears importance for other studies on the neurobiological mechanisms underlying swim stress, but also may explain the somewhat disparate results published in the literature. Thus, whereas Rueter and Jacobs describe a rise in hippocampal extracellular serotonin and 5-HIAA levels caused by swimming for 30 min in water of 30–35 °C (during the dark phase of the light-dark cycle, (Rueter and Jacobs, 1996), Kirby and colleagues found no effect on serotonin and a significant decrease in hippocampal 5-HIAA when exposing animals to 30-min forced swimming at 21–22 °C (Kirby et al., 1995). Water temperature may not be the only factor influencing the hippocampal serotonin response to swim stress. In a set of experiments in which rats were attached to a swivel-counterbalance arm system via a plastic collar around their neck (and not via a peg on their head as in the above-described experiments), we found that swim stress (15 min, 25 °C) results in a dramatic increase in hippocampal extracellular serotonin (about 900% of baseline; Linthorst et al., 2002). The relatively high standard error of this response prompted us to look at the behaviour of the rats during the forced swim session. It was found that only animals that dive during the swim stress show this exaggerated response (mean serotonin response in diving rats is 1500% of baseline). Interestingly, this diving-associated serotonin response was prevented by intracerebroventricular pre-treatment of the animals with the nonselective CRF receptor antagonist D-Phe-CRF12-41. We hypothesise that wearing a plastic collar around their neck may have led to a, CRF receptor-dependent, panic-like response in rats that dive to the bottom of the container during the swim session (Linthorst et al., 2002).

To conclude this section, we will discuss the effects of immune stress on hippocampal serotonin. The stressors reviewed above comprise examples of psychological and combined psychological/physical stressful challenges. In contrast, immune stress represents a serious and immediate physiological threat to the organism and is, therefore, often described as a so-called systemic stressor. Systemic stressors are stressors processed directly at the level of lower brain structures such as the brain stem and the hypothalamic nuclei without involving higher brain regions such as the cortex and hippocampus. However, although indeed having profound effects at the level of the hypothalamus and preoptic area (e.g. with respect to noradrenaline release; Linthorst et al., 1995a), immune stress also affects serotonergic neurotransmission in higher limbic structures. Intraperitoneal administration of bacterial endotoxin (100 μg/kg body weight) causes a marked and long-lasting (over 6 h) increase in hippocampal extracellular levels of serotonin and 5-HIAA (Linthorst et al., 1995b). Similar effects were found by peripheral (Merali et al., 1997) and central administration of interleukin-1β (Linthorst et al., 1995b) and interleukin-2 (Pauli et al., 1998); in contrast, TNF-alpha had no effect on hippocampal serotonin and 5-HIAA (Pauli et al., 1998). Whether the effects of interleukins on hippocampal serotonin involve activation of the raphe nuclei or are generated locally at the hippocampal level has not been clarified yet. The observation that local administration of interleukin-1β into the hippocampus via retrodialysis increases extracellular serotonin in this nucleus suggests that local mechanisms could be involved (Linthorst et al., 1994). Based on the marked effects of endotoxin and interleukins on hippocampal serotonin levels, we conclude that immune stress also possesses a clear-cut psychological component. This conclusion is underscored by the profound behavioural changes observed during inflammation and infection, summarised under the term 'sickness behaviour' (Hart, 1988; Dantzer et al., 1991; Reul and Linthorst, 2000).

2.2. Repeated and chronic stress

As mentioned, it is thought that stressful life events may precipitate in the development of a psychiatric disorder in vulnerable subjects. Such life events may happen acutely (e.g. the loss of a spouse, a serious car accident), but they often cause long-term stress in the individuals concerned. Furthermore, people experiencing (or perceiving) chronic stress are at a higher risk to develop a psychiatric disorder, as exemplified by the observation that depression often occurs in persons caring for their spouse who is suffering from dementia (Redinbaugh et al., 1995; Waite et al., 2004). Although we first will need to know how acute stress is being processed by the brain (see above), next the neurobiological processes involved in the processing of chronic stress will have to be clarified. Unfortunately, the information on the effects of chronic stress on hippocampal extracellular levels of serotonin is rather scarce; the fact that microdialysis can only be performed for a limited number of days may have contributed to this. However, there are a few interesting studies suggesting that exposure to chronic stress results in changes in hippocampal serotonergic neurotransmission. In all these studies, microdialysis is only performed at the end of the chronic stress procedure, leaving us, at present, without information on the dynamics of changes in serotonin during the chronic stress experience. The best characterised model so far (with respect to hippocampal serotonin) is the isolation rearing model as applied by Marsden and colleagues in which rats are either group-housed or singly housed for six weeks immediately after weaning. As may be

expected, isolation rearing has significant consequences for the behaviour of the animals. There are no differences in baseline serotonin levels in the dorsal hippocampus between isolation- and group-reared rats. However, whereas group-reared rats show a marked increase in hippocampal serotonin upon exposure to an elevated x-maze and footshock stress, these stressors were without effect in isolation-reared animals (Bickerdike et al., 1993; Muchimapura et al., 2002). In contrast, Storey et al. recently reported that, exposing rats repeatedly to an elevated open platform increased baseline levels (i.e. levels when not on the platform) of serotonin and 5-HIAA significantly in the dorsal hippocampus (Storey et al., 2006). Furthermore, whereas acute exposure to an elevated platform in naïve animals had no effect on dorsal hippocampal extracellular levels of serotonin, these levels were elevated when rats were tested on the tenth day of daily exposure to the platform.

Under normal conditions, there is a close relationship between hippocampal extracellular concentrations of serotonin and the sleep-wake stage of an animal; similar relationships are also observed in other brain regions. Thus, highest extracellular concentrations of serotonin are found during (active) waking. Serotonin levels decrease during slow wave sleep and are lowest during rapid eye movement sleep (Park et al., 1999; Peñalva et al., 2003). Furthermore, recently, we found that during a 4-hour sleep deprivation period (which is highly stressful for animals as evidenced by the rise in hippocampal corticosterone levels, see Fig. 2) hippocampal serotonin levels are increased (Peñalva et al., 2003). However, during rebound sleep the normal relationship between serotonin and sleep-wake stage is resumed. Interestingly, a recent microdialysis study by Gronli et al. shows that subjecting rats to a chronic mild stress paradigm causes a disruption of the serotonin-sleep stage relationship (Gronli et al., 2007); unfortunately by which mechanisms such disruption is brought about is presently unknown.

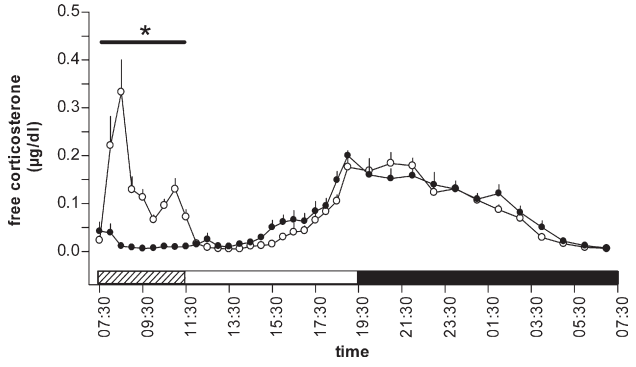

Fig. 2. Effect of a 4-hour sleep deprivation period on hippocampal free corticosterone levels as assessed by *in vivo* microdialysis in male Wistar rats. The closed circles represent the baseline day, whereas the open circles represent the sleep deprivation day (both the baseline and the sleep deprivation day samples were collected in the same animals). 30- and 60-min samples were collected during the light and dark phase of the diurnal rhythm, respectively. The hatched box indicates the sleep deprivation period, the black box the dark phase of the light-dark cycle. Mean+SEM (n=6). *, Significant effect of sleep deprivation (ANOVA with repeated measures). Note the diurnal rhythm in free corticosterone levels over the day-night cycle. From Peñalva et al. (2003) with the permission of Wiley-Blackwell Publishing Ltd. (please see this original paper for experimental details).

2.3. Stress and hippocampal serotonin in mutant mouse models

The vulnerability for developing a psychiatric disorder due to (chronic) stress may be caused by changes at the level of the genome. Implementing mutant mouse models may therefore be a valuable strategy to obtain insight into the neurobiology underlying successful and unsuccessful stress adaptation. In depression and anxiety, changes at the level of the HPA axis may involve hyperactivity of the CRF system and changes in MR and GR function. In a first study, using *in vivo* microdialysis, we demonstrated that genetic knockdown of GR (using an antisense transgene strategy; Pepin et al., 1992) decreases brain free corticosterone levels (see also Section 4) towards the dark period of the light-dark cycle (Linthorst et al., 2000); an effect which apparently can worsen the impact of GR dysfunction (Karanth et al., 1997; Barden et al., 1997). Furthermore, it was found that impairment of GR results in an exaggerated response in hippocampal extracellular serotonin when these mice are exposed to a predator rat. Interestingly, although the serotonin response to rat exposure is increased in GR-impaired mice, the HPA axis response is absent (Linthorst et al., 2000). The latter observation is in agreement with a recent study by Froger et al. demonstrating that a chronic mild stress paradigm increases plasma corticosterone levels in control but not in GR-impaired mice (Froger et al., 2004). These findings suggest that a life-long dysfunctioning of GR precipitates not only in aberrant HPA axis functioning under baseline and stress conditions but also in altered stress responsivity of serotonin at the level of the hippocampus. Several research groups have generated mouse lines with a changed expression (deletion or overexpression) of MR and GR (Tronche et al., 1999; Wei et al., 2004; Boyle et al., 2005; Rozeboom et al., 2007; see also Howell and Muglia, 2006), and in some of these mouse lines alterations in the serotonin system have been detected. For instance, mice overexpressing MR in the forebrain show enhanced expression of 5-HT_{1A} receptor mRNA and binding in the CA1 region of the hippocampus (Rozeboom et al., 2007). The same group also demonstrated increased 5-HT_{1A} mRNA expression in the dentate gyrus, but not the dorsal and median raphe nuclei, of mice with an overexpression of GR in the forebrain (Wei et al., 2004). Unfortunately, no information is available on the regulation of basal and stress-induced serotonin release and metabolism in the hippocampus or other brain regions of these mutant mouse lines; future microdialysis studies in GR- and MR-altered mice would therefore significantly contribute to our understanding of the relationship between stress, the HPA axis and serotonin.

In a second series of studies we investigated the consequences of genetic deletion of CRF receptor type 1 (CRF1) for the hippocampal serotonin system. Microdialysis studies on the diurnal rhythm demonstrated that hippocampal levels of 5-HIAA, but not serotonin, are significantly elevated during both the light and dark phase in CRF1 mutant mice (Peñalva et al., 2002). These findings are of considerable interest as it is thought that extracellular levels of 5-HIAA are an index for (metabolism of) newly-synthesised, unreleased, serotonin (Grahame-Smith, 1974; Kuhn et al., 1986; Peñalva et al.,

2002). Thus, life-long CRF1 knockout may result in enhanced synthesis of serotonin at least in the raphe–hippocampal system. When submitted to 10-min forced swim stress at 25 °C, CRF1 mutant mice show an enhanced increase in hippocampal extracellular serotonin, whereas the 5-HIAA response was not different from wildtype control animals (Peñalva et al., 2002). Similar as for MR and GR, other mouse models with genetic deletion or overexpression of various CRF system parameters have been generated (e.g. CRF overexpressing, CRF2 knock-out; Keck et al., 2005), but, apart from the above-described study, microdialysis studies for serotonin have not been performed yet. Given the profound effects of CRF and the urocortins on hippocampal extracellular serotonin and 5-HIAA in the rat (Linthorst et al., 2002; Oshima et al., 2003; De Groote et al., 2005), such studies may be highly relevant. Taken together, our observations in GR-impaired and CRF1 mutant mice demonstrate that a life-long genetic change in one component of the HPA axis may have profound knock-on effects not only for the HPA axis itself but also for serotonergic neurotransmission.

3. Effects of acute and chronic stress on GABA in the hippocampus

GABA is the main inhibitory neurotransmitter in the brain and plays a key role in stress-related psychiatric disease, sleep disorders and alcohol dependence. With respect to major depression, it has been found that the levels of GABA in the cerebrospinal fluid of depressed patients are decreased (Gold et al., 1980; Gerner and Hare, 1981). Furthermore, brain imaging studies have revealed that treatment with SSRIs normalises GABA levels in the occipital cortex of depressed patients (Sanacora et al., 2002). Most evidence, however, points to a dysregulation of GABAergic neurotransmission at the level of GABAA receptors in depression and anxiety; this notion is further substantiated by the widespread therapeutical use of benzodiazepines (Nutt and Malizia, 2001). In the past, microdialysis of GABA was controversial (Timmerman and Westerink, 1997; Rea et al., 2005). It was propounded by several research groups that measuring extracellular levels of GABA was not very useful because GABA would only be involved in direct synaptic processes and, therefore, any GABA measured outside the synapse (i.e. in the extracellular fluid) would be just spilled-over. However, recently, elegant electrophysiology studies have shown that GABA, in addition to fast synaptic transmission, also exerts a form of tonic inhibition by acting on (high affinity) GABAA receptors (and putatively GABAB receptors; Scanziani, 2000) located extrasynaptically (for review see Semyanov et al., 2004 and Farrant and Nusser, 2005). To date, tonic inhibition has been demonstrated in the hippocampus and the cerebellum. These new findings, together with the role of GABA in stress-related psychiatric disease, demonstrate the importance of characterising the effects of stress on extracellular levels of GABA. In a recent microdialysis study, we demonstrated that hippocampal extracellular levels of GABA are responsive to a variety of neuropharmacological manipulations, i.e. retrodialysis of the re-uptake inhibitor nipecotic acid and elevated levels of K^+ both increase extracellular levels of GABA, whereas GABA levels decrease after retrodialysis of the sodium channel blocker tetrodotoxin and the GABAB receptor agonist baclofen (De Groote and Linthorst, 2007). Similar findings have been reported by Rea and colleagues (Rea et al., 2005), who have also addressed the importance of careful selection of the pH conditions for the separation of GABA using HPLC. Importantly, in the same study we found that hippocampal extracellular levels of GABA respond to stress in a stressor-dependent manner (De Groote and Linthorst, 2007). When rats were exposed to a novel environment, hippocampal GABA levels increased rapidly and remained elevated during the total novelty period. Similarly, forced swimming for 15 min at 35 °C increased hippocampal extracellular GABA. In contrast, however, forced swim stress at 25 °C caused a marked decrease in GABA levels, which may, as discussed above for serotonin, be related to the increased physical impact of swimming at low water temperatures (i.e. the profound decrease in body temperature). Increased extracellular levels of hippocampal GABA due to novelty stress were also found by Bianchi and colleagues (Bianchi et al., 2003). The effect in their study was however larger (300% of baseline as compared to 120% of baseline in our study) which may be explained by the addition of the cholinesterase inhibitor physostigmine to the perfusion fluid in the study of Bianchi et al. Interestingly, in the rat chronic mild stress has been found to decrease extracellular GABA levels in the hippocampus (Gronli et al., 2007). Despite the limited available data, it may be concluded that stress does impact on extracellular levels of GABA in the hippocampus. Given the putatively important role of GABAergic tonic inhibition in the regulation of hippocampal output, further microdialysis studies on the effects of stress on extracellular GABA, and on the mediators involved, will be essential.

4. Microdialysis to study the hypothalamic–pituitary–adrenocortical axis

As described earlier in this review, dysregulation of HPA axis activity seems to play an important role in the aetiology of stress-related psychiatric disorders. Thus, it is essential to increase our insight into normal and aberrant functioning of the HPA axis and, particularly, to elucidate how glucocorticoids affect brain function via stimulation of MR and GR. To study the effects of manipulations (pharmacological, behavioural, stress, etc.) on the end-product of the HPA axis, i.e. corticosterone in rodents, usually blood samples are taken via a cannula in the jugular vein or via a cut in the tail, or, trunk blood is collected after decapitation of the animal. Clearly these methods have certain disadvantages. The number of blood samples that can be collected via the jugular vein is limited and keeping cannulas patent over several days can prove to be difficult. Tail bleeding causes pain and discomfort (also because of being restraint) for the animals and therefore levels of corticosterone may become affected by the method of collection per se. Also in this case, the number of samples that can be collected is rather restricted. By nature, sampling of trunk blood after decapitation results only in one sample and thus the number of animals needed to study the time course of a manipulation-induced corticosterone response

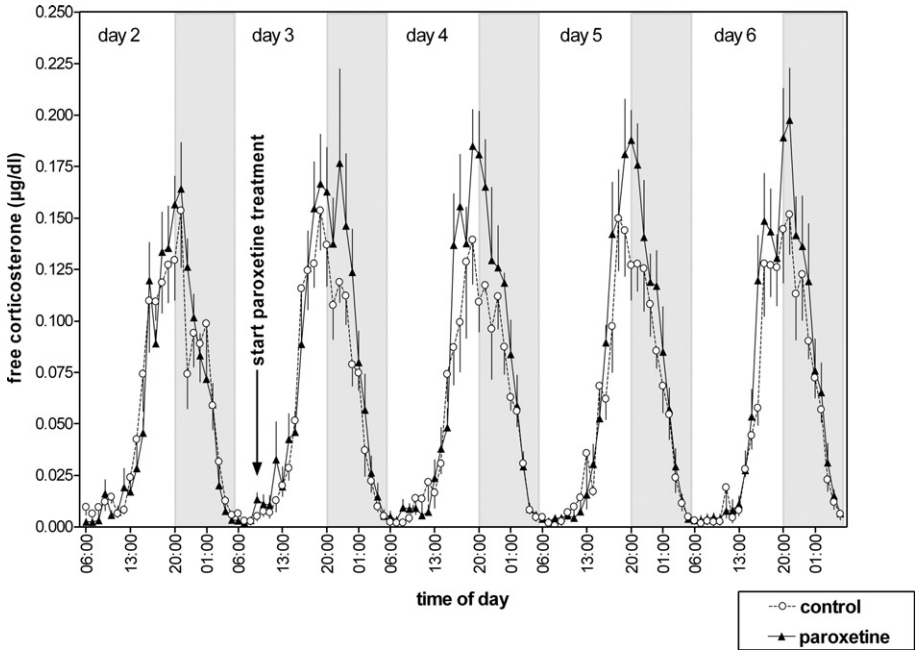

Fig. 3. Effect of a 4-day treatment with the SSRI paroxetine on hippocampal extracellular levels of free corticosterone as assessed by *in vivo* microdialysis in male Wistar rats. Rats were equipped for microdialysis in the hippocampus by implantation of a guide cannula (CMA12, CMA Microdialysis/AB, Stockholm, Sweden) under halothane anaesthesia. Eight days after surgery, a microdialysis probe (CMA12, 4 mm) was inserted into the hippocampus (via the guide cannula) under a short-lasting halothane anaesthesia and animals were connected to a swivel-counterbalance arm system. Two days after the insertion of the probe, twenty-four 60-min samples were collected to assess the diurnal rhythm of free corticosterone (day 2). On day 3, animals were given paroxetine in their drinking water (at 09:00 h; $n=7$, closed triangles) or were given normal, fresh tap water (control group, $n=5$, open circles). Water and paroxetine solutions were re-freshed daily and drinking bottles were weighted to determine the dose of paroxetine. Animals consumed on average 6 mg/kg bodyweight paroxetine per day. Paroxetine treatment continued for four days during which microdialysis samples were collected in 60-min intervals. Free corticosterone levels in the dialysates were determined by radioimmunoassay. Values represent mean+SEM. For results of the statistical analysis please see text. The dark period of the light-dark cycle is indicated by the shaded areas (lights off between 20:00–06:00 h). The experimental protocols were approved by the Ethical Committee on Animal Care and Use of the Government of Bavaria, Germany.

may become very large. To circumvent these problems, we decided in the early 1990s to measure corticosterone directly in the brain using *in vivo* microdialysis and a highly sensitive radioimmunoassay method. Given that in the brain binding proteins (e.g. corticosterone binding globulin) are absent in the extracellular fluid, dialysate corticosterone levels represent the free, unbound (and thus biologically active) fraction of this hormone (Reul et al., 2000). This is in contrast to routine measurements in plasma in which usually the total (bound + free) amount of corticosterone is assessed. Thus, the physiological significance of measuring free corticosterone levels directly in the brain is that these represent the levels actually 'seen' by neurons expressing MR and GR. Furthermore, the stress-free nature of the microdialysis method, as established in our laboratory, provides the unique opportunity to collect samples for corticosterone over extended periods of time, under basal and stressful conditions, without the animal noticing it. We were the first to describe that it is indeed possible to monitor free corticosterone levels in brain regions such as the hippocampus (Linthorst et al., 1994) and preoptic area (Linthorst et al., 1995a) under baseline and stress conditions. We could also extend these measurements to freely-behaving normal and mutant mice (Linthorst et al., 2000; Peñalva et al., 2002; Oshima et al., 2003).

Hippocampal free corticosterone levels show a clear diurnal rhythm with low levels in the morning and a rise towards the evening as demonstrated in Fig. 2. This figure also shows that free corticosterone levels in rats show a biphasic increase during a 4-hour sleep deprivation period which started at the beginning of the light period. Interestingly, after termination of the sleep deprivation (and during rebound sleep) free corticosterone levels decrease to baseline and the normal diurnal rhythm is resumed (Peñalva et al., 2003; Fig. 2). The power of the microdialysis of corticosterone is further demonstrated in Fig. 3. Antidepressants such as amitriptyline, moclobemide and tianeptine have been found to change HPA axis regulation in terms of both plasma corticosterone levels and corticosteroid receptor expression (Reul et al., 1994; Reul et al., 1993; Brady et al., 1991; Seckl and Fink, 1992; Yau et al., 2004; Droste et al., 2006). Because it has been observed that the effects of antidepressants on the HPA axis change over the course of chronic treatment regimes (Reul et al., 1993; Steckler et al., 1999), we wanted to know whether paroxetine, an SSRI, would influence corticosterone levels during the first few days of treatment. Therefore, male Wistar rats were surgically prepared for microdialysis in the hippocampus by implantation of a guide cannula, basically as described before (Linthorst et al., 1995b), and were allowed to recover for at least seven days before insertion of the microdialysis probe. Free corticosterone levels were measured on one baseline day (i.e. the second day after insertion of the probe), after which rats were either given paroxetine in their drinking water or continued to receive normal tap drinking water. Corticosterone levels were monitored for a further 4 days

to assess the acute and sub-chronic effects of the antidepressant drug. Fig. 3 shows that paroxetine (daily dose as calculated based on volume consumed is 6 mg/kg body weight) causes a small, but consistent and significant increase in hippocampal extracellular levels of free corticosterone, especially during the first part of the dark period, as compared to control treatment (ANOVA with repeated measures (using 4 time levels per day), effect of time: $F(19,190)=78.07$, $P\leq 0.0005$; interaction treatment and time: $F(19,190)=1.81$, $P<0.05$). These results indicate that antidepressant treatment, at least during the early phase of treatment, stimulates HPA axis activity. Importantly, these findings are in line with observations that the antidepressant amitriptyline transiently decreases the size of the thymus (a glucocorticoid-sensitive immune organ) during the first seven days of treatment (Steckler et al., 1999), demonstrating the biological impact of the elevated free hormone levels (although at present it cannot be excluded that amitryptiline and/or paroxetine may (also) have direct effects at the level of the thymus). Moreover, these data show that microdialysis can be used to monitor free corticosterone levels over extended periods of time in stress-free, freely-behaving animals. The usefulness of microdialysis in stress research is also underscored by literature reports of other groups (Azzi et al., 1998; Kitchener et al., 2004; Keeney et al., 2006). Finally, it is important to note that due to (1) the repeated measures design of microdialysis experiments and (2) the possibility of splitting samples for concurrent measurements of corticosterone and various neurotransmitters, fewer animals will need to be used to study the exact kinetics and dynamics of the stress response. As such microdialysis represents a significant contribution to the 3R's of animal welfare (Russell and Burch, 1959).

5. Conclusions and future directions

The microdialysis data discussed in this review clearly demonstrate the marked stress-responsiveness of both serotonergic and GABAergic neurotransmission in the hippocampus. Importantly, they also show that the exact shape of the neurotransmitter response is determined by the nature of the stressor, i.e. the balance between the psychological and physical aspects of the stressful challenge. Presently, the picture is most complete and comprehensive for the effects of acute stress (on serotonin), whereas information on chronic stress is limited. One of the main challenges for future research, therefore, lies in the design of studies to unravel the complex impact of chronic stress on serotonin and GABA in the hippocampus. Moreover, it is important to further understand how changes in HPA axis regulation (e.g. at the MR and GR level) may precipitate in altered hippocampal neurotransmission. As outlined in this review, *in vivo* microdialysis is an extremely powerful tool in research on stress physiology. However, experimenters need to take several measures to ensure that the stress associated with the procedure itself is minimized. Thus, post-surgical intervals should be long enough for the animals to recover (and thus guide cannulas should be used) and great attention should be given to the swivel/counterbalance arm system and cage to assure maximal behavioural freedom of the animal (including full rearing). Furthermore, animals should be carefully handled daily to reduce any experimenter-induced stress and arousal. Measurement of free corticosterone levels in dialysates is an easy way to check whether optimal microdialysis procedures are being used. Sensitive analytical methods will allow to study the temporal dynamics at a high definition and the measurement of different molecules in the dialysates. In this way, serotonin, GABA and free corticosterone can be measured in each dialysate within one animal. Thus, the strength of modern *in vivo* microdialysis is that relationships between neurotransmission, HPA axis activity and behaviour can be assessed within the same animal, which will without doubt not only reduce the number of experimental animals needed, but will provide stronger data and increase our understanding of stress physiology and behaviour.

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