The olfactory bulbectomized rat model of depression: on the role of dietary tryptophan and serotonergic functioning

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De olfactoire bulbectomie rat als model voor depressie:
de rol van tryptofaan in de voeding en serotonerg functioneren

(met een samenvatting in het Nederlands)

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Ik heb een steen verlegd
In een rivier op aarde
Nu weet ik dat ik nooit zal zijn vergeten
Ik leverde het bewijs van mijn bestaan
Omdat door het verleggen van die ene steen
De stroom nooit meer die zelfde weg zal gaan

Bram Vermeulen

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Chapter 1

Major depressive disorder (MDD) is one of the most prevalent psychiatric disorders with a lifetime prevalence as high as 13%. The World Health Organization has reported that MDD is the fourth most disabling health problem world wide, and it is predicted that it will be the second most disabling condition worldwide by 2020. At present more than 20 different drugs are approved for the treatment of MDD, but most compounds have been available for several decades and the majority can be divided into two major classes based on their pharmacological mechanism of action. Despite the large number of clinically effective antidepressants, our understanding of how these drugs exert their effect, and how to develop better treatments has progressed little.
Decreased levels of 5-hydroxytryptamine (5-HT, serotonin) in plasma and reduced 5-hydroxyindole acetic acid (5-HIAA) levels in CSF of depressed patients, together with altered responses in neuroendocrine tests and tryptophan depletion paradigms of patients with MDD, have suggested that an impairment in the central serotonin (5-HT) function plays a central role in the pathogenesis of MDD. Based on this so-called serotonin hypothesis of depression antidepressants have been developed, of which the selective serotonin reuptake inhibitors (SSRIs) arre currently one of the mainstays in the treatment of MDD. However, how these drugs exert their effects is still largely unknown. A better understanding of the pathophysiology of MDD might be achieved by the use of animal models, of which several have been developed, mainly on the basis of effects of current antidepressants on behavioral parameters. To bridge the gap between exploratory animal models of depression and clinical research a better understanding of the mechanism underlying these models is a prerequisite.

CENTRAL SEROTONERGIC SYSTEM

Organization of serotonergic pathways

The serotonin (5-hydroxytryptamine, 5-HT) system is one of the most extensive neurotransmitter system in the vertebrate CNS. However, its pattern of innervation is neither ubiquitous nor nonspecific. Neurons containing 5-HT are restricted to clusters of cells around the midline of the pons and upper brain stem; this is known as the raphe area of the midbrain.
The organization of the superior raphe projections indicates a rostrocaudal encephalotopy (Imai et al, 1986). For a schematic drawing of the location of the serotonergic nuclei and their major projections in the brain, see fig 1.

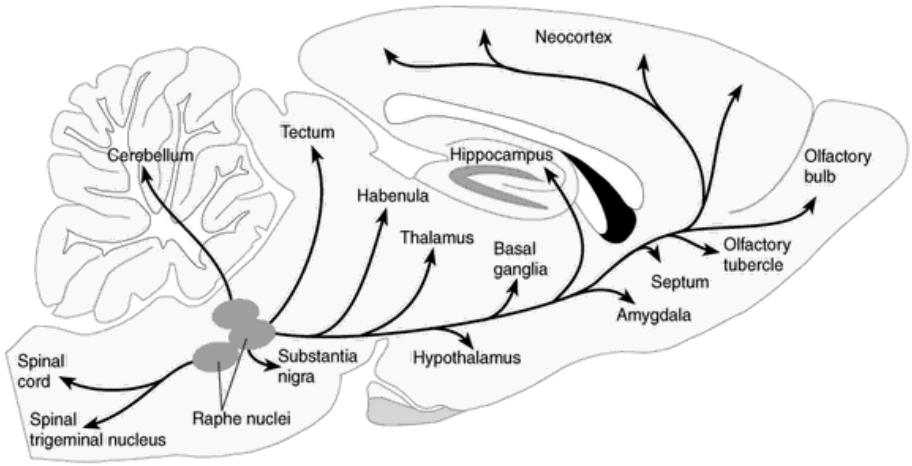

Fig. 1 Schematic drawing of the location of the serotonergic nuclei and their major projections in the brain.

Forebrain serotonin is derived nearly entirely from neurons located in the dorsal (DRN) and median raphe nuclei (MRN) of the midbrain. The rostral group (DRN and CLN) projects to the basal ganglia-motor systems (e.g. corpus striatum, substantia nigra and nucleus accumbens), whereas the caudal group (MRN and the interfascicular aspect of the DRN) projects to limbic structures (e.g. hippocampus, cingulate cortex and septal nuclei). The amygdala receives input from both groups, with the basolateral nucleus, which has projections to corpus striatum, belonging to the rostral group. It has been shown by Andersen et al (1983) that the dorsal raphe nucleus also projects to the olfactory bulbs. A heavy projection from the dorsal and median raphe nuclei to the main olfactory bulb was shown by both retrograde and anterograde tracing techniques (McLean and Shipley, 1987). The raphe nuclei with overlapping terminal areas have a sizeable number of collateralized neurons to these terminal areas. This confirms and extends reports showing that serotonergic neurons are highly collateralized with innervations to more than one terminal area (Van der Kooy and Hattori, 1980, De Olmos and Heimer, 1980).

Box 1. In vivo cerebral microdialysis

Since the 1980s the technique of in-vivo brain microdialysis has been frequently used as a method to study the in vivo release of endogenous neurotransmitters. The effect of amphetamine on dopamine release and metabolism was one of the first studies undertaken with the in-vivo microdialysis technique (Zetterstrom et al, 1983). This study was soon followed by studies on extracellular 5-HT (Kalén et al, 1988). Since then, microdialysis studies have provided important information on the functioning of the serotonergic system, and have contributed to the understanding of the in-vivo effects of antidepressants on extracellular 5-HT. The technique implies implantation of a small probe with a semi-permeable membrane in a specific brain area, after which the probe is perfused with an artificial cerebrospinal fluid. Neurochemicals that passively diffuse across the membrane are collected in the perfusion fluid, and are subsequently measured by means of high pressure liquid chromatography with electrochemical detection (HPLC-ECD). Studies indicating tetrodotoxin-sensitivity and calcium dependency of neurotransmitter in dialysates indicated that extracellular 5-HT is of neuronal origin. Moreover, of autoreceptor-related changes in neurotransmitter output is an additional argument to conclude that the sampled neurotransmitter is directly related to neurotransmission. The amount of 5-HT in dialysate samples is determined by the recovery through the probe, which is dependent on a range of in-vitro and in-vivo parameters such as membrane characteristics perfusate composition, perfusate flow rate, active tip of the membrane but also diffusion of the neurotransmitter in the region of the probe. Therefore 5-HT as measured with microdialysis is only a reflection of true extracellular concentrations.

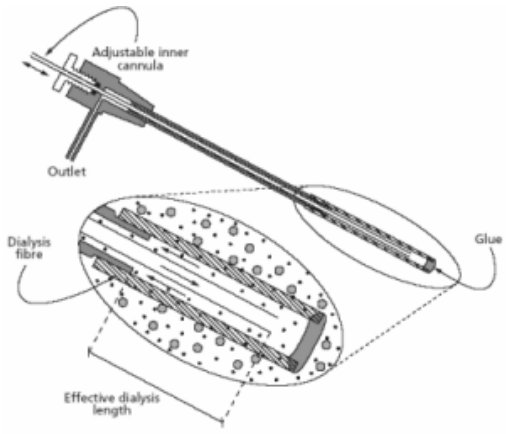

Fig. 2 Schematic drawing of a microdialysis probe

Serotonin synthesis

5-HT is an indoleamine neurotransmitter which is synthesized within the nerve ending from the essential amino acid L-tryptophan (fig 3, for reviews on serotonin synthesis see Fernstrom, 1983; Kema et al, 2000). Tryptophan, which is primarily derived from dietary sources, is unique among the amino acids that play a role as precursor for neurotransmitter synthesis in that it is for about 85% bound to plasma proteins. This means that only a small fraction of the plasma tryptophan is available for uptake into the brain as precursor for 5-HT synthesis. In the periphery, tryptophan may be metabolized in the liver via the kynurenine pathway. Biosynthesis of 5-HT represents only a minor metabolic route for tryptophan. Under normal conditions, it accounts for only 2% of ingested tryptophan, leading to a daily production of about 10 mg 5-HT (Sandyk, 1992).
Free tryptophan is transported into the brain and nerve terminal by an active transport mechanism which it shares with tyrosine and a number of other essential amino acids. Hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase, is the first and rate-limiting step in serotonin synthesis. Tryptophan hydroxylase is not saturated by its substrate under basal conditions, implicating that if the brain concentration of tryptophan rises, 5-HT synthesis will increase. Formation of serotonin occurs by decarboxylation of 5-HTP. This reaction is catalyzed by aromatic-L-amino acid decarboxylase (AADC) and uses pyridoxal-5-phosphate (the active form of vitamin B6) as coenzyme (Boman, 1988).

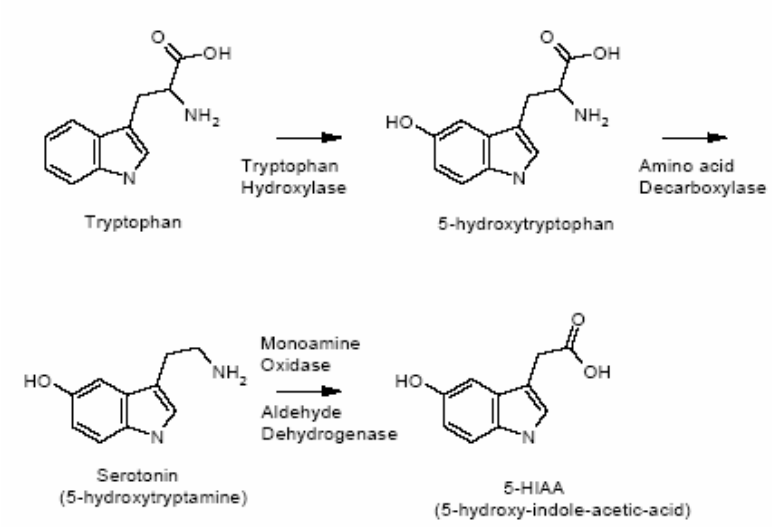

Fig 3. Synthesis and metabolism of serotonin.

There is evidence that the compartimentalisation of 5-HT in the nerve terminal is important in regulating its synthesis. It appears that 5-HT is synthesized in excess of normal physiological requirements and that some of the amine which is not immediately transported into the storage vesicle is metabolised by interneuronal monoamine oxidase. Another mechanism controlling 5-HT synthesis is based on stimulation of the 5-HT autoreceptor in the nerve terminal.
5-HT is metabolised by monoamine oxidase by a process of oxidative deamination to yield 5-hydroxyindoleacetic acid (5-HIAA). In the pineal gland, 5-HT is o-methylated to form melatonin, which plays a role in the regulation of the circadian rhythm.
Serotonin synthesis rate is obviously dependent on tryptophan hydroxylase and AADC activities, and tryptophan availability. Since AADC activity is about 75 times higher than tryptophan hydroxylase activity, 5-HTP formation is thought to be the rate limiting step. Availability of tryptophan also influences the serotonin synthesis rate. Because of its amphipathic nature circulating tryptophan is partially bound to plasma albumin. Its uptake
into the brain is known to be dependent on the plasma free tryptophan concentration. Consequently, processes that influence the equilibrium between the free and protein-bound form of plasma tryptophan (e.g. free fatty acids, certain drugs) modify the ability for uptake in the brain and thereby affect serotonin synthesis.

Regulation of serotonin transmission

Serotonin mediates a wide range of physiological functions by interacting with multiple receptors, and these receptors have been implicated in the pathology of several psychopathological conditions. To date, at least 14 5-HT receptor subtypes have been identified, which have been classified into seven receptor families (5-HT_{1-7}) on the basis of their structural, functional and to some extent pharmacological characteristics (for review see Barnes and Sharp, 1999). Most of the 5-HT receptor subtypes are G-protein coupled metabotropic receptors, with the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel. However, the intracellular signal transduction differs between 5-HT receptor families. 5-HT_1 receptors couple negatively to adenyl cyclase, whereas 5-$HT_{4,5,6,7}$ receptors couple positively to adenyl cyclase. 5-HT_2 receptors are coupled posistively to inositoltriphosphate.

5-HT_{1A} receptor
The 5-HT_{1A} receptor was the first 5-HT receptor to be fully sequenced. The rat 5-HT_{1A} receptor has 89% homology with the human 5-HT_{1A} receptor. The distribution of 5-HT_{1A} receptors in the brain has been mapped extensively by

receptor autoradiography, showing that the density of 5-HT_{1A} binding sites is high in limbic brain areas, notably hippocampus, lateral septum, cortical areas (particularly cingulated and entorhinal cortex) and also the mesencephalic raphe nuclei (both dorsal and median raphe nuclei). In contrast, levels of 5-HT_{1A} binding sites in basal ganglia and cerebellum are hardly detectable. 5-HT_{1A} receptors are located both postsynaptic to 5-HT neurons (in forebrain regions) and also on 5-HT cell bodies in the mesencephalic and medullary raphe nuclei (fig 3) (for review see Barnes and Sharp, 1999)

The somatodendritic 5-HT_{1A} autoreceptor is aimed at controlling 5-HT levels. Under normal circumstances, an increase in 5-HT levels will activate somatodendritic 5-HT_{1A} receptors in the raphe nucleus, which will in turn reduce the firing frequency of 5-HT neurons and subsequently diminish 5-HT release at the nerve terminals.

Neuroendocrine studies in rats have found that 5-HT_{1A} receptor agonists cause an elevation of plasma ACTH, corticosteroids and prolactin (e.g. Gilbert et al, 1988; Gartside et al, 1990) and in man there is also increased secretion of growth hormone (Cowen et al, 1990). Both human and animals work show that these neuroendocrine responses are blocked by 5-HT_{1A} receptor antagonists (Gilbert et al, 1988; Cowen et al, 1990; Gartside et al, 1990; Critchley et al, 1994). Data showing that the ACTH response is intact in rats with 5-HT lesions suggest that it is mediated by postsynaptic 5-HT_{1A} receptors (Fuller, 1996).

5-HT_{1B} receptor

5-HT_{1B} receptors were one of the first 5-HT_1-like receptors to be described in rats (Zifa and Fillion, 1992; Glennon and Westkaemper, 1993). At the same time two homologous human 5-HT_1 receptor clones were iolated on the basis of their sequence homology with the orphan receptor (canine RDC4 gene) which was suspected to be a 5-HT receptor. When expressed both clones demonstrated the pharmacology of the originally defined 5-HT_{1D} site and were termed 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ (Hamblin and Metcalf, 1991; Levy et al, 1992; Weinshank et al, 1992). It was later shown that the distribution and second messenger coupling of 5-HT_{1B} receptors in rodent brain was similar to that of 5-$HT_{1D\beta}$ receptors in mammalian brain, leading to the speculation that the rodent 5-HT_{1B} and human 5-$HT_{1D\beta}$ receptors might constitute species variants of the same receptor. With the subsequent discovery of a rat gene which was homologous to the human 5-$HT_{1D\alpha}$ receptor and encodes a gene with a 5-HT_{1D} binding site profile (Hamblin et al, 1992), the 5-$HT_{1D\alpha}$ receptor was renamed 5-HT_{1D} (Hartig et al, 1996).

5-HT_{1B} receptors were initially identified in rodent brain using radioligand binding techniques and were defined as sites labelled with [^{3}H]5-HT with low affinity for spiperone. 5-HT_{1B} receptors are located presynaptically (fig 4), where they control the release of 5-HT, and postsynaptically, where the highest density of 5-HT_{1B} receptors in rat and mouse brain is found in the substantia nigra, globus pallidus,

and dorsal subiculum (Zifa and Fillion, 1992). There is now convincing evidence that the 5-HT_{1B} receptor functions as a 5-HT autoreceptor at the 5-HT nerve terminal (for review see Middlemiss and Hutson, 1990, Bühlen et al, 1996).

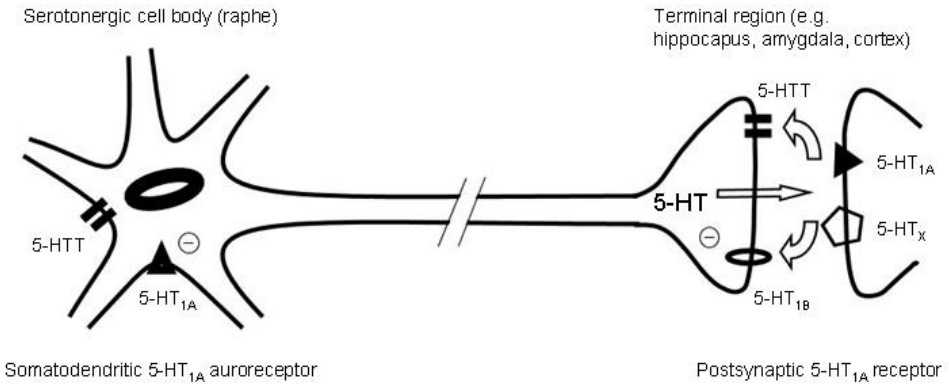

Fig 4. Diagram showing the anatomical location and functional role of 5-HT receptor subtypes

5-HT transporter

Following release, 5-HT is actively cleared from the synaptic cleft by a high-affnity transporter located on presysnaptic neuronal membranes (Kuhar et al, 1972; Kanner and Schuldiner, 1987; O'Reilly and Reith, 1988). The 5-HT transporter (5-HTT) is the pharmacological target for various therapeutic substances such as tricyclic antidepressants and SSRIs. However also abused substances such as stimulants as amphetamine and its derivatives block 5-HT uptake and promote release. 5-HTT immunoreactive fibres are widely distributed through the brain, with the highest densities in regions that receive dense serotonergic innervation, such as the cerebral cortex and the CA1 and CA3 regions of the hippocampus. Immunopositive staining for the 5-HTT showed that it was also present in the cell bodies of the raphe nuclei (Ojan, 1995; Sur, 1996).

OLFACTORY BULBECTOMY

The olfactory bulbectomized (OBX) rat is an animal model that meets most of the criteria of a valid model for depression. The olfactory bulbs are bilataral extensions of the rostral telencephalon and constitute 4% of total brain mass in an adult rat (Cain, 1974). Extensive efferent connections with limbic and higher brain centres would implicate more far-reaching effects of olfactory bulbectomy than anosmia alone. Indeed, bilateral removal of the olfactory bulbs results in a phenotype modelling several of the symptoms seen in major depression, including many of the behavioral, neurochemical, neuroendocrine and immune alterations (for reviews see Leonard and Tuite, 1981; Kelly et al, 1997 and Harkin et al, 2003). Whereas other models of depression such as learned helplessness and chronic mild stress (for review see Jesberger and Richardson, 1985; Willner et al, 2002) react to acute antidepressant treatment, OBX is unique in its sensitivity to chronic, but not acute administration of clinically efficacious antidepressants (Jesberger and Richardson, 1988; Van Riezen and Leonard, 1990; Cryan and Mombereau, 2004).

Behavioral changes

Early reports have shown an increase in locomotor activity in bulbectomized animals (Klein and Brown, 1969; Sieck, 1972). Since then, bulbectomy has consistently been shown to produce an increase in locomotor activity when rats are placed in a stressful novel environment such as the open field (Van Riezen et al, 1977; Cairncross, 1979; for review see Kelly et al, 1997). This effect does not seem to be related to loss of smell, as it has been shown that the performance in the open field of animals with peripherally induced anosmia is not different from controls (Sieck and Baumbach, 1974; Van Riezen et al, 1977). 24 h measurements have shown increased nocturnal activity of the OBX rat compared to control animals (Giardina and Radek, 1991; O'Halloran et al, 1993).

Removal of the olfactory bulbs has also been shown to result in learning deficits (for review see Van Riezen and Leonard, 1990). Impaired Morris water maze learning and a deficiency in the radial arm maze have been reported (Redmond et al, 1994; Hall and Macrides, 1983). Furthermore, bulbectomized animals demonstrate learning deficits in both step-down passive avoidance (Joly and Sanger, 1986) and step-through passive avoidance (Van Riezen et al, 1976).

Finally, several other behavioral abnormalities are associated with removal of the olfactory bulbs. Muricide, increased incidence in cannibalism of young by female OBX rats, intermale aggression, territorial aggression and a reduction in sexual behavior have been reported (Lumia et al, 1987; Tyler and Gorski, 1980). However, these behavioral abnormalities cannot be explained merely by the loss of olfaction (Leonard and Tuite, 1981).

Box 2. Olfactory Bulbectomy

Animals are fixed in a stereotactic instrument and a skin incision is made to expose the skull overlying the bulbs. Two burr holes are drilled (each 2 mm diameter) over the right and left olfactory bulbs, respectively, each 1.5 mm from the midline of the frontal bone and 6.7 mm anterior to bregma. The bulbs are cut and removed by suction, care being taken to avoid damage to the frontal cortex. The burr holes are then filled with haemostatic sponge and the incision sutured. Control (sham operated) animals are treated in a similar manner, except that the bulbs are not aspirated. The extent of the lesion is assessed at the end of the study. This can be done visually, and animals that show either incomplete removal of the bulbs or damage to other brain areas must be excluded from subsequent analysis.

A period of 2 weeks is usually allowed for recovery from the surgical procedure and is optimal for the development of the bulbectomy syndrome (Van Riezen and Leonard, 1990). The

olfactory bulbs are bilateral extensions of the rostral telencephalon and constitute 4% of the total brain mass in an adult rat (Cain, 1974). Extensive efferent connections with limbic and higher brain centers would implicate more far-reaching effects of olfactory bulbectomy than anosmia alone (fig). As a result, it is not surprising to find that a cascade of changes emerges following the disruption of connections between the bulbs and other brain regions. These changes resemble many of the changes found in major depression. The efficacy of chronic SSRI treatment on bulbectomy induced deficits has provided further evidence for the bulbectomized rat as a valuable animal model of depression.

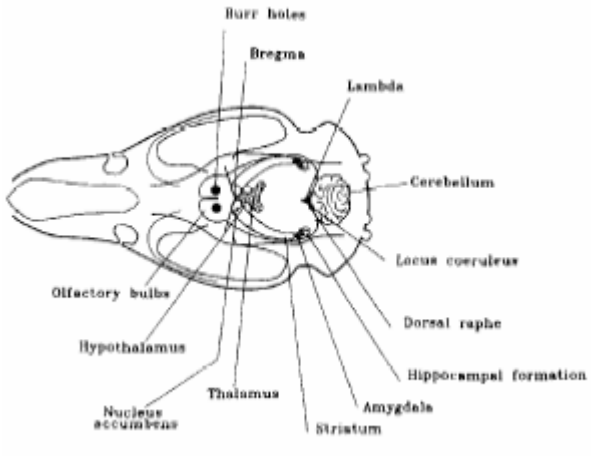

Fig.5 Schematic representation of the location of the burr holes relative to the main anatomical features of the rat skull and position of the olfactory bulb relative to important brain regions that are disrupted following bulbectomy. (Kelly et al, 1997)

Neuroanatomical changes

Bilateral removal of the olfactory bulbs induces a number of macroscopic changes in the brain, including a pronounced enlargement of the lateral and 3rd ventricle. Magnetic resonance imaging also revealed alterations in signal intensities in cortical, hippocampal, caudate and amygdaloid regions (Wrynn et al, 2000a).
Neuronal degeneration and remodelling have been reported following bulbectomy. Hall and Macrides (1983) reported reductions in dendritic branching, in the number of dendritic spines and in the number of synapses of both spines and shafts of dendrites within the pyriform cortex following bulbectomy. Bulbectomy was later reported to reduce dendritic spine density in CA1, CA3 and dentate gyrus of the hippocampus (Norrholm, 2001). Olfactory bulbectomy induces neuronal cell death manifested as an increased number of pyknotic neurons, predominantly in the DRN, but also in the locus coeruleus (Nesterova, 1997).

Neurochemical changes

Serotonin
There is substantial evidence that a deficiency in the central serotonergic system plays a role in the developemt of MDD (for review see Elwueghi, 2004). Earlier studies have detected reduced levels of CSF 5-Hydroxyindole-acetic acid (5-HIAA) in depressed suicide victims (for review see Smith and Cowen, 1997). Depressed patients also exhibit reduced plasma concentrations of serotonin (5-HT) and reduced platelet 5-HT uptake in comparison to control subjects (Sarrias et al, 1987; Meltzer and Arora, 1991, Quintana, 1991). Furthermore, dietary restriction of tryptophan in patients whose depression has remitted after a treatment with selective serotonin reuptake inhibitors (SSRIs) induces a relapse in depressive symptoms (Delgado et al, 1990, Bell et al, 2001). Drawing on these findings, there has been considerable interest in the role of 5-HT in the mechanism of action of OBX. Jancsar and Leonard (1984) found decreased tissue 5-HT content in amygdaloid cortex and midbrain following bulbectomy and Redmond (1997) showed lower tissue 5-HT concentration in the frontal cortex of bulbectomized animals. Several studies have reported an accompanying increase in 5-HIAA concentrations in amgdaloid cortex (Jancsar and Leonard, 1984) and frontal cortex (Zhou et al, 1998). However, Connor et al (1999) reported significantly reduced basal 5-HIAA levels in the nucleus accumbens.
Reduced platelet serotonin uptake (Butler et al, 1988) and a decrease in [3H]imipramine binding have been found in pons and hippocampus (Jesberger and Richardson, 1986; Stockert et al, 1988), whereas an increase in [^{3}H]imipramine binding has been reported in midbrain (Jesberger and Richardson, 1986).

Watanabe et al (2003) found increased 5-HT synthesis in frontal cortex and hippocampus, but a decrease in the raphe nuclei. Zhou et al (1998) reported an increase in the density of 5-HT transporters and tryptophan hydroxylase concentration in the frontal cortex of OBX rats. Moreover, 5-HT_2 receptors up-regulated following bulbectomy (Gurevich et al, 1993). These changes may reflect an upregulation of serotonergic transmission in response to neuronal degeneration.

Norepinephrine
Studies regarding the effect of olfactory bulbectomy on norepinephrine (NE) levels are not equivocal. Several studies indicate a reduction in NE and NE turnover (summarized in Van Riezen and Leonard, 1990), and more recently a reduction of NE content in frontal cortex and hypothalamus of bulbectomized rats has been found (Song and Leonard, 1995; Redmond et al, 1997). However, Broekkamp et al (1986) failed to find changes in amygdala and pyriform cortex.
Functional responses, such as clonidine-induced hypothermia and salbutamol-induced drinking responses, do not differ between OBX animals and SHAM controls (Redmond, 1995).

Dopamine
Jancsar and Leonard (1984) have found decreased dopamine (DA) turnover in the midbrain of bulbectomized rats, whereas turnover in the amygdaloid cortex was unchanged. In line with these findings, Redmond et al (1997) found decreased DA levels in the frontal cortex of bulbectomized rats. Recently, Masini et al (2004) found that OBX increased DA release in both dorsal and ventral striatum. However, metabolite levels or turnover were not affected.

Neuroendocrine changes

Glucocorticoid secretion has been investigated following olfactory bulbectomy, but has yielded conflicting results. Song et al (1994) reported normal secretion of corticosterone to occur in OBX rats during the light phase. However, during the dark phase they observed an extended hypersecretion, suggesting an altered circadian secretion of corticosterone. There are some reports of raised levels of both basal and stress-induced glucocorticoids (Cairncross et al, 1979, Catarelli and Demael, 1986). However, Broekkamp et al (1986) suggested that OBX animals show an increased corticosteroid response to stress rather than an increase in basal levels. It is feasible that compensatory changes in bulbectomized animals are sufficient to maintain normal functioning under normal circumstances. However, under stressful conditions, a bulbectomized animal may show an inability to adapt to its environment, as was first suggested by Leonard and Tuite (1981).

Immunological changes

Olfactory bulbectomy induces immunological changes (for review see Song and Leonard, 1995). Reduction in the number and proliferation of lymphocytes have been reported (Song and Leonard, 1994; Song et al, 1994), as well as a reduction in neutrophil phagocytosis (O'Neill et al, 1987). Mononuclear cell phagocytosis was increased (Kelly et al, 1991), as well as monocyte proliferation (Song and Leonard, 1995) and neutrophil number (Song and Leonard, 1994). Bulbectomized animals show an increase in positive acute phase proteins, whereas negative acute phase proteins are decreased (Song and Leonard, 1994). Also, a reduction in the relative weigth of the thymus and spleen, which are immune-related organs, have been found following bulbectomy (Song and Leonard, 1993).

Antidepressant treatment of OBX rats

Chronic treatment with clinically effective antidepressants attenuates a large proportion of the changes associated with olfactory bulbectomy. Contrary to most other animal model of depression, the hyperactivity in the open field displayed by OBX rats responds selectively to chronic antidepressant treatment, thus mimicking the clinical time course of antidepressant action (Van Riezen and Leonard, 1990). To date, there have been no reports of any antidepressants, including putative candidates, which will attenuate the hyperactivity of OBX rats in the open field following acute administration (Cryan et al, 1997, 1998; Harkin et al, 1999).
Chronic antidepressant treatment has also been shown to reverse the alterations in 5-HT and 5-HIAA brain tissue levels (Jancsar and Leonard, 1984; Lumia et al, 1992; Song and Leonard, 1995). Also, changes in platelet 5-HT uptake, [^{3}H] imipramine binding and 5-HT_{2C} receptor number have been shown to normalize following antidepressant treatment (Butler et al, 1988; Stockert et al, 1988; Earley et al, 1994).
Reduced dendritic spine density in the hippocampus of bulbectomized animals is reversed by chronic antidepressant treatment (Norrholm et al, 2001). Chronic antidepressant treatment also counteracts the degenerative process of neurons in the DR and LC and improves the functional condition of surviving neurons in both structures (Nesterova et al, 1997).

Box 3. Differential reinforcement of low rate 72 s

The Differential reinforcement of low rate (DRL) responding is a behavioral operant condition procedure in which an animal must learn to withhold responses to a lever for a certain time (typically 72 sec) in order to obtain reinforcement. The DRL-72 s test has been proposed as a behavioral screening model in rodents to identify compounds with antidepressant potential in man (McGuire and Seiden, 1980; O'Donnel and Seiden, 1983). Indeed, SSRIs have demonstrated beneficial effects in the DRL-72 s procedure (Sokolowski and Seiden, 1990; Olivier et al, 1993).
DRL experiments were conducted in eight rat operant chambers with stainless steel grid floors (MED associates, Georgia, USA), enclosed in ventilated sound-attenuating cubicles. Each chamber was equipped with a retractable lever, next to a food cup in which a pellet dispenser delivered 45 mg food pellets (Noyes Company, Lancaster, USA). Initially, rats were trained to press the response lever for food reinforcements in an autoshaping procedure. When all rats reliably pressed the lever, a DRL-12s schedule was presented, in which rats had to wait at least 12 sec between successive lever presses in order to obtain food reinforcement. Subsequently, the schedule requirement was increased every session in steps of 12 s until the DRL-72s schedule was in effect. Each session started with the illumination of the house light and the presentation of the lever. Pressing the lever resulted in delivery of a food pellet when the inter-response time was longer than the required DRL time.

MAJOR DEPRESSIVE DISORDER

Many of the changes seen in bulbectomized animals can be correlated to abnormalities observed in patients with major depression (MDD). Depressed patients exhibit reduced plasma concentration of 5-HT and reduced platelet 5-HT uptake in comparison to control subjects (Sarrias et al, 1987; Meltzer and Arora, 1991; Quintana, 1991). Clinical studies have shown that rapid lowering of brain 5-HT function, using the TRP-depletion method, can precipitate depressive symptoms in individuals in remission after successful treatment with SSRIs (Smith et al, 1997), supporting a role for 5-HT in the mechanism of action of these drugs. On a macroscopic level, Drevets (2001) showed increased ventricular size and decreased frontal lobe, caudate and amygdaloid volumes as well as lesions in the frontal lobes and basal ganglia of depressed patients. Also, amygdaloid blood flow and metabolism show a positive correlation to depression severity (Drevets, 1998). Post-mortem 5-HTT binding has been shown to be decreased in the ventral prefrontal cortex of suicides as well as in the prefrontal cortex of subjects with a history of depression (Mann et al, 2000).

Furthermore, some patients with MDD show hypersecretion of cortisol throughout the 24-h cycle and hence the diurnal rhythm exhibits a flattened profile. This hypersecretion of cortisol is not suppressed by administration of dexamethasone (Carrol et al, 1982), possibly due to abnormalities in negative feedback at multiple levels in the HPA axis (Holsboer et al, 1995).
Patients with MDD exhibit a range of immunological abnormalities. For instance, reduced mitogen-stimulated lymphocyte proliferation (Kronfol and House, 1989) and increased mononuclear phagocytotic poroliferation (McAdams and Leonard, 1993) have been reported. Also, positive acute phase proteins are increased and negative acute phase proteins are decreased in depressed patients (Maes, 1995).

In summary, the olfactory bulbectomized rat model of depression has shown to be an effective means of screening for candidate drugs with potential antidepressant activity, and a means of elucidating the neurobiological mechanism of action of effective treatments. Futhermore, it is a model for exploring the links between neurotransmitter, behavioral, endocrine and immune systems and their role in the pathophysiology of major depression.

AIMS AND OUTLINE

The aims of this thesis are three-fold. First, to assess the effects of dietary tryptophan content on serotonergic functioning in healthy animals. Second, to determine the effects of bilateral removal of the olfactory bulbs on functioning of the serotonin system, and third, to explore the effects of dietary tryptophan supplementation on bulbectomized animals.
Chapter 2 reports on several important considerations concerning experimental setup when using microdialysis. The effects of the length of the interval between surgery and perfusion on 5-HT were studied as well as the effect of the use of a single probe on two consecutive days as opposed to the use of two different bilaterally implanted probes. In **chapter 3** of this thesis, the neurochemical effects of long-term alterations in dietary intake of tryptophan were measured using in-vivo microdialysis. Extracellular basal levels of 5-HT were measured in the dorsal hippocampus of rats receiving low, normal or high levels of tryptophan in their diet. Fluvoxamine, an SSRI, and fenfluramine, a 5-HT releasing agent, were administered locally by reversed microdialysis into the dorsal hippocampus to measure the release and releasable pool of 5-HT, respectively. Furthermore, behavioral effects were assessed by using the differential reinforcement of low rate 72 s (DRL-72 s) procedure, an established paradigm to measure depressive-like behavior in animals.
The effects of bilateral removal of the olfactory bulbs on functioning of the serotonergic system are described in **chapter 4**. In-vivo microdialysis was

performed in rats that underwent SHAM or OBX surgery. Basal extracellular levels of 5-HT were measured in the basolateral amygdala (BLA) 2 weeks after bulbectomy and in the dorsal hippocampus (DH) 2 weeks and 5 months after bulbectomy. In addition, 5-HT synthesis, the amount of 5-HT available for release and the effects of a 5-HT uptake inhibitor on extracellular 5-HT levels in OBX rats as compared to SHAM operated animals were investigated. In addition, the effects of OBX on changes in body weight and locomotor activity in the open field were measured regularly over a period of 20 weeks following removal of the bulbs. **Chapter 5** describes the compensatory changes in serotonergic functioning of bulbectomized animals. Fluvoxamine and the 5-HT_{1B} receptor agonist CP93129 were infused locally into the DH of rats that underwent SHAM or OBX surgery to assess alteration in the 5-HT transporter (5-HTT) and the synaptic 5-HT_{1B} receptor, respectively. Flesinoxan a 5-HT_{1A} receptor agonist was infused locally in the median raphe (MR) to study the effects of bulbectomy on functioning of the somatodendritic 5-HT_{1A} receptor. Systemic administration of fluvoxamine in combination with the 5-HT_{1A} receptor antagonist WAY100.635 was carried out to verify effects found on the somatodendritic 5-HT_{1A} receptor. The effects of enhancement of serotonergic functioning by chronic tryptophan supplementation on several of the alterations seen in bulbectomized animals are reported in **chapter 6**. Locomotor activity of SHAM and OBX animals was measured using the open field. The effects on functioning of the somatodendritic 5-HT_{1A} receptor were assessed using microdialysis. Using quantitative PCR, gene expression of several genes relevant for serotonergic functioning was measured. Finally, corticosterone levels were measured over a 24 h period. In **chapter 7**, the main findings are discussed and concluded with future directions.

Methodological aspects of microdialysis in rat brain: effects of post-surgery interval and repeated perfusions

Chapter 2

Summary

Brain microdialysis is an invasive sampling technique and will always cause damage to nervous tissue. The trauma of implantation of a microdialysis probe causes edema and various other inflammatory changes and it has also been shown that perfusion as such also has an effect on the course of tissue reactions. Taken together, two important considerations concerning experimental setup of the microdialysis arise. First, the interval between surgery and perfusion has to be optimalised. Second, to reduce the number of animals, multiple microdialysis sessions in the same animals are common. The present study describes several important considerations concerning experimental setup when using microdialysis. The effects of the length of the interval between surgery and perfusion on 5-HT were studied as well as the effect of the use of a single probe on two consecutive days as opposed to the use of two different bilaterally implanted probes. It was found that an interval between surgery and experiment of up to 8 days did not affect the results of reuptake blockade with SSRIs, if expressed as percentage of the basal level. Basal levels of 5-HT as well as the absolute effect of SSRIs asdministration, however, increased with longer intervals. It was therefore not possible to compare absolute levels and effects at different post-surgery intervals. We also showed that repeated use of the same probe on two consecutive days did not result in reproducible data when results are expressed as absolute values; 5-HT levels were lower on the second test day. However, when two probes are used with the second probe implanted on the contralateral side, experiments can be reproducible conducted on two consecutive days. The present data do suggest though, that when it comes to relative increases of 5-HT, repeated perfusion through the same probe might be an option.

Introduction

Since its invention 2 decades ago the in-vivo microdialysis technique (Zetterman, 1983) has gained great acceptance for studies of physiological and pharmacological processes in the central nervous system. However, brain microdialysis is an invasive sampling technique and will always cause damage to nervous tissue. The possible artifacts of the microdialysis technique and methods to evaluate the possible neuronal origin of sampled transmitter were reviewed by Westerink and Timmerman (1998). Using telemetric recordings, Drijfhout et al (1995) showed pronounced effects of microdialysis surgery on amplitude and rhythmicity of the temperature and activity patterns which are still present 6-7 days after surgery.

The trauma of implantation of a microdialysis probe causes edema and various other inflammatory changes. Formation of eicosanoids, local disturbances in cerebral blood flow and glucose metabolism have been detected following intracerebral implantation of a microdialysis probe (Benveniste et al, 1987; Yergey et al, 1990). These changes were more or less normalized one day after surgery. Histological evaluation revealed that gliosis usually started 2 or 3 days after implantation of the probe, with the reactions being confined to a very small region around the probe (Benveniste et al, 1987; Shuaib et al, 1990). Perfusion as such also had an effect on the course of tissue reactions (De Lange et al, 1995). Taken together, two important considerations concerning experimental setup of the microdialysis arise. First, the interval between surgery and perfusion has to be optimalised; animals must be allowed sufficient time to recover from implantation of the probe without introducing artifacts due to gliosis. Second, to reduce the number of animals, multiple microdialysis sessions in the same animals are common. However, as De Lange et al (1995) described, perfusion itself could alter neuronal function, thereby introducing differences between consecutive days of microdialysis.

In the present study the effects of dietary tryptophan alterations on extracellular 5-HT levels in the hippocampus of rats were investigated. In order to circumvent effects of surgery on eating behavior, it is essential to allow animals to recover after implantation of the probe. However, as described above, the interval between surgery and the microdialysis experiment should not be too long, as this will result in long-term tissue reactions.
The first aim of the present study was therefore to determine whether an interval of 2, 5 or 8 days between surgery and experiment had an effect on basal extracellular 5-HT levels and the effect of local administration of an SSRI on 5-HT levels. The second aim was to test the effect of three diets on extracellular 5-HT levels on two consecutive days after implantation of the probe. One week after surgery, basal extracellular levels and the effect of local administration of an SSRI were measured

on two consecutive days. In the one group of animals the two measurements were done through the same probe implanted in the dorsal hippocampus and in another group the second measurement was done by using a second probe implanted in the dorsal hippocampus at the contralateral side.

Materials and methods

Animals
Male Sprague-Dawley rats (Harlan, Zeist, The Netherlands) weighing approximately 250g at the time of OBX or sham operation were housed two per cage under standard laboratory conditions (22-24˚C, 12/12h light/dark cycle (lights on from 0600), food and water ad libitum) for at least one week until surgery. After implantation of the microdialysis probe, animals were housed separately. One group of animals was left to recover 2, 5 or 8 days before microdialysis experiments were carried out. A separate group of animals was randomly assigned to one of three dietary conditions, containing different amounts of tryptophan. In this group, microdialysis experiments were carried out one week after implantation of the probe. All experiments were performed in accordance with the governmental guidelines for care and use of laboratory animals and were approved by the Ethical Committee for Animal research of the Medical Faculty of Utrecht University.

Surgery
Rats were anesthetized with chloral hydrate (400 mg/kg, ip) and placed in a stereotactic instrument (Kopf). Lidocaine (5%) was applied in the incision as local anesthetic. A concentric microdialysis probe was implanted at the following coordinates: tooth bar set at +5; A: -3.9 mm; L: -2.9 mm; V: -5.5 mm from bregma and skull surface (Paxinos and Watson, 1997). The exposed length of the probes was 2 mm (i.d. 220 μm; o.d. 310 μm). In one group of animals a second probe was placed contralaterally in the DH at the following coordinates: tooth bar set at +5; A: -3.9 mm; L: +2.9 mm; V: -5.5 mm from bregma and skull surface The probes were secured in place with dental cement and three anchor screws in the skull.

Diets
In one of the groups of animals, rats were randomly assigned to one of three dietary conditions. These consisted of three diets differing in L-tryptophan (TRP) content. All diets were manufactured by Numico, Wageningen, The Netherlands. The control diet contained 0.24 g TRP/100 g. The amounts of TRP in the low and high diets were 10 and 200 % of the control diet, respectively. To keep the total amount of amino acid in all three diets equal, the changes in tryptophan content were counterbalanced by adjusting the amounts of leucine, isoleucine and valine. Animals on TRP low normal or high diet received the diet for 1 week. Animals on

TRP low diet received TRP low diet for 4 days prior to experimentation to prevent excessive weight loss.

Microdialysis experiments

In one group of animals, experiments were performed 2, 5 or 8 days after implantation of the probe in conscious and freely moving animals. The probes were perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$, and 1 mM $MgCl_2$) using a Harvard Microinfusion pump (Harvard Scientific, USA) at a constant flow rate of 1.5 µl/min. A dual channel swivel (Harvard scientific, USA) was used to allow the animals relatively unrestricted movement.

Directly after connecting animals to the infusion pump, 30 min samples were collected into vials containing 15 µl 0.1M acetic acid. Fluvoxamine (10 µM) was dissolved in the perfusion fluid and applied for 120 min. Subsequently, 1 µM tetrodotoxin was infused through the probe for 30 min.

A second group of animals received one of three diets for one week after implantation of the probe (s). In half of these animals, one probe was implanted in the dorsal hippocampus, in the other half, two probes were implanted bilaterally in the hippocampus. Experiments were performed one week after implantation of the probe and samples were collected starting three hours after connecting the animals to the infusion pump. In the first group the perfusion was done through the same probe on both days. In the latter group the contralateral probe was used on the second day of experimentation. Local infusion of drugs was performed by switching syringes after one hour of baseline sampling. Fluvoxamine (10 µM) was dissolved in the perfusion fluid and applied for 120 min.

Samples were collected automatically using an Univentor 820 cooled autosampler (set at 8˚C) and were subsequently frozen at -80˚C until analysis. In the figures the period of local infusion is corrected for the lagtime of the microdialysis system.

Drugs and chemicals

All reagents were from Merck (Darmstadt, Germany) except for heptanesulfonic acid sodium salt (Kodak, USA) and methanol (Riedel-de Haën, Germany). Fluvoxamine was generously donated by Solvay Pharmaceuticals, Weesp, The Netherlands.

Analytical procedure

Analysis of 5-HT and 5-HIAA was performed by HPLC with electrochemical detection. Briefly, 20 µl samples were injected into a high performance liquid chromatograph (LKB, Woerden, The Netherlands) equipped with a 10 cm reversed phase column (Hypersil RP-18, 3 µM, 2.0 mm, Shandon) and an electrochemical detector (Antec Leyden BV, Leiden, The Netherlands) at a potential setting of 600 mV versus an Ag/AgCl reference electrode. A column oven (LKB), set at 40˚C, was used for both the column and the electrochemical detector. The mobile phase

consisted of 5 g/l $(NH_4)_2SO_4$, 50 mg/l heptane sulfonic acid sodium salt 500 mg/l EDTA, 5% methanol and 30 µl/L triethylamine adjusted to pH 4.65 with 30 µl acetic acid. The flow rate was 0.4 ml/min. The detection limit for 5-HT was 0.5 fmol/20 µl sample (signal/noise ratio=2). The cooling temperature of the vial collector as set at 8˚C.

Histology
Following the termination of each experiment, animals were anesthetized with chloral hydrate (400 mg/kg, ip) and decapitated. The brains were then fixed in a 5% formaldehyde solution and the area of insertion of the microdialysis probe was cut into 150 µm slices. The position of the probe was verified microscopically by the track of the probe through the brain. Data were discarded if bulbs were not completely removed, if the frontal cortex was damaged, or the dialysis probe was not in the vicinity of the region aimed at. Probes were considered placed correctly if they were within a range of 0.2 mm of the intended position.

Data analysis and statistics.
All data are expressed as mean ± SEM. Values for the first two consecutive samples were averaged to calculate basal levels. Data were analyzed with multivariate analysis of variance (MANOVA) with time as within factor and delay (2, 5 or 8 days) or diet (control, TRP low, TRP high) as between factor. When appropriate, data were broken down in group and comparisons of the AUC (Area Under the Curve) were made using a t-test. The significance level for all analyses was set at 5%. In the time-figures the start of local infusion of drugs (time point zero) is corrected for the lag-time of the microdialysis system.

Results

Microdialysis 2, 5 or 8 days after surgery
Directly after connecting the animals to the microdialysis setup, extracellular levels of 5-HT were relatively high in all three groups. Levels of 5-HT decreased gradually in all groups reaching steady state levels around two to three hours after start of the infusion (fig. 1). Extracellular basal 5-HT levels on day 2, 5 and 8 after surgery are shown in table 1. Multivariate analysis showed a significant difference in extracellular 5-HT between days with increasing 5-HT levels with longer intervals between surgery and microdialysis ($F_{(2,21)}$=49.9, $p<0.001$). 5-HT levels increased significantly between 2 and 5 days after surgery ($p=0.009$, $n=15$) and also between 5 and 8 days after surgery ($p=0.001$, $n=16$).

	Interval surgery-microdialysis			One probe		Two probes	
Diet	2 days	5 days	8 days	day 1	day 2	day 1	day 2
TRP low				2.9 ± 0.5	2.5 ± 0.5	2.7 ± 0.5	3.0 ± 0.5
Control	1.2 ± 0.1	1.9 ± 0.2	3.0 ± 0.3	4.4 ± 0.3	2.1 ± 0.5	4.6 ± 0.5	5.0 ± 0.4
TRP high				10.1 ± 0.9	2.6 ± 0.4	10.8 ± 0.8	10.2 ± 0.7

Table 1 Extracellular basal 5-HT levels in the dorsal hippocampus on day 2, 5 or 8 after implantation of the probe, and the effect of microdialysis on two consecutative days using the same (one) or (two) different probes.

Administration of 10 μM of fluvoxamine through the dialysis probe resulted in an increase in 5-HT levels on all three days. Maximum levels of 5-HT were reached after 60 min and amounted to 5.3 ± 0.4, 8.1 ± 0.5 and 1.6 ± 0.5 fmol/fraction on day 2, 5 and 8 after surgery, respectively. Multivariate analysis showed that the 5-HT increase was larger when the interval between surgery and microdialysis was longer ($F_{(10,34)}$=9.4, p<0.001). Breakdown of the data on interval showed that the effect of fluvoxamine was significantly larger at 5 days than at 2 days post-surgery ($F_{(5,9)}$=58.4, p<0.001) and it was also larger at 8 days than 5 days post surgery ($F_{(5,10)}$=6.1, p=0.008).

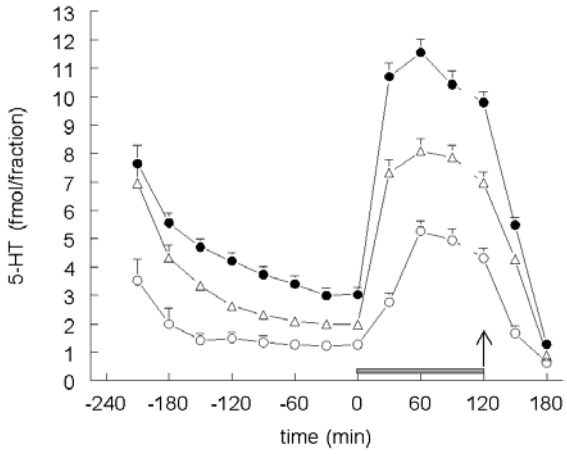

Fig 1 Microdialysis in the DH 2 (○, n=8), 5 (△, n=7)or 8 (●, n=9) days after probe implantation. Data are shown as mean absolute values in 30 min dialysate samples ± SEM. At time point zero, 10 μM fluvoxamine is infused into the DH as shown by the bar. The arrow indicates local infusion of 1μM TTX.

Fig. 2A shows the absolute increase of 5-HT after local administration of fluvoxamine. Multivariate analysis of the AUC values confirmed an increase of 5-HT in all three groups ($F_{(2,21)}$=100.8, p<0.001). Breakdown of the data on interval showed that the absolute increase was significantly larger at 8 days after surgery as compared to 5 days after surgery (p<0.001, n=16) and also at 5 days as compared to 2 days after surgery (p<0.001, n=15).
However, when data were calculated as percentage of basal values, no difference in effect of fluvoxamine was found. When 120 min after the start of local infusion of fluvoxamine, 1 μM tetrodotoxin (TTX) was infused (as shown by the arrow in fig. 1), 5-HT levels significantly decreased. Levels of 5-HT 60 min after TTX amounted to 0.63 ± 0.3, 0.89 ± 0.2 and 1.1 ± 0.3 fmol/fraction at 2, 5 and 8 days after surgery, respectively. Multivariate analysis showed no significant difference between the levels after TTX.

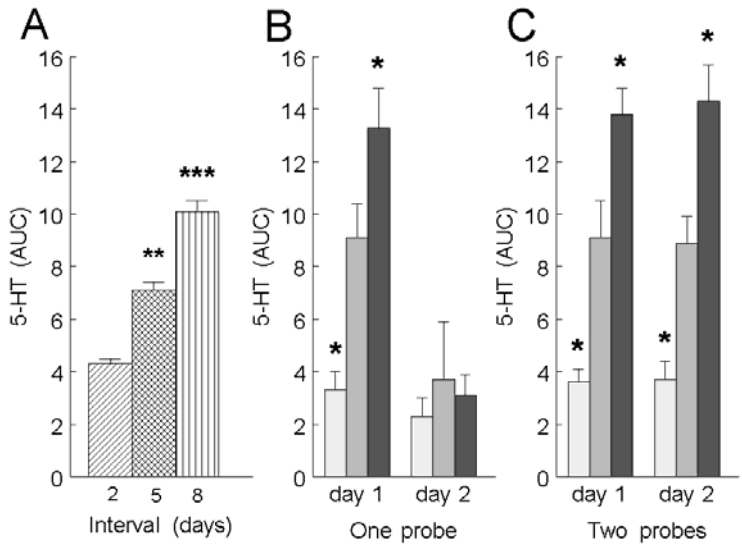

Fig. 2 Absolute increase of 5-HT levels after local administration of fluvoxamine. **A** Absolute increase in 5-HT on day 2, 5 or 8 after surgery. **B** Absolute increase in 5-HT on day one and day two of perfusion via the same probe in animals receiving TRP low (□, n=6), control (■, n=6) or TRP high (■, n=6) diet. **C** Absolute increase in 5-HT on day one and day two of perfusion via two probes in animals receiving TRP low (□, n=6), control (■, n=6) or TRP high (■, n=6) diet.
* p<0.005, TRP low or TRP high diet significantly different from control.
** p<0.005, 5 days significantly different from 2 days.
*** p<0.005, 8 days significantly different from 2 and 5 days.

Microdialysis in the same probe on two consecutive days

Figs. 2B and 3 show the effects of local administration of 10 μM fluvoxamine on 5-HT levels in animals receiving TRP low, control or TRP high diet. Microdialysis was performed using the same probe on two consecutive days. Extracellular basal levels of 5-HT are shown in table 1. Basal levels of 5-HT were significantly decreased after TRP low diet ($p=0.027$, n=12) and significantly increased after TRP high diet ($p<0.001$, n=12), compared to control diet. On the second day of dialysis, extracellular basal levels of 5-HT were lower in all groups and amounted to 2.5 ± 0.5, 2.1 ± 0.5 and 2.6 ± 0.4 fmol/fraction for TRP low, control and TRP high diet, respectively. The basal 5-HT levels on the second day of dialysis were not different. On day one, local administration of 10 μM fluvoxamine significantly increased extracellular 5-HT levels in all three groups (fig 3A), reaching maximum levels after 60 min (time $F_{(5,11)}=33.4$, $p<0.001$). Multivariate analysis showed that the effect of fluvoxamine was diet-dependent (diet $F_{(2,15)}=91.7$, $p<0.001$; time x diet interaction $F_{(10,22)}=8.1$, $p<0.001$). Breakdown of the data on diet showed that the effect of fluvoxamine was attenuated in animals receiving TRP low diet compared to controls ($F_{(1,10)}=22.5$, $p=0.001$). The effect of fluvoxamine was larger in animals receiving TRP high diet compared to controls ($F_{(1,10)}=84.3$, $p<0.001$). These data absolute increase in 5-HT after infusion of fluvoxamine.

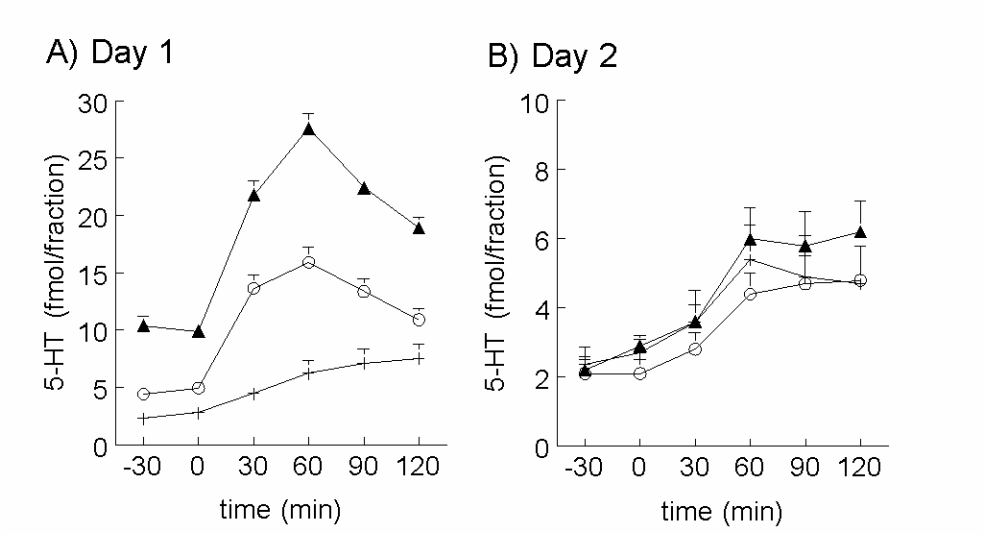

Fig. 3 Time course of the effects of local administration of 10 μM fluvoxamine into the DH of animals receiving TRP low (+, n=6), control (○, n=6) or TRP high (▲, n=6) diet. Data are shown as mean absolute values in 30 min dialysate samples ± SEM. **A** Day one of perfusion. **B** Second day of perfusion; in the same probe as day one. 10 μM fluvoxamine was infused from time point zero, as indicated by the bar.

Multivariate analysis of the AUC values (fig 2B) showed a main effect of diet ($F_{(2,15)}$=15.8, p<0.001). Contrast analysis revealed that the amount of 5-HT increase in animals receiving TRP low diet was smaller than in controls (p=0.005, n=12) whereas the increase in 5-HT of animals receiving TPP high diet was larger than in controls (p=0.035, n=12).
On the second day, fluvoxamine also increased extracellular 5-HT levels in all three dietary groups (fig 3B), reaching maximum levels of 5-HT after 60 min ($F_{(5,11)}$=12.5, p<0.001). However, in contrast to the first day, no effect of the diet on the total increase of 5-HT was found (fig 2B).
When data were calculated as percentage of basal value, total increase of 5-HT amounted to 224 ± 23, 321 ± 45 and 240 ± 25 % for TRP low, control and TRP high diet, respectively on the first day of dialysis. On the second dialysis day, total increase of 5-HT amounted to 179 ± 24, 232 ± 27 and 222 ± 28 % for TRP low, control and TRP high diet, respectively. Neither day one nor day two revealed a main effect of diet. Total increase of 5-HT for the three diets separately did not differ between day one and day two when calculated as percentage. The data indicate that although on day two the absolute levels of 5-HT are not similar to 5-HT levels on day one, the relative effect of administration of fluvoxamine still renders a similar effect.

Bilateral probe implantation
Fig 4 shows the effects of local administration of 10 μM fluvoxamine on extracellular 5-HT levels in animals receiving TRP low, control or TRP high diet. Microdialysis was performed on two consecutive days, using either the left or right probe the first day and the contralateral probe on the second day of dialysis. Multivariate analysis showed that extracellular basal 5-HT levels were not dependent on the day of perfusion, but did indicate a main effect of diet. Contrast analysis showed that basal 5-HT levels were decreased following a TRP low diet (p<0.001, n=12) and increased following a TRP high diet (p<0.001, n=12) compared to control diet.
Local administration of 10μM fluvoxamine significantly increased 5-HT levels in all groups (fig 4A and B), reaching maximum levels after 60 min ($F_{(5,26)}$=71.2, p<0.001). Multivariate analysis revealed a significant effect of diet on fluvoxamine induced 5-HT release ($F_{(10,52)}$=13.8, p<0.001), but no effects of the day of dialysis or an interaction between day and diet were found. Breakdown of the data on diet showed that the effect of fluvoxamine on 5-HT release was attenuated in animals receiving a TRP low diet ($F_{(5,18)}$=31.5, p<0.001), whereas it was increased in animals receiving a TRP high diet ($F_{(5,18)}$=9.2, p<0.001) compared to control diet.

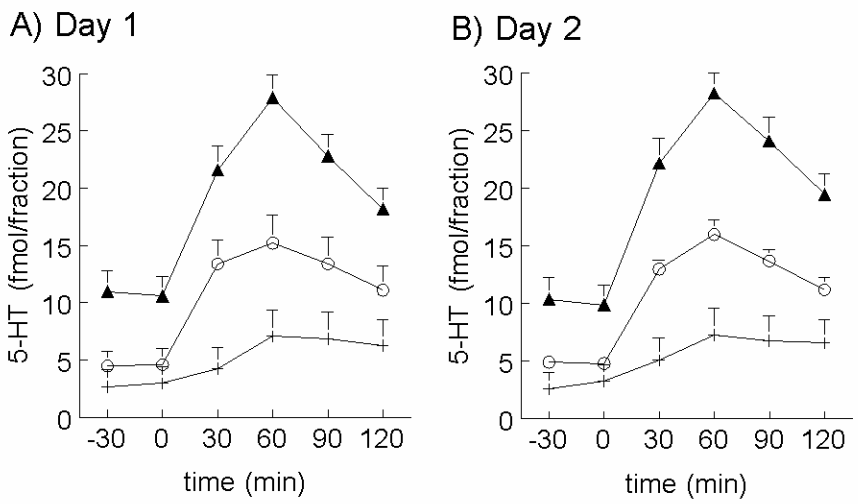

Fig. 4 Time course of the effects of local administration of fluvoxamine into the DH of animals receiving TRP low (+, n=6), control (○, n=6) or TRP high (▲, n=6) diet. Data are shown as mean absolute values in 30 min dialysate samples ± SEM. **A** Day one of perfusion. **B** Second day of perfusion, in the contralaterally placed probe. 10 μM fluvoxamine was infused from time point zero, as indicated by the bar.

Multivariate analysis of the absolute increase of 5-HT again showed a main effect of diet ($F_{(2,30)}$=40.1, p<0.001) and no effect of the day of dialysis (fig 2C). Contrast analysis revealed that the amount of 5-HT increase animals in receiving TRP low diet was smaller than controls (<0.001, n=24) whereas the increase in 5-HT in animals receiving TPP high diet was larger than controls (p=0.001, n=24).

Discussion

The results of the present study showed that reproducible microdialysis can be performed up to day 8 after implantation of the microdialysis probe. It was also shown that repeated perfusion through a single probe altered the results in such a manner that although the relative effects of administration of an SSRI are similar on both days, the absolute levels of 5-HT are not. When a contralaterally placed probe is used on the second day of dialysis, absolute levels of 5-HT and the effects of administration of an SSRI are reproducible, both as absolute and as relative values.

After implantation of a microdialysis probe, various tissue reactions take place. The trauma of the implantation causes edema and various other inflammatory changes. Granulocytes and monocytes migrate to damaged tissue and monocytes proliferate into macrophages being responsible for wound healing. Gaps caused by loss of cells are repaired by fibrosis and gliosis. Astrocytes that are activated will finally form a glial barrier around the probe (Benveniste and Diemer, 1987). De Lange et al (1995) studied the morphological changes around the probe by means of semi quantitative histology. The reactions to the presence of the probe as such were minimal. However, after repeated perfusion procedures, tissue scores of hypercellularity and infiltration of granulocytes increased.

In the present study we found that extracellular basal 5-HT levels increased with increased intervals between surgery and the microdialysis experiment. The absolute increase in 5-HT after local fluvoxamine administration also increased with longer intervals. However, the 5-HT increase relative to basal 5-HT values did not change. This indicates that although the recovery of 5-HT via the probe changes over time, probably due to processes initiated by the surgery, the effect of a pharmacological intervention does not change over time. This is in line with findings by De Lange et al (1995), who studied the morphological changes around the probe by means of semi quantitative histology. The reactions to the presence of the probe as such were minimal. Administration of TTX in the DH confirmed that the 5-HT measured by microdialysis in these experiments was of neuronal origin.
These findings are of particular interest when studying dietary interventions. Animals consume less food and tend to lose weight the first few days after implantation of the microdialysis probe (Van der Stelt et al, unpublished data). This might obscure the effects of dietary alterations. To interpret dietary effects correctly, it is essential to allow the animals sufficient time to recover and resume their normal feeding pattern. However, this period should not be too long as to limit tissue alterations. Based on the present findings, we have chosen for a recovery period of one week when dietary interventions are to be studied.

To limit the number of experimental animals, it is desirable to perform multiple experiments in the same animals. One of the easiest ways is to perform microdialysis in one probe on two consecutive days. In the present study, animals received TRP low, control or TRP high diet for one week to assess effects of repeated perfusion on extracellular basal 5-HT levels. On day one of perfusion, a diet dependent effect was found on basal 5-HT levels. However, on the second perfusion day, basal 5-HT levels had decreased in all diet groups and were no longer different. This implicates that when using the same probe twice, it is not possible to reproducible detect changes in basal levels of 5-HT.
Local infusion of the SSRI fluvoxamine increased 5-HT levels in a diet-dependent manner on the first day of dialysis. On the second dialysis day the SSRI induced

increase in extracellular 5-HT was no longer diet-dependent; absolute increases in 5-HT levels after fluvoxamine administration was similar for all three dietary conditions. This finding shows that problems in reproducing absolute levels of 5-HT arise when measuring repeatedly through the same probe.
When the second day of perfusion was performed in a contralateral probe, absolute baseline levels of 5-HT were reproducible, as was the effect of fluvoxamine on 5-HT release.
Several explanations can be advanced to explain these effects of multiple perfusion. It is feasible that perfusion of the microdialysis probe induces alterations in the characteristics of the dialysis membrane, resulting in a different recovery on the second day of dialysis. This may explain the difference in absolute basal extracellular 5-HT levels between the two measurements. Another possible explanation is that perfusion induces alterations in the tissue surrounding the probe. A contralaterally placed probe does produce similar measurements the second experimentation day, therefore changes appear to be restricted to the area surrounding the probe.

The present study shows that an interval between surgery and microdialysis of up to 8 days renders reproducible results when the effects of a reuptake blocker on 5-HT are measured. However, one should bear in mind that basal levels of 5-HT and also the absolute effect of an SSRI increase with longer intervals. It is therefore not possible to compare absolute levels and effects at different post-surgery intervals.
We have also shown that for studies involving the measurement of absolute 5-HT levels, it is not possible to use the same probe twice on two consecutive days. However, it is possible to use two probes implanted in the same animal on two consecutive days. The present data do suggest though, that for studies of the relative increase of 5-HT after SSRI administration, repeated perfusions are possible.

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Effects of dietary tryptophan variations on extracellular serotonin in the dorsal hippocampus of rats

Chapter 3

Summary

There is evidence to suggest that the efficacy of selective serotonin reuptake inhibitors (SSRIs) in depression is dependent on the availability of serotonin (5-HT) in the brain. Moreover, there is circumstantial eveidence suggesting that plasma tryptophan (TRP) levels can predict response to SSRIs. These findings suggest that dietary TRP variations may affect the efficacy of SSRIs in major depression. The objective of the present study was to study the neurochemical and behavioral effects of dietary TRP variations in rats. In vivo microdialysis in the hippocampus was performed in conscious rats randomly assigned to receive a diet containing low, normal or high levels of TRP. Basal levels of 5-HT and 5-HIAA were measured using HPLC. Fenfluramine and fluvoxamine were infused locally to determine the effect of the diet on 5-HT availability and release. In a parallel group of rats, the differential reinforcement of low rate 72 sec (DRL-72s) schedule was used to assess the behavioral effects of the dietary manipulations. 5-HT and 5-HIAA levels were significantly decreased after TRP low diet and 5-HT, but not 5-HIAA, levels were significantly increased after TRP high diet. 5-HT release after fluvoxamine and fenfluramine was significantly diminished in rats on a TRP low diet, and significantly enhanced after fenfluramine in rats on a TRP high diet. DRL-72 performance in rats was decreased by a TRP low diet, whereas a TRP high diet increased DRL performance similar to fluvoxamine administration. The amount of 5-HT released after a 5-HT releasing agent and the effect of an SSRI on extracellular 5-HT are dependent on the nutritional availability of TRP. Moreover, increased availability of TRP affects behavior in a manner similar to SSRI administration. These findings suggest that nutritional factors have behavioral and neurochemical effects, relevant for the treatment of depression.

Introduction

There is considerable clinical evidence that serotonin (5-HT) containing pathways in the central nervous system play a significant role in the pathophysiology of major depression. In line with this notion, selective serotonin reuptake inhibitors (SSRIs) are currently one of the mainstays for the treatment of major depression. There is also evidence that dietary manipulations leading to acute reductions in brain 5-HT neurotransmission may induce mood lowering effects in some healthy individuals (Neumeister et al, 2002) and that depressed patients recovered with SSRIs may experience significant clinical relapses following tryptophan (TRP) depletion (Delgado et al, 1990; Bell et al, 2001). Several studies have also found that plasma TRP levels are decreased in drug-free depressed patients (Anderson et al, 1990). Moreover, patients with an eating disorder, who usually have lowered levels of plasma TRP, often suffer from co-morbid depression (Cowen and Smith, 1999). There is also a higher prevalence of depression in patients with viral infections or cancer undergoing cytokine therapies. It has been shown that cytokines reduce plasma TRP levels and that the severity of depression in patients treated with cytokines is positively correlated with the reduction in plasma TRP (Capuron et al, 2002). These findings suggest that reductions in plasma TRP may be one of the factors triggering or maintaining major depression. By the same token, one may assume that dietary manipulations may affect on one hand severity of depressive symptoms and on the other the efficacy of antidepressants, particularly of SSRIs.

Animal data have confirmed that acute depletion of TRP impairs 5-HT availability in the brain. Thus, Young et al (1985) showed that acute administration of a TRP-deficient amino-acid mixture led to rapid decreases of plasma TRP levels. Using in vivo microdialysis, Bel and Artigas (1996) found that acute TRP depletion also led to a significant decline in extracellular brain 5-HT levels in rats pre-treated with the SSRI fluvoxamine for two weeks, but not in control animals. On the other hand, acute administration of 5-HT precursors, TRP and 5-hydroxytryptophan (5-HTP), caused a significant increase in extracellular 5-HT brain levels in food deprived but not in fed animals (Meeusen et al, 1996). Finally, Sharp et al (1992) reported that TRP pre-treatment significantly augmented the electrically evoked hippocampal 5-HT release in anaesthetized rats, but TRP alone had no effect on basal 5-HT output in this study. Information on the long-term effects of dietary manipulations on brain 5-HT levels is scarce. Franklin et al (1999) reported that a long-term low TRP diet reduced total plasma TRP and whole brain 5-HT content together with a functional upregulation of 5-HT_{2c} receptors, while Fadda et al (2000) reported a decrease in frontocortical 5-HT release following a TRP-free diet for three days.

The aims of the present study were threefold. First, to measure whether long-term alterations in dietary intake of TRP could influence basal extracellular 5-HT levels in the dorsal hippocampus of rats. Second, to determine whether these dietary manipulations changed the release of 5-HT and third, to investigate the behavioral consequences of these dietary manipulations. Neurochemical effects were measured by means of microdialysis in freely moving rats. Behavioral effects were assessed by using the Differential Reinforcement of Low Rate after 72 seconds (DRL-72s) procedure (McGuire and Seiden, 1980; O'Donnel and Seiden, 1983). Fluvoxamine, an SSRI, and fenfluramine, a 5-HT releasing agent, were administered locally by reversed microdialysis into the dorsal hippocampus to measure the release and releasable pool of 5-HT, respectively.

Materials and methods

Animals

For the microdialysis procedure, male Sprague-Dawley rats (Harlan, The Netherlands) weighing 250-300g at the time of the experiment were housed two per cage under standard laboratory conditions (22-24˚C, 12/12h light/dark cycle; lights on at 7:00, food and water ad libitum) for at least one week until surgery. After implantation of the microdialysis probe the animals were housed separately. For the DRL-72s procedure, rats were housed in groups of 4 animals per cage under standard laboratory conditions, except for reversed light/dark cycle (lights on at 19:00). Microdialysis experiments were performed during the light period and behavioral experiments during the dark period. Throughout the behavioral experiments, rats were kept on a food restriction schedule, which provided 15 g of diet food (Research Diets, Wijk bij Duurstede, The Netherlands) per rat per day, to increase motivation for the DRL-72s procedure Tap water was freely available in the home cages. All experiments were in accordance with the governmental guidelines for care and use of laboratory animals and were approved by the Animal Ethics Committee of the Faculties of Pharmacy and Medicine of Utrecht University.

Surgery

Rats were anesthetized with chloral hydrate (400mg/kg, ip) and placed in a stereotactic instrument (Kopf). Lidocaine (5%) was applied in the incision as local anesthetic. A concentric microdialysis probe was implanted in the dorsal hippocampus at the following coordinates: tooth bar set at +5; A: -3.9mm; L: -2.9mm; V: -5.5mm from bregma and skull surface (Paxinos and Watson, 1997). The exposed length of the probes was 2 mm (id 220µm; od 310µm). The probes were secured in place with dental cement and three anchor screws in the skull. Rats were then left for one week to recover.

Diets

Rats were randomly assigned to one of three dietary conditions. These consisted of three diets differing in L-tryptophan (TRP) content. All diets were manufactured by Numico, Wageningen, The Netherlands. The control diet contained 0.24 g TRP/100 g. The amounts of TRP in the low and high diets were 10 and 200% of the control diet, respectively. To keep the total amount of amino acid in all three diets equal, the changes in TRP content were counterbalanced by adjusting the amounts of Leucine, Isoleucine and Valine. Animals on TRP normal or TRP high diet received the diet for one week. Animals on TRP low diet received TRP low diet for 4 days prior to experimentation to prevent excessive weight loss.

Microdialysis experiments

Experiments were performed on day 7 after surgery in conscious and freely moving animals. The probes were perfused with Ringer solution (147mM NaCl, 4mM KCl, 2.3mM $CaCl_2$, and 1mM $MgCl_2$) using a Harvard Microinfusion pump (Harvard Scientific, USA) at a constant flow rate of 1.5µl/min. A dual channel swivel (Harvard scientific, USA) was used to allow the animals relatively unrestricted movement. Three hours after connecting the animals to the infusion pump, 30-min samples were collected into vials containing 15µl of 0.1M acetic acid. Samples were collected automatically using a Univentor 820 cooled autosampler (set at 8˚C) and were subsequently frozen at -80˚C until analysis.

Local infusion of drugs was performed by switching syringes after one hour of baseline sampling. Tetrodotoxin (TTX, 1 µM) and fluvoxamine (3 and 10 µM) were dissolved in the perfusion fluid and applied for 120 min. Fenfluramine (500µM) was also dissolved in the perfusion fluid and applied for 240 min.

Blood and CSF sampling

Sampling of blood and CSF for amino acid analysis was done during the light phase under chloralhydrate anesthesia (400 mg/kg, i.p.), in a separate group of animals on the same diets. Blood was obtained by heart puncture and CSF was withdrawn by cisternal puncture.

Drugs and chemicals

All reagents were from Merck (Darmstadt, Germany) except for heptanesulfonic acid sodium salt (Kodak, USA) and methanol (Riedel-de Haën, Germany). Fluvoxamine was generously donated by Solvay Pharmaceuticals B.V., Weesp, The Netherlands. Fenfluramine was obtained from Research Biochemicals International (Natick, USA).

Analytical procedure

Analysis of 5-HT and 5-HIAA was performed by HPLC with electrochemical detection. Briefly, 20µl samples were injected into a high performance liquid

chromatograph (LKB, Woerden, The Netherlands) equipped with a 10 cm reversed phase column (Hypersil RP-18, 3µM, 2.0mm, Shandon) and an electrochemical detector (Antec Leyden BV, Leiden, The Netherlands) at a potential setting of 600 mV versus an Ag/AgCl reference electrode. A column oven (LKB) set at 40˚C was used for both the column and the electrochemical detector. The mobile phase consisted of 5g/l $(NH_4)_2SO_4$, 50mg/l heptane sulfonic acid sodium salt 500mg/l EDTA, 5% methanol and 30µl/L triethylamine adjusted to pH 4.65 with 30µl acetic acid. The flow rate was 0.4ml/min. The detection limit for 5-HT was 0.5fmol/20µl sample (signal/noise ratio=2).
Amino acids were measured by HPLC with fluorescence detection. The system comprised an Applied Biosystems detector, a Pharmacia pump, a 25cm, 4.6mm LC-8DB column (Supelco) and a Gilson HPLC. The mobile phase consisted of 0.1M sodium phosphate and 47% methanol at a pH of 5.6. The flow rate was 1.0 ml/min.
To obtain plasma free TRP levels, plasma was first passed through a 30.000 molecular weight cut-off filter unit using centrifugation, separating free TRP from the fraction bound to albumin. Whole brain tissue samples were homogenized and deproteinised with 2 volumes of 30% trichloroacetic acid, followed by centrifugation prior to direct injection into the HPLC.

Data analysis and statistics.
Levels of 5-HT and 5-HIAA for the first two consecutive microdialysis samples were averaged to calculate the basal levels. Neurochemical and behavioral data were analyzed with multivariate analysis of variance (MANOVA) with time as within factor and treatment (diet or drug) as between factor. When appropriate, data were broken down on treatment, and comparisons were made by simple contrasts. The significance level for all analyses was set at 5%. In the microdialysis figures, the start of local infusion of drugs (time point zero) is corrected for the lag-time of the microdialysis system.

Histology
Following the termination of each experiment, animals were anesthetized with chloral hydrate (400 mg/kg, ip) and decapitated. The brains were removed, fixed in a 5% formaldehyde solution and the area of interest was cut into 150 µm slices. The position of the probe was verified microscopically by the track of the probe through the brain. Data were discarded if the dialysis probe was not in the vicinity of the region aimed at. Probes were considered placed correctly if they were within a range of 0.2mm of the intended position.

DRL-72s training and testing

The Differential reinforcement of low rate responding is a behavioral operant condition procedure in which an animal must learn to withhold responses to a lever for a certain time (typically 72 sec) in order to obtain reinforcement. The DRL-72s test has been proposed as a behavioral screening model in rodents to identify compounds with antidepressant potential in man (McGuire and Seiden, 1980; O'Donnel and Seiden, 1983).

DRL experiments were conducted in eight rat operant chambers with stainless steel grid floors (MED associates, Georgia, USA), enclosed in ventilated sound-attenuating cubicles. Each chamber was equipped with a retractable lever, next to a food cup in which a pellet dispenser delivered 45 mg food pellets (Noyes Company, Lancaster, USA). A red house light (50 lux) was located above the food cup. An IBM computer equipped with MED-PC software version 2.06 (MED associates, Georgia, USA) controlled experimental sessions and recorded data.

The DRL procedure was adapted from O'Donnel and Seiden (1983). Initially, rats were trained to press the response lever for food reinforcements in an autoshaping procedure. When all rats reliably pressed the lever, a DRL-12s schedule was presented, in which rats had to wait at least 12 sec between successive lever presses in order to obtain food reinforcement. Subsequently, the schedule requirement was increased every session in steps of 12 s until the DRL-72s schedule was in effect. Each session started with the illumination of the house light and the presentation of the lever. Pressing the lever resulted in delivery of a food pellet when the inter-response time was longer than the required DRL time. Daily training sessions lasted 60 min and were typically conducted 5 days per week. Animals were kept on the TRP-normal diet and trained until performance on the DRL-72s schedule had stabilized, before diet and drug tests were performed.

First, the effects of TRP diets on DRL-72s performance were assessed by feeding rats different diets on alternate days according to a 5-day schedule (either N-L-N-H-N or N-H-N-L-N). Rats received 15 g of diet food approximately 22 h before the start of the daily DRL-72s session. Data obtained after feeding the TRP normal diet (3 sessions) were averaged. Second, the effects of feeding the TRP high diet for 3 consecutive days were compared to 3 days of the TRP normal diet. Rats were tested in the DRL-72s procedure 22 h after the third daily meal. Half the rats first received the normal diet and half the rats first received the high diet. Finally, the effects of fluvoxamine administration on DRL performance were assessed. Test sessions were conducted twice a week, with at least 2 days in between, while training sessions continued on intervening days. Doses were tested according to a within-subject latin square design. Fluvoxamine was dissolved in 0.9% saline and injected orally in a volume of 2 ml/kg, with an injection-test interval of 60 min. During the period of fluvoxamine testing, rats were fed on the TRP normal diet.

Results

Body weight and food intake
Body weight of all animals was recorded every day from one week before surgery up to the day of the experiment. Mean body weight of the animals on the day of surgery was 273 ± 16 g. Multivariate ANOVA showed no difference between treatment groups. Mean basal food intake was 19.2 ± 2.1g per rat per day. Multivariate ANOVA did not reveal statistically significant differences in food intake between the treatment groups.

Amino acid levels after chronic depletion or supplementation of tryptophan
Amino acids were measured in blood (total and free (i.e. not bound to albumin), cerebrospinal fluid, brain tissue (hippocampus) and extracellular fluid. The results are summarized in table 1. Total plasma TRP levels were significantly decreased after a TRP low diet ($F=12.428$, $p=0.017$). The ratio of TRP to the sum of the Large Neutral Amino Acids (LNAA; Leucine, Isoleucine and Valine) was significantly decreased after both the TRP low ($F=10.563$, $p=0.017$) and TRP high diet ($F=9.751$, $p=0.011$) as compared to the control diet.

	Tryptophan			TRP/ΣLNAA ($x10^{-2}$)		
	TRP low	TRP normal	TRP high	TRP low	TRP normal	TRP high
Plasma total (μM)	32.6 ± 1.5*	40.9 ± 1.1	34.8 ± 3.0	6.7 ± 0.4 *	9.2 ± 0.6	7.5 ± 0.3
Plasma free (μM)	3.4 ± 0.2	4.7 ± 0.6	5.2 ± 0.8	0.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
CSF (μM)	0.68 ± 0.05	0.97 ± 0.09	0.82 ± 0.14	3.6 ± 0.2	4.9 ± 0.4	4.1 ± 0.5
Brain (μg/g protein)	31.3 ± 1.9	31.8 ± 4.0	35.8 ± 5.6	5.5 ± 0.5	5.7 ± 0.7	7.4 ± 1.1
Extracellular (μM)	0.26 ± 0.19	0.56 ± 0.17	0.38 ± 0.06	3.7 ± 1.1	4.7 ± 0.8	5.1 ± 0.8

Table 1 Levels of TRP and TRP/ΣLNAA in plasma (total and free TRP), CSF, brain tissue and dialysate after feeding on three different TRP diets: TRP low, TRP normal or TRP high.
* $p<0.05$, statistically different from TRP normal diet.

Baseline values of 5-HT and 5-HIAA after chronic depletion or supplementation of tryptophan.
Mean baseline extracellular 5-HT levels in the dorsal hippocampus amounted to 4.71 ± 0.5 fmol/fraction (mean ± SEM, n=24) for the TRP normal group (fig 1). Chronic administration of the TRP low diet (4 days) decreased basal extracellular 5-HT levels to 2.68 ± 0.4 fmol/fraction (n=27). Chronic administration of the TRP

high diet (7 days) increased basal extracellular 5-HT levels to 9.06 ± 0.9 fmol/fraction (n=23). Multivariate ANOVA revealed a statistically significant effect of the diet ($F=34.422$, $p<0.001$). Contrast analysis showed that both the low and the high diet were different from controls ($F=10.051$, $p=0.002$ and $F=14.837$, $p<0.001$, respectively).
Mean baseline extracellular 5-HIAA levels amounted to 2.51 ± 0.3 pmol/fraction (n=24) for the control diet. Chronic administration of the TRP low diet decreased basal extracellular 5-HIAA levels to 1.36 ± 0.2 pmol/fraction (n=27), whereas chronic administration of the TRP high diet caused a slight increase in basal extracellular 5-HIAA levels to 2.87 ± 0.2 pmol/fraction (n=23). Multivariate ANOVA revealed a statistically significant effect of diet ($F=11.235$, $p<0.001$). Contrast analysis showed that the low diet was different from controls ($F=9.603$, $p=0.002$), whereas the TRP high diet was not statistically different from controls.

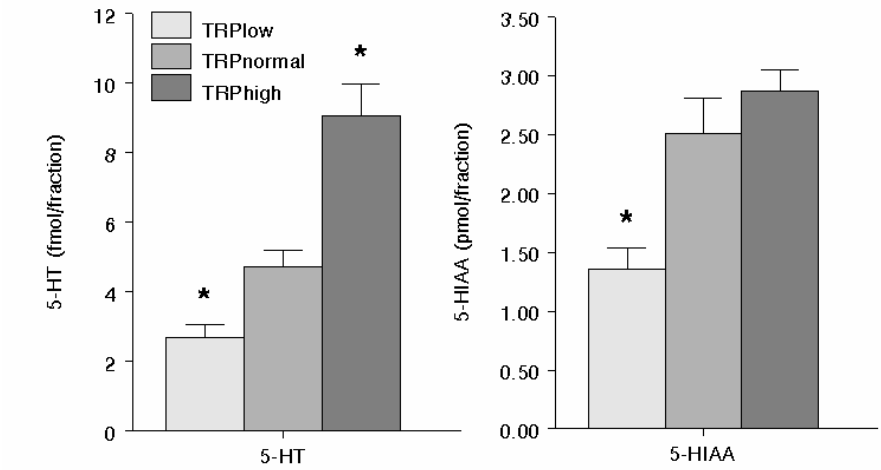

Fig.1 Basal levels of 5-HT and 5-HIAA in the dorsal hippocampus after TRP diet. Data are presented as absolute values ± SEM for the three diets: TRP low (*n*=27;), TRP normal (*n*=24;) and TRP high (*n*=23;).
* $p<0.01$, significantly different from TRP normal diet.

Local infusion of TTX
Local infusion of 1μM tetrodotoxin (TTX) into the dorsal hippocampus of rats receiving TRP normal or TRP high diet caused a statistically significant decrease in extracellular 5-HT levels. Minimal levels of 5-HT were obtained after 90 min and amounted to 1.08 ± 0.2 fmol and 1.16 ± 0.2 fmol for the TRP normal and TRP high diet, respectively. Contrast analysis showed no significant difference between minimal levels in the two conditions.

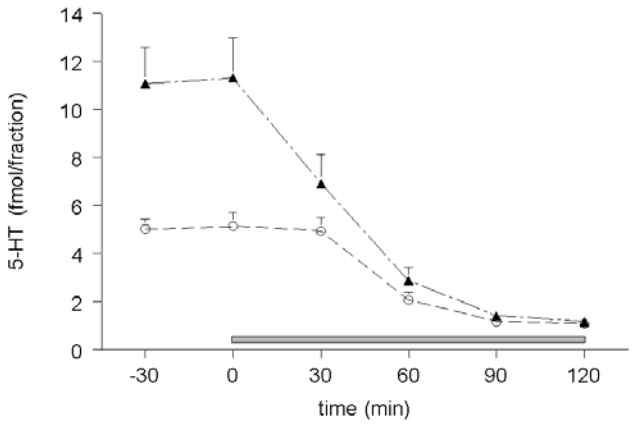

Fig. 2 Time course of the effect of local administration of TTX into the dorsal hippocampus after TRP normal diet (*n*=6; ○) or TRP high diet (*n*=6; ▲). Data are expressed as mean absolute values in 30 min dialysate samples ± SEM. At time point zero, 1 µM TTX was infused as indicated by the bar.

Local infusion of fenfluramine

Local infusion of 500µM fenfluramine into the dorsal hippocampus caused a transient but statistically significant increase in extracellular 5-HT levels in all three diet groups (fig 2). Maximum values of 5-HT amounted to 17.34 ± 4.0 fmol, 37.92 ± 2.7 fmol and 71.62 ± 12.3 fmol for the TRP low, TRP normal and TRP high diet, respectively. Absolute increases amounted to 14.18 ± 3.8 fmol, 34.54 ± 2.5 fmol and 62.43 ± 10.1 fmol for the TRP low, TRP normal and TRP high diet, respectively. One-way ANOVA showed a significant effect of diet ($F=14.033$, $p<0.001$). Contrast analyses showed that both high and low TRP diet were different from controls ($F=5.607$, $p=0.018$ and $F=9.127$, $p=0.003$).

Local infusion of fluvoxamine

Local infusion of 10µM fluvoxamine into the dorsal hippocampus caused an increase in 5-HT levels in all three diet groups (fig 3). Maximum levels of 5-HT were reached after 90 min and amounted to 7.47 ± 1.1 fmol, 13.64 ± 1.4 fmol and 22.17±1.8 fmol for the TRP low, TRP normal and TRP high diet, respectively. The absolute increase of 5-HT amounted to 3.59 ± 0.9 fmol, 7.96 ± 0.8 fmol and 11.24 ± 1.4 fmol for the TRP low, TRP normal and TRP high diet, respectively. Statistical analysis showed a significant main effect of diet ($F=15.042$, $p<0.001$). Contrast analyses revealed that the TRP low diet was significantly lower than controls ($F=6.870$, $p=0.009$). There was a trend towards an increase of 5-HT release in the TRP high diet as compared to controls ($p=0.070$).

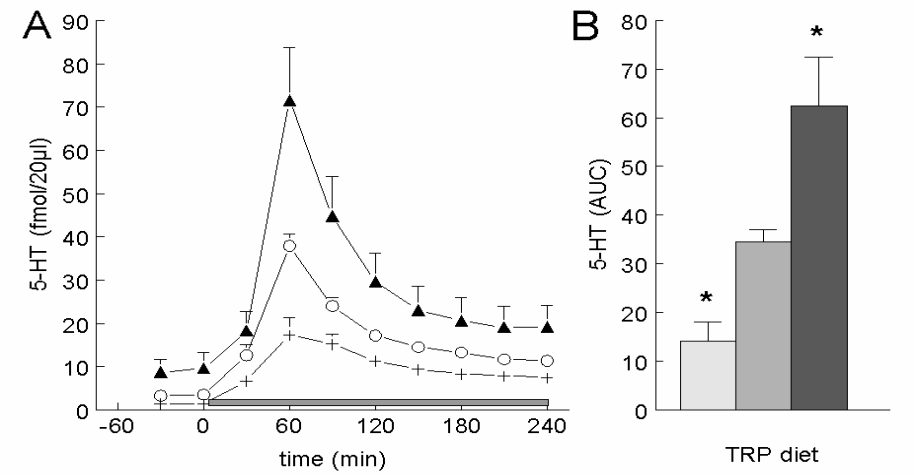

Fig. 3 Local administration of fenfluramine into the dorsal hippocampus. Data are expressed as mean absolute values in 30 min dialysate samples ± SEM. **A** Time course of the effects of fenfluramine on extracellular 5-HT after a TRP diet: TRP low (*n*=9; +), TRP normal (*n*=10; ○) and TRP high (*n*=10; ▲). At time point zero, 500 µM fenfluramine was infused as indicated by the bar. **B** Effects of fenfluramine on 5-HT expressed as mean absolute increase ± SEM for the three diets: TRP low ( ), TRP normal ( ) and TRP high ( ). * p<0.01, significantly different from TRP normal diet.

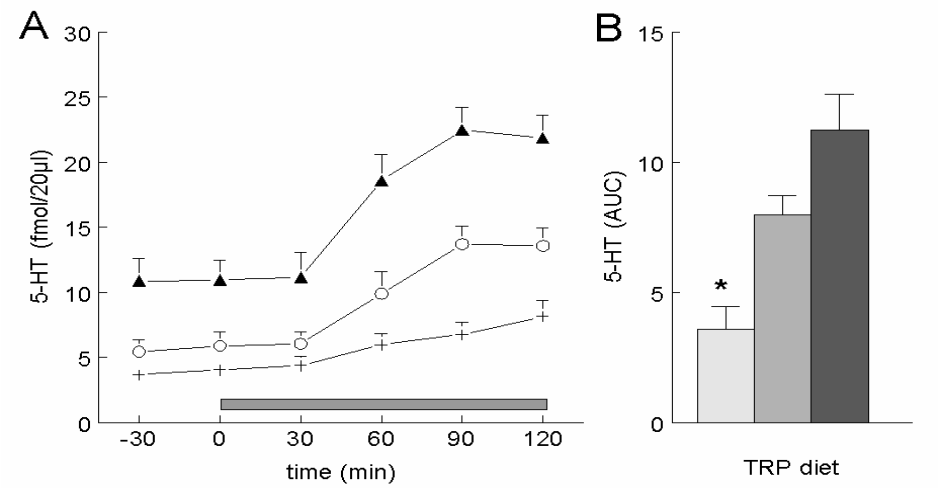

Fig. 4 Local administration of fluvoxamine into the dorsal hippocampus. Data are expressed as mean absolute values in 30 min dialysate samples ± SEM. **A** Time course of the effects fluvoxamine on extracellular 5-HT after a TRP diet: TRP low (*n*=11; +), TRP normal (*n*=9; ○) and TRP high (*n*=9; ▲).. At time point zero, 10 µM fluvoxamine was infused as indicated by the bar. **B** Effects of fluvoxamine on 5-HT expressed as mean absolute increase ± SEM for the three diets: TRP low ( ), TRP normal ( ) and TRP high ( ). * p<0.01, significantly different from TRP normal diet.

DRL-72s procedure

The effects of different TRP diets and oral fluvoxamine administration on DRL-72s performance in rats are shown in figure 5. After 1 day of diet, a significant main effect of diet on DRL-72s performance was observed with a repeated measures analysis of variance ($F = 8.67$, $p < 0.005$). Post-hoc analyses indicated that rats received less reinforcements after the TRP low diet compared to both other diets (both $p < 0.01$). After 3 days of TRP high diet, DRL-72s performance was improved compared to the TRP normal diet ($F = 10.40$, $p < 0.02$). Oral Fluvoxamine administration in rats on a normal diet induced a dose-dependent improvement of DRL-72s performance ($F = 8.14$, $p < 0.01$). The 20 mg/kg dose of fluvoxamine increased the number of reinforcements compared to both saline and 10 mg/kg of fluvoxamine dose (both $p < 0.02$).

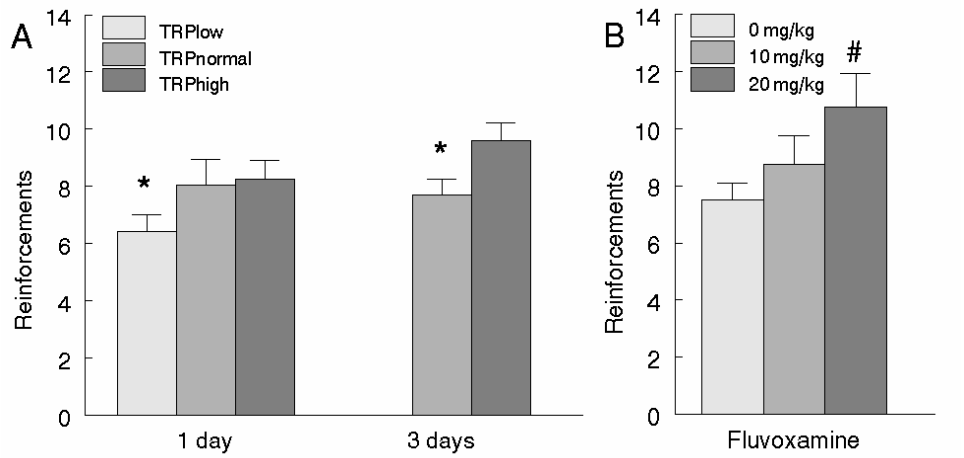

Fig. 5 **A** Effects of TRP diets for 1 and 3 days and **B** administration of oral fluvoxamine on DRL-72 performance in rats, expressed as the mean number of food reinforcements obtained during a 1h test session.

* $p<0.05$, significantly different from TRP normal diet.

\# $p< 0.05$, significantly different from vehicle and 10 mg/kg of fluvoxamine

Discussion

The major finding of this study is that extracellular levels of 5-HT in the rat dorsal hippocampus and the effect of SSRIs on the 5-HT output in this region can be manipulated by modifying the TRP content of the diet. Extracellular 5-HT levels were significantly increased after feeding with a TRP high diet for one week, and were significantly decreased after providing a TRP low diet for four days. Local administration of fenfluramine, a 5-HT releasing agent, or fluvoxamine, an SSRI,

increased extracellular 5-HT levels diet-dependently. DRL-72s performance was decreased after one day on TRP low diet, whereas three days on TRP high diet showed an increase in DRL-72s performance comparable to the effects of oral fluvoxamine administration.

The diets used in this study contained a balanced mixture of amino acids. The amounts of the amino acids that compete with TRP for the same transporter were adjusted in the TRP low and high diet, to maintain similar levels of amino acids in all three diets. Tagliamonte et al (1973) and Biggio et al (1974) reported that the amount of free plasma TRP is an indicator for TRP available for transport into the brain. Therefore, we measured plasma (total and free), CSF and brain (total and extracellular) TRP levels during dieting. Surprisingly, however, no consistent changes in TRP levels were found in this study, except a small decrease in total plasma TRP levels during the TRP low diet, despite significant changes in extracellular brain 5-HT levels in the brain following the TRP low and high diet. These findings are at variance with studies investigating the effects of acute TRP administration. Thus, Sarna et al (1991) found that acute administration of a high dose of TRP caused a parallel increase in brain TRP and 5-HIAA levels in rat striatum and cerebellum, as measured by microdialysis. Likewise, Moja et al (1989) showed that acute administration of a TRP-free amino acid mixture led to a decrease in plasma and brain TRP and a parallel decrease in brain 5-HT and 5-HIAA. Franklin and Cowen (2001) reported that a TRP low diet also led to a significant reduction in plasma total TRP levels for the first two weeks of treatment, whereas plasma free TRP levels were decreased during the first week of treatment only. The absence of a relationship between TRP levels and extracellular 5-HT concentrations in the hippocampus in the present study, suggests that small changes in the TRP content of the diet may affect 5-HT output in the brain without causing measurable effects in plasma TRP levels. Another explanation might be that TRP levels return more quickly to control levels than extracellular 5-HT levels in the brain. Microdialysis experiments were conducted during the light phase to obtain more steady state extracellular 5-HT levels. However, animals are more active and ingest most of their food during the dark phase. By the same reason, behavioral experiments were performed in the dark phase. TTX administration showed a statistically significant decrease in extracellular 5-HT levels in rats receiving TRP normal and TRP high diet. The levels of extracellular 5-HT after 90 min of TTX administration were not different between the two diets, indicating that the increase in basal levels of 5-HT after TRP high diet is of neuronal origin.

Because the effect of a TRP low diet was expected to have a greater effect on 5-HT synthesis than the TRP high diet, animals in the TRP low diet group received a control diet for three days followed by the TRP low diet for four days, while the

TRP high diet group received the TRP diet for seven days. Animals receiving the TRP low diet took on average slightly less food than control animals, and food intake after surgery was restored more slowly than in the control and TRP high group. At the time of the experiments, however, body weights between the three treatment groups were not statistically different, excluding explanations in terms of body weight regulation.

Local administration of the releaser fenfluramine led to a diet-dependent increase in 5-HT levels, suggesting that the amount of 5-HT available for release can be modulated by varying the TRP intake. These findings suggest that after a TRP high diet there is not only an increase in basal release of 5-HT, but also an increase in the synthesis and storage of 5-HT. Likewise, after a TRP low diet, there is less synthesis of 5-HT. This is in line with data from Westerink and de Vries (1991), who found an increased accumulation of 5-hydroxytryptophan (5-HTP) after TRP administration.

The effects of the TRP diets on extracellular 5-HT levels in the brain are corroborated by the behavioral tests. The TRP diets differentially affected DRL-72s performance, with the TRP low diet deteriorating and the TRP high diet improving the subsequent DRL-72s task performance. The effects of the TRP low diet were already present after 1 day, whereas the effect of the TRP high diet was observed after 3 days of dieting. Since rats were kept on a food restriction schedule, it might be hypothesized that only the cumulative effect of several days of TRP supplementation resulted in measurable effects on behavior in the DRL-72s task. In line with previous reports demonstrating beneficial effects of SSRI's in the DRL-72s procedure (Sokolowski and Seiden, 1990, Olivier et al, 1993), we found that the SSRI fluvoxamine induced a dose-dependent improvement in DRL performance. The DRL-72s has been proposed as a model for depression based on the activity of standard antidepressants on the rates of responding and reinforcement (McGuire and Seiden, 1980; O'Donnel and Seiden, 1983). The effect of the TRP high diet on DRL performance in the present study is similar to the effect of oral fluvoxamine administration of 20 mg/kg, suggesting that TRP supplementation may have antidepressant potential. It is of note in this respect, that the increase in extracellular 5-HT following a TRP high diet in this study is in the same order of magnitude as the 5-HT increase following oral administration of 30 mg/kg of fluvoxamine observed previously in our laboratory (Bosker et al, 1995).

The behavioral findings are also consistent with the microdialysis experiments conducted in this study. Local administration of fluvoxamine led to diet-dependent alterations in extracellular 5-HT levels, supporting the idea that 5-HT release is directly dependent on TRP availability. The dependency of 5-HT release on TRP availability may also have clinical consequences for efficacy of SSRIs. SSRIs

supposedly elicit their effect by inhibiting reuptake of 5-HT into the presynaptic neuron, however, as shown in this study, the effect of fluvoxamine on 5-HT release is highly dependent on the amount of TRP in the diet. A decreased availability of TRP significantly reduced the 5-HT output, whereas supplementation of the food with TRP resulted in a larger 5-HT output in the hippocampus. This would implicate that food intake might influence the efficacy of SSRIs. This is important for the clinical practice because patients suffering from depression often have decreased appetite and as a consequence an impaired food intake. In keeping with this notion, Ferguson (1999) reported that SSRIs are not effective in patients with anorexia nervosa who are underweight. Impaired TRP intake in these patients, resulting in an impaired synthesis of 5-HT, may account for this lack of effect. Indeed, Cowen and Smith (1999) found that in healthy women moderate dieting for three weeks lowered plasma TRP concentrations and impaired brain 5-HT neurotransmission as measured by serotonergic challenges in neuroendocrine tests.

A decrease in 5-HT synthesis may also be involved in the development of depressive symptoms in patients on cytokine therapy for cancer or viral diseases (Capuron et al 2002). It is suggested that immunotherapy induces depressive symptoms through indoleamine 2,3 dioxygenase (IDO) induction and IDO-mediated TRP depletion. Indeed, Capuron et al (2002) showed that serum concentrations of TRP as well as the TRP/LNAA ratio decreased significantly following cytokine therapy, due to an increased TRP catabolism. The development and severity of depressive symptoms were positively correlated with the magnitude of the decreases in TRP concentrations during treatment.
Clinical studies have shown that rapid lowering of brain 5-HT function can precipitate depressive symptoms in individuals who are vulnerable to major depressive disorder (Smith et al 1997). This finding supports a role for 5-HT function in the etiology of depression. Furthermore, Delgado et al (1990) and Aberg-Wistedt et al (1998) found that rapid TRP depletion caused a depressive relapse in remitted patients on SSRI treatment but not in healthy volunteers. Plasma TRP levels were negatively correlated with the depression score, suggesting that the therapeutic effects of some antidepressant drugs may be dependent on 5-HT availability. Plasma TRP levels have also been shown to predict response to SSRI's. Thus, Lucini et al (1996) suggest that the TRP/LNAA ratio could be of predictive value to antidepressant response and Nielsen et al (1991) reported a significant correlation between baseline plasma TRP/LNAA ratio and the severity of depression after paroxetine treatment.

In conclusion, the findings in this study suggest that nutritional factors play an important role in the biosynthesis of 5-HT. Increasing 5-HT levels by increasing the availability of TRP might augment the therapeutic efficacy of SSRIs, whereas malnutrition may render patients refractory to SSRI treatment.

Acknowledgements
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Permanent deficits in serotonergic functioning of olfactory bulbectomized rats: an in vivo microdialysis study

Chapter 4

Summary

Bilateral removal of the olfactory bulbs (OBX) in rats results in a complex constellation of behavioral, neurochemical, neuroendocrine and neuroimmune alterations, many of which are also reported in patients with Major Depressive Disorder (MDD). Drawing on clinical findings, there has been considerable interest in the role of serotonin in the mechanism of action of OBX. However, to date there has been no report of direct measurement of serotonergic functioning of bulbectomized animals using microdialysis. The objective of this study was to study the effects of olfactory bulbectomy on functioning of the serotonergic system and on hyperactivity. In vivo microdialysis was performed in conscious rats that underwent OBX or SHAM surgery. Alterations in the functioning of the serotonergic system were assessed by administration of fluvoxamine, fenfluramine and NSD-1015. Animals were also repeatedly tested in an open field. OBX decreased basal extracellular levels by decreasing the releasable pool of serotonin (5-HT) in the basolateral amygdala 2 weeks after surgery and in the dorsal hippocampus 2 weeks and 5 months after surgery. OBX animals showed a lower rate of 5-HT synthesis under basal conditions. However, the capacity of the system to synthesize 5-HT was not affected. OBX rats were chronically hyperactive in the open field. This hyperactivity remained after successive testing indicating permanent behavioral changes. To our knowledge, this is the first study that has directly investigated the effects of bulbectomy on the serotonergic system using microdialysis. This study shows that OBX has profound and long-lasting effects on serotonergic functioning and on activity levels and is therefore considered an intriguing and promising animal model for affective processes in the brain.

Introduction

The olfactory bulbectomized (OBX) rat is considered to be one of the best animal models of depression in terms of construct validity. OBX results in a complex constellation of behavioral, neurochemical, neuroendocrine and neuroimmune alterations, many of which reflect symptoms reported in patients with major depression (MDD) (for reviews see Leonard and Tuite, 1981; Kelly et al, 1997 and Harkin et al, 2003). Whereas other models of depression such as learned helplessness and chronic mild stress (for review see Jesberger and Richardson, 1985; Willner et al, 2002) react to acute antidepressant treatment, OBX is unique in its sensitivity to chronic, but not acute administration of clinically efficacious antidepressants (Jesberger and Richardson, 1988; Van Riezen and Leonard, 1990; Cryan and Mombereau, 2004).

Many clinically effective antidepressants are currently available, of which selective serotonin reuptake inhibitors (SSRIs) are currently one of the mainstays for the treatment of MDD. Based on the efficacy of these antidepressants, considerable clinical evidence has been found that serotonin (5-HT) containing pathways in the central nervous system play a significant role in the pathophysiology of MDD (for reviews see Meltzer and Lowy, 1987; Mann 1999 and Elhwuegi, 2003). Drawing on these findings, there has been considerable interest in the role of 5-HT in the mechanism of action of OBX. Decreased brain tissue levels of 5-HT were found in several regions of the brain of bulbectomized animals (Jancsar and Leonard, 1984; Redmond et al, 1997). Both an increase and a decrease in brain tissue 5-HIAA levels has been reported (Jancsar and Leonard, 1984; Zhou et al, 1998; Connor et al, 1999). Zhou et al (1998) have also found alterations in 5-HT transporter density and tryptophan hydroxylase concentrations. However, the changes presented in the literature are sometimes contradictory and the precise mechanism of these changes is still obscure.

In the present study we investigated the effects of OBX on the serotonergic system using in vivo microdialysis in freely moving rats. We measured the basal extracellular levels of 5-HT in the basolateral amygdala (BLA) 2 weeks after bulbectomy and in the dorsal hippocampus (DH) 2 weeks and 5 months after bulbectomy. In addition, we investigated 5-HT synthesis, the amount of 5-HT available for release and the effects of a 5-HT uptake inhibitor on the extracellular 5-HT levels in OBX rats as compared to SHAM operated animals. In a separate group of rats we studied the effects of OBX on activity in an open field 2, 11,16 and 20 weeks after surgery and also determined body weight development over the same period.

Materials and methods

Animals
Male Sprague-Dawley rats (Harlan, Zeist, The Netherlands) weighing approximately 250g at the time of OBX or sham operation were housed two per cage under standard laboratory conditions (22-24˚C, 12/12h light/dark cycle (lights on from 0600), food and water ad libitum) for at least one week until olfactory bulbectomy (OBX) or SHAM operation. Animals were left to recover for at least two weeks before implantation of the microdialysis probe. After implantation of the probe animals were housed separately. All experiments were performed in accordance with the governmental guidelines for care and use of laboratory animals and were approved by the Ethical Committee for Animal research of the Medical Faculty of Utrecht University.

Surgery
For the OBX procedure rats were anesthetized with chloral hydrate (400 mg/kg, ip) and placed in a stereotactic instrument (Kopf). Lidocaine (5%) was applied in the incision as local anesthetic. Two burr holes (2 mm diameter, 8 mm anterior to bregma) were drilled either side, 2 mm from the midline of the frontal bone overlying the olfactory bulbs. The bulbs were aspirated by means of a blunt hypodermic needle attached to a water pump, taking care not to damage the frontal cortex. Prevention of blood loss from the burr holes was achieved by filling them with haemostatic sponge. Sham-operated animals were treated similarly, except that the olfactory bulbs were not removed. After a minimum of two weeks a concentric microdialysis probe was implanted in either the dorsal hippocampus (DH) or the basolateral amygdala (BLA). The coordinates for the DH were: tooth bar set at +5; A: -3.9 mm; L: -2.9 mm; V: -5.5 mm from bregma and skull surface (Paxinos and Watson, 1997). The exposed tip length of the probes was 2 mm (id 220 μm; od 310 μm). The coordinates for the BLA were: tooth bar set at –3.5; A: -2.6 mm; L: -4.2 mm; V: -9.1 mm from bregma and skull surface. The exposed tip length was 1 mm. The probes were secured in place with dental cement and three anchor screws in the skull.

Body weights and behavioral testing
Body weight and locomotor activity were measured in a separate group of 29 animals. Body weight was recorded once a week from the time of arrival. Locomotor activity was measured using a brightly lit open field (72x72cm). Animals were tested once before surgery (14 Shams and 15 OBX) and again 2, 11, 16 and 20 weeks after surgery. Testing lasted 30 min, during which locomotor activity was recorded using the EthoVision video tracking system. Data were broken down in 2-min time bins to allow for analysis of habituation.

Microdialysis experiments

Experiments were performed the day after implantation of the probe in conscious and freely moving animals. The probes were perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$, and 1 mM $MgCl_2$) using a Harvard Microinfusion pump (Harvard Scientific, USA) at a constant flow rate of 1.5 µl/min. A dual channel swivel (Harvard scientific, USA) was used to allow the animals relatively unrestricted movement. Three hours after connecting the animals to the infusion pump, 30-minsamples (for 5-HT, 5-HIAA, NE and DA) or 15-min samples (for 5-HTP) were collected into vials containing 15 or 7.5 µl of 0.1M acetic acid, respectively. Samples were collected automatically using an Univentor 820 cooled autosampler (set at 8˚C) and were subsequently frozen at -80˚C until analysis.

Local infusion of drugs was performed by switching syringes after one hour of baseline sampling. Fenfluramine (500 µM), fluvoxamine (10 µM) or NSD-1015 (10 µM) was dissolved in the perfusion fluid. Fenfluramine and fluvoxamine were applied for 240 min, NSD-1015 for 120 min. In the figures the period of local infusion is corrected for the lagtime of the microdialysis system.

Drugs and chemicals

All reagents were from Merck (Darmstadt, Germany) except for heptanesulfonic acid sodium salt (Kodak, USA) and methanol (Riedel-de Haën, Germany). Fluvoxamine was generously donated by Solvay Pharmaceuticals, Weesp, The Netherlands. Fenfluramine was obtained from Research Biochemicals International (Natick, USA). NSD-1015 from Sigma-Aldrich (Zwijndrecht, The Netherlands) and L-TRP from Numico Research BV (Wageningen, The Netherlands)

Analytical procedure

Analysis of 5-HT, 5-HIAA and DA was performed by HPLC with electrochemical detection. Briefly, 20 µl samples were injected into a high performance liquid chromatograph (LKB, Woerden, The Netherlands) equipped with a 10 cm reversed phase column (Hypersil RP-18, 3 µM, 2.0 mm, Shandon) and an electrochemical detector (Antec Leyden BV, Leiden, The Netherlands) at a potential setting of 600 mV versus an Ag/AgCl reference electrode. A column oven (LKB), set at 40˚C, was used for both the column and the electrochemical detector. The mobile phase consisted of 5 g/l $(NH_4)_2SO_4$, 50 mg/l heptane sulfonic acid sodium salt 500 mg/l EDTA, 5% methanol and 30 µl/L triethylamine adjusted to pH 4.65 with 30 µl acetic acid. The flow rate was 0.4 ml/min. The detection limit for 5-HT was 0.5 fmol/20 µl sample, and 0.5 pmol/20 µl sample for 5-HIAA and 0.5 fmol/20 µl sample for DA (signal/noise ratio 2).

For the analysis of the NE concentration in the samples the mobile phase consisted of 0.05 M Na acetate/acetic acid buffer pH 4.35, 5% methanol, 450 mg/l heptanesulfonic acid sodium salt and 100 mg/l EDTA. For the analysis of NE a

HPLC equipped with a 25cm x 2mm reversed phase column was used (Supelco DB-RP8, 5µm). The flow rate was set at 0.3 ml/min. The detection limit for NE was 0.5 fmol/20 µl sample (signal/noise ratio 2).
Analysis of 5-HTP was performed by HPLC with electrochemical detection. Briefly, 20 µl samples were injected into a high performance liquid chromatograph (LKB, Woerden, The Netherlands) equipped with a 10 cm reversed phase column (Inertsil ODS-3, 10x0.2) and an electrochemical detector (Antec Leyden BV, Leiden, The Netherlands) at a potential setting of 520 mV versus an Ag/AgCl reference electrode. A column oven (LKB) set at 30˚C was used for both the column and the electrochemical detector. The mobile phase consisted of 100 mg/L EDTA, 2.5% methanol, and 100 mg/L heptane sulfonic acid sodium salt adjusted to pH 4.0 with 0.05M NaAc/acetic acid. The flow rate was 0.3 ml/min. The cooling temperature of the vial collector was set at 8˚C.

Histology
Following the termination of each experiment, animals were anesthetized with chloral hydrate (400 mg/kg, ip) and decapitated. The brains were removed and the OBX lesion was verified. The brains were then fixed in a 5% formaldehyde solution and the area of insertion of the microdialysis probe was cut into 150 µm slices. The position of the probe was verified microscopically by the track of the probe through the brain. Data were discarded if bulbs were not completely removed, if the frontal cortex was damaged, or the dialysis probe was not in the vicinity of the region aimed at. Probes were considered placed correctly if they were within a range of 0.2 mm of the intended position.

Data analysis and statistics.
All data are expressed as mean ± SEM. Values for the first two consecutive samples were averaged to calculate basal levels. Data were analyzed with multivariate analysis of variance (MANOVA) with time as within factor and group (OBX/SHAM) as between factor. When appropriate, data were broken down in group and comparisons of the AUC (Area Under the Curve) were made using a t-test. The significance level for all analyses was set at 5%. In the time-figures the start of local infusion of drugs (time point zero) is corrected for the lag-time of the microdialysis system.

Results

Body weight
Mean body weight of the animals at the time of bulbectomy was 298 ± 3 g (n=29). All animals lost weight during the first three days after surgery, but subsequently gained weight again. Overall analysis showed that OBX animals were significantly

lighter than SHAM controls ($F_{(1,27)}$=4.37, p=0.046) during the 18 weeks the animals were monitored (fig 1).

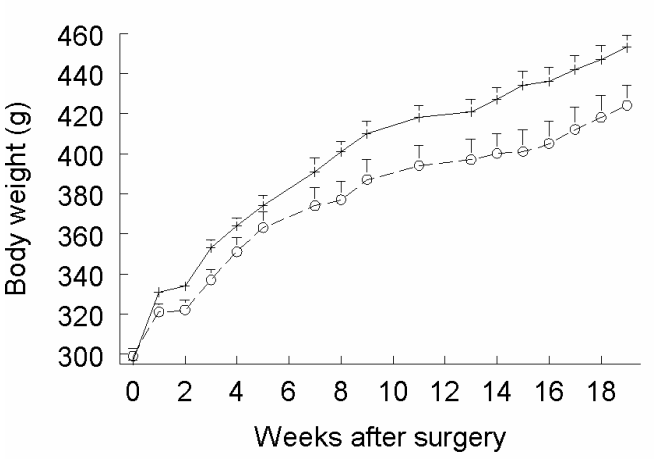

Fig 1. Body weight gain following of SHAM (n=15, +) and OBX (n=14, o) animals. Data are expressed as mean body weight (g) ± SEM.

Open field activity

Locomotor activity before bulbectomy did not differ between treatment groups (fig 2). Total distance traveled during the 30 min pre-test was 7544 ± 417 cm for the SHAM group and 8199 ± 589 cm for the OBX animals ($F_{(1,27)}$=0.84, n.s.). Two weeks after surgery, OBX animals were significantly more active than SHAM controls in the open field. Total distance amounted to 9225 ± 675 cm for SHAM and 14480 ± 1064 cm for OBX ($F_{(1,27)}$=20.3, p<0.001), respectively. OBX animals remained more active than SHAM controls when tested 11, 16 and 20 weeks after surgery ($F_{(1,27)}$=14.0, p=0.001; $F_{(1,27)}$=9.1, p=0.005 and $F_{(1,27)}$=6.9, p=0.014 respectively).

Baseline levels

Mean baseline extracellular 5-HT, 5-HIAA, NE and DA levels after SHAM and OBX treatment are shown in table 1. Basal levels of 5-HT, but not 5-HIAA, NE or DA, were decreased in the BLA of the OBX group two weeks after surgery compared to SHAM operated controls (p=0.001). The decreased levels of 5-HT were also found in the DH (p=0.003). Five months after OBX, 5-HT levels were still significantly lower in OBX compared to SHAM operated animals (p<0.001).

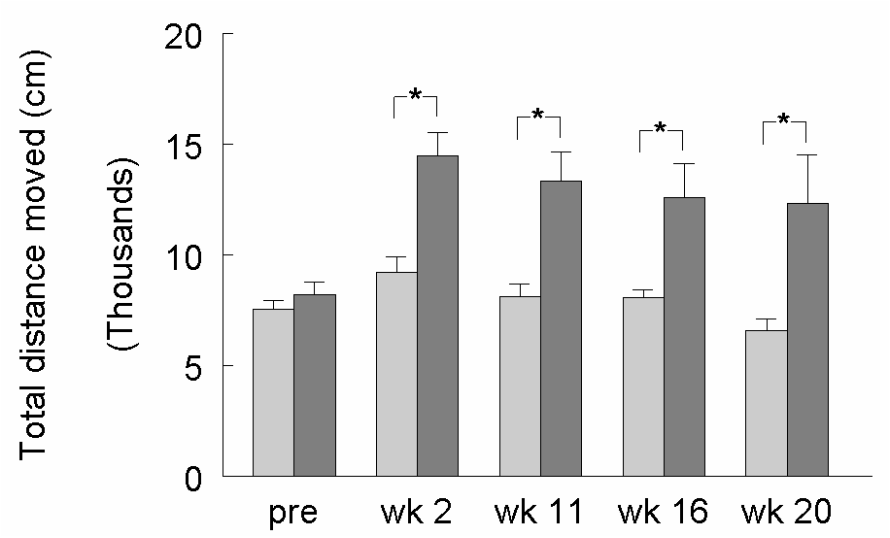

*Fig 2.*Open field activity, presented as total distance moved (cm) ± SEM during the 30 min test session. SHAM (n=15, ■) and OBX (n=14, ■) animals were tested before surgery (pre) and 2,11 and 16 weeks after surgery.
* p<0.005, OBX significantly different from SHAM

	Basolateral Amygdala 2 weeks		Dorsal Hippocampus 2 weeks		Dorsal Hippocampus 5 months	
	SHAM (n=19)	OBX (n=19)	SHAM (n=14)	OBX (n=14)	SHAM (n=17)	OBX (n=17)
5-HT (fmol/20µl)	**7.1 ± 0.6**	**3.6 ± 0.3 ***	**7.5 ± 0.9**	**3.7 ± 0.6 ***	**10.1 ± 1.0**	**5.1 ± 0.6 ***
5-HIAA (pmol/20µl)	4.8 ± 0.7	5.4 ± 0.5	4.9 ± 0.5	4.5 ± 0.3	5.3 ± 0.3	4.9 ± 0.3
NE (fmol/20µl)	1.78 ± 0.28	1.48 ± 0.13	0.96 ± 0.09	0.98 ± 0.09	n.a.	n.a.
DA (fmol/20µl)	1.12 ± 0.31	0.94 ± 0.13	0.76 ± 0.32	0.75 ± 0.23	0.73 ± 0.24	0.82 ± 0.11

Table 1. Basal levels of 5-HT, 5-HIAA, NE and DA ± SEM in basolateral amygdala (BLA) and dorsal hippocampus (DH) of SHAM and OBX animals (n.a.=data not available)
* p<0.005, OBX statistically different from SHAM

5-HT transporter blockade

The SSRI fluvoxamine was infused locally to further explore 5-HT release following bulbectomy. Local infusion of fluvoxamine (10 µM) significantly

increased extracellular 5-HT levels in both the BLA and DH of SHAM and OBX animals. Maximum levels of 5-HT were reached after 120 min for all groups. Multivariate analysis showed that the 5-HT increase in the BLA was smaller in OBX animals than in SHAM controls (fig 3A) (time $F_{(9,10)}$=18,7, p<0.001, group $F_{(1,18)}$=19.4, p<0.001 and time x group interaction $F_{(9,10)}$=6.3, p=0.004).

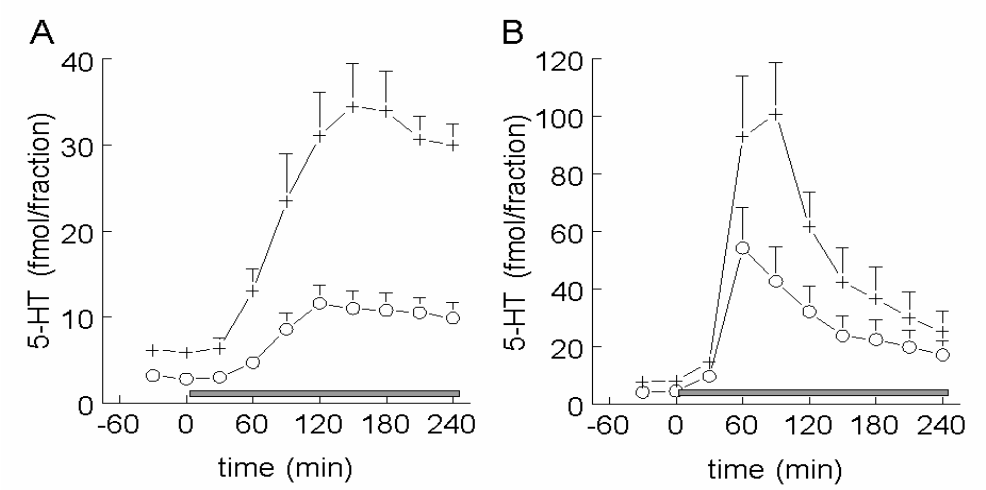

Fig 3. Local administration of fluvoxamine and fenfluramine into the BLA. Data are expressed as mean absolute values in 30 min dialysate samples ± SEM. **A** Time course of the effects of fluvoxamine on extracellular 5-HT in SHAM (n=10, +) and OBX (n=10, o) animals. At time point zero, 10 μM fluvoxamine was infused as indicated by the bar. **B** Time course of the effects of fenfluramine on extracellular 5-HT in SHAM (n=9, +) and OBX (n=9, o) animals. At time point zero, 500 μM fenfluramine was infused as indicated by the bar.

A similar effect was found in the DH 2 weeks after bulbectomy (time $F_{(9,4)}$=22.9, p<0.001, group $F_{(1,12)}$=16.6, p=0.002, and time x group interaction $F_{(9,4)}$=5.8, p=0.036) and this effect was still present in the DH 5 months after surgery (time $F_{(9,7)}$=15.8, p=0.001, group $F_{(1,15)}$=12.6, p=0.003, and time x group interaction $F_{(9,7)}$=3.1, p=0.039), indicating that the effects were long-lasting.
Analysis of the AUC (fig 4A) showed that the 5-HT increase was significantly smaller in the OBX group compared to SHAM operated controls for the BLA at 2 weeks (p=0.001, n=20) and for the DH at 2 weeks (p=0.004, n=14) and 5 months (p=0.032, n=17), indicating that the amount of 5-HT available for reuptake is decreased after bulbectomy.
Local administration of fluvoxamine significantly decreased 5-HIAA levels in the BLA (time $F_{(1,18)}$=24.8, p<0.001) and in the DH both 2 weeks and 5 months after surgery (time $F_{(1,12)}$=74.6, p<0.001 and $F_{(1,15)}$=46.2, p<0.001, respectively). No

difference between the decrease of 5-HIAA levels was found between SHAM and OBX animals. No effects of local administration of 10 μM fluvoxamine on extracellular DA levels were found.

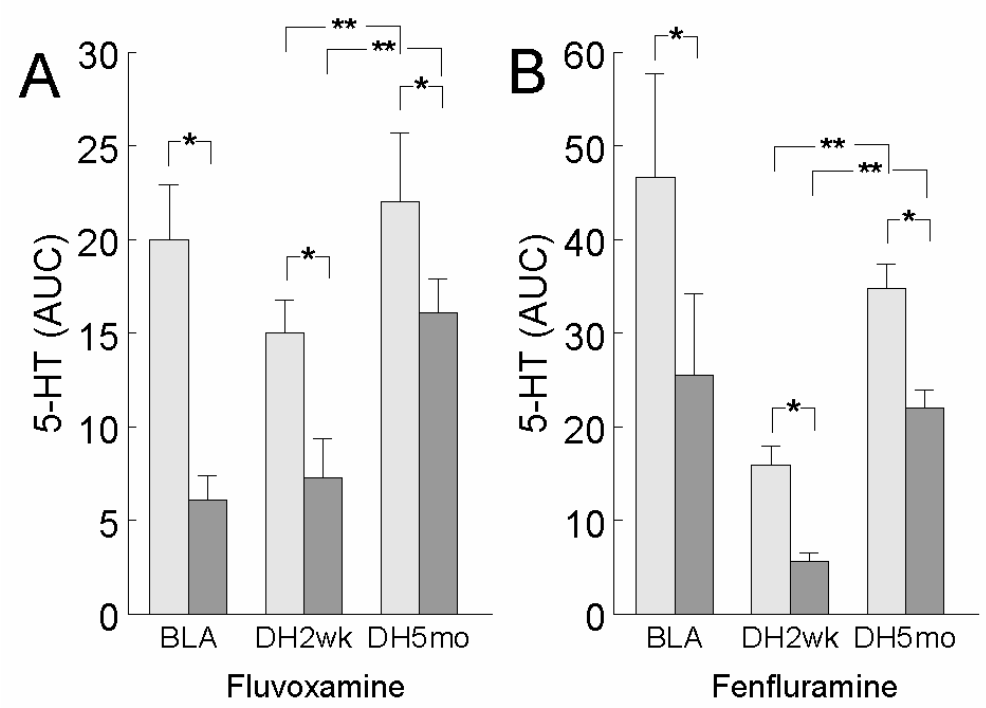

Fig 4. Local administration of fluvoxamine and fenfluramine into the BLA and DH. Data are expressed as AUC ± SEM. **A** Effect of 10 μM fluvoxamine on 5-HT of SHAM (■) and OBX (■) animals in the BLA at 2 wk and in the DH at 2 wk and 5 mo. **B** Effect of 500 μM fenfluramine on 5-HT in the BLA at 2 wk and in the DH at 2 wk and 5 mo.
***** $p<0.005$, OBX significantly different from SHAM
****** $p<0.005$, DH at 5 months significantly different from 2 weeks

Releasable pool of 5-HT

To assess the effect of bulbectomy on the releasable pool of 5-HT, fenfluramine (500 μM) was infused locally in the DH and BLA. Fenfluramine significantly increased 5-HT levels significantly in DH and BLA of SHAM and OBX animals. Maximum levels of 5-HT were reached after 60 min in all groups. In the BLA, multivariate analysis showed a significant effect of time ($F_{(9,8)}=20.4$, $p<0.001$), group ($F_{(1,16)}=28.3$, $p<0.001$) and a time x group interaction ($F_{(9,8)}=7.5$, $p=0.005$) (fig 3B), indicating that the 5-HT increase was larger in SHAM than in OBX animals. Similar results were obtained in the DH 2 weeks after surgery (time

$F_{(1,12)}$=20.2, p=0.001, group $F_{(9,4)}$=33.2, p=0.002, and a time x group interaction $F_{(9,4)}$=5.6, p=0.028). 5 months after surgery the difference between SHAM and OBX animals was still present (time $F_{(9,7)}$=18.4, p<0.001, group $F_{(1,15)}$=13.0, p=0.003 and time x group interaction $F_{(9.,7)}$=4.5, p=0.030).
Analysis of the AUC (fig 4B) revealed that the amount of 5-HT increase after fenfluramine administration was significantly smaller in the OBX group compared to SHAM controls for the BLA at 2 weeks (p<0.001, n=18) and for the DH at 2 weeks (p=0.001, n=14) and 5 months (p=0.013, n=17). These results indicate that the amount of 5-HT that is available for release is decreased in these brain areas following bulbectomy and that these effects are long-lasting.
Local administration of fenfluramine significantly increased levels of 5-HIAA (2 weeks after surgery: BLA time $F_{(9,8)}$=5.9, p<0.001; DH time $F_{(9,3)}$=7.4, p<0.001; 5 months after surgery: DH time $F_{(9,7)}$=6.1, p<0.001) and DA (2 weeks after surgery: BLA time $F_{(9,8)}$=8.1, p=0.003; DH time $F_{(9,3)}$=6.8, p=0.002; 5 months after surgery: DH time $F_{(9,7)}$=7.2, p<0.001) No difference was found in increase of 5-HIAA levels between SHAM and OBX animals.

Effects on 5-HT synthesis
The decarboxylase inhibitor NSD-1015 was infused locally in the DH and BLA surgery to assess the effects of bulbectomy on 5-HT synthesis. Basal levels of 5-HTP were below detection limit. Local infusion of NSD-1015 increased 5-HTP levels reaching steady-state levels after 1 h. Analysis of the steady state levels by t-test showed significantly lower levels of 5-HTP in OBX animals compared to SHAM operated controls in both the DH (p<0.001) and the BLA (p=0.002) 2 weeks after surgery (Fig 5A).
Oral administration of 100 mg/kg L-TRP further increased 5-HTP levels, reaching maximum levels after 1 h in all groups. Multivariate analysis showed that L-TRP significantly increased 5-HTP levels in the BLA in both SHAM and OBX animals (time $F_{(9,2)}$=12.9, p=0.001). The amount of 5-HTP increase was similar between SHAM and OBX. Similar results were found in the DH 2 weeks after surgery (time $F_{(9,2)}$=91.8, p<0.001).
Analysis of the AUC (fig 5B) indicated that there was no difference in amount of increase of 5-HTP levels after oral TRP administration (100 mg/kg) between OBX and SHAM animals. These results suggest that although the basal synthesis of 5-HT is diminished after OBX, the capacity of the system to synthesize 5-HT is not affected.

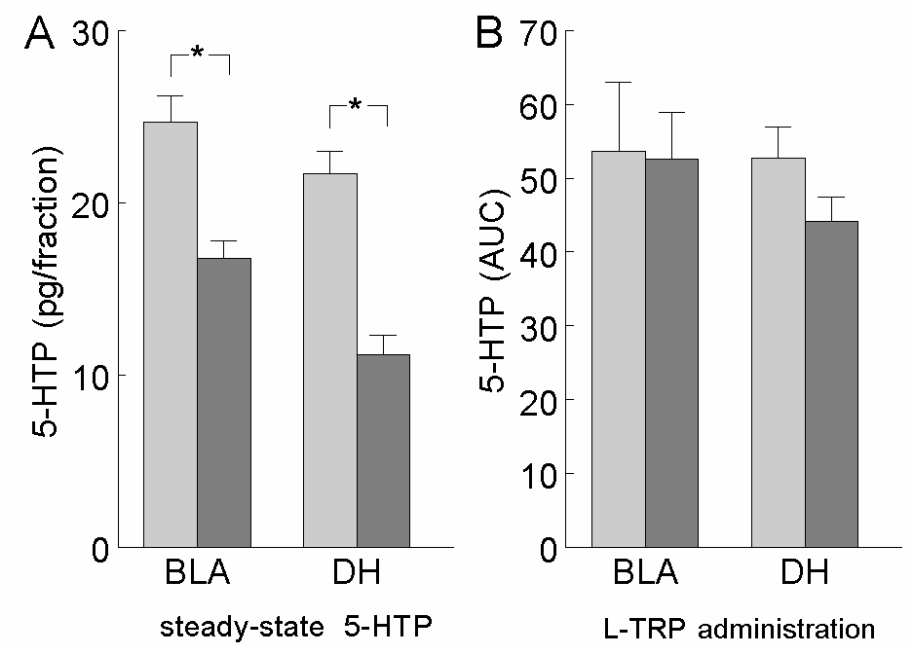

Fig 5. Local administration of NSD-1015 in the BLA and DH of SHAM (n=7, ■) and OBX (n=7, ■) animals. **A** Steady-state levels of 5-HTP during continuous infusion of 10 μM NSD-1015. Data are expressed as mean absolute value calculated over three 15 min samples ± SEM. **B** Effect of oral administration of 100 mg/kg L-TRP on extracellular 5-HTP in BLA and DH. Data are expressed as AUC ± SEM.
* $p<0.005$, OBX statistically different from SHAM

Discussion

This is the first microdialysis study reporting on extracellular 5-HT levels in OBX rats. The major finding of this study is that olfactory bulbectomy in rats results in selective alteration in the functioning of the serotonergic system. Basal extracellular levels of 5-HT, but not of 5-HIAA, NE and DA were decreased following olfactory bulbectomy in both the dorsal hippocampus (DH) and the basolateral amygdala (BLA) 2 weeks after surgery. Measurements in the DH 5 months after surgery revealed that this effect was long-lasting. Local administration of fenfluramine, a 5-HT releasing agent, and fluvoxamine, an SSRI, revealed that OBX caused an attenuated increase in 5-HT levels, while administration of NSD-1015, a decarboxylase inhibitor, suggested a lower rate of

synthesis under basal conditions in OBX animals. This difference in 5-HTP synthesis was no longer present when the availability of L-TRP was increased.

One of the most consistent findings in the OBX model is the increase in locomotor activity. Bulbectomy has consistently been shown to produce an increase in locomotor activity in novel environments such as the open field in several studies (Van Riezen et al 1977, Cairncross et al 1979, for review see Kelly et al, 1997). Van Riezen et al (1977) showed that the performance in the open field of peripherally anosmic rats was not significantly different from controls. Therefore, the alterations observed after ablation of the olfactory bulbs are not due to anosmia per se. This hyperactivity is significantly attenuated by chronic treatment with antidepressants (Mar et al 2000) but not by psychotropic drugs lacking antidepressant activity. Therefore, the open field test is commonly used as signal for antidepressant activity. In keeping with these previous findings, OBX animals in the present study showed increased locomotion in the open field compared to SHAM operated animals. The hyperactivity phenomenon appears to be a chronic characteristic of the OBX animal, as it is still present 16 weeks after surgery. Although habituation over time and with repeated testing occurs, the difference between SHAM and OBX remains. Recently, it was found that dopamine levels in the ventral and dorsal striatum were enhanced in OBX rats compared to shams, whereas norepinephrine was decreased and 5-HIAA unaltered (Masini et al, 2004). This dopaminergic overactivity might be directly related to the behavioral hyperactivity of OBX rats in an open field. It is unclear how the decreased 5-HT functioning in the amygdala and/or hippocampus relates to the increased dopaminergic functioning in the striatum. A second phenomenon that has been consistently reported in bulbectomized animals is reduced body weight (for review see Kelly et al, 1997). In keeping with these reports we have found an overall reduced body weight of OBX animals compared to SHAM operated controls, which is still present up to 20 weeks after surgery.

The decrease in extracellular basal 5-HT levels after bulbectomy found in the present study is in line with lower brain 5-HT content found in other studies. Jancsar and Leonard (1984) found decreased tissue content of 5-HT in amygdaloid cortex and midbrain following bulbectomy and Redmond et al (1997) showed a lower tissue 5-HT concentration in the frontal cortex of OBX animals. These findings are consistent with observations in depressed patients, showing decreased plasma and platelet 5-HT content (Sarrias et al , 1987). Butler et al (1988) found decreased platelet 5-HT uptake and increased synaptosomal 5-HT uptake, suggesting increased clearance from the synapse, which could account for decreased 5-HT levels. Clinical studies have shown that rapid lowering of brain 5-HT function, using the TRP-depletion method, can precipitate depressive symptoms in individuals who are in remission after successful treatment with

SSRIs (Smith et al, 1997), supporting a role for 5-HT in the mechanism of action of these drugs.

At variance with previous studies, we did not find changes in extracellular 5-HIAA levels in the DH or BLA. Jancsar and Leonard (1984) reported lower brain tissue 5-HIAA concentrations in the amygdaloid cortex and midbrain of OBX animals, and Connor et al (1999) report significantly reduced basal 5-HIAA levels in the accumbens. However, Zhou et al (1998) found higher 5-HIAA concentrations in the frontal cortex. Concentrations of 5-HIAA in CSF have been extensively studied in depressed patients. However, no significant differences in CSF 5-HIAA between depressed patients and normal controls have been found. In their review Meltzer and Lowy (1987) conclude that it is very difficult to draw any valid conclusions on 5-HT turnover in depression on the basis of CSF 5-HIAA data. At present , there is only overwhelming evidence that low CSF 5-HIAA levels are related to impulsivity rather than to mood alterations (Faustman et al 1991).

Despite changes in extracellular 5-HT levels, we did not find changes in extracellular NE or DA levels. Data on the effects of OBX on NE concentrations in the brain are not equivocal. Jancsar and Leonard (1984) found decreased NE levels in the amygdaloid cortex and decreased DA levels in the midbrain. However, Broekkamp et al (1986) failed to confirm these findings. They suggested that the OBX paradigm is primarily a serotonergic syndrome, given the fact that the effects of subchronic treatment with imipramine and mianserin were reversed by acute administration of the 5-HT_2 receptor antagonist metergoline (Broekkamp et al 1980). In their review, Kelly et al (1997), suggested that discrepant NE findings after OBX may be related to methodological problems rather than to differences in NE regulation in the brains of OBX animals. It cannot be excluded, though, that NE or DA alterations do occur in other brain regions. Recently, Masini et al (2004) found that OBX increases DA release in both dorsal and ventral striatum. However, metabolite levels or turnover were not affected.

The present finding raises the question regarding the functional consequences of these 5-HT abnormalities in OBX rats. To test this we blocked the 5-HT transporter in the DH and BLA by local infusion of the SSRI fluvoxamine. The increase in extracellular 5-HT levels was blunted in OBX rats, suggesting that either the 5-HTT density is enhanced or the amount of 5-HT released is diminished. Indeed, Zhou et al (1998) have found increased 5-HTT density in the frontal cortex of bulbectomized rats. However, the frontal cortex is associated with increased 5-HT innervation (Zhou et al, 1998; Watanabe, 2003), whereas in the present study we found a decrease in 5-HT functioning in the BLA and DH. Local infusion of the 5-HT releaser fenfluramine also showed an attenuated increase in 5-HT levels OBX animals. Taken together, and considering the decrease in extracellular basal 5-HT

levels, these data suggest that both the amount of 5-HT released and the amount of 5-HT available for release are decreased following OBX.

A possible explanation for decreased extracellular 5-HT levels might be a deficit in 5-HT synthesis in the bulbectomized rat. Jancsar and Leonard (1984) found decreased 5-HT turnover in the amygdaloid cortex, but not in midbrain of OBX animals. In line with this notion, we found that administration of NSD-1015, a decarboxylase inhibitor (in analogy to Westerink and the Vries, 1991), revealed an attenuated 5-HTP accumulation in the OBX group as compared to SHAM controls, suggesting a decreased rate of synthesis of 5-HT after OBX. In contrast, Watanabe et al (2003) found increased 5-HT synthesis in frontal cortex and hippocampus, but a decrease in the raphe nuclei. They suggest that this can be accounted for by collateral sprouting and synaptogenesis following bulbectomy, which is most prominent in the frontal cortex (Zhou et al, 1998). Interestingly, systemic administration of a high dose of L-TRP (100 mg/kg), resulted in similar increases in 5-HTP accumulation in both OBX and SHAM operated rats. This suggests that, despite an apparently impaired 5-HT synthesis under basal conditions, the capacity of the system to synthesize 5-HT is not affected. A parsimonious explanation might be that bulbectomy decreased TRP availability in the brain. In a previous study, we have shown that extracellular 5-HT levels are highly dependent on dietary TRP intake (Van der Stelt et al, 2004) and OBX rats gain less weight. There is also evidence that plasma TRP levels are decreased in patients with MDD (Cowen 1989, Quintana 1992), and Delgado et al (1990) and Aberg-Wistedt (1998) found that rapid TRP depletion to cause depressive relapse in remitted patients on SSRI treatment.

Another possible explanation for the decreased rate of 5-HT synthesis could be a loss of serotonergic fibers. McLean and Shipley (1987) showed that the raphe nuclei are the sole source of serotonergic input into the olfactory bulb. Bilateral removal of the bulbs could cause retrograde degeneration of neurons in various regions projecting to the bulbs as well as anterograde degeneration of neurons normally receiving projections from the bulbs. Indeed, Nesterova et al (1997) showed substantial loss of raphe neurons following bulbectomy. Neuronal degeneration has also been found in the hippocampus and the amygdala, which are projection areas of the medial and dorsal raphe, respectively (Carlsen et al, 1982). In concordance with these findings, Wrynn et al (2000a) used in-vivo magnetic resonance imaging to demonstrate decreased signal intensity in the cortex (frontal, occipital and cingulate), caudate and amygdala. OBX animals also exhibit pronounced enlargement of the lateral and 3rd ventricles, which correlates with decreased hippocampal volume. The amygdala and hippocampus have been implicated in depression. Drevets (1998) showed that amygdaloid blood flow and metabolism show a positive correlation to depression severity. This region has also

been proposed as a site for the behavioral alterations (Jesberger and Richardson, 1985) and antidepressant action in the OBX model (Wrynn et al, 2000b). Kellner et al (1986) have shown that there is a correlation between ventricle size and cognitive impairment in depression, and it is suggested by Kelly et al (1997) that impaired hippocampal function as a consequence of ventricular impairment may underlie spatial memory deficits in bulbectomized animals.
A smaller number of serotonergic neurons could explain decreased synthesis of 5-HT and consequently a reduction in release. However, if indeed less neurons are available for synthesis, a smaller increase in 5-HTP accumulation following TRP loading would be expected. This was not the case, suggesting that either there is no loss of functional neurons, or there is an overcapacity of the remaining neurons to synthesize 5-HT. Another possible explanation could be that removal of the olfactory bulbs has changed feedback mechanisms directed at maintaining homeostasis of the serotonergic system. Alterations in serotonin receptors and serotonin transporter have been proposed to be involved in depression (Lopez-Figuerou et al, 2004; Caspi et al 2003).

In summary, the present study shows long lasting deficits in 5-HT functioning in DH and BLA of bulbectomized rats. To our knowledge, this is the first study showing the effects of bulbectomy on extracellular levels of serotonin in the brain. Although the precise mechanism by which bilateral removal of the olfactory bulbs causes this decrease in extracellular 5-HT levels is unknown, the findings do support the validity of OBX as an intriguing and promising animal model for affective processes in the brain.

Acknowledgements
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Compensatory changes in serotonergic functioning following olfactory bulbectomy in rats: an in vivo microdialysis study

Chapter 5

Summary

The olfactory bulbectomized (OBX) rat stands out from other animal models of depression in terms of predictive and construct validity. Many of the changes seen after bulbectomy reflect symptoms reported in patients with major depression. Based on the clinical efficacy of SSRIs in the treatment of depression, there has been considerable interest in the role of serotonin in the brain mechanisms involved in of OBX. Putative degeneration of serotonergic neurons following bulbectomy could induce adaptive processes in the functioning of the serotonergic system. In vivo microdialysis was performed in the dorsal hippocampus (DH) and median raphe (MR) of conscious rats that underwent OBX or SHAM surgery. The SSRI Fluvoxamine, the 5-HT_{1B} receptor agonist CP 93129 and the 5-HT_{1A} receptor agonist flesinoxan were infused locally in the DH or MR to assess alterations in the 5-HT transporter (5-HTT), the terminal 5-HT_{1B} receptor and the somatodendritic 5-HT_{1A} receptor, respectively. Systemic administration of fluvoxamine in combination with WAY100.635 was carried out to verify the effects found on the 5-HT_{1A} receptor. Two weeks after OBX surgery, extracellular basal levels of 5-HT in DH and MR were significantly decreased. OBX also induced desensitization of somatodendritic 5-HT_{1A} autoreceptors. No alterations were found in the efficacy of 5-HTTs or on the functioning of 5-HT_{1B} receptors. Bilateral removal of the olfactory bulbs leads to neuronal adaptive changes in serotonergic functioning. These changes show similarity to alterations observed in depressed patients, strengthening the notion that OBX is a promising model for studying affective processes in the brain.

Introduction

There is substantial evidence that a deficiency in the central serotonergic system plays a role in the development of major depression (MDD) (for review see Elhwuegi, 2004). Earlier studies have detected reduced levels of CSF 5-Hydroxyindole-acetic acid (5-HIAA) in depressed suicide victims (for review see Smith and Cowen, 1997). Depressed patients also exhibit reduced plasma concentrations of serotonin (5-HT) and reduced platelet 5-HT uptake in comparison to control subjects (Sarrias et al, 1987; Meltzer and Arora, 1991, Quintana, 1991). Furthermore, dietary restriction of tryptophan in patients whose depression has remitted after a treatment with selective serotonin reuptake inhibitors (SSRIs) induces a relapse in symptoms Delgado et al, 1990, Bell et al, 2001). Neuroendocrine challenge tests and more recently functional neuroimaging studies support the notion that 5-HT alterations may play a key role in the pathophysiology of major depression (Drevets et al, 2000; Fujita et al 2000; Bhagwagar et al, 2002). Finally, drugs that target the serotonin transporter site, by inhibiting the reuptake of 5-HT, have shown to be effective antidepressants.
A better understanding of the pathophysiology of major depression might be achieved by the use of animal models. A major obstacle in research on depression has been the lack of good animal models.

The olfactory bulbectomy (OBX) in rats is an animal model that meets most of the criteria of a valid model for depression. It results in a phenotype modeling several of the symptoms seen in major depression, including many of the behavioral, neurochemical, neuroendocrine and immune alterations (for reviews see Leonard and Tuite, 1981; Kelly et al, 1997 and Harkin et al, 2003). In contrast to most other animal models of depression, chronic rather than acute administration of antidepressants results in a reversal of most of the alterations (Jesberger and Richardson, 1988; Van Riezen and Leonard, 1990.
The precise mechanism responsible for the abnormalities in OBX rats is still a matter of debate, but several studies have linked OBX to changes in the 5-HT system. Decreased brain tissue levels of 5-HT were found in several brain regions (Jancsar and Leonard, 1984, Redmond et al, 1997). Both a decrease and an increase in brain 5-HIAA levels have been reported (Jancsar and Leonard, 1984; Zhou et al, 1998, Connor et al, 1999). Jesberger and Richardson (1986) have found lower [^{3}H]imipramine binding in pons and hippocampus of bulbectomized rats, whereas Zhou et al (1998) found increased 5-HT transporter density in the frontal cortex. Butler et al (1988) found decreased platelet 5-HT uptake and increased synaptosomal 5-HT uptake following bulbectomy.

In a previous study (Van der Stelt et al, submitted) we have shown that olfactory bulbectomy leads to long-lasting and profound deficits in serotonergic functioning.

Basal release and the amount of 5-HT available for release were decreased, as well as basal 5-HT synthesis. A putative explanation could be that bilateral removal of the olfactory bulbs causes neuronal degeneration, leading to adaptive processes in the serotonergic system aimed at maintaining physiological levels of 5-HT. In the present study we used in-vivo microdialysis to investigate the effects of olfactory bulbectomy on several of these processes. Basal 5-HT release was measured in the dorsal hippocampus (DH) and median raphe nucleus (MR), following local and systemic administration of an SSRI. In addition, the effects of bulbectomy on the terminal 5-HT_{1B} and the somatodendritic 5-HT_{1A} autoreceptors were assessed.

Materials and methods

Animals

Male Sprague-Dawley rats (Harlan, Zeist, The Netherlands) weighing approximately 250g at the time of OBX or SHAM operation, were housed two per cage under standard laboratory conditions (22-24˚C, 12/12h light/dark cycle, food and water ad libitum) for at least one week until olfactory bulbectomy (OBX) or SHAM operation. Animals were left to recover for at least two weeks before implantation of the microdialysis probe. After implantation of the probe animals were housed separately. All experiments were performed in accordance with the governmental guidelines for care and use of laboratory animals and were approved by the Ethical Committee for Animal research of the Medical Faculty of Utrecht University.

Surgery

For the OBX procedure rats were anesthetized with chloral hydrate (400 mg/kg, ip) and placed in a stereotactic instrument (Kopf). Lidocaine (5%) was applied in the incision as local anesthetic. Two burr holes (2mm diameter, 8 mm anterior to bregma) were drilled either side, 2mm from the midline of the frontal bone overlying the olfactory bulbs. The bulbs were aspirated by means of a blunt hypodermic needle attached to a water pump, taking care not to damage the frontal cortex. Prevention of blood loss from the burr holes was achieved by filling them with haemostatic sponge. Sham-operated animals were treated similarly, except that the olfactory bulbs were not removed. After a minimum of two weeks a concentric microdialysis probe was implanted in either the dorsal hippocampus (DH) or the median raphe nucleus (MR). The coordinates for the DH were: tooth bar set at +5; A: -3.9 mm; L: -2.9 mm; V: -5.5 mm from bregma and skull surface (Paxinos and Watson, 1997). The exposed tip length of the probes was 2 mm (id 220 μm; od 310 μm). The coordinates for the MR were: tooth bar set at -3.5; A: -7.8 mm; L: -2.0 mm; V: -7.0 mm from bregma and skull surface. The exposed tip length for the MR was 1 mm and the probe was implanted at an angle of 10º. The

probes were secured in place with dental cement and three anchor screws in the skull.

Microdialysis experiments
Experiments were performed the day after implantation of the probe in conscious and freely moving animals. The probes were perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$, and 1 mM $MgCl_2$) using a Harvard Microinfusion pump (Harvard Scientific, USA) at a constant flow rate of 1.5 µl/min. A dual channel swivel (Harvard scientific, USA) was used to allow the animals relatively unrestricted movement. Three hours after connecting the animals to the infusion pump, 15 (MR) or 30 min (DH) samples were collected into vials containing 7.5 or 15 µl of 0.1 M acetic acid, respectively. Samples were collected automatically using an Univentor 820 cooled autosampler (set at 8˚C) and were subsequently frozen at -80˚C until analysis.
Local infusion of drugs was performed by switching syringes after one hour of baseline sampling. Fluvoxamine (3, 10 and 30 µM), CP9219 (1 µM) and flesinoxan (1µM) were dissolved in the perfusion fluid. Fluvoxamine was applied for 240 min. CP for 120 min and flesinoxan for 30 min. WAY 100.635 was injected 15 min prior to systemic fluvoxamine administration. In the figures the period of local infusion is corrected for the lagtime of the microdialysis system.

Drugs and chemicals
All reagents and drugs were from Merck (Darmstadt, Germany) except for heptanesulfonic acid sodium salt (Kodak, USA) and methanol (Riedel-de Haën, Germany). Fluvoxamine was generously donated by Solvay Pharmaceuticals (Weesp, The Netherlands).

Analytical procedure
Analysis of 5-HT was performed by HPLC with electrochemical detection. Briefly, 20 µl samples were injected into a high performance liquid chromatograph (LKB, Woerden, The Netherlands) equipped with a 10 cm reversed phase column (Hypersil RP-18, 3 µM, 2.0 mm, Shandon) and an electrochemical detector (Antec Leyden BV, Leiden, The Netherlands) at a potential setting of 600 mV versus an Ag/AgCl reference electrode. A column oven (LKB), set at 40˚C, was used for both the column and the electrochemical detector. The mobile phase consisted of 5 g/l $(NH_4)_2SO_4$, 50 mg/l heptane sulfonic acid sodium salt 500 mg/l EDTA, 5% methanol and 30 µl/L triethylamine adjusted to pH 4.65 with 30 µl acetic acid. The flow rate was 0.4 ml/min. The detection limit for 5-HT was 0.5 fmol/20 µl sample (signal/noise ratio=2).

Histology
Following the termination of each experiment, animals were anesthetized with chloral hydrate (400 mg/kg, ip) and decapitated. The brains were removed and the OBX lesion was verified. The brains were then fixed in a 5% formaldehyde solution and the area of insertion of the microdialysis probe was cut into 150 μm slices. The position of the probe was verified microscopically by the track of the probe through the brain. Data were discarded if bulbs were not completely removed, if the frontal cortex was damaged, or the dialysis probe was not in the vicinity of the region aimed at. Probes were considered placed correctly if they were within a range of 0.2 mm of the intended position.

Data analysis and statistics.
All data are expressed as mean ± SEM. Values for the first two consecutive samples were averaged to calculate basal levels. AUC (area under the curve) was calculated as a measure of total 5-HT increase. Data were analyzed with multivariate analysis of variance (MANOVA) with time as within factor and group (OBX/SHAM) or treatment as between factor. When appropriate, data were broken down in group and comparisons were made using a t-test. The significance level for all analyses was set at 5%. In the time-figures the start of local infusion of drugs (time point zero) is corrected for the lag-time of the microdialysis system.

Results

Body weight
Mean body weight of the animals at the time of bulbectomy was 279 ± 4 g (n=63). All animals lost weight during the first three days after surgery, but subsequently gained weight. Overall analysis showed that OBX animals were significantly lighter than SHAM operated controls during the two weeks the animals were monitored ($F_{(1,61)}$=3.8, p=0.025).

Baseline levels
Mean extracellular baseline levels of 5-HT were decreased in DH and MR of OBX animals compared to SHAM operated controls. Baseline levels of 5-HT in the DH amounted to 7.2 ± 0.9 and 2.9 ± 0.6 fmol/fraction for SHAM and OBX groups ($p<0.001$, n=50), respectively. Differences between baseline levels were even more pronounced in the MR. Here 5-HT levels amounted to 81.4 ± 9.6 and 9.5 ± 1.2 fmol/fraction for SHAM and OBX groups ($P<0.01$, n=14), respectively.

Efficacy of 5-HT transporter blockade
Fluvoxamine (3, 10 and 30 μM), was administered locally in the DH through the microdialysis probe to assess the functioning of the 5-HT transporter (5-HTT). Fig

1A illustrates the time course of local infusion of 10 µM fluvoxamine. All concentrations significantly increased 5-HT levels in both SHAM and OBX animals, reaching maximum levels between 60 and 90 min after the start of the infusion (time $F_{(9,19)}$=22.1, p<0.001). The increase was smaller in OBX animals than in SHAM controls (time x group $F_{(9,19)}$=2.6, p=0.037). However, no interaction effect was found between time, group and dose.
Fig 1B shows the absolute increase in 5-HT for all three doses of fluvoxamine. Multivariate analysis of the AUC values confirms a significant increase in 5-HT levels in both SHAM and OBX animals (group $F_{(1,27)}$=10.6, p=0.003 and dose $F_{(2,27)}$=6.9, p=0.004). Again, no interaction between group and dose was found, suggesting that the two dose-response lines were parallel. Univariate analysis revealed a significant increase between 3 and 10 µM in both groups (p=0.016 for SHAM and p=0.035 for OBX), while between 10 and 30 µM fluvoxamine no significant increase in either SHAM (p=0.734) or OBX animals (p=0.089) was found. This suggests that in both groups plateau levels were attained.

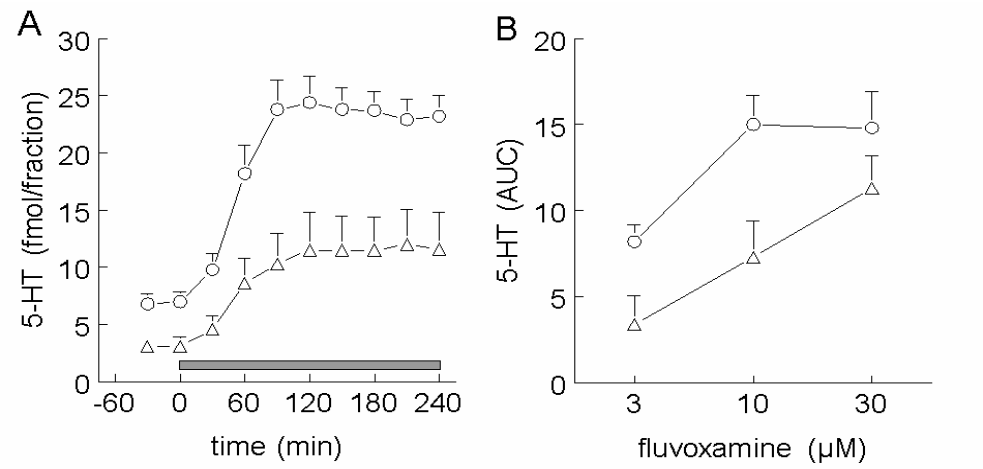

Fig 1. Local administration of fluvoxamine into the DH. **A** Time course of the effects of 10 µM fluvoxamine on extracellular 5-HT in SHAM (n=7, ○) and OBX (n=8, △) animals. Data are expressed as mean absolute values in 30 min dialysate samples ± SEM. At time point zero, 10 µM fluvoxamine was infused as indicated by the bar. **B** Effects of local administration of 3, 10 and 30 µM fluvoxamine on extracellular 5-HT in SHAM (○) and OBX (△) animals. Data are shown as AUC ± SEM.

Effects on the terminal 5-HT$_{1B}$ receptor

1 µM CP 93129, a 5-HT$_{1B}$ receptor agonist, was administered locally in the DH with 3 µM fluvoxamine present in the ringer. Time course of the effect of infusion of CP93129 is shown in fig 2. Basal levels of 5-HT amounted to 14.1 ± 0.9 and 6.8

± 1.6 fmol/fraction for SHAM and OBX animals, respectively ($p<0.001$, n=13). Multivariate analysis showed a significant effect of time ($F_{(5,7)}=20.7$, $p<0.001$) and group ($F_{(1,11)}=9.1$, $p=0.012$), but no interaction between time and group was found. The absolute decrease in 5-HT was smaller in the OBX group than in the SHAM group (4.9 and 8.8 fmol, respectively). But, there was no statistically significant difference between the two groups ($F_{(1,11)}=4.2$, $p=0.063$). When the decrease in 5-HT levels is calculated as percentage decrease from basal levels, the total amount of decrease is similar for SHAM and OBX animals (57.6 ± 7.4 and 56.8 ± 8.5, respectively).

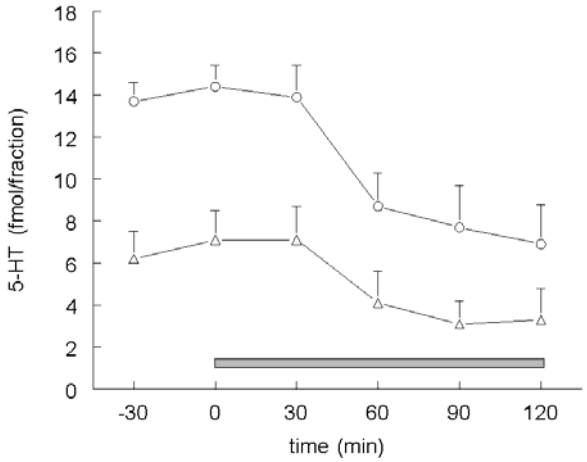

Fig 2. Local administration of CP 93129 into the DH. 3µM fluvoxamine is present in the ringer. Data are expressed as mean absolute values in 30 min dialysate samples ± SEM. Shown is the time course of the effect of local infusion of 1 µM CP on extracellular 5-HT levels in SHAM (n=6, ○) and OBX (n=7, △) animals. At time point zero, 1 µM CP was infused as indicated by the bar.

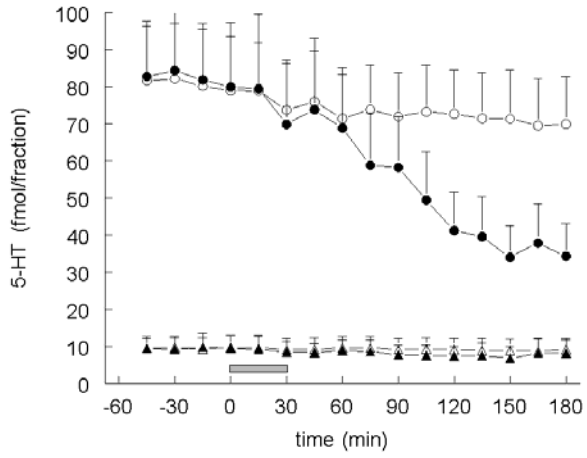

Fig 3. Local administration of flesinoxan in the MR. Time course of the effects of local infusion of ringer (open symbols, SHAM ○, n=6 and OBX △, n=7) or 1 µM flesinoxan (filled symbols, SHAM ●, n=6 and OBX ▲, n=7) on extracellular 5-HT in the MR. Data are expressed as mean absolute values in 15 min dialysate samples ± SEM. 1 µM flesinoxan was infused for 30 min from time point zero, as indicated by the bar.

Effects on the somatodendritic 5-HT$_{1A}$ receptor

The 5-HT$_{1A}$ receptor agonist flesinoxan (1μM) or saline was infused locally in the median raphe nucleus (MR) for 30 min and 5-HT levels were measured for 180 min (fig 3). Multivariate analysis showed that 5-HT levels were significantly decreased (time $F_{(15,8)}$=13.8, p<0.001), and that the decrease in 5-HT was smaller in OBX than in SHAM animals (time x group $F_{(15,8)}$=10.1, p=0.001). Also, the decrease was larger after administration of flesinoxan than saline (time x treatment $F_{(15, 8)}$=19.1, p<0.001). A significant interaction between time, group and treatment was found, indicating that flesinoxan has a different effect in SHAM and OBX animals ($F_{(15,8)}$=17.1, p<0.001). Breakdown of the data by groups showed that flesinoxan significantly decreased 5-HT levels in SHAM ($F_{(1,10)}$=7.8, p<0.001) animals, but not in OBX.

Effects of systemic fluvoxamine administration with and without 5-HT$_{1A}$ receptor antagonist WAY 100.635.

To further assess the effects of bulbectomy on functioning of the 5-HT$_{1A}$ autoreceptor, 10 mg/kg fluvoxamine was administered systemically (i.p.) with and without WAY 100.635 (0.05 mg/kg, s.c). 5-HT levels were significantly increased in both SHAM and OBX animals, reaching maximum levels after approximately 60 min (time $F_{(9,29)}$=72.7, p<0.001). Time course of the effects is shown in fig 4A. Multivariate analysis revealed a smaller 5-HT increase in SHAM compared to OBX animals (time x group $F_{(9,29)}$=6.8, p<0.001) as well as a difference in 5-HT increase depending on treatment (saline, fluvoxamine or fluvoxamine and WAY 100.635) (time x treatment $F_{(18,58)}$=16.1, p<0.001). A significant interaction between time, group and treatment was found ($F_{(18,58)}$=3.7, p<0.001), indicating that SHAM and OBX animals react differently to administration of fluvoxamine with or without WAY 100.635.

When data were broken down on group, multivariate analysis showed that co-administration of WAY 100.635 with fluvoxamine further increased 5-HT levels in SHAM animals ($F_{(9,3)}$=5.4, p=0.023), but not in OBX animals ($F_{(9,8)}$=3.1, p=0.107).

Fig 4B shows the absolute increase in 5-HT levels after fluvoxamine administration and after co-administration of WAY 100.635. Multivariate analysis of the AUC values shows that there is no difference in 5-HT increase between SHAM and OBX animals after systemic fluvoxamine administration. When data are broken down on group, co-administration of WAY significantly increases 5-HT levels in SHAM animals compared to administration of fluvoxamine alone (p=0.030, n=13), but has no effect in OBX animals.

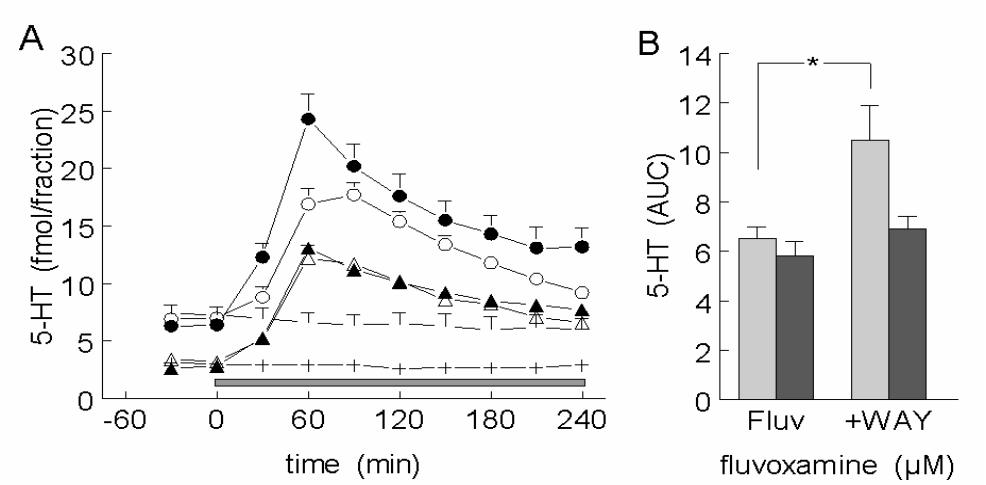

Fig 4. Systemic administration of fluvoxamine. **A** Time course of the effects of systemic administration of saline (SHAM no symbol, n=5 and OBX +, n=7), 10 mg/kg fluvoxamine (open symbols, SHAM ○, n=7 and OBX △, n=8) or 10 mg/kg fluvoxamine and 0.05 mg/kg WAY 100.635 (filled symbols, SHAM ●, n=7 and OBX ▲, n=10) on extracellular 5-HT in the DH of SHAM and OBX animals. Data are shown as mean absolute values in 30 min dialysate samples ± SEM. **B** Absolute increase of 5-HT levels after systemic administration of 10 mg/kg fluvoxamine in combination with WAY in SHAM (■) and OBX (■) animals. Data are expressed as AUC ± SEM.
* $p<0.005$, co-administration of WAY statistically different from fluvoxamine alone

Discussion

The present study investigated the effects of bilateral removal of the olfactory bulbs on functioning of the serotonergic system. The data indicate that OBX decreases extracellular basal 5-HT levels in the dorsal hippocampus (DH) and median raphe nucleus (MR). We also found that somatodendritic 5-HT_{1A} receptors are desensitized following OBX. No alterations were found in the efficacy of the 5-HT transporter (5-HTT) or in the functioning of the terminal 5-HT_{1B} autoreceptor.

This study has replicated findings from a previous study by our group (Van der Stelt et al, submitted), indicating that extracellular basal 5-HT levels are decreased in DH and basolateral amygdala (BLA) following OBX. In the latter study we have also shown that 5-HT release and 5-HT synthesis were decreased, whereas the capacity of the system to synthesize 5-HT was not affected. Removal of the olfactory bulbs initiates a sequence of structural and functional alterations in

different brain areas. Wrynn et al (2000a) used magnetic resonance imaging to reveal alterations in signal intensities in cortical, hippocampal, caudate and amygdaloid regions. Such changes parallel those reported in depressed patients, including increased ventricular size, decreased frontal lobe, caudate and amygdaloid volumes as well as lesions in the frontal lobes and basal ganglia (Drevets, 2001).
McLean and Shipley (1987) showed that the raphe nuclei are the sole source of serotonergic input into the olfactory bulbs. Bilateral removal of the bulbs could cause retrograde degeneration of neurons in various regions projecting to the bulbs as well as anterograde degeneration of neurons normally receiving projections from the bulbs. Indeed, Nesterova et al (1997) showed substantial loss of raphe neurons following bulbectomy and Norrholm and Ouimet (2001) reported reduced spine density in CA1, CA3 and dentate gyrus of the hippocampus following bulbectomy. This loss of serotonergic fibers could account for the dramatic decrease in extracellular basal 5-HT levels that was found in the MR and its projection areas. Degeneration of the raphe nuclei could cause adaptive changes in the functioning of the serotonergic system, aimed at maintaining physiological levels of 5-HT.

The present study showed a decrease of extracellular basal 5-HT levels in DH and MR, which is in line with lower brain 5-HT content found in other studies. Jancsar and Leonard (1984) found decreased tissue content of 5-HT in amygdala and midbrain following bulbectomy and Redmond et al (1997) showed a lower tissue concentration in the frontal cortex of OBX animals. A possible consequence of degeneration of serotonergic fibers and the resulting decrease in 5-HT levels, could be adaptive changes in the efficacy of the 5-HT transporter (5-HTT). It might be expected that olfactory bulbectomy results in downregulation of the 5-HTT. To assess the functioning of the 5-HTT, a dose-response curve was made using three concentrations of the SSRI fluvoxamine. The resulting 5-HT increase is larger in SHAM animals than in OBX animals. This can be explained by the fact that the basal release of 5-HT is lower in OBX animals. SHAM animals show saturation of the 5-HTT at a concentration of 10 μM fluvoxamine; when a higher concentration fluvoxamine is administered, no additional increase in 5-HT levels is found. The dose-response curve for OBX animals seems to be different in that 5-HT levels further rise from 10- 30 μM fluvoxamine, suggesting a shift in the dose-response curve, but the statistics do not confirm this. The most likely explanation is, therefore, that the difference in the height of the curves is caused by decreased 5-HT release, rather than a change in 5-HTT efficacy. In contrast to these findings, Zhou et al (1998) have found increased 5-HTT density in the frontal cortex of bulbectomized rats, but not in other brain areas. They suggest that lesioning the fibers projecting to the bulbs stimulates collateral sprouting and synaptogenesis, especially in the frontal cortex, leading to selective 5-HT hyperinnervation of the cortex.

Other feedback mechanisms aimed at controlling 5-HT levels are associated with modulation of neuronal activity (somatodendritic 5-HT_{1A} receptors) and neurotransmitter release (terminal 5-HT_{1B} receptors). 5-HT_{1B} receptors serve as 5-HT autoreceptors at 5-HT nerve terminals (for review see Middlemiss and Hutson, 1990). Under normal circumstances, activation of the 5-HT_{1B} receptor by increased levels of 5-HT inhibits transmitter release, thereby controlling 5-HT neuronal activity, release, and thus 5-HT levels. The decreased basal 5-HT levels found in bulbectomized animals could be a consequence of an upregulation of the 5-HT_{1B} receptor. However, the 5-HT_{1B} receptor could also be downregulated as a means to increase 5-HT levels. In the presence of fluvoxamine, 1 μM CP 9129, a selective 5-HT_{1B} receptor agonist, was infused locally in the DH to assess the functionality of the 5-HT_{1B} receptor. Both SHAM and OBX animals showed a significant decrease in 5-HT levels, indicating the presence of a functional 5-HT_{1B} receptor. When the absolute decrease in 5-HT levels, as calculated by AUC, is compared between the two treatment groups, there is a trend towards a larger effect in SHAM animals compared to OBX animals. However, basal release is significantly decreased in OBX animals and should be taken into account when comparing the effects. When the total amount of decrease is related to the basal levels of 5-HT, there is no difference in relative effect between the two groups, indicating that the sensitivity of the terminal 5-HT_{1B} receptor has not changed.

Like the 5-HT_{1B} receptor, the 5-HT_{1A} autoreceptor is aimed at controlling 5-HT levels. Under normal circumstances, an increase in 5-HT levels will activate somatodendritic 5-HT_{1A} receptors in the raphe nucleus, which will in turn reduce the firing frequency of 5-HT neurons. To assess the effects of bulbectomy on functioning of the somatodendritic 5-HT_{1A} receptor, flesinoxan, a 5-HT_{1A} receptor agonist, was infused locally into the MR. SHAM operated animals showed a significant decrease in 5-HT levels after flesinoxan administration. However, in the OBX group, although there was a slight decrease in 5-HT levels, no significant effect of flesinoxan was found, indicating that 5-HT_{1A} receptors in the MR are desensitized in OBX rats. Desensitization may result from decreased levels of 5-HT found in OBX animals. Desensitization of somatodendritic 5-HT_{1A} receptors is a means to maintain normal 5-HT levels when synthesis or release is decreased.

Since the effect of systemic SSRI treatment on 5-HT levels in projection areas like the DH is in part dependent on the 5-HT_{1A} receptor feed-back regulation, one might expect the effect of SSRIs on 5-HT levels in the DH of OBX animals to be smaller after systemic administration than after local administration into the DH. Normally, stimulation of somatodendritic 5-HT_{1A} autoreceptors reduces the effect on 5-HT release in the DH following systemic SSRI treatment by reducing the firing rate. Desensitization of somatodendritic 5-HT_{1A} receptors reduces the amount of

negative feedback, thus resulting in higher concentrations of extracellular 5-HT. Indeed, when fluvoxamine was administered systemically to OBX animals, the increase in 5-HT levels is similar to the increase seen in SHAM operated controls, despite the fact that basal extracellular levels are significantly lower after bulbectomy. When WAY 100.635, a 5-HT_{1A} receptor antagonist, was co-administered with fluvoxamine, levels of 5-HT increased significantly in SHAM animals compared to administration of fluvoxamine alone. However, in OBX animals, no extra increase in 5-HT levels after co-administration of WAY was seen, supporting the previous findings that olfactory bulbectomy leads to desensitization of somatodendritic 5-HT_{1A} receptors.

The present findings resemble observations in depressed patients that plasma and platelet 5-HT levels are decreased (Sarrias et al, 1987; Meltzer and Arora, 1991; Quintana, 1992). Studies in patients with major depression have also shown that rapid lowering of brain 5-HT synthesis by using the tryptophan depletion paradigm, can precipitate depressive symptoms in individuals who are in remission after successful treatment with SSRIs (Smith et al, 1997), suggesting a role for 5-HT in the mechanism of action of these drugs. Alterations in 5-HT receptors and 5-HT transporters have also been proposed to be involved in depression (Caspi et al, 2003; Lopez-Figueroa et al, 2004). Chronic administration of effective antidepressant treatment, including ECT, induces upregulation of 5-HT_{1A} receptors. Several studies have shown that depressed patients have blunted physiological and neuroendocrine responses to 5-HT_{1A} receptor agonists (Lesch et al, 1990; Meltzer and Maes, 1995, Shapira et al, 2000). Post mortem studies have revealed decreased 5-HT_{1A} receptor binding in depressed patients (Lopez et al, 1998; Stockmeier et al, 1998, Drevets et al, 2000). Lemonde et al (2003) suggest that impaired repression of the 5-HT_{1A} receptor gene decreases serotonergic transmission, predisposing an individual to depression and suicide. Lopez-Figueroa et al (2004) found a decrease of 5-HT_{1A} mRNA in the dorsolateral prefrontal cortex and hippocampus of patients with MDD. Cheetham et al (1990) found increased cortical 5-HT_{1A} receptors and a decreased density of 5-HT_{1A} receptors in hippocampus and amygdala of depressed suicide victims. Finally, Cowen (2000) suggests that unmedicated patients with MDD have decreased sensitivity of both pre-and postsynaptic 5-HT_{1A} receptors. These findings in depressed patients bear a striking resemblance to findings in the present study, showing decreased extracellular levels of 5-HT and desensitization of somatodendritic 5-HT_{1A} receptors in OBX rats. These findings support the notion that the olfactory bulbectomized rat is a promising model for the study of affective processes in the brain.

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Effects of dietary tryptophan supplementation in the olfactory bulbectomized rat model of depression

Chapter 6

Summary

The olfactory bulbectomized (OBX) rat displays a phenotype modeling several of the symptoms seen in major depression, including behavioral, neurochemical, neuroendocrine and immune alterations. It is suggested that serotonin (5-HT) may be involved in the mechanism of action of at least some successful antidepressant treatments and that correction of an underlying 5-HT dysfunction is linked to the initiation and maintenance of clinical remission from depression.
In this study functioning of the 5-HT system was enhanced by chronic dietary tryptophan supplementation. In animals on a control diet, locomotor activity in OBX animals was enhanced compared to SHAM animals. In vivo microdialysis showed decreased extracellular basal levels of 5-HT in the DH of bulbectomized rats, as well as desensitization of somatodendritic 5-HT_{1A} receptors. Using qPCR, we also found an increased expression of 5-HT_{1A} receptor mRNA in the hippocampus. In the amygdala a decrease in GABA-$A_{\alpha 1}$ subunit mRNA expression was found, as well as an increase in MAO-A mRNA expression. In contrast to earlier findings, bulbectomy did not affect the circadian pattern of corticosterone secretion. Chronic supplementation of tryptophan in the diet increased extracellular basal 5-HT levels and reversed the desensitization of the somatodendritic 5-HT_{1A} receptor in OBX animals. No effect of the diet was found on locomotor activity or on corticosterone secretion, suggesting a dissociation between neurochemical and behavioral or endocrine parameters.

Introduction

Bilateral removal of the olfactory bulbs (OBX) in rats results in a phenotype modeling several of the symptoms seen in major depression, including many of the behavioral, neurochemical, neuroendocrine and immune alterations (for reviews see Leonard and Tuite, 1981; Kelly et al, 1997 and Harkin et al, 2003). Whereas other models of depression are sensitive to acute antidepressant treatment, the OBX model is unique in that it is sensitive to chronic rather than acute treatment with antidepressants (Jesberger and Richardson, 1988, Van Riezen and Leonard, 1990, Cryan and Mombereau, 2004), thus mimicking the time course of the efficacy of antidepressants in patients with major depressive disorder (MDD).

One of the most consistent behavioral changes reported in OBX rats is the increase in activity when rats are placed in a stressful novel environment, such as the 'open field'. Also, deficits in passive avoidance have been found (Sieck, 1972, Van Riezen and Leonard, 1990). Some studies have reported alterations in circadian secretion of corticosterone (Song et al, 1994; Marcilhac et al, 1997), but no alterations in ACTH have been reported. However, Broekkamp et al (1980) suggested that OBX animals show an increased corticosteroid response to stress rather than an increase in basal levels. Based on the efficacy of selective serotonin reuptake inhibitors (SSRIs) in the treatment of major depression and the reversal of abnormalities in OBX animals, the role of serotonin (5-HT) in the bulbectomy syndrome has attracted considerable interest. A decrease in brain tissue 5-HT levels has been reported to occur in several brain regions (Jancsar and Leonard, 1984; Redmond et al, 1997), whereas both a decrease and an increase in brain tissue 5-HIAA levels have been found (Jancsar and Leonard, 1984; Zhou et al, 1998, Connor et al, 1999). In a previous study (Van der Stelt et al, submitted) we have shown that removal of the olfactory bulbs decreased extracellular basal 5-HT levels in dorsal hippocampus, basolateral amygdala and median raphe nucleus. We also showed that whereas basal 5-HT synthesis was decreased in OBX animals, the capacity of the system to synthesize 5-HT was not diminished. Moreover, the somatodendritic 5-HT_{1A} receptor was desensitized.

Clinical studies have shown that rapid lowering of brain 5-HT function, using the TRP-depletion method, can precipitate depressive symptoms in individuals who are in remission after successful treatment with SSRIs (Smith et al, 1997; Delgado et al, 1990). This suggests that 5-HT may be involved in the mechanism of action of antidepressants and that a correction of an underlying 5-HT dysfunction may be linked to the initiation and maintenance of clinical remission. Nutritional factors play an important role in the biosynthesis of 5-HT as is demonstrated by the finding that in rats chronic supplementation of the diet with L-tryptophan results in larger 5-HT output in the hippocampus (Van der Stelt et al, 2004). Considering

these facts, it was hypothesized that increasing 5-HT neurotransmission by chronic tryptophan supplementation, could reverse or diminish some or all of the effects of olfactory bulbectomy in rats.

The present study investigated the effects of chronic administration of a TRP-high diet on several of the alterations seen in bulbectomized rats. Locomotor activity was measured using the open field. In vivo microdialysis was used to assess the effects on the somatodendritic 5-HT_{1A} receptor. Using quantitative PCR, gene expression of several genes relevant for serotonergic functioning was measured. Finally, corticosterone levels were measured over a 24h period.

Materials and Methods

Animals
Male Sprague-Dawley rats (Harlan, Zeist, The Netherlands) weighing approximately 250g at the time of OBX or SHAM operation were housed four per cage under standard laboratory conditions (22-24˚C, 12/12h light/dark cycle, food and water ad libitum) for at least one week until olfactory bulbectomy (OBX) or SHAM operation. Animals were left to recover for at least two weeks before the experiments were started. After implantation of the probe animals were housed separately. All experiments were performed in accordance with the governmental guidelines for care and use of laboratory animals and were approved by the Ethical Committee for Animal research of the Medical Faculty of Utrecht University.

Surgery
For the OBX procedure rats were anesthetized with chloral hydrate (400 mg/kg, ip) and placed in a stereotactic instrument (Kopf). Lidocaine (5%) was applied in the incision as local anesthetic. Two burr holes (2mm diameter, 8 mm anterior to bregma) were drilled either side, 2mm from the midline of the frontal bone overlying the olfactory bulbs. The bulbs were aspirated by means of a blunt hypodermic needle attached to a water pump, taking care not to damage the frontal cortex. Prevention of blood loss from the burr holes was achieved by filling them with haemostatic sponge. Sham-operated animals were treated similarly, except that the olfactory bulbs were not removed. After a minimum of two weeks a concentric microdialysis probe was implanted in either the dorsal hippocampus (DH) at the following coordinates: tooth bar set at +5; A: -3.9 mm; L: -2.9 mm; V: -5.5 mm from bregma and skull surface (Paxinos and Watson, 1997). The exposed tip length of the probes was 2 mm (id 220 μm; od 310 μm). The probes were secured in place with dental cement and three anchor screws in the skull.

Diets
Two weeks after surgery, rats were randomly assigned to one of two dietary conditions. These consisted of two diets differing in L-tryptophan (TRP) content. The diets were manufactured by Numico, Wageningen, The Netherlands. The control diet contained 0.24 g TRP/100g. The amount of TRP in the High diet was 200 % of the control diet. To maintain equal amount of amino acids in both diets, the change in TRP content was counterbalanced by adjusting the amount of leucine, isoleucine and valine.

Behavioral testing
Locomotor activity was measured using a brightly lit open field (72x72cm). Animals were tested 4 weeks after SHAM or OBX surgery, after receiving control or TRP high diet for 2 weeks. Testing lasted 30 min, during which locomotor activity was recorded using the EthoVision video tracking system. Data were broken down in 2-min time bins to allow for analysis of habituation.

Microdialysis experiments
Experiments were performed the day after implantation of the probe in conscious and freely moving animals. The probes were perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$, and 1 mM $MgCl_2$) using a Harvard Microinfusion pump (Harvard Scientific, USA) at a constant flow rate of 1.5 µl/min. A dual channel swivel (Harvard scientific, USA) was used to allow the animals relatively unrestricted movement. Three hours after connecting the animals to the infusion pump, 30 min (DH) samples were collected into vials containing 15 µl of 0.1 M acetic acid. Samples were collected automatically using an Univentor 820 cooled autosampler (set at 8˚C) and were subsequently frozen at -80˚C until analysis.
After one hour of baseline sampling, fluvoxamine was administered i.p. WAY 100.635 was injected 15 min prior to systemic fluvoxamine administration. In the figures the period of local infusion is corrected for the lagtime of the microdialysis system.

Analytical procedure
Analysis of 5-HT was performed by HPLC with electrochemical detection. Briefly, 20 µl samples were injected into a high performance liquid chromatograph (LKB, Woerden, The Netherlands) equipped with a 10 cm reversed phase column (Hypersil RP-18, 3 µM, 2.0 mm, Shandon) and an electrochemical detector (Antec Leyden BV, Leiden, The Netherlands) at a potential setting of 600 mV versus an Ag/AgCl reference electrode. A column oven (LKB), set at 40˚C, was used for both the column and the electrochemical detector. The mobile phase consisted of 5 g/l $(NH_4)_2SO_4$, 50 mg/l heptane sulfonic acid sodium salt 500 mg/l EDTA, 5% methanol and 30 µl/L triethylamine adjusted to pH 4.65 with 30 µl acetic acid. The

flow rate was 0.4 ml/min. The detection limit for 5-HT was 0.5 fmol/20 µl sample (signal/noise ratio=2).

Quantitative PCR analysis

Rats were decapitated six weeks after SHAM or OBX surgery. The brains were removed and transferred 10 ml RNAlater (Ambion) and stored at 4°C for 48. The amygdala and hippocampus were dissected and stored in 1 ml RNAlater at -20°C until further use. After thawing, the excess RNAlater was removed and RNA was isolated from the sample using the GenElute RNA isolation kit from Sigma. All procedures were performed on ice and in a cooled microcentrifuge. Tissue samples were passed through a 22G syringe to obtain optimal homogenation. The OD260nm to OD280nm ratio (1.8-2) was used to assess the purity of the isolated RNA. 0.5 µg RNA in a total of 20 µl was reverse transcribed using the enhanced avian first strand synthesis kit from Sigma. 0.5 units enhanced avian reverse transcriptase were used per reaction. To prevent RNA degradation, 1 µl Superase-In (Ambion) was added. The RT reaction was performed at 45°C for 2 h.

A real time PCR reaction was performed on 0.1 to 0.5 µl of the cDNA samples in the presence of the Sybr Green I dye in a total volume of 20 µl, using the Absolute QPCR sybr Green kit of ABgene (Epson, UK). To determine the efficiency of a given PCR reaction, real time PCR reactions were performed on five 2-fold dilution of a mixture of the obtained cDNA samples. The following protocol was used for all PCR reactions: 15 min 95°C, followed by 40 cycles consisting of 15 s at 95°C, and 60 s at 60°C. To ensure that a new developed PCR reaction resulted in the expected product the size of the product was checked by agarose electrophoresis. Primers were developed using the Primer Express software (Applied Biosystems, see table 1 for sequences). We strived for primer pairs that hybridized on both sites of an intron. The efficiency (E) of the PCR reaction was calculated by linear regression of the obtained standard curve and the formula $E = (2^{1/dCt}-1) \times 100\%$ (dCt is the slope of the standard curve. From the obtained threshold cycle values (Ct), we calculated the relative initial amount of the selected cDNAs using the following formula: [amount at Ct] = [initial amount] x E^{Ct} . Finally to correct for differences within the initial RNA samples, we normalized the measured cDNA levels against the levels of GAPDH.

gene	FP	RP
5-HT_{1A}R	CCAACTATCTCATCGGCTCCTT	CTGGCCCAGGGTCCACTT
5-HT_{1B}R	TATTTACCCACCCTGCTCCTCAT	CGTGGATCCTGGAGAGTCTGTTA
GABA-$A_{\square 1}$	GCAAAAGCGTGGTTCCAGAA	CGGTTTTGTCTCAGGCTTGACT
GABA-$A_{\square 2}$	ATCGCTGTTTGTTACGCGTT	TCATGACGGAGCCTTTCTCTT
MAO-A	CATGAAACACTATGAGTGCAAATAC	TCTCAGGTGGAAGCTCTGGTT
Subst.-P	ATCGGTGCCAACGATGATCTA	GAACTGCTGAGGCTTGGGTCT
NK-1	CATCTACTGTTGCCTCAACGACA	TCCAGCCCCTCATAATCACCT
Tyrosine hydroxylase	GCCAAGGACAAGCTCAGGAA	AGTACGTCAATGGCCAGTGTG
GAPDH	GGCTGCCTTCTCTTGTGACAA	CTCAGCCTTGACTGTGCCATT

Table 1. Sequences of the primers used for the quantification of mRNAs using real time PCR.

Chemical determinations

For the 24-h corticosterone measurements, blood was collected by tailcut every 4 hours starting at 06.00h. Plasma was separated by centrifugation (3000rpm for 10 min at 4°C) and measured in duplicate using a standard radioimmunoassay (RIA) for corticosterone (rat, mice) (MP Biomedicals inc; formerly ICN Biomedicals inc, Zoetermeer, The Netherlands).

Drugs and chemicals

All reagents were from Merck (Darmstadt, Germany) except for heptanesulfonic acid sodium salt (Kodak, USA) and methanol (Riedel-de Haën, Germany). Fluvoxamine was generously donated by Solvay Pharmaceuticals, Weesp, The Netherlands.

Histology

Following the termination of each experiment, animals were anesthetized with chloral hydrate (400 mg/kg, ip) and decapitated. The brains were removed and the OBX lesion was verified. The brains were then fixed in a 5% formaldehyde solution and the area of insertion of the microdialysis probe was cut into 150 µm slices. The position of the probe was verified microscopically by the track of the probe through the brain. Data were discarded if bulbs were not completely removed, if the frontal cortex was damaged, or the dialysis probe was not in the vicinity of the region aimed at. Probes were considered placed correctly if they were within a range of 0.2 mm of the intended position.

Data analysis and statistics.
All data are expressed as mean ± SEM. For the microdialysis experiments, values for the first two consecutive samples were averaged to calculate basal levels. Microdialysis data, behavioral data and corticosterone levels were analyzed with multivariate analysis of variance (MANOVA) with time as within factor and group (OBX/SHAM), diet (control/TRP high) and treatment (saline, fluvoxamine, WAY 100.635) as between factor (according to experimental setup). When appropriate, data were broken down in group and comparisons of the AUC (Area Under the Curve) were made using a t-test. In the time-figures the start of local infusion of drugs (time point zero) is corrected for the lag-time of the microdialysis system. For the mRNA expression data an ANOVA was performed for amygdala and hippocampus on the normalized expression levels. The significance level for all analyses was set at 5%.

Results

Open field activity
Multivariate analysis of the locomotor activity showed a decrease of activity of all animals during the 30 min test (time $F_{(14,18)}=17.5$, $p<0.001$). OBX animals showed significantly higher locomotor activity than SHAM animals (time x group $F_{(14,18)}=4.2$, $p=0.002$). However, no significant effect of the diet was found on locomotor activity (fig 1A). Analysis of the total distance moved during the 30 min test (fig 1B) showed that this was significantly higher in OBX animals (group $F_{(1,31)}=10.9$, $p=0.002$). Again, no effect of the diet was found on total distance.

Baseline 5-HT levels
2 weeks after surgery, extracellular basal 5-HT levels for animals receiving the control diet were significantly decreased in OBX animals compared to SHAM controls (8.7 ± 0.4 fmol/fraction for SHAM and 4.1 ± 0.2 for OBX, $p<0.001$, $n=23$). SHAM animals receiving TRP supplementation had significantly elevated basal 5-HT levels (16.8 ± 0.6 fmol/fraction, $p<0.001$, $n=21$), as did OBX animals receiving TRP supplementation (12.0 ± 1.0 fmol/fraction, $p<0.001$, $n=27$). In the animals receiving TRP supplementation, OBX animals still had significantly lower basal 5-HT levels than SHAM animals on the same diet ($p<0.001$, $n=25$)

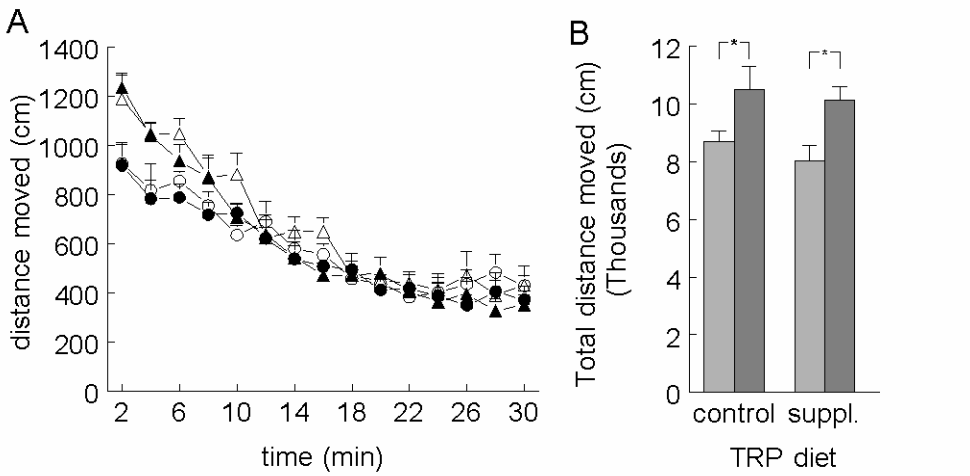

Fig 1. **A** Open field activity, presented as distance moved (cm) ± SEM in 2 min time-bins during the 30 min test session. Animals were tested after two weeks of control diet (open symbols; SHAM ○, n=8; OBX △, n=9) or tryptophan supplementation (filled symbols; SHAM ●, n=9; OBX ▲, n=9). **B** Total distance moved (cm) ± SEM during the 30 min test session for SHAM ( ) and OBX ( ) animals.
* p<0.005, OBX significantly different from SHAM

Somatodendritic 5-HT$_{1A}$ receptor

To assess the effects of TRP supplementation on functioning of the 5-HT$_{1A}$ autoreceptor in SHAM and OBX animals, 10 mg/kg fluvoxamine was administered systemically (i.p.) with and without WAY 100.635 (0.05 mg/kg, s.c). 5-HT levels were significantly increased in both SHAM and OBX animals, reaching maximum levels after 60 min (time $F_{(9,32)}$=209.2, p<0.001). Fig 2A and 2B show the time course of the effects in SHAM and OBX animals, respectively. Multivariate analysis showed that co-administration of WAY increased 5-HT levels further than administration of fluvoxamine alone (time x treatment $F_{(9,32)}$=9.3, p<0.001). 5-HT levels were increased more in SHAM animals than in OBX (time x surgery $F_{(9,32)}$=4.1, p=0.001) and the TRP-rich diet caused a larger increase in 5-HT levels than the control diet (time x diet $F_{(9,32)}$=29.5, p<0.001).

To determine the effect of co-administration of WAY, data were broken down on surgery (SHAM/OBX) and diet (Control/High). Univariate analysis showed that co-administration of WAY further increased 5-HT levels in SHAM animals receiving the control diet ($F_{(9,1)}$=6.3, p<0.001). However, in OBX animals receiving the control diet, no extra increase was found. After 2 weeks of a TRP-rich diet, co-administration of WAY further increased 5-HT levels in both SHAM ($F_{(9,1)}$=15.5,

p<0.001) and OBX animals ($F_{(9,5)}$=6.7, p<0.001), with no significant difference between SHAM and OBX.

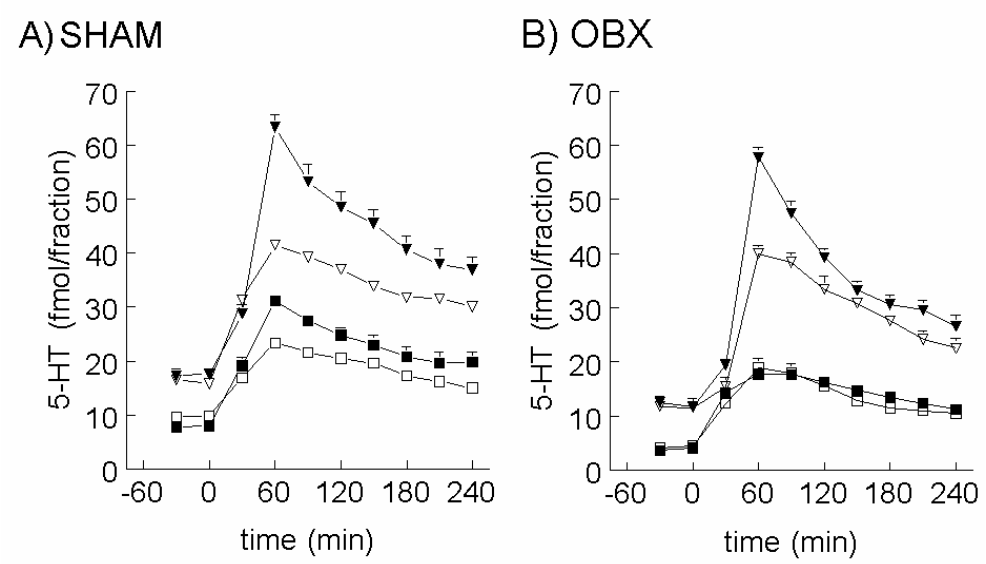

Fig 2. **A** Time course of the effect of systemic administration of fluvoxamine without (open symbols) or with WAY 100.635 (filled symbols) on 5-HT levels in the DH of SHAM animals receiving control diet (□, n=5; ■, n=6) or tryptophan supplementation (▽, n=5; ▼, n=5). **B** Time course of the effect of systemic administration of fluvoxamine without (open symbols) or with WAY 100.635 (filled symbols) on 5-HT levels in the DH of OBX animals receiving control diet (□, n=6; ■, n=6) or tryptophan supplementation (▽, n=7; ▼, n=8). Data are expressed as mean absolute values of 5-HT in 15 min dialysate samples ± SEM. Fluvoxamine and WAY were administered at time point zero.

Fig 3 shows the absolute increase in 5-HT levels after fluvoxamine administration and after co-administration of WAY 100.635. Comparison of the AUCs showed that although co-administration of WAY 100.635 augmented 5-HT in SHAM animals (p=0.007), no extra augmentation was found in OBX animals. After chronic TRP supplementation, 5-HT levels increased after fluvoxamine both with and without WAY 100.635 in both SHAM (p<0.001 and p=0.001) and OBX animals (p=0.009 and p<0.001). The effect of administration of fluvoxamine alone and the effect of administration of fluvoxamine with WAY 100.635 were not different between SHAM and OBX animals after TRP supplementation.

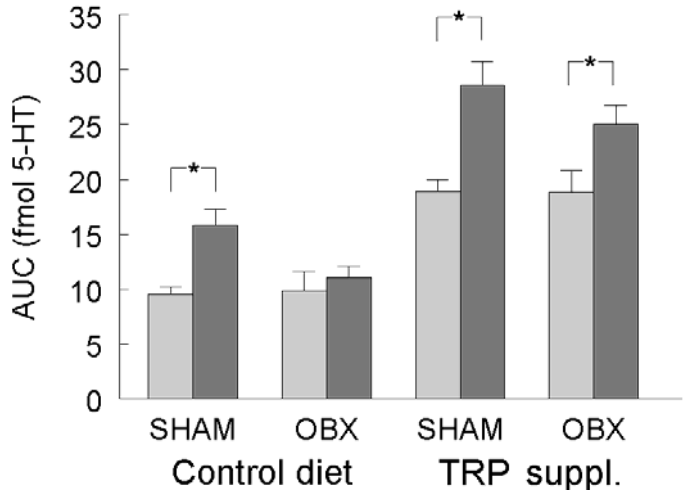

Fig 3. Absolute increase of 5-HT levels after systemic administration of fluvoxamine with (■)or without (□) WAY in SHAM and OBX animals receiving control diet or TRP supplementation. Data are expressed as AUC ± SEM. * $p<0.005$, co-administration of WAY statistically different from fluvoxamine alone.

mRNA expression

The results of the q-PCR analysis are summarized in table 2. Expression of the genes for the 5-HT transporter, tryptophan hydroxylase, tyrosine hydroxylase and 2,3 dioxygenase were below the detection limit in the amygdala and hippocampus. In the amygdala, OBX animals showed a significant reduction in the expression of GABA-$A_{\alpha 1}$ subunit mRNA($p=0.030$, $n=12$) compared to SHAM animals, as well as an increase in the expression of MAO-A mRNA ($p<0.001$, $n=12$). In the hippocampus in increase in the expression of postsynaptic 5-HT_{1A} receptor mRNA was found ($p=0.047$, $n=11$).

Circadian corticosterone levels

Levels of corticosterone in a 24-h period are shown in fig 4. Multivariate analysis of the 24-h corticosterone measurements showed that corticosterone levels were significantly elevated during the dark period (time $F_{(1,31)}=79.6$, $p<0.001$). No effects of surgery (SHAM/OBX) or diet (control/high) were found on the 24h corticosterone cycle.

	Amygdala		Hippocampus	
	SHAM (n=6)	OBX (n=6)	SHAM (n=5)	OBX (n=6)
5-HT_{1A}R	0.045 ± 0.021	0.034 ± 0.006	**0.031 ± 0.008**	**0.120 ± 0.036 ***
5-HT_{1B}R	18.9 ± 2.1	15.9 ± 3.3	5.8 ± 1.4	8.7 ± 1.3
GABA-$A_{\alpha 1}$	**844.4 ± 92.4**	**567 ± 51.9 ***	177.7 ± 50.6	243.2 ± 25.2
GABA-$A_{\alpha 2}$	394.3 ± 35.7	455.8 ± 81.0	168.8 ± 37.6	189.1 ± 31.4
MAO-A	**11.2 ± 0.9**	**16.9 ± 0.4 ***	17.5 ± 4.4	9.7 ± 1.6
Substance-P	3.2 ± 0.9	1.6 ± 0.3	0.49 ± 0.31	0.52 ± 0.39
NK-1	0.055 ± 0.008	0.042 ± 0.009	0.027 ± 0.005	0.051 ± 0.019

Table 2. Mean mRNA expression ± SEM in amygdala and hippocampus of SHAM and OBX animals. Genes that differed significantly between SHAM and OBX are shown in **bold**.
* $p<0.05$, OBX statistically different from SHAM

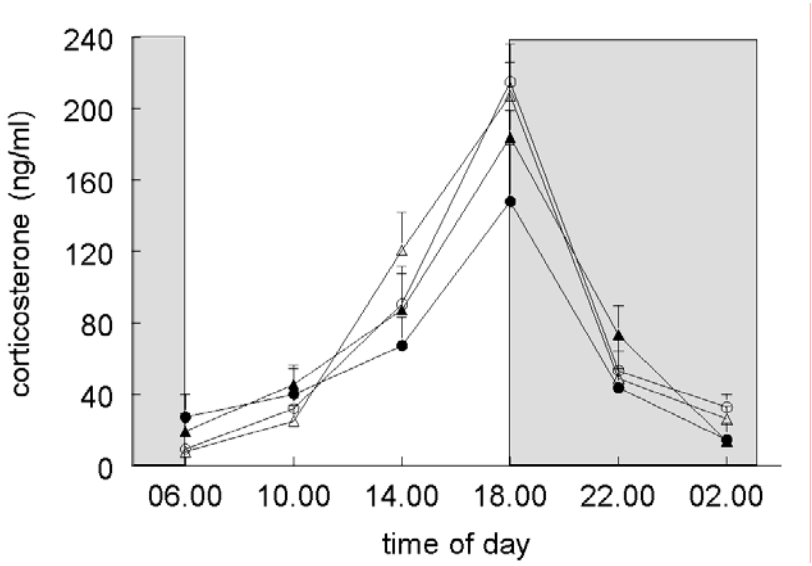

Fig 4. Circadian pattern of corticosterone secretion in animals receiving control diet (open symbols; SHAM ○, n=8; OBX △, n=9) or tryptophan supplementation (filled symbols; SHAM ●, n=9; OBX ▲, n=9). Plasma corticosterone was measured at 4 h intervals starting at 06:00. Results are presented as mean corticosterone concentration ± SEM. The shaded area represents the dark period during the 24 h cycle.

Discussion

In the present study we investigated the effects of a TRP rich diet on bulbectomized animals. OBX significantly decreased basal levels of 5-HT in the DH and induced desensitization of the somatodendritic 5-HT_{1A} receptor. Chronic TRP supplementation significantly increased basal 5-HT levels in both SHAM and OBX animals and reduced the desensitization of 5-HT_{1A} receptors in OBX animals. 24-h corticosterone measurement showed no difference between SHAM and OBX. qPCR showed a decrease in expression of GABA-$A_{\alpha 1}$ subunit mRNA and an increase of MAO-A mRNA expression in the amygdala and an increase of postsynaptic 5-HT_{1A} receptor mRNA in the hippocampus.

The present study showed an increase in locomotor activity of bulbectomized animals, which is consistent with early reports of behavioral changes in bulbectomized animals (Sieck, 1972). Since then, bulbectomy has consistently been shown to produce an increase in locomotor activity in novel environments such as the open field in several studies (Van Riezen et al, 1977; Cairncross et al, 1979, for review see Kelly et al, 1997). Van Riezen et al (1977) showed that the performance in the open field of peripherally anosmic rats was not different from controls. Therefore the alterations observed after bilateral removal of the olfactory bulbs are not due to anosmia per se. This hyperactivity is significantly attenuated by chronic treatment with antidepressants (Van Riezen et al, 1990; Mar et al, 2000), thereby mimicking the time course of effects of these drugs in depressed patients. Psychotropic drugs lacking antidepressant activity do not attenuate hyperactivity. Therefore, the open field test is commonly used as signal for antidepressant activity.
It was expected that since chronic supplementation of TRP in the diet augments the effects of antidepressants on 5-HT, this would also affect the activity in the open field. However, we did not find any effects of chronic TRP supplementation on locomotion, neither SHAM nor OBX animals. It might be argued, though, that the hyperactivity seen in OBX animals is primarily mediated by dopaminergic rather than serotonergic pathways, whereas chronic tryptophan supplementation selectively increases 5-HT levels (Van der Stelt et al, 2004). Redmond et al (1997) found decreased levels of 5-HT, NE and DA in the frontal cortex of OBX rats. Chronic administration of dizocilpine, a NMDA receptor antagonist which also blocks the reuptake of NE and DA, was found to attenuate the OBX-related deficit in DA, with effect on 5-HT or NE levels. Chronic dizocilpine reversed the OBX-induced hyperactivity in the open field, suggesting this is a primarily dopaminergic phenomenon. To gain more insight into the precise mechanism, changes in dopaminergic functioning and the relationship between dopamine and serotonin neurotransmission after bulbectomy should be studied.

Using in-vivo microdialysis, we confirmed earlier findings (Van der Stelt et al, submitted) showing decreased extracellular levels of 5-HT in the DH of bulbectomized animals. The effect of systemic SSRI treatment on 5-HT levels in projection areas like the DH is in part dependent on the 5-HT_{1A} receptor feed-back regulation. Normally, stimulation of the somatodendritic 5-HT_{1A} autoreceptor reduces the effect on 5-HT release in the DH following systemic SSRI treatment by reducing the firing rate. Desensitization of the somatodendritic 5-HT_{1A} receptor reduces the amount of negative feedback, resulting in higher concentrations of extracellular 5-HT. The present study replicated these findings of somatodendritic 5-HT_{1A} receptor desensitization following bulbectomy. In line with this finding, we found that fluvoxamine, administered systemically to animals receiving the control diet, increased 5-HT levels in OBX animals and SHAM operated controls to the same extent, despite the fact that basal extracellular levels were significantly lower after bulbectomy. When WAY 100.635, a 5-HT_{1A} receptor antagonist, was co-administered with fluvoxamine, 5-HT levels increased significantly in SHAM animals, as compared to fluvoxamine alone, while in OBX animals, no further increase in 5-HT levels after co-administration of WAY was seen, supporting our previous findings that olfactory bulbectomy leads to desensitization of the somatodendritic 5-HT_{1A} receptor.
After receiving chronic tryptophan supplementation, basal 5-HT levels were increased in all animals. The amount of 5-HT released after administration of an SSRI was also increased in both groups, indicating that 5-HT release is increased following a TRP-rich diet. Contrary to OBX animals receiving a normal diet, WAY 100.635 in OBX animals receiving a TRP-rich diet caused augmentation of 5-HT levels as compared to fluvoxamine alone. This suggests that chronic tryptophan supplementation can reverse the desensitization of the somatodendritic 5-HT_{1A} receptor found in bulbectomized animals.

In contrast to the desensitization of the somatodendritic 5-HT_{1A} autoreceptor, postsynaptic 5-HT_{1A} receptor mRNA expression was increased in the hippocampus of bulbectomized rats as measured with qPCR. It is conceivable that alterations in the 5-HT_{1A} receptor functioning are directed at maintaining normal levels of 5-HT, therefore negative feedback via the autoreceptor is reduced. Likewise, to maintain similar physiological responses, it is also feasible that among other adaptive processes, the sensitivity of the postsynaptic 5-HT_{1A} receptor will increase.
No alterations were found in the degree of expression of the 5-HT_{1B} receptor mRNA. This is in line with earlier findings by our group (Van der Stelt et al, submitted). Using microdialysis we showed that bulbectomy did not alter terminal 5-HT_{1B} receptor functioning.
In the present study we also found increased levels of monoamine oxidase A (MAO-A) mRNA in the amygdala. MAO-A preferentially catalyses the oxidative

deamination of 5-HT and NE. In a previous study we found decreased levels of 5-HT in the amygdala of bulbectomized rats, but levels of 5-HIAA were not changed (Van der Stelt et al, submitted). An increase in MAO-A levels could account for the lack of effect of bulbectomy on 5-HIAA levels despite a decrease in 5-HT levels. Likewise, MAO-A knockout mice show elevated brain concentrations of 5-HT and NE (Cases et al, 1995). MAO-A may be involved in the pathophysiology of depression and panic disorder, since MAO-A inhibitors are effective in these conditions.

GABA-$A_{\alpha 1}$ subunit mRNA expression was also found to be decreased in the amygdala of OBX rats. The amygdala is considered to be a key structure in mediating emotions such as fear and anxiety and amygdaloid nuclei have been shown to mediate the effects of $GABA_A$ receptor ligands on anxiety (Sanders et al, 1995). Interestingly, $5\text{-}HT_{1A}$ receptor knockout mice exhibit increased anxiety as indicated by decreased exploratory behavior in multiple paradigms (Heisler et al, 1998; Parks et al, 1998; Rambos et al, 1998). Depending on the genetic background, $5\text{-}HT_{1A}$ receptor knockout mice show disturbances in the $GABA_A$-benzodiazepine receptor system in the brain, including downregulation of GABA-$A_{\alpha 1}$ and GABA-$A_{\alpha 2}$ subunits in the amygdala (Olivier et al, 2001).

When interpreting the present data one has to bear in mind that although attempts have been made to correlate protein levels with mRNA expression levels, but these have been with variable success. Several reasons for this absence of correlation have been proposed. First, there are many complicated and varied post-transcriptional mechanisms involved in synthesizing protein from mRNA that are not yet sufficiently well defined. Second, it is proposed that proteins have very different half-lives as the result of varied protein synthesis and degradation. Finally, there is a significant amount of error and noise in both protein and mRNA experiments that limit the ability to get a clear picture (for review see Greenbaum et al, 2003).

In the present study an attempt was made to determine differences in mRNA expression of several proteins relevant for serotonergic functioning in bulbectomized animals. Although the present data do not allow for a comparison of mRNA levels coding for different proteins, comparisons of mRNA coding for a single protein were made between SHAM and OBX animals. However, such a comparison can only be made on the assumption that processes affecting transcription are not affected by bulbectomy.

Basal levels of corticosterone as measured during a 24-h diurnal cycle were not different between SHAM and OBX animals. Glucocorticoid secretion has been investigated following olfactory bulbectomy, but has yielded conflicting results with some reports of raised levels of both basal and stress-induced glucocorticoids (Cairncross et al, 1977; Cattarelli and Demael, 1986). However, Broekkamp et al (1980) suggested that OBX animals show an increased corticosteroid response to

stress rather than an increase in basal levels, which could be explained by the compensatory changes in the somatodendritic 5-HT_{1A} receptor and the postsynaptic 5-HT_{1A} receptor. Song et al (1994) reported normal secretion of corticosterone to occur in OBX rats during the light phase. However, during the dark phase they observed an extended hypersecretion, suggesting an altered circadian secretion of corticosterone. Patients with major depression show a hypersecretion of cortisol throughout the 24-h cycle and hence the diurnal rhythm has been shown to exhibit a flattened profile. This hypersecretion of cortisol is not suppressed by administration of dexamethasone (Carrol et al, 1982), possibly due to abnormalities in negative feedback at multiple levels in the HPA axis (Holsboer et al, 1995). 5-HT pathways modulate HPA responses to stress and play a key role in the maintenance of the circadian glucocorticoid rhythm (Meltzer et al, 1987; Fuller, 1992). Drawing on these facts, it is feasible that the compensatory changes seen in bulbectomized animals are sufficient to maintain normal functioning under normal circumstances. However, under stressful conditions, bulbectomized animals may show an inability to adapt to its environment, as was first suggested by Leonard and Tuite (1981).

In conclusion, the present study showed that bulbectomy induced desensitization of the somatodendritic 5-HT_{1A} receptor as well as sensitization of the postsynaptic 5-HT_{1A} receptor. Chronic tryptophan supplementation normalized basal 5-HT levels as well as the somatodendritic 5-HT_{1A} receptor. However, this normalization was not reflected in the open field or on corticosterone levels, suggesting a dissociation between neurochemical and behavioral or endocrine parameters.

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General discussion

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- Behavioral effects

Permanent deficits and compensatory changes in bulbectomized rats
- Alterations in neurotransmitter functioning

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- Effects of bulbectomy on norepinephrine and dopamine
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Conclusions and future directions

Chapter 7

The aims of this thesis were three-fold. First, the effects of variations in dietary tryptophan content on serotonergic functioning were investigated. Second, the effects of bilateral removal of the olfactory bulbs on functioning of the serotonergic system were assessed. Third, it was investigated to what extent enhancement of serotonergic functioning via chronic tryptophan supplementation would change several of the alterations seen in bulbectomized animals.

DIETARY TRYPTOPHAN VARIATIONS

Serotonergic functioning

Using in-vivo microdialysis, it was shown that extracellular levels of 5-HT in the rat dorsal hippocampus (DH) and the effect of an SSRI on 5-HT output in this region can be manipulated by modifying the tryptophan content of the diet. It has been suggested that the amount of free plasma tryptophan is an indicator for tryptophan transport into the brain (Tagliamonte et al, 1973; Biggio et al, 1974). Also, it has been found that acute tryptophan administration or depletion causes parallel effects in plasma and brain tryptophan levels and levels of 5-HT and 5-HIAA in the brain (Moja et al, 1989; Sarna et al, 1991). However, chronic alteration of dietary tryptophan intake did not affect tryptophan levels in the present study, despite significant changes in extracellular 5-HT levels following TRP high and TRP low diets (chapter 3). It is suggested that TRP levels return more quickly to control levels than extracellular 5-HT levels in the brain. Microdialysis experiments were performed in the light phase, whereas rats ingest most of their food during the dark phase.
Local administration of the releaser fenfluramine led to a diet-dependent increase in 5-HT levels in the DH, suggesting that the amount of 5-HT available for release can be modulated by varying tryptophan intake. Moreover, fluvoxamine administration also led to a diet-dependent increase in extracellular 5-HT levels, supporting the idea that 5-HT release is directly dependent on TRP availability. The dependency of 5-HT release on TRP intake may also have clinical consequences for efficacy of SSRIs. SSRIs supposedly elicit their effect by inhibiting reuptake of 5-HT into the presynaptic neuron, thus rendering more 5-HT available in the synapse for neurotransmission. However, as shown in chapter 3, decreased TRP availability reduced 5-HT output, which may result in lower efficacy of SSRI treatment.

Behavioral effects of dietary tryptophan variation

The effects of the TRP diets on extracellular 5-HT levels in the brain were corroborated by the behavioral tests. The amount of TRP in the diet differentially affected DRL-72 s performance, with the TRP low diet deteriorating and the TRP high diet improving the DRL-72 s task performance (chapter 3). In line with previous reports demonstrating beneficial effects of SSRIs in the DRL-72 s procedure (Sokolowski and Seiden, 1990; Olivier et al, 1993), we found that the SSRI fluvoxamine induced a dose-dependent improvement in DRL performance. The DRL-72 s has been proposed as a model for depression based on the activity of standard antidepressants on the rate of responding and reinforcement (McGuire and Seiden, 1980, O'Donnel and Seiden, 1983). The effect of a TRP high diet on DRL-72 s performance in the present study is similar to the effect of oral administration of 20 mg/kg fluvoxamine, suggesting that tryptophan supplementation may have antidepressant potential. It is of note in this respect, that the increase in extracellular 5-HT following a TRP high diet in this study is in the same order of magnitude as the 5-HT increase following oral administration of 30 mg/kg of fluvoxamine observed previously in our laboratory (Bosker et al, 1995).

PERMANENT DEFICITS AND COMPENSATORY CHANGES IN BULBECTOMIZED ANIMALS

Alterations in neurotransmitter functioning

Extracellular 5-HT and 5-HIAA levels

The present research showed that bilateral removal of the olfactory bulbs results in selective alteration in the functioning of the serotonin system. OBX animals showed decreased basal extracellular levels of 5-HT in DH, basolateral amygdala (BLA) (chapter 4) and median raphe nucleus (MR) (chapter 5). This is in line with lower brain 5-HT content found in other studies. Jancsar and Leonard (1984) found decreased tissue content of 5-HT in midbrain and amygdaloid cortex following olfactory bulbectomy. Redmond et al (1997) showed lower 5-HT tissue concentrations in the frontal cortex of OBX animals. These findings are consistent with observations in depressed patients, showing decreased plasma and platelet 5-HT content (Sarrias et al, 1997). The changes in extracellular 5-HT levels were not reflected in extracellular 5-HIAA levels in the DH or BLA. This is at variance with previous studies, showing lower 5-HIAA concentrations in the amygdaloid cortex and midbrain (Jancsar and Leonard, 1984) and in the n. accumbens (Connor et al, 1999) of bulbectomized animals. Early studies have detected reduced levels of CSF 5-HIAA in depressed suicide victims (for review see Smith and Cowen, 1997).

However, at present there is evidence that low CSF 5-HIAA levels are related to impulsivity rather than to mood alterations (Faustman et al, 1991).

Blockade of the 5-HT transporter (5-HTT) in the BLA and DH by local infusion of the SSRI fluvoxamine showed that the increase in extracellular 5-HT levels was attenuated in OBX animals. Moreover, local infusion of the releaser fenfluramine also showed an attenuated increase in 5-HT levels in bulbectomized animals. Taken together, and considering the decrease in extracellular basal 5-HT levels, these data suggest that both the amount of 5-HT release and the amount available for release are decreased following OBX.

Effects of bulbectomy on norepinephrine and dopamine
Extracellular basal levels of NE and DA were investigated in the DH and BLA, but no alterations in basal levels of NE and DA nor in the effects of fluvoxamine and fenfluramine on NE and DA as a result of bulbectomy were found. Data on the effects of OBX on NE and DA concentrations in the brain are not equivocal. Decreased levels of NE were found in the amygdaloid cortex (Jancsar and Leonard, 1984) and frontal cortex (Redmond et al, 1997). Decreased DA levels were found in the midbrain (Jancsar and Leonard, 1984) and frontal cortex (Redmond et al, 1997). However, these findings were not confirmed by Broekkamp et al (1980), who suggested that the OBX paradigm is primarily a serotonergic syndrome, given the fact that the effects of subchronic treatment with imipramine and mianserine were reversed by acute administration of the 5-HT_2 receptor antagonist metergoline (Broekkamp et al, 1980). Kelly et al (1997) suggested that discrepant NE findings after OBX may be related to methodological problems rather than to differences in NE regulation in the brains of OBX animals. It cannot be excluded, though, that NE or DA alterations do occur in other brain regions. Recently, Masini et al (2004) found that OBX increases DA release in both dorsal and ventral striatum, without affecting metabolite levels or turnover.

5-HT synthesis
A possible explanation for decreased 5-HT release and availability might be a reduced 5-HT synthesis. Administration of NSD-1015, a decarboxylase inhibitor, revealed an attenuated 5-HTP accumulation in the DH of bulbectomized animals compared to controls, suggesting a decreased rate of 5-HT synthesis after OBX. This is in line with decreased 5-HT turnover in the amygdaloid cortex (Jancsar and Leonard, 1984) and raphe nuclei (Watanabe et al, 2003) of bulbectomized animals. In contrast, Jancsar and Leonard (1984) did not find any effects on 5-HT synthesis in the midbrain and Watanabe et al (2003) reported increased 5-HT synthesis in frontal cortex and hippocampus following bulbectomy. They suggest that this increase is the result of collateral sprouting and synaptogenesis following bulbectomy, which is most prominent in the frontal cortex (Zhou et al, 1998).

Of interest is the finding that systemic administration of a high dose of L-tryptophan resulted in similar increases in 5-HTP accumulation in both SHAM and OBX animals. This suggests that, despite an apparently impaired 5-HT synthesis under basal conditions, the capacity of the system to synthesize 5-HT is not affected. This is of particular interest, since we have shown that dietary tryptophan content can influence extracellular 5-HT levels. It is feasible that dietary tryptophan supplementation might therefore be a means of increasing extracellular 5-HT levels and restoring normal 5-HT functioning.

A possible explanation for the decreased rate of 5-HT synthesis could be a loss of serotonergic neurons. Bilateral removal of the bulbs could cause retrograde degeneration of neurons in various regions projecting to the bulbs as well as anterograde degeneration of neurons normally receiving projections from the bulbs. The raphe nuclei are the sole source of serotonergic input into the olfactory bulb (McLean and Shipley, 1987) and indeed substantial loss of raphe neurons is found following bulbectomy (Nesterova et al, 1997). Likewise, neuronal degeneration has also been found in the amygdala and hippocampus, which are projection areas of the dorsal and median raphe, respectively (Carlsen et al, 1982). Moreover, Norrholm and Ouimet (2001) reported reduced spine density in CA1, CA3 and dentate gyrus of the hippocampus following bulbectomy. On a macroscopic level, changes in signal intensities in cortical, hippocampal, caudate and amygdaloid regions have been shown using magnetic resonance imaging (Wrynn et al, 2000a). Degeneration of serotonergic neurons could cause adaptive changes in the functioning of the serotonergic system, which are aimed at maintaining physiological levels of 5-HT.

5-HT transporter

As mentioned in chapter 1, a means of regulating extracellular 5-HT levels is by reuptake of 5-HT from the synapse via the 5-HT transporter (5-HTT). It might be expected that olfactory bulbectomy results in downregulation of the 5-HTT, in order to increase the amount of 5-HT available for neurotransmission. Local administration of different concentration of fluvoxamine showed a parallel increase in 5-HT levels, the increase being larger in SHAM animals than in bulbectomized animals (chapter 5). The most likely explanation for a difference in the height of the curves is the decreased 5-HT released found in OBX animals, thereby suggesting that the efficacy of the 5-HTT has not changed. Zhou et al (1998) have found increased 5-HTT density in the frontal cortex of bulbectomized animals, but not in other brain areas. They suggest that lesioning the fibers projecting to the bulbs stimulates collateral sprouting and synaptogenenesis, especially in the frontal cortex, leading to selective 5-HT hyperinnervation of this brain area.

Terminal 5-HT$_{1B}$ autoreceptor

Other feedback mechanisms aimed at controlling 5-HT levels are associated with modulation of neuronal activity (the somatodendritic 5-HT$_{1A}$ receptor) and neurotransmitter release (the terminal 5-HT$_{1B}$ receptor). As mentioned in the introduction, the 5-HT$_{1B}$ receptor serves as a 5-HT autoreceptor at the 5-HT terminal (for review see Middlemiss and Hutson, 1990). Under normal circumstances, activation of the 5-HT$_{1B}$ receptor by increased levels of 5-HT inhibits transmitter release, thereby controlling 5-HT levels. OBX animals show decreased basal 5-HT levels, which could be a consequence of upregulation of the 5-HT$_{1B}$ receptor. However, by the same means the 5-HT$_{1B}$ receptor could also be downregulated as a means to increase 5-HT levels. Using the 5-HT$_{1B}$ receptor agonist CP-93129 we detected the presence of a functional 5-HT receptor in both SHAM and OBX animals. Moreover, the efficacy of the terminal 5-HT$_{1B}$ receptor was not affected by removal of the olfactory bulbs.

Somatodendritic 5-HT$_{1A}$ autoreceptor

Like the 5-HT$_{1B}$ receptor, the somatodendritic 5-HT$_{1A}$ receptor is aimed at controlling 5-HT levels. Under normal circumstances, an increase in 5-HT levels will activate somatodendritic 5-HT$_{1A}$ receptors in the raphe nucleus, which will in turn reduce the firing frequency of 5-HT neurons (chapter 1). Local administration of the 5-HT$_{1A}$ receptor agonist flesinoxan in the median raphe nucleus (MR) decreased 5-HT levels in SHAM animals but had no effect of 5-HT levels in OBX animals, indicating that 5-HT$_{1A}$ receptors in the MR are desensitized after bulbectomy (chapter 5). It is feasible that desensitization of somatodendritic 5-HT$_{1A}$ receptors results from decreased levels of 5-HT in OBX animals, as it results in a reduction of negative feedback on the firing frequency. By this means, desensitization of somatodendritic 5-HT$_{1A}$ receptors is aimed at maintaining normal 5-HT levels when synthesis or release is decreased.

Since the effect of systemic SSRI treatment on 5-HT levels in projection areas like the DH is in part dependent on the 5-HT$_{1A}$ receptor feed-back regulation, one might expect the effect of SSRIs on 5-HT levels in the DH of OBX animals to be smaller after systemic administration than after local administration into the DH. Normally, stimulation of somatodendritic 5-HT$_{1A}$ autoreceptors reduces the effect on 5-HT release in the DH following systemic SSRI treatment by reducing the firing rate. Desensitization of somatodendritic 5-HT$_{1A}$ receptors reduces the amount of negative feedback, thus resulting in higher concentrations of extracellular 5-HT. Indeed, when fluvoxamine was administered systemically to OBX animals, the increase in 5-HT levels is similar to the increase seen in SHAM operated controls, despite the fact that basal extracellular levels are significantly lower after bulbectomy (chapters 5, 6). When WAY 100.635, a 5-HT$_{1A}$ receptor antagonist, was co-administered with fluvoxamine, levels of 5-HT increased significantly in

SHAM animals compared to administration of fluvoxamine alone (chapter 5). However, in OBX animals, no extra increase in 5-HT levels after co-administration of WAY was seen, supporting the previous findings that olfactory bulbectomy leads to desensitization of somatodendritic 5-HT_{1A} receptors.

Postsynaptic 5-HT_{1A} receptor
Using quantitative PCR, the expression of several genes relevant for serotonergic functioning was determined in hippocampus and amygdala. In contrast to desensitization of the somatodendritic 5-HT_{1A} autoreceptor, postsynaptic 5-HT_{1A} receptor expression was found to be increased in the hippocampus of bulbectomized rats (chapter 6). It can be argued that adaptive processes in sensitivity or expression of receptors are aimed at maintaining physiological responses. For instance, desensitization of somatodendritic 5-HT_{1A} autoreceptors will reduce the amount of negative feedback, thereby aiding in achieving higher levels of 5-HT. Likewise, upregulation of the postsynaptic 5-HT_{1A} receptor will result in an enhanced physiological response even though 5-HT levels are decreased, as is the case in bulbectomized animals.
The discrepancy between pre- and postsynaptic 5-HT_{1A} receptors could explain the lack of difference in basal corticosterone levels between SHAM and OBX animals. It is feasible that the compensatory changes seen in bulbectomized animals are sufficient to maintain normal functioning under normal circumstances. However, as was first suggested by Leonard and Tuite (1981), it can be argued that under stressful conditions bulbectomized animals may show an inability to adapt to their environment,

Body weight and locomotor activity

Early reports of changes in bulbectomized animals have shown reduced body weight of OBX animals compared to SHAM controls and this has consistently been found since (for review see Kelly et al, 1997). In keeping with these reports we have found an overall reduced body weight of OBX animals compared to SHAM controls, which is still present up to 20 weeks after surgery (chapter 4).
A second phenomenon that has been consistently reported in bulbectomized animals is the increase in locomotor activity. Bulbectomized animals show an increase in locomotor activity when placed in a novel, stressful environment, such as the ‘open field’ (Van Riezen et al, 1977, Cairncross et al, 1979; for review see Kelly et al, 1997). Van Riezen et al (1977) showed that the performance in the open field of peripherally anosmic rats was not different from controls. Therefore the alterations observed after bilateral removal of the olfactory bulbs are not due to anosmia per se. In keeping with these previous findings, OBX animals in the present study showed increased locomotion in the open field compared to SHAM

operated controls (chapter 4, 6). The hyperactivity phenomenon appears to be of a chronic character in the OBX animal, as it is still present 20 weeks after surgery (chapter 4). Although habituation over time and with testing occurs, the difference between SHAM and OBX remains.

Corticosterone cycle

Levels of corticosterone as measured during the 24-h diurnal cycle were significantly elevated during the dark period in both SHAM and OBX animals, with no difference in levels between the two groups. Investigation of glucocorticoid secretion following bulbectomy has yielded conflicting results with some reports of raised levels of both basal and stress-induced glucocorticoids (Cairncross et al, 1977; Cattarelli and Demael, 1986). However, Broekkamp et al (1980) suggested that OBX animals show an increased corticosteroid response to stress rather than an increase in basal levels. Song et al (1994) reported normal secretion of corticosterone to occur in OBX rats during the light phase. However, during the dark phase they observed hypersecretion, suggesting an altered circadian secretion of corticosterone. Patients with major depression show hypersecretion of cortisol throughout the 24-h cycle and hence the diurnal rhythm has been shown to exhibit a flattened profile. This hypersecretion of cortisol is not suppressed by administration of dexamethasone (Carrol et al, 1982), possibly due to abnormalities in negative feedback at multiple levels in the HPA axis (Holsboer et al, 1995). 5-HT pathways modulate HPA responses to stress and play a key role in the maintenance of the circadian glucocorticoid rhythm (Meltzer et al, 1987; Fuller, 1992). Drawing on these facts, it is feasible that the compensatory changes seen in bulbectomized animals are sufficient to maintain normal functioning under normal circumstances. However, under stressful conditions, bulbectomized animals may show an inability to adapt to its environment, as was first suggested by Leonard and Tuite (1981).

mRNA expression of genes relevant for serotonergic functioning

The expression of several genes relevant for serotonergic functioning was measured using qPCR (chapter 6). The increase in postsynaptic 5-HT_{1A} receptor mRNA expression has been described previously. The absence of an effect of bulbectomy on 5-HT_{1B} receptor mRNA expression (chapter 6) is consistent with the absence of an effect of bulbectomy on terminal 5-HT_{1B} receptor functioning (chapter 4).
The degree of expression of monoamine oxidase-A (MAO-A) mRNA was increased in the amygdala of bulbectomized rats. MAO-A preferentially catalyses

the oxidative deamination of 5-HT and NE. An increase in MAO-A levels could account for the lack of bulbectomy on 5-HIAA levels despite a decrease in 5-HT levels (chapter 4). MAO-A may be involved in the pathophysiology of depression and panic disorder, since MAO-A inhibitors are effective in treating these conditions.

EFFECTS OF ENHANCEMENT OF SEROTONIN TRANSMISSION ON BULBECTOMIZED RATS

A deficiency in the functioning of the central serotonergic system has been implicated in the development of major depression by numerous studies (for review see Smith and Cowen, 1997). Furthermore, it has been shown that dietary restrictions of tryptophan in patients whose depression has remitted after treatment with selective serotonin inhibitors (SSRIs), induces a relapse in symptoms (Delgado et al, 1990; Bell et al, 2001).This suggests that 5-HT may be involved in the mechanism of action of at least some successful antidepressant treatments and that correction of an underlying 5-HT dysfunction is linked to the initiation and maintenance of clinical remission. The results in chapter 3 support the notion that 5-HT release is directly dependent on TRP availability. This dependency of 5-HT release on TRP intake may also have clinical consequences for efficacy of SSRIs. SSRIs supposedly elicit their effect by inhibiting reuptake of 5-HT into the presysnaptic neuron, thus enhancing serotonergic neurotransmission. However, as shown in chapter 3, decreased TRP availability reduced 5-HT output, which may result in lower efficacy of SSRI treatment. Likewise, SSRIs might be more effective if the amount of 5-HT released is increased via dietary tryptophan supplementation.
As described in chapter 4 and 5, bulbectomized animals show deficits in serotonergic functioning and chronic dietary tryptophan supplementation enhances serotonergic functioning in control animals. Taken together, these data suggest that chronic dietary tryptophan supplementation in bulbectomized animals could increase 5-HT levels and enhance serotonergic functioning, thereby reversing or restoring some or all of the alterations found in bulbectomized animals.

Effects on serotonergic functioning

Chronic tryptophan supplementation increased extracellular basal 5-HT levels in both SHAM and OBX animals (chapter 6). It was also found that the effect of systemic SSRI administration on 5-HT levels was increased in both groups, indicating that chronic tryptophan supplementation increases 5-HT release not only in SHAM animals but also in bulbectomized animals. Contrary to OBX animals

receiving a normal diet, systemic administration of fluvoxamine together with WAY 100.635 in OBX animals receiving a TRP-rich diet caused augmentation of 5-HT levels compared to fluvoxamine alone. This suggests that chronic tryptophan supplementation can reverse the desensitization of somatodendritic 5-HT_{1A} receptors found in bulbectomized animals.

Locomotor activity

The hyperactivity of bulbectomized animals in the open field is significantly attenuated by chronic treatment with antidepressants (Van Riezen et al, 1990; Mar et al, 2000), thereby mimicking the time course of effects of these drugs in depressed patients. To date, there have been no reports of the action of any antidepressive agent, including putative candidates, which will attenuate the hyperactivity of OBX rats in the open field following acute administration (Cryan et al, 1997, 1998; Harkin et al, 1999). Also, it has been shown that psychotropic drugs lacking antidepressant activity do not attenuate hyperactivity. Therefore, the open field test is commonly used as a signal for antidepressant activity.
Although bulbectomized animals were hyperactive in the open field test (chapters 3, 5), chronic dietary tryptophan supplementation did not attenuate this hyperactivity, suggesting that tryptophan supplementation does not have antidepressant potential. Masini et al (2004) suggest that the dopaminergic hyperactivity that they found in ventral and dorsal striatum is directly related to the behavioral hyperactivity of bulbectomized animals in an open field. In contrast, Redmond et al (1997) found decreased levels of 5-HT, NE and DA in the frontal cortex of bulbectomized rats. Chronic administration of dizocilpine, an NMDA receptor antagonist which also blocks the reuptake of NE and DA, was found to attenuate the OBX-related deficit in DA, without affecting NE or 5-HT levels. Also it was found that chronic dizocilpine reversed the hyperactivity in the open field, suggesting that this is a primarily dopaminergic phenomenon.

The acute pharmacological effects of SSRIs are essentially confined to blockade of 5-HT reuptake. This action occurs within hours of SSRI administration; however, the therapeutic effect of SSRIs can take several weeks to manifest. From this it has been argued that repeated SSRI treatment results in neuroadaptive changes in 5-HT and other receptors, which ultimately produce the antidepressant effect (Blier and de Montigny, 1994). Indeed, studies in animals and humans have shown that SSRI treatment decreased sensitivity of both pre- and postsynaptic 5-HT_{1A} receptors (Cowen, 1998; Sargent et al, 1997).
Indirect actions of SSRIs on other neurotransmitters cannot be ruled out. Infusion of relatively high nanomolar concentrations of 5-HT in the nucleus accumbens facilitates the release of DA (Parsons and Justice, 1993). Also it has been suggested

that activation of postsynaptic 5-HT_{1A} receptors increases DA release in a brain region specific manner (Sakaue et al, 2000; Ago et al, 2003). Antidepressants have been shown to exert different effects on dopaminergic functioning depending on whether they are administered acutely or repeatedly . Drugs with different primary targets for their mechanism of action (such as fluoxetine, clomipramine, imipramine, desipramine) all increase dopamine release in different brain areas after single administration (Tanda et al, 1994; Tiihonen et al, 1996; Penttil et al, 2004). On the other hand, SSRIs have been shown to inhibit dopaminergic neurotransmission upon chronic administration (Tiihonen et al, 1996; Damsa et al, 2004; Penttil et al, 2004). It is suggested therefore that the modulatory effects of antidepressants on dopaminergic neurotransmission also depend on de duration of treatment (acute versus multiple dosing).
Taken together, it might be suggested that the effects of SSRIs on locomotor activity in the bulbectomized rat could result from neuroadaptive changes, possibly mediated via the postsynaptic 5-HT_{1A} receptor. The question still remains why enhancement of serotonergic functioning by dietary tryptophan supplementation does not attenuate the hyperactivity in OBX rats, despite the fact that it does normalize basal 5-HT levels and 5-HT release. A possible explanation is that the increase in extracellular 5-HT after dietary tryptophan is not sufficient to induce the neuroadaptive changes. The increases in extracellular 5-HT levels after administration of a SSRI is substantially greater than the increases observed after a high TRP diet. Possibly these higher 5-HT levels are a prerequisite for the neuroadaptive changes purported to be implicated in the locomotor effects. In the present study we did show increased postsynaptic 5-HT_{1A} receptor mRNA expression in bulbectomized animals, but the effect of tryptophan supplementation remains to be elucidated. In depressed patients, tryptophan administration has been shown to enhance the antidepressant effects of TCAs, MAOIs and SSRIs manifested by a more rapid onset of antidepressant action (Ayuso and Lopez-Ibor, 1971; Young, 1986; Young, 1991; Levitan et al, 2000). The efficacy of tryptophan given alone has been the subject of several investigations. Overall, the studies are not unequivocal as to the efficacy of tryptophan (for reviews see Young, 1986; Sandyk, 1992). Studies on the effects of dietary tryptophan addition to SSRI treatment on the locomotion in OBX rats would shed more light on this issue.

METHODOLOGICAL CONSIDERATIONS

Microdialysis

Intracerebral microdialysis is a technique that permits in-vivo monitoring of concentrations of neurotransmitters and administration of drugs at specific regions of the brain. One of the major concerns with this technique is that implantation of the microdialysis probe may evoke inflammation and subsequent healing which

may affect the results of microdialysis experiments. Repeated experiments in the same animal can alter basal levels of (in this case) 5-HT, however, when testing the effects of different drugs, the relative effects on 5-HT levels are comparable between test sessions. It is also important to take into consideration that when investigating the effects of chronic dietary alterations, it is essential for the animals to consume a normal amount of food prior to experimentation. Therefore the procedure of probe implantation and the interval between surgery and microdialysis experiments should be carefully considered (chapter 2).

mRNA expression and protein levels

Proteins are synthesized on the basis of a DNA blueprint. Briefly, parts of the DNA are transcribed into mRNA which is then translated into protein. Attempts have been made to correlate protein levels with mRNA expression levels, but these have been with variable success. Several reasons for this absence of correlation have been proposed. First, there are many complicated and varied post-transcriptional mechanisms involved in synthesizing protein from mRNA that are not yet sufficiently well defined. Second, it is proposed that proteins have very different half-lives as the result of varied protein synthesis and degradation. Finally, there is a significant amount of error and noise in both protein and mRNA experiments that limit the ability to get a clear picture (for review see Greenbaum et al, 2003).

In the present study (chapter 6) an attempt was made to determine differences in mRNA expression of several proteins relevant for serotonergic functioning in bulbectomized animals. Although the present data do not allow for a comparison of mRNA levels coding for different proteins, comparisons of mRNA coding for a single protein were made between SHAM and OBX animals. However, such a comparison can only be made on the assumption that processes affecting transcription are not affected by bulbectomy.

CONCLUSIONS AND FUTURE DIRECTIONS

The present research describes a range of alterations following bilateral removal of the olfactory bulbs in rats. The most profound changes consist of decreased basal levels of 5-HT accompanied by desensitization of the somatodendritic 5-HT_{1A} autoreceptor. Chronic dietary tryptophan supplementation was found to enhance serotonergic neurotransmission in normal animals as well as normalize basal 5-HT levels and functioning of the somatodendritic 5-HT_{1A} receptor in bulbectomized animals. However, this normalization was not reflected in the open field or on corticosterone levels, suggesting a dissociation between neurochemical and behavioral or endocrine parameters.

The present research suggests that although bulbectomized animals are able to function well under normal circumstances, their ability to adapt to novel, stressful conditions might be affected. Therefore, future research should be directed at the effects of stressful stimuli, either as a behavioral or pharmacological challenge, on functioning of bulbectomized animals.
Furthermore, no alterations in dopaminergic and noradrenergic functioning were found in the present studies. However, several of the changes found in bulbectomized animals, such as the hyperactivity in the open field, do suggest a role for dopamine in this model. The drugs used in the present research and the brain areas studied were aimed at describing the effect of bulbectomy on serotonergic functioning. To further elucidate the role of dopamine and norepinephrine in the olfactory bulbectomy syndrome, brain areas that are relevant for dopaminergic cq noradrenergic neurotransmission should be studied using selective drugs.
Finally, an effort should be made to try to correlate brain neurochemistry to behavior in a direct manner, for instance by monitoring neurotransmitter functioning during behavioral testing.

The research described in this thesis supports the validity of the olfactory bulbectomized rat as an intriguing model for affective processes in the brain. Further investigation of the neurochemical, behavioral, endocrine and immune alterations, and their interactions and correlations in bulbectomized animals may help to gain more insight into the etiology and treatment of major depressive disorder.

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Summary

Major depressive disorder (MDD) is one of the most prevalent psychiatric disorders with a lifetime prevalence as high as 13%. Currently more than 20 different drugs are approved to treat depression, but half of these compounds have been available for decades and the majority can be grouped into two classes based on their pharmacological mechanism. Of these the selective serotonin reuptake inhibitors (SSRIs) are currently one of the mainstays in the treatment of MDD. Based on the efficacy of these antidepressants, considerable clinical evidence has been found that serotonin (5-HT) containing pathways in the central nervous system play a significant role in the pathophysiology of MDD.
Animal models of depression might be helpful in understanding the pathophysiology of MDD. The olfactory bulbectomy (OBX) in rats is an animal model that meets most of the criteria of a valid model for depression and as such may help to gain more insight in the etiology of depression and the development of novel antidepressants.

In **chapter 1** relevant literature is described. The serotonergic system is an extensive neurotransmitter system in the vertebrate CNS. Cell bodies of serotonergic neurons are located in the raphe nuclei and project to many brain areas, including the olfactory bulbs. 5-HT mediates a wide range of physiological effects and exerts its function through interaction with at least 14 different 5-HT receptor subtypes. 5-HT_{1A} receptors are located both postsynaptic to 5-HT neurons (in forebrain regions) and also on 5-HT neurons at the level of the soma and dendrites in the raphe nuclei. The latter receptor, commonly termed somatodendritic 5-HT_{1A} autoreceptor, aims at controlling 5-HT neuronal activity. Activation of this receptor by 5-HT will reduce the firing frequency of 5-HT neurons and subsequently diminish 5-HT release at the nerve terminals. 5-HT_{1B} receptors are located presynaptically, where they control 5-HT release, and postsynaptically mainly in the globus pallidus, dorsal subiculum and substantia nigra.
This chapter also describes the behavioral, neurochemical, endocrine and immune changes that have been reported in bulbectomized animals. One of the most consistent findings in bulbectomized rats is an increase in locomotor activity in a stressful, novel environment, such as the 'open field'. This hyperactivity has been shown not to be related to loss of smell. Macroscopic changes in the cortex, hippocampus, caudatus and amygdala have also been reported following bulbectomy as well as neuronal degeneration and remodeling. Alterations in 5-HT levels have been found, with most studies reporting decreased levels of 5-HT. However, there are also few reports of increased 5-HT levels. Norepinephrine (NE) and dopamine (DA) have also been studied following bulbectomy, but have yielded conflicting results. Stress-induced glucocorticoids are increased, but both an

increase or no alteration of basal levels of corticosterone have been reported. Immune changes found in bulbectomized animals correlate closely with immune changes found in depressed patients, as do many of the other effects of olfactory bulbectomy in rats. Many of the changes found in olfactory bulbectomized rats have been shown to respond selectively to chronic treatment with clinically effective antidepressants.

Chapter 2 reports on some methodological aspects of the microdialysis procedure used throughout this thesis. Important issues were the maximal interval between implantation of the microdialysis probe and microdialysis experiment without a significant effect on 5-HT functioning and the effect of multiple experiments through the same probe on 5-HT levels. It was found that an interval between surgery and experiment of up to 8 days did not affect the results of reuptake blockade with SSRIs, if expressed as percentage of the basal level. Basal levels of 5-HT as well as the absolute effect of SSRIs administration, however, increased with longer intervals. It was therefore not possible to compare absolute levels and effects at different post-surgery intervals. We also showed that repeated use of the same probe on two consecutive days did not result in reproducible data when results are expressed as absolute values; 5-HT levels were lower on the second test day. However, when two probes are used with the second probe implanted on the contralateral side, experiments can be reproducible conducted on two consecutive days. The present data do suggest though, that when it comes to relative increases of 5-HT, repeated perfusion through the same probe might be an option.

A deficiency in the functioning of the central serotonergic system has been implicated in the development of MDD. Furthermore, it has been shown that dietary restrictions of tryptophan in patients whose depression has remitted after treatment with SSRIs, induce a relapse in some patients. **Chapter 3** describes the effects of chronic dietary tryptophan on serotonergic functioning. Using in-vivo microdialysis, it was shown that extracellular levels of 5-HT in the rat dorsal hippocampus (DH) and the effects of the SSRI fluvoxamine on the 5-HT output in this region can be manipulated by modifying the tryptophan content of the diet. Furthermore, local administration of the 5-HT releaser fenfluramine led to a diet-dependent increase in 5-HT levels, suggesting that the amount of 5-HT available for release can be modulated by varying tryptophan intake. The effects of the TRP diets on extracellular 5-HT levels in the brain were corroborated by behavioral tests. The amount of tryptophan in the diet differentially affected DRL-72 s performance, with the TRP low diet deteriorating and the TRP high diet improving the DRL-72 task performance. The latter effect is similar to the effect of oral administration of 20 mg/kg of fluvoxamine, suggesting that tryptophan supplementation may augment the effect of antidepressants.

The second part of this thesis describes the permanent deficits and compensatory changes following olfactory bulbectomy. In **chapter 4** it was shown that basal levels of 5-HT, but not of 5-HIAA, NE and DA, were decreased in both the dorsal hippocampus (DH) and basolateral amygdala (BLA) two weeks after bulbectomy. Measurements in the DH five months after surgery revealed that this effect was long-lasting. Local administration of fenfluramine and fluvoxamine revealed that OBX caused an attenuated increase in 5-HT levels, while administration of NSD-1015, a decarboxylase inhibitor, suggested a lower rate of synthesis under basal conditions in bulbectomized animals. Systemic administration of a high dose of L-TRP resulted in a similar increase in 5-HTP accumulation in SHAM and OBX animals, suggesting that the capacity of the system to synthesize 5-HT is not affected.

Chapter 5 describes the adaptive processes in functioning of the serotonergic system resulting from possible degeneration of serotonergic neurons following removal of the olfactory bulbs. Decreased extracellular basal 5-HT levels in the DH were confirmed and decreased extracellular 5-HT levels were also found in the median raphe nucleus (MR). Local administration of different concentrations of fluvoxamine into the DH showed a parallel increase in 5-HT levels, the basal level and maximum increase being smaller in OBX animals than in SHAM operated controls. The most likely explanation for the smaller increase in 5-HT following SSRI administration in OBX animals is the lower efflux of 5-HT. The parallelism of the of the dose-response curves suggests that the efficacy of the 5-HT transporter had not changed. In the presence of fluvoxamine, 1 μM CP 9129, a selective 5-HT_{1B} receptor agonist, was infused locally in the DH to assess the functionality of the 5-HT_{1B} receptor. Both SHAM and OBX animals showed a significant decrease in 5-HT levels, indicating the presence of a functional 5-HT_{1B} receptor. When the total amount of decrease was related to the basal levels of 5-HT, there was no difference in relative effect between the two groups, indicating that the sensitivity of the terminal 5-HT_{1B} receptor has not changed. To assess the effects of bulbectomy on functioning of the somatodendritic 5-HT_{1A} receptor, flesinoxan, a 5-HT_{1A} receptor agonist, was infused locally into the median raphe nucleus (MR). SHAM operated animals showed a significant decrease in 5-HT levels after flesinoxan administration. However, in the OBX group, no significant effect of flesinoxan was found, indicating that 5-HT_{1A} receptors in the MR are desensitized in OBX rats. Systemic administration of fluvoxamine to OBX animals resulted in an increase in 5-HT levels similar to the increase seen in SHAM operated controls, despite the fact that basal extracellular levels are significantly lower after bulbectomy. When WAY 100.635, a 5-HT_{1A} receptor antagonist, was co-administered with fluvoxamine, levels of 5-HT increased significantly in SHAM animals compared to administration of fluvoxamine alone. However, in OBX animals, no extra increase in 5-HT levels after co-administration of WAY

was seen, supporting the previous findings that olfactory bulbectomy leads to desensitization of somatodendritic 5-HT_{1A} receptors.

In the last part of this thesis the effects of enhancement of serotonergic functioning on bulbectomized animals is described. **Chapter 6** describes the effects of a TRP rich diet on bulbectomized animals. OBX rats displayed hyperactivity in the open field, which was not attenuated by chronic dietary tryptophan supplementation. Using microdialysis, we confirmed earlier findings that OBX significantly decreased basal levels of 5-HT in the DH and induced desensitization of the somatodendritic 5-HT_{1A} receptor. Chronic TRP supplementation significantly increased basal 5-HT levels in both SHAM and OBX animals and reduced the desensitization of 5-HT_{1A} receptors in OBX animals. 24-h corticosterone measurement showed no difference between SHAM and OBX. qPCR showed a decrease in expression of GABA-$A_{\alpha 1}$ subunit mRNA and an increase of MAO-A mRNA expression in the amygdala and an increase of postsynaptic 5-HT_{1A} receptor mRNA in the hippocampus.

The main findings are discussed in **chapter 7**. The most profound neurochemical changes in bulbectomized animals reported in this thesis are decreased basal extracellular levels of 5-HT accompanied by a desensitization of the somatodendritic 5-HT_{1A} autoreceptor. Chronic dietary tryptophan supplementation was found to enhance serotonergic neurotransmission in normal animals and to normalize basal extracellular 5-HT levels and somatodendritic 5-HT_{1A} receptor function in bulbectomized animals. Interestingly, this normalization at the level of 5-HT release was not reflected in the open field, suggesting a dissociation between neurochemical and behavioral endpoint.
In conclusion the research described in this thesis supports the validity of the olfactory bulbectomized rat as a model for affective processes in the brain. Further investigation of the neurochemical, behavioral, endocrine and immune alterations, and their interactions and correlations in bulbectomized animals may help to gain more insight into the etiology and treatment of major depressive disorder.

Samenvatting

Depressie is een van de meest voorkomende psychiatrische aandoeningen met een prevalentie van 13%. Momenteel zijn er meer dan 20 verschillende erkende medicijnen voor de behandeling van depressie, maar de helft van de beastanddelen hiervan is al decennia beschikbaar en het grootste deel kan op grond van hun farmacologische werkingsmechanisme worden onderverdeeld in twee groepen. Van deze medicijnen worden momenteel voornamelijk de selectieve serotonine heropname remmers (SSRIs) gebruikt voor de behandeling van depressie. Uitgaande van de werkzaamheid van deze antidepressiva is er een substantiële hoeveelheid klinisch bewijs gevonden dat serotonine (5-HT) bevattende banen in het centraal zenuwstelsel een grote rol spelen in de pathofysiologie van depressie.
Diermodellen voor depressie kunnen nuttig zijn voor een beter begrip van de pathofysiologie van depressie. De olfactoire bulbecomie (OBX) in ratten is een diermodel dat voldoet aan de meeste criteria van een valide model voor depressie en zou als zodanig meer inzicht kunnen verschaffen in de etiologie van depressie en het ontwikkelen van nieuwe antidepressiva.

In **hoofdstuk 1** wordt een overzicht gegeven van de relevante literatuur. Het serotonerg systeem is een uitgebreid neurotransmitter systeem in het centraal zenuwstelsel van zoogdieren. Cellichamen van serotonerge neuronen zijn gelokaliseerd in de raphe kernen en projecteren naar vele hersengebieden, waaronder de olfactoire bulbi. 5-HT medieert een groot aantal fysiologische effecten door interactie met minstens 14 verschillende 5-HT receptor subtypes. 5-HT_{1A} receptoren bevinden zich zowel postsynaptisch van 5-HT neuronen (in de voorhersenen) als op 5-HT neuronen zelf op het soma en de dendrieten in de raphe kernen. Deze laatstgenoemde receptor, die somatodendritische 5-HT_{1A} autoreceptor wordt genoemd, is gericht op het reguleren van 5-HT neuronale activiteit. Activatie van deze receptor door 5-HT verlaagt de vuurfrequentie van 5-HT neuronen en vermindert hiermee de afgifte van 5-HT in het zenuwuiteinde. 5-HT_{1B} receptoren bevinden zich presynaptisch, waar zij 5-HT afgifte reguleren, en postsynaptisch voornamelijk in de globus pallidus, dorsale subiculum en substantia nigra.
Dit hoofdstuk beschrijft tevens de gedragsmatige, neurochemische, endocrine en immunologische veranderingen die zijn gevonden in olfactoire bulbectomie dieren. Een van de meest consistente bevindingen is een verhoogde locomotorische activiteit in een stressvolle, nieuwe omgeving, zoals bijvoorbeeld het 'open veld'. Het is aangetoond dat deze hyperactiviteit niet gerelateerd is aan het verlies van reukvermogen. Macroscopische veranderingen in de cortex, hippocampus, caudatus en amygdala zijn ook gemeld na bulbectomie, evenals neuronale degenaratie en herstructurering. Er zijn afwijkingen in 5-HT niveaus gevonden, waarbij de meeste studies een afname van 5-HT rapporteren. Noradrenaline (NA) en dopamine (DA) zijn ook bestudeerd na bulbectomie, maar met tegenstrijdige

resultaten. Stress- geïnduceerde glucocorticoïden zijn verhoogd, terwijl zowel een toename of geen verandering in basale corticosteron niveaus is gevonden. Immunologische veranderingen in bulbectomie dieren correleren met immunologische veranderingen in depressieve patienten, evenals veel van de andere effecten van olfactoire bulbectomie in ratten. Veel van de veranderingen in gebulbectomiseerde ratten zijn gevoelig voor chronische behandeling met klinisch effectieve antidepressiva.

In **hoofdstuk 2** worden enkele methodologische aspecten van de microdialyse procedure, gebruikt in dit proefschrift, behandeld. Belangrijke punten hierin zijn het maximale interval tussen implantatie van de microdialyse probe en het microdialsye experiment, zonder hierbij 5-HT functioneren te beïnvloeden, en het effect van meerdere experimenten gebruikmakend van dezelfde probe op 5-HT niveaus. Het is aangetoond dat een interval tussen operatie en experiment van 8 dagen het effect van heropname remming met een SSRI niet beïnvloedde wanneer de resultaten werden weergegeven als percentage van de basale niveaus. Het is echter ook gebleken dat de absolute basale niveaus van 5-HT evenals het het absolute effect van SSRI toediening toenamen bij langere intervals. Daardoor was het niet mogelijk absolute waarden en effecten te vergelijken bij verschillende post-operatie intervals. Herhaaldelijk gebruik van dezelfde probe op twee opeenvolgende dagen leverde geen reproduceerbare data op wanneer de data werden weergegeven als absolute getallen; 5-HT niveaus waren lager op de tweede dag. Wanneer echter twee verschillende probes gebruikt werden, waarbij de tweede probe contralateraal aan de eerste geplaatst werd, konden experimenten reproduceerbaar worden uitgevoerd op twee opeenvolgende dagen. Deze data suggereren echter wel dat wanneer gekeken wordt naar relatieve toenames van 5-HT, het wel mogelijk is om meerdere malen door dezelfde probe te perfuseren.

Een afwijkinging in het functionergen van het serotonerg systeem is geïmpliceerd in het onstaan van depressie. Tevens is het aangetoond dat restrictie van de hoeveelheid tryptofaan (TRP) in het dieet van patienten wiens depressie is behandeld met SSRIs een terugval in sommige patienten kan veroorzaken. **Hoofdstuk 3** beschrijft de effecten van chronische tryptofaan toevoeging aan het dieet op het functioneren van het serotonerg systeem. Gebruikmakend van in-vivo microdialyse werd aangetoond dat de extracellulaire hoeveelheid 5-HT in de dorsale hippocampus (DH) en de effecten van de SSRI fluvoxamine op 5-HT afgifte in dit gebied beïnvloed konden worden door het tryptofaan gehalte van het dieet te veranderen. Lokale toediening van de releaser fenfluramine leidde tot een dieet-afhankelijke toename van 5-HT, wat suggereert dat de hoeveelheid 5-HT die beschikbaar is voor afgifte gemoduleerd kan worden door de tryptofaan inname te veranderen. De effecten van de tryptofaan dieten op extracellulaire 5-HT niveaus werden bevestigd door de gedragstesten. De hoeveelheid tryptofaan in het dieet had

een verschillend effect op de prestatie in de DRL-72s test, waarbij het TRP laag dieet een verslechtering in de prestatie liet zien en het TRP hoog dieet een verbetering. Dit laatste effect was vergelijkbaar met het effect van orale toediening van 20 mg/kg fluvoxamine, wat suggereert dat tryptofaan toevoeging aan het dieet het effect van antidepressiva zou kunnen versterken.

Het tweede deel van dit proefschift beschrijft de permanente afwijkingen en compensatoire veranderingen als gevolg van olfactoire bulbectomie. **Hoofdstuk 4** beschrijft een verlaging van de basale niveaus van 5-HT, maar niet van 5-HIAA, NA en DA in zowel de dorsale hippocampus (DH) als de basolaterale amygdala (BLA) twee weken na bulbectomie. Metingen in de DH 5 maanden na de operatie toonden aan dat dit effect langdurig is. Door middel van lokale toediening van fenfluramine en fluvoxamine werd aangetoond dat OBX een verminderde toename in 5-HT niveaus tot gevolg heeft. Toediening van NSD-1015, een decarboxylase remmer, suggereerde dat onder basal condities de synthese snelheid verlaagd is in bulbectomie dieren. Systemische toediening van een hoge dosis L-TRP leidde tot een vergelijkbare toename in 5-HTP accumulatie in SHAM en OBX dieren, wat erop wijst dat de capaciteit van het systeem om 5-HT te synthetiseren niet veranderd is.
Hoofdstuk 5 beschrijft de aanpassingen in het functioneren van het serotonerg systeem als gevolg van mogelijke degeneratie van serotonerge neuronen na verwijdering van de olfactoire bulbi. Verlaagde extracellulaire 5-HT nieveaus in de DH werden bevestigd en tevens aangetoond in de mediane raphe kern (MR). Locale toediening van verschillende concentraties fluvoxamine veroorzaakte een parallele toename in 5-HT gehaltes, waarbij de basale niveaus en de maximale toename kleiner waren in OBX dieren dan in SHAM geopereerde controle dieren. De meest waarschijnlijke verklaring voor de kleinere toename in 5-HT na toediening van een SSRI in OBX dieren is de lagere 5-HT afgifte. Het feit dat de twee dosis-respons curves parallel lopen suggereert dat de gevoeligheid van de 5-HT transporter niet veranderd is. 1 μM CP9129, een selectieve 5-HT_{1B} antagonist, werd in aanwezigheid van fluvoxamine lokaal in de DH toegediend om de functionaliteit van de 5-HT_{1B} receptor te bepalen. Zowel SHAM als OBX dieren lieten een significante verlaging van 5-HT niveaus zien, wat erop wijst dat er in beide gevallen een functionele 5-HT_{1B} receptor aanwezig is. Wanneer de totale afname gerelateerd werd aan de basale 5-HT niveaus, werd er geen verschil in relatief effect gevonden tussen de twee groepen, wat erop duidt dat de gevoeligheid van de 5-HT_{1B} autoreceptor niet is veranderd. Om de effecten van bulbectomie op het functioneren van de somatodendritische 5-Ht_{1A} receptor te bepalen, werd flesinoxan, een 5-HT_{1A} receptor agonist, lokaal in de MR toegediend. SHAM geopereerde dieren lieten een significante verlaging van 5-HT niveaus zien na flesinoxan toediening. Echter, in de OBX groep werd geen significxant effect van flesinoxan gevonden, wat aangeeft dat 5-HT_{1A} receptoren in de MR

gedesensitiseerd zijn in OBX ratten. Systemische toediening van fluvoxamine gaf een vergelijkbare stijging van 5-HT niveaus in SHAM en OBX dieren, ondanks het feit dat de basale extracellulaire niveaus lager zijn na bulbectomie. Wanneer WAY 100.635, een 5-HT_{1A} receptor antagonist, samen met fluvoxamine werd toegediend, namen 5-HT niveaus in SHAM dieren significant toe vergeleken met toediening van alleen fluvoxamine. In OBX dieren werd echter geen extra toename van 5-HT gevonden na gelijktijdige toediening van WAY 100.635, wat de voorgaande bevindingen ondersteunt dat olfactoire bulbectomie leidt tot desensitisatie van de somatodendritische 5-HT_{1A} receptor.

In het laatste deel van dit proefschrift worden de effecten van verhoging van de activiteit van het serotonerg systeem in bulbectomie dieren beschreven. **Hoofdstuk 6** beschrijft het effect van een tryptofaan rijk dieet op bulbectomie dieren. OBX dieren vertoonden hyperactiviteit in het open veld, welke niet verminderd werd na chronische tryptofaan suppletie. Gebruikmakend van microdialyse zijn eerdere bevindingen bevestigd dat bulbectomie leidt tot verlaging van basale 5-HT niveaus in de DH en desensitisatie van de somatodendritische 5-HT_{1A} receptor induceert. Chronische tryptofaan toevoeging deed de basale 5-HT niveaus significant toenemen in zowel SHAM als OBX dieren en verminderde de desensitisatie van 5-HT_{1A} receptoren in OBX dieren. Metingen van het 24-uurs corticosteron ritme lieten geen verschillen zien tussen SHAM en OBX dieren. Met qPCR werd in de amygdala een afname in de expressie van GABA-Aα1 subunit mRNA gevonden en toename in de expressie van MAO-A mRNA. In de hippocampus werd een toename in de expressie van postsynaptische 5-HT_{1A} receptor mRNA gevonden.

De belangrijkste resultaten worden besproken in **hoofdstuk 7**. De grootste neurochemische veranderingen in bulbectomie dieren besproken in dit proefschrift zijn de verlaagde basale extracellulaire 5-HT niveaus samen met een desensitisatie van de somatodendritische 5-HT_{1A} receptor. Chronische tryoptofaan toevoeging aan het dieet verhoogde serotonerge neurotransmissie in gezonde dieren en normaliseerde extracellulaire basale 5-HT niveaus en somatodendritische 5-HT_{1A} receptor functie in bulbectomie dieren. Interessant is dat deze normalisatie van 5-HT niet weerspiegeled werd in het open veld, wat suggereert dat er een dissociatie tussen neurochemische en gedragsmatige veranderingen zou kunnen zijn.
Samenvattend ondersteunt het onderzoek in dit proefschrift de validiteit van de olfactoire bulbectomie rat als een model voor stemmingsstoornissen. Verder onderzoek naar de neurochemische, gedragsmatige, endocriene en immunologische veranderingen, en hun inetractie en correlatie in bulbectomie dieren zou meer inzicht kunnen geven in het ontstaan en de behandeling van depressie.

About the author

Hiske Marije van der Stelt was born on August 21st 1976 in Amsterdam, The Netherlands. In 1994 she graduated from highschool and received her VWO certificate at the Hermann Wesselink College in Amstelveen. From 1994 untill 1999 she studied Medical Biology at Utrecht University. She participated in research projects on transmitter systems and cognitive flexibility at the National Brain Institute, Amsterdam (supervised by Dr. M.G.P. Feenstra and Dr. J.P.C. de Bruin) and on feedback regulation of serotonin neurons at the department of Psychiatry, University Medical Center Utrecht (supervised by Prof. Dr. H.G.M. Westenberg).
The research resulting in this thesis started in June 2000 at this same department and in collaboration with Prof. Dr. B. Olivier at the Department of Psychopharmacology, Utrecht University.

List of publications

Full papers

Van der Stelt HM, Broersen LM, Olivier B, Westenberg HGM (2004) Effects of dietary tryptophan variations on extracellular serotonin in the dorsal hippocampus of rats. *Psychopharmacology* (Berl). 172: 137-144

Van der Stelt HM, Olivier B, Westenberg HGM; Permanent deficits in serotonergic functioning of olfactory bulbectomized rats: an in vivo microdialysis study. Submitted

Van der Stelt HM, Olivier B, Westenberg HGM; Compensatory changes in serotonergic functioning following olfactory bulbectomy in rats: an in vivo microdialysis study. Submitted

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