Effect of pharmacological treatment on serotonergic function in depression

ARAM EL KHOURY

Stockholm 2002
"Om Glädje och Sorg"

Er glädje är er sorg utan mask.
Samma källa, från vilken ert skratt porlar,
har ofta varit fyld med era tårar.
Ju mer sorgen urholkar er varelse, desto mer glädje kan ni rymma.
Är inte den luta, som lugnar er ande,
samma stycke trä som urholkades med knivar?
Då ni är lyckliga, skåda djupt in i era hjärtan och ni skall finna att endast det,
som berett er sorg, nu ger er glädje.
Då ni är sorgsna, blicka åter in i ert hjärta, och ni skall se att ni i verkligheten
gråter över det, som en gång skänkte er glädje.
Tillsammans kommer de, och när den ene sitter ensam med er vid bordet,
kom då ihåg, att den andre sover i er säng.
I sanning, ni är som en våg mellan er sorg och er glädje.
Endast då vågskålarna är tomma, är ni stilla och i jämvikt.
När skattmästaren lyfter er för att väga sitt guld och sitt silver,
måste er glädje eller er sorg, antingen stiga eller falla.
(ur PROFETEN – Khalil Gibran 1923)

To Maria,
Edessa and Simona
ABSTRACT

Since its identification in raphe neurons of the central nervous system (CNS), the biogenic amine serotonin (5-hydroxytryptamin, 5-HT) has been implicated in the etiology and pathophysiology of affective disorders, such as depression. It has been proposed that enhancement of central serotonergic transmission may underlie the therapeutic response to different types of antidepressant drugs. Furthermore, dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis has been documented repeatedly in patients with major depression.

The overall aim of this thesis was to investigate the effects of pharmacological treatment with different agents on central serotonergic mechanisms and indices of endocrine activity in depression.

The 5-HT parameters studied in this work were the active 5-HT uptake, the \[^{3}H\]paroxetine binding to the 5-HT transporter and the \[^{3}H\]LSD binding to 5-HT\(_{2A}\) receptors. Since direct studies of the living human brain are associated with great methodological difficulties, blood platelets, which share several biochemical and pharmacological properties with serotonergic nerve endings, provide useful peripheral model systems in the investigations of certain central serotonergic neuron dynamics. Additionally, plasma levels of cortisol, prolactin and dehydroepiandrosterone (DHEA) were measured in order to assess some aspects of the HPA axis.

Medication-free depressed patients showed significantly lowered platelet 5-HT uptake and elevated plasma cortisol concentrations than healthy controls. However, no differences were noted in the density of 5-HT transporters and 5-HT\(_{2A}\) receptors or in plasma levels of prolactin and DHEA between these two groups. Lowered 5-HT uptake and hypercortisolemia found in depressive illness may reflect a reduced central serotonergic activity during the state of illness or represent a biological marker of vulnerability and postulate hyperactivity of the HPA system. Interestingly, lowered velocity and affinity of platelet 5-HT uptake and higher basal values of plasma cortisol and DHEA were observed in patients who had suffered from multiple depressions compared to patients with first depressive episodes. These differences may be explained by a persisting modulation of the 5-HT uptake, caused by earlier use of antidepressant drugs with 5-HT inhibiting properties or the possibility that repeated depressive illness may cause some alterations in the serotonergic machinery. They may also postulate the existence of an association between a past history of depressive episodes with some alterations in the serotonergic machinery and the function of the HPA system. These changes, which may be caused by repeated depressive illness or earlier antidepressant treatment, provide additional aspects to the complexity of depressive illness. Finally, pharmacological inhibition of central serotonin uptake appears to be an effective antidepressant treatment and alterations of the HPA axis may be in pace with the mood-normalizing effects of antidepressant therapy.

**Key words:** serotonin, depression, platelets, HPA-axis, intracellular calcium, lithium, SSRI, NRI

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# TABLE OF CONTENTS

## ABBREVIATIONS

<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

## PAPERS INCLUDED IN THIS THESIS

<table>
<thead>
<tr>
<th>PAPERS INCLUDED IN THIS THESIS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

## INTRODUCTION

<table>
<thead>
<tr>
<th>INTRODUCTION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

### The serotonergic system

<table>
<thead>
<tr>
<th>The serotonergic system</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>The origin, distribution and function of serotonin</td>
<td>3</td>
</tr>
<tr>
<td>Serotonin receptor subtypes</td>
<td>4</td>
</tr>
<tr>
<td>The serotonin transporter</td>
<td>4</td>
</tr>
</tbody>
</table>

### The hypothalamic-pituitary-adrenal (HPA) axis

<table>
<thead>
<tr>
<th>The hypothalamic-pituitary-adrenal (HPA) axis</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

### Mood disorders

<table>
<thead>
<tr>
<th>Mood disorders</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

### Serotonergic mechanisms in depression

<table>
<thead>
<tr>
<th>Serotonergic mechanisms in depression</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

### Platelets as a peripheral model of central serotonergic neurons

<table>
<thead>
<tr>
<th>Platelets as a peripheral model of central serotonergic neurons</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

### Platelet indices in depressive disorders

<table>
<thead>
<tr>
<th>Platelet indices in depressive disorders</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

#### The 5-HT uptake

<table>
<thead>
<tr>
<th>The 5-HT uptake</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

#### The [H]paroxetine binding

<table>
<thead>
<tr>
<th>The [H]paroxetine binding</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

#### The [H]LSD binding

<table>
<thead>
<tr>
<th>The [H]LSD binding</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

### Calcium homeostasis and mood disorders

<table>
<thead>
<tr>
<th>Calcium homeostasis and mood disorders</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

### Effects of antidepressant treatment on platelet serotonergic indices

<table>
<thead>
<tr>
<th>Effects of antidepressant treatment on platelet serotonergic indices</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
</tr>
</tbody>
</table>

#### Lithium

<table>
<thead>
<tr>
<th>Lithium</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
</tr>
</tbody>
</table>

#### Selective serotonin reuptake inhibitors

<table>
<thead>
<tr>
<th>Selective serotonin reuptake inhibitors</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
</tr>
</tbody>
</table>

#### Selective noradrenaline reuptake inhibitors

<table>
<thead>
<tr>
<th>Selective noradrenaline reuptake inhibitors</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
</tr>
</tbody>
</table>

## AIMS OF THE THESIS

<table>
<thead>
<tr>
<th>AIMS OF THE THESIS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13</td>
</tr>
</tbody>
</table>

## MATERIALS & METHODS

<table>
<thead>
<tr>
<th>MATERIALS &amp; METHODS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
</tbody>
</table>

### Patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
</tbody>
</table>

### Controls

<table>
<thead>
<tr>
<th>Controls</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
</tbody>
</table>

### Ethical consideration

<table>
<thead>
<tr>
<th>Ethical consideration</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
</tbody>
</table>

### Clinical ratings

<table>
<thead>
<tr>
<th>Clinical ratings</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

### Blood sampling

<table>
<thead>
<tr>
<th>Blood sampling</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

### Determination of platelet [H]paroxetine and [H]LSD-binding

<table>
<thead>
<tr>
<th>Determination of platelet [H]paroxetine and [H]LSD-binding</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16</td>
</tr>
</tbody>
</table>

### Measurement of platelet 5-HT uptake

<table>
<thead>
<tr>
<th>Measurement of platelet 5-HT uptake</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

ACTH  adrenocorticotropic hormone
ANOVA  analysis of variance
ATP  adenosine triphosphate
AVP  arginine-vasopressin
BBB  blood-brain barrier
Bmax [3H]LSD  maximum density of serotonin receptors
Bmax [3H]paroxetine  maximum density of serotonin transporters
cAMP  cyclic 3’, 5’ adenosine-monophosphate
CNS  central nervous system
CRH  corticotropin-releasing hormone
CSF  cerebrospinal fluid
DHEA  dehydroepiandrosterone
DSM-IV  Diagnostic and Statistical Manual of Mental Disorders, 4th ed.
ECT  Electroconvulsive therapy
HOST  hypoosmotic shock treatment
HRDS  Hamilton Rating Scale for Depression
i.e.  (lat. Id est) that is
Kd  dissociation constant
Km  Michaelis-Menten constant, affinity constant
L-Trp  tryptophan
MADRS  Montgomery-Asberg Depression Rating Scale
MAO  monoamine oxidase
NA  noradrenaline
NRI  selective noradrenaline reuptake inhibitor
PGE1  prostaglandin E1
PRP  platelet-rich plasma
PTH  parathyroid hormone
RIA  radioimmunoassay
SSRI  selective serotonin reuptake inhibitor
TCA  tricyclic antidepressant
TSH  thyroid-stimulating hormone
Vmax  maximum velocity of serotonin uptake
5-HIAA  5-hydroxyindoleacetic acid
5-HT  5-hydroxytryptamine, serotonin
5-HTP  5-hydroxytryptophan
PAPERS INCLUDED IN THIS THESIS

This thesis is based on the following papers and manuscripts, which are referred to in the text by their Roman numerals I-V:


INTRODUCTION

"En del människor är så imponerade av vad vetenskapen vet, att de glömmer bort vad den inte vet. Andra är så mycket mer intresserade av vad den inte vet, att de förringar dess prestationer."

Bertrand Russel

THE SEROTONERGIC SYSTEM

The origin, distribution and function of serotonin

In 1948, a tonic factor released from platelets during blood clotting was isolated, and purified from serum, and identified as a monoamine named serotonin (5-hydroxytryptamine, 5-HT), due to its origin in serum and its tensing action on smooth muscle, by Rapport and colleagues. A few years later, Twargo and Page (1953) reported that 5-HT was present in the mammalian brain, Amin et al. (1954) demonstrated its widespread distribution within the central nervous system, and Bogdanski et al. (1956) hypothesized that 5-HT might serve as a chemical mediator in brain.

Using fluorescence histochemical techniques, Dahlström and Fuxe (1964) showed that the central serotonergic system has its center in clusters of serotonin-containing cell bodies that are located within the raphe nuclei near the midline of the brain stem. The axons of the serotonergic system project to nearly all parts of the CNS – to the entire neocortex, thalamus, hypothalamus, limbic structures, reticular formation, locus ceruleus, cerebellum and spinal cord (Figure 1) - and this widespread distribution of the terminals gives the system a good basis for acting in a modulatory fashion (Aghajanian et al 1987; Kaplan and Sadock, 1995). The important depots for 5-HT in mammals are the enterochromaffin cells in the gastrointestinal mucosa (about 95% (~10 mg) of total body 5-HT), the brain (about 1-2% of the total 5-HT content is found in the CNS) and platelets (Gershon, 1985; Lambert et al., 1995). Lower levels of 5-HT have also been detected in other tissues, notably in heart, kidney spleen and thyroid (Essman, 1978).

Since 5-HT cannot readily pass the blood-brain barrier (BBB), the serotonergic neurons in the brain must produce their own transmitter. The indoleamine 5-HT is synthesized from the essential amino acid tryptophan (L-Trp), which originates from the diet, via 5-hydroxytryptophan (5-HP) (Figure 2). The rate-limiting step in the 5-HT biosynthesis is the activity of brain tryptophan hydroxylase, but also the availability of L-Trp in brain is an important determinant (Cooper et al. 1991). The biosynthesis of 5-HT occurs in most of the tissues in which it is stored, with the exception for platelets where it has its origin mainly in the gastrointestinal tract. Intracellular 5-HT not
protected by vesicular storage is catabolized by the degradative enzyme monoamine oxidase (MAO) to the main metabolite 5-hydroxyindoleacetic acid (5-HIAA), which is secreted in the urine.

The serotonergic system modulates a wide variety of basic physiological functions such as sleep, appetite, sexual behavior, pain perception and circadian rhythms, but it has also been implicated in the regulation of aggressive behavior, depressive illness, anxiety and panic attacks (Meltzer and Lowy 1987).

**Serotonin receptor subtypes**

The serotonergic system has over a dozen 5-HT receptor subtypes expressed both in the periphery and throughout the CNS. The first 5-HT receptor was described by Gaddum and Picarelli (1957) and all the 5-HT receptors, with the exception of the 5-HT3 receptor that belongs to the family of transmitter-operated ion channels, belong to the G protein-coupled superfamily of neurotransmitter receptors (Hoyer et al, 1994). The 5-HT receptors are largely postsynaptic receptors in the 5-HT system’s terminal field. However, 5-HT1A receptors exist also as autoreceptors on the soma and dendrites of serotonergic neurons. The existence of so many receptor subtypes for a single transmitter permits a great diversity of signaling so that the same neurotransmitter can produce very different effects on different neurons and on different part of the same neuron (Mann, 1999). Not all of the 5-HT receptors have identified physiological roles in the brain (Saudou and Hen, 1994). With respect to mood disorders, the 5-HT1A receptor have been hypothesized to have a role in depression, anxiety and panic disorder (Baldwin and Rudge, 1995) and the 5-HT2A receptor has been proposed in the pathogenesis of major depression, migraine, aggression and suicidal behavior (Mann, 1999).

**The serotonin transporter**

The 5-HT transporter associated with the membrane of the 5-HT neurons is a very efficient system for the re-uptake of the released 5-HT molecules (Marcusson and Ross, 1990). It is a carrier system localized on presynaptic axon terminals and on the cell bodies of serotonergic neurons. The 5-HT transport process is saturable, requires energy, is temperature and sodium (Na+) dependent and is inhibited by specific uptake inhibitors (Ross, 1982).
Figure 1. Serotonergic pathways in the human brain

Figure 2. The serotonergic nerve ending
THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS

The HPA axis is a dynamic, auto-regulating, endocrine system, involving both central (hypothalamus) and peripheral tissues (pituitary and adrenal cortex) and provides a cascade of processes exerting positive and negative control at several levels (Figure 3). By controlling the secretion of stress hormones (glucocorticoids), it functions to restore deviations from homeostasis and contributes to the regulation of energy metabolism on a daily basis (Ganong, 1985).

The hypothalamus releases corticotropin-releasing hormone (CRH), which is transported to the anterior pituitary where it stimulates adrenocorticotropic hormone (ACTH) secretion. ACTH is then released into the general circulation and is carried out to the adrenal cortex, where it stimulates the production and the release of the glucocorticoid cortisol. In addition to cortisol, the adrenal cortex produces several other steroid hormones, such as the mineralcorticoid aldosterone and several estrogens and androgens (Kaplan and Sadock, 1995). Cortisol is the primary stress-response hormone and, directly or through its effects on other hormones, functions in the regulation of carbohydrate, protein, and lipid metabolism, maintains vascular reactivity to catecholamines, and modulates peripheral blood cell counts. Although the underlying neuropathological mechanisms are not yet fully understood, alterations in cortisol levels result in pronounced neuropsychiatric disturbances, presenting mainly as increased concentrations of cortisol in plasma, urine, and the CSF in patients with major depression (Gold et al., 1988; Owens and Nemeroff, 1993; Holsboer and Barden, 1996).

Prolactin, a hormone secreted from the lactotroph cells in the anterior pituitary gland, has also been used as an accessible indirect index of central neurotransmitter function in studies of affective disorder (Nicholas et al., 1998). The neurotransmitters dopamine and 5-HT are considered to play a central role in regulating prolactin release. While dopamine acts as powerful inhibitor, 5-HT has been attributed a stimulatory role in prolactin release. In terms of the pathophysiology of depression, 5-HT is the most relevant molecule in controlling prolactin release. Antidepressant agents have been demonstrated to improve HPA axis in depressive patients and this improvement was associated with a reduction in symptom severity (Plotsky et al., 1998).
Another adrenal steroid of relevance in this context is the adrenocorticotropic hormone (ACTH)-regulated steroid dehydroepiandrosterone (DHEA). It is quantitatively the most abundant circulating steroid in humans and mammals and it possesses anti-glucocorticoid properties (Kalimi et al., 1994). DHEA antagonizes action to certain effects of cortisol in the brain (Kalimi et al., 1994) and the ratio of DHEA to cortisol was found lowered in depressed patients compared to controls (Osran et al., 1993; Goodyer et al., 1998). Furthermore, decreases in DHEA concentrations were associated with improvement in mood and functioning in depressed patients following antidepressant treatment (Fabian et al., 2001).

Figure 3. The HPA axis
MOOD DISORDERS

According to the 4th edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association, 1994), mood disorders are divided in two categories:

1. **Major depressive disorder**, MDD, sometimes called unipolar depression, is the most common mood disorder in the general population, with a lifetime prevalence of 10-25% for women and 5-12% for men. It may manifest as a single episode or more often as recurrent episodes. The majority of patients who recover from an episode will have a recurrence: 60% within 5 years and 75% within 10 years (Montano, 1994)

2. **Bipolar disorder**, previously called manic-depressive disorder, consists of at least one excited manic (bipolar I) or hypomanic episode (bipolar II) and one or more major depressive episodes. Life time prevalence for bipolar I and bipolar II disorders are 0.4-1.6% and 0.5%, respectively.

Criteria for Major Depressive Episode describe disturbances in mood, psychomotor activity, cognitive and vegetative spheres and include the following nine symptoms: depressed mood, diminished interest, significant weight loss, insomnia or hypersomnia, psychomotor agitation, fatigue, feeling of worthlessness, diminished ability to think or concentrate, and suicidal ideation.

SEROTONERGIC MECHANISMS IN DEPRESSION

One major theory of the pathophysiology of depression, “the indoleamine hypothesis” formulated by Coppen (1967) in England and Lapin and Oxenkrug (1969) in Russia, has been in focus for the last thirty years. A large body of evidence has demonstrated that depressive illness may be associated with a deficit in central serotonergic transmission. Reduced levels of 5-HT in whole blood or platelets were found in unipolar depressed or melancholic patients (Coppen et al., 1976; Sarrias et al., 1987). Low concentrations of 5-HIAA, the principal metabolite of brain 5-HT, have been demonstrated in the cerebrospinal fluid (CSF) and in postmortem brain tissue taken from depressed and suicidal patients (Åsberg et al., 1976; Ohmori et al., 1992). Furthermore, numerous studies have shown that antidepressant treatment causes alterations in the reuptake, metabolism, and turnover of 5-HT in the brain (Bertilsson et al. 1980; Linniola et al., 1984, Bjerkenstedt 1985).
PLATELETS AS A PERIPHERAL MODEL OF CENTRAL SEROTONERGIC NEURONS

Human blood platelets are anucleated cells, with a discoid shape approximately 2-3 µm in diameter, derived from bone marrow megakaryocytes by a process of cytoplasmic fragmentation (White and Gerrard, 1982). Platelets play a central role in homeostasis and thrombosis. They also participate more broadly in other body responses to injury, reflecting their evolutionary heritage as inflammatory cells (Weksler, 1992). The main part of 5-HT in the blood is stored in the platelets (20-60 ng per 10^8 platelets).

The human platelet has many morphological, biochemical and pharmacological characteristics similar to those in serotonergic neurons. These similarities include kinetic and other characteristics of cellular processes by which 5-HT is concentrated, stored in the 5-HT storage vesicle, released, and metabolized by MAO. Blood platelets also possess 5-HT_2 receptors which, when activated by 5-HT, produce platelet shape change and partial platelet aggregation (Pletcher et al., 1979; Da Prada et al., 1988). Additionally, the primary structure of the human platelet 5-HT uptake site is identical with the human brain 5-HT transporter (Lesch et al., 1993). The multitude of similarities between the two cell types may be explained by a common origin of platelets and serotonergic neurons from the embryonic ectoderm (Campbell et al., 1981) and could be viewed as a phylogenetic remnant. Due to these similarities and the fact that direct studies of details of serotonergic mechanisms and monoaminergic disturbances in the living human brain are, for obvious reasons, associated with great methodological difficulties, blood platelets has been extensively used as easily available extracerebral model systems in the investigations of some aspects of the central serotonergic neuron dynamics (Lingjærde 1990).

PLATELET INDICES IN DEPRESSIVE DISORDERS

The most intensively studied 5-HT parameters in platelets have been the active 5-HT uptake, the [³H]imipramine and [³H]paroxetine binding and the [³H]LSD binding.

The 5-HT uptake

Kinetic studies of the 5-HT transport into the platelet of depressive patients have generally demonstrated a reduction of the rate of 5-HT transport (V_max), but unaltered affinity of 5-HT for the platelet membrane carrier (K_m) (Tuomisto et al., 1979; Malmgren et al., 1989, Neuger et al., 1999).
The \[^3\text{H}\]paroxetine binding

Investigations concerning the presynaptic serotonin transporter have been performed using tritiated imipramine or paroxetine. It has been suggested that \[^3\text{H}\]paroxetine may be a more selective ligand for labeling the serotonin transporter (Mellerup and Plenge, 1986). The binding is described by two parameters \(B_{\text{max}}\) (the density of binding site) and the dissociation constant \(K_d\). Results from different studies of the density of binding sites for \[^3\text{H}\]paroxetine in platelets in depression have not been as consistent as the 5-HT uptake results showing reduced (Nemeroff et al., 1994), unchanged (Lawrence et al., 1994) as well as increased density (Neuger et al., 1999), whereas the dissociation constant \(K_d\) is normal.

The \[^3\text{H}\]LSD binding

Investigations of postsynaptic 5-HT\(_{2A}\) receptors have most commonly been performed using tritiated LSD. Similarly to the \[^3\text{H}\]paroxetine binding, this binding is described by the two parameters \(B_{\text{max}}\) for the 5-HT\(_{2A}\) receptor density and the dissociation constant \(K_d\). Several studies have reported elevated 5-HT\(_{2A}\) receptor density in blood platelets and in postmortem brain tissue of depressed patients and suicides (Arora and Meltzer, 1989; Pandey et al., 1990; Yates et al., 1990), whereas the dissociation constant \(K_d\) is normal. It has been suggested that upregulation of 5-HT\(_{2A}\) receptors may be associated with aggression and suicidal ideation (Hrdina et al., 1993; Pandey et al., 1995).

Calcium homeostasis and mood disorders

Intracellular ionized calcium (Ca\(^{2+}\)) is involved in the regulation of many neuronal mechanisms such as synthesis and release of neurotransmitters and regulation of receptor mechanisms (Dubovsky et al., 1992; Helmeste and Tang, 1998). Several studies have suggested that abnormalities of calcium metabolism, with alterations of the complex mechanisms of Ca\(^{2+}\) homeostasis resulting in an elevated calcium signal, are associated with mood disorders (Meltzer, 1986; Dubovsky et al., 1991). Investigations of possible mechanisms of action of lithium have, at least in part, been focusing on changes in intracellular calcium dynamics and enhanced serum levels of Ca\(^{2+}\) (hypercalcemia) has been suggested to be associated with lithium treatment (Meltzer, 1986; Manji et al., 1995).
EFFECTS OF ANTIDEPRESSANT TREATMENT ON SEROTONERGIC PARAMETERS

Several lines of preclinical and clinical evidence have shown that enhancement of central serotonergic transmission may underlie the therapeutic response to different types of antidepressant drugs (Blier et al., 1990; Cowen, 1990). One of the aims of the studies outlined in this thesis was to investigate the effects of different antidepressant treatments on serotonergic parameters. The antidepressant treatments selected were the mood stabilizing agent lithium, the three highly selective 5-HT reuptake inhibiting compounds citalopram, paroxetine and sertraline and the highly selective noradrenaline reuptake inhibiting compound reboxetine.

Lithium

Although, the monovalent cation lithium has been introduced in psychiatry for more than 50 years (Cade, 1949) as an agent of therapeutic relevance in the prophylaxis and treatment of affective disorders, its mode of action at the molecular level and the biochemical mechanisms related to its clinical effect remain unclear. Results from animal studies have indicated enhanced brain serotonergic activity (Blier and De Montigny, 1985) and facilitated release of 5-HT from nerve terminals (Treiser et al., 1981) following lithium treatment. Human studies on platelet 5-HT uptake have indicated that lithium therapy enhanced and restored lowered V_max in depressed patients without affecting K_m (Coppen et al., 1980; Meltzer et al., 1983). However, the effect of lithium on platelet 5-HT transporter and postsynaptic 5-HT_{2A} receptors is less consistent.

Selective 5-HT reuptake inhibitors (SSRIs)

Highly selective compounds with inhibition of 5-HT reuptake as the primary action leading to their therapeutic efficacy were introduced into clinical use in the 1980s. SSRIs have a relative lack of affinity for other neurotransmitter receptors, including noradrenaline (alpha1-receptors), acetylcholine (muscarinic receptors) and histamine (H1-receptors). These compounds possess approximately equivalent antidepressant efficacy as the tricyclic drugs (TCAs), but have a much more improved safety and tolerability profile (Fuller, 1992). Within the class of SSRIs, the compounds are very different from one another in chemical structure and vary in their pharmacology and in their pharmacokinetics (Stahl, 1998).
Due to a marked degree of heterogeneity of design and methodology, there are discrepancies between results from studies examining the effect of treatment with SSRIs on platelet serotonergic indices. Animal studies have shown decreased density of 5-HT\textsubscript{2} receptors in the rat brain after chronic treatment with citalopram and paroxetine (Klimek et al., 1994, Maj et al., 1996). Treatment of depressed patients with paroxetine and fluoxetine caused a marked decrease in platelet 5-HT uptake but no alterations in the density of 5-HT-transporters or 5-HT\textsubscript{2A} receptors (Lawrence et al., 1994; Bakish et al., 1997). In panic patients, citalopram treatment caused reduced density ($B_{\text{max}}$) of 5-HT\textsubscript{2A} receptors as well as an increase in the affinity ($K_{\text{m}}$) and a decrease in the rate ($V_{\text{max}}$) for platelet 5-HT uptake (Neuger et al., 2000).

**Selective noradrenaline reuptake inhibitors (NRIs)**

Alterations in central noradrenaline (NA) function have also been implicated in the pathophysiology of affective disorders (Thase and Howland, 1995). Reboxetine is a non-tricyclic antidepressant drug that potently and selectively inhibits NA uptake (Wong et al., 2000) and has no affinity of relevance for the serotonin and dopamine uptake sites or for the muscarinic and adrenergic receptors. In general, NRIs, have been shown to modify certain noradrenergic parameters, but not serotonergic indices (Frazer, 2000).
AIMS OF THE THESIS

The overall aim of this thesis was to investigate the effects of pharmacological treatment with different compounds on certain indices of the serotonergic function in depression. The specific objectives of this work were to:

- investigate activity of the HPA system and the relationships between age and changes in function of the HPA axis in women with bipolar disorder.

- elucidate the effects of long-term lithium administration on central serotonergic mechanisms and intracellular calcium mobilization in patients with major depression.

- elucidate the impact of different pharmacological agents, which selectively inhibit the reuptake of serotonin (SSRI) or noradrenaline (NRI), on central serotonergic function and indices of endocrine activity in major depression.
MATERIALS & METHODS

“En neurutiker är en man som bygger luftslott. 
En psykotisk människa bor i det, 
Psykiatern är den som tar emot hyran.”
Jerome Lawrence

PATIENTS

Outpatients in the depressed phase of their illness were recruited from the Mood Disorder Clinics at the Department of Psychiatric Clinics at St. Göran’s Hospital and Danderyd’s Hospital, Stockholm, and the University Hospitals at Lund and Umeå. To be included, patients should not have received any psychotropic drug (with the exception of benzodiazepines) during the last eight weeks preceding the study. Past history of suicide attempts or current high suicide risk, physical illness such as heart, kidney or liver disease and signs and symptoms of drug abuse, neurological, or organic mental disorder were exclusion criteria.

The euthymic lithium treated patients were recruited from patients with recurring mood disorders treated at the Mood Disorder Clinic of Psychiatry, St. Göran’s Hospital according to specific inclusion criteria, such as being in remission for at least one year and not receiving any additional psychotropic drug or synthetic thyroid hormone agents.

CONTROLS

The control subjects were healthy volunteers, recruited from the hospital staff and their acquaintances, without heredity for affective disorders or history of drug and alcohol abuse, matched for sex, age and season. The physical and mental health of the volunteers was judged by their self-assessment and that of the examining physician, in addition to the evidence of physical examination and psychiatric review. Exclusion criteria were somatic illness and a family history of mental disorders.

ETHICAL CONSIDERATIONS

The studies were approved by the Research Ethics Committee at Karolinska Hospital and were described both orally and in writing to all subjects. Written informed consent was obtained from the patients prior to their inclusion in the studies.
**CLINICAL RATINGS**

The diagnoses were established according to DSM IV criteria for Major Depressive Disorder (American Psychiatric Association, 1994). The severity of depression in Paper I-IV was assessed using the 10-item Montgomery-Asberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979) (maximum 60 points) alone. In Paper V, patients were also rated on the 21-item Hamilton Rating Scale for Depression (HRDS) (Hamilton, 1960). In the treatment studies (Paper I and V) a reduction of at least 50% on the MADRS and HRDS total score versus baseline was considered an index of response to antidepressant treatment.

**BLOOD SAMPLING**

The subjects remained in a supine position for 30 min before blood samplings. Fasting blood was drawn from an antecubital vein early in the morning (between 07.00 and 9.00) and used as follows:

- 60 ml of blood was used for determination of $[^3H]$paroxetine and $[^3H]$LSD-binding to platelet membranes (Paper I, II and V).
- 40 ml of blood was used for measurement of platelet $[^{14}C]$5-HT uptake (Paper I and II).
- 30 ml for determination of basal intracellular calcium in resting platelets and thrombin-induced ionized calcium in aequorin-loaded platelets (Paper III).
- 20 ml for measurement of cAMP-levels after PGE$_1$-stimulation (Paper III).
- 20 ml for hormone measures in plasma (Paper I-V).
- 10 ml for determination of platelet 5-HT$_2$ and alpha$_2$-receptor function in whole blood, by measurement of 5-HT and NA potentiated collagen-induced platelet secretion of ATP (Paper II).

Additional analyses were measurement of plasma drug concentrations (Paper I-V) and determination of plasma TSH, PTH, amino acids and electrolytes (Paper III).
DETERMINATION OF PLATELET [3H]PAROXETINE AND [3H]LSD-BINDING
Platelet serotonin transporters and 5-HT2A receptor binding were studied using the ligands [3H]paroxetine and [3H]LSD, respectively. The method for preparation of platelet membranes and analysis of [3H]paroxetine and [3H]LSD-binding has been described in detail in study II.

Specific binding for [3H]paroxetine and [3H]LSD was defined as the difference between total binding and binding in the presence of clomipramine and spiperone, respectively. Each determination was performed in duplicate. The kinetic parameters Bmax and Kd of [3H]paroxetine and [3H]LSD were derived from computerized Scatchard plots with linear regression analysis by the method of least squares. The protein concentration was determined using the method described by Smith et al. (1985).

MEASUREMENT OF PLATELET 5-HT UPTAKE
The method for determination of platelet 5-HT uptake, determined - within one hour after blood sampling - in undiluted platelet-rich-plasma (PRP) at 37 °C, is described in detail in study II. The net uptake was calculated after subtraction of passive uptake, assessed in samples incubated with 10 µM imipramine as an inhibitor of the saturable transport, from total 5-HT uptake, determined using 5 concentrations in duplicate of [14C]5-HT ranging from 0.1 to 5 µM. The kinetic parameters Vmax and Km were calculated from computerized individual Eadie-Hofstee plots (Zivin and Waud, 1982).

DETERMINATION OF BASAL INTRACELLULAR CALCIUM IN RESTING PLATELETS AND THROMBIN-INDUCED IONIZED CALCIUM IN AEOQUORIN-LOADED PLATELETS
The method for platelet membrane preparation and loading of the Ca²⁺-sensitive photoprotein aequorin using the HOST-technique to study platelet intracellular calcium is described in detail in study III. As an agonist, thrombin was used in duplicates. The loss in luminescence activity of intracellular aequorin was indicated photometrically by measurement of the peak light signal. [Ca²⁺], in resting platelets was determined as the difference between baseline tracings evoked by 1 ml of calcium-containing buffer with or without aequorin-loaded platelets. Calibration of the light signals and calculations of results were done as described by Malmgren et al. (1992).
MEASUREMENT OF CAMP-LEVELS AFTER PGE1-STIMULATION
The method for platelet membrane preparation and measurement of cAMP activity in PRP stimulated with prostaglandin E1 (PGE1) is described in detail in study III. Labeled cAMP ([8-3H]ATP) was used and quantitative analyses of basal and stimulated total counts were determined by counting the radioactivity in duplicates of tritated cAMP in a liquid scintillator.

HORMONE MEASURES IN PLASMA
Plasma levels of cortisol were measured in fasting morning blood, sampled at 10-min. intervals (0, 10 and 20 min) using the radioimmunoassay (RIA) technique. The mean level of cortisol was calculated from the three values obtained from each subject. Plasma levels of prolactin and DHEA were assayed separately in duplicate using validated immunoradiometric kits.

WHOLE BLOOD AGGREGATION
The in vitro method for determination of 5-HT and NA potentiated collagen induced platelet secretion of ATP in whole blood at 37 °C, as a measure for platelet 5-HT2- and alpha2-receptor function, is described in detail in study II. Human platelets respond to 5-HT with a shape change and only a weak, reversible aggregation (Baumgartner and Born, 1968). However, 5-HT largely amplifies the human platelet aggregation response to various agonists, including collagen, thrombin, ADP, and NA. The induced second and irreversible wave of aggregation results in intragranular product release of measurable ATP (De Clerck, 1984). Quantification of secreted ATP was carried out photometrically by measurements of the light signal from a known quantity of ATP added to a similarly diluted whole blood sample and determination of the ratio between sample and standard reflections.
STATISTICAL METHODS

Statistic analyses were performed using the StatView 4.0 software ('92 edition, SAS Institute Inc., Cary, NC, USA) in study I-III and V and the Statistica 5.5 software ('00 edition, StatSoft, Inc., Tulsa, USA) in study IV. Parametric statistics were used for the majority of data, but in small samples or in case of absence of a normal distribution, non-parametric statistics were used. The significance of differences in group means at baseline and after treatment or between group means was determined using Student’s t-test (one or two tailed) for dependent and independent variables, Mann-Whitney’s non-parametric tests for paired and grouped data, or factorial ANOVA when applicable. Correlations were evaluated using simple and multiple regression analyses of variance (ANOVA). P-values were corrected according to Bonferroni for multiple comparison procedures and by Fisher’s PLSD post hoc test when implicated.

All values are presented as means ± SD and in all statistics p<0.05 was considered statistically significant.
RESULTS AND DISCUSSION

"Man gör alltid bäst i att uttrycka sig rakt på sak, utan att vilja bevisa för mycket. Ty alla bevis, som vi frambringar, är endast variationer av våra meningar, och de som menar annorlunda, bör varken på det ena eller det andra.”

Johann Wolfgang von Goethe

SEROTONERGIC FUNCTION IN MAJOR DEPRESSION (Paper I and V)

Reduced platelet serotonergic function has been a very consistent finding related to the pathophysiology of depressive illness. With respect to platelet presynaptic 5-HT indices at baseline in patients and healthy controls, our finding of lowered velocity for platelet serotonin uptake (V_max) in depressed patients is in conformity with most published investigations (Tuomisto et al., 1979; Arora and Meltzer, 1988; Neuger et al., 1999). Possible interpretation from this finding is that a lowered 5-HT uptake may reflect a reduced central serotonergic activity during the state of illness or represent a biological marker of vulnerability.

Furthermore, consistent with previous literature (Lawrence et al., 1993, D’Hondt et al., 1994; Iy et al., 1994; Nankai et al., 1994, Rosel et al. 1997), no significant variations were apparent between depressed patients and healthy controls in our investigations of the two measures of platelet [3H]paroxetine binding sites: the serotonin transporter density (B_max) and the equilibrium dissociation constant (K_d). These sites on blood platelets have analogous properties to the substrate recognition sites for 5-HT uptake found in the brain (Habert et al., 1986) and may thus serve as peripheral models for central 5-HT function. Our studies provide no support for the findings of reduced number of platelet 5-HT uptake sites in depression by some investigators (Nemeroff et al., 1994; Sallee et al., 1998).

A role for the 5-HT_{2A} receptor has been proposed in the pathogenesis of major depression. In conformity with McBride et al. (1994), but in conflict with the findings of other investigators (Arora and Meltzer, 1989; Pandey et al., 1990; Hrdina et al., 1995) no differences were noted in 5-HT_{2A} receptor density or equilibrium dissociation constant values (i.e. B_max and K_d of platelet [3H]LSD-binding) between depressed patients and healthy controls. Some studies have reported higher levels of 5-HT_{2A} receptors in the brain of suicide victims and on the platelet of depressed individuals who have attempted suicide (see Mann 1998 for a review). Therefore, a possible explanation...
close at hand to why we were unable to demonstrate a difference in 5-HT₂A receptor density is that patients with a past history of suicide attempts or current high risk by Investigator judgment were excluded from our studies.

THE HPA SYSTEM IN MAJOR DEPRESSION (Paper IV and V)

Disturbances in the hypothalamic-pituitary-adrenal (HPA) system have been extensively studied in the investigation of the biological origin of affective disorders. Our finding of elevated plasma cortisol concentrations in medication-free depressed patients in comparison with healthy control individuals (study IV) confirms results in earlier investigations (Pfohl et al., 1985; Linkowski et al., 1985; Heuser, 1998). Hypercortisolemia found in depressive illness postulate in its turn hyperactivity of the HPA system. The hypercortisolaemic depressed patient is able to sustain an increased secretion of corticosteroid hormones due to an increased central drive either mediated by hypothalamic corticotropin-releasing hormone (CRH) and/or by arginine-vasopressin (AVP) (Young et al., 1994) and an impaired negative feedback control of the HPA axis (Carroll et al., 1981).

Due to the wide inter-individual and intra-individual variability of basal prolactin level (Cohen, 1983) and time effect on circadian prolactin release, studies of basal prolactin levels have revealed no consistent changes associated with affective disorder. In agreement with other reports (Rubin et al., 1989; Jarrett et al., 1987; Kjellman et al., 1985), our data showed that plasma prolactin levels in non-medicated depressed patients were not different from those in healthy individuals.

Several studies have found decreased levels of the ACTH-regulated steroid DHEA(S) or decreased ratios of cortisol to DHEA(S) in depressed patients (Legrain et al., 1995; Goodyer et al., 1998). In study V, we were unfortunately unable to investigate mean plasma DHEA levels in the total control group, since we lack of data from male subjects due to technical mishap and could therefore not compare data from patients and controls.
EFFECT OF EARLIER DEPRESSIVE EPISODES ON SEROTONERGIC FUNCTION AND HORMONE MEASURES (Paper I and V)

When data in study I and V were split up according to depressive episodes, we found some interesting differences in basal values of serotonergic indices and the hormone measures.

In study I, lowered $V_{\text{max}}$ and increased $K_m$ for platelet 5-HT uptake were observed in patients who had suffered from multiple depressions compared to patients who suffered from their first depression. The lowered velocity and affinity of the serotonin uptake in this group of patients may be explained by a persisting modulation of the 5-HT uptake caused by earlier use of antidepressant drugs with 5-HT inhibiting properties or the possibility that repeated depressive illness may cause some alterations in the serotonergic machinery.

Furthermore, an interesting observation in study V was the difference in basal values of plasma cortisol and DHEA between patients who had suffered from their first depression and patients who had had multiple depressive episodes. The higher values of these two measures in the latter group indicate a greater disturbance of HPA axis in patients with multiple episodes of illness and may postulate the existence of an association between a past history of depressive episodes with dysregulation of the HPA system.

EFFECT OF TREATMENT ON SEROTONERGIC FUNCTION (Paper I, II and V)

Long-term lithium treatment

The finding in study II of a correlation between plasma lithium and the kinetic variable for the 5-HT uptake velocity ($V_{\text{max}}$) suggests that lithium has a net ameliorating impact on the 5-HT uptake and implies that when plasma lithium is kept at a steady state at recommended plasma levels (0.50 – 1.20 mM) any changes in the 5-HT uptake may be counteracted by lithium. The finding is in line with an earlier investigation by Malmgren et al. (1991) who found that the seasonal rhythm in platelet 5-HT uptake was extinguished suggesting that lithium may stabilize the uptake and render it resistant to change.
Another interesting observation in the same study was the increased number of platelet 5-HT2 receptors with lithium treatment. These data suggest that the ability of lithium to stimulate an increase in the density of 5-HT2 receptors, may be particularly relevant to its mode of action in order to affect the 5-HT neurotransmission. Furthermore, concerning the functional activity of the 5-HT2 receptor system as assayed using platelet aggregation techniques, our findings indicate that lithium-treated patients show a significant greater response when compared with controls and suggest a modulation in the sensitivity of platelet 5-HT2 receptors in patients undergoing prophylactic lithium treatment. However, no effects on the density and affinity of [3H]paroxetine binding sites was detected. Our results are in line with other investigators (Plenge et al., 1992) indicating that long-term lithium treatment does not produce changes in the 5-HT transporter density.

**Antidepressant treatment with SSRIs**

Treatment with paroxetine and sertraline (study I) caused a significant decrease in the affinity but a marked suppression of the velocity of the 5-HT uptake. Pharmacological inhibition of central 5-HT uptake is an effective antidepressant treatment and this finding, in agreement with other reports (Bakish et al., 1997; Neuger et al., 2000), is most likely explained by the known inhibitory effects of SSRIs on 5-HT reuptake. The dual effect on $K_m$ and $V_{max}$ indicates that both compounds act as noncompetitive inhibitors of the 5-HT uptake.

Results from our studies (I and V) examining the effect of antidepressive therapy on platelet serotonin transporters and serotonin receptors have been ambiguous. In disagreement with Arora and Meltzer (1988) and Wägner et al. (1990), but confirming previous reports (Bakish et al., 1997; Neuger et al., 2000) 5-HT transporter density was not altered following treatment with paroxetine, sertraline or citalopram. This could be explained by the use of the more selective ligand [3H]paroxetine in our studies rather than [3H]imipramine. In the case of $K_d$, consistent with other studies (Lawrence et al., 1994; Bakish et al., 1997) paroxetine caused an increase, while no alterations were detected following treatment with either sertraline or citalopram. However, since these data were derived from patients who all responded to treatment, the differences in effect of the SSRIs may only be academic and hence without clinical relevance. The finding in study I of an interaction between the drug effect on $K_d$ and earlier depression suggests
that the differences may be due to antidepressant drugs or drug metabolites remaining in the platelet membrane preparations from earlier treatment.

In conformity with the results by Hrdina et al. (1997), treatment with paroxetine, sertraline or citalopram was not associated with any changes in the density and affinity of 5-HT$_{2A}$ receptors in platelets. Modulation of postsynaptic serotonergic functions, such as a reduction in platelet 5-HT$_{2A}$-receptor density and affinity, following 6 months of citalopram treatment was shown to have an anxiolytic effect and an influence on mood in patients with panic disorders (Neuger et al., 2000). However, since neither pre- nor post-treatment platelet density and affinity of 5-HT$_{2A}$ receptors appeared to relate to clinical response to treatment, our results indicate that treatment with SSRIs does not impact upon these specific aspects of 5-HT function in major depression.

**Antidepressant treatment with NRIs**

Treatment with the selective noradrenaline reuptake inhibitor reboxetine (study V) was not associated with any changes in the density and affinity of 5-HT transporter or 5-HT$_{2A}$ receptors in platelets. Since these data were derived from patients who all responded to treatment, our results indicate that effects on these aspects of the serotonergic system may not in fact be relevant to the antidepressant activity of reboxetine.

**Effect of treatment on the HPA system**

In study IV and V, we found in line with previous reports (Greden et al., 1983; Holsboer and Barden, 1996; Plotsky et al., 1998) that long-term medication with the mood stabilizing agent lithium as well as short-term citalopram treatment and 6 months of treatment with either citalopram or reboxetine caused a significant decrease in plasma cortisol concentration. Since these changes occurred in association with the improvement of the patient’s clinical state, it is tempting to postulate that changes of the HPA-axis in depressive illness may be in pace with the mood-normalizing effects of antidepressant treatment.

The finding in study IV of lowered plasma prolactin levels in long-term lithium treated patients is consistent with the results of Bastürk et al. (2001) but in conflict with those
of Lang Nielsen et al. (1977). Our finding may be explained by the net inhibitory impact of lithium’s augmentative effects on dopaminergic activity (since the major control of prolactin release is via the inhibitory effects of dopamine) and serotonergic neurotransmission (due to the role of the 5-HT_{1A} and 5-HT_{2} family of receptors in prolactin release) in the central nervous system (Wood and Goodwin, 1987; Hamon et al., 1990; Cowen, 1992).

In accordance with Fabian et al. (2001) and Takebayashi et al. (1998) a significant decrease in plasma DHEA levels in depressed patients was found following treatment with citalopram and reboxetine (study V). Mechanisms of decreased plasma DHEA following treatment in depressed patients could be partly explained by the action of DHEA as an antagonist to glucocorticoids (Wolf and Kirschbaum, 1999; Wolkowitz et al., 1997). However, in conflict with other investigators (Goodyer et al., 1998; Scott et al., 1999), we failed to demonstrate alterations in the cortisol/DHEA ratio following antidepressant treatment. Since a decrease in both plasma cortisol and DHEA was detected, it appears as changes in DHEA may be parallel to those in cortisol.

**Effect of Lithium Treatment on Intracellular Calcium Mobilization**

In study III, total serum calcium and ionized serum calcium levels were higher in female bipolar patients on long-term lithium treatment than in controls and there was a significant correlation between these two measures. In the patient group, serum lithium concentration correlated positively with stimulated levels of intracellular calcium in platelets. These findings indicate that lithium administration affects calcium metabolism in patients with bipolar affective disorder inducing mild hypercalcemia and a dose dependent normalization of calcium mobilization.
CONCLUSIONS

"Innan man är riktigt säker på en sak, är man gärna tvärsäker.”
Arne Hirdman

- The lowered $V_{\text{max}}$ for platelet 5-HT uptake found in depressive patients proposes that depression is associated with a disturbance in central serotonergic activity. The lowered 5-HT uptake may represent a biological marker of serotonergic vulnerability during the state of illness.

- Earlier depressive episodes exert an impact on the serotonergic activity in depressive illness due to the possibility that repeated depressive illness may cause some alterations in the 5-HT machinery or a persisting modulation of the 5-HT uptake caused by earlier use of antidepressant drugs with 5-HT inhibiting properties.

- Pharmacological inhibition of central 5-HT uptake is an effective antidepressant treatment.
  a) The mood stabilizing effect of lithium may be attained by a net ameliorating impact on the 5-HT uptake and a dual influence on postsynaptic serotonergic structures.

  b) The dual effects of sertraline and paroxetine on $K_{\text{m}}$ and $V_{\text{max}}$ indicate that these compounds act as noncompetitive inhibitors of the 5-HT uptake. However, the differences in the impact on the 5-HT transporter may only be academic but could also have some clinical relevance since these compounds at comparable doses display differences in safety and tolerability (i.e. adverse effect profile).

- Elevated morning plasma cortisol concentrations in depressed patients postulates hyperactivity of the HPA system in depressive illness. Alterations of the HPA-axis may be simultaneous with the mood-normalizing effects of antidepressant treatment.

- Lithium administration affects calcium metabolism in patients with bipolar disorder inducing mild hypercalcemia and a dose dependent normalized calcium mobilization.
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