Transcription Factor AP-2 in Relation to Personality and Antidepressant Drugs

BY

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Abstract

The CNS monoaminergic systems are considered as the head engine regulating neuropsychiatric functions and personality. Transcription factor AP-2 is known to be essential for the development of the brainstem including the monoaminergic nuclei, and has the ability to regulate many genes in the monoaminergic systems. The ability of transcription factors to regulate specific gene expression, has lately made them hot candidates as drug targets. In this thesis, results indicating a role of AP-2 in the molecular effects of the antidepressant drugs citalopram and phenelzine, are presented.

A polymorphism in the second intron of the gene encoding AP-2β has previously been associated with anxiety-related personality traits as estimated by the Karolinska Scales of Personality (KSP). In this thesis, results confirming this association, gained by using a larger material and several different personality scales, are presented. Furthermore, data is presented showing an association between the activity of platelet monoamine oxidase, a trait-dependent marker for personality, and the genotype of the AP-2β intron 2 polymorphism.

The functional importance of the AP-2β intron 2 polymorphism has not yet been elucidated. Included in this thesis are results showing that the AP-2β intron 2 polymorphism is not in linkage disequilibrium with the only other described polymorphism in the AP-2β gene, i.e. in the AP-2β promoter (-67 G/A). Introns have in several studies been shown to include binding sites for regulatory proteins, and thus, to be important in transcriptional regulation. Results are presented demonstrating that one human brain nuclear protein binds only to the long variant of the AP-2β intron 2 polymorphism. If this protein is involved in the regulation of the AP-2β gene, it would affect the expression levels of the AP-2β protein.

In general, this thesis further establishes the role of transcription factor AP-2 as a regulatory factor of importance for personality and monoaminergic functions.

Keywords: Transcription factors, serotonin, AP-2, antidepressants, CNS, personality

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Till mamma och pappa
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


II. **Berggard C**, Damberg M, Oreland L. Brainstem levels of transcription factor AP-2 in rat are changed after treatment with phenelzine, but not with citalopram. Submitted to *BMC Pharmacology, BioMedCentral*, 2004.


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<th>Full Form</th>
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<tr>
<td>AADC</td>
<td>Aromatic amino acid decarboxylase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-2</td>
<td>Activating Protein-2</td>
</tr>
<tr>
<td>ATP</td>
<td>2’-Deoxyadenosine 5’-triphosphate</td>
</tr>
<tr>
<td>β-PEA</td>
<td>2-Phenylethylamine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CTP</td>
<td>2’-Deoxycytidine 5’-triphosphate</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DβH</td>
<td>Dopamine β-hydroxylase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOPA</td>
<td>Dihydroxyphenylalanine</td>
</tr>
<tr>
<td>DOPAC</td>
<td>Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic Mobility Shift Assay</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavine adenine nucleotide</td>
</tr>
<tr>
<td>GTP</td>
<td>2’-Deoxyguanosine 5’-triphosphate</td>
</tr>
<tr>
<td>HeLa</td>
<td>Immortal Henrietta Lack’s cells</td>
</tr>
<tr>
<td>HP5i</td>
<td>Health-Relevant 5- Factor Personality Inventory</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-Hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>HPA-axis</td>
<td>Hypothalamic-pituitary-adrenal-axis</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanilllic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine, serotonin</td>
</tr>
<tr>
<td>5-HTT</td>
<td>5-Hydroxytryptamine transporter</td>
</tr>
<tr>
<td>5-HTP</td>
<td>5-Hydroxytryptophan</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>KSP</td>
<td>Karolinska Scales of Personality</td>
</tr>
<tr>
<td>LC</td>
<td>locus coeruleus</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
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<tr>
<td>MAO-I</td>
<td>Monoamine oxidase inhibitor</td>
</tr>
<tr>
<td>MHPG</td>
<td>3-methoxy-4-hydroxyphenylglycol</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NEO-PI-R</td>
<td>Revised NEO Personality Inventory</td>
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<tr>
<td>NRI</td>
<td>Noradrenaline reuptake inhibitor</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pc</td>
<td>Post coitum</td>
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<tr>
<td>PLSD</td>
<td>Fisher’s Protected Least Significant Difference</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneously</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package Software System</td>
</tr>
<tr>
<td>SSP</td>
<td>Swedish Universities Scales of Personality</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
</tr>
<tr>
<td>TCI</td>
<td>Temperament and Character Inventory</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TTP</td>
<td>2’-Deoxythymidine 5’-triphosphate</td>
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1. INTRODUCTION

1.1 The CNS serotonergic system

1.1.1 The serotonergic cell nuclei and their projections

A cornerstone in the area of neuropsychopharmacology is the ability of CNS monoamines, i.e., serotonin (5-hydroxytryptamine, 5-HT), noradrenaline (NA) and dopamine (DA), to modulate neuropsychiatric functions, such as personality and mental states. A head player among the monoamines is in this aspect the neurotransmitter serotonin, which system is widely distributed throughout the brain. The cell bodies of the serotonergic neurons are located in clusters along the midline of the brainstem, from the midbrain to the medulla. These clusters are referred to as the raphe nuclei. The rostrally located nuclei project, via the medial forebrain bundle, to many parts of the cortex, hippocampus, basal ganglia, hypothalamus and the limbic system. The caudally located nuclei project to the cerebellum, medulla and spinal cord (Figure 1). The 5-HT neurons are highly bifurcated, which indicates that they have the possibility to influence several brain regions simultaneously (Lucki, 1998).
1.1.2 The serotonin synapse- serotonin metabolism and the 5-HT receptors

Serotonin is derived from the amino acid tryptophan, which in the neurons is assimilated from the diet via the blood. Tryptophan is converted to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase. The 5-hydroxytryptophan is then converted by the enzyme L aromatic acid decarboxylase to 5-hydroxytryptamine (5-HT), serotonin. The factors mainly regulating the synthesis of serotonin are thought to be the availability of tryptophan and the activity of the tryptophan hydroxylase enzyme.

Serotonergic neurotransmission is regulated in a complex manner, involving multiple serotonergic receptor sub-types, both in the nerve terminals and in the cell bodies. Today, more than fourteen 5-HT receptors have been identified (Lesch, 2001). The CNS 5-HT₂ and the 5-HT₃ receptors are excitatory, while the CNS 5-HT₁ receptors mediate an
inhibitory effect. For instance, the presynaptic 5-HT\textsubscript{1A} receptors mediate negative feedback inhibition of the release of serotonin from the cell bodies. The receptors are all G-protein coupled, except for the 5-HT\textsubscript{3} receptors, which are ligand-gated ion channels. The serotonin transporter, 5-HTT, is responsible for the reuptake of serotonin from the synaptic cleft.

Serotonin is metabolized by monoamine oxidase (MAO, further described in section 1.6) to 5-hydroxyindolacetaldehyde, which is then converted by aldehyde dehydrogenase to 5-hydroxyindolacetic acid (5-HIAA) that is excreted in the urine.

1.1.3 The serotonergic system and behavior

Functions associated with 5-HT pathways include control of mood and emotion, control of sleep/wakefulness, control of sensory pathways including nociception, control of body temperature, sexual behavior, appetite, vomiting and several behavioral responses. Variability in serotonergic gene expression has been shown to influence complex behavior like anxiety and aggression (Lesch and Heils, 2000, Lesch et al., 2003) and a serotonergic component has been associated with several anxiety disorders like obsessive compulsive disorder, panic disorder, social phobia and generalized anxiety disorder (McDonough and Kennedy, 2002; Kent et al., 1998; Furmark et al., 2004). Also eating disorders, depression and premenstrual dysphoria are likely to include a serotonergic component (Brewerton, 1995; Lucki, 1998; Eriksson et al., 2002). Alterations in 5-HT function have also been associated with several impulsiveness-related disorders like substance and alcohol abuse, gambling and attention-deficit disorder (Lucki, 1998).

Low concentration of CSF 5-HIAA has in several studies been observed in depressed patients, but subsequent studies have shown that this association occurs preferentially in patients with a history of suicidal behavior (for a review see Brown and Goodwin, 1986; Tuinier et al., 1995; Asberg, 1997). Since low CSF 5-HIAA also has been associated with suicidal behavior in patients with alcoholism, adjustment disorder and schizophrenia (see Brown and Goodwin, 1986), this indicates that it is more likely that the low CSF 5-HIAA levels seen in depressed patients are associated with suicidal behavior per se, rather than
with the depressive state (see Asberg, 1997). Asberg, 1997 discusses the possibility that the association between suicide and serotonin may partly be explained by aggression dyscontrol.

The serotonin system is clearly involved in many behavioral functions, but it is important to notice that it is unlikely that a single neurotransmitter alone is responsible for complex behavioral processes. Since the monoaminergic systems and other neurotransmitter systems are closely interacting, activation of one system affects the others in specific complex patterns resulting in different behavioral effects.

1.2 The CNS noradrenergic and dopaminergic systems

The noradrenergic cell bodies are located mainly in clusters in the pons and medulla. One of these clusters, the locus coeruleus (LC), is responsible for the majority of the noradrenaline released in the brain. The cell bodies project to several parts of the brain, including the cerebral cortex, hippocampus, limbic system, hypothalamus and the cerebellum. The noradrenergic transmission is mainly important for wakefulness and alertness (“arousal”) and for regulation of blood pressure.

There are two main dopaminergic pathways. One of these is the nigrostriatal pathway, which projects from cell bodies in the substantia nigra to the corpus striatum and which is important for motor control. The other is the mesolimbic/mesocortical pathway, which projects from cell bodies in the midbrain to the nucleus accumbens in the limbic system and to the cortex. The latter pathway is involved in emotion and reward systems.

Both noradrenaline and dopamine are synthesized from the amino acid tyrosine. Tyrosine is converted by the enzyme tyrosine hydroxylase to dihydroxyphenylalanine (DOPA), which then is converted by the enzyme DOPA decarboxylase to dopamine. The enzyme dopamine β-hydroxylase (DβH) then converts dopamine to noradrenaline. Degradation of noradrenaline and dopamine is performed by the enzymes MAO (further described at section 1.6) and catechol-O-methyltransferase (COMT). The main metabolite of
noradrenaline is 3-methoxy-4-hydroxyphenylglycol (MHPG) and of dopamine homovanillic acid (HVA). These are excreted in the urine.

The noradrenergic and dopaminergic receptors are all G-protein coupled. The two major subtypes of noradrenergic receptors are the $\alpha$-adrenoceptors ($\alpha_1$, which activates PLC and $\alpha_2$, which decreases cAMP) and the $\beta$-adrenoceptors ($\beta_1$, $\beta_2$, $\beta_3$, which all increase cAMP). The dopaminergic receptors consist of two main families; the D1 receptors (D1, D5), which increase cAMP and the D2 (D2, D3, D4) receptors, which decrease cAMP and/or increase IP3.

Both the noradrenergic and dopaminergic systems are likely to be involved in the expression of personality. However, what particular personality traits that are influenced are not entirely evaluated.

1.3 Transcription factors

1.3.1 What is a transcription factor?

In the transcriptional process DNA is copied to mRNA by a transcriptional machinery consisting of RNA polymerase and DNA binding proteins (transcription factors). There are two main classes of transcription factors, the general and the gene-specific transcription factors. The general transcription factors are common factors for all genes. Together with RNA polymerase these form the general transcriptional machinery (Figure 1) that is required for initiating the transcriptional process. The action of the general transcriptional machinery results in a low level background activity of transcription (basal transcription). The gene-specific transcription factors bind to upstream specific binding sites and cause bending of the DNA in such a way that the gene specific transcription factors can interact with the general transcriptional machinery, thereby activating or inhibiting the basal transcription (Figure 1). The gene-specific transcription factors often act in clusters, involving several different transcription factors and transcriptional co-factors. The action of the gene-specific transcription factors results in a high level of transcriptional activity. Since different
genes include different binding sites for such transcription factors together with the fact that these transcription factors and co-factors often are tissue-specific, one can get a highly specific expression of different genes in different tissues or cell-types.

When discussing transcription factors in general, one usually refers to the gene-specific transcription factors, this is also the case during the rest of this thesis.

Figure 2. A promoter region of a gene with gene-specific transcription factors interacting with the general transcriptional machinery (picture from Alberts et al., Molecular biology of the cell, third edition, 1994).

1.3.2 Transcription factors as potential drug targets

Transcription factors have increasingly been found to play vital roles in the development of many human pathophysiology, such as developmental disorders, cardiac disorders, immune disorders and cancer (Papavassiliou, 1998). It is also very likely that mental conditions, such as affective disorders may result from a dysregulation of neuron specific gene expression caused by an inappropriate activation or inactivation of regulatory proteins, including transcription factors (Lesch and Heils, 2000). Thus, transcription factors are interesting novel targets for drug development and several authors discuss the benefits of using this approach (Butt and Karathanasis, 1995; Pennypacker, 1995;
Papavassiliou et al., 1998; Emery et al., 2001). When using drugs directed at a protein acting upstream of a transcription factor in the signal-transduction pathway, the drug may affect the transcription factor in an indirect way. Problems with this kind of approach are e.g. the extensive "cross-talk" among signaling proteins, for instance the possibility of enzymes having multiple substrates, making this a risky approach. In contrast, drugs directly targeting gene-specific transcription factors could effectively overcome these problems by exerting a direct action on gene expression (Emery et al., 2001; Papavassiliou, 1998), thereby yielding fewer side effects.

1.4 Transcription factor family AP-2

1.4.1 Structure and function of transcription factor AP-2

The AP-2 family of transcription factors consists of a group of related proteins translated from different genes and with a molecular weight of around 50 kD. There are today five different isoforms identified; AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε (Williams et al., 1988; Moser et al., 1995; Chazaud et al., 1996; Zhao et al., 2001; Wang et al., 2004). The AP-2δ and AP-2ε isoforms were identified 2001 and 2004, respectively (Zhao et al., 2001; Wang et al., 2004), and there have been very few studies regarding structural and functional differences between these and the other isoforms. Thus, the properties of the AP-2 isoforms described here mainly refer to the other AP-2 isoforms. Transcription factor AP-2α was purified and cloned from HeLa cells (Mitchell et al., 1987; Williams et al., 1988) and the gene encoding AP-2α is located on chromosome 6p24 (Kawanishi et al., 2000). Transcription factor AP-2β was cloned and characterized by Moser et al., 1995 and the gene of AP-2β is located on chromosome 6p12-p21.1. The AP-2 isoforms have a unique structure consisting of an amino-terminal proline- and glutamine-rich transcriptional activation domain and a complex basic helix-span-helix motif necessary and sufficient for dimerization and site-specific DNA binding (Williams and Tjian, 1991a, 1991b). Moreover, they have been reported to exhibit very similar biochemical properties. They differ in their N-terminal transcription activation domains, but show high conservation (75-85%) within their C-terminal DNA binding and dimerization domains, and they all bind GC-rich sequences (Bosher et al., 1996). The DNA sequences 5'-
(G/C)CCCA(G/C)(G/C)(G/C)-3’ and the palindromic sequences 5’-GCCNNNGGC-3’ are considered as consensus AP-2 binding sites (Williams and Tjian, 1991a; Roesler et al., 1988). Transcriptional activation mediated by AP-2 can be induced by two different signal transduction pathways; the phorbol-ester and diacylglycerol activated protein kinase C pathway and the cAMP-dependent protein kinase A pathway (Imagawa et al., 1987). Several genes in the monoaminergic systems have binding sites for AP-2 in their regulatory regions e.g. the genes encoding dopamine β-hydroxylase (DβH) (McMahon et al., 1992; Greco et al., 1995; Kim HS et al., 2001), dopamine transporter (Gene Bank acc. Nr. U13956), dopamine D1A receptor (Healy et al., 1997), rat aromatic L-amino acid decarboxylase (Hahn et al., 1993), 5-HT transporter (Bradely and Blakely, 1997), rat 5-HT2A receptor (Du et al., 1994), tryptophan hydroxylase (Cote et al., 2002), tyrosine hydroxylase (TH) (Kim HS et al., 2001) and choline acetyltransferase (Quirin-Stricker et al., 1997; Baskin et al., 1997). A directly regulatory function of AP-2 has been shown for the genes encoding TH and DβH (Kim HS et al., 2001). The isoforms AP-2α and AP-2β have been shown to be abundant in the brain, both during embryonic development of the brain and in the adult brain (see below). In the mouse brain, AP-2γ has been shown to be co-expressed with AP-2α and AP-2β in several brain regions. However, the expression level of AP-2γ in the brain was, compared with the levels of the other isoforms, shown to be very low (Moser et al., 1995; Oulad-Abdelghani et al., 1996; Shimada et al., 1999).

1.4.2 AP-2 and brain embryonic development

In the mammalian central nervous system transcription factor AP-2 is one of the critical regulatory factors for neural gene expression and neural development. Thus, studies on mice have identified the isoforms AP-2α and AP-2β as important regulators of gene expression during embryonic development of many neural tissues including the brainstem, where the monoaminergic nuclei are located (Mitchell et al., 1991; Moser et al., 1997a). Moser et al., 1997 reported that the genes of AP-2α and AP-2β were expressed with different temporal and spatial patterns during mouse embryonic development. The genes of the AP-2α and AP-2β isoforms were shown to be co-expressed day 8 post coitum (pc) in the head mesenchyme and extraembryonic trophoblast, and also in the primordia of the midbrain, hindbrain and spinal cord. The
expression patterns of AP-2α and AP-2β were identical until day 10, but from day 11 the expression patterns were different. Expression of AP-2α was mainly seen in the limb buds, dorsal root ganglia and tooth germs, whereas expression of AP-2β was mainly seen in the midbrain, sympathetic ganglia, adrenal medulla and dorsal root ganglia. Both genes were expressed in the skin, facial mesenchyme, spinal cord, cerebellum and the renal tubular epithelia.

1.4.3 AP-2 in the adult brain

Expression patterns of the AP-2α, AP-2β and AP-2γ isoforms have been studied in adult mice (Shimada et al., 1999). A high expression of all studied isoforms was seen in the cerebellum Purkinje cells and in the hippocampus. The expression of AP-2γ mRNA was relatively weak compared to the other isoforms and the AP-2β mRNA expression was higher than that of AP-2α in many brain regions. The AP-2α and AP-2β mRNA expression was specifically high in the olfactory bulb, hippocampus, brainstem, cerebellum and cerebral cortex. In many brain regions AP-2α and AP-2β mRNAs were co-expressed, but each isoform had a slightly different expression pattern (Shimada et al., 1999).

Recently, our research group found several regionally specific correlations between AP-2α and AP-2β and specific monoamines in the rat forebrain. AP-2α levels correlated positively to levels of 5-hydroxyindoleaceticacid (5-HIAA), dihydrophenylaceticacid (DOPAC) and noradrenaline in the frontal cortex and to 5-HT levels in the hippocampus, and AP-2β levels correlated positively to 5-HT turnover and dopamine metabolism in the frontal cortex and to 5-HIAA and noradrenaline levels in the septum (Damberg et al., 2001a). One may speculate, according to these findings, that AP-2 not only is an important regulatory factor during embryonic development of the brain, but that this transcription factor also has an important regulatory function in the adult brain.
1.4.4 Polymorphisms in the AP-2β gene

The gene encoding AP-2β includes two polymorphic regions. One is located in the promoter, 67 bp upstream of exon 1 (G/A), and the other is located in intron 2 between nucleic acid residues 12593 and 12612, close to the 3’ splice site of exon 2. The latter polymorphism consists of a tetranucleotide repeat of CAAA repeated either four or five times (Moser et al., 1997b). The AP-2β intron 2 polymorphism has been associated, in two independent studies, with anxiety-related personality traits (Damberg et al., 2000a; Paper IV). This polymorphism has also been linked to binge-eating disorder (Damberg et al., 2001b), a condition characterized with impaired impulsive control (Brewerton, 1995).

Furthermore, the AP-2β intron 2 genotype has been associated with platelet monoamine oxidase (platelet-MAO) activity (Paper III), that previously has been shown to be associated with personality characteristics such as sensation seeking, impulsiveness and monotony avoidance (Oreland and Hallman, 1995; Schalling et al., 1987). Platelet-MAO activity is further described below. Moreover, the AP-2β intron 2 genotype is associated with CSF-levels of homovanillic acid (HVA) (Damberg et al., 2004). However, AP-2β genotype is not associated with premenstrual dysphoric disorder in women (Damberg et al., submitted manuscript 2004) or with schizophrenia (Jönsso et al., 2002).

Data obtained with the AP-2β intron 2 polymorphism suggests that AP-2β influences functions in the central nervous system and one can speculate that these events take place especially during development of the brainstem neurotransmitter systems and therefore give rise to specific personality characteristics in the adult. The possible functional importance of this variable region has not yet been elucidated. Since this polymorphism is located in a non-coding region one possibility would be that it is in linkage disequilibrium with another polymorphism, most probably located in a regulatory region. In paper V, we showed that the AP-2β intron 2 polymorphism does not seem to be in linkage disequilibrium with the only other yet described polymorphism in the AP-2β gene, i.e. the AP-2β promoter polymorphism (-67 G/A). Another mechanism explaining a functional relevance of the AP-2β intron 2 polymorphism would be that the polymorphic region contained binding sites for regulatory proteins. Examples on regulatory factors binding to non-coding regions and having a direct effect on gene expression have been demonstrated.
in several studies (Van Haasteren et al., 2000; Morishita et al., 2001; Kim CH et al., 2001; Takimoto and El-Deiry, 2000; Scohy et al., 2000; Müller et al., 1998; Surinya et al., 1998; Zhang et al., 1997; Bergers et al., 1995). In Paper V, we demonstrate that one human brain nuclear protein binds to the long variant of the polymorphism, but not to the short variant. We speculate that this protein is of functional relevance for the regulation of the expression of the AP-2β gene. This issue is further discussed in section 2.5 and in Paper V.

1.5 Depression and antidepressants

1.5.1 Theories of depression

During the years of research on mental disorders a number of different factors and mechanisms have been experimentally shown to be altered during conditions of depression. This has given rise to several theories on what causes this state. The most common theories involve defects in the monoamine and/or neuropeptide systems, in the expression or function of transcription factors or growth factors, or describe alterations in the function of the HPA-axis. Most probably, all these factors are somehow involved in the state of depression. However, the views on what is initially causing the disorder are still controversial. Though, the most common view is that imbalances in the CNS monoaminergic systems are the primary reason for developing this condition. Harro and Oreland, 2001, discuss that a low tonic activity in the locus coeruleus NA system, either caused by a constitutional weakness of this system or through a dysregulation of it by neuropeptides such as galanin, substance P or neuropeptide Y, can be the cause of the depressive state. Since there are interindividual structural differences in the monoaminergic systems, some individuals might be more vulnerable for developing depression than others, e.g. as an effect of stress.

1.5.2 Antidepressant drugs

Since the serotonin system is crucial for the control of mood and emotion, this is a natural target system when developing drugs for different kinds of mental conditions. Many
antidepressant drugs interact with targets involved in the serotonergic system. One example is the selective serotonin reuptake inhibitors (SSRIs) that are directed against the serotonin transporters, thereby blocking the reuptake of serotonin from the synaptic cleft. These are today frequently used for many other diagnoses than depression, i.e. panic and anxiety disorders, obsessive compulsive disorder, phobias, eating disorders and aggressive behavior. Drug targets involved in the noradrenergic system are also common for drugs used when treating depression, e.g. noradrenaline reuptake inhibitors (NRIs). Clinically, SSRIs and NRIs seems to be equally effective as antidepressants (Baldwin, 2001). However, individual differences in therapeutic response and severity of side-effects may occur between patients and it is therefore important to make the treatment as individually based as possible (Baldwin, 2001). Irrespective of which drug, or combination of drugs that is used, there is a delay of 2-6 weeks before therapeutic effect is noticed. This initial lag-period is usually associated with several side-effects. However, many of these fade away with the appearance of the therapeutic effect. The main classes of antidepressants, their respective drug targets and some examples of each drug are listed below:

- **Tricyclic antidepressants (TCA)**, inhibit the 5-HT/NA reuptake, e.g. imipramine, desipramine and clomipramine
- **Selective serotonin reuptake inhibitors (SSRIs)**, inhibit the 5-HT reuptake, e.g. fluoxetine, paroxetine, citalopram, sertraline and fluvoxamine
- **Selective noradrenaline reuptake inhibitors (NRIs)**, inhibit the NA reuptake, e.g. reboxetine and maprotiline
- **Monoamine oxidase inhibitors (MAO-Is)**, inhibit MAO-A or MAO-A/B, e.g. phenelzine (MAO-A/B), moklobemide (MAO-A)
- **Miscellaneous antidepressants**, e.g. venlafaxine (inhibits 5-HT/NA reuptake and also non-selective receptor-blocking effects) and mirtazapine (blocks $\alpha_2$, 5-HT$_2$ and 5-HT$_3$ receptors)
Our research group previously analysed the effects on transcription factor AP-2 in rat whole brain after subchronic (10 days) administration of three different types of antidepressants; citalopram, imipramine and lithium. We found a decrease in the DNA-binding activity of AP-2 in rats treated with citalopram, imipramine or lithium for 10 days. Moreover, citalopram and imipramine decreased the amount of AP-2α, but only citalopram decreased the levels of AP-2β (Damberg et al., 2000b). Recently, we also reported that the DNA-binding activity of AP-2 and the levels of AP-2α and AP-2β were increased in rat whole brain after 10 days of treatment with phenelzine (Damberg et al., 2003). Further studies with regard to this subject are presented in Paper I and Paper II.

1.5.3 Animal models of depression

When trying to evaluate the neurochemical mechanism of depression (as well as of other clinical states) one is limited by the lack of good animal models. There are obvious problems to produce animals with a condition that is identical with human depression. However, there are several procedures where one tries to evoke a behavioral state that in many aspects resembles human depression (such as withdrawal from social interaction, loss of appetite or reduced motor activity) (Porsolt, 2000). One of the most commonly used animal models of depression is the chronic mild stress model (CMS). In this model, animals (rats or mice) are exposed to different unpredictable stressful conditions such as mild uncontrollable foot shock, forced swimming in cold water, changes in the housing conditions, food and water deprivation, reversal of light/dark periods and exposure to noise and bright light. After exposure to these stressors for 2 to 3 weeks, rats show behavioral changes that are maintained for months and that resemble depressive symptoms (Charney et al., 1999). These symptoms include both changes in psychomotor behavior, as evidenced by reduced open field activity, and also reduced sensitivity to rewards, as evidenced by a decrease in the consumption of sucrose solution in comparison to tap water (Willner et al., 1992). Long-term treatment with various antidepressants has been shown to restore the reduced sucrose intake produced by the CMS conditions (Charney et al., 1999). This has e.g. been shown after treatment with citalopram, where significant increase in sucrose intake is seen after 2 weeks (Papp and Sanchez, 2001,
Montgomery et al., 2001). Other animal models of depression are e.g. tail suspension, learned helplessness and olfactory bulbectomy (Porsolt, 2000).

1.6 Platelet monoamine oxidase (platelet-MAO)

In the human central nervous system both monoamine oxidase (MAO) type A and type B are expressed. They are encoded by two genes closely linked in opposite direction on the X chromosome (Shih and Chen., 1999; Grimsby et al., 1991; Lan et al., 1989). These are flavine-containing mitochondrial enzymes which oxidatively deaminate the neurotransmitters dopamine, noradrenaline, and serotonin as well as exogenous monoamines. MAO-A prefers the substrates 5-HT and NA while MAO-B prefers phenylethylamine (PEA). DA is equally preferred by both MAO isoforms. MAO-A and MAO-B are co-expressed in many tissues, but in platelets only the MAO-B type is expressed. Platelet-MAO has the same amino acid sequence as brain MAO-B (Chen et al., 1993). However, platelet-MAO activity does not seem to correlate with brain MAO-B activity (Winblad et al., 1979; Young et al., 1986). The enzyme activity varies between individuals, but remains stable during lifetime, but a slight increase can be seen after the age of 40 (Murphy et al., 1976, Bridge et al., 1985, Bagdy and Rihmer., 1986). In humans, women have 10-20 % higher platelet-MAO activity than men (Murphy et al., 1978). A high degree of heritability has been reported in several family and twin studies (Pedersen et al., 1993).

Structural differences of the MAOB gene have been shown to be of minor importance (Girmen et al., 1992, Garpenstrand et al., 2000) for the specific enzymatic activity. Several studies confirm that the expression of the MAOB gene rather is controlled at a transcriptional level (Zhu et al., 1992; Grimsby et al., 1990; Ekblom et al., 1996; Shih and Chen, 2004).

Low platelet-MAO activity has, in clinical studies, been shown to correlate with personality characteristics such as sensation seeking, impulsiveness and monotony avoidance (Oreland and Hallman, 1995; Schalling et al., 1987) and also with alcoholism and alcohol intake in non-human primates (von Knorring and Oreland, 1995; Fahlke et
al., 2002). Furthermore, studies on non-human primates have reported associations between platelet-MAO and behavior (Redmond et al., 1979; Fahlke et al., 2002), findings in support of what has been shown in human studies. However, it is important to notice that induced inhibition of platelet-MAO does not result in major alterations in temperament, and knock-out mice deficient in MAO-B do not display changed behavior (Grimsby et al., 1997). Different explanations on how platelet-MAO is correlated to personality are discussed by Oreland and Hallman, 1995. They conclude that the most probable explanation is that platelet-MAO can be considered as a genetic marker that is regulated by the same mechanisms regulating some brain function(s) of importance for personality.
2. PRESENT INVESTIGATION

2.1 Aims of the thesis:

Paper I
To analyse the DNA-binding activity of transcription factor AP-2 and the levels of AP-2\(\alpha\) and AP-2\(\beta\) in rat whole brain after treatment with citalopram for different time periods.

Paper II
To analyse the levels of AP-2\(\alpha\) and AP-2\(\beta\) in rat brainstem after treatment with phenelzine or citalopram for different time periods.

Paper III
To investigate if the human AP-2\(\beta\) intron 2 polymorphism is associated with catalytic activity of platelet-MAO in healthy volunteers.

Paper IV
To, by using five different personality questionnaires, further analyse the relationship between the human AP-2\(\beta\) intron 2 polymorphism and anxiety-related personality traits in healthy volunteers.

Paper V
To investigate if the AP-2\(\beta\) intron 2 and the AP-2\(\beta\) promoter (-67 G/A) polymorphisms are in linkage disequilibrium. Also to study if the AP-2\(\beta\) promoter (-67 G/A) polymorphism is associated with platelet-MAO activity in healthy volunteers. Furthermore, to analyse if human brain nuclear proteins are binding to the AP-2\(\beta\) intron 2 polymorphic region, and if a different binding pattern can be observed for the different variants of the AP-2\(\beta\) intron 2 polymorphism.
2.2 Methodological considerations

2.2.1 Animals and treatment paradigms

In Paper I, adult male Sprague Dawley rats (10 weeks of age, B & K Universal AB, Sollentuna, Sweden) were housed in groups of five and maintained on a 12 hour light/dark cycle with food and water freely available. The animals were administered citalopram (10 mg/kg) subcutaneously with daily injections for 1, 3, 7 and 21 days. In Paper II, the animals were housed in groups of three and the animals were administered citalopram (10 mg/kg) or phenelzine (10 mg/kg) subcutaneously with daily injections for 1, 7 and 21 days. In both Paper I and Paper II, control animals were administered saline injections in the same volume as that given the drug treated animals. Both citalopram and phenelzine were dissolved in saline (NaCl, 9 mg/ml). All animals were sacrificed 24 hours after their last injection. In Paper I, a group of untreated animals (n=5) was included and these were sacrificed after 21 days.

In Paper I, the cerebrum was dissected after sacrifice and the right hemisphere was used for extraction of nuclear proteins for subsequent EMSA and ELISA analyses. In Paper II, the brainstem was dissected after sacrifice and used for extraction of nuclear proteins for subsequent ELISA analysis.

The drugs used for these studies were obtained by the following sources: saline (NaCl) from Pharmacia & Upjohn, Uppsala, Sweden; citalopram from H.Lundbeck AB, Helsingborg, Sweden; phenelzine from Sigma, Sweden.

The animal studies were carried out with permission from the local animal ethics committee in Uppsala, Sweden.

2.2.2 Preparation of nuclear extracts

In Paper I and Paper II, nuclear proteins were extracted from rat whole brain and rat brainstem, respectively. In Paper V, nuclear proteins were extracted from human frontal
cortex. The brain tissue was homogenized in 3 ml buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, pH 7.9). The homogenate was incubated on ice for 15 minutes. To this 125 µl Nonidet P40 was added, and the homogenate was centrifuged for 30 seconds at 14000 rpm in 4°C. The pellet was resuspended in 500 µl buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 7.9). Thereafter, the tubes were put on a shaker for 15 minutes and centrifuged at 14000 rpm for 5 minutes (4°C). The supernatant, including the nuclear proteins, was aliquoted and stored at -80°C. The protein concentrations for all nuclear extracts were determined by a method by Lowry at al., 1951. The concentration of nuclear extracts was in Paper I ~12 µg/µl, in Paper II ~8 µg/µl and in Paper V ~3 µg/µl.

2.2.3 Electrophoretic mobility shift assay (EMSA)

EMSA analyses were performed in Paper I and Paper V. From a mix of proteins, proteins binding to a specific DNA sequence can be selected by the use of a radiolabelled probe corresponding to the specific DNA sequence. The EMSA assay is based on the fact that a radiolabelled DNA probe binding to a protein can be identified by its increased molecular mass, determined by nondenaturation polyacrylamide gel electrophoresis.

Radiolabeling of the ds-DNA probes was done according to the same protocol in Paper I and V. In Paper I, the ds-probe included the consensus sequence of AP-2 and had the following sequence: 5´- GATCGAACTGACCGCCCGGCTTCT-3´. In Paper V, the two ds-probes included the two different variants of the AP-2β intron 2 polymorphism and 9 bp on each side of this region; long variant of the polymorphism: 5´-AACCACAAA-[CAAA]5-AAAGACCAC-3´ and short variant of the polymorphism: 5´-AACCACAAA-[CAAA]4-AAAGACCAC-3´. All probes were 5´-end labelled with T4 polynucleotide kinase. The labeling reaction was carried out in 20 µl of 0.5 M TRIS-HCl, pH 7.6, 100 mM MgCl₂, 100 mM 2-mercaptoethanol (United States Biochemical, Cleveland, Ohio), containing 10 pmol of AP-2β intron 2 ds-DNA probe, 2 µl (20 µCi) γ-32-dATP (Amersham, Buckinghamshire, UK) and 9 units T4 polynucleotide kinase (United States Biochemical, Cleveland, Ohio). The labeling mixture was incubated for 60 minutes at 37°C and the reaction was terminated on ice.
In order to separate unincorporated $\gamma$-$^{32}$P-dATP from the labelled probes two different protocols were used in Paper I and Paper V. In Paper I, the labeling mix was electrophoresed on a 8 % non-denaturing polyacrylamide gel containing 8 ml 30 % acrylamide 37.5:1, 19 ml water, 3 ml 10 x TBE, 70 µl TEMED and 500 µl 10 % ammoniumpersulphate. As running buffer 0.25 x TBE was used. The electrophoresis was run at 500 V for 3 hours at room temperature. The gel was attached to an autoradiography film for one minute and developed by autoradiography. The band representing the double-stranded AP-2 oligo was excised from the gel and purified by ethanol precipitation. In Paper V, the unincorporated $\gamma$-$^{32}$P-dATP was separated from the labelled probes using spin columns (QIAquick®Nucleotide Removal Kit, Qiagen, GmdH, Hilden, Germany). The amount of radioactivity was measured in a scintillation counter. Both in Paper I and Paper V, the labelled probes had a specific activity of $\sim 2 \times 10^5$ cpm/pmol DNA.

The EMSA binding reaction was carried out in a total volume of 20 µl containing 0.5 mM EDTA, 10 mM TRIS-HCl, 5 mM MgCl$_2$, 50 mM NaCl and 1 mM DTT, 1 pmol labelled ds-DNA probe, 1 µg poly (dC-dI) and to this $\sim 24$ µg nuclear extract was added in Paper I and $\sim 6$ µg nuclear extract was added in Paper V. The binding reaction mixture was incubated on ice for 30 minutes. A 4 % non-denaturing polyacrylamide gel containing 4 ml 30 % acrylamide 37.5:1, 25 ml water, 0.75 ml 10 x TBE, 70 µl TEMED and 500 µl 10 % ammoniumpersulphate was used in the electrophoresis. The electrophoresis was run at 200 V for 3 hours at 4 °C and 0.25 x TBE was used as running buffer. The gel was removed and dried for 45 minutes on a 3 MM paper, thereafter the dried gel was attached to an autoradiography film and exposed for 1-2 days in -80 °C.

In Paper I, the DNA binding activity of AP-2 was quantified. For each gel a known amount of radioactive ds-probe was added in three wells as a reference. For each treatment group optical density (OD), reflecting the DNA binding activity of AP-2, was estimated for the main band (AP-2), and the three bands corresponding to the reference, using Image-Pro Plus 4.0 software. As an internal standard procedure the ratio between the amount of protein bound to the labelled probe in the main band and the mean value of
the reference bands in each gel was calculated and used as a value of the relative DNA binding activity. Each rat was analysed three times for accuracy.

In Paper I, specificity was estimated by competition with different concentrations (100-1000 pmol) unlabelled AP-2 consensus probe. The binding of AP-2 to the labelled probe was then shown to be reduced with increasing concentrations of unlabelled probe. To further analyse the specificity of the binding reaction 50 x unlabelled CREB consensus probe was added to some binding reactions. This did not affect the binding of AP-2 to the labelled probe. In Paper V, specificity was determined by competition with different concentrations (100-1000 pmol) of unlabelled long and short variants of the AP-2β intron 2 polymorphic region. The proteins binding to the labelled long and short probes were then shown to be reduced with increasing concentration of unlabelled long and short probe, respectively.

All probes used were HPLC purified and purchased from Thermo BioSciences, Germany.

2.2.4 Enzyme-Linked Immunosorbent assay (ELISA)

In Paper I and Paper II, ELISA was used to analyse the levels of AP-2α and AP-2β in nuclear extracts from rat whole brain and rat brainstem, respectively. 96-well microtiter plates were coated (50 µl/ well) with nuclear extracts (10 µg/ml in Paper I and II) diluted in 50 mM Carbonate-Bicarbonate buffer pH 9.0. The plates were covered with parafilm and incubated overnight at 4°C. Antigen solution was then removed and 200 µl blocking buffer (PBS, 1 % BSA) was added to each well and the plates were incubated for two hours in room temperature. Following this the blocking buffer was removed and the plates were washed with PBS. Primary antibody (50 µl, goat polyclonal AP-2α and AP-2β, 15 µg/ml respectively, SDS Biosciences, Falkenberg, Sweden) diluted in blocking buffer was then added and the plates incubated overnight at 4°C. After incubation the antibody was removed and the plates were washed three times with Wash buffer I (PBS, 0.05 % Tween-20). Secondary antibody (Donkey anti-goat IgG AP conjugated, SDS Biosciences, Falkenberg, Sweden) diluted 1:350 in blocking buffer, was then added (50 µl) to each well and the plates were incubated for two hours in room temperature. After
removal of the secondary antibody the plates were washed three times with Wash buffer I, and once with Wash buffer II (10 mM diethanolamine, 0.5 mM MgCl₂, pH 9.5). Thereafter, 50 µl substrate (Phosphatase substrate, 5 mg tablets, Sigma, Sweden, diluted in 5 ml Wash buffer II) was added to each well. The reaction continued for 30 minutes and was terminated by adding 50 µl of 0.1 M EDTA, pH 7.5. The plates were analysed in an ELISA reader (Molecular Devices, Thermo Max) at optical density (OD) 405/490. The OD of the AP-2 isoforms for each rat was correlated to a value in a standard curve, where known concentrations of antibody were plotted against optical density. The value form the standard curve was then divided with the concentration of total protein in the nuclear extracts. The quota was used as a value of the relative amount of AP-2α and AP-2β protein. All animals were analysed three times in Paper I and twice in Paper II for accuracy.

2.2.5 Subjects and estimation of personality

In Paper III, 158 male and 64 female subjects were analysed with regard to AP-2β intron 2 genotype and platelet MAO-B activity. The male subjects were healthy blood-donors recruited at the Uppsala University Hospital. The female subjects consisted of two groups of women. One group of women was recruited by an advertisement in the Swedish magazine Amelia® and consisted of women with binge-eating episodes (n=32) and the other group consisted of healthy female volunteers (n=32). Since tobacco smoke can inhibit MAO activity only non-smoking individuals were included in the study. No differences in platelet MAO could be observed between the two female groups, i.e. 14.18±4.45 and 14.08±3.16, F=0.11 and p=0.915 (mean MAO in nmol/10^10 platelets/min ±SD). Therefore, these groups were merged into one group of females (n=64).

In Paper V, 155 of the males used in paper III were analysed with regard to AP-2β promoter (-67 G/A) genotype. Three of the originally 158 samples analysed in paper III were excluded because of technical difficulties when genotyping the AP-2β promoter polymorphism.
In Paper IV, 207 male and 163 female subjects were analysed with regard to AP-2β intron 2 genotype and personality traits. All subjects were unrelated Caucasians living in Stockholm. The subjects included both re-examined healthy controls from previous psychiatric studies and individuals selected from the general population. The age range of the participants was 20 to 83 years (mean age 42.3 years). Some of the subjects (n=137; 64 men, 73 women) had been included in a previous study on the relationship between AP-2β intron 2 genotype and personality traits estimated by Karolinska Scales of Personality (KSP). These individuals were excluded in the separate replication analyses regarding personality traits estimated by KSP in the present study. For estimating personality traits, the subjects completed personality questionnaires assessing one or several of the following personality inventories: KSP, Swedish Universities Scales of Personality (SSP), Health-Relevant 5-factor Personality Inventory (HP5i), Temperament and Character Inventory (TCI) and Revised NEO Personality Inventory (NEO-PI-R). KSP, SSP and HP5i are derived from a common pool of items. Thus, some of the personality scales in the KSP, SSP and HP5i are highly correlated. TCI is composed of 7 main scales, 6 of them being in turn composed of 5 main scales, each in turn built up of 3-5 subscales. Similarly, NEO-PI-R is composed of 5 main scales, each in turn built up by 6 subscales. There is a high correlation between the main scales and their subscales in both the TCI and NEO-PI-R, respectively. Thus, several scales from each of the 5 inventories are highly correlated with scales and subscales in other inventories.

2.2.6 Blood sampling and preparation of DNA

Blood samples of approximately 7 ml were drawn into Vacutainer® tubes containing ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson, Franklin Lakes, NJ, USA) for prevention of clotting. From each sample 700 µl was pipetted into Eppendorf tubes and genomic DNA was extracted using Qiamp® DNA extraction kit (Qiagen GmdH, Hilden, Germany). The DNA solution was stored at -20 °C. For measurement of platelet-MAO activity (see 2.2.8) the remaining blood was low speed centrifuged within 24 hours to prepare platelet rich plasma. The platelet concentration was measured electronically in a Thrombocounter-C® (Coulter Electronics Ltd., Luton, UK) and the platelet rich plasma stored at -80 °C.
In Paper III and Paper IV, polymerase chain reaction (PCR) was used for genotyping the AP-2β intron 2 polymorphism. The primers used were AP-2βF (5’-CCTACCACCAGAGCCAGGACCC-3’) and AP-2βR (5’-CCCCCCTCCAGAAGCATTCCT-3’). The PCR reaction mix (30 µl) contained 100 ng genomic DNA, PCR buffer (200 mM Tris-HCl, pH 8.4), 400 µM dNTP (100 µM each of dATP, dCTP, dTTP, dGTP), 2 pmol of each primer, 5 % DMSO, 1 % W-1 buffer and 5 units Taq polymerase (Life Technologies). The PCR reaction was amplified through 30 cycles on a GeneAmp 9700® (Applied Biosystems) and each cycle consisted of a 95°C denaturation step for 60 seconds, a 57°C annealing step for 60 seconds and finally a 72°C elongation step for 60 seconds.

The PCR products had a size of 370 or 366 bp and were analysed on a 4 % denaturing polyacrylamide gel containing 25 g UREA, 33 ml water, 3.1 ml 20 x GTB (1.78 M TRIS, 0.58 M taurine and 10.7 mM EDTA), 8 ml acrylamide (19:1 acrylamide:bisacrylamide), 400 µl 10 % ammoniumpersulphate and 80 µl TEMED. The electrophoresis was run at 1.7 kV for 3 hours at 4 °C. The PCR products were denaturated at 98 °C for 2 minutes before loading. As running buffer 1 x GTB was used. After electrophoresis, the PCR products were detected by silver staining according to a protocol by Bassam et al., 1991.

In order to confirm that the specific PCR products corresponded to the expected regions of the AP-2β gene, the PCR-products were cloned using a pGEM®-T Easy Vector System (Promega Corporation, Madison, Wi). T-vector DNA was prepared from bacteria culture using a Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, Madison, Wi), and the DNA was stored at -20°C. Sequence analysis on a capillary gel was performed using a BigDye™ Terminator Cycle Sequencing Ready Reaction kit (ABI PRISM™, Perkin Elmer, Foster City, CA, USA) with AmpliTaq® DNA polymerase on a
ABI PRISM™ 310 Genetic Analyzer (ABI PRISM™, Perkin Elmer, Foster City, CA, USA).

In Paper V, PCR and sequencing were used for genotyping the AP-2β promoter (-67 G/A) polymorphism. PCR amplification was performed in a 50 µl volume of reaction mixture containing 100 ng genomic DNA, 10 x Pfu DNA polymerase reaction buffer (200 mM TRIS-HCl, 20 mM MgSO₄, 100 mM KCl, 100 mM (NH₄)₂SO₄, 1% Triton® X-100, 1mg/ml nuclease-free BSA pH 8.8, Stratagene, USA), 640 µM dNTP (160 µM each of dATP, dCTP, dTTP, dGTP), 20 pmol of each primer and 5 units of PfuTurbo®Hotstart DNA proofreading polymerase (Stratagene, USA). The primers used were AP-2β-F: 5´-TGCATACAAAACACCTAAAATAACAAGC-3´ and AP-2β-R: 5´-TAAAACGAGAAAAAGCCCTGACTTTATCAG -3´ (Thermo BioSciences, Germany). The PCR reaction was amplified through 45 cycles on a GeneAmp 9700® (Applied Biosystems) and each cycle consisted of a 95°C denaturation step for 30 seconds, a 55°C annealing step for 45 seconds and finally a 72°C elongation step for 2 minutes. The PCR products were purified from a 1 % agarose gel using Gel Extraction kit (Qiagen, Hilden, Germany). The AP-2β promoter polymorphism was genotyped by sequencing using ABI PRISM Dye Terminator cycle sequencing kit according to the manufacturers recommendations (Applied Biosystems, Stockholm, Sweden) and analysed on an ABI PRISM-310 Automated Sequencher (Applied Biosystems). As sequence primer we used 5´- CTTTGCTGAGGCTTACTTGTC -3´. All PCR reactions were performed on a GeneAmp® PCR System 9700 (Applied Biosystems).

2.2.8 Estimation of platelet-MAO activity

When analysing platelet-MAO activity (Paper III) a radiometric assay with 14C-labelled 2-phenylethylamine (β-PEA) and/or tryptamine (Try) as substrates was used. This assay was previously described by Hallman et al., 1987. The samples of platelet rich plasma were, before analysis, thawed and sonicated at 0°C during 4 x 10 seconds with intervals of 5 seconds for lysis of the platelets containing the MAO enzyme. Of the sonicated plasma, 50 µl was added to 50 µl of 0.1 mM 14C-β-PEA (0.5 µCi/ml) or 50 µl of 0.1 mM 14C-Try (0.5 µCi/ml) in 0.1 M sodium phosphate buffer (pH 7.8). The reaction mixture
was incubated at 37°C for 4 minutes, and the reaction was terminated by the addition of 30 µl 1 M HCl. After this, the radioactive aldehyde product was extracted, under vigorous shaking for 30 seconds, into 750 µl toulene:ethylacetate (1:1, vol/vol). Thereafter, the samples were centrifuged at room temperature for 5 minutes at 1000 x g. Of the organic phase, 500 µl, including the aldehyde product, was added to 8 ml scintillation fluid and the amount of radioactive aldehyde product, for each sample, was quantified by scintillation analysis. Enzyme activity is expressed as nmol of substrate oxidized per 10^{10} platelets per minute. In order to increase the reliability of the assay two substrates, β-PEA and Try, were used in parallel, and all samples were analysed blindly and in duplicates.

2.2.9 Statistical analyses

In Paper I, the statistical comparisons between citalopram- and saline-treated rats, with regard to DNA binding activity of AP-2 and amounts of AP-2α and AP-2β protein, for each time-point were analysed using Mann–Whitney U-test. In Paper II, the statistical comparisons between drug-treated and saline-treated animals, with regard to amounts of AP-2α and AP-2β protein, for each time-point were analysed using unpaired t-test.

In both Paper I and Paper II, analysis of variance (ANOVA) and Fisher’s protected least significant difference (PLSD) test were used when comparing the group of untreated animals to all treatment groups, and also when testing if any of the groups of saline-treated animals differed in DNA-binding activity or AP-2α and AP-2β protein amounts. ANOVA and PLSD test were also used in Paper III when analysing the AP-2β intron 2 genotype in relation to platelet-MAO activity and in Paper IV when analysing the relation between AP-2β intron 2 genotype and scores on the personality questionnaires. Furthermore, ANOVA and PLSD test were used in Paper V when analysing the genotype of the AP-2β promoter polymorphism in relation to platelet-MAO activity.

All calculations described above were performed using StatView 5.0 software (SAS Institute, Cary, NC, USA).
In Paper IV, correction for multiple testing was performed using Bonferroni correction and power was estimated in accordance with published methods (Cohen, 1988; Erdfelder et al., 1996).

In order to, in Paper V, investigate if the AP-2β promoter polymorphism and the AP-2β intron 2 polymorphism are in linkage disequilibrium we used Linear-by-Linear association test. Statistical Package Software System (SPSS) 11.0 was used for calculation.

All results have been considered statistically significant when p<0.05.

2.3 Summary of papers

2.3.1 Paper I
Molecular mechanisms in the monoaminergic systems are likely to play an important role in the mechanism of action of antidepressant drugs. Many genes encoding proteins in the monoaminergic systems have binding sites for transcription factor AP-2 in their regulatory regions. Our research group have previously shown that the DNA-binding activity of AP-2 and the levels of AP-2α and AP-2β were down-regulated in rat whole brain after 10 days of treatment with several different antidepressants, e.g., the SSRI citalopram (Damberg et al., 2000b). In Paper I, we analysed the effect of citalopram on AP-2 in rat whole brain in a time-dependent manner. Rats were treated with citalopram (10 mg/kg/day, s.c.) for 1, 3, 7 or 21 days. Control animals were treated for the same time periods with saline (NaCl, 9 mg/ml). One group of untreated animals was also included in the study. The results showed that the DNA-binding activity of AP-2 and the levels of AP-2α and AP-2β were down-regulated after 7 days of treatment, but were back to control levels after 21 days of treatment with citalopram. The levels of AP-2α were also down-regulated after 3 days of treatment with citalopram. For the group of untreated animals the DNA-binding activity of AP-2 and the levels of AP-2α and AP-2β were higher than in all treated animal groups. The results of this study are in line with previous published data. Furthermore, these results indicate that the effect on AP-2 after subchronic treatment with citalopram is temporal and occur before the therapeutic effect
of citalopram is manifested. We speculate in this study that the decrease in AP-2 is involved in the neuronal adaptations that are needed to gain the antidepressant effect of citalopram.

2.3.2 Paper II
This study is a follow-up on Paper I, and also on another study where we showed that the DNA-binding activity of AP-2 and the levels of AP-2α and AP-2β in rat whole brain were up-regulated after treatment with the MAO-I phenelzine for 10 days (Damberg et al., 2003). In Paper II, we analysed the levels of AP-2α and AP-2β in rat brainstem, that includes the monoaminergic cell nuclei, after treatment with citalopram (10 mg/kg/day, s.c.) or phenelzine (10 mg/kg/day, s.c.) for 1, 7 or 21 days. Controls were treated with saline for the same time-periods. The results showed that neither treatment with citalopram for 1, 7 nor 21 days did affect the AP-2α and AP-2β levels in the rat brainstem. Treatment with phenelzine, however, increased the levels of both AP-2α and AP-2β in the rat brainstem after 7 days of treatment, but after 21 days of treatment the levels had returned to control levels. Regarding citalopram, these results implicate that the decrease in DNA binding activity of AP-2 and levels of AP-2α and AP-2β in rat whole brain previously seen after treatment with citalopram is not localized to the brainstem. Previous obtained data regarding AP-2 in relation to the monoaminergic systems suggests that AP-2 is a regulatory factor of importance in these systems. Thus, one can speculate that the decrease in AP-2 levels in rat whole brain seen after citalopram treatment occurs in the monoaminergic terminal projection areas. The data regarding phenelzine treatment suggests that the increase in AP-2 levels previously seen in rat whole brain after subchronic treatment with phenelzine, at least partly, is located in the brainstem. Since the change in AP-2, both after citalopram and phenelzine treatment, was temporal and coincide in time with the initial side-effects of antidepressants, it is also possible that the changes in AP-2 somehow may be involved in these side-effects.

2.3.3 Paper III
By having in mind the previously described association between platelet-MAO and specific personality characteristics (Schalling et al., 1987), it can be proposed that the same developmental mechanisms that regulate the platelet-MAO expression also regulate
CNS proteins influencing complex functions like personality. Since the intron 2 polymorphism in the AP-2β gene also is associated with certain personality characteristics (Damberg et al., 2000a; Paper IV), in addition to the fact that many genes in the monoaminergic systems, like the MAOB gene, have potential binding sites for AP-2 in their regulatory regions, we wanted, in Paper III, to analyse if these markers are associated with each other. Healthy, non-smoking male (n=158) and female (n=64) volunteers were analysed with regard to AP-2β intron 2 genotype and platelet-MAO activity. The results of this study showed that the individuals that were homozygotes for the long AP-2β intron 2 allele had lower platelet-MAO activity than the individuals that were heterozygotes or homozygotes for the short AP-2β intron 2 allele. These results indicate that transcription factor AP-2β in some way can be involved in the regulation of the MAOB gene.

2.3.4 Paper IV
In a previous study by our research group, we analysed the relationship between the AP-2β intron 2 genotype and personality traits as estimated by Karolinska Scales of Personality (KSP) (Damberg et al., 2000a). It was found in this study that the long allele of the AP-2β intron 2 polymorphism was associated with low levels of anxiety-related personality traits in women. In Paper IV, we wanted to, on a larger material and by using several different personality scales when estimating personality traits, confirm the previous data. Healthy, male (n=270) and female (n=163) volunteers were analysed with regard to AP-2β intron 2 genotype and personality. Personality was estimated using five different personality scales: KSP, Swedish Universities Scales of Personality (SSP), Health-Relevant 5- Factor Personality Inventory (HP5i), Temperament and Character Inventory (TCI), and the Revised NEO Personality Inventory (NEO-PI-R). Women homozygous for the long AP-2β intron 2 allele were shown to have lower "muscular tension" (KSP), "somatic trait anxiety" (SSP), "trait irritability" (SSP), "mistrust" (SSP) and "negative affectivity" (HP5i) than women heterozygous or homozygous for the short allele of the AP-2β intron 2 allele. For the male subjects, however, no associations were found between the AP-2β intron 2 genotype and personality traits for any of the studied personality scales. The results of this study are in accordance with previous published
data, and they further support the notion that AP-2β is a factor of importance for personality.

2.3.5 Paper V
Previously obtained associations between the AP-2β intron 2 genotype and personality traits (Damberg et al., 2000a; Paper IV) and with activity of platelet-MAO (Paper III), suggest that the AP-2β intron 2 polymorphism may be functional. One explanation of the associations to the AP-2β intron 2 polymorphism would be that it is in linkage disequilibrium with another polymorphism in a regulatory region. In Paper V, we analysed if the AP-2β intron 2 polymorphism is in linkage disequilibrium with the only other described polymorphism in the AP-2β gene, i.e., in the promoter region (-67 G/A). The same subjects analysed in Paper III with regard to the AP-2β intron 2 polymorphism were now analysed with regard to the AP-2β (-67 G/A) promoter polymorphism (n=155). Using Linear-by-linear test we found that the AP-2β intron 2 polymorphism and the AP-2β (-67 G/A) promoter polymorphism were not in linkage disequilibrium. We also showed that the AP-2β promoter (-67 G/A) is not associated with platelet-MAO activity. Another possibility that could explain the previous associations to the AP-2β intron 2 genotype would be if different regulatory proteins bind to the different variants of the AP-2β intron 2 polymorphism. This may then result in different expression levels of the AP-2β protein. In Paper V, we analysed, using EMSA, the binding pattern of human brain nuclear proteins to labeled probes of the different variants of the AP-2β intron 2 polymorphic region. We found that one protein bound only to the long variant of the AP-2β intron 2 polymorphic region, and not to the short. Thus, the possibility exists that this protein is involved in the regulation of the AP-2β gene and that different variants of the AP-2β intron 2 polymorphism yield different expression levels of the AP-2β protein. Functional expression studies are needed to evaluate this possibility.
2.4 Conclusions

The main conclusions of this thesis are:

- Transcription factor AP-2 is involved in the molecular mechanisms of the antidepressant drugs citalopram (SSRI) and phenelzine (MAO-I). The effect on AP-2 of these drugs is temporal and occurs in time before the expected therapeutic effect of the drugs is manifested.

- The genotype of AP-2β intron 2 is associated with platelet-MAO activity in healthy volunteers.

- The genotype of AP-2β intron 2 is associated with anxiety-related personality traits in women.

- The AP-2β intron 2 polymorphism is not in linkage disequilibrium with a polymorphism in the AP-2β promoter (-67 G/A), and the AP-2β promoter (-67 G/A) polymorphism is not associated with platelet-MAO activity.

- There is a different binding pattern of human brain nuclear proteins to the different variants of the AP-2β intron 2 polymorphism. This implies that the different variants of the AP-2β intron 2 polymorphism may yield different expression levels of the AP-2β protein. Functional expression studies, however, are needed to confirm this.
2.5 General discussion

Results of studies on transcription factor AP-2 in relation to molecular mechanisms in the brain monoaminergic systems are presented in this thesis. The monoaminergic systems are known to be of great importance for personality and neuropsychiatric disorders. Transcription factor AP-2 has been shown to be an essential regulatory factor during embryonic development of many parts of the brain, including the brainstem where the monoaminergic nuclei are located. Moreover, many genes encoding proteins involved in the monoaminergic systems, have binding sites for AP-2 in their regulatory regions. Furthermore, AP-2 has been shown to correlate with many monoaminergic parameters in the rat brainstem. Taken together, these results implicate transcription factor AP-2 to be a factor of importance not only during development of the brain, but also for regulation of neuronal activity in the adult brain.

Regarding the data obtained in Paper I and Paper II, the different responses on AP-2 after treatment with citalopram and phenelzine, respectively, must be explained by the different molecular mechanisms of the drugs. Differences in molecular mechanisms between SSRIs and MAO-Is have been observed at receptor level, e.g., a decreased function of terminal 5-HT autoreceptors is observed after treatment with SSRIs (e.g., citalopram), but not with MAO-Is (Blier et al., 1988). However, the complicated picture of the molecular mechanisms of antidepressants makes it impossible to, based on the current data, exactly point out the position of AP-2 in these mechanisms. It is very likely, though, that the changes in AP-2 are involved at a receptor- or enzyme level. The importance of transcription factors as new drug targets has been discussed by many authors (Butt and Karathanasis 1995, Pennypacker 1995, Papavassiliou et al 1998, Emery et al 2001). The ability of transcription factors to regulate gene expression in a specific way, together with fact that the transcription factors and co-factors often are tissue-specific, yields promising opportunities for a future new generation of specific and effective drugs with a high degree of precision.

In Paper III we report that AP-2β intron 2 genotype is associated with platelet-MAO activity. Several possibilities can explain this association. It has been shown that AP-2, in
a tissue-specific way, in addition to binding to its binding site, also has the ability of binding other transcription factors, such as SP1, and enhance transcription via the binding sites of these transcription factors (Pena et al., 1999). Since SP1 is known to regulate the expression of the MAOB gene (for a review see Shih and Chen., 2004), it is possible that AP-2 may be involved in the SP1 mediated regulation. Another possibility is that AP-2 regulates the expression of a protein, e.g. a transcriptional co-factor, of importance for the regulation of the MAOB gene.

Previous obtained data regarding the AP-2β intron 2 polymorphism, in addition to the association between this genotype and platelet MAO activity (Paper III) and anxiety-related personality traits in women (Paper IV), suggest that this intron polymorphism may be functional. The most likely explanation would be that this polymorphism is in linkage disequilibrium with another polymorphism in a regulatory region. However, in Paper V we show that the AP-2β intron 2 polymorphism is not in linkage disequilibrium with the only other described polymorphism in the AP-2β gene, i.e., in the AP-2β promoter (-67 G/A). In addition to these results we, in Paper V, demonstrate that one human brain nuclear protein binds to the long variant of the polymorphism, but not to the short. The observation of a difference in the binding pattern of nuclear proteins (including transcription factors) to the different variants of the polymorphism, in combination with all reports on the relevance of introns in transcriptional regulation (e.g. see Van Haasteren et al., 2000; Morishita et al., 2001; Kim CH et al., 2001; Takimoto and El-Deiry, 2000; Scohy et al., 2000; Müller et al., 1998; Surinya et al., 1998; Zhang et al., 1997; Bergers et al., 1995), make it tempting to speculate that this protein is of importance for regulation of the AP-2β expression. This would imply that the different variants of the AP-2β intron 2 polymorphism yield different expression levels of the AP-2β protein. However, functional studies on this matter are necessary to verify this hypothesis. Since the AP-2β intron 2 polymorphism is located close to the splice site of exon 2, the protein binding the long variant of the polymorphism may also be involved in splicing of the gene.

It is unlikely that a single factor, or polymorphism in a monoaminergic candidate gene, is responsible for major changes in psychiatric phenotypes. Rather, these changes are caused by a combination of genetic mechanisms involving several candidate genes. Transcription
factors, such as AP-2, have the ability to simultaneously regulate the expression of many different genes, e.g., monoaminergic candidate genes. Thus, some phenotypes may be explained by polymorphisms in genes encoding transcription factors. To increase the knowledge of transcription factors in relation to neuropsychiatric conditions and personality, further studies on polymorphisms in transcription factor genes, and also on the functional mechanisms of transcription factors, are needed.
3. SUMMARY IN SWEDISH, SVENSK SAMMANFATTNING


Delarbete I

kontroller. AP-2α-nivåerna var även nedreglerade efter 3 dagars behandling. Dessa resultat är i linje med tidigare resultat av vår forskargrupp. Efter 21 dagars behandling var det ingen skillnad mellan citaloprambehandlade djur och kontrolldjuren, dvs. nedregleringen av AP-2 efter 7 dagars citaloprambehandling var temporär. Vi spekulerar, baserat på data från denna studie, att AP-2 är involverad i den molekylära verkningsmekanismen för citalopram.

**Delarbete II**

Denna studie bygger på delarbete I samt på en tidigare studie av vår forskargrupp som visar att behandling med MAO-inhibitorn fenelzin, som är ett annat typ av antidepressivt läkemedel än citalopram som undersöcktes i delarbete I, uppreglerar den DNA-bindande aktiviteten av AP-2 samt nivåerna av AP-2α och AP-2β i helhjärna hos råttor efter 10 dagars behandling. I delarbete II behandlades råttor med citalopram och fenelzin (kontroller med koksaltlösning) i 1, 7 och 21 dagar. Efter avlivning dissekterades hjärnstammen ut och nukleära extrakt preparerades fram. De monoaminerga cellkärnorna finns i hjärnstammen och eftersom AP-2 har bindingsställen i flera monoaminerga gener samt att vi tidigare sett kopplingar mellan nivåer av AP-2α och AP-2β och flera monoaminerga parametrar valde vi att i denna studie undersöka hjärnstammen istället för hela hjärnan (som i delarbete I). De nukleära extrakten användes för med ELISA undersöka nivåerna av AP-2α och AP-2β protein. Vid behandling med citalopram fann vi ingen skillnad i vare sig AP-2α eller AP-2β nivåer mellan behandlade djur och kontroller. Behandling med fenelzin, å andra sidan, visade sig uppreglera AP-2α och AP-2β nivåerna efter 7 dagar jämfört med kontrollgruppen men efter 21 dagar var de fenelzinbehandlade djuren åter på kontrollnivå igen. Baserat på resultat från denna studie samt delarbete I spekulerar vi i att den nedreglering av AP-2α och AP-2β nivåerna som vi tidigare sett efter 7 och 10 dagars citaloprambehandling sker i någon annan region än i hjärnstammen, sannolikt i de monoaminerga projektionsområdena. Den uppreglering av AP-2α och AP-2β nivåerna som vi såg i hjärnstammen efter 7 dagars behandling med fenelzin stämmer väl överens med tidigare resultat. Vi spekulerar i att denna uppreglering är involverad i presynaptiska mekanismer i de monoaminerga kärnorna i hjärnstammen och vidare att uppregleringen av AP-2α och AP-2β kan vara involverad i de antidepressiva
verkningsmekanismerna eller i de bieffekter som uppkommer under de första behandlingsveckorna.

**Delarbete III**


**Delarbete IV**

personlighet och AP-2β intron 2 genotyp. Av dessa var 163 kvinnor och 270 män. Personligheten uppskattades med hjälp av fem olika personlighetsskattningsskalar; KSP, Swedish Universities Scales of Personality (SSP), Health-Relevant 5- Factor Personality Inventory (HP5i), Temperament and Character Inventory (TCI), och the Revised NEO Personality Inventory (NEO-PI-R). Kvinnor som var homozygoter för den långa AP-2β allelen visade sig ha lägre ”muscular tension” (KSP), ”somatic trait anxiety” (SSP), ”trait irritability” (SSP), ”mistrust” (SSP) och ”negative affectivity” (HP5i) än kvinnor som är heterozygoter eller homozygoter för den korta allelen. För männen däremot, fanns det inga kopplingar mellan AP-2β genotyp och något personlighetsdrag för någon av de olika personlighetsskalorna. Dessa resultat bekräftar tidigare data och tyder på att AP-2β genotyp är en faktor av betydelse för personlighet.

**Delarbete V**

Att vi sett kopplingar mellan AP-2β intron 2 genotyp och bland annat personlighet (se delarbete IV) och MAO i blodplättar (delarbete III) visar att möjligheten finns att AP-2β intron 2 polymorfismen är funktionell. Vi ville i delarbete V bland annat undersöka om de kopplingar till AP-2β intron 2 polymorfismen som vi tidigare hittat beror på att AP-2β intron 2 genotypen samuttrycks med en annan polymorfism i AP-2β genen som i sig skulle kunna vara funktionell. Den enda polymorfismen i AP-2β genen som man hittills hittat förutom i intron 2 ligger i promotorregionen, -67 G/A. Vi undersökte därför om AP-2β intron 2 polymorfismen och AP-2β promotorpolymorfismen är samuttryckta. Vi använde 155 av de individer som i delarbete III genotypades med avseende på AP-2β intron 2 polymorfismen, för att i delarbete V genotypa dem med avseende på AP-2β promotorpolymorfismen. Med hjälp av ett statistiskt datorprogram visade vi att dessa polymorfismen inte var samuttryckta. En annan möjlighet som skulle kunna förklara de samband med AP-2β intron 2 polymorfismen som vi tidigare sett är om olika regulatoriska proteiner binder till de olika varianterna av polymorfismen, vilket skulle kunna resultera i olika uttrycksnivåer av AP-2β proteinet. Även detta undersöcktes i delarbete V. Med hjälp av EMSA (Elecrophoretic Mobility Shift Assay) undersökte vi bindningsmönstret mellan nukleära proteiner från humanhjärna och inmärkt probe av de olika varianterna av polymorfismen. Vi fann att ett protein band endast till den långa varianten av AP-2β intron 2 polymorfismen. Fyndet innebär att möjligheten finns att detta
protein är involverat i regleringen av uttrycket av AP-2β proteinen, dvs. att beroende på vilken genotyp av AP-2β intron 2 polymorfismen man har får man olika uttrycksnivåer av AP-2β proteinen. Studier av den funktionella betydelsen av proteinen krävs dock för att bekräfta detta.
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