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Transcription Factor AP-2 in Relation to Serotonergic Functions in the Central Nervous System

BY

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ABSTRACT

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Eukaryotic gene transcription plays a regulatory role in mammalian developmental processes. It has been shown that transcriptional control is an important mechanism for specification of neurotransmitter phenotypes. In the mammalian central nervous system, the transcription factor AP-2 family is one of the critical regulatory factors for neural gene expression and neuronal development. It has been shown that several genes in the monoaminergic systems have AP-2 binding sites in regulatory regions, suggesting a regulatory role of AP-2 also in the adult brain. Brainstem monoamines are implicated in the expression of personality traits and imbalances in these systems may give rise to psychiatric disorders.

The gene encoding AP-2β includes a polymorphic region consisting of a tetranucleotide repeat of $[CAAA]_{4-5}$ in intron 2. Studies on AP-2β genotype in relation to personality and platelet MAO activity, a trait-dependant marker for personality, are presented in this thesis. Furthermore, correlations between brainstem levels of AP-2α and AP-2β and monoamine turnover in projection areas in rat forebrain are reported. These results strengthen the notion that the AP-2 family is important regulators of the monoaminergic systems in the adult brain. Furthermore, two studies are presented in this thesis with analyses indicating a role for AP-2 in the molecular mechanism of antidepressant drugs.

Altogether, this thesis presents data supporting our notion that the transcription factor AP-2 family is involved in the regulation of the monoaminergic systems both pre- and postnatally, and, therefore, might be involved in the pathophysiology of neuropsychiatric disorders.

Key Words: CNS, serotonin, transcription factors, AP-2, personality, antidepressants

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

I. **Damberg M**, Garpenstrand H, Alfredsson J, Ekblom J, Forslund K, Rylander G, and Oreland L. A polymorphic region in the human transcription factor AP-2β gene is associated with specific personality traits. *Molecular Psychiatry 2000 Mar;5(2):220-4.*

II. **Damberg M**, Garpenstrand H, Berggård C, Åsberg M, Hallman J, and Oreland L. The genotype of human transcription factor AP-2β is associated with platelet monoamine oxidase B activity. *Neuroscience Letters 2000 Sep 22;291(3):204-6*

III. **Damberg M**, Eller M, Tõnissar M, Oreland L, and Harro J. Levels of transcription factors AP-2α and AP-2β in the brainstem are correlated to monoamine turnover in the rat forebrain. *Neuroscience Letters 2001 Nov 2;313(1-2):102-4.*

IV. **Damberg M**, Ekblom J, and Oreland L. Chronic pharmacological treatment with certain antidepressants alters the expression and DNA-binding activity of transcription factor AP-2. *Life Sciences 2000 Dec 29;68(6):669-78.*

V. Berggård C, **Damberg M**, and Oreland L. Chronic citalopram treatment induces time-dependent changes in the expression and DNA-binding activity of transcription factor AP-2 in rat brain. *European Journal of Neuropsychopharmacology 2002, in press.*

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LIST OF ABBREVIATIONS

Some of the abbreviations present in the thesis are listed below.

AADC	Aromatic amino acid decarboxylase
ATP	2'-Deoxyadenosine 5´-triphosphate
β-PEA	2-Phenylethylamine
cDNA	Complementary DNA
ChAT	Choline acetyl transferase
CNS	Central nervous system
cpm	Counts per minute
CSF	Cerebrospinal fluid
CTP	2'-Deoxycytidine 5´-triphosphate
DA	Dopamine
DAT	Dopamine transporter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
FAD	Flavine adenine nucleotide
GTP	2'-Deoxyguanosine 5´-triphosphate
HeLa	Immortal Henrietta Lack's cells
HPLC	High pressure liquid chromatography
kDa	Kilo Dalton
kb	Kilo base pairs
KSP	Karolinska scales of personality
MAO	Monoamine oxidase
MAO-I	Monoamine oxidase inhibitors
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
OD	Optical density
PCR	Polymerase chain reaction
pc	Post coitum
RNA	Ribonucleic acid
SNARI	Selective noradrenaline reuptake inhibitors
SNRI	Serotonin and noradrenaline reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitors
TCA	Tricyclic antidepressant
TTP	2'-Deoxythymidine 5´-triphosphate
5-HIAA	5-Hydroxyindoleaceticacid
5-HT	5-Hydroxytryptamine, serotonin
5-HTT	5-Hydroxytryptamine transporter
5-HTP	5-Hydroxytryptophan

INTRODUCTION

A brief background

Over the last decades there has been an increasing optimism about applying molecular genetics to human behavior and disease. A large number of articles have been published showing associations of specific gene alleles to certain psychiatric syndromes, and still many studies on single candidate genes are being performed. In some cases these studies have yielded interesting results, but quite often the results have been difficult to reproduce, probably because the effect of a single gene polymorphism rarely results in a great change in the phenotype. Recently, researchers have started to discuss the possibility to identify combinations of genes that are all linked to one disease or syndrome, (for a review, see (Comings 1998). In the present thesis, we would like to extend this notion with the suggestion that the expression of several of these candidate genes is regulated by the same transcription factors. In that case we are focusing on proteins with an ability to regulate the expression of several previously identified candidate genes in the monoaminergic systems, that have been shown to be involved in the expression of personality traits and in the pathogenesis of psychiatric disorders.

Transcription factors are proteins with the ability to bind DNA, thereby regulating the expression of other genes. A number of transcription factors are critical during development of the brain, turning on and off gene expression at certain time points. For example, when considering development of the brainstem, where the serotonergic nuclei are located, specific transcription factors will control the synthesis and metabolism of serotonin by regulating the expression of genes encoding important enzymes, receptors and transporters within the serotonergic system.

The rapid development of the pharmaceutical screening technology has raised interest for rational targeting of transcription factors that are involved in human disease and in the molecular mechanisms of therapeutic agents (Peterson and Baichwal 1993). Many drugs used for pharmacotherapy of illnesses in the central nervous system seem to require transcriptional adaptation as part of their mechanism of action, e.g. antidepressants and neuroleptic agents.

One part of our research hypothesis is that if an organism has an abnormally, altered level of certain transcription factors during development of the brain this will

consequently give rise to an altered structural and biochemical milieu in the adult brain. Thus, if levels of transcription factors of importance for gene expression in the serotonergic system would be altered during development of the brain, or in the adult brain, it might predispose the development of affective disorders. Another part of our hypothesis is that transcription factors important for development and adult function of the brainstem may be downstream antidepressant drug targets, being responsible for the neuronal adaptations that occur before onset of the antidepressant effect.

In this thesis, the transcription factor AP-2 family has been investigated as a candidate gene in personality in humans, in relation to monoaminergic turnover in rat brain and as a downstream antidepressant drug target in rats.

The CNS serotonergic system

From the middle of the nineteenth century, scientists have been aware that a substance found in serum caused a powerful conctraction of smooth muscle organs, but it took more than a century before scientists succeeded in isolating this substance. The material isolated from serum was given the name ”serotonin”. Subsequently, when the material was purified and crystallised it was shown to be 5-hydroxytryptamine (5-HT). When 5-HT was first found within the mammalian CNS, a hypothesis was formed that various psychiatric disorders could be due to biochemical abnormalities in its metabolism. This theory was shown to be correct and is maintained by several research groups today.

Serotonergic neurons and their projections

Serotonin expressing cells in the adult central nervous system are located in the raphe nuclei, which are largely restricted to the basal plate of the pons and medulla in the brainstem (Rubenstein 1998). Most 5-HT-containing neurons are localised along the midline of the brainstem and send long axons to innervate a wide distribution of receiving areas throughout the nervous system, from the spinal cord to the cortex. The raphe nuclei contain two clusters of cells with different projection areas. The rostral raphe nuclei produce axonal projections to midbrain and forebrain regions, whereas the caudal raphe nuclei produce axons that descends to the spinal cord (Wallace, Petrusz et al. 1982;

Molliver 1987; Aitken and Tork 1988). Forebrain serotonin is derived nearly entirely from neurons located in the dorsal and median raphe nuclei of the midbrain. Prominent forebrain terminal regions include the hypothalamus, cortex, septum, hippocampus, amygdala, and the striatum. Furthermore, 5-HT neurons are highly bifurcated, indicating the function of several regions of the central nervous system simultaneously. These innervation patterns are relatively conserved throughout mammalian species, including man.

The pathway of serotonin synthesis and catabolism

Serotonin is found in many cells that are not neurons, such as blood platelets and immunologic mast cells. In fact, only about 1-2 % of whole body serotonin is found in the brain. Nevertheless, since 5-HT cannot cross the blood-brain barrier, it is obvious that brain cells must synthesise their own 5-HT. For brain cells, the first important step in the synthesis of 5-HT is the uptake of the amino acid tryptophan, which is the primary substrate for 5-HT synthesis, from the blood. Plasma tryptophan arises primarily from the diet. Following uptake, tryptophan is hydroxylated at the 5 position to form 5-hydroxytryptophan (5-HTP). The enzyme responsible for this rate-limiting reaction, tryptophan hydroxylase, occurs in low concentrations in most tissues, including the brain. 5-HTP is a short-lived intermediate and once synthesised from tryptophan, 5-HTP is almost immediately decarboxylated to yield serotonin. The enzyme responsible for this conversion is aromatic amino acid decarboxylase (AADC), which also has a function in the synthesis of dopamine in dopaminergic and noradrenergic neurons.

Serotonin nerve terminals possess a high affinity 5-HT re-uptake mechanism consiting of the serotonin transporter protein (5-HTT). As with the catecholamine-containing neurons, reuptake serves as major mechanism for the termination of action of synaptic serotonin and in maintaining transmitter homeostasis.

Serotonin is primarily metabolised by deamination of monoamine oxidase, an enzyme that will be discussed further on page 18. The product of this deamination, 5-hydroxyindoleacetaldehyde, is further oxidised to 5-hydroxyindoleacetic acid (5-HIAA). One can measure 5-HIAA in cerebrospinal fluid (CSF) by performing a lumbal puncture, and the level of 5-HIAA in CSF is a rough measure of 5-HT metabolism.

The spectrum of behaviors influenced by serotonin

Serotonin has been shown to influence a wide range of physiological systems, such as cardiovascular regulation, respiration, thermoregulation, and a variety of behavioral functions including sleep-wake cycles, appetite, aggression, sexual behavior, pain sensitivity and learning (Lucki 1998). Dysregulation of 5-HT neurotransmission has been found to influence a range of psychiatric disorders. Abnormalities in serotonergic neurotransmission have been reported to occur at several critical points in serotonin synthesis and transmission. Disorders with a likely serotonergic component include depression, a spectrum of anxiety disorders (generalised anxiety disorder, obsessive compulsive disorder, panic disorder, and social phobia), schizophrenia, and eating disorders such as anorexia nervosa, bulimia and binge-eating disorder (Hartmann, Kunig et al. 1993; Brewerton 1995; Brewerton and Jimerson 1996; Kent, Coplan et al. 1998; Lucki 1998; McDonough and Kennedy 2002; Sawa and Snyder 2002; Stein, Westenberg et al. 2002). In addition, a less structured range of impulse-control disorders or personality traits has been associated to 5-HT function, including substance abuse, gambling, obsessive control, and attention-deficit disorder (Lucki 1998). Studies on human subjects have investigated the association of CSF 5-HIAA levels with depression and other behavioral characteristics. It was shown that a subgroup of depressed patients displayed reduced concentrations of CSF 5-HIAA levels, for a review see (Brown and Goodwin 1986; Tuinier, Verhoeven et al. 1995; Asberg 1997). Subsequent studies suggested that low CSF 5-HIAA levels occured preferentially in depressed patients who had attempted suicide before hospital admission. A common behavioral characteristic or trait distinguishing patients with low CSF 5-HIAA was impulsive and destructive behaviors, particularly where aggression and violence were involved, irrespective of the psychiatric diagnostic category (Asberg, Nordstrom et al. 1986; Brown and Linnoila 1990).

The involvement of 5-HT in many behavioral functions has caused the speculation that 5-HT may have a capacity to integrate a variety of behavioral functions. A general theory of 5-HT function in behavior can help to account for why 5-HT appears to influence so many behaviors, but it is unlikely that 5-HT as a single neurotransmitter would be the principal or sole mediator of any of these behaviors. Most surely these

behaviors are expressed by the involvement of a pattern of neuronal changes and adaptations induced by a combination of neurotransmitters, such as dopamine, noradrenaline and 5-HT.

Serotonin receptors

A vast amount of new information has become available concerning the various 5-HT receptor subtypes and their functional and structural characteristics. Serotonin receptors are highly heterogenous, and gene cloning has lead to the discovery and recognition of previously unknown 5-HT receptors and has facilitated their classification. There are at least 14 subtypes of 5-HT receptors cloned from mammalian tissue (Lauder 1993) The majority of 5-HT receptors belong to the large family of receptors interacting with G-proteins, except for the 5-HT_3 receptor, which is a ligand-gated ion-channel. The 5-HT receptors belonging to the G-protein receptor superfamily are characterised by the presence of seven transmembrane domains and have the ability to alter G-protein-dependant processes. This group of 5-HT receptors can be divided into families based on their coupling to second messengers and amino acid sequence homology.

Another important recognition site for 5-HT is the serotonin transporter, which consists of 12 membrane-spanning domains. Transporter proteins are used as drug targets and a number of selective 5-HT re-uptake inhibitors have been developed by the pharmaceutical industry.

Transcription factors

General aspects of transcription factors

The process of transcription, whereby an RNA product is produced from the DNA, is an essential element in gene expression. An enormous amount of detailed information has been gathered concerning how genes in mammalian cells are transcribed into mRNA and further translated into proteins (Darnell 1982; Faisst and Meyer 1992; Latchman 1997). The basic components required for specific gene transcription in eukaryotes include an RNA synthesising enzyme called RNA polymerase. Eukaryotic RNA polymerases cannot

initiate transcription on their own. They require a set of DNA-binding proteins called general transcription factors, which must be assembled at the promoter before transcription can begin. Initiation of transcription can be up- or down-regulated by many sequence-specific eukaryotic transcription factors binding upstream of the transcription initiation site (Faisst and Meyer 1992), see figure 1.

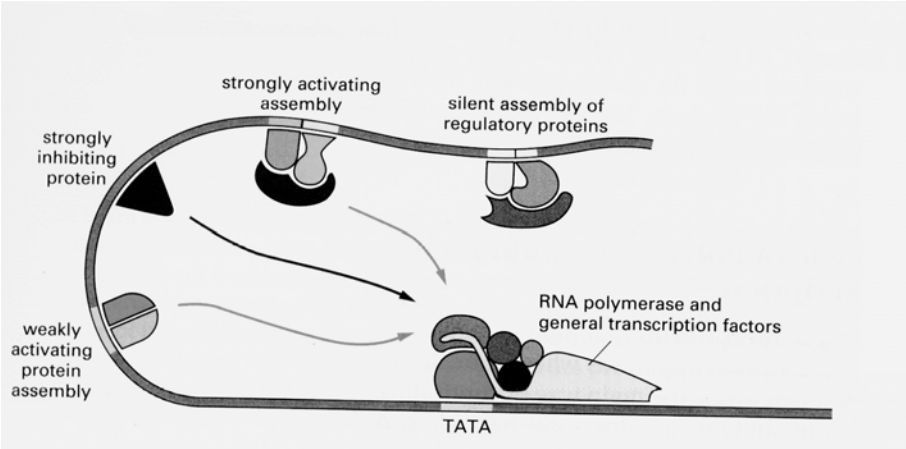

Figure 1. Integration of transcription factors at a promoter. (Alberts et al, Molecular biology of the cell, page 429).

Initiation of transcription by RNA polymerase II is a complex process that requires the orchestered function of several factors. Specific gene sequences are recognized by transcription factors that modulate RNA polymerase activity. Hundreds of such DNA-binding proteins, have been characterised during the last few years (Faisst and Meyer 1992). The temporal and spatial expression pattern of these factors, as well as their molecular role, is often completely unknown. In many cases these factors are expressed in a tissue-specific fashion. It is assumed that transcriptional changes are important in neuronal adaptive mechanisms in the adult brain, e.g. up- or down-regulation of receptors for neurotransmitters and synaptic remodelling. Many drugs used for pharmacotherapy of illnesses in the central nervous system seem to require transcriptional adaptation as part of their mechanism of action, e.g. antidepressants and neuroleptic agents.

Extrapolation of genomic data from primitive organisms suggests that there may be as many as 7000 genes encoding DNA-binding proteins in the human genome. Consequently, a high number of important transcription factors in the CNS remains to be discovered which provides a central research challenge for the future. A first step in this direction may be to identify abundant DNA-binding proteins that are restricted in their expression to the CNS.

Transcription factor AP-2 family

AP-2 (Activating protein-2) is a cell type-specific DNA-binding transcription factor family that has the ability to specifically regulate the expression of other genes in vertebrate organisms. AP-2 is a family of different, yet closely related, proteins with a molecular weight around 50 kDa. AP-2 family members are expressed from different genes. Four different isoforms of AP-2, i.e., AP-2α, AP-2ß, AP-2γ, and AP-2δ have been identified (Williams, Admon et al. 1988; Moser, Imhof et al. 1995; Chazaud, Oulad-Abdelghani et al. 1996; Oulad-Abdelghani, Bouillet et al. 1996; Zhao, Satoda et al. 2001). Accumulating evidence has established that AP-2 participates in many kinds of specific gene expression in mammals. Especially, AP-2 is one of the critical factors for neural gene expression (Mitchell, Timmons et al. 1991), and various neural genes contain AP-2 binding sites in their regulatory regions. All AP-2 isoforms reveal a unique modular structure consisting of an amino-terminal proline- and glutamine-rich transcriptional activation domain and a complex helix-span-helix motif necessary and sufficient for dimerisation and site-specific DNA binding (Williams and Tjian 1991; Williams and Tjian 1991). The proteins display similar biochemical properties and they differ in their N-terminal transcription activation domains, but show high conservation (75-85%) within their DNA binding and dimerisation domains. They all bind to GC-rich sequences in the genome (Bosher, Totty et al. 1996). The cis-acting DNA sequences 5'-(G/C)CCCA(G/C)(G/C)(G/C)-3' and the palindromic sequence 5'-GCCNNNGGC-3' are considered as consensus AP-2 binding sites for all AP-2 isoforms (Greco, Zellmer et al. 1995; Bosher, Totty et al. 1996). It is therefore possible that during evolution, different AP-2 genes have evolved from a common ancestor to allow greater flexibility in tissue-

specific gene regulation (Meier, Koedood et al. 1995), while their biochemical functions have been conserved.

Two different signal-transduction pathways influence the function of AP-2. AP-2 seems to mediate transcriptional activation in response to both the phorbol-ester- and diacylglycerol-activated protein kinase C, and the cAMP-dependent protein kinase A (Roesler, Vandenbark et al. 1988; Moser, Imhof et al. 1995). Moreover, AP-2 gene expression is subject to positive autoregulatory mechanisms with its own gene product (Bauer, Imhof et al. 1994).

Transcription factor AP-2α was purified and cloned from HeLa cells (Mitchell, Wang et al. 1987; Williams, Admon et al. 1988) and the gene encoding AP-2α is located on chromosome 6p24 (Kawanishi, Harada et al. 2000). In mice, there are several splice variants of AP-2α, named AP-2α variant 1-4, whose mRNAs were observed to overlap spatially and temporally in the mouse embryo (Meier, Koedood et al. 1995). Another AP-2α splice variant, named AP-2B, has been shown to be a negative regulator of the AP-2α gene (Buettner, Moser et al. 1994).

Transcription factor AP-2β was cloned and characterized by Moser et al 1995 (Moser, Imhof et al. 1995). The gene encoding AP-2β is located on chromosome 6p12-p21.1 and includes a polymorphic region consisting of a variable number of [CAAA] repeats located in the second intron between nucleotides 12593 and 12612, close to the 3'-splice site of exon 2. The [CAAA] sequence is repeated four or five times (Moser, Pscherer et al. 1997). The functional importance of this variable region has not yet been elucidated, but investigations regarding functionality is currently being performed by our research group. We have linked a specific genotype of this polymorphism to personality traits (paper I), platelet MAO activity (paper II) and binge-eating disorder in females (Damberg, Garpenstrand et al. 2001).

Transcription factor AP-2γ (also called AP-2.2) was cloned and characterised in 1996 (Oulad-Abdelghani, Bouillet et al. 1996). AP-2γ is co-expressed with the other AP-2 isoforms in several brain regions, and expression of AP-2γ is lowest among the AP-2 isoforms both during development and in the adult brain (Moser, Imhof et al. 1995; Oulad-Abdelghani, Bouillet et al. 1996; Shimada, Konishi et al. 1999).

Recently a fourth member of the AP-2 family, i.e. AP-2δ, was cloned from a mouse cDNA library (Zhao, Satoda et al. 2001). A partial predicted AP-2δ gene was identified in tandem with AP-2β on human chromosome 6p12-p21.1. The proline rich motif and critical residues in the transactivating domain, which are highly conserved in the AP-2 family and believed necessary for transactivation, were divergent in AP-2δ. The unique protein sequence and functional features of AP-2δ suggest mechanisms, besides tissue-specific AP-2 gene expression, for specific control of target gene activation. Regarding the structural and transactivational differencies between AP-2δ and the other AP-2 subtypes further studies are needed to indicate possible relevance for the development and function in the adult CNS.

Expression of AP-2 during development of the brain

Gene expression of the AP-2 family during development has been investigated. The mRNA of the subtypes AP-2α, AP-2β, and AP-2γ exhibits a distinct distribution pattern in the developing brain of mouse embryos. Altogether, transcription factor AP-2 has been identified as an important regulator of gene expression during embryonic development of many neural, neuroectodermal, and ectodermal tissues including the midbrain, hindbrain, spinal cord, cranial and dorsal root ganglia, facial mesenchyme, limb bud-mesenchyme, mesometa-nephric mesenchyme, and skin (Mitchell, Timmons et al. 1991).

Studies on mice have shown that expression of the most abundant isforms, i.e. AP-2α and AP-2β, starts at day 8 post coitum (pc) in the lateral head mesenchyme and in the extraembryonic trophoblast. Their expression patterns were identical until day 10 pc but diverged significantly during later stages of development (Mitchell, Timmons et al. 1991; Moser, Ruschoff et al. 1997). From day 11 forward, specific expression patterns of AP-2α and AP-2β mRNA were observed. A complex and dynamic expression pattern is most evident in the primordia of the midbrain, hindbrain, and medulla oblongata. In contrast to early stages until day 10 pc (when both mRNAs are detected at equal ratios), AP-2α expression in the midbrain ceases entirely at this point at late stages and is most prominent posterior of the midbrain-hindbrain junction. In contrast AP-2β signals become very prominent during later embryonic stages. However, both AP-2α and AP-2β mRNA

expression persists in adulthood in the cerebellum and the brainstem. This indicates that both genes are expressed with different temporal and spatial patterns during embryonic development. (Mitchell, Timmons et al. 1991; Moser, Ruschoff et al. 1997).

Expression of AP-2 in the adult brain

Studies on expression patterns in the adult CNS have previously only been performed in mice (Shimada, Konishi et al. 1999). We are currently performing studies on expression patterns in the adult rat brain. Interestingly, preliminary data obtained from our studies differ from the published data on mice, suggesting species differences in regional distribution of the AP-2 isoforms.

In mice, AP-2 was essentially expressed in most regions of the brain, the hippocampus and cerebellum Purkinje cells exhibited a relatively high concentration of transcripts of any of the investigated AP-2 subtypes. The expression of AP-2β mRNA was higher than that of AP-2α in many regions. Especially, the olfactory bulb, hippocampus, brainstem, cerebellum, and cerebral cortex contained an abundance of these mRNAs (Shimada, Konishi et al. 1999). It was shown that the expression of AP-2γ was weak throughout the brain. AP-2α and AP-2β were co-expressed in many regions of the adult mouse brain, each having its own specific intensity, but their precise distribution profiles were not exactly the same (Shimada, Konishi et al. 1999).

In our pilot study on adult rat brain we have used immunohistochemistry with specific AP-2α and AP-2β antibodies to analyse specific distribution patterns of the two isoforms in the adult rat brain. Our preliminary data shows that AP-2α is expressed in the hypothalamus, septum, thalamus, hippocampus, and frontal cortex. AP-2β seems to be expressed in the septum, thalamus, stria medullaris and in the frontal cortex. This suggests that there are species differencies regarding specific regional expression of the AP-2 subtypes. However, further analyses are needed to confirm these findings.

Transcription factor AP-2 and the monoaminergic systems

Identification of potential binding sites for transcription factors in target genes is a way to investigate relevance for a certain transcription factor to a certain gene family. If several

binding sites for a transcription factor is detected within a gene family one can assume that this specific transcription factor is somehow involved in the expression of the genes within that gene family. Since there are few functional studies indicating transcription factor AP-2 involvement in the expression of monoaminergic genes, we have based our research hypothesis on the fact that several genes in the monoaminergic systems display multiple binding sites for AP-2 in their regulatory regions. Although few data are available to date, it is highly likely that transcriptional events including transcription factors, are responsive to 5-HT receptor activation in the brain, possibly with effects on AP-2 (Bhat, Cole et al. 1992; Lucas, Segu et al. 1997). Furthermore, the involvement of AP-2 subtypes during development of the brainstem and the expression of AP-2 subtypes in the adult brainstem adds further interest to elucidate the involvement of AP-2 in the monoaminergic system. In paper III, we present positive correlations between brainstem levels of AP-2α and AP-2β to monoamine turnover in the rat forebrain. These data indicate a regulatory role for AP-2 in the adult monoaminergic systems.

As mentioned earlier, several of the genes involved in brainstem CNS transmitter systems, of fundamental importance for behavior, have multiple AP-2 binding sites in their regulatory regions, e.g., the genes encoding dopamine β-hydroxylase (*DβH*), dopamine transporter (*DAT*) (Gen Bank acc. no. U13956), dopamine D1A receptor ($D1_ADR$), aromatic L-amino acid decarboxylase (*AADC*), 5-HT transporter (*5-HTT*), rat 5-ht_{2a} receptor ($5\text{-}ht_{2a}\,rec$), tryptophan hydroxylase, tyrosin hydroxylase and choline acetyl transferase (*ChAT*) (Kobayashi, Kurosawa et al. 1989; McMahon, Kvetnansky et al. 1992; McMahon and Sabban 1992; Hahn, Hahn et al. 1993; Du, Wilcox et al. 1994; Greco, Zellmer et al. 1995; Baskin, Li et al. 1997; Bradley and Blakely 1997; Healy and O'Rourke 1997; Quirin-Stricker, Mauvais et al. 1997).

Since important genes encoding proteins in the monaminergic systems have binding sites for AP-2 in their regulatory regions, one might speculate that the expression of different isoforms of AP-2 influence mood and personality, not only due to their role during development of the brain, but also due to their function during adulthood.

Monoamine oxidase

General aspects of monoamine oxidases

Monoamine oxidase (MAO; E.C. 1.4.3.4) in mammals is in fact not a single enzyme but two mitochondrial enzymes divided into the separate forms MAO-A and MAO-B, which are encoded by different genes. The two isoenzymes are flavoproteins with flavine adenine nucleotide (FAD) as co-enzyme covalently bound to the C-terminal region (Oreland 1971). They have a molecular weight of approximately 65 kDa each, and display a 70 % sequence identity on the amino acid level (Shih and Chen 1999). These enzymes are important in catalysing the oxidative deamination of many exogenous and endogenous monoamines. The endogenous amines include the neurotransmitters dopamine (DA), 5-hydroxytryptamine/serotonin (5-HT), noradrenaline (NA), and adrenaline. Also trace amines such as β-phenylethylamine (β-PEA) and tyramine are deaminated by MAO.

MAO-A and MAO-B have different substrate preferences, inhibitor specificities, and tissue distribution. In the human brain, 5-HT and NA are mainly deaminated by MAO-A. Dopamine functions as a substrate for both enzyme isoforms. However, in the human brain deamination of DA is primarily catalysed by MAO-B (Oreland, Arai et al. 1983). The enzymes are mainly sub-cellularly localised to the inner surface of the outer mitochondrial membrane and most human tissues express both forms of MAO. For example, both MAO-A and MAO-B are abundant in the brain. However, in platelets and lymphocytes only the MAO-B isoform is expressed, and in the placenta only MAO-A is detected, (for a review see (Shih, Chen et al. 1999).

Clincial observations and genetics of platelet MAO

In human platelets MAO-B is exclusively expressed and has the same amino acid sequence as brain MAO-B (Chen, Wu et al. 1993). However, the specific activities of brain and platelet MAO seem not to be significantly correlated (Winblad, Gottfries et al. 1979; Young, Laws et al. 1986). The enzyme activity is characterised by a considerable variability between individuals and platelet MAO activity is stable during lifetime,

however with a possible minor increase after the age of 40. In humans, women have approximately 10-20 % higher platelet MAO activity when compared to men (Murphy, Donnelly et al. 1976).

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). In studies on non-human primates, associations between platelet MAO activity and behavior were reported, which confirmed the associations reported in humans (Redmond, Murphy et al. 1979). Investigations on the correlation between platelet MAO activity and neuropsychological measures strongly suggest platelet MAO as a stable biological marker for personality (af Klinteberg, Schalling et al. 1987; af Klinteberg, Oreland et al. 1990).

Both family and twin studies have shown a high degree of heritability of platelet MAO activity (Reveley, Reveley et al. 1983; Oxenstierna, Edman et al. 1986), and in the study of (Pedersen, Oreland et al. 1993) a heritability factor of about 0.75 was found for both males and females. In accordance with results in human studies, a strong genetic influence on platelet MAO activity has also been observed in rhesus monkeys (Murphy, Redmond et al. 1978). A few studies on structural differences of the MAOB gene in relation to specific enzymatic activity have been performed. However, these studies demonstrate that these differences seem to be of minor importance (Girmen, Baenziger et al. 1992; Garpenstrand, Ekblom et al. 2000). Most likely the regulation of MAO-B activity is governed at a transcriptional level, this process being controlled by several transcription factors (Zhu, Grimsby et al. 1992; Zhu, Chen et al. 1994). The specific characteristics of the human MAOB gene promoter have been described and several fragments have been investigated with reporter gene assays (Zhu, Grimsby et al. 1992; Zhu, Chen et al. 1994; Ekblom, Zhu et al. 1996; Ekblom, Garpenstrand et al. 1998). For example, the maximal promoter activity for MAOB has been observed for a 0.15 kb GC-rich sequence close to the first exon of the gene. We have also shown that two, yet unidentified, DNA-binding proteins, binding the 0.15 kb sequence, correlates to platelet MAO activity (Ekblom, Garpenstrand et al. 1998).

Antidepressants

General aspects of antidepressants

Over the years several different classes of antidepressants have evolved, etiher from empirical knowledge or from a direct strategy to develop a selective antidepressant drug. Seven major classes of antidepressants can be defined by their principal mechanisms of action, i) tricyclic antidepressants (TCA), e.g. *imipramin, klomipramin*, act by combined NE and 5-HT reuptake inhibiton, and have effects on multiple other neuronal receptors and fast sodium channels, ii) serotonin selective reuptake inhibitors (SSRI), e.g. *citalopram, fluoxetine*, work by inhibiting the reuptake of 5-HT, iii) selective noradrenaline reuptake inhibitors (SNARI), e.g. *reboxetin,* work by inhibiting the reuptake of NE, iv) serotonin noradrenaline reuptake inhibitor (SNRI), e.g. *venlafaxine*, act by combined NE and 5-HT reuptake inhibition, v) phenylpiperazine**,** e.g. *nefazodon*, are 5-HT_2 receptor blockers and display also 5-HT reuptake inhibition, vi) monoamineoxidase inhibitors (MAO-I), e.g. *moclobemide, phenelzin*, are either nonselective and irreversible or selective and/or reversible inhibitors of MAO-A or MAO-B, vii) other antidepressants,
e.g. *mirtazapine, mianserin*, have presynaptic noradrenergic alpha-2-receptor inhibition and postsynaptic 5-HT receptor inhibition.

It has been suggested that the therapeutic action of antidepressant drugs is directly related to their uptake-blocking capability. However, since the onset in therapeutic action of antidepressant drugs is usually delayed for several weeks, the hypothesis that the rapid, acute actions of these drugs in blocking NE and 5-HT reuptake are responsible for the long-term clinical antidepressant effects has been questioned. A common result of long-term treatment with different classes of antidepressants is an enhancement of monoaminergic synaptic transmission. This result is obtained in different ways by different antidepressant drug classes. Some drugs (TCA) are suggested to enhance 5-HT neurotransmission mainly by increasing the sensistivity of 5-HT_{1A} postsynaptic receptors. Other drugs (MAO-I and SSRI) mainly affect 5-HT autoreceptors that regulate the efficacy of neuronal firing or transmitter release, or both, in presynaptic neurons (Blier and de Montigny 1994; Mongeau, Blier et al. 1997). Elevation of NE and

5-HT levels in response to acute drug exposure is not consistent with the time course for the therapeutic action of antidepressant treatments, suggesting that their mechanism of action involves neuronal adaptations in addition to these acute effects (Sulser 1989; Hudson, Young et al. 1993; Duman, Heninger et al. 1994; Duman, Malberg et al. 1999) Several studies have shown that antidepressant treatment have effects on single transcription factors which might be responsible for the neuronal adaptations that are needed for the therapeutic effect to arise (Nibuya, Nestler et al. 1996; Manji, McNamara et al. 1999; Damberg, Ekblom et al. 2000; Thome, Sakai et al. 2000; Berggard, Damberg et al 2002 (Paper V)). Several research groups are currently discussing the fact that serotonergic gene transcriptional control regions, or transcription factors themselves could be future targets for antidepressant drugs (Hurley 1989; Butt and Karathanasis 1995; Heguy, Stewart et al. 1995; Pennypacker 1995; Nibuya, Nestler et al. 1996; Papavassiliou 1998; Popoli, Brunello et al. 2000; Damberg, Garpenstrand et al. 2001). Based on the growing body of evidence that expression variability of proteins that regulate the central 5-HT system is associated with complex behavioural traits, it specifically emphasises transcriptional control regions of serotonergic genes as potential targets for antidepressant drug development (Lesch and Heils 2000).

Selective serotonin reuptake inhibitors

In this thesis, studies on antidepressants were primarily performed with the SSRI citalopram (paper IV and paper V). Therefore, the following paragraph describes SSRIs more thoroghly than other classes of antidepressants.

Following their successful and effective use in the treatment of depression, the SSRIs are rapidly coming into use as first-line agents in treatment of anxiety disorders. SSRIs are currently indicated for use in panic disorder and obsessive compulsive disorder, and have been successfully employed in the treatment of social phobia, posttraumatic stress syndrome, eating disorders, and generalised anxiety disorder (Kent, Coplan et al. 1998). Although the putative antidepressant and anti-anxiety action of the SSRIs is generally understood to involve an increase in the extracellular serotonin concentrations in the synapse, resulting in increased postsynaptic receptor binding, this has not been clearly established. The SSRIs do block the reuptake of released serotonin, preventing it

from being transported back into the presynaptic neuron following discharge of the neuron. However, a straightforward increase in the synaptic concentration of serotonin may not be the end result, due to the presence of autoreceptors on the presynaptic neuron. These autoreceptors, which are located on both the cell body (5-HT_{1A}) and the axon (5-HT_{1D}), regulate the release of 5-HT and therefore effect the net amount of 5-HT available in the synapse. Long-term administration of SSRIs has been shown to desensitise these 5-HT autoreceptors, thereby increasing the availability of extracellular serotonin (Blier, Serrano et al. 1990; Lucki 1998). This may account for the latency to clinical efficacy, and provide support for an overall enhancement of 5-HT neurotransmission as the therapeutic mechansims of action of the SSRIs. Other suggestions regarding explanations of the latency in clinical efficacy is the need of neuronal adaptations that occur before the antidepressant effect is obtained. These neuronal adaptations might be regulated by transcription factors. Several studies have shown that single transcription factors are downstream molecular targets for SSRIs (Nibuya, Nestler et al. 1996; Thome, Sakai et al. 2000).

PRESENT INVESTIGATION

Aims of the present investigation

Paper I

To investigate the human AP-2β intron 2 polymorphism in relation to specific personality traits in healthy volunteers.

Paper II

To investigate the human AP-2β intron 2 polymorphism in relation to catalytic activity of platelet MAO in healthy volunteers.

Paper III

To analyse a possible correlation between brainstem levels of AP-2α and AP-2β and monoamine turnover in the forebrain of rats.

Paper IV

To investigate the effect of antidepressant treatment on the expression and DNA-binding activity of transcription factor AP-2 in rat brain.

Paper V

To analyse the effect of citalopram treatment on the expression and DNA-binding activity of AP-2 in rat brain in a time-dependent manner.

Methodological considerations

Blood sampling and preparation of DNA

Blood samples of approximately five ml were drawn into Vacutainer® tubes containting ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson, Franklin Lakes, NJ, USA) for prevention of clotting. From these samples, 700 µl of whole blood were pipetted into Eppendorf tubes and genomic DNA was subsequently extracted by use of Qiamp® DNA extraction kit (Qiagen GmdH, Hilden, Germany) and the DNA solution stored at –20°C until later use. For later estimation of trbc-MAO activity, platelet rich plasma was prepared by low speed centrifugation of the remaining blood within 24 hours. The platelet concentration was estimated electronically in a Thrombocounter-C® (Coulter Electronics Ltd., Luton, UK) and the platelet rich plasma was thereafter stored at – 80°C.

Genotyping procedure

For genotyping the AP-2β intron 2 polymorphism, polymerase chain reaction (PCR) was used. In the PCR, a large number of repeated cycles of synthesis of DNA, produce an exponentially increasing number of DNA between two short oligonucleotides, i.e. forward and reverse primers. These primers are located over the site where the variable region is located. Thus, differences in this intermediate DNA sequence can be detected when separating and visualising the amplified DNA sequence by electrophoresis. In the present investigation the following oligonucleotides were used as primers in the analysis of the AP-2β polymorphism:

AP-2β forward	5'-CCTACCACCAGAGCCAGGACCC-3'
AP-2β reverse	5'-CCCCCCCTCCAGAAGCATTCCT-3'

PCR protocols

For estimation of AP-2β genotypes, different PCR protocols were used in paper I and paper II. In paper I, the 20 µl reaction mixture contained 100 ng genomic DNA, PCR buffer (10mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$ pH 8.3, Boeringer Mannheim Scandinavia AB, Bromma, Sweden), 400 µM dNTP (100 µM each of dATP, dCTP, dTTP, dGTP), 20 pmol of each primer and 2 units Taq-DNA polymerase (Boeringer Mannheim Scandinavia AB, Bromma, Sweden). The PCR-reaction was amplified through 28 cycles on a RoboCycler®96 Temperature Cycler (Stratagene, La Jolla, CA) and each cycle consisted of a 95°C denaturation step for 60 seconds, a 56°C annealing step for 60 seconds and finally a 72°C elongation step for 60 seconds.

For genotyping in paper II, a 30 µl reaction mixture was used containing 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-DNA 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.

Electrophoresis

The PCR products (370 bp/366 bp) were analysed by electrophoresis on a 4 % denaturing polyacrylamide gel containing 25 g urea, 33 ml water, 3.1 ml of 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 % ammoniumpersulfate and 80 µl TEMED. The electrophoresis was run at 1.7 kV for 3 hours at 4°C. Prior to loading, the PCR-products were denaturated at 98°C for 2 minutes. Buffer used as running buffer was 1 x GTB. After electrophoresis, the PCR-products were detected by silver staining according to the protocol by Bassam and collaborators (Bassam, Caetano-Anolles et al. 1991).

Cloning and sequencing of PCR-products

In order to confirm that the correct region of the AP-2β gene was amplified, PCR-products from all genotypes 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 -20ºC. 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).

Subjects and estimation of personality traits

In paper I, subjects were volunteers living in the catchment area of the Karolinska hospital (Stockholm, Sweden). Individuals were randomly selected from the population register but stratified so as to obtain an approximately equal number of men and women within age intervals of five years. A total of 409 individuals were invited for a physical health examination as part of a survey initiated by the General Medicine Departement of the hospital. The 283 volunteers who consented to participate and appeared for the examination were asked, towards the end of the interview, which was conducted by a physician, whether they would like to participate in a subsequent psychiatric interview study. A total of 184 individuals, 88 men and 96 women, volunteered to participate in the psychiatric interview study. Out of the 184 individuals, 137 (64 men and 73 women) consented to deliver blood samples for determination of genetic markers. All subjects were unrelated individuals with no history of psychiatric disorders.

In paper II, the male subjects (n=156) were healthy blood-donors recruited at the Uppsala University Hospital. The female subjects consisted of two groups of women, one group of women with binge eating episodes (n=32) that were recruited by an advertisment in a Swedish magazine (Amelia®), and another group of healthy female volunteers (n=32). 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). Since the aim of this study was to analyse a possible association between AP-2β genotype and platelet MAO activity, these groups were merged into one group of females (n=64). All subjects were unrelated individuals with no history of psychiatric disorders.

Estimation of platelet MAO activity

Platelet MAO activities were analysed by a radiometric assay with ^{14}C-labelled 2-phenylethylamine (β-PEA) and/or tryptamine (Try) as substrates as previously described by Hallman and collaborators (Hallman, Oreland et al. 1987). Before analysis, the samples of platelet rich plasma were thawed and sonicated at 0°C during 4 x 10 seconds with intervals of 5 seconds for lysis of the enzyme containing platelets. Fifty µl of the sonicated plasma were added to 50 ml of 0.1 mM ^{14}C-β-PEA (0.5 µCi/ml) or 50 µl of 0.1 mM ^{14}C-Try (0.5 µCi/ml) in 0.1 M sodium phosphate nuffer (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. Thereafter, the radioactive aldehyde product formed was extracted, under vigorous shaking for 30 seconds, into 750 µl toulene:ethylacetate (1:1, vol/vol). The samples were then centrifuged at room temperature for 5 minutes at 1000 x g. 500 µl of the organic phase, including the aldehyde product, were pipetted into vials with 8 ml scintillation fluid and the amount of radioactive aldehyde product was subsequently quantified by scintillation analysis. Enzyme activity is expressed as nmol of substrate oxidised per 10^{10} platelets per minute. The two substrates, β-PEA and Try, were in used in parallell in order to increase the reliability of the assay, and all samples were analysed blindly and in duplicates.

Animals and treatment paradigms

In paper III, Male Wistar rats from the National Laboratory Animal Centre, Kuopio, Finland, weighing 286-360 g at the beginning of the experiment were used. Rats were

grouphoused after arrival and after two weeks of habituation housed individually in plastic cages with food and water ad libitum. A 12 hour light/dark cycle was applied.

In paper IV and V 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 ad libitum. In paper IV, rats were administered imipramine (10 mg/kg), citalopram (10 mg/kg), and LiCl (40 mg/kg) subcutaneously with daily injections. All drugs were dissolved in saline (NaCl, 9 mg/ml). The animals were sacrificed on day 11.

In paper V, rats were administered citalopram (10 mg/kg) subcutaneously for 1, 3, 7, and 21 days and they were sacrificed the day after their last injection. Sham treated animals recieved saline injections of the same volume as that given for drug treatments. After sacrifice the cerebrum was dissected and the right hemispheres were used for extraction of nuclear proteins for subsequent EMSA and ELISA analyses. The drugs used for these studies were obtained from the following sources: saline (NaCl, Pharmacia & Upjohn, Uppsala, Sweden), citalopram (H/S Lundbeck AB, Helsingborg, Sweden) and lithium chloride (KEBO, Stockholm, Sweden).

Preparation of nuclear extracts from rat brain

For preparation of nuclear proteins, different protocols were used in paper III, IV and V. In paper IV, nuclear proteins were extracted essentially according to the protocol by Dignam and co-workers (Dignam, Lebovitz et al. 1983). Rat brain (~700 mg) was homogenized in 2 ml of buffer A (10 M Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT). The homogenate was incubated on ice for 10 minutes. After incubation the homogenate was centrifuged for 15 minutes at 4000 x g at 4ºC. The nuclear pellets were resuspended in 1.5 ml of buffer B (20mM Hepes. pH 7.9, 25% glycerol, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) and lysed with a pestle. To this lysate 500 µl of buffer C (50 ml buffer B, 1.2 M KCl) was added. Incubation on ice for 30 minutes and occasional vortex mixing was followed by a high speed centrifugation at 25.000 x g for 30 minutes at 4ºC. The supernatant was dialysed against 200 ml of buffer D (20 mM Hepes, 20% glycerol, 100 mM KCl, 0.2 mM EDTA) for 5 hours at 4ºC. The nuclear protein aliqouts were frozen on dry ice and stored at -80ºC

until later use. Totalprotein concentration was determined for all nuclear extracts by the method of (Lowry et al 1951). The concentration of the nuclear extracts were ~2.0 µg/µl.

In paper V, rat brain (~700 mg) and in paper III rat brainstem (~500 mg) was homogenized in 3 ml buffer A (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM 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 30 seconds at 14000 rpm in 4°C. The pellet was resuspended in 500 µl buffer C (20 mM HEPES, 0.4 M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM 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, i.e. the nuclear proteins, was aliqouted and stored at -80°C. Total protein concentration was determined for all nuclear extracts by the method of (Lowry et al. 1951). The concentration of the nuclear extracts were ~12.0 µg /µl.

Electrophoretic Mobility Shift Assay

A binding sequence is a region of dsDNA that can bind a regulatory protein. It is therefore possible to identify binding sequences upstream of a cloned gene by searching the relevant region for protein-binding sites. Today, three major approaches are available for studying this, i.e., electrophoretic mobility shift assay (EMSA), DNAse I footprinting (Galas and Schmitz 1978) and Methylation Interference Assay (Siebenlist and Gilbert 1980). EMSA is a simple and sensitive method for determining interactions between protein and DNA. This assay was developed by Fried and Crothers (Fried and Crothers 1981) and Garner and Revsin (Garner and Revzin 1981) for analysing protein-DNA interactions. The assay is based on the fact that a radiolabelled dsDNA fragment that has a protein bound to it is identified by its increased molecular mass, determined by non-denaturing polyacrylamide gel electrophoresis. Unlike the DNA footprinting techniques that rely on the loss of a signal to determine protein-DNA interactions (negative assay), the EMSA yields a positive signal, the appearance of a DNA fragment with altered mobility. However, the EMSA doesent give a direct readout of the DNA nucleotides that the protein is recognizing. For this type of information, a higher resolution technique such

as DNAse I footprinting or methylation interference is necessary. In paper IV and paper V EMSA technique was used to analyse DNA binding activity of AP-2.

Labelling of AP-2 probe

The AP-2 consensus dsDNA oligos (5'-GATCGAACTGACCGCCCGCGGCCCGT-3') were 5´-end labelled with T4 polynucleotide kinase. The labelling reaction was carried out in 20 µl of 0.5 M Tris-HCl pH 7.6, 100 mM $MgCl_2$, 100 mM 2-mercaptoethanol (United States Biochemical, Cleveland, Ohio) containing 10 pmol of AP-2 dsDNA oligo, 2 µl (20 µCi) γ-^{32}P-dATP (Amersham, Buckinghamshire, UK) and 5 units T4 polynucleotide kinase (United States Biochemical, Cleveland, Ohio). The labelling mixture was incubated for 45 minutes at 37°C followed by heat deactivation at 65ºC for 5 minutes. In order to separate unincorporated γ-^{32}P-dATP from the labelled AP-2 oligos, the labelling mix was electrophoresed on a 8 % non-denaturing polyacrylamide gel containing 8 ml 30 % acrylamide 37.5:1, 19 ml water, 3 ml 10xTBE, 70 µl TEMED and 500 µl 10 % ammoniumpersulphate, 0.25xTBE was used as running buffer. 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. The amount of radioactivity was measured in a scintillation counter. Typically, the labelled probes had a specific activity of ~2 x 10^5 cpm/pmol DNA.

EMSA binding reaction

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-oligo, 1 µg poly (dC-dI) and ~8 µg nuclear extract. The binding reaction mixture was incubated on ice for 30 minutes. Following incubation, the reaction mixture was loaded on the gel. A 4 % non-denaturing polyacrylamide gel containing 4 ml 30 % acrylamide 37.5:1, 25 ml water, 0.75 ml 10xTBE, 70 µl TEMED and 500 µl 10 % ammoniumpersulphate was used in the electrophoresis, 0.25xTBE was used as running buffer. The electrophoresis was run at 100 V for 3 hours at 4ºC.

Quantification of DNA-binding activity of AP-2

For quantification of the DNA binding activity of AP-2, different protocols were used in paper IV and V. In paper IV, the gel was removed after electrophoresis and dried for 45 minutes on a 3MM paper, thereafter the dried gel was attached to a Phosphor Imager® screen (Molecular Dynamics, CA, USA) for 1-3 days i.e., this time of exposure is considered to be within the linear range of the screen, subsequently the gels were analysed according to the instructions of the manufacturer. For each treatment group optical density (OD), i.e. DNA-binding activity of AP-2, was determined for the main band (AP-2), and the band corresponding to free probe, using Molecular Dynamics ImageQuant™ 3.3 software. The OD of the main band and free probe were calculated by a standardised procedure where the entire volume of the bands were included in the analyses. As an internal standardisation procedure, the ratio between the amount of protein bound to labelled probe in the main band and the amount of free probe was calculated and used as a value of the relative DNA binding activity of AP-2.

In paper V, the gel was removed after electrophoresis and dried for 60 minutes on a 3MM paper. Therafter the dried gel was attached to an autoradiography film and exposed for 1-2 days at -80ºC. For each gel, a known amount of radioactivly labelled ds-probe was added in three of the wells as a reference. For each treatment group 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 standardisation 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 of AP-2.

In both studies each treatment group consisted of five animals and these were analysed twice for accuracy. The DNA-binding activity of AP-2 was analysed in relation to each of the different treatment groups.

Specificity of AP-2 binding reaction

The dsDNA probe used in the EMSA analyses in order to analyse the DNA binding activity of AP-2 has been used in many other studies of AP-2, and it has been confirmed that AP-2 binds to the probe by interacting with the AP-2 consensus site. The specificity of the AP-2 binding was determined by competition with different concentrations (100 pmol - 1000pmol) of cold AP-2, mutated AP-2 (5'-GATCGAACTGACCGC**TT**GCGGCCCGT-3': mutated nucleotides are in bold), or AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3'), see figure 2. From the figure below it is obvious that excess of cold AP-2 probe competes with AP-2 binding to the labelled probe. In contrast, excess of a cold mutated AP-2 probe or a cold AP-1 probe does not affect AP-2 binding to the labelled probe.

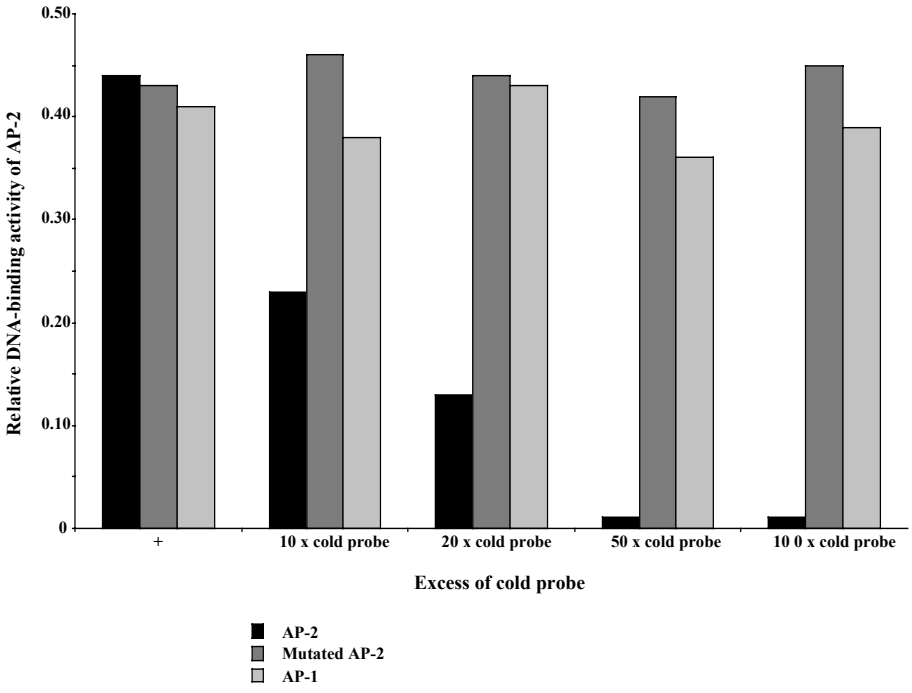

Figure 2. Specificity of the AP-2 binding reaction used in the EMSA analyses.

Enzyme-Linked Immunosorbent Assay

In paper IV we analysed levels of AP-2α and AP-2β according to the following protocol. A 96-well microtiterplate was coated with 50 µl (1µg/µl) affinity-purified polyclonal antibody (AP-2α, AP-2β, Santa Cruz Biotechnology) in 0.05 M carbonate-bicarbonate buffer pH 9.6. The plates were incubated overnight at 4°C covered with parafilm in a humidified chamber. Following the incubation the plates were washed three times with PBS (9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCL). After washing 200 µl blocking buffer (PBS containing 1 % BSA) was added to all wells in order to block non-specific binding. The plates were incubated for 60 minutes at room temperature. 50 µl nuclear protein homogenate (total protein concentration 50 µg protein/ml in PBS-T (0.5 ml Tween 20 to 1000 ml BSA) containing 0.5 % PBS) was added to the plates, and incubated for 60 minutes at room temperature. The nuclear protein homogenates were removed and the plates were washed once with 200 µl PBS-T containing 0.5% BSA. 50 µl anti-rat IgG, (Horseradish Peroxidase Conjugated Rabbit Affinity Isolated Antibody) diluted 1:500 with PBS-T containing 0.5% BSA, was added to all wells except those used for blank values. The plates were incubated for two hours in dark at room temperature. Secondary antibody was removed and the plates were washed three times with PBS-T and three times with water. Plates were slapped dry against a paper sheet. 100 µl TMB (3,3',5,5'-tetramethylbenzidine, DAKO one-step substrate system) was added to all wells After incubation in dark for 20 minutes, the reaction was stopped by adding 100 µl 4M HCL. The plates were analysed on a microtiter plate reader (Molecular Devices, Thermo Max) at 450 nm. The amount of AP-2 IgG was calculated from a standard curve for rat IgG. The amount of AP-2α and AP-2β was analyzed in relation to each of the different treatment groups. Each treatment group consisted of five rats and each rat was analysed six times for accuracy.

Due to optimization experiments we found that the method used in paper IV was not optimal to measure levels of AP-2. This made us optimise the protocol for analyses in paper V. To ensure that the results gained in paper IV were reliable we re-analysed the rat brain nuclear extracts used in study IV with the new optimised ELISA protocol. This confirmatory experiment showed that the results presented in paper IV was

reliable for citalopram and imipramine treated rats. However, lithium-chloride treated rats displayed lower levels of both AP-2α and AP-2β when compared to saline treated rats All treatment groups displayed significantly lower AP-2α and AP-2β levels than saline groups, $p<0.0001$ for all comparisons. Rawdata for both ELISA methods is presented in the following table.

Group (n=5)	AP-2α (paper IV)	AP-2β (paper IV)	AP-2α (re-analysed)	AP-2β (re-analysed)
Citalopram	2.81×10^{-4} ($SD \pm 5.1 \times 10^{-5}$)	3.70×10^{-4} ($SD \pm 6.2 \times 10^{-5}$)	1.32×10^{-4} ($SD \pm 1.5 \times 10^{-5}$)	9.15×10^{-5} ($SD \pm 7.6 \times 10^{-6}$)
Imipramin	4.69×10^{-4} ($SD \pm 4.7 \times 10^{-5}$)	4.08×10^{-4} ($SD \pm 4.4 \times 10^{-5}$)	1.21×10^{-4} ($SD \pm 1.2 \times 10^{-5}$)	8.45×10^{-5} ($SD \pm 8.7 \times 10^{-6}$)
Lithium-chloride	1.01×10^{-3} ($SD \pm 1.1 \times 10^{-5}$)	4.15×10^{-4} ($SD \pm 4.8 \times 10^{-5}$)	1.20×10^{-4} ($SD \pm 1.2 \times 10^{-5}$)	8.57×10^{-5} ($SD \pm 1.2 \times 10^{-5}$)
Saline	1.07×10^{-3} ($SD \pm 4.7 \times 10^{-5}$)	4.50×10^{-4} ($SD \pm 6.6 \times 10^{-5}$)	1.78×10^{-4} ($SD \pm 1.8 \times 10^{-5}$)	1.46×10^{-4} ($SD \pm 2.2 \times 10^{-5}$)

In paper V the ELISA protocol was the following. A 96-well microtiterplate was coated with 50 µl (0.06 µg/ml) nuclear extract diluted in 50 mM Carbonate-Biscarbonate buffer, pH 9.0. The plate was covered with parafilm and incubated overnigh at 4°C. Following the incubation the antigen solution was removed and 200 ml blocking buffer (PBS and 1% BSA) was added to each well and the plates were incubated for two hours at room temperature. The blocking buffer was removed and the plates were washed with PBS. Thereafter, the primary antibody (goat polyklonal AP-2α and AP-2β, 15 µl/ml respectively, Santa Cruz Biotechnology) diluted in blocking buffer was added. To each well 50 ml was added and the plate was incubated overnight at 4°C. After incubation the antibody was removed and the plate was washed three times with Wash buffer I (PBS, 0.05% Tween-20). Thereafter the secondary antibody (Donkey anti-goat IgG AP conjugated, SDS) diluted 1:350 in blocking buffer was added. To each well 50 ml was added and the plate was incubated for two hours at room temperature. After removal of the secondary antibody, the plate was washed three times with wash buffer I and once with wash buffer II (10mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.5). Therafter, 50 ml substrate (Phosphate substrate, 5 mg tablets, Sigma) diluted in 5 ml Wash buffer II was

added to each well. The reaction continued for 10-20 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 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 are plotted against optical denisty. The value from the standard curve was then divided with the concentration of the total protein in the nuclear extracts. The quota was used as a relative amount of AP-2α and AP-2β. Each treatment group consisted of five rats and each rat was analysed twice for accuracy.

High Performance Liquid Chromatography

Monoamines and their metabolites were estimated by HPLC with electrochemical detection. The rat brain tissues were homogenised with Bandelin Sonoplus ultrasonic homogeniser (Bandelin Electronic, Germany) in ice cold solution (5-30 ul/mg tissue) of 0.09 M perchloric acid containing 5 mM sodium bisulfite and 0.04 mM EDTA for avoiding oxidation, and dihydroxybenzylamine as internal standard. The homogenate was then centrifuged at 17000 x g for 20 min at 4°C. Aliquots (10 – 30 ul) of the supernatant obtained were chromotographed on a Lichrospher 100 RP-18 column (250 x 3 mm; 5 um) protected by a Supersphere RP18 (10 x 2 mm; 4 um) guard column. The separation was done in isocratic elution mode at column temperature 30°C using the mobile phase containing 0.05 M citric acid buffer at pH 3.6, 0.9 mM sodium octylsulfonate, 0.3 mM triethylamine, 0.02 mM EDTA, 1 mM KCl and 8-10 % acetonitrile. The chromatography system consisted of a HP 1100 series isocratic pump, a thermostated autosampler, a thermostated column compartment and an HP 1049 electrochemical detector (Hewlett Packard, Germany) with glassy carbon electrode. The measurements were done at an electrode potential of + 0.6 V versus the Ag/AgCl reference electrode.

Statistical analyses

The statistical comparisons between AP-2β genotype and KSP personality scores (paper I), and between AP-2β genotype and platelet MAO activity (paper II), were done by use

of analysis of variance (ANOVA) and Fisher's Protected Least Significant Difference (PLSD) Test. In paper III, The statistical comparisons between AP-2α and AP-2β levels were done by regression analyses. In paper IV, the statistical comparisons between antidepressant treated rats and controls were done by the use of analysis of variance (ANOVA) and Fisher's Protected Least Significant Difference (PLSD) Test. In paper V, the statistical comparisons between citalopram treated rats and saline treated rats for each time point were analysed using Mann-Whitney U test, testing equality between the treatment groups for each time point. When comparing the group of untreated rats to all treatment groups, analysis of variance (ANOVA) and Fisher's Protected Least Significant Difference (PLSD) Test were used. The statistical comparisons between citalopram treated rats and controls with regard to DNA-binding activity and protein levels, analysis of variance (ANOVA) and Fisher's Protected Least Significant Difference (PLSD) Test were used. All calculations were performed using StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). Results have been considered statistically significant when $p<0.05$.

Summary of papers included in the thesis

Paper I

Since the midbrain contains structures important for variables as mood and personality, we investigated if the genotype of AP-2β was associated with personality as estimated by the Karolinska Scales of Personality (KSP) (Damberg, Garpenstrand et al. 2000).

AP-2β genotype and KSP scores were determined for 137 Caucasian volunteers (73 females and 64 males). The outcome of this study showed that several personality traits were significantly associated with AP-2β genotype. The observed frequencies of genotypes in our sample were $[CAAA]_5$ 38.7%, $[CAAA]_{4\text{-}5}$ 47.4 %, and $[CAAA]_4$ 13.9 %. The personality traits muscular tension, guilt, somatic anxiety, psychastenia, and indirect aggression were significantly associated with homozygosity for the long $[CAAA]_5$ allele.

Females homozygous for the long allele displayed lower scores for anxiety-related personality traits and indirect aggression. In contrast, males homozygous for the long allele displayed higher scores for indirect aggression.

In conclusion, the associations between KSP personality traits and the AP-2β polymorphism suggest that AP-2β influences functions in the central nervous system. We speculate that these events take place especially during development of the midbrain neurotransmitter systems and therefore give rise to specific personality characteristics in the adult.

Paper II

With regard to the previously described association between platelet MAO activity and certain personality characteristics, it has been proposed that common developmental mechanisms may regulate the enzyme expression as well as other proteins involved in complex CNS functions. The association of both platelet MAO activity and genotype of AP-2β with personality characteristics might suggest that these two markers in some way are linked to each other. Moreover, since the regulatory regions of the MAOB gene, as well as several other genes of the central monoaminergic systems, contain potential binding sites for AP-2, we tested the hypothesis that transcription factor AP-2β is connected to the expression of the human *MAOB* gene in megakaryocytes, as estimated by platelet MAO activity (Damberg, Garpenstrand et al. 2000).

Male (n=158) and female subjects (n=64) were analysed with regard to AP-2β intron 2 genotype and trbc MAO activity. When analysing the male individuals we observed that those homozygous for the long allele displayed significantly lower platelet MAO activity as compared to those with one or two short alleles, ($F = 18.054$, $p < 0.0001$), see figure 1. Similarly, women homozygous for the long allele had lower platelet MAO activity than those with one or two short alleles, ($F = 6.163$, $p = 0.0158$), see figure 3. Based on the present data we suggest that intron 2 genotype of AP-2β is of relevance for the regulation of platelet MAO protein expression.

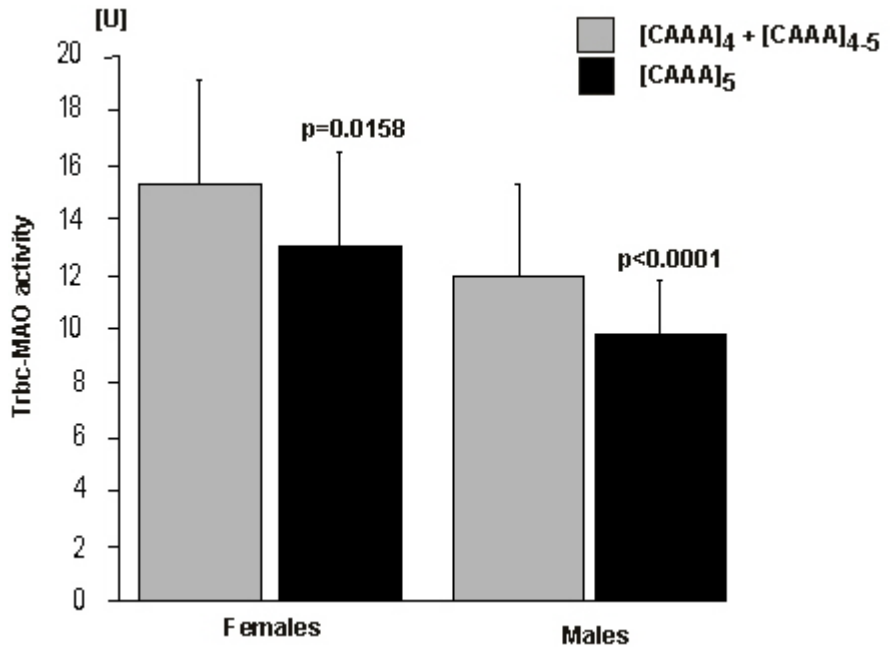

Figure 3. Platelet MAO activity and AP-2β genotype in 158 male and 64 female subjects.

Paper III

Eukaryotic gene transcription plays a regulatory role in mammalian developmental processes such as phenotypic specification of terminally differentiated cell types. It has been shown that transcriptional control is a critical mechanism for specification of neurotransmitter phenotypes.

In order to investigate if the levels of transcription factor AP-2α and AP-2β are correlated to monoamine metabolism, rats (n=9) were sacrificed and the brainstem was dissected. Analysis of AP-2α and AP-2β levels was performed with Enzyme-Linked Immuno-Sorbent Assay (ELISA). Monoamine levels, i.e. serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), dihydroxyphenylacetic acid

(DOPAC), homovanillic acid (HVA), and noradrenaline (NA) were determined by HPLC.

AP-2α and AP-2β correlated positively to several monoamines, and interestingly AP-2β correlated positively to serotonin turnover in the frontal cortex (r^2=0.61, p = 0.014), see figure 4.

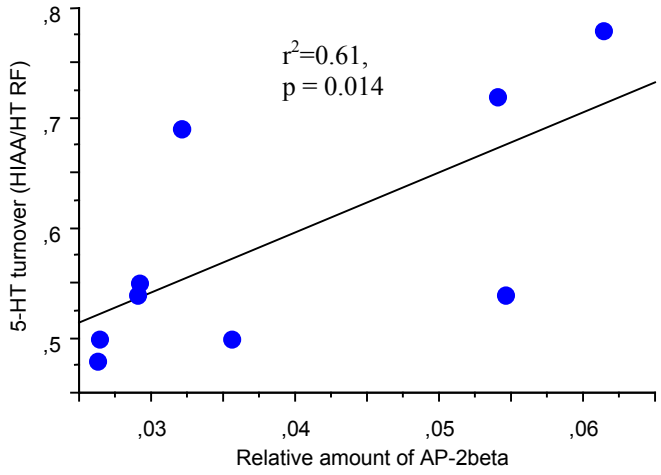

Figure 4. Brainstem levels of AP-2β correlates positively to 5-HT turnover in the rat frontal cortex.

Hence, it seems as high levels of transcription factor AP-2 drive the expression of target serotonergic genes in order to increase the serotonergic activity in the brain regions analysed. The fact that AP-2α and AP-2β were correlated to serotonin measures suggests a prominent role of AP-2 to regulate serotonergic gene expression in the brainstem raphe nuclei, which can affect the release and metabolism of 5-HT in the nerve terminals. The present study shows that AP-2α and AP-2β are correlated to monoamine levels in rat forebrain. One might speculate that transcriptional control, e.g. excerted by AP-2, would

be of importance for developmental determination of the size, i.e. number of neurons of different neurotransmitter systems, see (Damberg, Garpenstrand et al. 2001).

Paper IV

Several of the genes in the serotonergic and the dopaminergic systems have consensus binding sites for the AP-2 transcription factor family in their regulatory regions. Imbalances in these systems have been implicated in many psychiatric disorders, including depression and bipolar affective disorder. In paper IV, we report that chronic administration of three different classes of antidepressant drugs modulates the level and DNA-binding activity of AP-2 in the rat brain (Damberg, Ekblom et al. 2000). Chronic administration of citalopram (10 mg/kg), imipramin (10 mg/kg), and lithium-chloride (40 mg/kg) significantly decreased DNA-binding activity of AP-2 as estimated by electrophoretic mobility shift assay. Furthermore, citalopram and imipramin significantly decreased the level of AP-2α protein as determined by ELISA. In addition, citalopram significantly decreased the level of AP-2ß protein. In contrast, chronic administration of lithium-chloride did not affect the levels of the two AP-2 isoforms.

Since we observe effects from antidepressant drug treatment on AP-2, which has the ability to regulate monoaminergic genes, we speculate that the downstream effect of antidepressants on AP-2 will give rise to adaptations and alterations regarding expression of AP-2 target genes. Thus, subsequent changes in specific expression levels of monoaminergic genes might be dependent on the antidepressant effect on a number of transcription factors, including AP-2.

Paper V

This study is a follow-up study on paper IV. We have in this study analysed the effect of citalopram (10 mg/kg) on transcription factor AP-2 family in a time-dependant manner. In paper IV, we presented a decrease in DNA-binding activity and protein levels of AP-2α and AP-2β after 10 days of citalopram treatment. In order to investigate both acute and chronic effects on AP-2, we have, in this study, injected rats subcutaneously with daily injections of citalopram for 1, 3, 7, and 21 days. We used two groups of rats as controls,

i.e. one group of rats recieving saline injections for 1, 3, 7, and 21 days, and one naive group of rats recieveing no injections. As in paper IV, we used ELISA for protein level analyses, and EMSA for estimation of DNA-binding activity.

After 7 days of citalopram treatment, DNA-binding activity of AP-2 was decreased. Levels of AP-2α were decreased after 3 and 7 days of treatment. Levels of AP-2β were decreased after 7 days of treatment. These data are in line with data obtained in paper IV. After 21 days of treatment we observed no difference between control animals and citaolpram treatmed animals. The group of rats that did not recieve any injections displayed higher levels of AP-2α and AP-2β as well as higher DNA-binding activity of AP-2. This study further suggest transcription factor AP-2 to be a downstream molecular target for citalopram, possibly being involved in neuronal adaptations that occur before onset of the antidepressant effect. It could also be that the decrease in AP-2α and AP-2β levels, and DNA-binding activity of AP-2, after 7 days of treatment in fact delays onset of the antidepressant effect.

General discussion

The results from the different studies included in this thesis suggest transcription factor AP-2 to be involved in central serotonergic neurotransmission. Furthermore, AP-2 seems to be a downstream molecular target for antidepressant drugs. Based on the presented data, the gene encoding the human AP-2β protein is suggested to be a novel candidate gene in personality and neuropsychiatric disorders.

Since AP-2 has been identified as an important regulator of gene expression during embryonic development of many neural tissues, especially the brainstem, one would assume that if the expression of AP-2 itself is altered during certain phases during development, the adult brain would, as a consequence, have an altered structural and biochemical milieu with regard to the monoaminergic systems. The dopamine and serotonin cell bodies are mainly located within the brainstem of an adult brain and are of fundamental importance for personality and a variety of psychiatric disorders, for reviews see (Hartmann, Kunig et al. 1993, Cravchik and Goldman 2000). Central dopamine and serotonin systems are crucial for most brain functions and properties of those systems

have been implicated in the constitutional part of personality (Zuckerman 1993; Depue and Collins 1999), as well as in psychiatric disorders, including eating disorders and substance abuse (Brewerton 1995; Koob and Nestler 1997; Koob 1998). In this thesis we show positive correlations between brainstem levels of AP-2α and AP-2β and forebrain monoamine levels in adult rats. Though AP-2α and AP-2β were correlated to serotonin measures, we suggest a prominent role of AP-2 to regulate serotonergic gene expression in the brainstem raphe nuclei, which can affect the release and metabolism of 5-HT in the nerve terminals.

Regarding the association between platelet MAO activity and AP-2β genotype, several possible explanations occur. In our hypothesis on the connection between platelet MAO activity and personality, we have previously suggested that e.g. the number of neurons, preferentially serotonergic, is co-regulated with trbc MAO. This suggestion was based on early findings that there are marked strain-dependent variations in number of midbrain dopamine neurons in mice (Baker, Joh et al. 1980), and that these variations correlated with inter-strain differences in behavior (Fink and Reis 1981). Regardless of whether transcription factors, such as the AP-2 family, regulate the expression of the number of monoamine neurons, or a variety of candidate genes within the monoamine systems, or both, we would like to emphasise the role of transcription factors, besides polymorphisms in monoaminergic candidate genes, when explaining inter-individual differences in temperament and psychiatric vulnerability.

At the molecular level, psychiatric disorders may result from altered regulation of neuron-specific gene expression and may eventually be characterised by disruption of intra- and interneuronal signalling through inappropriative activation or inactivation of regulatory factors, including nuclear transcription factors. It has for a long time been suggested that small molecules may interfere with gene transcription. However, in what manner this is accomplished is still largely unknown. Increasing knowledge of the molecular background of psychiatric disorders and identification of important intracellular mechanisms in specific mental illnesses is leading the way for drug design, targeting individual genes or their corresponding proteins. It is very likely that identification of transcription factors involved in regulation of genes encoding proteins in the serotonin system will be an important research challenge for the future. Numerous recent articles are optimistic, considering it probable that the ”transcription-based

approach" will yield a valuable contribution, and that this approach will lead to the development of a new generation of specific and effective pharmaceutical agents that can modulate human metabolism with a high degree of precision (Hurley 1989; Butt and Karathanasis 1995; Heguy, Stewart et al. 1995; Pennypacker 1995; Papavassiliou 1998). In this thesis, we present data linking the transcription factor AP-2 family to the serotonin system. Furthermore, two studies included here report that transcription factor AP-2 is a downstream target for antidepressant drugs. An increased understanding of the function of AP-2 and other transcription factors and their involvement in vulnerability for psychiatric disease, could in the future make it possible to identify compounds that exert their therapeutic action at the transcriptional level.

Conclusions

The main conclusions of this thesis are:

- Transcription factor AP-2β is involved in the expression of specific personality traits in humans.

- Transcription factor AP-2β intron 2 genotype is associated to platelet MAO activity in male and female volunteers.

- Levels of transcription factor AP-2α and AP-2β are positively correlated to monoamine turnover in the rat forebrain.

- Transcription factor AP-2 is involved in the molecular mechanims of different antidepressant drugs such as citalopram (SSRI), imipramin (TCA) and lithium-chloride.

SUMMARY IN SWEDISH, SAMMANFATTNING PÅ SVENSKA

I denna avhandling presenteras resultat av studier på transkriptionsfaktorfamiljen AP-2 i relation till molekylära mekanismer som är kopplade till personlighetskarakteristika och psykiatrisk vulnerabilitet. Vidare presenteras resultat av studier på AP-2 i relation till antidepressiv behandling. Vi har studerat en variabel region (polymorfism) i genen som kodar för transkriptionsfaktor AP-2β. Denna polymorfism har inte tidigare analyserats i relation till personlighet och neuropsykiatriska tillstånd. Vi har som första forskargrupp lyckats påvisa en koppling mellan en transkriptionsfaktor och specifika personlighetsdrag hos friska volontärer. Aktiviteten av monoaminoxidas i trombocyter har i tidigare studier visats vara en biologisk markör för personlighet. Den variabla regionen i AP-2β visade sig vara kopplad till låg trombocyt MAO aktivitet. Specifika nivåer av AP-2α och AP-2β i hjärnstammen analyserades i relation till monoaminnivåer i framhjärnan hos råtta och det visade sig att de båda transkriptionsfaktorerna korrelerade till specifika monoaminnivåer. Kronisk och subkronisk antidepressiv behandling på råtta visade sig påverka både nivåerna och den funktionella DNA-bindande aktiviteten hos AP-2 familjen. Generellt visar avhandlingen att transkriptionsfaktorfamiljen AP-2 verkar vara involverad i) vid uttryck av vissa personlighetsdrag hos friska volontärer ii) i regleringen av trombocyt MAO aktivitet, iii) i omsättningen av monoaminer i framhjärnan hos råtta, och iv) i antidepressiva läkemedels molekylära verkningsmekansimer. Nedan följer kortfattade sammanfattningar för respektive delarbeten som ingår i avhandlingen.

Delarbete I

Grundläggande personlighetsdrag och risk för psykiatriska sjukdomar beror i hög utsträckning på ännu ej klarlagda genetiska faktorer. Transkriptionsfaktor AP-2β är starkt involverad i hjärnstammens utveckling och fungerar även i den adulta hjärnan som ett viktigt reglerande protein för gener som ingår i de monoaminerga systemen. De monoaminerga systemen har tidigare visats vara involverade i både personlighet och neuropsykiatriska sjukdomar. I intron 2 i genen som kodar för AP-2β finns en variabel region som består av en repeterad sekvens, [CAAA], som upprepas fyra eller fem gånger. Funktionen av denna intronpolymorfism är ännu ej fastställd. I det aktuella arbetet

undersökte vi 137 friska volontärer för att se om vi kunde koppla genotypen av AP-2β till specifika personlighetsdrag. De friska volontärerna hade genomgått psykiatrisk undersökning och deras personlighet hade skattats med hjälp av Karolinska Scales of Personality (KSP). Individerna som analyserades delades upp i män och kvinnor och de analyserades även tillsammans.

Sammantaget visade det sig att de individer som var homozygoter för den långa allelen $[CAAA]_5$ uppvisade lägre ”muscular tension” och ”guilt”. De män som var homozygoter för den långa allelen uppvisade högre ”indirect aggression”. De kvinnor som var homozygoter för den långa allelen uppvisade låga ångest-relaterade personlighetsdrag, låg ”guilt” och låg ”indirect aggression”. Baserat på dessa resultat spekulerar vi att transkriptionsfaktor AP-2β är involverad i molekylära mekanismer kopplade till personlighet.

Delarbete II

Hos däggdjur är monoaminoxidaser (MAO) centrala vid metabolismen av både endogena och exogena monoaminer. MAO existerar i två former, MAO-A och MAO-B, och hos människa kodas båda dessa protein av separata gener på X-kromosomen. Förändrade MAO-B aktiviteter har tidigare kopplats till flera neurologiska sjukdomar som t ex, Parkinson’s och Huntington’s sjukdomar. I trombocyter (trbc) finns endast MAO-B formen av enzymet och trbc-MAO aktiviteten står under stark, men ännu ej helt klarlagd, genetisk kontroll. Trbc-MAO aktiviteten har tidigare rapporterats var förhöjd vid vissa neurodegenerativa sjukdomar samt inverst korrelera till personlighetsdrag som ”sensationssökande” och ”impulsivitet”.

I promotorregionen uppströms av genen som kodar för MAO-B finns ett potentiellt bindningsställe för transkriptionsfaktor AP-2. Detta tillsammans med resultatet i delarbete I gjorde att vi i denna studie undersökte om den långa allelen $[CAAA]_5$ av AP-2β, var kopplad tilll trbc-MAO aktivitet. Detta genomfördes på friska icke-rökande män (158 st) och kvinnor (64 st). Tobaksrökning har tidigare visats hämma trbc-MAO aktivitet och därför exkluderades de individer som var rökare ur studien.

De individer som var homozygoter för den långa AP-2β allelen uppvisade signifikant lägre trbc-MAO aktivitet än de individer som var heterozygoter eller

homozygoter för den korta allelen. Baserat på resultaten i denna studie spekulerar vi att transkriptionsfaktor AP-2β är involverad i regleringen av genen som kodar för MAO-B.

Delarbete III

Transkriptionsfaktorerna AP-2α och AP-2β är involverade i den embryonala utvecklingen av olika delar av hjärnan och de är även involverade i regleringen av ett flertal monoaminerga gener i den adulta hjärnan. Ett flertal monoaminerga gener har bindningsställen för AP-2 i regulatoriska regioner. I hjärnstammen finns de monoaminerga kärnorna som skickar projektioner fram till framhjärnan, och i dessa kärnor agerar transkriptionsfaktor AP-2 tillsammans med andra transkriptionsfaktorer. I den aktuella studien analyserade vi kopplingen mellan nivåer av AP-2α och AP-2β i hjärnstammen i råtta (n=9) och monoaminnivåer i frontala cortex, septum och i hippocampus. För att analysera proteinmängd av AP-2α och AP-2β användes Enzyme-Linked Immunosorbent Assay (ELISA) och för att analysera nivåer av monoaminer användes High Performance Liquid Chromatography (HPLC).

Vi hittade ett flertal positiva korrelationer mellan AP-2α och AP-2β nivåer och monoaminnivåer. AP-2α visade sig korrelera med 5-HIAA och DOPAC nivåer i frontala cortex och till 5-HT i hippocampus. AP-2β visade sig korrelera med serotoninomsättning (5-HIAA/5-HT) och dopaminomsättning (HVA+ DOPAC/DA) i frontala cortex och med 5-HIAA och NA i septum. Denna studie bekräftar vår hypotes om att transkriptionsfaktorfamiljen AP-2 är involverad i regleringen av de monoaminerga systemen i hjärnan och vi spekulerar därför att AP-2 är involverad i patofysiologin vid personlighetsförändringar och neuropsykiatriska sjukdomar.

Delarbete IV

Ett flertal studier har undersökt de molekylära verkningsmekanismerna av antidepressiva läkemedel. Det finns starka bevis på att monoaminerg neurotransmission spelar en viktig roll i den terapeutiska effekten av antidepressiva läkemedel. Då transkriptionsfaktorfamiljen AP-2 har bindningsställen i regulatoriska regioner i ett flertal monoaminerga gener har vi undersökt om AP-2 är involverad i den molekylära verkningsmekanismen av citalopram (serotoninselektiv återupptagshämmare, SSRI),

imipramin (tricykliskt antidepressivum (TCA) och litiumklorid. Vi behandlade råttor, fem i varje grupp, med en subkutan injektion dagligen i 10 dagar med ovanstående läkemedel. Vi hade en kontrollgrupp som fick motsvarande behandling med koksaltlösning. Dag 11 avlivades djuren och hjärnan dissekerades fram. Den högra hjärnhemisfären användes för att preparera nukleära ektrakt innehållande transkriptionsfaktorer och andra nukleära proteiner. För att analysera proteinmängd av AP-2α och AP-2β användes Enzyme-Linked Immunosorbent Assay (ELISA) och för att analysera den DNA-bindande aktiviteten för AP-2 användes Electrophoretic Mobility Shift Assay (EMSA).

10 dagars behandling med citalopram, imipramin och litiumklorid nedreglerade den DNA-bindande aktiviteten för AP-2. Proteinnivåerna av AP-2α visade sig också vara signifikant lägre efter behandling med citalopram och imipramin än i kontrolldjuren. Vidare hade citaloprambehandling en nedreglerande effekt även på AP-2β nivåerna jämfört med kontrolldjuren. Litiumkloridbehandling påverkade ej nivåerna av AP-2α eller AP-2β. Baserat på dessa resultat spekulerar vi att AP-2 är en gemensam molekylär måltavla för antidepressiva läkemedel.

Delarbete V

Denna studie är en uppföljningsstudie på delarbete IV. Vi har i denna studie analyserat vilken effekt citalopram (10 mg/kg) har på transkriptionsfaktorfamiljen AP-2 över en tidskurva. I delarbete IV såg vi en nedreglerande effekt på AP-2 efter 10 dagars behandling. Här behandlade vi råttor, fem i varje grupp, med en subkutan injektion dagligen i 1, 3, 7, 21 dagar med citalopram och vi hade en kontrollgrupp som fick motsvarande behandling med koksaltlösning. Då djuren hade avlivats och hjärnan dissekerats fram så användes den högra hjärnhemisfären för att preparera nukleära ektrakt innehållande transkriptionsfaktorer och andra nukleära proteiner. För att analysera proteinmängd av AP-2α och AP-2β användes Enzyme-Linked Immunosorbent Assay (ELISA) och för att analysera den DNA-bindande aktiviteten för AP-2 användes Electrophoretic Mobility Shift Assay (EMSA).

Efter 7 dagars behandling så hade den DNA-bindande aktiviteten av AP-2 nedreglerats. Nivåerna av AP-2α var nedreglerade efter 3 och 7 dagars behandling. Nivåerna av AP-2β var nedreglerade efter 7 dagars behandling. Dessa resultat var i linje

med delarbete IV. Efter 21 dagars behandling hade AP-2 nivåerna fanns ingen skillnad mellan de citaloprambehandlade djuren och kontrolldjuren. Baserat på dessa resultat spekulerar vi att det finns ytterligare bevis för att AP-2 verkar vara involverad i den molekylära verkningsmekanismen för citalopram. Det skulle även kunna vara så att nedregleringen av AP-2 nivåer och DNA-bindande aktivitet efter 7 dagars behandling motverkar initiering av den antidepressiva effekten.

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