REGULATION OF SIGNAL TRANSDUCTION IN THE STRIATUM BY TYPICAL AND ATYPICAL ANTIPSYCHOTIC DRUGS

Kerstin Håkansson

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Cover;
Louis le Brocquy (b. 1916)
*Isolated Being*, 1962
oil on canvas, 152 x 91 cm.
Coll. Dublin City Gallery The Hugh Lane
Courtesy Pierre le Brocquy

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TO ANTON

“The only interesting answers are those which destroy the questions”
Susan Sontag
Abstract

The only effective therapy for schizophrenia is based on the use of antipsychotic drugs. These substances act as dopamine D2 receptor antagonists and can be classified as typical or atypical. Typical antipsychotics, e.g. haloperidol, reduce the positive symptoms of schizophrenia, but have no effect in treating the negative symptoms of the disease. In addition, prolonged use of these drugs often causes extrapyramidal side effects (EPS). Treatment with the atypical antipsychotic, clozapine, ameliorates both the negative and the positive symptoms of schizophrenia with a much lower incidence of EPS. However, the use of clozapine is limited by the occurrence of agranulocytosis in about one percent of the patients.

The aim of this thesis is to examine the effects produced by a haloperidol and clozapine on the state of phosphorylation of proteins involved in postsynaptic and presynaptic transmission within the basal ganglia, a group of brain structures critically involved in motor control. The results of these studies show the existence of differences in the effects produced by haloperidol and clozapine, which may help to identify specific molecular determinants involved in the generation of EPS. Acute administration of haloperidol or clozapine increases the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) at the cAMP-dependent protein kinase (PKA) site (Thr34). This phosphorylation converts DARPP-32 into an inhibitor of protein phosphatase-1 (PP-1), thereby amplifying cAMP/PKA-mediated responses. Haloperidol also stimulates the phosphorylation of the two mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases 1 and 2 (Erk1/2). In addition, haloperidol phosphorylates and activates the transcription factors CAMP response element binding protein (CREB) and Elk-1. In contrast, clozapine significantly reduces the phosphorylation and activation of Erk1/2, CREB and Elk-1. The stimulation of Erk1/2 phosphorylation produced by haloperidol is not prevented by genetic inactivation of DARPP-32, indicating that the regulation exerted by haloperidol on the MAPK cascade is independent of activation of the cAMP/PKA/DARPP-32 cascade. Repeated administration of haloperidol for 14 days followed by a challenge dose of the same drug on day 15, led to a reduction in the stimulation of Erk1/2 phosphorylation, suggesting the development of tolerance after chronic treatment. In contrast, the ability of clozapine to decrease Erk 1/2 phosphorylation was not affected by prolonged administration. AMPA receptors are widely distributed in the brain, and are regulated by phosphorylation on different subunits. The GluR1 subunit is phosphorylated by PKA at Ser845, and by calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) at Ser 831. Haloperidol produced a transient and dose-dependent increase GluR1 phosphorylation at Ser845, without affecting Ser831 phosphorylation. In the striatum, PKA is activated via dopamine D2 receptors in striatonigral neurons and via adenosine A2A receptors in striatopallidal neurons. Blockade of A2A, but not of D1, receptors prevented haloperidol from increasing Ser845 phosphorylation. The ability of haloperidol to phosphorylate Ser845 was also abolished by genetic inactivation of DARPP-32. This indicates that haloperidol regulates the state of phosphorylation of GluR1 at Ser845 in striatopallidal neurons and that this effect depends both on PKA activation and PP-1 inhibition (achieved via DARPP-32 phosphorylation at Thr34). Clozapine had no effect on Ser845, but it produced a long-lasting (≥120 min) decrease in Ser831 phosphorylation. In the basal ganglia, antipsychotics block dopamine D2 autoreceptors, thereby stimulating dopamine synthesis and release. The turnover of dopamine depends on the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamines, which is regulated by phosphorylation at multiple seryl residues. Acute administration of haloperidol, but not clozapine, stimulated the phosphorylation of TH at Ser19, Ser 31 and Ser40. This effect was abolished in dopamine D2-receptor knockout mice. Moreover, blockade of Erk1/2 activation by SL327 prevented haloperidol induced phosphorylation of TH at Ser 31 and Ser40, showing that MAPK are involved in TH regulation in vivo.

Keywords: adenosine A2A receptor, basal ganglia, cAMP-dependent protein kinase, clozapine, DARPP-32, dopamine D2 receptor, extracellular signal-regulated protein kinases 1 and 2, extrapyramidal side effects, glutamate AMPA receptor, haloperidol, knockout mouse, phosphorylation, schizophrenia, tyrosine hydroxylase.

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   *Manuscript*

III. Håkansson K., Snyder G., Usiello A., Borrelli E., Greengard P. and Fisone G. Haloperidol-induced increase in striatal Erk1/2 phosphorylation is dependent on dopamine D₂ receptors but not on the cAMP/PKA/DARPP-32 cascade
   *Manuscript*

IV. Håkansson K., Pozzi L., Usiello A., Haycock E., Borrelli E. and Fisone G (2004). Regulation of striatal tyrosine hydroxylase phosphorylation by acute and chronic haloperidol
   *Eur J Neuroscience* 20:1108-1112
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## Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AC</td>
<td>Adenelyl cyclase</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-isoxazole-acid</td>
</tr>
<tr>
<td>B-Raf</td>
<td>B-Raf proto-oncogene serine/threonine protein kinase</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium/calmodulin dependent protein kinase II</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>Calcium response element binding protein</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>D₁</td>
<td>Dopamine D₁ receptor subtype</td>
</tr>
<tr>
<td>D₂</td>
<td>Dopamine D₂ receptor subtype</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>Dopamine and cAMP regulated phosphoprotein of 32 kDa</td>
</tr>
<tr>
<td>Elk-1</td>
<td>Ets-domain protein Elk-1</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extra cellular regulated protein kinase 1 and 2 (MAPK)</td>
</tr>
<tr>
<td>FRA</td>
<td>Fos related antigen</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GluR1</td>
<td>Glutamate receptor 1 (AMPA receptor subunit)</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus pallidus external segment</td>
</tr>
<tr>
<td>GPi</td>
<td>Globus pallidus internal segment</td>
</tr>
<tr>
<td>IEG</td>
<td>Immediate early gene</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase (e.g. ERK1/2)</td>
</tr>
<tr>
<td>MAPKK</td>
<td>Mitogen activated protein kinase kinase (e.g. MEK1/2)</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen activated protein kinase kinase kinase (e.g. Raf-1)</td>
</tr>
<tr>
<td>MEK1/2</td>
<td>MAPK-Erk-kinase 1/2</td>
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<tr>
<td>MKP-3</td>
<td>MAP kinase phosphatases 3</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartate</td>
</tr>
<tr>
<td>NRG1</td>
<td>neuregulin-1</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine; “angel dust”</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PP-1</td>
<td>Protein phosphatase 1</td>
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<td>PP-2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PP-2B</td>
<td>Protein phosphatase 2B</td>
</tr>
<tr>
<td>PPI</td>
<td>Prepulse inhibition</td>
</tr>
<tr>
<td>Raf-1</td>
<td>RAF-proto-oncogene serine/threonine kinase 1</td>
</tr>
<tr>
<td>Rsk</td>
<td>Ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNpr</td>
<td>Substantia nigra pars reticulata</td>
</tr>
<tr>
<td>SRE</td>
<td>Serum response element</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>STEP</td>
<td>Striatal enriched phosphatase</td>
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<tr>
<td>STN</td>
<td>Subthalamic nucleus</td>
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1. Introduction

1.1 Schizophrenia

At the end of the 19th century, the German psychiatrist Emil Kraepelin described a mental disorder, which he referred to as dementia praecox (Kraepelin 1896). The same disorder was later named schizophrenia (from Greek schizein, to split, and phren, mind) by the Swiss psychiatrist, Eugen Bleuler (Bleuler 1911). Schizophrenia is a devastating psychotic disease affecting about one percent of the human population worldwide. The onset of symptoms in schizophrenia usually begins in late adolescence or early adulthood and continues throughout life. The burden of schizophrenia, not only to the patients and their families but also, to society is extremely high. For instance, about 10% of affected individuals will eventually commit suicide (Siris 2001) and up to 90% of schizophrenic patients are unemployed (Castner et al. 2004).

The most striking clinical features of schizophrenia consist of hallucinations (false perceptions) and delusions (false believes). These symptoms are classified as positive, since they represent mental phenomena that unaffected individuals do not experience. In addition to the positive symptoms, schizophrenic patients also exhibit loss of normal behaviour, generally referred to as negative symptoms. For instance, affected individuals may show social withdrawal, flattening of affect, apathy and loss of motivation. In addition, cognitive dysfunctions including attention deficits, as well as impaired executive functions and working memory are today regarded as core features of the illness (Andreasen 1997; Weinberger and Gallhofer 1997).

Information processing appears to be impaired in schizophrenic patients, as shown by studies employing the prepulse inhibition (PPI) paradigm. PPI is a cross-species measure of a sensorimotor gating phenomenon, in which a weak stimulus reduces the startling effect of a subsequent intense stimulus (Graham 1975). Reduction of PPI has been reported in patients affected by psychiatric disorders associated with dopaminergic dysfunction, including schizophrenia (cf. section 1.1.2) (Braff et al. 1992). Schizophrenic patients may
also display movement abnormalities such as catatonia, stereotypy, and abnormal posture and limb movements (Gupta et al. 1995).

1.1.1 Neuropathological changes associated with schizophrenia

Structural brain abnormalities have been extensively documented in schizophrenic individuals; however, this evidence is diverse and in some case conflicting. Some studies have reported reduction in cortical neuropil (Black et al. 2004), reduced density of axon terminals from cortical inhibitory interneurons (chandelier cells) (Woo et al. 1998) and enlargement of the third and lateral ventricles (van Haren et al. 2004). Neuropathological abnormalities affecting the hippocampus, such as a reduction of pyramidal neurons (Jeste and Lohr 1989), cellular disarray (Kovelman and Scheibel 1984), and reduced tissue volume (Heckers et al. 1991) have also been observed in schizophrenic patients. Other areas that might be involved in the pathology of schizophrenia are the thalamus, the basal ganglia, the temporal and parietal association cortices and limbic areas including the entorhinal and cingulate cortex (Bunney et al. 1997; Kasai et al. 2002). Most studies have reported absence of gliosis, suggesting that schizophrenia is a neurodevelopmental rather than a neurodegenerative disorder (Kasai et al. 2002).

A large body of evidence also suggests that alterations in several neurotransmitter systems including dopamine, glutamate and serotonin might be involved in the generation of schizophrenia (see sections 1.1.2, 1.1.3 and 1.2.2) (Mackay et al. 1980; Iversen et al. 1983; Laruelle et al. 2003). In addition, genetic factors, located at multiple loci, and environmental factors, including viral infections and psychosocial stress, may contribute to the disease (Tsuang 2000; McGuffin et al. 2001). For instance, the incidence of schizophrenia among monozygotic twins is nearly 50%, whereas the risk is only about 14% in dizygotic twins (Tsuang 2000). In summary, the broad range of abnormalities associated with schizophrenia and its undefined etiology suggest that this disease consists of a group of illnesses rather than one single disorder.
Dopamine and the dopamine hypothesis of schizophrenia

Dopamine is the predominant catecholamine in the brain. The majority of dopamine cell bodies are found in the substantia nigra pars compacta (SNpc; A9) and in the ventral tegmental area (VTA; A10). A smaller fraction of dopaminergic neurons is also found in the retrorubral area (A8) and in the tuberoinfundibular system of the hypothalamus (Dahlström and Fuxe 1964). Neurons in the SNpc project mainly to the dorsal striatum via the nigrostriatal pathway and are involved in the regulation of voluntary, goal-directed, movements. The neurons of the VTA, project to the prefrontal cortex (PFC) and form the mesocortical pathway, which is involved in cognitive functions. In addition, VTA neurons, project to the ventral striatum (nucleus accumbens), amygdala, hippocampus and olfactory tubercle, forming the mesolimbic pathway, which regulates motivation and reward.

Dopamine acts by binding to five subtypes of heptahelical, G-protein coupled receptors, which have been divided into two groups. The D₁-like group comprises D₁ and D₅ receptors, which are positively coupled to adenylyl cyclase and production of cAMP. The D₂-like group comprises D₂, D₃ and D₄ receptors, which are coupled to inhibition of adenylyl cyclase and reduction of cAMP (Stoof and Kebabian 1981). In addition, alternative mRNA splicing generates two isoforms of the dopamine D₂ receptor, named long (D₂L) and short (D₂S) isoforms, which differ by an insertion of 29 amino acids in the third intracellular loop of the D₂L receptor (Dal Toso et al. 1989).

The dopamine hypothesis of schizophrenia was formulated almost 40 years ago by Van Rossum and postulated that schizophrenia is related to increased dopaminergic transmission (Van Rossum 1967). Currently, a revised version of this hypothesis is based on the idea that the positive symptoms of schizophrenia involve primarily dopamine hyperactivity in mesolimbic areas, whereas the negative symptoms originate from reduced dopaminergic transmission in the mesocortical projections to the PFC (Lewis and Lieberman 2000). These imbalances in dopaminergic transmission at the level of the mesocorticollimbic system have been proposed to occur as a result of a reduction of
cortical glutamatergic signalling in the same circuits (Laruelle et al. 2003; Moghaddam 2003) (cf. section 1.1.3).

Several lines of evidence support the involvement of abnormal dopaminergic transmission in schizophrenia. In fact, the therapeutic efficacy of antipsychotic drugs has been correlated to their ability to antagonize dopamine D₂ receptors (Seeman et al. 1975; Creese et al. 1976). In addition, amphetamine, a psychostimulant that increases dopamine release, produces psychotic-like behaviours resembling those of schizophrenia (Angrist et al. 1974; Sato 1992). Furthermore, amphetamine and apomorphine, a dopamine receptor agonist, disrupt PPI (Mansbach et al. 1988), an effect prevented by antipsychotic drugs (Mansbach et al. 1988; Swerdlow and Geyer 1993). In support of the dopamine hypothesis, it has also been shown that schizophrenic patients show increased dopamine D₂ receptor occupancy during a psychotic period (Abi-Dargham et al. 2000).

The evidence in support of the dopamine hypothesis of schizophrenia, however, remains for the most part strictly pharmacological, as a direct demonstration of abnormal dopaminergic transmission in schizophrenia is still lacking. Indeed, most studies have failed to find altered dopamine levels or dopamine metabolites in schizophrenic individuals (Pickar et al. 1990; Issa et al. 1994b; Issa et al. 1994a; Miller et al. 1996). In addition, blockade of dopamine D₂ receptors relieves only part of the symptoms of schizophrenia, and typical antipsychotic drugs like haloperidol might even worsen the negative symptoms of the disease (Meltzer 1999; Castner et al. 2000), probably by further decreasing dopaminergic transmission in the PFC (cf. above). Whatever the primary cause, the interplay between dopaminergic and glutamatergic transmission is such that alterations in one of them will ultimately affect the other. Investigations of the pathology of schizophrenia have therefore extended beyond dopamine in search for other neurotransmitter systems.

1.1.3 The glutamate hypothesis of schizophrenia

The glutamate hypothesis of schizophrenia is largely based on pharmacological evidence. For instance, administration of the dissociative anaesthetic, phencyclidine (PCP or “angel dust”), a
non-competitive antagonist at glutamate NMDA receptors, produces psychotic symptoms in healthy individuals and exacerbates schizophrenic symptoms in patients (Itil et al. 1967). Previous studies have also shown that ketamine (a PCP analog) produces transient psychosis, disrupted affect and cognitive deficits similar to those observed in schizophrenia (Tamminga et al. 1995; Lahti et al. 2001). In addition, transgenic mice that express only 5-10% of the normal levels of NMDA receptors, express a wide range of symptoms reminiscent of schizophrenia, like stereotypies, hyperactivity and social isolation, which are ameliorated by antipsychotic drugs (Mohn et al. 1999).

Post-mortem and genetic studies have generally failed to find alterations in NMDA receptor density (Goff and Coyle 2001; Moghaddam 2003). However, some studies have reported altered NMDA receptor subunit composition in hippocampus and PFC of schizophrenic patients (Akbarian et al. 1996; Gao et al. 2000). A study of an Icelandic population identified neuregulin-1 (NRG1) as a candidate gene for schizophrenia (Stefansson et al. 2002). NRG1 is involved in the regulation of NMDA receptors. Indeed, mutant mice heterozygous for NRG1, or its receptor ErbB4, express fewer functional NMDA receptors and exhibit behaviour similar to mice treated with NMDA antagonists (Stefansson et al. 2002). Other reports showed altered mRNA expression of glutamate AMPA and kainate receptors in the brain of schizophrenic patients (Eastwood et al. 1995; Gao et al. 2000; Ibrahim et al. 2000; Meador-Woodruff and Healy 2000). Finally, reduced concentration of glutamate has been reported in the cerebrospinal fluid of schizophrenic patients (Macciardi et al. 1990; but see also Perry 1982).

1.2 ANTIPSYCHOTIC DRUGS

The first drugs shown to ameliorate schizophrenia were reserpine, a substance that produces a long-lasting depletion of biogenic amines by interfering with their uptake-storage mechanisms, and chlorpromazine, a dopamine receptor antagonist. Prior to the discovery of these drugs, in the 1950s, psychotic patients were typically hospitalized for long periods, and were subjected to extreme procedures such as frontal lobotomy. The initial success of
chlorpromazine stimulated the development of other antipsychotic drugs, which still represent the only effective treatment for schizophrenia. Antipsychotic drugs are divided into two categories, typical and atypical, based on their pharmacological profile and their ability to cause extra pyramidal side effects (EPS) (see section 1.2.1, 1.2.2 and 1.3).

1.2.1 Typical antipsychotic drugs – Haloperidol

Typical antipsychotics, exemplified by haloperidol, are able to reduce the positive symptoms of schizophrenia, but show only moderate effect on the negative or cognitive symptoms of the disease. Pharmacologically, all typical antipsychotic drugs share the ability to antagonize dopamine D$_2$ receptors. This feature is one of the most compelling facts at the basis of the dopamine hypothesis of schizophrenia (see section 1.1.2). Haloperidol binds with high affinity to dopamine D$_2$ receptors and, during treatment, the dopamine D$_2$ receptors occupancy of this drug is comprised between 70 and 90% (Farde et al. 1989; Farde et al. 1992; Nordstrom et al. 1995). Although acute administration of haloperidol or chlorpromazine produces a partial antipsychotic effect, prolonged treatment for six to eight weeks is generally necessary to obtain full therapeutic efficacy. Thus, long-term blockade of D$_2$ receptors, results in adaptive changes, probably mediated by regulation of gene transcription (cf. section 1.5.3 and 4.3), that ultimately result in full antipsychotic effect.

Treatment with typical antipsychotic drugs results in the appearance of unwanted and sometimes irreversible movement abnormalities, or EPS. These side effects are most likely produced by blockade of dopamine D$_2$ receptors, although it has been proposed that the percent of receptor occupancy necessary to generate EPS is higher than that necessary to produce therapeutic effects (Seeman 2002). Another common side effect of typical antipsychotic drugs is elevated plasma levels of prolactin, a hormone responsible for lactation and secretion of progesterone. Temperature dysregulation may also occur due to D$_2$ receptor blockade (Boulay et al. 1999).
A major problem of treatment with typical antipsychotics is that a considerable group of patients (10-30%) does not respond to these drugs. Moreover, people initially responding may eventually relapse within one to two years, while still on medication. One third of such a refractory group of patients has been shown to benefit from treatment with clozapine, an atypical antipsychotic drug that improves both positive and negative symptoms (see next section) (Kane et al. 1988).

1.2.2 Atypical antipsychotic drugs – Clozapine

Clozapine is the prototype of an atypical antipsychotic. These drugs can generally be defined based on the following criteria: 1) lower tendency to induce EPS, 2) minimal elevation of prolactin, and 3) efficacy in a subgroup of patients refractory to treatment with typical antipsychotic drugs. In animal studies, atypical antipsychotic drugs are defined by their ability to produce antipsychotic effects (i.e. blockade of amphetamine- or apomorphine-induced hyperlocomotion) and EPS (i.e. catalepsy) at clearly distinct doses (Fig. 1) (Ögren 1996).

Fig.1. Typical and atypical antipsychotic drugs differ in their ability to separate between antipsychotic effect and EPS. The difference between doses necessary to block dopamine agonist-induced hyperlocomotion (regarded as an index of antipsychotic effect) and those that induce catalepsy (regarded as an index of EPS) is larger for atypical antipsychotic drugs. Adapted from (Ögren 1996).
Clozapine was first introduced in Europe in the early 70’s, and in the United States in 1989. Other compounds introduced more recently and defined as atypical antipsychotics, include risperidone, olanzapine, quetiapine, aripiprazole and ziprasidone. These drugs have lower affinity for dopamine D\textsubscript{2} receptors as compared to haloperidol, but high affinity for serotonin 5-HT\textsubscript{2A} receptors (Table 1). It has been proposed that the higher 5-HT\textsubscript{2A}/D\textsubscript{2}-affinity ratio of atypical antipsychotic drugs is at the basis of their better clinical outcome (Meltzer et al. 1989). Although 5-HT\textsubscript{2A} receptor antagonism may contribute to the therapeutic efficacy of atypical antipsychotics, it might not be sufficient by itself to improve all symptoms of schizophrenia (Meltzer 1999).

The lower affinity of atypical antipsychotic drugs for dopamine D\textsubscript{2} receptors is most likely responsible for their low to moderate propensity to induce EPS. Striatal D\textsubscript{2} receptor occupancy during clozapine treatment ranges between 32 and 63%, as compared to 78 to 85% occupancy reported for haloperidol (Talvik et al. 2001). Clozapine has a moderate antagonistic effect on dopamine D\textsubscript{1} and D\textsubscript{2} receptor, but binds with high affinity to D\textsubscript{3} and D\textsubscript{4} dopamine receptors (Deutch et al. 1991). In addition, clozapine antagonizes various other receptors, including \alpha\textsubscript{1}-adrenergic, H\textsubscript{1}-histaminergic and muscarinic acetylcholine receptors (Table 1).

The complex pharmacological profile of clozapine may contribute to its unique therapeutic properties, but also to its numerous side effects. The main obstacle to the use of clozapine, is the development in approximately 1% of the patients of agranulocytosis, a potentially lethal condition marked by a reduction of leukocytes, which imposes frequent monitoring of the patients (Alvir and Lieberman 1994). For this reason, clozapine is generally not considered a first-choice antipsychotic drug (American Diabetes Association, 2004). Other side effects with atypical antipsychotic treatment are much more common and consist of moderate to severe weight gain, diabetes mellitus Type II and elevated plasma levels of cholesterol (Umbricht et al. 1994; Henderson et al. 2000; Wirshing et al. 2002; American Diabetes Association et al. 2004).
Table 1. Equilibrium dissociation constants for antipsychotic drugs at human brain receptors\(^1\). (Adapted from Richelson and Souder 2000).

<table>
<thead>
<tr>
<th></th>
<th>HAL</th>
<th>CLOZ</th>
<th>OLZ</th>
<th>RPD</th>
<th>QTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine D(_2)</td>
<td>2.6±0.5</td>
<td>210±30</td>
<td>20±3</td>
<td>3.77±0.04</td>
<td>770±30</td>
</tr>
<tr>
<td>α(_1)-adrenergic</td>
<td>17±1</td>
<td>6.8±0.8</td>
<td>44±4</td>
<td>2.7±0.3</td>
<td>8.1±0.9</td>
</tr>
<tr>
<td>α(_2)-adrenergic</td>
<td>600±100</td>
<td>15±0.6</td>
<td>280±30</td>
<td>8±1</td>
<td>80±10</td>
</tr>
<tr>
<td>Histamine H(_1)</td>
<td>260±20</td>
<td>3.1±0.5</td>
<td>0.087±0.005</td>
<td>5.2±0.5</td>
<td>19±1</td>
</tr>
<tr>
<td>Muscarinic</td>
<td>&gt;10000</td>
<td>9±1</td>
<td>36±5</td>
<td>34000±3000</td>
<td>1400±200</td>
</tr>
<tr>
<td>5-HT(_1)A</td>
<td>1800±300</td>
<td>160±20</td>
<td>610±80</td>
<td>190±20</td>
<td>300±20</td>
</tr>
<tr>
<td>5-HT(_2)A</td>
<td>61±3</td>
<td>2.59±0.01</td>
<td>1.48±0.05</td>
<td>0.15±0.02</td>
<td>31.4±4</td>
</tr>
</tbody>
</table>

\(^1\) Values are geometric means ± SEM (nM). When means are presented, compounds were tested in at least, three independent experiments. HAL: haloperidol, CLZ: clozapine, OLZ: olanzapine, RPD: risperidone, QTP: quetiapine

1.3 EXTRA-PYRAMIDAL SIDE EFFECTS

Treatment with antipsychotic drugs has revolutionized the therapy of schizophrenia, but it has also been lined with serious side effects that have limited their use. It is now accepted, that blockade of dopamine D\(_2\) receptors results not only in antipsychotic action, but also in the production of abnormal involuntary movements, or EPS. Acute EPS can occur within hours or days from the beginning of drug treatment and include akathisia, acute dystonia, parkinsonism and neuroleptic malignant syndrome (Table 2). Other EPS, like tardive dyskinesia (TD), are only observed after prolonged (months to years) treatment. It has been estimated that approximately 90% of patients treated with conventional neuroleptics, like haloperidol, develop drug-induced EPS (parkinsonism, dystonia and akathisia) and 20-30 % develop TD (Casey 1998). In fact, it has been suggested that the high incidence of EPS observed in association with the use of haloperidol may be related to the ability of this drug to stimulate immediately early gene (IEG) expression in the dorsal striatum (Pisa and Schranz 1988; Robertson and Fibiger 1992) (see section 1.5.3).

Atypical antipsychotics have a much lower propensity than haloperidol to induce EPS. Thus, chronic treatment with clozapine is associated with no or very low probability of contracting EPS. However, the risk of EPS persists even when using atypical
antipsychotics. For instance, risperidone induces EPS with relatively high frequency (Tarsy et al. 2002). Some of the motor side effects produced by haloperidol can be reversed by lowering the dose, by administering anticholinergic agents or by switching to an atypical antipsychotic drug. Others like TD are potentially irreversible, exacerbated by drug withdrawal, and insensitive to anticholinergic treatment. However, several studies have reported that clozapine might alleviate drug-induced TD and dystonia (reviewed in (Tarsy et al. 2002)).

Table 2. Antipsychotic-induced EPS

<table>
<thead>
<tr>
<th>EPS</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parkinsonism</td>
<td>Rigidity, resting tremor, bradykinesia, shuffling gait.</td>
</tr>
<tr>
<td>Acute dystonia</td>
<td>Spastic muscle contraction in neck, back, face and tongue.</td>
</tr>
<tr>
<td>Akathisia</td>
<td>Motor restlessness, anxiety-like syndrome.</td>
</tr>
<tr>
<td>Tardive dyskinesia</td>
<td>Involuntary movements usually seen as oro-facial dyskinesia (excessivé rapid movements of mouth and tongue), trunk and extremities, observed after chronic treatment, potentially irreversible. Few treatment options.</td>
</tr>
<tr>
<td>Neuroleptic malignant syndrome</td>
<td>Catatonia, stupor, fever, unstable blood pressure etc. can be fatal.</td>
</tr>
</tbody>
</table>

The choroid-like features of TD have led to the suggestion that this disorder might be related to dopamine super-sensitivity that develops as an adaptation to persistent D₂ receptor blockade in the striatum (Klawans and Rubovits 1972). Another explanation of TD is provided by the “neuronal degeneration hypothesis”, which postulates that the irreversible nature of this EPS is due to a neurotoxic process induced by antipsychotic drugs (Cadet and Lohr 1989; Elkashef and Wyatt 1999). Despite a great deal of research, the specific neuroadaptive changes responsible for the development of TD remain to be identified.

Although there is a high risk factor of EPS, associated with typical antipsychotic treatment, spontaneous EPS identical to
antipsychotic-induced dyskinesia have been reported in antipsychotic-naïve patients (Chatterjee et al. 1995; Puri et al. 1999), and appear to develop specifically in schizophrenic subjects (Fenton et al. 1997).

1.4 General Organization of the Basal Ganglia

The basal ganglia are a group of interconnected subcortical nuclei, involved in the regulation of psychomotor behaviour, including voluntary movements, reward mechanisms, attention and memory. The striatum is the principal input structure of the basal ganglia and is subdivided into a dorsal part, consisting of the caudate-putamen, and a ventral part, consisting of the nucleus accumbens. The striatum receives excitatory glutamatergic input from cerebral cortex, thalamus and limbic areas, like amygdala and hippocampus. In addition, striatal neurons receive modulatory inputs from dopaminergic and serotonergic neurons (Gerfen 1992; Bolam et al. 2000), as well as from recurrent axon collaterals and local interneurons that release GABA or acetylcholine (Kawaguchi 1997).

One major anatomical feature of the striatum is the presence of morphologically distinct compartments, named patch and matrix. The striatal matrix is rich in cholinergic and somatostatin-containing neurons, whereas the patch compartment displays high levels of opiates and substance P (Gerfen 1992).

The vast majority of striatal neurons (≈95%) are GABAergic medium spiny neurons. Approximately half of these neurons co-express GABA, substance P and dynorphine and project directly to the substantia nigra pars reticulata (SNpr)/globus pallidus internal segment (Gpi; entopeduncular nucleus in rodents). The remaining medium spiny neurons co-express GABA and enkephalin, and project indirectly to SNpr/GPi, via globus pallidus external segment (GPe) (Fig. 2). Striatal medium spiny neurons are normally quiescent, but respond rapidly to excitatory inputs from cortex and thalamus. In the dorsal striatum, the two subpopulations of projection neurons exhibit opposing effects on motor activity via regulation of thalamocortical neurons. Activation of the direct pathway facilitates locomotion by disinhibiting thalamocortical neurons, whereas activation of the indirect pathway reduces motor
activity by enhancing inhibition of the thalamocortical pathway (Gerfen 1992) (Fig. 2).

**Fig. 2.** Diagram illustrating the functional organization of the basal ganglia. The striatum receives an excitatory glutamatergic (black) input from cerebral cortex and a modulatory dopaminergic input from the SNpc. GABAergic (light grey) medium spiny neurons innervate either directly or indirectly the SNpr/GPi (see text). Cholinergic and GABA-ergic interneurons (IN) innervates medium spiny neurons. The neurons of the direct pathway preferentially express dopamine D₁ receptors whereas the neurons of the indirect pathway express dopamine D₂ and adenosine A₂A receptors. STN; subthalamic nucleus

The striatal medium spiny neurons of the direct and the indirect pathway express high levels of dopamine receptors. Considerable morphological and functional evidence indicates that the neurons of the direct pathway preferentially express dopamine D₁ receptors, whereas the neurons of the indirect pathway are enriched in dopamine D₂ receptors (Gerfen 1992). In addition, the neurons of the indirect pathway selectively express the A₂A receptor subtype for the neuromodulator adenosine (Fink et al. 1992; Schiffman and Vanderhaeghen 1993).

1.5 **Signal Transduction**

The ability of antipsychotic medications to antagonize dopamine D₂ receptor transmission appears to be critical for their therapeutic efficacy (cf. above). However, the same pharmacological effect,
particularly when exerted at the level of the basal ganglia, is most likely involved in the generation of EPS. In order to understand the mechanisms underlying the clinical properties, as well as the undesired side effects of antipsychotic drugs, it is necessary to consider some of the signalling pathways potentially involved in their effects.

1.5.1 The cAMP/PKA/DARPP-32-cascade

Dopamine D₂ receptors are coupled to Gi/o proteins, which modulate potassium and calcium channels, and inhibit adenylyl cyclase (Picetti et al. 1997). The latter effect results in reduced synthesis of cAMP, the second messenger responsible for the activation of protein kinase A (PKA). In the absence of cAMP, PKA is an inactive heterotetramer composed of two catalytic and two regulatory subunits (Taylor et al. 1990). Binding of cAMP to the regulatory subunits causes the dissociation of the catalytic subunits, which phosphorylate a large number of downstream target proteins involved in the regulation of the state of excitability of nerve cells, including ion channels (e.g. voltage-dependent calcium and sodium channels) and neurotransmitter receptors (i.e. GABA and glutamate receptors). PKA can also translocate to the nucleus (Hagiwara et al. 1993) where it regulates transcription via phosphorylation and activation of the cAMP-response element binding protein (CREB) (see section 1.5.3).

The dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) is enriched in both striatonigral and striatopallidal neurons (Ouimet et al. 1998), where it acts as a modulator of the cAMP/PKA pathway (Fienberg et al. 1998). DARPP-32 plays an important role in striatal dopaminergic transmission. Activation of the cAMP cascade in medium spiny neurons leads to PKA-catalyzed phosphorylation of DARPP-32 at Thr34 (Nishi et al. 1997; Svenningsson et al. 1998). PhosphoThr34-DARPP-32 amplifies the effects of PKA by inhibiting protein phosphatase-1 (PP-1) and reducing dephosphorylation of downstream target proteins, such as voltage-dependent calcium and sodium channels, NMDA, AMPA, and GABA_A receptors (Fig. 3) (Greengard et al. 1999).
In striatopallidal neurons, the state of phosphorylation of DARPP-32 is determined to a large extent, by the opposite actions of adenosine A$_{2A}$ receptors, which stimulate, whereas dopamine D$_2$ receptors inhibit, the production of cAMP. Blockade of dopamine D$_2$ receptors by haloperidol results in disinhibition of PKA and increased phosphorylation of DARPP-32 at Thr34. PhosphoThr34-DARPP-32 strengthens the effects of activation of the cAMP/PKA pathway by inhibiting PP-1, thereby promoting phosphorylation of downstream target proteins, including glutamate AMPA and NMDA receptors, and voltage-gated calcium channels (VGCC). AC; adenylyl cyclase (see text for further details). The calcium/calmodulin-dependent protein phosphatase, calcineurin (PP2B) dephosphorylates DARPP-32 at Thr34.

Fig. 3. Schematic representation of the regulation of DARPP-32 in striatopallidal medium spiny neurons. Adenosine A$_{2A}$ receptors stimulate, whereas dopamine D$_2$ receptors inhibit, the production of cAMP. Blockade of dopamine D$_2$ receptors by haloperidol results in disinhibition of PKA and increased phosphorylation of DARPP-32 at Thr34. PhosphoThr34-DARPP-32 strengthens the effects of activation of the cAMP/PKA pathway by inhibiting PP-1, thereby promoting phosphorylation of downstream target proteins, including glutamate AMPA and NMDA receptors, and voltage-gated calcium channels (VGCC). AC; adenylyl cyclase (see text for further details). The calcium/calmodulin-dependent protein phosphatase, calcineurin (PP2B) dephosphorylates DARPP-32 at Thr34.
dopamine D<sub>2</sub> receptor antagonist, is attenuated in DARPP-32-null mice (Fienberg et al. 1998).

1.5.2 The mitogen activated protein kinases - Erk1/2

The extracellular signal-regulated kinases 1 and 2 (Erk1/2) are the best characterized members of the mitogen activated protein kinases (MAPK), a family of kinases involved in various neuronal activities including cell growth, gene transcription, synaptic plasticity and memory formation. Activation of Erk1 (44 kDa) and Erk2 (42 kDa) is mediated by phosphorylation at Thr202 and Tyr204, catalysed by the dual specificity kinase MEK1/2 (MAPK/Erk kinase), which is in turn phosphorylated/activated by MAPK kinase kinases, such as B-Raf and Raf-1 (Fig. 4). The state of phosphorylation of Erk1/2 is further controlled by different protein phosphatases, including the striatal enriched protein phosphatase (STEP) and dual-specificity threonine/tyrosine phosphatases (i.e. MKP3) (Haneda et al. 1999; Camps et al. 2000; Takaki et al. 2001; Paul et al. 2003).

Activation of ionotropic, metabotropic or neurotrophic receptors stimulates small G-proteins like Ras and Rap-1, which in turn regulate the activity of the Raf/MEK/Erk signalling cascade. One mechanism by which this occurs is via regulation of guanine nucleotide exchange factors (GEF’s) and GTPase activating proteins (GAP’s), many of them controlled by second messengers like calcium and cAMP (Grewal et al. 1999) (Fig. 4).

In the striatum, stimulation of glutamatergic corticostriatal afferents increases intracellular calcium via activation of NMDA receptors. This, in turn, activates the MAPK/Erk pathway, leading to phosphorylation of transcription factors, such as CREB and Elk-1 (cf. section 1.5.3) (Sgambato et al. 1998a). Phosphorylation of Erk is also triggered by administration of psychostimulants, such as cocaine and amphetamine. This effect appears to depend on NMDA receptors as well as on dopamine D<sub>1</sub> receptor-mediated activation of the cAMP/PKA/DARPP-32 cascade (Valjent et al. 2005). Stimulation of Erk phosphorylation has also been shown to occur following systemic administration of the dopamine D<sub>2</sub> receptor antagonist eticlopride. Even in this case, activation of NMDA receptors is required, as the effect of eticlopride is prevented by
administration of MK-801, a non-competitive NMDA receptor antagonist (Gerfen et al. 2002).

Several downstream targets for Erk1/2 have been described. Erk-dependent phosphorylation of RSKs [which belong to the pp90 ribosomal S6 kinase (pp90RSK) family of serine/threonine kinases] results in the translocation of these kinases to the nucleus, where they regulate CREB phosphorylation (Chen et al. 1992; Xing et al. 1996). Erk1/2 also activate serum response element (SRE)-driven gene expression via phosphorylation of the transcription factor Elk-1 (Fig. 5) (Vanhoutte et al. 1999; Thiels et al. 2002). In addition, Erk1/2 phosphorylate Kv4.2 A-type potassium channels, which are abundantly expressed in the striatum and hippocampus (Varga et al. 2000).

1.5.3 Regulation of gene transcription - CREB and Elk-1

Many drugs, including antipsychotic and antidepressant agents, exert a full therapeutic effect only after prolonged use. It is believed
that this slow onset is a consequence of adaptive changes affecting gene expression. These changes, which are also responsible for the generation of many side effects, require activation of a number of transcription factors, many of which are regulated by phosphorylation.

A large number of genes, including the proto-oncogene c-fos, are regulated by CREB (Sheng et al. 1990). CREB binds as a dimer (Yamamoto et al. 1988) to its DNA target sequence, the calcium- and cAMP response element (CRE) (Montminy et al. 1986; Johnson et al. 1997), and is activated by phosphorylation at a seryl residue in position 133 (Gonzalez and Montminy 1989) (Fig. 5). Several kinases, including PKA, protein kinase C (PKC), calcium/calmodulin protein kinases (CaMKs), Erk and RSKs have been shown to phosphorylate CREB, either directly or indirectly, in neuronal cells (Shaywitz and Greenberg 1999). In addition, CREB phosphorylation is controlled by several protein phosphatases, including PP-1, protein phosphatase-2A and protein phosphatase-2B (PP-2B, also named calcineurin) (Bito et al. 1996; Choe et al. 2004). Amplitude and mode of calcium entry are important factors that control the kinetics of CREB-phosphorylation and contribute to signal-specific IEG expression (Bading et al. 1997; Ginty 1997; Liu and Graybiel 1998).

Phosphorylation of CREB might not be sufficient to activate IEG expression. Thus, binding and activation of adaptor proteins such as CREB-binding protein (CBP) (Kwok et al. 1994), as well as other transcription factors such as serum response factor (SRF) and members of the p62TCF family (i.e. Elk-1; see below) might also be required (Bonni et al. 1995; Janknecht and Nordheim 1996).

The ternary complex factor Elk-1 is highly expressed in the CNS, and particularly in striatal neurons (Sgambato et al. 1998b). Elk-1 is phosphorylated at Ser383 and Ser389 by MAPKs, including Erk1/2 (Vanhoutte et al. 1999), c-Jun N-terminal/stress activated protein kinases (JNK/SAPK), and p38 (Whitmarsh et al. 1997). Elk-1 dephosphorylation is catalysed by the same phosphatases involved in the regulation of CREB (i.e. PP1, protein phosphatase-2A and calcineurin) (Tian and Karin 1999; Choe et al. 2004). Phosphorylation increases the ability of Elk-1 to form a ternary complex with SRE and SRF (Gille et al. 1992), thereby potentiating gene (e.g. c-fos) transcription (Marais et al. 1993).
The IEG *c-fos* is induced by many stimuli and is commonly used as a marker of cell activation (Sagar et al. 1988). In rodents and non-human primates, typical antipsychotics, like haloperidol, stimulate *c-fos* expression in the dorsal and ventral striatum (Robertson and Fibiger 1992; Deutch et al. 1996), but not in the prefrontal cortex (Robertson and Fibiger 1992). In the dorsal striatum, haloperidol also stimulates the expression of ΔFosB, a stable Fos related antigen (FRA) that accumulates in the brain after chronic drug treatment (Atkins et al. 1999). The increase in striatal *c-fos* expression produced by haloperidol is dependent on phosphorylation of CREB (Konradi and Heckers 1995) and is reduced by blockade of NMDA receptors with MK-801 (Ziolkowska and Hollt 1993). Haloperidol-induced *c-fos* expression (particularly in the medial and lateral caudate-putamen) is also reduced by administration of scopolamine, an acetylcholine muscarinic receptor antagonist (Guo et al. 1992).

In contrast to haloperidol, clozapine (10-30 mg/kg) stimulates *c-fos* expression in prefrontal cortex and nucleus accumbens, but not in dorsal striatum (Robertson and Fibiger 1992; Deutch and Duman 1996; Guo et al. 1998). It should be noted, however, that the effects of clozapine on IEG expression are observed at relatively high doses.
and that lower doses of this drug (e.g. 7.5 mg/kg) do not produce any changes in c-fos expression in the prefrontal cortex (Young et al. 1998). The lack of effect of clozapine on IEG expression in the caudate-putamen indicates a lower propensity to affect gene expression in this brain region and may be related to the minimal incidence of EPS of this drug (Pisa and Schranz 1988; Robertson and Fibiger 1992; Deutch 1994).

1.5.4 Regulation of tyrosine hydroxylase

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of catecholamines. Short-term TH activity is regulated via phosphorylation at multiple seryl residues, i.e. Ser19, Ser31 and Ser40, through activation of numerous protein kinases, including PKA, CaMKs, and MAPKs. Previous studies have shown that, in the striatum, phosphorylation at Ser31 and Ser40, but not at Ser19, is associated with increased TH activity (Harada et al. 1996; Lindgren et al. 2000; Lindgren et al. 2002).

In the basal ganglia, dopamine transmission is controlled by D₂ autoreceptors located on the cell bodies and terminals of nigrostriatal and mesocorticolimbic neurons. Activation of such autoreceptors reduces neuronal firing rate, thereby inhibiting dopamine release and synthesis (Fig. 6). Studies performed in striatal slices have shown that TH activity is specifically regulated by dopamine D₂S receptors, which are preferentially expressed in midbrain dopaminergic neurons. Reduced dopamine synthesis may in turn, depress dopamine transmission by limiting the amount of releasable neurotransmitter.

Acute administration of haloperidol stimulates dopamine synthesis, turnover and release in the striatum (Zivkovic et al. 1975; Zetterström et al. 1984; Imperato and Di Chiara 1985; Rayevsky et al. 1995). However, prolonged treatment with haloperidol reduces the ability of this drug to stimulate dopamine turnover in the striatum (Chiodo and Bunney 1983; Ichikawa and Meltzer 1991) and decreases spontaneous firing of midbrain dopaminergic neurons (Bunney and Grace 1978), a phenomenon that has been proposed to participate in the generation of EPS (Grace et al. 1997). Clozapine is also capable to stimulate dopamine release and turnover in the striatum (Ichikawa and Meltzer 1991). However, this effect is
exerted at high doses (10-30 mg/kg) and is less pronounced than that observed with haloperidol (Anden and Stock 1973; Zivkovic et al. 1975; Rayevsky et al. 1995). Previous studies reported that haloperidol and clozapine both increase TH phosphorylation at Ser19, Ser31 and Ser40 (Salvatore et al. 2001).

**Fig. 6.** Regulation of TH phosphorylation by haloperidol. Dopamine D<sub>2S</sub> autoreceptors inhibit the firing rate of midbrain dopaminergic neurons. Blockade of these receptors by haloperidol produces a depolarization that activates the MAPK cascade, leading to the sequential phosphorylation of MEK and Erk1/2. Activation of Erk1/2 results in phosphorylation of TH at Ser31 and Ser40, thereby increasing enzymatic activity and dopamine synthesis. SL327 prevents these effects by blocking MEK. Haloperidol also removes the inhibitory tone exerted by D<sub>2S</sub> receptors on cAMP production, thereby promoting PKA-catalyzed phosphorylation of TH at Ser40.
2. Specific Aims

The goal of this thesis is to examine the effects produced by antipsychotic drugs on the state of phosphorylation of proteins involved in striatal postsynaptic and presynaptic transmission. The specific aims are:

- To study the regulation of the cAMP/PKA/DARPP-32 cascade by haloperidol and clozapine.
- To examine the signalling pathways involved in the regulation of CREB and Elk-1 phosphorylation exerted by haloperidol and clozapine.
- To investigate the effects of acute and chronic treatment with haloperidol and clozapine on TH and Erk1/2 phosphorylation.
- To study the effects of typical and atypical antipsychotics on the state of phosphorylation of the AMPA GluR1 receptor.
3. METHODS

3.1 ANIMALS

The studies described in this thesis were performed using:

- C57BL/6 mice from Scanbur/B&K (Stockholm, Sweden), M&B (Ry, Denmark), or Charles River (Kingston, NY, USA) (Paper I-IV).
- DARPP-32-knockout mice (Fienberg et al. 1998) generated from the offspring of DARPP-32/- X DARPP-32/- mating pairs, which were backcrossed for at least 20 generations on a C57BL/6 background.
- Thr34→Ala DARPP-32 mutant mice (Svenningsson et al. 2003) obtained from heterozygous animals generated from C57BL/6 X 129SV hybrids bred for one generation on a C57BL/6 background.
- Dopamine D2 receptor-knockout mice, generated as described in (Baik et al. 1995).

All experiments were approved by the Swedish Animal Welfare Agency.

3.2 DOSES OF ANTIPSYCHOTIC DRUGS

The doses of haloperidol and clozapine used in this thesis were chosen based on clinical doses. Haloperidol is administered to psychotic patients at doses comprised between 2 and 40 mg/day (Sacristan et al. 2000). Assuming a body weight of 70 kg, these doses correspond to approximately 0.03 to 0.6 mg/kg. Individuals treated with clozapine receive doses of 300 to 450 mg/day (doses >600 mg/day may be associated with seizures) (Kinon et al. 2004), which correspond to about 5.0 mg/kg.

3.3 DETERMINATION OF PHOSPHORYLATED PROTEINS

Phosphoproteins were determined by Western blotting as described in detail in Paper I-IV. In Paper II, two different methods were used
for tissue extraction and phosphoprotein measurement. These methods gave indistinguishable results. The different antibodies used for western blotting are described in Table 3.

Table 3. Antibodies used for western blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Concentration used</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-Thr34-DARPP-32</td>
<td>Mouse</td>
<td>1:500-1:1000</td>
<td>Gift from Prof. Paul Greengard</td>
</tr>
<tr>
<td>Total-DARPP-32</td>
<td>Rabbit</td>
<td>1:5000</td>
<td>Upstate, Cell sign.</td>
</tr>
<tr>
<td>P-Ser845-GluR1</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Upstate, Cell sign.</td>
</tr>
<tr>
<td>P-Ser831-GluR1</td>
<td>Rabbit</td>
<td>1:400</td>
<td>Upstate, Cell sign.</td>
</tr>
<tr>
<td>Total-GluR1</td>
<td>Rabbit</td>
<td>1:60000</td>
<td>Upstate, Cell sign.</td>
</tr>
<tr>
<td>P-Thr202/Tyr204-Erk1/2</td>
<td>Rabbit</td>
<td>1:1500</td>
<td>Upstate, Cell sign.</td>
</tr>
<tr>
<td>Total-Erk1/2</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>Upstate, Cell sign.</td>
</tr>
<tr>
<td>P-Ser133-CREB</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>Upstate, Cell sign.</td>
</tr>
<tr>
<td>Total-CREB</td>
<td>Rabbit</td>
<td>1:3000</td>
<td>Upstate, Cell sign.</td>
</tr>
<tr>
<td>P-Ser383-Elk-1</td>
<td>Mouse</td>
<td>1:200</td>
<td>Upstate, Cell sign.</td>
</tr>
<tr>
<td>P-Ser19-TH</td>
<td>Rabbit</td>
<td>1:40000</td>
<td>Gift from Prof. John Haycock</td>
</tr>
<tr>
<td>P-Ser31-TH</td>
<td>Rabbit</td>
<td>1:20000</td>
<td>Gift from Prof. John Haycock</td>
</tr>
<tr>
<td>P-Ser40-TH</td>
<td>Rabbit</td>
<td>1:10000</td>
<td>Gift from Prof. John Haycock</td>
</tr>
<tr>
<td>Total-TH</td>
<td>Rabbit</td>
<td>1:10000</td>
<td>Gift from Prof. Tomas Hökfelt</td>
</tr>
</tbody>
</table>
4. Results and discussion

4.1. Regulation of cAMP signalling by antipsychotic drugs (Paper I and II)

4.1.1 Haloperidol and clozapine stimulate DARPP-32 phosphorylation at Thr34

In Paper I, we show that administration of haloperidol (0.2 mg/kg), clozapine (5 mg/kg), or the selective dopamine D₂ receptor antagonist eticlopride (0.5 mg/kg), stimulates the phosphorylation of DARPP-32 at the PKA site, Thr34. The increases in phosphoThr34-DARPP-32 produced by haloperidol and eticlopride lasted for 30 and 60 min, respectively, whereas the increase produced by clozapine lasted for at least one hour. The ability of haloperidol and eticlopride to stimulate DARPP-32 phosphorylation is most likely due to blockade of postsynaptic dopamine D₂ receptors, which exert a tonic inhibition on cAMP production and PKA activity in striatal medium spiny neurons (Stoof and Kebabian 1981). A similar mechanism may also be responsible for the increase produced by clozapine on Thr34 phosphorylation. In fact, clozapine appears to attain a significant degree of dopamine D₂ receptor occupancy in vivo (Suhara et al. 2002). However, in Paper II we found that clozapine failed to stimulate PKA-dependent phosphorylation of AMPA GluR1 (see section 4.1.2). Thus, it is possible that the sustained increase in DARPP-32 phosphorylation produced by clozapine at Thr34 does not occur via disinhibition of PKA. For instance, clozapine may act by suppressing Thr34 dephosphorylation through inhibition of calcineurin activity, exerted via reduction of intracellular calcium signalling (cf. Fig. 3) (Gong et al. 1996; Park et al. 2001).

4.1.2 Haloperidol, but not clozapine, stimulates GluR1 phosphorylation at Ser845

In Paper I, we obtained evidence indicating that treatment with antipsychotic drugs results in activation of the cAMP/PKA/DARPP-32 cascade, in striatal medium spiny neurons. One target for
phosphorylation catalysed by PKA is the GluR1 subunit of the glutamate AMPA receptor. Therefore, in Paper II, we examined whether administration of antipsychotic drugs changed the state of phosphorylation of GluR1 at the PKA site, Ser845. Haloperidol and eticlopride (both used at the dose of 0.5 mg/kg) stimulated Ser845 phosphorylation. In contrast clozapine (5 mg/kg) did not alter Ser845-GluR1 phosphorylation.

4.1.3 Haloperidol induced phosphorylation of GluR1 is mediated by activation of the cAMP/PKA/DARPP-32 cascade in striatopallidal neurons

The ability of haloperidol to activate the cAMP/PKA/DARPP-32 cascade (cf. section 4.1.1; Paper I) raised the possibility that this effect may mediate the stimulation of GluR1 phosphorylation at Ser845 produced by the typical antipsychotic. Previous work showed that the increase in DARPP-32 phosphorylation at Thr34 induced by eticlopride depends on intact adenosine A2A receptor transmission (Svenningsson et al. 2000) (cf. section 1.5.1). Therefore, we examined the effect of the selective A2A receptor antagonist, KW-6002 (3 mg/kg), on haloperidol-induced DARPP-32 and GluR1 phosphorylation. We found that KW-6002 blocked the ability of haloperidol to increase both DARPP-32 phosphorylation at Thr34 and GluR1 phosphorylation at Ser845 (Paper II). These results indicated that haloperidol stimulates GluR1 phosphorylation specifically in the striatal neurons of the indirect pathway, which selectively express A2A receptors (cf. section 1.4; Fig. 2). They also suggest that the increase in GluR1 phosphorylation produced by haloperidol depends on the concomitant phosphorylation of DARPP-32 at Thr34. To check this possibility, we examined the effect of haloperidol in DARPP-32 knockout mice and Thr34→Ala mutant mice. We found that, in these mice, haloperidol did not produce any effect on Ser845 phosphorylation. We therefore concluded that haloperidol increases the levels of phosphoSer845-GluR1 in striatopallidal neurons by: 1) stimulating phosphorylation via disinhibition of PKA and 2) suppressing dephosphorylation via phosphoThr34-DARPP-32-mediated inhibition of PP-1.
4.1.4 Atypical antipsychotics differ in their ability to regulate GluR1 phosphorylation

In Paper II, we tested a number of drugs generally classified as atypical antipsychotics for their ability to regulate GluR1 phosphorylation. Our results indicate that risperidone (3 mg/kg, s.c.), olanzapine (1 mg/kg, i.p.), quetiapine (30 mg/kg, i.p.) and aripiprazole (30 mg/kg, i.p.) all induced a significant increase in Ser845 phosphorylation. However, the effects of these drugs were in general less prominent than that produced by haloperidol, most likely due to their lower potency as dopamine D₂ receptor antagonists. Thus, among the different antipsychotic drugs tested in this study, only clozapine did not produce any significant change in Ser845 phosphorylation (cf. section 4.1.2).

GluR1 is phosphorylated at Ser831 by PKC and CaMKII. In our studies, we found that clozapine induced a long-lasting decrease in Ser831 phosphorylation. In contrast, neither haloperidol nor the other atypical antipsychotics (i.e. risperidone, olanzapine, quetiapine and aripiprazole) affected Ser831 phosphorylation. The distinctive biochemical effects of clozapine on GluR1 phosphorylation further emphasize the unique therapeutical properties of this atypical antipsychotic. Phosphorylation of Ser845 may be related to the different propensity to generate EPS (cf. section 5). For instance, among atypical antipsychotics, clozapine, which does not increase Ser845 phosphorylation, shows virtually no tendency to induce motor side effects. In contrast, risperidone, which produces a considerable increase in Ser845 phosphorylation, shows a higher propensity to generate EPS.

4.2. Regulation of Erk-MAPK cascade by antipsychotic drugs (Paper I and III)

4.2.1 Haloperidol stimulates Erk1/2 phosphorylation via blockade of dopamine D₂ receptors

In Paper I and III, we found that a single injection of haloperidol produced a time and dose-dependent increase in Erk1/2 phosphorylation. Haloperidol was most effective in increasing the
levels of phosphoErk1, which were maximally enhanced 15 min following drug administration and still elevated after one hour. Haloperidol-induced phosphorylation of Erk2, which is the predominant isoform in the striatum, was more modest and returned to basal levels 30 min after injection.

In agreement with previous findings (Gerfen et al. 2002) (cf. section 1.5.2), the effect of haloperidol on Erk1/2 phosphorylation was mimicked by systemic administration of the selective dopamine D2 receptor antagonist, eticlopride. It is likely that, as in the case of eticlopride, the ability of haloperidol to stimulate Erk1/2 phosphorylation depends on activation of NMDA receptors. Indeed, dopamine D2 receptors are present on both glutamatergic corticostriatal nerve terminals and striatal medium spiny neurons, where they inhibit glutamatergic transmission. Furthermore, quinpirole inhibited Erk1/2 phosphorylation produced, in vivo, by stimulation of corticostriatal fibers (Gerfen et al. 2002). It is therefore likely that haloperidol and eticlopride increase Erk phosphorylation by removing the inhibitory tone exerted by dopamine D2 receptors on striatal glutamatergic transmission. The consequent activation of glutamate receptors (including NMDA and AMPA receptors) would increase intracellular calcium and promote Erk phosphorylation.

In Paper III, we show that the haloperidol-induced stimulation of Erk1/2 phosphorylation is abolished in mice with a targeted deletion of the dopamine D2 receptor. It should be pointed out that, activation of D2 receptors in brain slices (Yan et al. 1999) or cell cultures (Welsh et al. 1998; Brami-Cherrier et al. 2002; Takeuchi and Fukunaga 2004), has been shown to stimulate Erk1/2 phosphorylation, (but see also (Banihashemi and Albert 2002; Liu et al. 2002)). In vivo, however, this effect was shown to be restricted to cholinergic interneurons, which only make up less than 5% of striatal neurons (Gerfen et al. 2002).

4.2.2 Haloperidol-induced stimulation of Erk1/2 phosphorylation is independent of activation of adenosine A2A or dopamine D1 receptors.

Previous evidence indicates that the ability of a dopamine D2 receptor antagonist to stimulate the cAMP/PKA pathway depends
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on intact adenosine A$_{2A}$ receptor transmission (Svenningsson et al. 2000). This observation was confirmed in Paper II, in which we showed that treatment with KW-6002, a selective adenosine A$_{2A}$ receptors antagonist, prevented the increase in DARPP-32 phosphorylation at Thr34 induced by 0.5 mg/kg of haloperidol. In the same set of experiments, KW-6002 did not affect haloperidol-induced Erk1/2 phosphorylation, suggesting that the functional interaction between dopamine D$_2$ and adenosine A$_{2A}$ receptors is limited to the regulation of the cAMP/PKA/DARPP-32 pathway.

Administration of haloperidol stimulates dopamine release by blocking inhibitory D$_2$ autoreceptors located on the dopaminergic terminals that innervate striatal medium spiny neurons (Imperato and Di Chiara 1985; Zetterström et al. 1986). It was therefore possible that the increase in Erk1/2 phosphorylation produced by the typical antipsychotic was due to indirect activation of postsynaptic dopamine D$_1$ receptors. However, pre-treatment with SCH23390 (0.15 mg/kg), a selective dopamine D$_1$ receptor antagonist did not affect the increase in Erk1/2 phosphorylation induced by haloperidol (0.5 mg/kg) (Paper II). Thus, haloperidol-induced increase in Erk1/2 phosphorylation is not attributable to the ability of this drug to stimulate dopamine release.

4.2.3 Haloperidol-induced stimulation of Erk1/2 phosphorylation is independent of cAMP/PKA/DARPP-32 cascade activation

The experiments described in section 4.2.3 indicate that the activation of the MAPK pathway produced by haloperidol is independent of the concomitant activation of the cAMP/PKA/DARPP-32 cascade. To further test this hypothesis, we examined the ability of haloperidol to stimulate Erk phosphorylation in DARPP-32 knockout mice and in Thr34→Ala mutant mice. We found that haloperidol failed to increase Erk1/2 phosphorylation in both strains of genetically altered mice (Paper II). Thus, it appears that activation of cAMP/PKA/DARPP-32 pathway and MAPK pathway produced by blockade of dopamine D$_2$ receptors are functionally distinct events.

In a recent study, Valjent et al. (Valjent et al. 2005) reported that the increase in Erk phosphorylation induced by psychostimulants,
such as cocaine and amphetamine, is abolished in DARPP-32 knockout mice and in Thr34→Ala mutant mice. Psychostimulant-induced phosphorylation of Erk is dependent on activation of dopamine D₁ receptors, which are preferentially expressed on the striatal neurons of the direct pathway (Fig. 2). In contrast, the increase in Erk phosphorylation produced by haloperidol is most likely occurring in the striatal neurons of the indirect pathway, which selectively express dopamine D₂ receptors (Fig. 2). Therefore, it appears that the regulation of Erk phosphorylation occurs via different mechanisms (one dependent on and the other one independent of concomitant activation of the cAMP pathway) in distinct population of striatal projection neurons.

4.2.4 Clozapine decreases Erk1/2 phosphorylation and this effect is independent of dopamine D₂ receptors

In contrast to haloperidol, the atypical antipsychotic clozapine produced a decrease on Erk1/2 phosphorylation that persisted for at least one hour after drug administration (Paper I). Clozapine reduced phosphorylation of both Erk isoforms, with the strongest effect being exerted on Erk2. In Paper III, it is shown that the decrease in Erk1/2 phosphorylation produced by clozapine is still present in dopamine D₂ receptor-null mice. Therefore, the negative regulation exerted by clozapine on Erk phosphorylation appears to be independent of a possible antagonistic action exerted by the atypical antipsychotic on dopamine D₂ receptors. In Paper I, it is also shown that administration of clozapine prevents haloperidol from increasing phosphorylation of Erk1/2 (cf. section 5).

The precise mechanisms underlying the negative regulation exerted by clozapine on Erk phosphorylation remain to be elucidated (see Conclusions and Future Directions). It is likely that one or more of the numerous pharmacological effects of clozapine (cf. section 1.2.2 and Table 1) may be involved. Interestingly, it has been reported that clozapine inhibits voltage-gated calcium channels in adrenal chromaffin cells (Park et al. 2001), an effect which may lead to reduced Erk phosphorylation. A possible negative regulation exerted on intracellular calcium would also explain the stimulation produced by clozapine on DARPP-32 phosphorylation (see section 4.1.1; Paper I).
4.2.5 Development of tolerance to haloperidol-induced increase, but not to clozapine-induced decrease, of Erk1/2 phosphorylation

The therapeutic effect of antipsychotic drugs, as well as their side effects, can only be seen after prolonged treatment. Since the Erk-MAPK cascade is known to be involved in the regulation of gene expression, we investigated the effect of chronic (14 days) treatment with haloperidol (0.5 mg/kg) and clozapine (5 mg/kg) on Erk1/2 phosphorylation. Basal Erk1/2 phosphorylation was not affected by repeated administration of haloperidol or clozapine (Paper II). However, one day after the suspension of the chronic treatment (day 15) the ability of a challenge dose (0.5 mg/kg) of haloperidol to stimulate Erk1/2 phosphorylation was decreased by almost 50 percent. In contrast, prolonged treatment with clozapine, did not affect the ability of this drug to decrease Erk1/2 phosphorylation. These results are in agreement with other studies, in which tolerance to chronic treatment with haloperidol, but not with clozapine, has been observed at the striatal level (Chiodo and Bunney 1983; Ichikawa and Meltzer 1990).

4.3 Regulation of CREB and Elk-1 phosphorylation by antipsychotic drugs (Paper I)

4.3.1 Haloperidol and clozapine exert opposite effects on CREB and Elk-1 phosphorylation

As shown in Paper I, haloperidol and eticlopride induce similar increases in the state of phosphorylation of CREB at Ser 133. The effect of both drugs was maximal at 15 min and returned to basal levels 60 min after injection. It has been proposed that the ability of haloperidol to phosphorylate CREB, thereby activating CREB-driven gene expression, is due to removal of the inhibitory tone exerted by dopamine D₂ receptors on the cAMP/PKA pathway (Konradi and Heckers 1995). More recent studies, however, suggests that PKA activates CREB in a calcium-dependent fashion, via stimulation of the phosphorylation of NMDA receptors, and activation of L-type voltage-gated calcium channels (Rajadhyaksah,
1999; Leveque, 2000). Thus, the increase produced by haloperidol on CREB phosphorylation might be due to blockade of inhibitory D₂ autoreceptors located on glutamatergic terminals, increased glutamate release and activation of NMDA receptors (cf. section 4.2.1). Haloperidol may also increase CREB phosphorylation by promoting glutamate transmission and calcium signalling at the postsynaptic level (i.e. in medium spiny neurons), via activation of the cAMP/PKA/DARPP-32 pathway and phosphorylation of AMPA receptors (see section 4.1.2; Paper II), NMDA receptors (Snyder et al. 1998), and L-type-voltage-gated calcium channels (Surmeier et al. 1995).

In contrast to haloperidol, clozapine produced a long-lasting (≥ 60 min) decrease (≈50%) in CREB phosphorylation (Paper I). A similar regulation was exerted on Elk-1. Thus, haloperidol produced a transient increase in Elk-1 phosphorylation, whereas clozapine decreased Elk-1 phosphorylation. The inhibitory effect observed on CREB and Elk-1 phosphorylation after clozapine treatment, is in line with previous work showing that clozapine is much less effective than haloperidol in inducing IEG expression (determined as Fos-immunoreactivity) in the dorsal striatum (Robertson and Fibiger 1992; Deutch and Duman 1996; Guo et al. 1998). Clozapine was also able to prevent haloperidol from stimulating CREB phosphorylation (as well as Erk phosphorylation; see section 4.2.1 and Paper I), an effect which may be related to the ability of this atypical antipsychotic to abolish haloperidol-induced catalepsy and c-fos expression (Young et al. 1999). The mechanism by which clozapine reduces CREB phosphorylation is still unresolved. However, as in the case of Erk phosphorylation, a reduction in intracellular calcium may be one of the factors involved (cf. section 4.2.1).

4.3.2 Blockade of Erk-MAPK cascade by SL327 prevents haloperidol from stimulating Elk-1, but does not affect CREB phosphorylation

It has been reported that, in the striatum, Erk1/2 activation is required for glutamate-mediated increases in CREB and Elk-1 phosphorylation (Sgambato et al. 1998a; Vanhoutte et al. 1999). In Paper I, we show that haloperidol enhances the phosphorylation of
Results and discussion

Erk1/2, and that this effect is paralleled by increased CREB and Elk-1 phosphorylation. To test whether the increase in CREB and Elk-1 phosphorylation triggered by haloperidol was mediated by its ability to activate Erk1/2, we employed SL327, a drug that abolishes Erk1/2 activation by blocking MEK-catalyzed phosphorylation. As shown in Paper I, pre-treatment with SL327, abolished haloperidol-induced increase in Elk-1 phosphorylation, without affecting CREB phosphorylation. These results suggest that haloperidol regulates SRE- and CRE-mediated gene expression by targeting different protein kinases. These findings are consistent with other studies showing that an increase in intracellular calcium, most likely mediated via NMDA receptors, is able to trigger Erk-catalysed phosphorylation of Elk, without activating CREB (Johnson et al. 1997).

4.4 Regulation of Tyrosine Hydroxylase Phosphorylation by Antipsychotic Drugs (Paper IV)

4.4.1 Haloperidol stimulates TH phosphorylation via blockade of dopamine D₂ receptors

In Paper IV, we show that a single dose of haloperidol (0.5 mg/kg) increases the state of phosphorylation of TH at Ser31 and Ser40. A similar effect had previously been reported using 2 mg/kg of haloperidol (Salvatore et al. 2000). We found that a lower dose (0.1 mg/kg) of haloperidol was still able to increase Ser31 phosphorylation, but did not affect Ser40 phosphorylation (data not shown). The ability of haloperidol to stimulate TH phosphorylation, provides a possible mechanism by which this drug enhance dopamine synthesis, turnover and release in the striatum (Anden et al. 1970; Zivkovic et al. 1975; Bunney and Grace 1978; Nissbrandt et al. 1989).

The ability of haloperidol to stimulate TH phosphorylation at Ser31 and Ser40 is abolished in dopamine D₂ receptor knockout mice. This observation suggests that haloperidol regulates dopamine synthesis and release by antagonizing somatodendritic inhibitory dopamine D₂ autoreceptors located on midbrain dopaminergic
neurons (Fig. 6) (Pucak and Grace 1994, 1996). Blockade of such an inhibitory feedback would result in increased firing rate, depolarization of dopaminergic terminals and increased phosphorylation of TH. Indeed, previous work carried out in striatal slices has shown that depolarization stimulates TH phosphorylation and activity (Lindgren et al. 2002). It should be noted, however, that our studies cannot exclude the possibility that haloperidol regulates the activity of midbrain dopaminergic neurons in the SNpc via blockade of striatal postsynaptic dopamine D$_2$ receptors and activation of a feed-forward loop.

4.4.2 Haloperidol stimulates TH phosphorylation via activation of Erk1/2

In a previous study, Lindgren, et al., (2002) showed that high potassium-induced depolarization of rat striatal slices, stimulates TH phosphorylation at Ser31 and Ser40 via activation of Erk1/2 (Lindgren et al. 2002). Since administration of haloperidol produced a similar pattern of TH phosphorylation, we investigated whether this effect is mediated via activation of Erk. We found that pretreatment with SL327, abolished haloperidol-induced TH phosphorylation at Ser31, and produced a 50% decrease in Ser40 phosphorylation. SL327 did not affect the increase produced by haloperidol on Ser19 phosphorylation. Virtually identical results were obtained using the selective dopamine D$_2$ receptor antagonist, eticlopride. We therefore concluded that blockade of dopamine D$_2$ receptors, results in depolarization induced-increase of Erk1/2, which mediates the increase in TH phosphorylation at Ser31, and part of that occurring at Ser40. The residual phosphorylation at Ser40 produced by haloperidol in the presence of SL327 is most likely due to blockade of dopamine D$_{2S}$ autoreceptors located on striatal dopaminergic terminals and disinhibition of PKA (Lindgren et al. 2001) (Fig. 6).

4.4.3 Clozapine fails to stimulate TH phosphorylation in the striatum

It has been reported that clozapine, at the dose of 30 mg/kg, stimulates the phosphorylation of striatal TH, mainly on Ser19 and
Ser40 (Salvatore et al. 2000). In Paper IV, we tested a lower dose of clozapine (5 mg/kg), which was previously shown to increase DARPP-32 phosphorylation at Thr34 in medium spiny neurons (see section 4.1.1) and induce high dopamine D$_2$ receptor occupancy in the primate striatum (Suhara et al. 2002). Acute or chronic (14 days) administration of this dose of clozapine did not produce any changes in TH phosphorylation. The effect on chronic clozapine treatment is line with previous findings that chronic treatment with clozapine (21 days x 20 mg/kg) does not alter basal striatal DA or DOPAC levels (Blaha and Lane 1987; Ichikawa and Meltzer 1990). Thus, our result are in line with the idea that clozapine is less efficient than haloperidol in stimulating dopamine turnover in the striatum (Anden et al. 1970; Zivkovic et al. 1975; Rayevsky et al. 1995).

4.4.4 Development of tolerance to haloperidol-induced increase in TH phosphorylation at Ser40

Several studies have reported that repeated administration of haloperidol reduces the ability of this drug to stimulate TH activity and dopamine turnover (Scatton 1977; Tissari et al. 1979; Ichikawa and Meltzer 1991). Furthermore, prolonged treatment with haloperidol reduces spontaneous firing of nigrostriatal dopaminergic neurons, a phenomenon referred to as a depolarization block (Bunney and Grace 1978; Chiodo and Bunney 1983; Grace et al. 1997), and regarded as one possible mechanism by which haloperidol induces EPS. We found that prolonged treatment with haloperidol (0.5 mg/kg) for 14 days (one injection/day) altered the basal levels of Ser31 phosphorylation and reduced the ability of haloperidol to stimulate TH phosphorylation at Ser40, without affecting the total amount of TH protein. The blunted effect of haloperidol on Ser40 phosphorylation, as well as the reduced basal levels of phosphoSer31-TH, might be a result of the depolarization block produced by the prolonged antagonism at dopamine D$_2$ receptors exerted by chronic haloperidol.
Conclusions and future perspectives

One important finding of the studies presented in this thesis is that administration of haloperidol and clozapine results in markedly different changes in protein phosphorylation within the striatum (cf. Table 3). Haloperidol appears to produce a substantial activation of the cAMP/PKA/DARPP-32 pathway, which leads to PKA-dependent phosphorylation of AMPA GluR1 at Ser845. In contrast, clozapine, although able to promote DARPP-32 phosphorylation at Thr34, does not alter phosphorylation of GluR1 at Ser845. The inefficiency of clozapine to activate the cAMP/PKA pathway is most likely due to the weaker dopamine D$_2$ antagonistic properties of this drug, particularly when compared to haloperidol. The lack of clozapine effect on GluR1 phosphorylation may be related to its lower liability to produce EPS. Indeed, increased GluR1 phosphorylation in striatopallidal neurons may facilitate excitatory glutamate transmission at the level of the indirect pathway, thereby suppressing motor activity (cf. Fig. 2 and section 1.4) and perhaps concurring to generate catalepsy, which is regarded as an index of EPS. Future studies utilizing mice bearing specific mutations that affect phosphorylation of GluR1 at Ser845 may help to elucidate this point.

Table 3. Summary of the effects exerted by haloperidol (HAL; 0.5 mg/kg) and clozapine (CLZ; 5.0 mg/kg) on protein phosphorylation.

<table>
<thead>
<tr>
<th>Phospho-protein</th>
<th>T34 DARPP-32</th>
<th>Erk 1/2 CREB</th>
<th>Elk-1 S845 GluR1</th>
<th>S831 GluR1</th>
<th>S31 TH</th>
<th>S40 TH</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAL</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<td>CLZ</td>
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<td>−</td>
</tr>
</tbody>
</table>

The opposite regulation exerted by haloperidol and clozapine on striatal Erk phosphorylation may represent another important factor involved in the different propensity of these drugs to induce EPS. The possibility to assess the involvement of the Erk cascade and of other signaling pathways in the generation of antipsychotic-induced...
motor disturbances requires the development of a reliable mouse model of EPS. Vacuous chewing movements (VCM) have been previously utilized in the rat to assess the propensity of antipsychotic drugs to induce tardive dyskinesia, an important component of EPS. Future studies will be necessary to set up and validate pharmacologically a similar model in the mouse. This, in turn, will make it possible to test different strains of genetically modified mice to elucidate signalling abnormalities associated with and possibly involved in EPS.

The studies presented in this thesis are based on biochemical determinations aiming at elucidating differences in the mechanism of action of antipsychotic drugs at the level of the striatal formation. One crucial future direction will be to characterize the effects of haloperidol, clozapine and other drugs at the level of other brain structures involved in psychotic disorders, such as prefrontal cortex and hippocampus. It will also be important to examine changes in protein phosphorylation induced by pharmacological interventions used to reproduce psychotic symptoms (cf. section 1.1.3). Treatment with PCP is regarded as a possible model of schizophrenia. It will therefore be interesting to examine the effect of acute and chronic administration of PCP on the state of phosphorylation of various signalling components. Moreover, it will be important to determine the ability of different antipsychotic medications to revert possible changes in protein phosphorylation produced by PCP.

Finally, one important aspect still to investigate is the identification of the mechanisms underlying the unique biochemical properties displayed by clozapine in these studies. We have hypothesized that clozapine reduces Erk, CREB and Elk-1 phosphorylation via suppression of calcium-dependent signalling. The specific pharmacological effects (e.g. blockade of specific serotonin, muscarinic or adrenergic receptors) that may be involved in this action remain to be tested.
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“Mum, it’s really quite simple, look this is the way a mouse brain works”
Anton, 5 years old
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