RECEPTOR AND CELLULAR MECHANISMS OF ANTIPSYCHOTIC DRUGS

By

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ABSTRACT

The mechanisms contributing to the atypical clinical profile of clozapine remain uncertain. To address this question, Fos-immunostaining in combination with drug manipulation, brain lesions and in situ hybridization histochemistry were used to examine neurotransmitter systems involved in the effects of antipsychotic drugs. This research program employed these techniques (1) to determine receptor mechanisms mediating clozapine-induced c-fos expression in the forebrain and (2) to characterize the phenotypes of neurons targeted by clozapine and the typical antipsychotic haloperidol.

In the first experiment, scopolamine, a muscarinic receptor antagonist, attenuated haloperidol-induced Fos immunoreactivity in the striatum. This suggests that haloperidol-induced Fos induction in the striatum is modulated by muscarinic cholinergic mechanisms, and that the antimuscarinic action of clozapine may contribute to its failure to increase Fos induction in the striatum.

5,7-dihydroxytryptamine lesions of the medial forebrain bundle or 6-hydroxydopamine lesions of the dorsal noradrenergic bundle produced extensive serotonin and noradrenaline depletions in the forebrain, respectively. However, neither type of lesion affected clozapine-induced c-fos expression in the rat forebrain, suggesting that neither serotonergic nor noradrenergic mechanisms are involved in this action of clozapine.

7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7-OHDPAT), a D3 receptor-preferring agonist, attenuated clozapine-induced c-fos expression in the nucleus accumbens (NAc), lateral septum (LSN) and the major island of Calleja (ICjM), without affecting the medial prefrontal cortex (mPFC). Quinpirole, which has similar affinities for D3 and D4 receptors, produced a small but significant attenuation of clozapine’s effects in the mPFC and blocked
clozapine's actions in the ICjM, NAC and LSN. Given the different affinities of quinpirole and 7-OHDPAT for D2, D3 and D4 receptors, these data suggest that clozapine-induced \textit{c-fos} expression in the ICjM, NAc and LSN is due to its antagonist actions at D3 receptors, while antagonist actions at D4 receptors may contribute, in part, to the Fos induction in the mPFC.

\textit{In situ} hybridization for D3 receptor mRNA confirmed that most of the clozapine-induced Fos positive neurons in the ICjM, and the majority in the NAc and LSN express D3 receptor mRNA. In contrast, haloperidol-induced Fos positive neurons rarely expressed D3 mRNA in any brain region. Further studies demonstrated that clozapine increased \textit{c-fos} expression in both enkephalin (Enk) and dynorphin (Dyn) containing neurons in the NAc and LSN. Haloperidol also increased \textit{c-fos} expression in Enk and Dyn neurons, albeit in a different pattern. This suggests that while some Enk and/or Dyn neurons targeted by clozapine express D3 receptors, others do not.
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For my parents, who have suffered too much from the second world war, the civil war, and abnormal political movements in their lives, yet always tried their best for their children.

For my friends who have shared both my good times and hard times,

All their invaluable encouragement and generous help have supported a Chinese woman studying overseas, alone...
CHAPTER I. ANTIPSYCHOTIC DRUGS: INTRODUCTION AND OVERVIEW

1. History of Antipsychotics: Typical and Atypical

Antipsychotic or neuroleptic drugs are used to treat severe psychiatric illnesses, the psychoses [Baldessarini, 1990]. Psychosis is a form of mental illness wherein a person’s abilities to think, remember, communicate, respond emotionally, interpret reality and behave appropriately are impaired to the extent that he (she) is unable to meet the ordinary demands of life [American Psychiatric Association, 1984]. The most prevalent psychotic illness, commonly known as schizophrenia, is characterized by a spectrum of symptoms that typically include: disordered thought, social withdrawal, hallucinations (both auditory and visual), delusions of persecution (paranoia) and bizarre behaviour. These symptoms are sometimes categorized as “positive” (e.g. hallucinations) and “negative” (e.g. social withdrawal and apathy). The etiology of schizophrenia is unknown. In view of newer understandings of neurochemistry and of the effects of a variety of psychotropic medications on the symptoms of psychotic disorders, there is increasing evidence to suggest that schizophrenia is a chemical brain disorder; specifically, a manifestation of an abnormality of neurotransmission in the central nervous system (CNS) [Snyder et al, 1974a; Carlsson & Carlsson, 1990b; Lieberman & Koreen, 1993].

Human awareness of insanity is quite ancient and was recorded as early as biblical times [Ronsner, 1978]. Modern psychiatric treatment can be viewed as having begun with Phillippe Pinel’s release of the insane from their chains at Bicetre in the late 1700s [Morgan, 1985]. Clearly seeing the mentally ill as suffering from a medical illness, Pinel
described them in a medical model. By the end of the 1800s, mental illness became a legitimate area of study within medicine and great advances in the field of psychiatry were made [Spitzka, 1887]. Approximately a century ago, the English physician and biochemist, J.L.W. Thudichum, began to study the chemical composition of the bovine brain; these studies formed the early foundation of modern neurochemistry. Thudichum proposed that many forms of insanity were caused by the generation of toxic substances within the body [Kety, 1978]. About a half-century after the momentous hypothesis of this early neurochemist, other investigators began to search for abnormal chemicals in the biological fluids of psychotic individuals. One such early investigator was H.H. DeJong, who isolated a substance (which he termed “catatonin”) from the urine of schizophrenic patients. While DeJong hoped that “catatonin” would be a clue to the biochemical etiology of psychosis, he was disappointed to find that the abnormal substances in the urine correlated with coffee and tobacco consumption by the patients and could more properly be identified as metabolites of caffeine and nicotine rather than as specific psychotogenic substances. In the 1950s and 1960s, a number of investigators also tried to identify abnormal substances in the blood and urine of psychotic individuals [Sankar, 1962]. Unfortunately, the various chromatographic spots identified represented primarily metabolites of the drug chlorpromazine, rather than any naturally generated psychotogenic substances. Throughout the late 1950s and the 1960s, a variety of investigators injected blood serum from schizophrenic patients into animals, in an attempt to observe modifications of behavior or interactions with a variety of neurotransmitters. These early investigators proposed a distinct difference between serum from normals and that from schizophrenics but again failed to prove a distinct biochemical etiology for psychosis.
Although early attempts to determine a chemical etiology in psychosis were unsuccessful, all contributed to our understanding of neurochemistry and psychopharmacology and helped to pave the way for the modern view of schizophrenia as a manifestation of abnormal neurochemistry.

One of the most fruitful areas of investigation supporting current thinking about the etiology of psychotic illness is an outgrowth of the administration of a variety of medications to medical and psychiatric patients. In 1931, Sen and Bose published the first report of the use of *Rauwolfia serpentina* in the treatment of insanity [Giachetti & Shore, 1978]. Insulin shock, pentylenetetrazol-induced convulsions and electroconvulsive therapy followed in 1933, 1934 and 1937, respectively. Beginning in 1948 with the discovery of the hallucinogenic effects of lysergic acid diethylamide (LSD) and continuing for a period of nearly 20 years, investigations of chemically induced psychotic behavior in man and animals were central to the development of the embryonic science of biological psychiatry [Hollister, 1968]. Reserpine was isolated in the early 1950s from the root of the plant *Rauwolfia serpentina*. The marked antihypertensive effect of reserpine, combined with its tranquilizing activity, led to its use in the treatment of schizophrenia. Although reserpine produced a calming effect and a reduction in psychotic symptoms in schizophrenia and other psychotic illnesses [Sulser & Bass, 1968], adverse side effects such as depression and suicidal feelings led to its relatively short, although important, career as an early antipsychotic drug.

The introduction of chlorpromazine in 1952 marked the beginning of modern psychopharmacology [Caldwell, 1978; Morgan, 1985]. Prior to 1950, psychopharmacology was limited to the study of bromides, paraldehyde, chloralhydrate
and barbiturates. These compounds had sedative effects on patients but had little or no effect upon their psychotic thinking processes and bizarre behavior. The effects of chlorpromazine on psychiatric patients showed that, for the first time, the active symptoms of schizophrenia, i.e., hallucinations, delusions and agitation could be brought under control without excessive sedation [Caldwell, 1978]. Since then, pharmacotherapy has become the treatment of choice for schizophrenia. Over the subsequent two decades a large number of other antipsychotic drugs was developed. The main groups of these antipsychotics include phenothiazines, thioxanthenes, butyrophenones, diphenylbutylpiperidines, dibenzazepines, substituted benzamides and miscellaneous compounds. Among them, one of the most widely used antipsychotics is haloperidol, a drug of the butyrophenone class.

The use of classical (or typical) antipsychotic drugs, as exemplified by the phenothiazines, thioxanthines, butyrophenones and diphenylbutylpiperidines, has been a landmark in the pharmacotherapy of schizophrenia and other psychotic disorders. However, these drugs also have a spectrum of adverse effects including hypotension and neurological (most notably parkinsonism, dyskinesia, and akathisia), dermatological and hematological side-effects [Morgan, 1985]. In particular, the extrapyramidal side effects (EPS) were considered to be a necessary concomitant to the antipsychotic efficacy of these drugs and led to the drugs being termed 'neuroleptics' (WHO Tech. Rep. No. 371). The neuroleptic actions of these drugs were specified as (1) the effects on the extrapyramidal system, often resulting in a variety of EPS; (2) a lowering of the convulsive threshold, probably through actions on the hippocampus and (3) marked effects on the brain's reticular formation, the limbic system and the hypothalamus, which reduce excessive
psychomotor excitement and emotional tension and, frequently, produce a variety of changes in the autonomic nervous system function [Lehmann, 1965]. Therefore, if a potential neuroleptic compound failed to induce EPS, it was not considered for further development, because neuroleptics had been defined as drugs capable of producing such side effects. One such compound was clozapine, which was first investigated in Europe in the mid-1960’s [Bente et al, 1966a, 1966b; Gross & Langner, 1966; Hippius, 1989]. Clozapine, despite being an effective antipsychotic drug, does not produce EPS associated with the so-called classical/typical neuroleptics such as haloperidol. Consequently, the manufacturers of clozapine were hesitant to introduce the drug. Furthermore, some pharmacologists did not consider it to be a neuroleptic, not because it lacked effectiveness, but because it lacked these adverse side-effects. Others, however, considered clozapine to be a pioneering drug because it refutes the hypothesis of a connection between clinical efficacy and the generation of EPS [Hippius & Stille, 1973; Stille & Hippius, 1971]. Clozapine’s status as an antipsychotic drug which did not produce EPS was consolidated by further studies [Berzewski et al, 1969; Angst et al, 1971a; 1971b] and as more became known as to the specific anatomical sites of actions of antipsychotic drugs [Anden, 1972; Borison & Diamond, 1983; Borison et al, 1983; Chiodo & Bunney, 1983; White & Wang, 1983; Seeman & Ulpian, 1983; Carlsson, 1988], the requirement of EPS for therapeutic efficacy of neuroleptics was further questioned. Consequently, more putative antipsychotic drugs with lower or without the typical profile were developed [Leysen et al, 1988; Lewander et al, 1990; Svartengren & Simonsson, 1990; Hyttel et al, 1992; Wadworth & Heel, 1991]. This led to the development of criteria to define an “atypical compound”. In preclinical testing, the criteria applied to define “atypical” include: (1) efficacy in standard
antipsychotic screening paradigms (e.g. antagonism of dopamine (DA) agonist-induced stereotypes, conditioned avoidance response); (2) no induction of catalepsy; (3) no up-regulation of DA D2 receptors and (4) no development of tolerance to increased DA turnover or depolarization block of A9 DA neurons with chronic treatment. In clinical studies, the following criteria have been used to define atypical antipsychotics: (1) antipsychotic efficacy; (2) no or markedly reduced induction of acute EPS and tardive dyskinesia (TD) and (3) no elevation of prolactin [Lieberman, 1993].

In the mid-1970's, reports documented a 1-2% incidence of clozapine-induced agranulocytosis in patients [Amsler et al 1977; Krupp & Barnes, 1989]. However, therapeutic interest in clozapine continued, primarily because this drug was consistently reported to be a superior antipsychotic for treatment-resistant schizophrenic patients [Fisher-Cornellson & Ferner, 1976; Shopsin et al, 1979; Claghorn et al 1987; Kane et al, 1988, 1989]. Clozapine has recently been approved for use in the United States and Canada on the condition of the establishment of a patient monitoring system which includes weekly blood counts to detect reduced white blood cell counts.

Despite the drawbacks of clozapine, this atypical drug has provided (1) potential insights into the development of novel and effective antipsychotic drugs and (2) insights concerning the pathophysiology of schizophrenia. Therefore, determination of the mechanisms of action of atypical and typical neuroleptics is of importance and might provide critical clues for improving treatments. Research for a better understanding of the mechanisms which contribute to the therapeutic effects of antipsychotics include anatomical, pharmacological, biochemical, and electrophysiological studies as well as clinical investigations. This thesis will focus on pharmacological and biochemical
approaches to understanding the receptor and molecular mechanisms of these typical and atypical drugs. The brain sites important for the actions of antipsychotic drugs will also be briefly discussed. For the purpose of this thesis, the definition for atypical antipsychotics will focus on the clinical efficacy and the absence of EPS (i.e. parkinsonism), excluding akathisia. The prolactin response, the amelioration of negative symptoms, and issues pertaining to treatment-resistant patients will not be included as criteria for atypical drugs.

2. Sites of Action of Antipsychotic Drugs in the Brain

The considerable variety of biological effects which result from the central actions of neuroleptic drugs clearly indicates that there must be more than one site of action in the brain; several principal anatomical sites are thought to be involved. These brain sites are: (1) the brainstem, where sedation and effects on sensory input are thought to be mediated; (2) the hypothalamus, which affects body temperature, appetite, and endocrine systems; (3) the basal ganglia, where the EPS are likely to be mediated; (4) the limbic system, which affects emotion and mood and (5) the prefrontal cortex (PFC), which is involved in motivation, planning and attention. Also it is probable that antipsychotic drugs have some actions on the hippocampus and cerebral cortex, since they can influence seizure activity, and on the spinal cord, as they can affect spinal reflexes, although some of these actions could be mediated via descending influences from the brainstem. In addition, recent studies suggest that schizophrenia may be associated with structural and functional disturbances of the hippocampus, parahippocampal gyrus, entorhinal cortex, amygdala,
cingulate gyrus, septum, thalamic nuclei, and cerebral cortices [Bogerts, 1993; Andresen et al, 1994; Selemon et al, 1995; Silbersweig et al, 1995].

**a. The brainstem**

Studies on the effects of neuroleptic drugs in experimental animals showed that chlorpromazine caused a marked reduction in responsiveness to sensory stimuli without inducing appreciable sedation [Bradley & Hance, 1957]. These effects were ascribed to inhibition of the activation of the brainstem reticular activating system by afferent impulses from collaterals of the sensory pathways rather than to a direct depression of reticular neurons. Support for this hypothesis came from the finding that chlorpromazine had little effect on the threshold of arousal produced by direct electrical stimulation of the brainstem reticular formation, but caused marked elevations in thresholds for sensory-induced arousal [Bradley & Key, 1958]. It was also shown that this reduced responsiveness to sensory stimulation produced by chlorpromazine closely resembled the process of habituation, in which responses to non-significant stimuli are progressively reduced and eventually lost. This neuroleptic-induced reduction of sensory influences on the brainstem led to the suggestion that these drugs produced a state of "pharmacological deafferentiation" of the brainstem [Bradley, 1965].

In other experiments, Bunney and colleagues [1973] demonstrated that intravenously administered d-amphetamine markedly decreased the spontaneous activity of dopaminergic neurons in the substantia nigra (A9) and ventral tegmental area (A10). Acute treatment with antipsychotic phenothiazines and haloperidol increased the firing rate of these DA neurons and reversed the d-amphetamine depression [Bunney et al, 1973;
Chiodo & Bunney, 1983]. However, chronic treatment with haloperidol, sulpiride and
chlorpromazine, typical neuroleptics which cause EPS, markedly reduced the number of
active DA neurons below control levels in both the substantia nigra (SN) and the ventral
tegmental area (VTA) [Chiodo & Bunney, 1983; White & Wang, 1983]. In contrast,
repeated treatment with clozapine resulted in the depolarization inactivation of A10 DA
neurons but not of A9 cells [Chiodo, & Bunney, 1983; White & Wang, 1983]. These data
indicate that atypical drugs which lack the EPS liability only inactivate A10 DA neurons
whereas neuroleptics which can induce EPS produce depolarization inactivation of both A9
and A10 DA neurons. It was thus suggested that the time-dependent development of A9
DA inactivation induced by repeated neuroleptic treatment may provide a mechanism for
understanding the delayed onset of EPS often observed with these drugs [Chiodo &
Bunney, 1983]. Since all antipsychotic drugs tested produced depolarization inactivation
in A10 neurons, it was suggested that the development of a state of antipsychotic-induced
depolarization blockade in A10 midbrain DA neurons might be related to the delayed onset
of clinical efficacy [Chiodo & Bunney, 1983; White & Wang, 1983; Chiodo & Bunney,
1985].

b. The hypothalamus

Many neuroleptic drugs cause increased secretion of prolactin and decreased
release of growth hormone [Bradley, 1986]. The hypothalamus exerts humoral control
over adenohypophysial function and can stimulate or inhibit the release of hormones by the
pituitary. The anterior lobe mammatrophs of the pituitary control lactation via prolactin
release, and dopamine acting on D2 receptors in this area acts as the prolactin release
inhibitory factor. Hypophysial portal blood has been found to contain significant quantities of dopamine. Also, it has been demonstrated that dopamine D2 receptors regulate the release of DA in the rat basal hypothalamus and the neurointermediate lobe of the pituitary gland [Plantje et al, 1987]. Thus, it is thought that an antagonist action by neuroleptic drugs at D2 receptors in the hypothalamus and pituitary is responsible for the increased levels of prolactin and decreased levels of growth hormone associated with the use of these drugs.

c. The basal ganglia

Of the many side-effects produced by neuroleptic drugs, their ability to induce parkinsonism is perhaps the most notable. Except for clozapine, all antipsychotic drugs currently available have been found to cause EPS to some extent. These effects are most marked in the case of the butyrophenones (e.g. haloperidol). Because nearly all of the clinically used antipsychotics produce EPS, a great deal of interest has centered on the actions of these drugs in the basal ganglia, notably the caudate nucleus, putamen, globus pallidus and allied nuclei, which play a crucial role in the control of posture and movement. Parkinson’s disease (PD) has long been known to be a disorder of the basal ganglia: DA is drastically reduced in the striatum [Hornykiewicz, 1966]. Current understanding of the role of DA deficiency in this region in the pathogenesis of PD, the demonstration that neuroleptic agents are antagonists at DA receptors, and the striking resemblance between the clinical manifestations of Parkinson’s disease and the neurological side effects of neuroleptic drugs, all point to a deficiency of DA activity as the basis of some neuroleptic-induced EPS.
Initially, it was assumed that the antipsychotic actions of the neuroleptic drugs were inextricably linked to their ability to produce EPS, possibly by an action at a common site. However, this assumption is now being questioned since atypical antipsychotic drugs have lower potential to produce EPS. The most clear-cut differences among antipsychotic medications lie not in their therapeutic efficacy, which are basically equivalent, but in their side effects. Additionally, the presence of PD does not appear either to protect patients from nor to predispose them to schizophrenia [Bradley, 1986]. Therefore, it is possible that these two main actions of neuroleptic drugs, i.e. the antipsychotic action and the ability to induce symptoms of PD, may be mediated by actions at different sites in the brain [American Psychiatric Association, 1980]. Since the main function of the nigrostriatal dopaminergic pathway is to mediate motor performance, and the degeneration of this pathway is responsible for the symptoms of idiopathic PD, it is usually assumed that the neuroleptic drugs produce EPS by blockade of dopamine receptors in the basal ganglia (see discussion below). On the other hand, the antipsychotic effects of neuroleptic drugs has been postulated to be mediated at another brain site, the limbic forebrain including the nucleus accumbens (NAc) [Anden, 1972; Borison & Diamond, 1983; Borison et al, 1983; Chiodo & Bunney, 1983; White & Wang, 1983].

The concept of site specificity of drug actions has been supported by pharmacological and biochemical evidence. As discussed above, electrophysiological studies have demonstrated that repeated treatment with typical neuroleptics produces a decrease in the number of spontaneously active DA cells in the SN pars compacta and the VTA, while the atypical drug clozapine only reduces the number of spontaneously firing DA neurons in the VTA [Chiodo & Bunney, 1983; 1985; Grace & Bunney, 1984; White &
Wang, 1983]. More recent studies have attempted to relate these electrophysiological results to neurochemical measures of extracellular DA concentrations in forebrain DA terminal regions such as the striatum and NAc. *In vivo* microdialysis studies suggest that acute administration of different antipsychotic drugs may differentially increase the extracellular concentration of DA in mesotelencephalic systems [Moghaddam & Bunney, 1990b]. Haloperidol and sulpiride were more effective in releasing DA from the terminals of the nigrostriatal DA system while clozapine had a preferential effect on the mesolimbic and particularly the mesocortical DA systems [Moghaddam & Bunney, 1990a, 1990b]. Other studies have shown that both acute and chronic treatment with haloperidol increased 3-methoxytyramine (3-MT) levels, a reliable index of DA release in the striatum and NAc, while acute or chronic clozapine treatment either failed to increase 3-MT accumulation in the striatum and NAc, or reduced 3-MT in the striatum [Karoum & Egan, 1992; Egan et al, 1991]. These data suggest that the different effects of haloperidol and clozapine on DA release in the striatum and NAc may contribute to their different therapeutic profiles. Studies on DA turnover also show that typical and atypical drugs have different effects in the striatum and NAc. For example, haloperidol significantly increased DA turnover in both the NAc and striatum [Csernansky et al, 1993]. Increases in DA turnover in both the NAc and striatum are a reliable characteristic of typical neuroleptics [Scatton, 1977]. In contrast, clozapine and other atypical drugs produce preferential elevations of DA turnover in the NAc [Csernansky et al, 1993]. These results suggest that the striatum is the site for typical neuroleptic drugs to induce EPS.
d. The mesolimbic system

The mesolimbic dopaminergic system has its origin in cell bodies in the VTA. These neurons project to the mesial component of the limbic system, the NAc, part of the amygdala and hippocampus, the lateral septal nuclei, the nuclei of the stria terminalis, and the mesial frontal anterior cingulate and entorhinal cortices [Bradford, 1986]. Among the projections of the mesolimbic system, those to the NAc are thought to be particularly important. This nucleus is a convergence site for inputs from the anterior cingulate cortex, entorhinal cortex, hippocampus, amygdala and parts of the temporal lobe [Alexander, 1986]. Within the NAc, the terminal regions of these inputs overlap with one another and with the mesolimbic DA afferents [Cador et al, 1991]. Therefore, the mesolimbic dopaminergic projections are thought to modulate this convergent flow of neural activity and thereby transform the information conveyed by the NAc to the frontal lobes and limbic structures. The role of the mesolimbic system in emotions and memory, and the similarity between schizophrenia and certain types of psychomotor (limbic system) epilepsy in which disturbances of thought and perception are prominent, have led to the postulate that the symptoms of schizophrenia may be related to the mesolimbic components of the dopaminergic system [Stevens, 1973; Carlsson, 1974].

As discussed above, there is considerable electrophysiological, pharmacological and biochemical evidence to support the conclusion that both typical and atypical antipsychotic drugs exert common effects in the NAc [Chiodo & Bunney, 1983; White & Wang, 1983; Grace & Bunney, 1984; Moghaddam & Bunney, 1990a; 1990b; Karoum & Egan, 1992; Csernansdy et al, 1993]. Recently, the possibility that antipsychotic drugs might produce their therapeutic effects by an action on the mesolimbic dopaminergic
system has been investigated by examining the effects of antipsychotic drugs on
electrotonic transmission in subregions of the striatal complex [O'Donnell & Grace, 1995; Onn & Grace, 1995]. Electrotonic transmission is a mode of communication between sets of two or more cells in which apposed membranes are connected by gap junctions. Gap junctions are composed of aggregates of low-resistance intermembranous channels [Loewenstein, 1981] which do not appear to be rigid structures. Their permeability can vary to produce either “open” or “closed” states in response to electrical or chemical events [Baux et al, 1987; Bennett, et al, 1988; Rao et al, 1987; Spray et al, 1979]. It has been found that electrical transmission exists in the mammalian brain, in areas including the SN [Grace & Bunney, 1983], hippocampus [MacVicar & Dudek, 1981] and striatum [Walsh et al, 1989]. Electrical coupling, as demonstrated by simultaneous intracellular recording from two cells in close proximity, as well as dye-coupling, has been used extensively as indirect evidence for the existence of this type of communication [Dudek et al, 1983; Llinas, 1985; Stewart, 1978]. In contrast to transmission at chemical synapses, signals in electrotonic junctions were thought to be transmitted between the coupled neurons through gap junction channels, without intervention of neurotransmitters [Bennett, 1977].

However, increasing evidence has shown that electrotonic transmission is not passive in nature but can be modulated dynamically by such factors as pH [Church & Baimbridge, 1991], intracellular calcium levels [Rose & Loewenstein, 1975; Ramon et al, 1985; Spray et al, 1985; Rao et al, 1987] and synaptic transmitters [Cepeda et al, 1989; O'Donnell & Grace, 1993; Onn et al, 1991; Piccolino et al, 1984]. For example, it has been demonstrated that dopaminergic modulation of electrical transmission takes place in the striatum, where the incidence of dye coupling can be increased by DA-depleting lesions.
made prior to in vitro recordings [Cepeda et al, 1989] or by systemic administration of DA agonists and antagonists [O'Donnell & Grace, 1993; Onn et al, 1991]. In vivo recordings have also shown that the administration of D1 and D2 agonists can dramatically increase the degree of dye coupling between neurons in the core and shell regions of the NAc [O'Donnell & Grace, 1993; Onn & Grace, 1994]. These findings suggest that DA, in addition to its actions on the membrane of single neurons, may also affect neuronal integration by regulating the transfer of information between adjacent neurons in the NAc.

Using in vivo intracellular recording and dye labeling techniques, the effects of antipsychotic drugs on dye coupling were examined in different regions of the striatum [O'Donnell & Grace, 1995; Onn & Grace, 1995]. Rats treated repeatedly with antipsychotic drugs were found to exhibit a high incidence of dye coupling that occurred in a drug- and region-specific manner in the brain. Both clozapine and haloperidol increased dye coupling in the limbic-associated accumbens shell region whereas only haloperidol increased dye coupling in the motor-related striatal matrix and accumbens core regions [O'Donnell & Grace, 1995; Onn & Grace, 1995]. In addition, these changes were not observed in response to DA receptor blockade by acute drug administration or when haloperidol was administered for a short period, which is not sufficient to induce DA cell depolarization inactivation. Therefore, alterations in dye coupling appears to be correlated temporally with the development of antipsychotic-induced DA neuron depolarization blockade [Chiodo & Bunney, 1983; White & Wang, 1983]. Although the precise mechanisms through which antipsychotics regulate electrotonic transmission in the brain remain unknown, these drugs may alleviate the profound disturbances in cognitive function of schizophrenics by producing sustained alterations in junctional resistance. In addition,
the fact that both typical and atypical drugs target neurons in the accumbens shell region suggests that this may be a site of therapeutic action of these drugs. In contrast, the selective modulation of coupling in the dorsal striatum by haloperidol may be relevant to the motor side-effects of this drug.

e. The prefrontal cortex

The PFC is involved in motivation, planning, attention, and the organization of behavior. At the end of the 19th century, Emil Kraepelin speculated that psychiatric diseases might be localized to specific regions that were consistent with the clinical presentation, suggesting that the PFC and the temporal cortex would be the most probable sites of abnormality [Kraepelin, 1923; 1971]. Kraepelin believed that dementia praecox (schizophrenia) had an organic base, citing as evidence autopsy reports of nerve cell damage in the frontal and temporal lobes [Kraepelin, 1923; 1971]. Modern investigators have confirmed Kraepelin’s early speculations about prefrontal and temporal cortex and have also highlighted interactions with other regions such as the thalamus, hippocampal formation, and cingulate gyrus [Weinberger et al, 1988; Andreasen et al, 1994; Selemon et al, 1995; Shenton et al, 1992].

It has been postulated that the mesocortical dopaminergic system is important for the normal functions of the dorsolateral PFC [Brozowski et al, 1979; Scatton et al, 1982; Weinberger et al, 1986; 1987; 1988]. Depletion of DA in the prefrontal cortex impairs the performance of monkeys in cognitive tasks, similar to the effect of ablating the PFC, and this cognitive defect can be reversed by giving the DA precursor L-dihydroxy-phenylalamine (L-DOPA) or the agonist apomorphine [Brozoski et al, 1979]. The
impaired performance was attributed to the substantial depletion of DA, since depletions of
noradrenaline (NA) or serotonin (5-HT) in combination with modest DA depletion failed
to produce significant impairments [Brozoski et al, 1979]. In fact, many patients with PD
suffer not only from a motor disorder, but also lack motivation and have flat affect and
reduced spontaneity, which may reflect a decrease in transmission in the mesocortical
dopaminergic pathways [Scatton et al, 1982]. These findings have led Weinberger to
suggest that a decrease in activity of the PFC may account for the negative symptoms of
schizophrenia [Weinberger, 1987]. In a recent study, Dolan et al [1995] used positron
emission tomography (PET) to examine the regulatory role of dopamine on cortical
function in normal subjects and unmedicated schizophrenic patients. In this study, normal
individuals showed increased blood flow in a prefrontal-thalamic-cingulate circuit during a
cognitive task, while the patients had a failure of activation of the anterior cingulate cortex
[Dolan et al, 1995]. However, this failure was reversed by apomorphine, a DA receptor
agonist: after apomorphine, the schizophrenic subjects displayed a significantly enhanced
cognitive activation of the anterior cingulate cortex relative to the controls [Dolan et al,
1995]. These results provide in vivo evidence that an impaired cognitive-task-induced
activation of the anterior cingulate cortex in schizophrenic patients can be significantly
modulated by a dopaminergic manipulation. They also suggest that in schizophrenia there
is a dysregulation in the dopaminergic modulation of cingulate neuronal activity with a
consequent impairment in the functional integration of more remote, but anatomically
connected, cortical regions [Dolan et al, 1995].

Other researchers have suggested that a dysfunction of the glutamatergic system
may underlie this brain disorder [Kim et al, 1980; 1983; Nishikawa et al, 1983; Carlsson &
Carlsson, 1990a; Sherman et al, 1991a; 1991b]. In the last few years, there has been a burgeoning interest in excitatory amino acids (EAA) and their role in schizophrenia. Although the involvement of glutamate (Glu) was proposed over a decade ago [Kim et al, 1980], significant interest developed only after the nature and function of the glutamatergic systems and their receptor subtypes were identified [Fagg & Foster, 1983; Fonnum, 1984]. Studies on the function of EAA such as Glu or L-aspartate in the brain have led to suggestions that disturbed EAA neurotransmission may play a role in the pathophysiology of psychiatric disorders [Cavalheiro et al, 1988]. An important contribution to this hypothesis is the observation that phencyclidine (PCP), a powerful psychotomimetic drug [Snyder, 1980b], is a noncompetitive antagonist of N-methyl-D-aspartic acid (NMDA) receptors [Anis et al, 1983; Sircar et al, 1987]. PCP and related drugs cause psychoses in humans [Allen & Young, 1978] and produce behaviors resembling the action of high doses of amphetamine in experimental animals [Freed et al, 1980]. Furthermore, PCP and dizocilpine induce pathomorphological changes in cingulate and retrosplenial cortices in rodents [Olney et al, 1989]; structural abnormalities in the cingulate cortex are also reported in individuals with schizophrenia [Benes et al, 1986]. Added impetus has come from studies that linked EAA-mediated neurotoxicity to various pathologic conditions including epilepsy, cerebrovascular ischemia, Huntington’s disease, and other neuropsychiatric disorders [Rothman & Olney, 1987; Young et al, 1988; Javitt & Zukin, 1991]. Several different theoretical models have been proposed that relate EAA dysfunction to schizophrenia. These models include: (1) activation of D2 receptors on corticostriatal glutamatergic nerve terminals (which inhibit Glu release) that may lead to deficient glutamatergic neurotransmission [Kornhuber & Kornhuber, 1986], (2) deficient
activity of the corticostriatal glutamatergic/aspartergic pathway [Deutsch et al, 1989; Carlsson & Carlsson, 1990a; 1990b], (3) abnormal genetic regulation of NMDA receptors, leading to a persistence of immature forms into adulthood and possibly resultant development of schizophrenic symptoms [Etienne & Baudry, 1990] and (4) disturbance in the normal development of cortical glutamatergic neurons in the frontal cortex and temporal lobe [Deakin et al, 1989].

Increasing evidence suggests that the PFC may be a major therapeutic target in the treatment of schizophrenia. To determine the region-selective effects of different antipsychotic drugs, extracellular DA and Glu concentrations have been examined in recent studies. Yamamoto and Cooperman [1994] demonstrated that antipsychotic drug-induced DA as well as Glu efflux in DA terminal fields are altered in a region-dependent manner. Thus, chronic treatment with clozapine selectively increased basal extracellular DA concentrations in the PFC, while haloperidol only increased extracellular concentrations of Glu in the striatum [Yamanoto & Cooperman, 1994]. Similarly, other studies have indicated that clozapine produces a sustained enhancement in extracellular DA levels in the PFC whereas haloperidol induces greater increases in dopaminergic tone in the striatum [Pehek & Yamamoto, 1994; Youngren et al, 1994; Youngren et al, 1995]. Therefore, the contrasting effects of atypical and typical antipsychotic drugs on cortical and striatal DA and Glu release may be relevant to their different clinical profiles.

Typical and atypical antipsychotic drugs also produce certain common effects in the PFC. Chronic treatment with clozapine, haloperidol, and remoxipride up-regulates D2 receptors in cortical areas of the rhesus monkey, including the frontal cortex, and down-regulate D1 receptors in the prefrontal and temporal association regions [Lidow &
Goldman-Pakic, 1994]. A recent study, however, suggested that haloperidol- and clozapine-induced changes in D1 and D2 mRNA expression in rat cortical areas were generally not overlapping. Thus, subchronic treatment of haloperidol caused downregulation of D2 receptor mRNA in most cortical regions and upregulated D1 receptor mRNA mainly in cingulate and piriform cortices, while clozapine upregulated D1 and D2 mRNA primarily in parietal cortex [Damask et al, 1996]. A common effect of atypical and typical drugs is that both inhibit GABA release from interneurons in the PFC. For example, clozapine markedly reduced extracellular GABA levels in the PFC [Bourdelais & Deutch, 1994], while haloperidol weakly but significantly decreased extracellular GABA levels in the PFC [Bourdelais & Deutch, 1994]. The common effects of antipsychotics with different pharmacological profiles in the PFC are consistent with the view that this structure is a therapeutic target in the pharmacological treatment of schizophrenia.

3. Receptor Mechanisms of Antipsychotics

Over the past twenty years, much attention has been paid to the receptor mechanisms which subserve the therapeutic actions of antipsychotic drugs. The goal of many studies has been to identify the relevant receptor or receptors with which atypical but not typical antipsychotics interact. Most studies have focused on the importance of dopaminergic, serotonergic, cholinergic, adrenergic, and sigma receptors [Deutch et al, 1991; Meltzer, 1991; Lieberman, 1993].
a. Dopamine D2 receptor

Among CNS neurotransmitter receptors, DA receptors have received the most intense scrutiny as candidate sites for neuroleptic action. Although in the 1950s and 1960s it was difficult to establish whether serotonergic, adrenergic or dopaminergic mechanisms are involved in the effects of antipsychotic drugs [Woolley & Shaw, 1954; Shore et al, 1955; Pletscher et al, 1955; Brodie et al, 1955; Carlsson & Lindqvist, 1963; Corrodi et al, 1967], many early studies focused on the role of 5-HT. It was concluded that depletion of brain 5-HT was responsible for the effect of reserpine on mental state [Brodie et al, 1955; Gaddum, 1954; Plescher et al, 1955; Shore et al, 1955; Woolley & Shaw, 1954].

Following the discovery that DA is a neurotransmitter in the basal ganglia [Bertler & Rosengren, 1959; Carlsson, 1959], Carlsson and colleagues reported that chlorpromazine and haloperidol did not deplete brain amines [Carlsson & Lindqvist, 1963]. Instead, these drugs enhanced the accumulation of the DA metabolite 3-MT and the NA metabolite normetanephrine in the brains of mice pretreated with a monoamine oxidase inhibitor. These drugs also increased striatal concentrations of acidic metabolites of DA [Anden et al, 1964]. This was suggested to be due to blockade of monoamine receptors with resultant compensatory neuronal activation leading to increased amine synthesis and metabolism [Carlsson & Lindqvist, 1963; Anden et al, 1964]. However, the specific role of DA receptor blockade in the action of neuroleptic drugs only became clear in 1970 when Anden et al [1970] showed that a wide range of neuroleptics all blocked DA receptors. The finding that antipsychotic drugs block DA receptors has led to the suggestion that an excess of dopaminergic transmission underlies some aspects of the pathogenesis of schizophrenia. This idea was supported by observations that drugs, such as amphetamine,
cocaine, and L-DOPA, that increase DA neurotransmission in the brain, cause a psychosis that resembles the paranoid subtype of schizophrenia [Siomopoulos, 1975; Snyder, 1973]. In addition, antipsychotic drugs reverse both the amphetamine psychosis in humans and the bizarre behavioral syndrome in monkeys [Angrist et al, 1975; Schechter & Cook, 1975; Siomopoulos, 1975]. The discovery that all neuroleptics in clinical use are DA receptor antagonists further suggested that an abnormality in the dopaminergic system might contribute to the pathology of schizophrenia [Creese et al, 1976; Meltzer & Stahl, 1976; Seeman et al, 1976]. The correlation between clinical efficacy and D2 binding potency has led to the hypothesis that the therapeutic actions of antipsychotic drugs are mediated by their D2 receptor antagonist properties [Clement-Cormier et al, 1974; Seeman et al, 1975; Snyder et al, 1975; Creese et al, 1976; Seeman et al, 1976; Kebabian & Calne, 1979; Peroutka & Snyder, 1980]. In contrast, there is a poor correlation between clinical efficacy and binding to a number of other receptors (such as muscarinic, histamine H1, 5-HT2, and adrenergic α1) [Peroutka & Snyder, 1980]. With the advent of PET, the concept that D2 antagonism is critical for the antipsychotic actions of neuroleptics has received some support. These studies showed that 65-89% of D2 receptors in subcortical regions of the brains of schizophrenics are occupied by typical neuroleptics such as chlorpromazine, flupenthixol, and haloperidol, or the atypical antipsychotics such as clozapine and sulpiride [Farde et al, 1988a; 1989]. These findings suggest that all antipsychotic drugs cause a substantial blockade of central D2 receptors in humans. A somewhat simplistic theory for the effects of neuroleptics has gained prominence: the antagonism of D2 receptors in limbic, mesocortical and hypothalamic systems may mediate the antipsychotic effects of
neuroleptics, while the antidopaminergic actions of these drugs in the basal ganglia account for their EPS [Baldessarini, 1990; Kandel, 1991; Meltzer, 1991].

The DA hypothesis has formed the basis for drug development for several decades. While there is extensive evidence showing that all clinically effective neuroleptic drugs block DA receptors, the observation that clozapine and other putative atypical antipsychotics have a lower affinity for the D2 site than do typical drugs questions the theory of D2 receptor blockade as the sole therapeutic mechanism of antipsychotics [Meltzer, 1989]. Further studies support the view that antipsychotic potency does not lie completely in D2 receptor blockade. For example, haloperidol produces 70% - 89% D2 receptor occupancy in the brain of patients, while clozapine only induces 38% - 63% D2 receptor occupancy [Farde et al, 1992]. In addition, the patients on typical neuroleptics showed poor therapeutic response despite D2 receptor blockade while clozapine, with less complete D2 receptor blockade, produced clinical improvement in patients [Pilowsky et al, 1992]. Further, a recent study showed that there was no difference in striatal D2 receptor occupancy by typical drugs in antipsychotic responders and non-responders [Pilowsky et al, 1993]. Clearly, these data indicate a limitation of the DA hypothesis and suggest that the D2 receptor mechanism is insufficient to account for the atypical profile. Instead, the distinct clinical profiles of atypical and typical antipsychotic drugs have led the reconceptualization of the neurochemical basis of schizophrenia to include a range of neurotransmitters in addition to DA [Deutch et al, 1991; Lieberman et al, 1993]. Actions at other neurotransmitter receptors and/or different subtypes of the D2 receptors in combination with D2 antagonism, may subserve the mode of action of atypical

b. Dopamine D1 receptor

Like D2 receptors, D1 receptors are expressed at high levels in the caudate putamen and NAc in both human and rat brain [Fremeau et al, 1991; Weiner et al, 1991; Meador-Woodruff et al, 1991; Mengod et al, 1992]. Clozapine differs from conventional neuroleptics in that it has higher affinity for D1 and lower affinity for D2 receptors than do conventional neuroleptics, which are relatively selective D2 antagonists. Differences between atypical and typical antipsychotic drugs in their effects on striatal DA receptor binding [O’Dell et al, 1990] and DA metabolism [Bartholini, 1976a, 1976b; Zetterstrom et al, 1985; Altar et al, 1988; Csernansky et al, 1993; Egan et al, 1991; Karoum & Egan, 1992; Moghaddam & Bunney, 1990a; 1990b] suggest that antipsychotic drugs may act through some mechanism related to D1 antagonism. Subchronic treatment with clozapine increases striatal D1 receptor binding but has no effect on D2 receptor binding, while haloperidol has the reverse effect [O’Dell, et al, 1990]. PET studies have shown that at clinically effective doses, clozapine occupies 40% - 50% of D1 receptors in the striatum of humans, while haloperidol and remoxipride occupy few or no D1 receptors [Farde et al, 1992]. These data indicate that clozapine may have significant effects on D1 receptors in vivo. Moreover, preclinical studies have suggested that selective D1 receptor antagonists might possess antipsychotic efficacy [Chipkin et al, 1988; Waddington, 1993; Gerlach et al, 1995]. For example, oral administration of the selective D1 antagonist SCH-39166 to non-human primates produces effects characteristic of antipsychotics without producing
the EPS which commonly accompany typical neuroleptic therapy in humans [McHugh & Coffin, 1991; Coffin et al, 1992]. Thus, in contrast to haloperidol, administration of SCH39166 for 14 weeks did not produce abnormal movements but did produce equivalent sedative effects [Coffin et al, 1992]. Other studies in rodents and monkeys have also raised the possibility that D1 antagonists may have antipsychotic efficacy and lower EPS liability [Waddington, 1988; Coffin et al, 1989; Chipkin et al, 1988; Trugman, et al, 1994; Daly & Waddington, 1994; Heal et al, 1994; Gerlach et al, 1995]. In rodents, D1 receptor antagonists meet the criteria for an antipsychotic effect, and in non-human primates they have a more potent anti-amphetamine effect than typical D2 antagonists, with less liability to produce EPS [Gerlach et al, 1995].

While these preclinical findings show the therapeutic potential of selective D1 antagonists, clinical trials do not support the prediction that D1 receptor antagonism produces antipsychotic effects in schizophrenics [de Beaurepaire et al, 1995; Den Boer et al, 1995; Karlsson et al, 1995]. Preliminary studies have shown that SCH39166, which shows up to 70% occupancy of D1 receptors in PET studies [Karlsson et al, 1995], does not produce significant clinical improvement in psychotic patients: no reduction of the Brief Psychiatric Rating Scale (BPRS) and the Clinical Global Impressions (CGI) scores have been observed [de Beaurepaire et al, 1995; Den Boer et al, 1995; Karlsson et al, 1995]. Although SCH39166 is not effective in reducing positive symptoms in schizophrenic patients, it seems to have an effect on negative symptoms [Den Boer et al, 1995]. An open trial with the D1 receptor antagonist NNC 01-0687 demonstrated some improvement in psychotic symptoms in most of the patients [Karle et al, 1995], and neither EPS nor an increase in the prolactin levels was observed [Gerlach et al, 1995]. This
suggests that NNC 01-0687 may have an therapeutic actions, especially on negative symptoms [Karle et al, 1995]. It is unknown why negative but not positive symptoms seem to be affected by D1 receptor antagonists. However, as indicated by Barnes and Gerlach [1995], further studies are needed to investigate the effects of selective D1 antagonists on negative symptoms.

c. Dopamine D3 and D4 receptors

A remarkable development in the field of DA receptor molecular biology was the recent discovery of three new subtypes: D3, D4, and D5 receptors [Sokoloff et al, 1990; Sunahara et al, 1991; Van Tol et al, 1991]. DA receptors are members of a large gene family of hormone/neurotransmitter receptors that exert their biological actions through signal transduction pathways that involve guanine nucleotide binding or G-proteins [Kaziro et al, 1991; Simon et al, 1991]. Based on molecular, biochemical and pharmacological criteria, D3 and D4 receptors belong to the D2 receptor family, while the D5 is D1-like receptor [Grandy & Civelli, 1992; Sibley & Monsma, Jr., 1992; Gingrich & Caron, 1993].

All of the DA subtypes share relatively high homology within their trans-membrane segments (TMS). The D1 and D5 receptors share 78% identity in their TMS. The D3 receptor shares a 75% identity, and the D4 receptor shares a 53% identity with the D2 receptor in the TMS. Also, all of the DA receptor subtypes share within their TMS several conserved residues, which are thought to be the minimal requirements for catecholamine binding: the two serine residues in the putative fifth TMS, which are involved in
recognition of the two hydroxyl groups of catecholamines, and the aspartic acid residue in
the third TMS, which interacts with the amine moiety in biogenic amines [Strader et al,
1987; 1989]. Structurally, the various genes of the DA receptor family can be divided into
two groups according to their organization: (1) intronless genes, (e.g. D1 and D5), in
which the coding nucleotide sequence is continuous, and (2) genes with their coding
sequence contained in discontinuous DNA segments (exons) interspersed among sequences
that do not form a part of the mature mRNA (introns). This latter group includes D2, D3
and D4 receptor genes [Gingrich & Caron, 1993; Schwartz et al, 1992]. The presence of
introns within the coding region of the D2-like receptors allows for the possibility of
alternate splicing of the exons. Indeed, splice variants of the D2 and D3 receptors have
been described. Two isoforms of the D2 receptor, which differ by 29 amino acids in the
third intracellular loop, are generated by alternate splicing of the 87 base pair exon between
introns 4 and 5. These isoforms, D2\(_{(444)}\) (or D2L for D2 long) and D2\(_{(415)}\) (or D2S for D2
short), have different regional patterns of expression but display identical pharmacology
[Dal Toso et al, 1989; Giros et al, 1989; Monsma et al, 1989]. Thus far, no functional
differences have been reliably attributed to the different D2 receptor splice variants.
Similarly, two truncated forms of D3 receptor mRNA, generated by alternative splicing,
have been detected in rat brain [Giros et al, 1991; Schwartz et al, 1992]. A recent study
reported a selective loss of D3 receptor mRNA transcripts in the parietal and motor cortices
of schizophrenics [Schmauss et al, 1993]. However, a shorter form of the D3 receptor
mRNA was still found in the regions of schizophrenic brains where D3 mRNA was lost
DA receptor subtypes use different intracellular signaling systems. Thus, DA may affect target cells through three major signaling pathways: adenylyl cyclase, phospholipase A₂ and phospholipase C (PLC) products [Gingrich & Caron, 1993; Grandy & Civelli, 1992; Schwartz et al, 1992]. The D₁ and D₅ receptors couple to the stimulation of adenylyl cyclase through a stimulatory G protein [Deary et al, 1990; Sunahara et al, 1991; Zhou et al, 1990]. In contrast, both short and long forms of the D₂ receptor couple to the inhibition of adenylyl cyclase through Gᵢ [Seeman & Grigoriadis, 1987; Dal Toso et al, 1989; Neve et al, 1989]. Also, via G-protein regulation, D₂ receptors can activate PLC and modulate potassium (K⁺) and calcium (Ca²⁺) channels in striatal neurons and transfected cells [Einhorn et al, 1990; 1991; Greif et al, 1995; Surmeier & Kitai, 1993; Valentijn et al, 1993; Vallar et al, 1990]. The second-messenger coupling of D₃ and D₄ receptors has not been well characterized, although it has been reported that D₃ receptors are coupled to G-proteins [Castro & Strange, 1993]. Likewise, the binding of DA at the D₄ receptor in COS-7 cells is sensitive to guanyl-nucleotides, suggesting coupling to endogenous G-proteins [Van Tol et al, 1991]. However, the second messenger systems for these D₂-like receptors remain essentially unknown.

Pharmacologically, DA receptor subtypes can be recognized as D₁-like or D₂-like. The D₁ selective agonist SKF 38393 and the antagonist SCH 23390 are almost equipotent at D₁ and D₅ receptors, but 100-10,000 times less potent at D₂-like receptors. Conversely, the D₂ antagonist sulpiride is approximately 1000-fold less potent at D₁ or D₅ receptors than at D₂ subtypes [Gingrich & Caron, 1993; Schwartz et al, 1992]. Interestingly, human D₅ receptors have 10-fold higher affinity for DA than D₁ receptors [Sunahara et al, 1991]. In contrast, each D₂-like subtypes possesses high affinity for
butyrophenone compounds such as spiperone (0.03 - 0.6 nM $K_D$) and haloperidol (0.45 - 9.8 nM $K_D$), and low affinity for benzazepines such as SKF 38393 (1800 - 9560 nM $K_D$) [Gingrich & Caron, 1993; Schwartz et al, 1992].

The existence of so many different DA receptor subtypes, produced by distinct genes or through alternative splicing, raises questions about the functional significance of this heterogeneity. One clue to this question may come from the observation that while the D2 receptor is widely expressed in all major dopaminceptive brain areas, this is not the case for the two other D2-like receptor subtypes. Localization studies of D3 receptor gene transcripts, using northern blot or PCR analysis as well as in situ hybridization histochemistry, indicate that D3 receptor mRNA is expressed predominantly in limbic brain areas including the olfactory tubercle, NAc, ICj and hypothalamus in rats [Sokoloff et al, 1990; Bouthenet et al, 1991; Landwehrmeyer et al, 1993b] and the NAc and ICj in human [Landwehrmeyer et al, 1993a]. This distribution has led to the hypothesis that D3 receptors mediate dopaminergic regulation of cognitive and emotional functions and thus may be relevant to the antipsychotic effects of DA receptor antagonists [Sokoloff et al, 1990; Sibley & Monsma, Jr., 1992]. Moreover, most antipsychotic drugs are well recognized by the D3 receptor, indicating that this receptor may be a target for antipsychotic drugs [Sokoloff et al, 1994]. Like the D3 receptor, D4 receptors are expressed at a lower level than D2 receptors. The areas of highest D4 mRNA expression includes the frontal cortex, midbrain, amygdala and medulla, with lower levels in the striatum in human [Van Tol, et al, 1991; Meador-Woodruff et al, 1996]. In rodent, high levels of D4 receptor mRNA is found in the cerebellum and peripheral tissues [Cohen et al, 1992; Suzuki et al, 1995]. Receptor binding analyses indicate that the affinity of clozapine
for the D4 receptor is one order of magnitude higher than for the D2 receptor [Van Tol et al, 1991], and that under therapeutic conditions, clozapine and olanzapine occupy more D4 receptors than D2 receptors [Seeman & Van Tol, 1994]. These data, therefore, suggest that D4 receptors may also be targets for atypical antipsychotic drugs. Clearly, it will be important to further investigate the role of D3/D4 receptors in the therapeutic effects of atypical neuroleptics.

d. 5-HT2, 5-HT3 and 5-HT6 receptors

The anatomical distribution of 5-HT2 receptors is similar in human and rat brain with the highest ligand labeling in the frontal, temporal and parietal cortices and hippocampus, and lower levels in the caudate putamen [Hoyer et al, 1986; Wong et al, 1987; Pompeiano et al, 1994; Wright et al, 1995]. In human, 5-HT3 receptors are present in the hippocampus, caudate putamen, NAc and amygdala [Bufton et al, 1993], while 5-HT6 receptors are expressed most prominently in the caudate putamen [Kohen et al, 1996]. In rodent, 5-HT3 receptors are highly expressed in the piriform, cingulate and entorhinal cortices and hippocampal formation [Tecott et al, 1993], and the 5-HT6 receptor is abundantly expressed in the striatum, olfactory tubercle, NAc and hippocampus [Ruat et al, 1993].

5-HT has long been suspected of involvement in schizophrenia. However, the importance and extent of 5-HT’s role in the pathophysiology of this illness is still unresolved. Two bodies of data provide the theoretical rationale for 5-HT receptor antagonism being important in the mechanism of action of antipsychotic drugs. First, several lines of evidence suggest that 5-HT agonists can induce some features of psychosis
[Gaddum, 1954; Wooley & Shaw, 1954; Langs & Barr, 1968; Fischman, 1983]. Second, nearly all antipsychotic drugs are at least moderately potent antagonists at the 5-HT2 receptor. Clozapine has potent effects at several 5-HT receptor subtypes including the 5-HT2, 5-HT3 and 5-HT6. The possible importance of the 5-HT2 antagonist properties of clozapine to explain its mechanism of action as an atypical antipsychotic drug has emerged from both preclinical and clinical studies [Meltzer, 1988; 1989; Meltzer et al, 1989a; 1989b]. However, it is unlikely that 5-HT2 antagonism alone is responsible for its antipsychotic efficacy. Meltzer and colleagues [1989a; 1989b] have proposed that a certain ratio of 5-HT2 to D2 receptor blockade may be required for antipsychotics to induce the beneficial effects of serotonin-dopamine interaction to counteract the EPS liability. Consistent with this hypothesis, PET studies have demonstrated that clozapine produced a very high 5-HT2 receptor occupancy (85-94%) in patients [Farde et al, 1994; Nordstrom et al, 1993] and a significantly lower D2 receptor occupancy (20%-67%) than do typical neuroleptics [Farde et al, 1989; 1992; 1994]. These data suggest that combined 5-HT2 and D2 receptor antagonism may be advantageous in treatment of schizophrenia. Risperidone, a new putatively atypical antipsychotic drug, has a higher affinity for 5-HT2 receptors than does any other antipsychotic agent, including clozapine [Reyntjens et al, 1986; Megens et al, 1994]. Unlike clozapine, which produces essentially no EPS even at high doses, risperidone is associated with dose-dependent EPS [Breier, 1995; Marder, 1992; Chouinard et al, 1993; Marder & Meibach, 1994]. PET studies showed that at a therapeutic dose (6 mg daily), risperidone produced 78% - 88% 5-HT2 receptor occupancy in the neocortex and 75% - 80% D2 receptor occupancy in the striatum [Farde et al, 1995]. This has led to the suggestion that, at certain doses, risperidone’s high 5-HT2 receptor affinity provides a
relative protection from EPS [Kapur et al, 1995; 1996]. However, at high doses, risperidone-induced D2 occupancy apparently exceeds the threshold such that the 5-HT2 receptor-mediated protection from EPS may be lost [Kapur et al, 1995; 1996]. Further studies are needed to determine if the advantageous clinical/side effect profiles of clozapine and risperidone are related to their effects on 5-HT2 receptors.

Other data indicate that clozapine has a relatively high affinity for 5-HT3 receptors [Hoyer et al, 1989; Schmidt & Peroutka, 1989]. Moreover, the 5-HT3 antagonists odansetron and zacopride have been claimed to demonstrate characteristics that are suggestive of antipsychotic potential, including modulation of mesolimbic DA activity [Costall et al, 1987; Imperato & Angelucci, 1988] and a reduction in abnormal motor movements in a patient with TD [Newcomer et al, 1992]. These data have led to the suggestion that atypical antipsychotic drugs differ from typical antipsychotic drugs by virtue of interactions with 5-HT3 receptors [Apud, 1993]. Other data, however, are clearly not compatible with this hypothesis [Greenshaw, 1993]. Loxapine, a typical neuroleptic agent which results in significant EPS, binds to 5-HT3 sites with approximately the same affinity as clozapine [Hoyer et al, 1989]. Furthermore, the distribution of 5-HT3 receptors is not consistent with the loci at which 5-HT3 antagonists are reported to act on central DA systems [Kilpatrick et al, 1988; 1989; Waeber et al, 1988; 1989]. Thus it appears unlikely that a high affinity for the 5-HT3 receptor is responsible for an atypical antipsychotic profile.

Recently, 5-HT6 receptor genes of both human and rat have been cloned [Kohen et al, 1996; Ruat et al, 1993]. Clozapine and several related atypical antipsychotic agents (e.g. rilapine, olanzepine, tiospirone, fluperlapine, clorotepine and zotepine) have high
affinities for 5-HT6 receptors [Roth et al, 1994; Kohen et al, 1996]. Also, it has been found that about 40% of clozapine binding sites in rat brain pharmacologically resemble the 5-HT6 receptor [Glatt et al, 1995]. These data suggest that antagonist action at 5-HT6 receptors may contribute to the atypical profile of clozapine.

e. Muscarinic cholinergic receptors

Five different subtypes of muscarinic receptors have been found in both human and rat brain [Bonner, 1989]. The distribution of muscarinic receptors is similar in human and rat brain with high expression levels in the striatum, hippocampus and amygdala, and very low levels in thalamic and brainstem areas [Cortes et al, 1986; Levey et al, 1991; Boundy et al, 1995].

There is considerable evidence that neuroleptic-induced EPS can be modulated by a central cholinergic mechanism [Costall, 1975; Klemm, 1985; Butkerait & Friedman, 1988]. Neuroleptic drugs that frequently induce EPS (e.g. haloperidol) are relatively weak blockers of muscarinic receptors, whereas antipsychotic drugs which have a low incidence of EPS (e.g. clozapine and thioridazine) are muscarinic receptor antagonists [Miller & Hiley, 1974; Snyder et al, 1974b; 1974c]. Moreover, the symptoms of PD and neuroleptic-induced EPS can be reduced by muscarinic receptor antagonists [Arana et al, 1988; Brown et al, 1990; Cedarbaum & Schleifer, 1990]. In a model of cholinergic / dopaminergic interactions in schizophrenia, Tandon and Greden [1989] proposed that cholinergic hyperactivity may underlie the pathophysiological mechanisms of negative symptoms in schizophrenia. In support of this view, a recent study has shown that clozapine has high affinity for all five subtypes of human muscarinic receptors [Bolden et al, 1991].
Electrophysiological experiments have demonstrated that chronic treatment with haloperidol decreases the number of spontaneously active A9 and A10 DA neurons, whereas clozapine only has this effect on A10 neurons [Chiodo & Bunney, 1985]. However, chronic co-administration of the muscarinic receptor antagonist trihexyphenidyl with haloperidol only results in depolarization inactivation of A10 neurons, similar to what is observed with clozapine [Chiodo & Bunney, 1985]. These data suggest that antimuscarinic actions of clozapine may, in part, mediate its differential effects on A9 and A10 DA neurons. Furthermore, preclinical and clinical research has provided support for a role of muscarinic blockade in clozapine’s reduction of negative symptoms as well as its lack of EPS [Kelley & Porter, 1994].

While these data suggest that the potent antagonism of muscarinic receptors may contribute to the unique therapeutic profile of clozapine, other factors may also be associated with the atypical properties of this drug. For example, animal studies have shown that haloperidol combined with muscarinic antagonist atropine only partly mimics the atypical pharmacological properties of clozapine [Sayers et al, 1976]. Clinical studies, on the other hand, have shown that clozapine provides better antipsychotic action in treatment-resistant patients and fewer parkinsonian side effects than a combination of haloperidol and benzotropine (a muscarinic receptor antagonist) [Kane et al, 1988]. In addition, while classical neuroleptics in combination with anticholinergic agents reduce the incidence of EPS, it does not reduce the risk of TD after long-term neuroleptic exposure [Klawans & Rubovits, 1974; Gerlach, 1977]. Therefore, it is likely that although clozapine is a potent cholinergic antagonist, this property of the drug is not sufficient to account for its atypical profile.
f. $\alpha_1$ noradrenergic receptors

$\alpha_1$ noradrenergic receptors are present in the neocortex and the dentate gyrus in human brain [Zilles et al, 1993], while in rat, the primary motor cortex and thalamic nuclei show the highest density of $\alpha_1$ receptors [Zilles et al, 1993]. One of the hypotheses that has been proposed to explain clozapine's atypical properties is its potent ability to block $\alpha_1$ noradrenergic receptors. Although virtually all antipsychotic drugs are antagonists at the $\alpha_1$ receptor [Cohen & Lipinski, 1986], the distinguishing features of clozapine is its relatively potent central anti-adrenergic actions and low its anti-dopaminergic activity [Baldessarini & Frankenburg, 1991; Baldessarini et al, 1992]. Biochemical studies have suggested that clozapine modulates levels of cGMP predominantly through its interaction with central adrenergic receptors [Rao et al, 1991]. Physiological studies have also demonstrated that co-administration of haloperidol and the $\alpha_1$ antagonist prazosin produce a differential effect on A9 and A10 neurons identical to that observed with clozapine treatment. Thus, chronic treatment with haloperidol in combination with prazosin produced depolarization inactivation only of A10 cells, suggesting that $\alpha_1$-blocking property of clozapine may partly contribute to its unique effect on midbrain DA neurons [Chiodo & Bunney, 1985]. Moreover, administration of prazosin in combination with haloperidol has been reported to alter DA release in the NAc but not in the striatum. This regionally specific response is similar to that produced by clozapine [Lane et al, 1988]. However, clinical trials, have not supported the suggestion that $\alpha_1$ antagonism contributes to the antipsychotic efficacy and the reduced EPS of atypical drugs [Hommer et al, 1984]. Moreover, actions at the $\alpha_1$ receptor are thought to underlie the propensity of
antipsychotics to produce cardiovascular side effects [Deutch et al, 1991; Jackson et al, 1994]. Thus, it seems unlikely that a potent noradrenergic blocking property can explain the atypical profile of clozapine.

g. Sigma receptors

Sigma (σ) binding sites have been found in limbic areas in the primate brain, including the orbitofrontal and cingulate cortices, parahippocampal and temporopolar gyri, amygdala and the hippocampal formation [Mash & Zabetian, 1992]. In guinea pig, σ receptors are present in the cerebellum, cingulate cortex, hippocampus, thalamus and the pons [Okuyama et al, 1995]. On the basis of both their activity profile in animal models and their indirect modulation of dopaminergic function, it has been proposed that sigma receptor ligands are potential antipsychotic drugs [Deutch et al, 1988; Walker et al, 1990; Snyder & Largent, 1989; Gilligan et al, 1992]. Several potential atypical antipsychotic drugs possess high affinity for σ receptors but only have moderate to poor affinity for the D2 receptor [Ferris et al, 1986; Largent et al, 1988; Taylor & Dekleva, 1987; Gilligan et al, 1992; Clissold et al, 1993; Shepard et al, 1994; Gilligan et al, 1994]. These observations raise the possibility that σ ligands are potential antipsychotic agents that do not act, at least directly, on D2 receptors and therefore will not produce undesirable side effects, such as TD. However, the high affinity of these ligands for the σ site is not shared by all atypical agents: clozapine is essentially devoid of activity at the σ receptor [Tam & Cook, 1984]. In addition, haloperidol has a very high affinity for the σ receptor [Tam & Cook, 1984]. It has been suggested that although remoxipride, which has high affinity for the σ site,
appears to be an atypical agent [Farde et al, 1988b], it may be effective as an antipsychotic
drug because of its D2 antagonist properties [Deutch et al, 1991; Meltzer, 1991]. Thus it
seems unlikely that antagonism at the σ receptor is related to an atypical profile.

Mechanisms which underlie the unique therapeutic profile of atypical antipsychotic
drugs such as clozapine remain elusive as clozapine does not appear to have any unique
actions on dopaminergic, serotonergic, cholinergic, adrenergic or sigma receptors which
can account for its atypical profile. Therefore, in addition to pharmacological studies,
additional attention should be devoted to elucidating the intracellular transduction
mechanisms that may contribute to the therapeutic effects of these drugs. Moreover, since
drug treatments may regulate gene expression of various transmitters, enzymes, and
proteins involved in signal transduction cascades, examining the differences between
atypical and typical drug-induced gene expression will be helpful in understanding
molecular mechanisms underlying the therapeutic effects of these antipsychotic drugs.

4. Molecular Mechanisms of Antipsychotics

Because the full therapeutic effects of antipsychotics generally take several weeks
to develop, their interactions with neurotransmitter receptors are probably only the initial
step in their actions. The therapeutic effects may result from postreceptor mechanisms that
occur in response to repetitive or chronic receptor occupancy by the drugs. In general, the
responses of neurons to environmental cues, including physiological and pharmacological
stimuli, can be divided into early and late responses. The early responses are mediated by activation of second-messenger systems by G-protein coupled receptors. All known second-messenger systems activate different protein kinases that in turn phosphorylate specific neuronal proteins [Nairn et al, 1985; Axelrod et al, 1988; Nishizuka, 1988; Garthwaite, 1991; Pelech & Sanghera, 1992; Berridge, 1993]. The physiological response activated in this manner is short-lived because of the balanced control of the phosphorylation state of cellular proteins via dephosphorylation by phosphatases [Alexander, 1990; Cohen, 1992; Kurosawa, 1994]. The long-term or late responses persist from hours to days and may be rendered permanent within the neuron in certain circumstances [Goelet et al, 1986; Black et al, 1987; Comb et al, 1987]. Changes in gene expression seem necessary for the long-term phenotypic changes [Goelet et al, 1986; Comb et al, 1987; Armstrong & Montminy, 1993; Bliss & Collingridge, 1993]. Therefore, an important candidate mechanism of post-receptor processes for drug-induced neuronal plasticity is the receptor-mediated regulation of neuronal gene expression. Studies of transcriptional regulatory proteins and their target genes have provided important insights into these post-receptor processes.

**a. Transcription factors: IEG proteins**

**(i) Immediate early genes (IEGs)**

Neuronal gene expression can be modulated by neurotrophic growth factors, neurotransmitters, membrane electrical activity and drugs: these signals begin a cascade of
events which culminates in postsynaptic changes in gene expression [Black et al, 1987; Cochran et al, 1983; Comb et al, 1987; Montarolo et al, 1986; Morgan & Curran, 1988]. These genes fall into two general classes: (1) the IEGs, whose transcription is activated rapidly and transiently within minutes of stimulation [Greenberg et al, 1985; Morgan & Curran, 1986; Bartel et al, 1989; Barzilai et al, 1989] and (2) the late response genes, whose expression is induced (or repressed) more slowly, over a time frame of hours, via a mechanism that is generally dependent on new protein synthesis [Merlie et al, 1984; Castellucci et al, 1988; Goldman et al, 1988; Barzilai et al, 1989; Offord & Catterall, 1989; Klarsfeld et al, 1989]. Several processes exist in eukaryotic cells for the regulation of gene expression in response to extracellular stimuli [Morgan & Curran, 1989; Struhl, 1991; Hughes & Dragunow, 1995]. One such strategy involves the rapid induction of a set of IEGs encoding transcription factors which then in turn regulate the expression of late response target genes. The products of the late response genes are then thought to serve more specific effector functions in the neuronal response.

IEGs were first characterized in nonneuronal cells in experiments aimed at identifying growth factor-responsive genes that might control the re-entry of G_0 resting cells into the cell cycle [Kelly et al, 1983; Bravo et al, 1985; Conchran et al, 1984; Greenberg & Ziff, 1984]. This work resulted in the discovery of a class of genes whose transcription is activated within minutes of addition of a growth factor. The c-fos and c-myc proto-oncogenes were among the first IEGs to be identified [Kelly et al, 1983; Greenberg & Ziff, 1984]. Since then, a large number of IEGs have been identified, including genes that are closely related to c-fos or c-jun and several other IEGs which contain homologies with, or structural motifs characteristic of, known transcription factor
genes (e.g. zif/268, nur77, and c-myc). In general, IEG expression is low or undetectable in quiescent cells but is rapidly induced at the transcriptional level within minutes of extracellular stimulation. This transcriptional induction is transient and independent of new protein synthesis, the subsequent shut-off of transcription requires new protein synthesis and the mRNAs transcribed from these genes often have a very short half-life. For instance, the transcriptional activation of c-fos occurs within 5 minutes and continues for 15-20 minutes [Greenberg & Ziff, 1984; Greenberg et al, 1985]; c-fos mRNA accumulates and reaches peak values at 30-45 minutes post-stimulation [Muller et al, 1984]; synthesis of c-fos protein (Fos) follows mRNA expression and is turned over with a half-life of about 2 hours [Muller et al, 1984; Curran et al 1984].

Of the approximately 100 IEGs which have been identified, the c-fos and c-jun gene families have been the most extensively characterized [Sheng & Greenberg, 1990; Morgan & Curran, 1991]. The c-fos gene encodes a 55 kDa nuclear protein, Fos, which has an apparent molecular weight of 62 kDa after post-translational modification [Curran et al, 1984]. Currently, five Fos (c-Fos, Fos-B[L], Fos-B[S], Fra-1, Fra-2) and three Jun (c-Jun, Jun-B, Jun-D) family members have been identified. Each has several regions of homology with either Fos or Jun, respectively, and all contain DNA-binding and leucine zipper domains, which allow strong protein-protein interactions to occur. IEG proteins of the Fos and Jun families bind to each other through hydrophobic interactions between the linear leucine crests of the two molecules to form dimeric transcription factor complexes [Halazonetis et al, 1988; Kouzarides & Ziff, 1988; Nakabeppu et al, 1988; Rauscher et al, 1988a; Curran & Franza, 1988]. There is a high degree of specificity among zipper containing proteins. Jun and Jun-related proteins form both heterodimeric and
homodimeric complexes, while Fos and Fos-related proteins only form stable heterodimers with members of the Jun family [Halazonetis et al, 1988; Nakabeppu et al, 1988; Cohen et al, 1989]. Jun is also capable of interacting with one of the CRE binding proteins, CRE-BP1, which has a leucine zipper. These heterodimeric and homodimeric complexes function as transcriptional regulators by interacting with DNA enhancer sequences related to the AP-1 and CRE motifs [Bohmann et al, 1987; Rauscher et al, 1988b; Curran & Franza, 1988]. All of the different combinations of Jun/Jun dimers and Fos/Jun dimers bind to the consensus AP-1 binding sites [Nakabeppu et al, 1988; Cohen et al, 1989], while Jun/CRE-BP1 has greater specificity for CRE sites [Macgregor et al, 1990]. The c-Fos/c-Jun complexes activate transcription of genes containing AP-1 sites [Chiu et al, 1988; Sassone-Corsi et al, 1988; Sonnenberg et al, 1989], while under some circumstances, the c-Fos/Jun-B complex represses transcription [Chiu et al, 1989; Schutte et al, 1989]. Such properties of c-fos and c-jun gene families illustrate particularly well how regulatory diversity and specificity might be generated by different combinations of distinct proteins.

The human central nervous system (CNS) contains about $10^{12}$ cells. The cells in the CNS are distinct with respect to which genes are expressed. In addition to this cellular specificity, gene regulatory patterns are constantly changing throughout development and in response to extracellular signals. As a rough estimate, probably between $10^4$ - $10^5$ genes are expressed, many of them in unique and unexpectedly complicated cellular patterns [Bier et al, 1989; McKay & Hockfield, 1982; Sutcliffe, 1988]. This enormous diversity and flexibility in gene expression patterns is accomplished with a relatively small number of transcription factors. Thus, only hundreds (or possibly a few thousands) of transcription factors exist. It is evident, therefore, that a “one regulatory protein per gene”
model, such as is frequently applied to prokaryotic organisms, is grossly inadequate. Instead, combinatorial action of transcriptional regulatory proteins is necessary for multicellular organisms to generate the requisite diversity in gene expression patterns. Thus, heterodimer formation between individual members of IEG protein families can provide an additional diversity mechanism that increases the number of DNA-binding transcription factors.

Transcriptional control of c-fos and other IEGs involves multiple second-messenger pathways that act on distinct upstream cis-acting regulatory DNA elements. Several studies have attempted to probe directly the second-messenger pathways involved in c-fos activation. In the pheochromocytoma (PC12) cell line, at least three distinct second-messenger systems can activate c-fos, namely those involving diacylglycerol-protein kinase C [Greenberg et al, 1986], cAMP [Greenberg et al, 1985], and calcium-calmodulin [Morgan & Curran, 1986]. The earliest known example of the induction of c-fos involved the opening of voltage-sensitive calcium channels (VSCCs) after membrane depolarization induced by nicotinic receptor activation in PC12 cells [Greenberg et al, 1986]. The increases in the concentration of intracellular Ca$^{2+}$ leads to the activation of mechanisms that then regulate the expression of IEGs [Morgan & Curran, 1986; Curran and Morgan, 1986]. In contrast, growth factor-mediated induction of c-fos gene expression in PC12 cells does not require the entry of extracellular Ca$^{2+}$ ions [Morgan & Curran, 1986; Sheng et al, 1990]. Protein kinase C (PKC) is thought to be involved in the pathway from growth factor activation of its receptor to c-fos induction. Thus, pretreatment of cells with phorbol-ester to deplete cellular PKC activity will reduce induction of c-fos in both 3T3 cells and adipocytes. Similarly, treatment of 3T3 cells with the protein kinase inhibitor, 2-
aminopurine, blocks \textit{c-fos} induction by serum [Zinn et al, 1988]. Recent studies have shown that in dentate gyrus neurons, NMDA and non-NMDA receptor-mediated increases in \textit{c-fos} mRNA involves Ca\textsuperscript{2+} influx via different routes. Using \textit{in situ} hybridization to measure \textit{c-fos} mRNA and fura-2 imaging to measure intracellular calcium (Ca\textsubscript{i}\textsuperscript{2+}) in individual dentate gyrus neurons, Lerea and colleagues [1992] found that activation of either NMDA or non-NMDA receptor subtypes is sufficient to induce a rapid and dramatic increase in both \textit{c-fos} mRNA and Ca\textsuperscript{2+} in these cells. Both effects can be blocked by the removal or chelation of extracellular calcium (Ca\textsubscript{e}\textsuperscript{2+}) [Lerea et al, 1992]. The increase in Ca\textsubscript{i}\textsuperscript{2+} induced by activating non-NMDA receptors is inhibited either by removal of extracellular sodium (Na\textsubscript{e}\textsuperscript{+}) or by addition of the voltage-sensitive Ca\textsuperscript{2+} channel blocker nifedipine. In contrast, the increase of Ca\textsubscript{i}\textsuperscript{2+} induced by activating NMDA receptors is not inhibited by either removal of Na\textsubscript{e}\textsuperscript{+} or addition of nifedipine [Lerea et al, 1992]. Consistent with these effects on Ca\textsubscript{i}\textsuperscript{2+}, nifedipine inhibits induction of \textit{c-fos} mRNA by non-NMDA, but not by NMDA receptor agonists [Lerea et al, 1992]. These findings indicate that Ca\textsuperscript{2+} serves as a second messenger which couples ionotropic excitatory amino acid (EAA) receptor subtypes with transcriptional activation of \textit{c-fos} mRNA. The route of Ca\textsuperscript{2+} entry into dentate neuron, however, depends on the EAA receptor subtype stimulated. Non-NMDA receptor activation results in Ca\textsuperscript{2+} influx indirectly via VSCCs, whereas NMDA receptor activation results in Ca\textsuperscript{2+} influx directly through the NMDA channel itself. Calmidazolium, the calmodulin antagonist, markedly inhibits kainic acid- but not NMDA-mediated increases of \textit{c-fos} mRNA [Lerea et al, 1993]. It is thus proposed that the dissociation in the mechanisms transducing the Ca\textsuperscript{2+} influx signals to the nucleus following NMDA and non-NMDA receptor activation is due to spatially distinct sites of Ca\textsuperscript{2+} entry,
which result in activation of different enzymes located at distinct sites in the cell [Lerea et al, 1993]. In addition, a recent study also demonstrated that activation of NMDA receptors triggers the transcriptional activation of several IEGs, such as c-fos and zif268 (also called NGFI-A) mRNAs in dentate gyrus neurons [Lerea et al, 1995]. The induction of both IEGs was blocked by structurally distinct inhibitors of phospholipase A₂. This enzyme catalyzes phospholipid degradation and formation of arachidonic acid, which is catalyzed to biologically active metabolites by multiple enzymes, including cyclooxygenase and lipoxygenase. Pretreatment of dentate gyrus neurons with selective inhibitors of cyclooxygenase attenuated NMDA induction of c-fos but not of NGFI-A [Lerea et al, 1995]. Conversely, structurally distinct inhibitors of lipoxygenase blocked NMDA induction of NGFI-A but not of c-fos [Lerea et al, 1995]. Therefore, after activation of NMDA receptors, the induction of two distinct IEGs, c-fos and NGFI-A is mediated by related, but divergent intracellular pathways: phospholipase A₂ and the arachidonic acid cascade play a pivotal role in NMDA receptor regulation of gene expression. It is hypothesized, therefore, that to gain specificity of long term consequences of glutamate receptor activation, distinct intracellular signaling pathways are necessary to regulate the initial induction of varying arrays of IEGs [Lerea et al, 1995].

Several regulatory regions located in the 5’untranslated region of c-fos have been demonstrated to play a role in controlling the induction of c-fos expression. The serum response element (SRE) has been described as a protein binding site required for the induction of c-fos expression by serum [Treisman, 1992]. The SRE consists of a set of transcription factor binding sites through which most PKC-dependent and -independent signaling occurs [Shaw et al, 1989; Rivera et al, 1990; Graham & Gilman, 1991; Marais et
al, 1993; Hill et al, 1993; Rivera et al, 1993]. Its central core binds a dimer of a protein known as the serum response factor (SRF) [Edwards, 1994]. The SRE, together with flanking DNA sequences, serves as the site of assembly of multiprotein complexes that include SRF, p62TCF/Elk-1, and several other transcription factors [Shaw et al, 1989; Hipskind et al, 1991; Hill et al, 1993]. Although the exact mechanism responsible for activation of c-fos transcription via the SRE is unclear, phosphorylation of the p62TCF/Elk-1 protein by microtubule-associated protein (MAP) kinase is believed to be a key event [Marais et al, 1993].

The Ca$^{2+}$ and cyclic AMP response element (Ca/CRE) is defined as a DNA sequence functionally distinct from the SRE that mediates rapid gene induction via elevated intracellular Ca$^{2+}$ and cAMP [Sassone-Corsi et al, 1988; Sheng et al, 1988; Berkowitz et al, 1989; Fisch et al, 1989]. The induction of c-fos mRNA by membrane depolarization and Ca$^{2+}$ influx has been demonstrated to occur through phosphorylation of the CRE binding protein (CREB), which binds to the Ca/CRE site in vitro [Sheng et al, 1990]. Thus, the Ca/CRE site has been shown to mediate transcriptional inducibility of c-fos by depolarization. Also, CREB is rapidly phosphorylated in response to depolarization or cAMP. Further, it has been found that CREB is the critical nuclear factor that interacts with Ca/CRE and that this interaction is essential for the Ca$^{2+}$ inducibility of this regulatory element in vivo [Sheng et al, 1990]. Therefore, the Ca/CRE mediates membrane depolarization- and calcium-induced c-fos transcription via phosphorylation of CREB. The sis-inducible element (SIE) was identified as the binding site for an inducible factor and was proposed to contribute to c-fos induction in cells exposed to platelet-derived growth factor, PDGF [Wagner et al, 1990]. Using transgenic mice carrying fos-lacZ fusion genes
with clustered point mutations in each of several distinct regulatory sequences, Robertson and colleagues [1995] demonstrated recently that fos-lacZ expression in either the CNS or in cultured neuronal cells required all of the regulatory elements tested, including the SIE, the SRE, the fos AP-1 (FAP) site, and the Ca/CRE. The analysis of fos-lacZ expression indicated that four major regulatory elements, the SIE, SRE, FAP, and Ca/CRE, are essential for the normal function of the c-fos promoter in vivo [Robertson et al, 1995]. Mutation of any single element resulted in a profound loss of tissue-specific and stimulus-evoked gene expression [Robertson et al, 1995]. Clearly, within the context of the intact c-fos promoter, the Ca/CRE, SRE, SIE, and FAP are required in combination for induction of c-fos expression in many neurons both in vitro and in vivo. These results suggest that physiological regulation of c-fos expression requires the concerted action of multiple control elements that direct the assembly of an interdependent transcription complex.

A potential role for IEGs in the nervous system was initially suggested by studies on PC12 cells. Transcription of many IEGs in PC12 cells is markedly induced by growth factors, neurotransmitters and electrical excitation [Greenberg et al, 1985; 1986; Morgan & Curran, 1986; Bartel et al, 1989]. Following on from these early studies in vitro, numerous studies have demonstrated that c-fos and other IEGs are induced during stimulation of the intact nervous system, suggesting that these genes are important regulators of neuronal cell responses in vivo. For example, increased expression of IEG mRNAs and proteins can be induced by seizure activity, kindling, hypoxic-ischemic stroke, nerve transection, long-term potentiation, stress, sensory stimulation, and activation of neurotransmitter receptors, including glutamate, cholinergic, adrenergic, serotonergic, dopaminergic, opiate, adenosine, neuropeptide, and hormone receptors [reviewed in Morgan & Curran, 1991;
Hughes & Dragunow, 1995]. These studies have led to the suggestion that IEG induction and Fos immunostaining can be used as a marker to map the pattern of postsynaptic stimulation within the intact nervous system with single-cell resolution [Hunt et al, 1987; Morgan et al, 1987; Sagar et al, 1988; Morgan & Curran, 1989; 1991]. Therefore, the induction of IEGs provides a novel avenue for research and a useful indicator with which the effects of pharmacological, electrical, and physiological stimuli may be traced in the nervous system.

(ii) Antipsychotics and IEG expression

In the search for the mechanisms underlying the actions of antipsychotic drugs, IEG mRNA expression (such as c-fos, c-jun, jun-B, jun-D, and zif268) and IEG protein immunohistochemistry (including Fos, FRAs, Fos-B, Jun, Jun-B, Jun-D and Zif268) have been advanced as markers (1) to identify the brain regions that are targets of antipsychotic drugs [Dragunow et al, 1990; Miller, 1990; MacGibbon et al, 1994; Cohen & Wan, 1995] and (2) to determine the regionally specific manner of the activation of IEGs induced by typical and atypical antipsychotics [Deutch et al, 1992; Nguyen et al, 1992; Robertson & Fibiger, 1992; Deutch, 1994; MacGibbon et al, 1994; Simpson & Morris, 1994; Fink-Jensen & Kristensen, 1994; Robertson et al, 1994; Semba et al, 1995].

Dragunow and colleagues [1990] first reported that haloperidol induced Fos- and FRA-immunoreactivities in the striatum. Further studies have shown that in response to acute administration of antipsychotic drugs, marked increases in IEG mRNAs (c-fos, zif268, jun-B, nur77) and proteins (Fos, FRA, Fos-B, Jun-B, Zif268) are induced in different brain regions such as the dorsolateral striatum, medial striatum, NAc, lateral
septal nucleus (LS), PFC, islands of Calleja (ICj) and thalamic midline nuclei [Deutch et al, 1992; Nguyen et al, 1992; Robertson & Fibiger, 1992; MacGibbon et al, 1994; Cohen & Wan, 1995]. These data suggest that these brain areas are involved in mediating the actions of antipsychotic drugs. Further, these studies revealed that typical and atypical antipsychotics exerted regionally distinct effects on c-fos expression in the brain: typical neuroleptics such as haloperidol and other D2 receptor antagonists increased c-fos expression in striatum, NAc and LS, while clozapine induced c-fos expression in NAc, LS, ICj, and PFC [Dragunow et al, 1990; Deutch et al, 1992; Nguyen et al, 1992; Robertson & Fibiger, 1992; Deutch, 1994; MacGibbon et al, 1994]. Studies also showed that haloperidol, but not clozapine, induced Fos-B, jun-B, and nur/77 expression in the striatum and NAc [MacGibbon et al, 1994]. This regional specificity of antipsychotic-induced IEG expression suggested that different patterns of neuronal activity are evoked by the two classes of neuroleptics and that the regionally different neuronal activities correlate with the different therapeutic effects of these drugs.

Neuroleptic-induced increases in c-fos expression in the dorsolateral striatum are thought to be reflective of the relatively high incidence of EPS in patients treated with these drugs. In screening a variety of antipsychotic drugs, it has been found that all typical antipsychotic drugs that have EPS liability also cause an increase in the number of Fos-like immunoreactive neurons in the dorsolateral striatum [Deutch et al, 1992; Robertson et al, 1994; Semba et al, 1995]. In contrast, clozapine and other atypical drugs produce minor or no elevations in Fos immunoreactivity in the dorsolateral striatum [Deutch et al, 1992; Robertson et al, 1994; Semba et al, 1995]. Further, the putative atypical drug risperidone, which lacks EPS liability at low doses but induces motor side effects at higher doses
[Claus et al, 1992], does not induce Fos induction in this brain structure at low doses whereas it does at higher doses [Deutch et al, 1994]. Thus the pattern of Fos induction in the striatum mirrors the clinical observations of EPS produced by this drug. Likewise, the atypical-like antipsychotic remoxipride, which has significantly lower EPS liability than most typical neuroleptics, does not increase Fos expression in the dorsolateral striatum at low doses [Deutch, 1992]. These data indicate that actions in the dorsolateral striatum correlate with EPS produced by neuroleptic drugs, suggesting that the ability of neuroleptics to increase Fos induction in this brain region may predict their potential for EPS. Further studies have shown that the atypical index (i.e. the difference in the number of Fos-positive nuclei between the NAc and dorsolateral striatum) is always positive for atypical antipsychotics but negative for typical drugs [Robertson et al, 1994]. Robertson and colleagues [1994] thus suggest that the atypical index may accurately predict the EPS liability of antipsychotic drugs.

While typical and atypical antipsychotics have markedly different effects on IEG induction in the dorsal striatum, they all increase c-fos expression in the NAc [Deutch et al, 1992; Nguyen et al, 1992; Robertson & Fibiger, 1992; Robertson et al, 1994]. This suggests that this region may be a common site of antipsychotic action. This view is bolstered by the finding that the inverse neuroleptic metoclopramide, which has a very high incidence of EPS but does not appear to have antipsychotic efficacy, does not increase Fos induction in the NAc shell but does increase the number of Fos-like immunoreactive neurons in the dorsal striatum in both rodents and nonhuman primates [Deutch 1994]. The observation that both typical and atypical antipsychotic drugs increase c-fos expression in
the NAc shell also suggests that this ventral striatal site is not the site at which clozapine acts to produce its unique therapeutic effects.

In neuropsychological, pharmacological, anatomical and imaging studies of schizophrenia, the PFC has been implicated as being an important site. Robertson and Fibiger [1992] reported that clozapine, but not haloperidol, increases Fos immunoreactivity in the PFC. Further studies showed that haloperidol and some other typical neuroleptics do not increase c-fos expression in the PFC [Deutch, 1994; Robertson et al, 1994]. In addition, high doses of haloperidol do not increase Fos protein levels in the PFC, suggesting the lack of response to haloperidol is not due to an inadequate dose [Deutch, 1994]. In contrast, clozapine and some other putative atypical antipsychotics markedly increase the number of Fos-like immunoreactive neurons and the amount of Fos protein in the PFC [Robertson & Fibiger, 1992; Deutch, 1994; Robertson et al, 1994; Fink-Jensen & Kristensen, 1994]. These data suggest that the failure of clozapine to induce c-fos expression in the dorsal striatum as well as its idiosyncratic actions in the PFC may be related to its unique therapeutic profile.

b. Target gene expression: Neuropeptides

As described above, physiologically relevant changes in synaptic activity in the CNS can lead to the induction of IEGs. These IEGs have been proposed to mediate transcriptional activation of late-onset genes in response to neuronal activity. For example, apomorphine-induced Fos and Fra mediate the long-term induction of striatal dynorphin (Dyn) expression in the 6- hydroxydopamine (6-OHDA) lesioned rat [Bronstein et al,
1994]. Also, induction of AP-1 (Fos/Jun) and other transcription factors (e.g. Fos/Jun-B heterodimer and Jun/Jun homodimer) can activate glutathione transferase and quinone reductase gene expression in mice [Bergelson et al, 1994; Bergelson & Daniel, 1994]. Likewise, haloperidol induces \( c\text{-}f_{os} \) expression and AP-1 binding activity in the striatum [Nguyen et al, 1992], suggesting that antipsychotic drug-induced IEG proteins may regulate the activities of various target genes, and this may contribute to the clinical profile of this drug. Therefore, a key question concerning the differences between the typical and atypical neuroleptics is the elucidation of the targets of transcriptional regulation by these drugs. Because most neuropeptide genes contain the consensus AP-1 binding sites [Sonnenberg et al, 1989; Goodman, 1990; Sheng & Greenberg, 1990; Kislauskis & Dobner, 1990; Bronstein et al, 1994], these peptide genes are among the best studied late-onset genes.

(i) Enkephalin (Enk)

The proenkephalin (PEnk) gene has long been proposed as an IEGP target (late-response gene). First, the 5' control region of the PEnk gene contains binding sites for several different transcription factors, including AP-1 [Comb et al, 1988]. Further, it has been reported that the PEnk gene may be a physiological target for Fos and Jun in the hippocampus [Sonnenberg, et al, 1989]. Because haloperidol-induced \( c\text{-}f_{os} \) expression is thought to be mediated by D2 receptors in striatal neurons which utilize Enk and GABA as neurotransmitters, it was suggested that the elevated \( c\text{-}f_{os} \) expression induced by haloperidol may serve to increase the transcription of Enk mRNA in striatopallidal neurons [Robertson & Fibiger, 1992]. Indeed, chronic and acute administration of haloperidol
increases levels of Enk mRNA and peptide in the striatum and NAc [Hong, et al, 1980; Romano et al, 1987; Robbins et al, 1991; Schiffmann & Vanderhaeghen, 1992; Marksteiner et al, 1992].

Several lines of evidence, however, do not support the view that PEnk is a target gene regulated by haloperidol-induced c-fos. First, the pattern of Enk mRNA expression is different from that of c-fos expression induced by antipsychotic drugs. It has been reported that both haloperidol and clozapine cause a significant increase in the levels of Enk mRNA in the striatum and NAc [Angulo et al, 1990]. Similarly, a recent study showed that continuous infusion of clozapine increases the levels of Enk mRNA in both striatum and NAc in mice [Zhang et al, 1995]. In contrast, haloperidol and clozapine produce distinct regional patterns of c-fos expression in the forebrain, suggesting that Enk is not the response target for Fos/Jun complexes. Another study showed that chronic clozapine treatment decreased the level of Enk mRNA in the striatum while haloperidol produced no change when compared with controls [Mercugliano & Chesselet, 1992]. This pattern of Enk mRNA expression, however, is still inconsistent with the pattern of Fos expression induced by haloperidol and clozapine and does not support Enk as a target gene for Fos induced by these drugs. Furthermore, a recent study has found that in the striatal nuclear extracts from rats treated with haloperidol, CREB-like proteins, rather than Fos, interact with the PEnk enhancer [Konradi et al, 1993]. This result suggests that haloperidol induces Enk mRNA in striatal neurons via CREB phosphorylation and that c-fos is not involved [Konradi et al, 1993]. Taken together, these data suggest that Enk is not the target gene regulated by antipsychotic-induced Fos protein in the brain.
(ii) Dynorphins (Dyn)

Dyn are biologically active opioid peptides derived from the precursor prodynorphin, which encodes α- and β-neoendorphin, Dyn A-(1-32), Dyn A-(1-17), Dyn A-(1-8), Dyn B-29 and Dyn B [Civelli et al, 1985; Spampinato & Ferri, 1991]. All of these peptides contain the leucine-Enk sequence at their N-termini and it is unclear whether the leucine-Enk pentapeptide is generated as an end product of proteolytic cleavage of Dyn [Zamir et al, 1984]. Prodynorphin-derived peptides are considered the endogenous ligands for kappa opioid receptors [James, 1986]. A large body of evidence suggests that these peptides act as neurotransmitters/neuromodulators in the brain or as neurohormones in the endocrine system [Vincent et al, 1982a; Goldstein et al, 1981; Herman et al, 1980; Friedman et al, 1981; Kromer et al, 1981; Morley & Levine, 1981; Przewlocki et al, 1983].

In the striatum, approximately half of the neurons of both the patches and the matrix contain Dyn/substance P (SP), while the remainder contain Enk [Vincent et al, 1982b; Besson et al, 1990; Gerfen & Young, 1988]: the striatonigral pathway contains SP and Dyn, while the striatopallidal pathway contains Enk [Gerfen et al, 1990; Gerfen et al, 1991]. It has been reported that DA differentially modulates the levels of these peptides. For example, the DA agonist apomorphine increases both Dyn and SP synthesis but has little effect on Enk [Gerfen et al, 1991; Li et al, 1986; 1987; 1988]. Denervation of the nigrostriatal dopaminergic system increases Enk peptide and mRNA levels but decreases or fails to alter Dyn and SP [Gerfen et al, 1991; Li et al, 1990; Normand et al, 1988].

The effects of antipsychotic drugs on levels of Dyn peptide and mRNA have also been studied. An early report showed that subchronic treatment of haloperidol slightly
reduced Dyn in the striatum, while clozapine had no effect in this brain region [Nylander & Terenius, 1986]. Further studies, however, have demonstrated that repeated treatment with haloperidol neither changes Dyn immunoreactivity [Li et al, 1986] nor affects Dyn mRNA levels in the striatum and NAc [Morris et al, 1988; Fox et al, 1994]. The effect of clozapine on Dyn mRNA levels in the forebrain has not been reported to date.

Several lines of evidence suggest that Dyn is not a target gene regulated by haloperidol-induced c-fos. First, haloperidol increases c-fos expression in the striatum [Nguyen et al, 1992; MacGibbon et al, 1994], while it does not affect either Dyn peptide nor its mRNA in this structure [Fox et al, 1994; Li et al, 1986; Morris et al, 1988]. Second, it has been shown that haloperidol induces Fos immunoreactivity in striatal D2-/Enk-expressing neurons [Robertson et al, 1992; Guo et al, 1996]. Third, Dyn is regulated by D1 receptors, which mediate c-fos and fra induction by amphetamine, cocaine and D1 agonists [Graybiel et al, 1990; Robertson et al, 1990; Young et al, 1991]. Finally, in DA-depleted animals, apomorphine-induced Fra has been demonstrated to co-localize with striatal Dyn neurons [Zhang et al, 1992]. Taken together, these data suggest that Dyn is not the response gene product for haloperidol-induced Fos protein. However, the effect of clozapine and clozapine-induced c-fos on Dyn expression remain to be further investigated.

(iii) Cholecystokinin (CCK)

CCK is a neuropeptide common to the central and peripheral nervous systems. In the mesolimbic system, CCK is co-stored in certain dopaminergic neurons [Hökfelt et al, 1980a; 1980b]. Depletion of DA modifies the CCK content of the NAc and striatum [Govoni et al, 1982], and DA modulates the release of CCK [Meyer & Krauss, 1983].
Conversely, CCK increases firing rates of DA neurons in the SN and the VTA [Skirboll et al, 1981]. This close association of CCK with DA has led to speculation about the possible role of CCK in schizophrenia [Hökfelt et al, 1980a; 1980b; Snyder, 1980a]. Neurochemical analyses have shown that CCK concentrations in the striatum and NAc are increased after either haloperidol or clozapine treatment [Frey, 1983]. Because the two classes of antipsychotics induce c-fos expression in a regionally specific manner, it is therefore improbable that CCK is the response gene product for drug-induced Fos and Fos-like proteins or that the induction of CCK accounts for the different profiles of typical and atypical drugs. More recent studies have reported that haloperidol does not induce any detectable changes in CCK peptide or mRNA expression in the striatum, NAc and midbrain [Radke et al, 1989; Cottingham et al, 1990; Schiffmann & Vanderhaeghen, 1992]. Therefore, these data suggest that CCK is not a target gene regulated by haloperidol-induced Fos protein in the forebrain.

(iv) Neurotensin (NT)

NT is a tridecapeptide that is heterogeneously distributed in the mammalian central nervous system, and has been implicated in a wide variety of functions [Uhl & Snyder, 1976; Kitabgi et al, 1977; Iversen et al, 1978; Young & Kuhar, 1981; Uhl, 1982]. Central NT pathways may play an important role in the etiology and/or pharmacotherapy of schizophrenia and other psychotic disorders [Nemeroff, 1980; Nemeroff et al, 1989]. NT has close anatomical and biochemical associations with brain DA systems [Quirion, 1983; Stowe et al, 1991; Fuxe et al, 1992a; 1992b] implicated in the etiology of schizophrenia [Seeman, 1987]. Further, drug-free schizophrenic patients have significantly lower
concentrations of NT in their cerebrospinal fluid as compared to controls [Widerlov et al, 1982; Lindstrom et al, 1988; Nemeroff et al, 1989]. When NT is administered centrally to rats, it has a pharmacological profile very similar to that of peripherally administered antipsychotic drugs [Nemeroff, 1980; Levant & Nemeroff, 1988]. These findings have led to the suggestion that NT may be an endogenous neuroleptic [Nemeroff, 1980]. Further studies have shown that there are marked differences in the degree to which typical and atypical neuroleptics augment striatal NT concentrations. Thus, acute or chronic administration of haloperidol and other neuroleptics increases NT concentrations in the NAc and striatum [Govoni et al, 1980; Frey et al, 1986; Letter et al, 1987; Eggerman & Zahm, 1988; Kilts et al, 1988; Nemeroff et al, 1992], while the concentration of NT is only markedly elevated in the NAc after clozapine [Kilts et al, 1988; Levant & Nemeroff, 1992; Nemeroff et al, 1992]. These finding suggest that neuroanatomically-selective enhancement of NT may contribute to the therapeutic and motoric side effects of these drugs: the clinical efficacy of antipsychotic drugs may be predicted by the increase in NT concentrations in the NAc, while the EPS liability may be predicted by alterations in NT levels in the striatum. Recent studies have demonstrated that neurotensin/neuromedin (NT/N) gene transcription is regulated by typical and atypical antipsychotics in a regionally specific manner: haloperidol and other typical drugs increased NT/N mRNA expression in the dorsolateral striatum and NAc, whereas clozapine and other atypical agents enhanced NT/N mRNA only in the NAc [Merchant et al, 1991; 1992; Williams et al, 1990; Merchant & Dorsa, 1993]. In addition, neuroleptic-induced increases in NT/N mRNA expression in the dorsolateral striatum are preceded by a rapid and transient activation of *c-fos* mRNA [Merchant & Dorsa, 1993], and the pattern of NT/N gene
expression produced by typical and atypical antipsychotics is strikingly similar to that of the \textit{c-fos} expression induced by these drugs [Merchant et al, 1992; Merchant & Dorsa, 1993]. These data, together with the observation that both the AP-1 site and CRE elements are present in the NT/N gene [Kislauskis & Dobner, 1990], suggests that \textit{c-fos} may be involved in transcription of the NT/N gene. Indeed, it has been found that neostriatal neurons coexpress NT/N and \textit{c-fos} mRNAs following acute haloperidol injection [Merchant & Miller, 1994]. Moreover, a \textit{c-fos} antisense oligomer specifically attenuated haloperidol-induced increases in NT/N mRNA expression in the dorsal striatum [Merchant, 1994; Robertson et al, 1995]. These results demonstrate a specific role of Fos in the regulation of NT/N gene expression in rat dorsal striatal neurons by haloperidol.

These data suggest that haloperidol-enhanced striatal NT/N gene expression may underlie the EPS of typical agents, and that increased NT/N expression in NAc induced by both clozapine and haloperidol may contribute to the antipsychotic effects of these drugs. Further identification of the target neuropeptide genes of antipsychotic-induced \textit{c-fos} and other IEGs will provide insights into the functional circuitry of the brain and the mechanisms of action of antipsychotic drugs.

5. Hypothesis and Strategies

The mechanisms that underlie the atypical profile of clozapine remain uncertain. Hypotheses that have been proposed to account for the mechanisms of the initial step of clozapine’s atypical actions include those that emphasize the different brain sites at which clozapine acts [Borison & Diamond, 1983; Borison et al, 1983; Chiodo & Bunney, 1983;
White & Wang, 1983; Seeman & Ulpian, 1983; Carlsson, 1988] and those that focus on distinct receptor(s) through which clozapine operates [Deutch et al, 1991; Meltzer, 1991; Lieberman & Koreen, 1993; Su, 1993; Jackson et al, 1994; Prinissen et al, 1994; Seeman & Van Tol, 1994; Sokoloff et al, 1994; Pere, 1995]. To date, atypical (e.g. clozapine) and typical (e.g. haloperidol) antipsychotics cannot be differentiated on the basis of pharmacological studies of receptors, including dopaminergic, serotonergic, cholinergic, adrenergic and sigma receptors. This implies that there might be more than one mechanism through which antipsychotics exert their actions. Moreover, recent data from molecular neurobiological studies reveal that most receptors and ion channels have different subtypes, each having a distinct anatomical distribution in the brain [Sibley & Monsma, Jr., 1992; Gingrich & Caron, 1993; Hosey, 1992; Wess, 1993; Caulfield, 1993; Teitler & Herrick-Davis, 1994; Pandey et al, 1995; Saxena, 1995; Lucas & Hen, 1995; Seeburg, 1993; Bockaert et al, 1993; Gallo et al, 1995; Wakamori et al, 1994; Dunlap et al, 1995; Varadi et al, 1995]. An emerging view is that both anatomical and pharmacological mechanisms contribute to the action of antipsychotic drugs. Specifically, it is hypothesized that the interaction of clozapine with regionally specific neurotransmitter receptors determines the unique therapeutic profile of this drug.

To address this hypothesis, one approach is to use a measure that will (1) reflect activation of brain neurons in response to clozapine or other antipsychotic drugs and (2) allow anatomical localization of neuronal activity at the cellular level. As discussed above, the induction of IEGs has provided a useful marker to map neuronal activities evoked by antipsychotic drugs. Because Fos immunohistochemistry permits a discrimination between pre- and post-synaptic elements in signal transduction pathways with single cell resolution,
it is reasonable to posit that drug-induced regionally specific Fos expression in the brain via selective neurotransmitter systems can be viewed as fingerprints for those neurotransmitter systems (i.e., to use Fos-immunostaining as a pharmacological activity indicator to examine neurotransmitter systems involved in the effects of antipsychotic drugs). Using Fos-immunohistochemistry, in combination with drug manipulations, brain lesions and in situ hybridization histochemistry, this thesis seeks to (1) determine receptor mechanisms of clozapine-induced c-fos expression in the forebrain and (2) characterize phenotypes of neurons targeted by clozapine and haloperidol.

Several strategies are employed in this study. First, Fos-positive neurons are used as markers for neurons targeted by clozapine or haloperidol. When combined with drug manipulations (e.g. muscarinic, 5-HT2, D3 and D4 receptor agonists) and neurotoxic lesions (e.g. 6-hydroxydopamine (6-OHDA) and 5,7-dihydroxytryptamine (5,7-DHT)), the effect of these manipulations on antipsychotic-induced Fos immunoreactivity reveals whether muscarinic, serotonergic, noradrenergic or dopaminergic receptor mechanisms are involved in clozapine’s or haloperidol’s effects. Further, when combined with in situ hybridization for particular receptor or peptide mRNAs, the co-localization of clozapine- or haloperidol-induced Fos-positive neurons with the receptor or peptide mRNA(s) provides an anatomical basis for characterizing the phenotype of cells targeted by these two antipsychotic drugs.
CHAPTER II. RECEPTOR MECHANISMS OF CLOZAPINE-INDUCED C-FOS EXPRESSION IN THE FOREBRAIN

1. Introduction

The mechanisms by which clozapine exerts its therapeutic effects without producing concomitant EPS remain elusive. Several studies have shown that typical and atypical antipsychotics have regionally different effects on c-fos expression in the forebrain: haloperidol induces c-fos expression in the NAc, LSN, and striatum, while clozapine increases the number of Fos-positive neurons in the NAc, LSN, and mPFC [Deutch et al, 1992; Nguyen et al, 1992; Robertson & Fibiger, 1992; MacGibbon et al, 1994]. This regional specificity of neuroleptic-induced IEG expression suggests that different patterns of neuronal activity are evoked by the two classes of antipsychotic drugs, and raises the possibility that these regionally different effects are related to the different clinical profiles of these drugs. Therefore, it is important to elucidate how such patterns are generated.

Haloperidol-induced striatal c-fos expression is thought to be D2 receptor mediated inasmuch as co-administration of a D2 agonist prevents the induction [Miller, 1990], and the selective D2 antagonist raclopride produces a regional pattern of Fos-like immunoreactivity that is indistinguishable from that produced by haloperidol [Robertson & Fibiger, 1992]. Because of the regional differences between clozapine- and haloperidol-induced c-fos expression in the brain, D2 receptor antagonism is not sufficient to account for the unique pattern produced by clozapine, even though D2 receptors are a common site of action for haloperidol and clozapine [Meltzer et al, 1989].
Clozapine is a potent muscarinic receptor antagonist with high affinity for all five subtypes of human muscarinic acetylcholine receptors [Bolden et al, 1991]. This raises the possibility that antimuscarinic actions of clozapine may contribute to the failure of this drug to induce c-fos expression in the striatum. If this is the case, administration of scopolamine, a muscarinic antagonist, might reduce haloperidol-induced striatal c-fos expression. Also, clozapine is an antagonist at 5-HT2 [Meltzer, 1989; Meltzer et al, 1989] and α1 noradrenergic receptors [Peroutka & Snyder, 1980; Lane et al, 1988; Baldessarini et al, 1992]; hence, it may induce c-fos expression via actions at these receptors. Indeed, Meltzer and colleagues have advanced the hypothesis that the ratio of 5-HT2 to D2 receptor blockade is a key variable that discriminates between typical and atypical antipsychotic drugs [Meltzer et al, 1989; Stockmeier et al, 1993], while Baldessarini et al [1992] have pointed out that a distinguishing feature of clozapine is its relatively potent central antiadrenergic actions and low antidopaminergic activity. In addition, interactions with D3 and D4 receptors, the two recently discovered D2-like receptor subtypes, are also candidate mechanisms for clozapine-induced c-fos expression in the brain. D3 receptors are expressed mainly in limbic brain areas, including the olfactory tubercle, NAc, ICj, and hypothalamus [Sokoloff et al, 1990]. Their predominant limbic distribution has led to the hypothesis that D3 receptors are involved in the regulation of cognitive and emotional functions and may thus be relevant to the therapeutic effects of antipsychotic drugs [Sokoloff et al, 1990; Sibley & Monsma, 1992]. Like D3 receptors, D4 receptors also have a unique regional distribution, with the highest level of expression occurring in the PFC [Mansour et al, 1991; Van Tol et al, 1991; O'Malley et al, 1992]. Moreover, the affinity of
clozapine for the D4 receptor is about 10 times higher than for the D2 receptor [Van Tol et al, 1991], raising the possibility that actions at D4 receptors contribute to clozapine's unique therapeutic profile.

In the experiments reported here, the effects of scopolamine on haloperidol-induced c-fos expression were examined in different brain structures. Furthermore, the extent to which serotonergic and/or noradrenergic mechanisms are involved in clozapine-induced c-fos expression was determined by examining the effects of prior lesions of either 5-HT- or NA-containing neurons on clozapine-induced c-fos expression in the brain. In addition, combined administration of the 5-HT2 receptor antagonist ritanserin and haloperidol was used to determine if concomitant blockade at D2 and 5-HT2 receptors would mimic clozapine’s actions on c-fos expression. To test the involvement of subtypes of the D2 receptor family in the actions of clozapine, the D3 receptor preferring agonist 7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7-OHDPAT), which has about 75- and 1000-fold lower affinity for D2 and D4 receptors, respectively, than for the D3 receptor [Levesque et al, 1992], and quinpirole, which has approximately equal affinity for D3 and D4 receptors and about 100-fold lower affinity for the D2 receptor [Levesque et al, 1992; Schwartz et al, 1992; Gingrich & Caron, 1993], were combined with clozapine administration.
2. Materials and Methods

a. Animals

Adult male Wistar rats (280-325 g) were kept under a 12 hr light/12 hr dark cycle, with free access to food and water. The rats were handled periodically for at least three to four days prior to the experiment.

b. Drug administration

Doses for different drugs used in the present experiments were those commonly used in such animal studies. To examine the effects of scopolamine on haloperidol-induced Fos-immunoreactivity in the forebrain, two groups of rats, each consisting of 9 animals, received subcutaneous (s.c.) injections of either scopolamine hydrobromide (Sigma, St Louis, MO; 2.5 and 5 mg/kg) or saline, 30 min before haloperidol (McNeil Pharmaceutical Canada LTD., Stouffville, Ont; 2 mg/kg). Two control groups, each composed of 4 animals, were injected with saline or scopolamine, respectively.

To determine whether serotonergic or noradrenergic mechanisms contribute to clozapine-induced Fos immunoreactivity in the mPFC and other brain regions, clozapine (H. Lundbeck, Kobenhavn-Valby; 20 mg/kg, s.c.) or vehicle (40 µl 20% acetic acid in 1 ml 0.9% saline) was injected into 5,7-DHT- (n=6) or 6-OHDA-lesioned (n=6) rats and sham control animals (n=6). Other unlesioned rats were injected with ritanserin (Research Biochemicals International, Natick, MA; 5 mg/kg, s.c., n=6) or saline (n=6), respectively, 30 min before haloperidol (1 mg/kg, s.c.) or vehicle injections.
In other experiments, quinpirole or 7-OHDPAT were co-administered with clozapine. Rats were injected with clozapine (20 mg/kg, s.c.) 15 min after the injection of quinpirole (RBI, Natick, MA; 1.0 mg/kg, s.c., n=7) or 7-OHDPAT (RBI, Natick, MA; 0.5 mg/kg, s.c., n=7). Control groups received quinpirole (1 mg/kg, s.c., n=6), 7-OHDPAT (0.5 mg/kg, s.c., n=6) or vehicle (n=6), respectively, following saline injection.

c. Brain lesions

Unilateral lesions of the serotonergic medial forebrain bundle (MFB) were made in 22 rats following surgical procedures [Paris et al, 1989]. The rats were pretreated with the NA and DA uptake inhibitor, nomifensine (Research Biochemicals International, Natick, MA; 15 mg/kg, i.p.), 30-40 min prior to the neurotoxin infusion. The rats were then anesthetized with sodium pentobarbital (Sigma, St Louis, MO; 60 mg/kg, i.p.) and placed in a stereotaxic apparatus. With the incisor bar set 3.3 mm below the interaural plane, the coordinates were: AP -3.8 mm; ML ±1.7 mm; DV -8.7 mm, relative to bregma [Paxinos & Watson, 1986]. The lesion side received an infusion of 5,7-DHT (Sigma, St Louis, MO; 8.0 μg in 3.0 μl of 0.1% ascorbic acid in 0.9% saline), and on the sham side a cannula was lowered to DV -1.5 mm without drug injection. The drug was infused via a 30-gauge cannula over 10 min (0.3 μl/min), and the cannula was left in place for an additional 5 min to allow for diffusion.

For bilateral lesions of the dorsal noradrenergic bundle (DNB), the following coordinates were used: AP +2.6 mm from the interaural line, ML ±1.1 mm from midline, DV + 3.7 mm from the interaural line, with the incisor bar set 3.3 mm below the interaural
plane [U’Prichard et al, 1980]. Twenty-two rats received 6-hydroxydopamine (6-OHDA, Sigma, St Louis, MO; 4 μg/side in 2 μl of 0.3% ascorbic acid in 0.9% saline), and the drug was infused bilaterally through 30-gauge cannulae at 1.0 μl/min for 2 min. Another 22 rats served as operated controls in which the cannulae were lowered bilaterally in the overlying cortex without infusion.

d. Neurochemical analysis

Two to three weeks after surgery, some of the lesioned rats were killed to determine the effects of 5,7-DHT MFB lesions (n=5) on 5-HT concentrations and 6-OHDA DNB lesions (n=5) on NA concentrations in the mPFC and other brain regions. The brains were removed and dissected on a cold Petri dish. The PFC, striatum and hippocampus were weighed and placed in a cold homogenizing solution (0.22 N perchloric acid, 0.05% EDTA-Na₂, and 0.15% sodium bisulfate). The tissues were sonicated for 30 sec and centrifuged at 32,500 g for 20 min (4°C), and the supernatants were stored at -80°C. Regional brain analysis of 5-HT, NA and DA was performed by reverse phase high-pressure liquid chromatography (HPLC) with electrochemical detection [Blackburn et al, 1986]. The flow rate of the mobile phase (33 mM sodium acetate, 0.9 mM octanesulfonic acid, 13% methanol, 0.036% EDTA, pH 3.6) was 0.7 ml/min. Standards (10⁻⁷ M of uric acid, adrenaline, NA, DA, 3-MT, 5-HT, dihydroxy-phenylacetic acid and 5-hydroxyindoleacetic acid) were injected into the HPLC-electrochemical detection system before and after running tissue samples. The tissue levels of 5-HT, NA, and DA were determined by comparing sample peaks with those of known standards. Each
measurement was taken as the average of two separate elutions of each sample, and was utilized for subsequent statistical analysis. The detection limit of the system was approximately 0.5 pmol/injection.

e. Fos immunohistochemistry

Two hours after the final drug injection, the rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardially with 200 ml of 0.9% saline followed by 150 ml 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brains were removed immediately after perfusion and placed in fresh fixative (4% PFA) for at least 12 h. Thirty-micrometer sections were cut from each brain using a Vibratome at room temperature. Unless otherwise specified, brain sections were processed at room temperature.

Drug-induced Fos protein was visualized using the immunohistochemical methods [Guo et al, 1992]. Sections were preincubated in 0.5% hydrogen peroxide in 0.02 M PBS for 15 min to remove endogenous peroxidase activity. After 3 washes in 0.02 M PBS, the sections were incubated with the primary antibody, sheep anti-Fos antibody (Cambridge Research Biochemicals, CRB OA-11-823 and CRB OA-11-824) diluted 1:2000 - 1:3000 in 0.02 M PBS containing 0.3% Triton X-100 and 0.02% azide. Sections were then washed three times with 0.02 M PBS and incubated with a biotinylated rabbit anti-sheep secondary antibody (Vector Laboratories; 1:200) for 1 h and subsequently washed 3 times with 0.02 M PBS. The sections were incubated for 1 h with 0.5% avidin-biotinylated horseradish peroxidase complex (ABC, Vector Laboratories) containing 0.3% Triton X-100. After two washes in 0.02 M PBS, the sections were rinsed in 0.2 M acetate buffer, pH
6.0. The reaction was visualized using the glucose oxidase-diaminobenzidine (DAB)-nickel method [Shu et al, 1988]. After 3 washes in 0.2 M acetate buffer, the sections were mounted on chrome alum-coated slides, dehydrated, and prepared for microscopic observations.

**f. Statistical analysis**

To determine the effects of either unilateral MFB 5,7-DHT or bilateral DNB 6-OHDA lesions on forebrain 5-HT, DA, or NA levels, HPLC-measured tissue concentrations of 5-HT, DA, and NA from lesioned or sham animals were analyzed using a Students two-tailed *t*-test.

The number of Fos-positive nuclei in the PFC, medial and lateral striatum, NAc, and LSN were counted with a 520 x 520 μm grid placed over these areas at 79x magnification. Fos-positive nuclei in the major island of Calleja were counted within a 63.5 x 190 μm grid at 125x magnification. The number of Fos-positive nuclei for each brain region was an average of those from three separate sections in each animal, and was subsequently used in the statistical analysis. Between-group differences in the number of Fos-positive nuclei within specified brain regions from animals for both lesion experiments and different drug administrations were evaluated by one-way ANOVA. Newman-Keuls' post hoc test was used to compare drug-induced changes in the number of Fos-positive nuclei in each brain area.
3. Results

a. Muscarinic Mechanisms

In agreement with previous studies, haloperidol increased the number of Fos-positive neurons in the striatum, NAc, and LSN (Fig. 1, 2A, C and E). Scopolamine significantly attenuated the haloperidol-induced increases in Fos expression in the medial and lateral aspects of the striatum (Fig. 1, 2A and B). The haloperidol-induced increases in the number of Fos-positive neurons in the LSN was also attenuated by scopolamine (Fig. 1, 2E and F). However, scopolamine did not significantly reduce the haloperidol-induced Fos expression in the NAc (Fig. 1, 2C and D). Compared to saline injections, scopolamine (2.5 mg and 5.0 mg/kg, i.p.) did not by itself alter the number Fos-positive nuclei in the striatum, NAc, or LSN (data not shown). Also, scopolamine either alone or in combination with haloperidol did not mimic clozapine-induced Fos immunoreactivity in the PFC. These data indicate that haloperidol-induced Fos immunoreactivity in the striatum has a muscarinic cholinergic component.

b. Serotonergic Mechanisms

(i) Effect of medial forebrain bundle lesions.

The effect of unilateral MFB lesions on the concentrations of 5-HT in the PFC and striatum are shown in Table 2. The concentration of DA in these two regions was also measured. As shown in Table 1, the 5,7-DHT lesions significantly reduced 5-HT tissue concentrations in the two brain regions assayed, without affecting the DA concentration in the PFC; 5-HT was decreased in both the PFC and the striatum by more than 91%. These
Fig. 1. Effects of saline (n = 4), haloperidol (2 mg/kg, n = 9), and scopolamine (2.5 mg/kg) plus haloperidol (2 mg/kg, n = 9) on the number of Fos-positive nuclei (mean ± S.E.M.) within a 520 x 520 μm area of the medial striatum (M. CPu), lateral striatum (L. CPu), nucleus accumbens (NAc) and lateral septal nucleus (LSN). * P < 0.001 vs. saline; ** P < 0.005 vs. haloperidol.
Fig. 2. Fos immunoreactivity in the lateral striatum (A, B), nucleus accumbens (C, D) and lateral septal nucleus (E, F) 2 hr after haloperidol (2 mg / kg; A, C, E) and scopolamine (2.5 mg / kg) plus haloperidol (2 mg / kg; B, D, F) treatment. Bar = 150 μm.
data show that unilateral MFB 5,7-DHT lesions specifically and extensively damaged 5-HT axons innervating the PFC and striatum on the lesioned side, without affecting DA axons which are also located in the MFB.

(ii) Effect of 5-HT depletion on clozapine-induced Fos induction in the forebrain.

To determine whether serotonergic mechanisms are involved in clozapine-induced Fos induction in the PFC, or contribute to the failure of this drug to induce c-fos expression in the striatum, clozapine-induced Fos immunoreactivity was examined in 5,7-DHT lesioned animals. As shown in Figure 3, depletion of 5-HT on the lesioned side did not affect the number of clozapine-induced Fos-positive neurons in the mPFC, NAc, and LSN, nor increased Fos immunoreactivity in the striatum, compared to the control side.

Using a similar strategy, a previous study demonstrated that lesions of the ascending mesotelencephalic dopaminergic projection abolished haloperidol-induced Fos induction in the striatum and the NAc [Robertson & Fibiger, 1992], suggesting that dopaminergic mechanisms mediate this effect. In contrast, the present results show that 5-HT depletions do not affect clozapine-induced increases in the number of Fos-positive neurons in the forebrain. These data suggest that serotonergic mechanisms are not involved in these effects of clozapine.

(iii) Effect of ritanserin on haloperidol-induced Fos induction in the forebrain.

Figure 4 shows that haloperidol (1mg/kg, s.c.) increased the number of Fos-positive neurons in the NAc, medial and lateral striatum, and LSN, and that ritanserin (5 mg/kg, s.c.) alone failed to influence Fos-like immunoreactivity in these structures.
Table 1. Effects of unilateral medial forebrain bundle (MFB) 5,7-DHT lesions on forebrain serotonin (5-HT) and dopamine (DA) levels.

<table>
<thead>
<tr>
<th>Region group</th>
<th>5-HT (pmol/mg)</th>
<th>DA (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>lesion</td>
</tr>
<tr>
<td>Prefrontal cortex</td>
<td>0.78 ± 0.10</td>
<td>0.07 ± 0.01*</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.90 ± 0.09</td>
<td>0.07 ± 0.01*</td>
</tr>
</tbody>
</table>

Data represent the mean (+SEM) tissue concentrations in 5-HT and DA following unilateral MFB 5,7-DHT lesion and sham surgery; numbers in parentheses represent % change from corresponding control; n = 6 per group.
* Significant (P < 0.001) difference from corresponding control.
Fig. 3. Effect of 5,7-DHT lesion in the MFB on clozapine-induced Fos-like immunoreactivity in the brain. 5-HT depletion in the forebrain produced by the lesion did not alter the number of Fos-positive nuclei induced by clozapine (20 mg / kg) in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc) and lateral septal nucleus (LSN). Neither sham nor neurotoxic lesions affected baseline levels of Fos-like immunoreactivity in the brain. Data represented as the mean ± S.E.M. * P < 0.001 vs. vehicle; n = 6 per treatment. M. CPu, medial striatum; L. CPu, lateral striatum.
Fig. 4. Effects of saline, ritanserin (5 mg / kg), haloperidol (1 mg / kg) and ritanserin (5 mg / kg) plus haloperidol (1 mg / kg) on the number of Fos-positive nuclei (mean ± S.E.M.) in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), medial striatum (M. CPu), lateral striatum (L. CPu) and lateral septal nucleus (LSN). * P < 0.001 vs. saline; n = 6 per treatment.
When combined with haloperidol, ritanserin failed to influence haloperidol-induced increases in the number of Fos-positive nuclei in the NAc, medial and lateral striatum, and LSN, and did not mimic clozapine-induced Fos induction in the mPFC. Consistent with the MFB lesion data, this result does not support the view that 5-HT2 receptor blockade contributes to clozapine’s effects on *c-fos* in the forebrain.

**c. Noradrenergic Mechanisms**

*(i) Effect of dorsal noradrenergic bundle lesions.*

In preliminary experiments, unilateral DNB lesions only decreased NA concentrations by 60-80% in the PFC and hippocampus (data not shown), suggesting that crossed NA projections might prevent sufficiently extensive effects of the lesion on NA concentrations. Consequently, bilateral DNB 6-OHDA lesions were utilized.

Table 2 shows the effects of bilateral 6-OHDA DNB lesions on forebrain NA and DA levels. The lesion caused extensive reductions in NA tissue concentrations in the forebrain, with a loss of 93.6% in the PFC and 94.7% in the hippocampus. In contrast, the 6-OHDA lesions did not affect DA concentration in the hippocampus, but did result in a small reduction in the PFC.

*(ii) Effect of NA depletion on clozapine-induced Fos induction in the forebrain.*

As shown in Figure 5, NA depletion did not alter baseline levels of *c-fos* expression compared to sham control animals. Also, bilateral DNB lesion-induced NA
Table 2. Effects of bilateral noradrenergic bundle (DNB) 6-OHDA lesions on forebrain noradrenaline (NA) and dopamine (DA) levels.

<table>
<thead>
<tr>
<th>Region</th>
<th>Group</th>
<th>NA (pmol/mg)</th>
<th>DA (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>1.44 ± 0.08</td>
<td>0.36 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>lesion</td>
<td>0.09 ± 0.01*</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>Prefrontal cortex</td>
<td>(-93.6%)</td>
<td></td>
<td>(-24.3%)</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>1.85 ± 0.10</td>
<td>0.022 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>lesion</td>
<td>0.10 ± 0.01*</td>
<td>0.023 ± 0.00</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>(-94.7%)</td>
<td></td>
<td>(+4.3%)</td>
</tr>
</tbody>
</table>

Data are the mean (±SEM) tissue concentrations in NA and DA after bilateral DNB 6-OHDA lesion or sham surgery; numbers in parentheses represent % change from corresponding control; n = 10 per group.

* Significant (P < 0.001) difference from corresponding control.
Fig. 5. Effect of 6-OHDA lesion of the DNB on clozapine-induced Fos-like immunoreactivity in the brain. NA depletion in the forebrain produced by neurotoxic lesions did not alter the number of Fos-positive nuclei induced by clozapine (20 mg / kg) in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and lateral septal nucleus (LSN). Baseline levels of Fos-immunoreactivity were not affected by either sham or neurotoxic lesion. Data represented as the mean ± S.E.M. * P < 0.001 vs. vehicle; n = 7 per group. M. CPu, medial striatum; L. CPu, lateral striatum.
depletion in the forebrain failed to affect clozapine-induced increases in the number of Fos-positive neurons in the mPFC, NAc, LSN, and other brain regions (Fig. 5). These results suggest that NA mechanisms do not contribute to clozapine’s effects on Fos induction in the forebrain.

\textit{d. D3/D4 receptor mechanisms}

\textit{(i) Clozapine-induced Fos-like immunoreactivity.}

Clozapine (20 mg/kg, s.c.) produced significant increases in Fos-like immunoreactivity in the mPFC, NAc, LSN, and the major island of Calleja (ICjM) (Figs 6 and 9). Fos-positive nuclei were scattered in a homogeneous manner throughout each of these structures (Figs 7 and 10) with the exception of the ICjM. Within the latter structure, the vast majority of nuclei were Fos-positive following clozapine (Fig. 8A).

\textit{(ii) Effects of 7-OHDPAT on clozapine-induced Fos induction.}

7-OHDPAT (0.5 mg/kg, s.c.) significantly reduced (78.7% and 51.6%) clozapine-induced increases in the number of Fos-positive neurons in the NAc (Figs. 6, 7C and D) and LSN (Figs 6, 7E and F), respectively. 7-OHDPAT also blocked clozapine-induced Fos immunoreactivity in the major island of Calleja (Figs 6 and 8C). However, this DA receptor agonist failed to influence the number of clozapine-induced Fos-positive neurons in the mPFC (Figs 6, 7A and B). These results suggest that clozapine’s effects in the ICjM, NAc, and LSN may be due to its antagonist actions at D3 receptors, and that D3
Fig. 6. Effects of clozapine (20 mg / kg) and 7-OHDPAT (0.5 mg / kg) plus clozapine (20 mg / kg) on the number of Fos-positive nuclei (mean ± S.E.M.) in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), medial striatum (M. PCu), lateral striatum (L. PCu), lateral septal nucleus (LSN) and major island of Calleja (ICjM). * P < 0.001 vs. clozapine; n = 6 per group.
Fig. 7. Photomicrographs of Fos-like immunoreactivity in the mPFC (A, B), NAc (C, D) and lateral septal nucleus (E, F) following clozapine (A, C, E) and 7-OHDPAT plus clozapine treatment (B, D, F). Scale bar = 100 μm.
Fig. 8. Photomicrographs of Fos-like immunoreactivity in the major island of Calleja (ICjM) after clozapine (A), quinpirole plus clozapine (B) and 7-OHDPAT plus clozapine (C) treatment. MS: medial septal nucleus. Scale bar = 46 μm.
receptor mechanisms do not contribute to clozapine-induced Fos-immunoreactivity in the mPFC.

(iii) Effects of quinpirole on clozapine-induced Fos induction.

Quinpirole (1 mg/kg, s.c.), which by itself did not affect Fos immunoreactivity in the forebrain (Fig. 9), produced small but statistically significant decreases in the number of clozapine-induced Fos-positive nuclei in the mPFC (Figs 9 and 10A,B). Quinpirole also blocked clozapine-induced Fos-immunoreactivity in the ICjM (Figs 8B and 9), and the NAc (Figs 9 and 10C, D), and had a large, although incomplete, effect in the LS (Figs 9 and 10E, F). These results, together with a report that quinpirole has about 75-fold higher affinity for D3 than the D2 receptor, are consistent with the view that the antagonist actions at D3 receptors contribute to clozapine-induced Fos immunoreactivity in the ICjM, NAc, and LSN. Because quinpirole has equal affinities for D3 and D4 receptors, the small but significant decrease by this agonist in clozapine-induced Fos immunoreactivity in the PFC suggests that actions on dopamine D4 receptors may partly contribute to clozapine's effect in this brain region.

(iv) Effects of 7-OHDPAT on haloperidol-induced Fos induction.

In contrast to its effects on clozapine-induced Fos-immunoreactivity in the forebrain, 7-OHDPAT (0.5 mg/kg, s.c.) failed to attenuate haloperidol-induced Fos induction in the NAc, medial and lateral striatum, and LSN (Fig. 11). Consequently, these data suggest that D3 receptor mechanisms are not involved in haloperidol-induced c-fos expression in the forebrain.
Fig. 9. Effects of vehicle (1 ml / kg), quinpirole (1 mg / kg), clozapine (20 mg / kg), and quinpirole (1 mg / kg) plus clozapine (20 mg / kg) on the number of Fos-positive nuclei (mean ± S.E.M.) in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), medial striatum (M. CPu), lateral striatum (L. CPu), lateral septal nucleus (LSN) and the major island of Calleja (ICjM). * P < 0.001 vs. quinpirole, + P < 0.007 vs. quinpirole plus clozapine.
Fig. 10. Photomicrographs of Fos-like immunoreactivity in the mPFC (A, B), NAc (C, D) and lateral septal nucleus (E, F) after clozapine (A, C, E) and quinpirole plus clozapine (B, D, F) treatment. Scale bar = 100 μm.
Fig. 11. Effects of haloperidol (1 mg / kg) and 7-OHDPAT (0.5 mg / kg) plus haloperidol (1 mg/ kg) on the number of Fos-positive nuclei (mean ± S.E.M.) in the nucleus accumbens (NAc), medial striatum (M. CPu), lateral striatum (L. CPu) and lateral septal nucleus (LSN).
Fig. 12. Effects of haloperidol (1 mg / kg) and quinpirole (1 mg / kg) plus haloperidol (1 mg / kg) on the number of Fos-positive nuclei (mean ± s.E.M.) in the nucleus accumbens (NAc), medial striatum (M. CPu), lateral striatum (L. CPu) and lateral septal nucleus (LSN).
(v) Effects of quinpirole on haloperidol-induced Fos induction.

Similar to the lack of effect of 7-OHDPAT, quinpirole (1 mg/kg, s.c.) failed to influence haloperidol-induced Fos-immunoreactivity in the NAc, medial and lateral striatum and LSN (Fig. 12).

4. Discussion

a. Muscarinic Mechanisms

In the present study, the muscarinic receptor antagonist scopolamine attenuated haloperidol-induced Fos immunoreactivity in striatal neurons (Fig. 1 and 2), suggesting that haloperidol-induced Fos induction in the striatum is modulated by muscarinic cholinergic mechanisms [Guo, et al, 1992b]. It is noteworthy in this regard, that a recent study showed that activation of muscarinic receptors increases Fos immunoreactivity in the striatum [Hughes & Dragunow, 1993]. Another study, however, reported that the muscarinic receptor antagonist atropine did not block haloperidol-induced c-fos mRNA expression in the striatum [Merchant & Dorsa, 1993], suggesting that muscarinic mechanisms are not involved in haloperidol-induced c-fos expression in this brain region.

NMDA receptor antagonists have been reported to block Fos-like protein translation, without blocking c-fos mRNA transcription [Hisanaga et al, 1992]. Thus, the non-competitive NMDA receptor antagonist MK-801 and competitive antagonists 4-(3-phosphonopropyl) piperazin-2-carboxylic acid (CPP) and 2-amino-7-phosphonoheptanoate
(APH) did not affect basic fibroblast growth factor (bFGF)-induced \(c\text{-}fos\) mRNA expression in cultured cortical neurons, suggesting that bFGF induces \(c\text{-}fos\) mRNA transcription via non-NMDA receptor-mediated mechanisms [Hisanaga et al, 1992]. However, these NMDA antagonists blocked bFGF-induced induction of Fos-like protein (FLP) [Hisanaga et al, 1992]. Furthermore, treatment of the cultures with these NMDA antagonists had no effect on total protein synthesis [Hisanaga et al, 1992]. Clearly, activation of NMDA receptors are required to translate Fos protein from the \(c\text{-}fos\) mRNA induced by bFGF. This raises the possibility that muscarinic receptor activation is not involved in haloperidol-induced \(c\text{-}fos\) expression, but is required for translation of Fos protein from the induced \(c\text{-}fos\) mRNA in striatal neurons.

Modulation of motor function is mediated in part by interactions between dopaminergic and cholinergic mechanisms in the extrapyramidal system [Costall & Naylor, 1975]. For example, both haloperidol-induced catalepsy and EPS are attenuated by muscarinic receptor antagonists [Arnt & Christensen, 1981; Brown, 1990; Butkerait & Friedman, 1988; Cedarbaum & Schleifer, 1990; Klemm, 1985]. Neurochemically, D2 receptor agonists decrease and antagonists increase acetylcholine (ACh) release in the striatum [Bertorelli & Consolo, 1990; Consolo et al, 1987; Damsma et al, 1990; Damsma et al, 1992; Drukarch et al, 1989; Drukarch et al, 1990]. Most striatal cholinergic neurons are known to be interneurons that express D2 receptors [Le Moine et al, 1990; Maclellan et al, 1994] and innervate striatal medium spiny neurons [Izzo & Bolam, 1988]. A recent study has shown that haloperidol selectively increases Fos immunoreactivity in the subpopulation of striatal medium spiny neurons that contain Enk and project to the globus pallidus [Robertson et al, 1992]. The fact that scopolamine attenuates haloperidol-induced
increases in striatal Fos immunoreactivity raises the possibility that these increases are mediated indirectly by the ability of this neuroleptic to increase ACh release which in turn acts on muscarinic receptors located on striatopallidal neurons. Alternatively, because striatopallidal neurons also contain D2 receptors [Gerfen & Young, 1988; Gerfen et al, 1990], ACh may serve a permissive function with respect to direct actions of the neuroleptic on these neurons. Regardless of the precise mechanisms by which haloperidol increases c-fos expression/Fos-immunoreactivity in striatum, the fact that scopolamine can attenuate haloperidol-induced Fos immunoreactivity, as well as neuroleptic-induced EPS and catalepsy, suggests that these phenomena are related. Specifically, these results are consistent with the hypothesis that neuroleptic-induced increases in striatal Fos immunoreactivity may be predictive of motoric side effects produced by antipsychotic drugs [Deutch et al, 1992; Nguyen et al, 1992; Robertson & Fibiger, 1992; Hughes & Dragunow, 1995]. It is interesting in this regard that the atypical neuroleptic clozapine, which lacks EPS at clinically effective doses, does not increase Fos immunoreactivity in the striatum [Deutch et al, 1992; Nguyen et al, 1992; MacGibbon et al, 1994; Robertson & Fibiger, 1992].

In contrast to its effects in the striatum, scopolamine failed to influence haloperidol-induced increases in the number of Fos-positive nuclei in the NAc (Figs. 1 and 2). This is notable in view of the fact that D2 receptors do not appear to exert the same potent inhibitory control over ACh release in the NAc as they do in the striatum [Henselmans & Stoof, 1991; Henselmans et al, 1991]. For example, Henselmans and Stoof [1991] have shown that there are clear rostrocaudal and lateromedial gradients with respect to D2 agonist-mediated inhibition of ACh release in the NAc, with a complete lack of inhibition
occurring in the caudomedial part of this structure. Moreover, a recent study showed that while most cholinergic neurons in the striatum (~95%) express D2 receptor mRNA, only about half of cholinergic neurons in the NAc express D2 receptor mRNA [Maclennan et al, 1994]. These findings suggest that the mechanisms by which haloperidol increases Fos immunoreactivity in the NAc do not have a cholinergic component and thus differ from those mediating this effect in the striatum. The present findings in the NAc are also potentially consistent with clinical observations indicating that antimuscarinic drugs do not block the antipsychotic efficacy of neuroleptics [Baldessarini, 1990].

Scopolamine also reduced the number of haloperidol-induced Fos-positive neurons in the lateral septal nucleus (Fig. 1 and 2). At present the receptor mechanisms by which haloperidol produces this effect in this nucleus are not known, although recent research suggests that a blockade of central DA receptors is not importantly involved [Robertson & Fibiger, 1992]. The present results clearly suggest that muscarinic receptors contribute to this effect in the LSN although the details by which this occurs cannot be specified at present.

b. Serotonergic Mechanisms

Because clozapine is a more potent antagonist at 5-HT2 receptors than at D2 receptors, Meltzer and colleagues [Meltzer et al, 1989; Meltzer et al, 1992; Stockmeier et al, 1993] have proposed that strong blockade at 5-HT2 receptors in the presence of weak blockade at D2 receptors contributes to the unique clinical profiles of clozapine and other atypical antipsychotic drugs. In order to determine if serotonergic or noradrenergic mechanisms play a role in the unique pattern of Fos immunoreactivity produced by
clozapine, the effects of extensive prior lesions of ascending serotonergic projections to the telencephalon were examined. The present results show that MFB 5,7-DHT lesions failed to influence the extent or distribution of clozapine-induced Fos-like immunoreactivity in the mPFC, NAc or LSN (Fig. 3). This contrasts with earlier observations that 6-OHDA lesions of the mesotelencephalic DA system block haloperidol-induced Fos immunoreactivity in the striatum and haloperidol- and clozapine-induced Fos induction in the NAc [Robertson & Fibiger, 1992]. The present lesion study therefore provides no support for the hypothesis that clozapine-induced c-fos expression in the mPFC or other brain regions is mediated by 5-HT2 receptor blockade. In agreement with the lesion study, the 5-HT2 antagonist ritanserin failed to mimic clozapine-induced increases in Fos-like immunoreactivity in the mPFC either by itself or when combined with haloperidol (Fig 4), again suggesting that 5-HT2 antagonism does not contribute to clozapine-induced c-fos expression in the mPFC or other brain regions.

Consistent with these findings, others have reported that co-administration of haloperidol (1 mg/kg) with ritanserin (1 and 3 mg/kg) does not mimic the effect of clozapine on Fos protein immunoreactivity in the forebrain [Fink-Jensen et al, 1995]. Likewise, ritanserin either alone or in combination with sulpiride does not induce Fos expression in the PFC [Deutch & Duman, 1996]. In addition, several studies have shown that the atypical behavioral effects of clozapine are not altered by changes in serotonergic transmission [Jaskiw et al, 1994; Jaskiw & Vrtunski, 1995]. For example, the differences in biting behaviors between haloperidol and clozapine treated rats persist after large and selective 5-HT depletions [Jaskiw & Vrtunski, 1995]. Furthermore, co-administration of clozapine (30 mg/kg) with the 5-HT2 agonist DOI (0.1, 1 and 5 mg/kg) does not induce
catalepsy in rats; similarly, ritanserin does not reduce haloperidol-induced catalepsy [Jaskiw et al, 1994]. These data suggest that clozapine’s preferential actions on mesolimbic DA systems do not depend on its effects on serotonergic transmission in the brain. Nevertheless, it remains possible that a certain ratio of 5-HT2 to D2 receptor blockade is important with respect to antipsychotic-induced c-fos expression in different brain regions. This issue will require an examination of the effects of a range of doses of ritanserin on haloperidol-induced c-fos expression and Fos-like immunoreactivity.

c. Noradrenergic Mechanisms

It has been suggested that the antagonist actions of clozapine at α1 [Baldessarini & Frankenburg, 1991; Baldessarini et al, 1992; Lane et al, 1988] or α2 noradrenergic receptors [Pickar et al, 1992] contribute to the atypical profile of clozapine. To determine whether noradrenergic mechanisms play a role in clozapine-induced Fos induction in the forebrain, the effects of extensive prior lesions of ascending noradrenergic projections to the telencephalon were examined. The bilateral DNB lesions failed to influence clozapine-induced Fos immunoreactivity in the mPFC, NAc, or lateral septal nucleus (Fig. 5), suggesting that noradrenergic mechanisms are not involved in clozapine’s effect.

Consistent with this study, another report has shown that α1 adrenergic receptor blockade does not account for clozapine’s effect [Fink-Jensen et al, 1995]. Thus, administration of the α1 noradrenergic receptor antagonist prazosin (0.3 and 1 ml/kg) does not increase Fos immunoreactivity in the forebrain; when co-administered with haloperidol (1 mg/kg), prazosin does not mimic clozapine’s effect on Fos immunoreactivity in the forebrain [Fink-
Jensen et al, 1995]. These results, therefore, suggest that clozapine-induced c-fos expression in the mPFC and other brain areas is not mediated by α1/α2 noradrenergic receptor blockade.

However, a recent study found that combined administration of the α1 antagonist phenoxybenzamine, the dopamine D1 antagonist SCH 39166, and the 5-HT2 antagonist ketanserin produced a profile similar to that of clozapine, suggesting that combined antagonisms of noradrenergic, dopaminergic, and serotonergic receptors may underlie the atypical profile of clozapine [Prinssen et al, 1994]. This raises the possibility that a certain ratio of α1 to D1 and 5-HT2 receptors is crucial for the unique c-fos expression pattern induced by clozapine.

d. Dopamine D3/D4 Receptor Mechanisms

The failure of haloperidol, a DA receptor antagonist with a higher affinity for D2 than for D3 or D4 receptors [Sokoloff, et al, 1990; Van Tol et al, 1991], to induce c-fos expression in the mPFC suggests that D2 receptor antagonism does not account for the effects of clozapine in this structure. However, the data reported here suggest that actions at subtypes of the D2 family of receptors, including D3 and perhaps D4 receptors, may contribute to clozapine’s effects on Fos immunoreactivity in the forebrain. Thus, the DA receptor agonist quinpirole, which in vitro has approximately equal affinities for D3 and D4 receptors but 100-fold lower affinity for D2 receptors [Gingrich & Caron, 1993; Levesque et al, 1992; Sokoloff et al, 1990; Schwartz et al, 1992], produced small but significant decreases in clozapine-induced Fos immunoreactivity in the mPFC and had
large effects in the ICjM, NAc, and LSN (Figs 8, 9 and 10). In contrast, the somewhat selective D3 receptor agonist 7-OHDPAT, which in vitro has about 75-fold lower affinity for D2 receptors and 1000-fold lower affinity for D4 receptors than for D3 receptor [Levesque et al, 1992; McElroy, 1994], significantly reduced the number of clozapine-induced Fos-like immunoreactive neurons in the ICjM, NAc, and LSN without having any effect in the mPFC (Figs 6, 7 and 8). These data suggest that actions at D3 receptors may mediate clozapine-induced Fos-immunoreactivity in the ICjM, NAc, and LSN, while its actions at D4 receptors may contribute to its actions in the mPFC. These working hypotheses are consistent with current anatomical information concerning the distributions of D3 and D4 receptors, the former being expressed primarily in limbic brain regions such as the NAc and the islands of Calleja [Bouthenet et al, 1991; Sokoloff et al, 1990; 1992], and the latter being most highly expressed in the frontal cortex and amygdala [O’Malley et al, 1992; Van Tol et al, 1991]. However, several caveats should be emphasized with respect to these interpretations. First, quinpirole produced only a small attenuation of clozapine-induced Fos immunoreactivity in the mPFC (Figs. 9 and 10). This suggests that while antagonist actions at D4 receptors may contribute to clozapine’s actions in the mPFC, the full spectrum of mechanisms by which it increases Fos immunoreactivity in this structure remains to be determined. Also, recent Western-blot data suggested that quinpirole (0.1 and 0.5 mg/kg) increased Fos expression in the PFC [Deutch & Duman, 1996]. This result is in contrast with the present study which shows that quinpirole (1 mg/kg) does not increase Fos immunoreactivity in the PFC. While the reason for the discrepancy is unclear, Deutch and Duman [1996] have suggested that clozapine-induced Fos expression in the PFC may due to its actions at a novel, yet unidentified DA receptor.
Second, despite its considerable selectivity to D3 receptors in *in vitro* assays, the extent to which the *in vivo* actions of 7-OHDPAT can be attributed to D3 receptors selectivity is uncertain [Freedman et al, 1994; Large & Stubbs, 1994a; 1994b]. Indeed, recent data suggest that it is difficult to differentiate between the *in vivo* activity of 7-OHDPAT at D2 and D3 receptors. Thus, *in vivo* displacement studies showed that 7-OHDPAT produced dose-dependent D2 receptor occupancy in rat brain: significant D2 occupancy was observed at a dose of 1 mg/kg [Levant et al, 1996]. Therefore, the blockade by this compound of clozapine’s effects may not be solely due to its D3 receptor activity.

Some data are not easily accommodated by the hypothesis that clozapine increases Fos-immunoreactivity in the ICjM, NAc, and LSN via actions at D3 receptors and in the mPFC via D4 receptor mechanisms. For example, *in vitro* studies have shown that haloperidol has near 2-fold higher affinity for D4 receptors than clozapine [Van Tol et al, 1991]. If D4 receptors partly contribute to clozapine’s effect in the mPFC, haloperidol should also produce Fos expression in this brain region. It is worth noting that haloperidol has 10-fold higher affinity for D2 than for D4 receptors [Van Tol et al, 1991]. Therefore, the dose used in the present study may not have been sufficient for haloperidol to have significant activity at D4 receptors. Receptor binding data showed that clozapine has two to three-fold higher affinity for D2 receptors than for D3 receptors [Gingrich & Caron, 1993; Sokoloff et al, 1990]. This appears to be inconsistent with the present results which suggest that clozapine increases Fos immunoreactivity in the ICjM, NAc, and LSN via D3 receptor antagonism, because at the same time it failed to produce the expected D2 receptor antagonist-mediated increases in the number of Fos-like positive neurons in the striatum. Given clozapine’s higher affinity for D2 than D3 receptors *in vitro*, its positive effects on
Fos immunoreactivity in the ICjM, NAc, and LSN in vivo indicate that its inability to increase the number of Fos-like immunoreactive neurons in the striatum by blocking D2 receptors was not due to insufficient dosage. The disparities between the results of previous receptor binding studies and the present results indicate that in vitro affinities of typical and atypical antipsychotics for DA receptors cannot by themselves account for the patterns of c-fos expression produced by these compounds. A number of other contributing factors must therefore be considered. First, it is possible that active metabolites that have affinities for the DA receptor subtypes that are different from the parent compounds contribute to the patterns of c-fos expression reported here. Second, the in vivo potencies of antipsychotic drugs in occupying central neurotransmitter receptors do not always correspond to their in vitro affinities for these receptors [Leysen et al, 1993; Meltzer et al, 1992; Sautel et al, 1995; Stockmeier et al, 1993]. It is worth noting in this regard that the conditions under which in vitro affinities are determined no doubt differ very substantially from those that exist in vivo [Prosser et al, 1989; Freedman et al, 1994; Large & Stubbs, 1994b; Sautel et al, 1995]. For instance, receptor binding displacement studies suggested that the in vivo striatal and mesolimbic Kd values of haloperidol for D2 receptors are 6- to 9-fold higher than the in vitro Kd values [Prosser et al, 1989]. Finally, multiple neurotransmitter receptors are probably involved in regulating c-fos mRNA transcription and Fos protein translation in individual neurons. For example and as mentioned above, the antimuscarinic drug scopolamine attenuates haloperidol-induced Fos immunoreactivity in the striatum [Guo et al, 1992]. Similarly, haloperidol-induced Fos immunoreactivity in the striatum can be reduced by competitive and noncompetitive
NMDA receptor antagonists [Dragunow et al, 1990; Boegman & Vincent, 1996] and adenosine A2 receptor antagonists [Boegman & Vincent, 1996].

Robertson and Fibiger [1992] reported that while 6-OHDA lesions of the mesotelencephalic DA system blocked clozapine-induced increases in the number of Fos-like immunoreactive neurons in the NAc, these lesions had no effect on clozapine’s actions in the mPFC and lateral septal nucleus. Indeed, it was on the basis of this observation that a non-dopaminergic mechanism was proposed for clozapine in these structures [Robertson & Fibiger, 1992]. However, the observation that unilateral 6-OHDA lesions of the mesotelencephalic DA system produce less than a 60% reduction of DA in the PFC [Deutch & Duman, 1996] suggests that more extensive cortical DA depletion may be required. The fact that quinpirole significantly attenuates clozapine-induced increases in Fos immunoreactivity in the mPFC and LSN (Figs. 9 and 10) suggests that dopaminergic mechanisms do contribute, at least in part, to clozapine’s actions in these structures. Also, it is worth noting that there is precedence for lesion-induced depletions of DA failing to block neurochemical actions of neuroleptics. For example, extensive (~ 95%) 6-OHDA lesions of the nigrostriatal projection fail to affect the ability of haloperidol to reduce striatal concentrations of acetylcholine [Fibiger & Grewaal, 1974). This raises the possibility that the effects of neuroleptics may not simply be due to blocking the actions of DA at DA receptors but that these compounds have a level of intrinsic activity, which perhaps only occurs in the denervated condition.

In contrast to their effects on clozapine induced Fos-immunoreactivity in the forebrain, neither 7-OHDPAT nor quinpirole affected haloperidol-induced increases in the number of Fos-like immunoreactive neurons (Figs. 11 and 12). The fact that haloperidol
does not induce \textit{c-fos} expression in the islands of Calleja, a structure which expresses D3 and D1 but not D2 receptors \cite{Bouthenet et al, 1991; Diaz et al, 1995; Fremeau et al, 1991; Landwehrmeyer et al, 1993a; Mengod et al, 1991; Sokoloff et al, 1990}, suggests that D3 receptor mechanisms are not involved in haloperidol’s effect on \textit{c-fos} expression in the forebrain. The failure of 7-OHDPAT to influence haloperidol-induced Fos immunoreactivity in different brain areas is consistent with the view that D2 but not D3 receptors mediate the actions of this neuroleptic. Since several lines of evidence have suggested that D2 antagonism mediates haloperidol-induced \textit{c-fos} expression in the forebrain, it was expected that quinpirole, which is also a D2 receptor agonist, might influence haloperidol-induced Fos immunoreactivity, at least in the striatum, regardless of its higher affinity \textit{in vitro} for D3 than D2 receptors. First, both haloperidol and other D2 receptor antagonists increase Fos induction in the striatum \cite{Dragunow et al, 1990; Robertson & Fibiger, 1992} and the selective D2 antagonist raclopride produces a pattern of Fos-like immunoreactivity that is indistinguishable from that produced by haloperidol \cite{Robertson & Fibiger, 1992}. Second, it has been demonstrated that most Fos-positive neurons induced by haloperidol are Enk-expressing neurons \cite{Robertson et al, 1992} which contain D2 receptor mRNA \cite{Gerfen et al, 1990; Le Moine et al, 1990}.

Somewhat surprisingly, the present results show that quinpirole failed to affect haloperidol’s effects in any of these brain regions. This contradicts a previous study which showed that haloperidol-induced \textit{c-fos} expression in the striatum was blocked by quinpirole, which has traditionally been used as a D2 receptor agonist \cite{Miller, 1990}. This disparity cannot be explained by differences in drug dosage. The present study used 1 mg/kg quinpirole vs. 1 mg/kg haloperidol, while the previous study used 1 mg/kg
quinpirole vs. 2.5 mg/kg haloperidol [Miller, 1990]. While explanations for this discrepancy are not obvious, it is worth noting that quinpirole (5 mg/kg) did not alter haloperidol-induced (1 mg/kg) NT/N mRNA expression [Merchant & Dorsa, 1993]. It has been demonstrated that haloperidol-induced \textit{c-fos} expression regulates downstream target NT/N gene expression [Merchant, 1994]. Thus, striatal neurons co-express \textit{c-fos} and NT/N mRNAs following haloperidol treatment [Merchant & Miller, 1994]. Furthermore, \textit{c-fos} antisense oligonucleotide attenuates haloperidol-induced increases in NT/N mRNA expression in the dorsal striatum [Merchant, 1994; Robertson et al, 1995]. Therefore, should quinpirole block haloperidol-induced \textit{c-fos} expression in the striatum, the effect of haloperidol-induced NT/N gene expression should also be blocked. However, it was not. Moreover, Merchant and Dorsa [1993] showed that the failure of quinpirole to attenuate haloperidol's effects is not due to insufficient dosage. In this latter study, the animals received 5 mg/kg quinpirole vs. 1 mg/kg haloperidol, i.e. on a molar basis, 10 times as much quinpirole as haloperidol (2 x 10^{-5} M quinpirole vs. 2 x 10^{-6} M haloperidol) [Merchant & Dorsa, 1993]. Therefore, this result is consistent with the present study, which suggests that quinpirole does not influence haloperidol-induced Fos immunoreactivity in the forebrain.

Nevertheless, the present result suggests that quinpirole may not be a sufficient pharmacological agent to test the D2 hypothesis of haloperidol's effect in the brain. Clearly, more selective D2 receptor agonists and other experimental approaches, such as the using of D2 receptor antisense oligonucleotides or D2 gene knock-outs, may be required to determine D2 receptor mechanisms of haloperidol-induced \textit{c-fos} expression in the forebrain. In addition, a recent study reported that haloperidol-induced Fos-positive
neurons in the NAc only account for about 5% of the total population of medium spiny neurons in this structure [Robertson & Jian, 1995]. Since about 50% of the medium spiny neurons is thought to express D2 receptors, this observation suggest that other factors, in addition to D2 receptor blockade, may also contribute to haloperidol-induced \( c-fos \) expression in the forebrain. Indeed, it has been reported that Fos induction by haloperidol in striatopallidal neurons is mediated in part through activation of NMDA glutamate receptors, adenosine A2 receptors and muscarinic receptors [Dragunow et al, 1990; Boegman & Vincent, 1996; Guo et al, 1992]. Therefore, as suggested by Boegman and Vincent [1996], even when the D2 receptor inhibition is attenuated, the collective actions of glutamate, adenosine and acetylcholine on the striatal neurons could produce sufficient intracellular calcium and/or cAMP to induce \( c-fos \).
CHAPTER III. CELLULAR MECHANISMS OF CLOZAPINE- AND HALOPERIDOL-INDUCED C-FOS EXPRESSION

1. Introduction

The distinct regional patterns of c-fos expression in response to clozapine and haloperidol raise questions about the phenotypic characteristics of the neurons that increase c-fos expression in response to these drugs. The present experiments sought to address this issue by determining the phenotypes of neurons in which clozapine increases Fos immunoreactivity.

While the mechanisms by which clozapine increases c-fos expression have yet to be elucidated, data from the previous chapter and other reports suggest that the antagonist actions of this drug at DA D3 receptors may be involved in its effects in the forebrain. Localization studies of the D3 receptor gene transcripts indicate that this receptor is mainly expressed in limbic brain regions such as the ICj, olfactory tubercle and NAc. The similarity between patterns of D3 receptor mRNA expression and clozapine-induced c-fos expression in the brain also suggests that D3 receptors are a substrate for clozapine’s actions. Further, since quinpirole and 7-OHDPAT block or attenuate clozapine-induced increases in the number of Fos-positive neurons in the ICjM, NAc and LSN, it has been proposed that clozapine increases c-fos expression in these structures primarily by blocking D3 receptors [Guo et al, 1995]. On the other hand, haloperidol has a twenty-fold higher affinity for D2 than for D3 receptors [Sokoloff et al, 1990], this neuroleptic may increase c-fos expression in the NAc and LSN via its antagonist actions at D2 receptors which, like D3 receptors, are also located in these structures. As a test of the hypothesis that clozapine
increases \textit{c-fos} expression in these forebrain structures via its actions at D3 receptors, \textit{in situ} hybridization histochemistry for D3 receptor mRNA was used in combination with Fos immunohistochemistry to determine if D3 receptors are expressed by neurons in which clozapine increases Fos immunoreactivity. Previous studies have demonstrate that D3 receptor mRNA is abundantly expressed in the ICj of rat [Sokoloff et al, 1990; Diaz et al, 1995] and human brain [Landwehrmeyer et al, 1993a]. The ICj is composed of clusters of granule cells located in the polymorph layer of the olfactory tubercle and between the septum and NAc [Fallon et al, 1978; Millhouse, 1987]. The term “islands of Calleja complex” (ICC) has been suggested, referring to a proposed functional unit of this nucleus. 

Within this unit, the granule cells tend to form cup-like structures enclosing a cell-poor hilus region, with a few large and medium sized neurons present in the hilus and surrounding neuropil [Fallon et al, 1978; Ribak & Fallon, 1982; Millhouse, 1987]. Although the physiological significance of the ICj remain to be understood, this structure provide a positive marker for D3 receptor mRNA expression. For comparative purposes, identical experiments were conducted with haloperidol.

In the striatum, medium spiny projection neurons, which represent 90-95% of all neurons in this structure, can be subdivided into two populations: (1) D2-expressing, enkephalinergic/neurotensinergic cells with projections to the external division of the globus pallidus, and (2) D1-expressing dynorphin/substance P-containing neurons which innervate the internal division of the globus pallidus (entopeduncular nucleus) and the SN pars reticulata [Anderson & Reiner, 1990; Beckstead & Kersey, 1985; Gerfen & Young, 1988; Gerfen et al, 1990; Le Moine et al, 1990; Sugimoto & Mizuno, 1987]. While haloperidol selectively increases \textit{c-fos} expression in striatopallidal enkephalinergic neurons
Robertson et al, 1992], at present, the peptidergic phenotype of neurons in the NAc and LSN that are targeted by haloperidol is not known. Similarly, the extent to which clozapine increases Fos immunoreactivity in enkephalinergic vs. dynorphinergic neurons in the NAc and LSN has not been investigated. To determine the peptidergic nature of neurons in different brain regions which show increased c-fos expression in response to clozapine or haloperidol, Fos immunohistochemistry was combined with in situ hybridization histochemistry using cDNA oligonucleotide probes for enkephalin or dynorphin.

2. Materials And Methods

a. Animals

Adult male Wistar rats (280-320 mg) were maintained on a 12 hr light/12 hr dark cycle, with free access to food and water. The rats were handled periodically for 4-5 days prior to the experiment.

b. Drug administration and brain section preparation

Clozapine (H. Lundbeck, Kobenhavn-Valby) and haloperidol (McNeil Pharmaceutical, Stouffville, Canada) were dissolved in 40 μl of 20% acetic acid and brought to final volume (1 ml) with 0.9% saline. Rats were injected subcutaneously with
clozapine (20 mg/kg) or haloperidol (1 mg/kg). Two hours after the injection, the rats were deeply anesthetized with pentobarbital and perfused with 0.9% saline (200 ml) followed by 4% PFA (150 ml) in 0.1 M PBS. The brains were removed immediately after perfusion and placed in fresh fixative (4% PFA) for at least 12 hours. Twenty or 25 μm sections were cut from each brain using a Vibratome. The sections were collected in autoclaved 0.02M PBS for immunostaining and in situ hybridization. In addition, 20-μm brain sections were cut from drug-free rats using a cryostat. The sections were mounted on diethyl pyrocarbonate (DEPC)-treated slides and stored at -20°C for in situ hybridization.

c. Fos- and Enk- immunostaining

For Fos-immunohistochemistry, all solutions were prepared with pyrogen-free, ultrapure water and autoclaved before using. Brain sections were processed at room temperature for Fos immunostaining as described above, using the avidin-biotin-complex (ABC) method [Guo et al, 1992a] under RNAase-free conditions. Free-floating sections were preincubated with 0.5% H₂O₂ for 15 min to remove endogenous peroxidase activity. After 3 washes in 0.02 M PBS, the sections were incubated with the primary antibody, sheep anti-Fos antibody (CRB OA-11-824; 1:3000 diluted) in 0.02 M PBS containing 0.3% Triton X-100 and 0.002% azide. Sections were then washed three times with 0.02 M PBS and incubated with a biotinylated rabbit anti-sheep secondary antibody (Vector Laboratories, Burlingame, CA; 1:200) containing 0.3% Triton X-100. Following 3 washes in 0.02 M PBS, the sections were incubated for 1 hr with 0.5% avidin-biotinylated horseradish peroxidase complex (ABC, Vector Laboratories, Burlingame, CA) in 0.3%
Triton X-100. After two washes in 0.02 M PBS the sections were rinsed in 0.2 M acetate buffer, pH 6.0. The sections were stained with 0.1% DAB in 0.02% H₂O₂, and then mounted on double chrome-alum-coated, DEPC-treated slides.

For enkephalin immunostaining, 5 adult male Wistar rats were injected with colchicine (Sigma, St Louis) bilaterally into the lateral ventricle [Kivipelto & Panula, 1986]. The coordinates were: AP +8.8 mm from the interaural line, ML ±1.4 mm from midline, DV +3.7 mm from the interaural line, with the incisor bar set 3.3 mm below the interaural plane. The drug (60 µg/side in 0.9% saline) was infused through 30-gauge cannulae at 1 µl/min for 6 min. Forty-eight hours after colchicine, the rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardially with 200 ml of 0.9% saline followed by 150 ml 4% PFA in 0.1 M PBS. Brains were removed immediately after perfusion and placed in fresh fixative (4% PFA) for at least 12 hr.

Thirty-µm sections were cut from each brain using a Vibratome. The free-floating sections were preincubated in 0.5% H₂O₂ in 0.02 M PBS for 15 min to remove endogenous peroxidase activity. After 3 washes in 0.02 M PBS, the sections were incubated with leu-Enk primary antibody (Incstar, Stillwater, MN) diluted 1:1500 in 0.02 M PBS containing 0.3% Triton X-100 for 48 hours at 4°C. Sections were then washed three times with 0.02 M PBS and incubated with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA; 1:200) in 0.3% Triton X-100 for 1 hr. Following 3 washes in 0.02 M PBS, the sections were incubated with 0.5% avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA; 1:200) containing 0.3% Triton X-100 for 1 hr. After two washes in 0.02 M PBS the sections were rinsed in 0.2 M acetate buffer, pH
6.0. The sections were then stained with DAB using the glucose oxidase-DAB-nickel
method [Shu et al, 1988] as described in chapter 2. After 3 washes in 0.2 M acetate buffer,
the sections were mounted on chrome alum-coated slides, dehydrated, and prepared for
microscopic observation.

d. Oligonucleotide probes

For D3 receptor mRNA in situ hybridization, two 35-mer oligonucleotides, derived
from the putative third cytoplasmic loop of rat D3 receptor, were synthesized (Bio-
technology Laboratory, University of British Columbia). The antisense oligonucleotide is
complementary to bases 901-935 of the rat D3 receptor [Sokoloff et al, 1990] and the sense
oligonucleotide is identical to the base sequence. The synthesized oligonucleotides were
purified by the butanol extraction method [Sawadogo & Van Dyke, 1991]. The
oligonucleotides dissolved in 100 µl of 30% NH₄OH were vortexed vigorously in a 1.5 ml
eppendorf tube with 1000 µl n-butanol (ACS reagent grade) for 20 sec, and then
centrifuged for 1 min at 12,000 rpm. The single H₂O-containing n-butanol phase was
discarded. The oligonucleotide pellets were redissolved in 100 µl H₂O and extracted with
1000 µl n-butanol as described above. Following n-butanol extraction, the pellets were
dried under vacuum and resuspended in H₂O before using. The Enk and Dyn oligo-
nucleotides [Young et al, 1986] were synthesized (Biotechnology Laboratory, University
of British Columbia) to be complementary to bases 382-421 and 870-909 respectively of
preproenkephalin A [Yoshikawa et al, 1984] and prodynorphin [Civelli et al, 1985]. These
oligonucleotides were purified with the butanol extraction method described above.
The oligonucleotides were tailed with $^{33}\text{P-dATP}$ (NEN Research Products, Boston, MA) or $^{35}\text{S-dATP}$ (NEN Research Products, Boston, MA) on the 3'-OH end by terminal deoxynucleotidyl transferase (TdT) (NEN Research Products, Boston, MA; Gibco BRL, Gaithersburg, MD) [Guo et al, 1996]. The reaction mixture contained 5 µl of 2 nmol/ml oligonucleotide, 7 µl of 5x TdT buffer (0.5 M potassium cacodylate, pH 7.2, 10 mM CoCl$_2$, 1 mM DTT), 5 µl of 12.5 mCi/ml $^{33}\text{P-dATP}$ or $^{35}\text{S-dATP}$, 15 µl H$_2$O, and 3 µl TdT. The reaction was carried out at 37°C for 30 min and terminated by adding 400 µl reagent A (NEN Research Products, Boston, MA) on ice. The labeled oligonucleotide probes were purified with NENSORB purification cartridges (NEN Research Products, Boston, MA).

e. In situ hybridization

For each hybridization experiment, brain sections from 5-6 drug-treated rats were used. Hybridizations were carried out as described by Lewis et al. [1988], with minor modifications [Guo et al, 1996a]. Sections were hybridized with one of the three probes described above ($5 \times 10^5$ - $1 \times 10^6$ cpm/section) at 37°C overnight in a buffer containing 5x SSPE (0.75 M NaCl, 0.05 M NaH$_2$PO$_4$, 0.0055 M EDTA, pH 7.4), 50% (vol/vol) deionized formamide, 10% (vol/vol) dextran sulfate, 1 x Denhardt's solution (0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), 0.25 mg/ml tRNA, and 0.2 mg/ml salmon sperm. The hybridization solution also contained 100 mM DTT (dithiothreitol) when a $^{35}\text{S-labeled}$ probe was used. After hybridization, the sections were washed at room temperature with 1 x SSC (saline sodium citrate buffer, 0.15 M NaCl, 0.015 sodium citrate, pH 7.2) for 15 min x 4, then with 0.5 x SSC (4 x 15 min), followed
by 4 x 15 min at 55-60°C with 0.5 x SSC. For 35S-labeled sections, washing solutions contained 14 mM β-mercaptoethanol. The sections were air dried and examined autoradiographically by apposition to X-ray film (X-Omat, Kodak). The exposures were 3-5 days for 33P- and 10-14 days for 35S-labeled slides. The slides were then dipped into Kodak NTB2 nuclear emulsion (1:1 diluted with 0.6 M ammonium acetate) at 42°C, and exposed for 12-16 days at -70°C (33P) or 3-5 weeks at room temperature (35S). After being developed in Kodak D-19 at 16°C for 4 min, the sections were fixed, dehydrated, and mounted for microscopic image analysis.

f. Data analysis

To determine whether clozapine- and haloperidol-induced Fos-positive neurons express D3 receptor, Enk, or Dyn mRNA, these in situ mRNA signals were digitized using image quantification analysis [Guo et al, 1996] with a MCID program (Imaging Research Inc., St. Catharines, Ontario). The density of in situ mRNA signal was measured as proportional grain area, i.e., grain area/scan area. For the major island of Calleja, in which cells are smaller than those in the striatal complex, a 91.9 μm² circle was used as the scanning area for each measured cell. For the other measured brain regions, scan area/cell = 153.8 μm². Circles with the same area were placed in the vicinity of Fos-positive neurons in different brain regions to measure the backgrounds (Fig. 13). In each brain region, the measured neurons were classified into two groups: neurons with a grain ratio (proportional grain area) at least two times higher than the mean background grain ratio were assigned to mRNA⁺/Fos⁺ (mRNA-positive/Fos-positive) group; Fos-positive neurons
Fig. 13. A. A dark-field digitized image of clozapine-induced Fos-positive neurons in the NAc after *in situ* hybridization with the $^{33}$P-labeled D3 antisense probe. Grain densities in individual neurons were measured within a certain area (red circles). Circles with the same area (green) were placed in the vicinity of Fos-positive neurons to measure the background. B. After scanning, the density of *in situ* mRNA signals in each circle was measured as proportional grain area, i.e., grain area (red)/scan area (blue).
which failed to meet this criterion were assigned to the Fos- (mRNA-negative/Fos-positive) group. Between-group differences in the mean grain ratio of the two groups of neurons and background within specified brain regions were evaluated by one way ANOVA. Newman-Keuls' post hoc test was performed to compare the density of in situ grains in Fos-positive neurons and background in each brain area.

To determine the distributions of Enk mRNA along the rostrocaudal axis in the striatal complex, the autoradiograms of Enk transcripts in different brain sections were digitized and analyzed with a MCID image program (Imaging Research Inc., St Catharines, Ontario) [Guo et al, 1996b]. The mean densities of enkephalin transcripts in each brain region, which were computed in 5 sections from 5 animals, were measured as relative optical densities (ROD).

3. Results

a. Distribution of D3 mRNA

The islands of Calleja were heavily labeled with the 33P-labeled D3 oligonucleotide antisense probe (Fig. 14A, B). At low magnification, a moderate density of D3 mRNA signal was also evident in the lateral septal nucleus (Fig. 14A, B) but not in the NAc (Fig. 14A). Labeling was also seen along the border between the third ventricle and lateral septum in Fig. 14A, but not in Fig. 14B. The former appears to be an artifact signal from residual, unwashed oligonucleotide probe bound between folded tissue along the ventricle. The 35S-labeled D3 antisense probe produced the same labeling pattern as the
Fig. 14. Pseudocolor coded dark-field digitized images of coronal sections showing the distributions of D3 receptor mRNA in rat brain by *in situ* hybridization. D3 receptor mRNA is expressed in the ICj, and, less abundantly, in the LSN (A, B). C. A brain section adjacent to A and B hybridized with the $^{33}$P-labeled D3 sense probe. The scale bar represents relative optical densities (ROD) of D3 receptor transcript levels in brain sections.
$^{33}$P-labeled probe. In adjacent sections, neither $^{33}$P- nor $^{35}$S-labeled D3 sense oligonucleotide probes resulted in significant signal in these brain regions (Fig. 14C).

(i) Clozapine-induced Fos colocalization.

At high magnification, the great majority of Fos-positive neurons in the ICjM met the criterion for positive labeling with the $^{33}$P-labeled D3 antisense probe (Fig. 15A), while the D3 sense probe only produced background labeling in the same structure (Fig. 15D). Similarly, some of the Fos-positive neurons in the NAc (Fig. 15B) and LSN (Fig. 15C) also showed significant labeling with the D3 antisense probe. This was confirmed by quantitative analysis of the autoradiographic data wherein the criterion for positive labeling was set as the grain ratio being at least twice as high as background (see Materials and Methods). As shown in Table 3, the grain ratio in Fos-positive neurons is significantly different from that of the background: the average density of D3 antisense labeling in clozapine-induced Fos-positive neurons which met this criterion was about 4 times greater than that of either background or Fos-positive neurons that failed to meet the D3 positive criterion ($D3^+$/Fos$^+$ grain ratios: 0.101 - 0.114; background grain ratios: 0.022 - 0.025; Fos$^+$ grain ratios: 0.027 - 0.030). This 3-4 fold difference in grain density between double labeled and single labeled or background measurements was seen in all of the experiments (Tables 3 - 8) and serves to confirm the validity of this quantitative approach. Table 3 shows that nearly all (94.5%) of the clozapine-induced Fos-positive neurons in the ICjM express D3 mRNA. Similarly, despite the lack of significant overall D3 labeling in the NAc at low magnification, the majority (69%) of Fos-positive neurons in this structure also expressed above background levels of D3 mRNA (Table 3). In the lateral septal nucleus,
Fig. 15. Dark-field views of rat brain sections after *in situ* hybridization with $^{33}$P-labeled D3 probes. In rats treated with clozapine (A-D), most Fos-positive neurons in the ICjM (A), and many in the NAc (B) and LSN (C) were labeled by the D3 antisense probe, while the D3 sense probe only produced few autoradiographic grains in the ICjM (D). In contrast, most haloperidol-induced Fos-positive neurons in the NAc (E) and LSN (F) did not contain D3 receptor mRNA.
Table 3. Proportion of clozapine-induced Fos-positive neurons that express D3 receptor mRNA in the forebrain.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Prefrontal Cortex</th>
<th>Nucleus Accumbens</th>
<th>Lateral Septum</th>
<th>Major Island of Calleja</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of neurons</td>
<td>Grain ratio&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>Number of neurons</td>
<td>Grain ratio&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
<td>0.101±0.005**</td>
<td>312</td>
<td>0.111±0.005**</td>
</tr>
<tr>
<td>D3&lt;sup&gt;+&lt;/sup&gt;/Fos&lt;sup&gt;+&lt;/sup&gt;</td>
<td>36</td>
<td>0.101±0.005**</td>
<td>216</td>
<td>0.111±0.005**</td>
</tr>
<tr>
<td>Fos&lt;sup&gt;+&lt;/sup&gt;</td>
<td>91</td>
<td>0.029±0.002</td>
<td>96</td>
<td>0.030±0.003</td>
</tr>
<tr>
<td>Background</td>
<td>30</td>
<td>0.025±0.002</td>
<td>60</td>
<td>0.025±0.002</td>
</tr>
<tr>
<td>Colocalized neurons (%)</td>
<td>28.3</td>
<td>69.2</td>
<td>72.9</td>
<td>94.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Grain ratio: proportional grain area, i.e. grain area/scan area. For the major island of Calleja, scan area/cell = 91.9 µm<sup>2</sup>. For the other brain regions, scan area/cell or background area = 153.8 µm<sup>2</sup>.

<sup>b</sup> Data represent the mean (± S.E.M.) proportional grain area of Fos-positive neurons or background areas.

<sup>c</sup> D3<sup>+</sup>: D3 receptor mRNA-positive; Fos<sup>+</sup>: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.

<sup>d</sup> Neurons with grain ratios less than two times the mean proportional grain area of the background were assigned to the Fos<sup>+</sup> group.

** Significantly different from background area and Fos<sup>+</sup> neurons ( P<0.001 ).
the majority (73%) of clozapine-induced Fos-positive neurons were also positive for D3 message. Also, a small population of the Fos-positive neurons in the PFC (28%) were labeled by the D3 antisense probe.

(ii) Haloperidol-induced Fos colocalization.

In contrast to clozapine, haloperidol-induced Fos positive neurons rarely colocalized with D3 message in the NAc (Fig. 15E), LSN (Fig.15F) or dorsal striatum. This was confirmed by the quantitative analysis (Table 4), which showed that only a small minority of haloperidol-induced Fos-positive neurons met the criterion for being D3 positive in the striatum (4%), NAc (9%) or LSN (6%).

In agreement with previous studies of D3 receptor mRNA distribution [Sokoloff et al, 1990; Bouthenet et al, 1991; Landwehrmeyer et al, 1993b; Diaz et al, 1995], the present study shows that D3 receptor mRNA was expressed in the ICj, NAc and LSN. Pharmacological studies have demonstrated that clozapine-induced Fos immunoreactivity in the ICjM, NAc, and LSN was blocked by D3 receptor agonists, suggesting that clozapine’s effects in these brain regions are due to its antagonist actions at D3 receptors [Guo et al, 1996]. In contrast, most haloperidol-induced Fos-positive neurons in the striatum, NAc, and LSN are not colocalized with D3 receptor mRNA, suggesting that haloperidol-induced Fos induction in different brain regions is not mediated by D3 receptors. This may also explain the observation that the D3 receptor agonist 7-OHDPAT and quinpirole, which has higher affinity for the D3 receptor than D2 receptors, did not block haloperidol-induced Fos induction in the forebrain.
Table 4. Proportion of haloperidol-induced Fos-positive neurons that express D3 receptor mRNA in the forebrain.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Striatum</th>
<th>Nucleus Accumbens</th>
<th>Lateral Septum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of neurons</td>
<td>Grain ratio&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>Number of neurons</td>
</tr>
<tr>
<td>Total</td>
<td>215</td>
<td>0.088±0.011**</td>
<td>220</td>
</tr>
<tr>
<td>D3&lt;sup&gt;c&lt;/sup&gt;/Fos&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9</td>
<td>0.024±0.002</td>
<td>19</td>
</tr>
<tr>
<td>Fos&lt;sup&gt;d&lt;/sup&gt;</td>
<td>206</td>
<td>0.022±0.002</td>
<td>201</td>
</tr>
<tr>
<td>Background</td>
<td>60</td>
<td>4.2</td>
<td>60</td>
</tr>
</tbody>
</table>

<sup>a</sup> Grain ratio: proportional grain area, i.e. grain area/scan area. For all measured brain regions, scan area/cell or background area = 153.8 \( \mu m^2 \).

<sup>b</sup> Data represent the mean (± S.E.M.) proportion grain area of Fos-positive neurons or background areas.

<sup>c</sup> D3*: D3 receptor mRNA-positive; Fos*: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.

<sup>d</sup> Neurons with grain ratios less than two times the mean proportional grain area of the background were assigned to in Fos* group.

** Significantly different from background area and Fos* neurons (P<0.001).
b. Distribution of enkephalin mRNA

In agreement with previous studies, Enk mRNA was prominent in the caudate-putamen, NAc, and olfactory tubercle [Young et al, 1986] (Fig. 16). A moderate density of Enk mRNA signal was also observed in the LSN (Fig. 16B-D) and the ventral limb of the nucleus of the diagonal band (VDB; Fig.16D). Also, rostrocaudal differences in Enk mRNA expression were observed in the striatum (Fig. 16A-D). Quantitative analysis of the autoradiographic densities demonstrated that Enk mRNA was uniformly expressed in the dorsal striatum along the rostrocaudal axis, while caudally it was less abundant in the ventral striatum, i.e. the NAc shell and the olfactory tubercle (Fig. 17). At high magnification, a large number of Enk-expressing cells were identified in the LSN (Fig. 18A), VDB, and medial septal nucleus, while most granule cells in the ICj were not labeled by the oligonucleotide probe (Fig. 18C). Most Enk mRNA-expressing neurons in these brain regions are medium-sized or large cells with less density of Enk transcript levels in individual neurons than those in the striatum (Fig. 18B), while small-sized cells were not labeled by the Enk probe (Fig. 18A). In agreement with the in situ hybridization histochemical study, immunocytochemistry demonstrated that leucine (Leu)-Enk-like immunoreactive neurons were distributed in all three (dorsal, intermediate, and ventral) subdivisions of the LSN (Fig. 19). This is consistent with a previous observation that methionine (Met)-Enk-like immunoreactive cell bodies are present in the LSN [Kivipelto & Panula, 1986]. These data, together with the finding that neurons in the LSN express Enk mRNA, suggest that PEnk-derived peptides play a role in neurotransmission in the LSN.
Fig. 16. Pseudocolor coded dark-field digitized images of coronal serial sections showing the distribution of enkephalin (Enk) mRNA expression in rat brain by *in situ* hybridization histochemistry. Scale bar represents relative optical densities (ROD) of Enk transcript levels in brain sections. Enk mRNA is most abundant in the striatum, NAc, and olfactory tubercle (A-D), while less abundant in the LSN (B-D). In the ventral striatum, Enk mRNA is not uniformly expressed along the rostrocaudal axis (A-D).
Fig. 17. A. Distribution of Enk mRNA along the rostrocaudal axis (Bregma 2.7 mm - 0.2 mm, Paxinos & Watson, 1986) in the striatal complex. Data are presented as mean ± S.E.M. density of Enk transcripts ($n = 5$). Enk mRNA is almost uniformly expressed in the dorsal striatum along the rostrocaudal axis, while being caudally less abundant in the NAc shell. B. Digitized image of a coronal section through the LSN (Bregma 0.7 mm, Paxinos & Watson, 1986) hybridized with the $^{35}$S-labeled Enk probe. Boxed areas indicate the measurements carried in (A) of Enk transcript densities in the striatum, NAc and LSN.
Fig. 18. Bright-field views of rat brain sections after in situ hybridization with $^{35}$S-labeled Enk probe. A. many medium-sized cells in the lateral septal nucleus (LSN) express Enk mRNA (solid arrows) with less density of Enk transcript levels in each neuron than that in the striatum (B), while most small-sized cells in the LSN (open arrows) do not contain Enk mRNA. C. Most granule cells in the islands of Calleja are not labeled by the Enk oligonucleotide probe. Bar = 16 μm.
Fig. 19. Enk-like immunoreactive neurons in the septal area of the rat. A. Low-power photomicrograph of the septal area showing the presence of Enk-positive neurons throughout the dorsal, intermediate and ventral divisions of the LSN, as well as in the medial septum and the ventral diagonal band. Bar = 100 μm. B. Details of (A) showing the strongly immunostained Enk-positive neurons and fibers in the ventral subdivision of the LSN. Bar = 25 μm.
(i) Clozapine-induced Fos colocalization.

In contrast to D3 mRNA, only a small minority (6%) of clozapine-induced Fos-positive neurons in the ICjM were labeled with the Enk oligonucleotide probe (Fig. 20C; Table 5). In contrast, a significant percentage of Fos positive neurons in the NAc (40%) and LSN (32%) met the criteria for being enkephalinergic (Fig. 20A, B). However, the vast majority (90%) of Fos-positive neurons in the mPFC of clozapine treated animals failed to meet this criterion and were therefore classified as non-enkephalinergic.

(ii) Haloperidol-induced Fos colocalization.

In the striatum, the vast majority (93%) of haloperidol-induced Fos-positive neurons were labeled with the Enk oligonucleotide probe (Table 6; Fig. 21A). In the NAc (Fig. 21B) and lateral septal nucleus (Fig. 21C), the number of Fos positive neurons meeting the criterion for being enkephalinergic was much smaller, being 46% in the NAc and 29% in the LSN (Table 6).

c. Distribution of Dynorphin mRNA

In accordance with previous reports [Young et al, 1986; Gerfen et al, 1988], the distribution of dynorphin mRNA was similar to that of enkephalin except that dynorphin transcripts were also found in the cerebral cortex and higher levels were observed in the ventral striatum than in the dorsal striatum.
Table 5. Proportion of clozapine-induced Fos-positive neurons that express enkephalin mRNA in the forebrain.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Prefrontal Cortex</th>
<th>Nucleus Accumbens</th>
<th>Lateral Septum</th>
<th>Major Island of Calleja</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of neurons</td>
<td>Number of neurons</td>
<td>Number of neurons</td>
<td>Number of neurons</td>
</tr>
<tr>
<td>Total</td>
<td>129</td>
<td>304</td>
<td>284</td>
<td>128</td>
</tr>
<tr>
<td>Enk&lt;sup&gt;c&lt;/sup&gt;/Fos&lt;sup&gt;d&lt;/sup&gt;</td>
<td>13</td>
<td>122</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>Fos&lt;sup&gt;d&lt;/sup&gt;</td>
<td>116</td>
<td>182</td>
<td>192</td>
<td>120</td>
</tr>
<tr>
<td>Background</td>
<td>30</td>
<td>55</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td>Colocalized neurons (%)</td>
<td>10.1</td>
<td>40.1</td>
<td>32.4</td>
<td>6.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Grain ratio: proportional grain area, i.e. grain area/scan area. For the major island of Calleja, scan area/cell = 91.9 µm<sup>2</sup>. For the other brain regions, scan area/cell or background area = 153.8 µm<sup>2</sup>.

<sup>b</sup> Data represent the mean (± S.E.M.) proportional grain area of Fos-positive neurons or background areas.

<sup>c</sup> Enk<sup>c</sup>: enkephalin mRNA-positive, Fos<sup>d</sup>: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.

<sup>d</sup> Neurons with grain ratios less than two times the mean proportional grain area of the background were assigned to Fos<sup>d</sup> group.

** Significantly different from background area and Fos<sup>d</sup> neurons (P<0.001).
Fig. 20. Bright-field views of brain sections after in situ hybridization with $^{35}$S-labeled Enk (A-C) or dynorphin (Dyn) probes. Many clozapine-induced Fos-positive neurons in the NAc (A, D) and LSN (B, E) express either Enk or Dyn mRNA. In the ICj, most clozapine-induced Fos-positive neurons do not express Enk mRNA (C), while many contain Dyn mRNA (F).
Table 6. Proportion of haloperidol-induced Fos-positive neurons that express enkephalin mRNA in the forebrain.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Striatum</th>
<th>Nucleus Accumbens</th>
<th>Lateral Septum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of neurons</td>
<td>Grain ratio&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>Number of neurons</td>
</tr>
<tr>
<td>Total</td>
<td>284</td>
<td>256</td>
<td>201</td>
</tr>
<tr>
<td>Enk&lt;sup&gt;+&lt;/sup&gt;/Fos&lt;sup&gt;+&lt;/sup&gt;</td>
<td>263</td>
<td>0.180±0.009**</td>
<td>118</td>
</tr>
<tr>
<td>Fos&lt;sup&gt;+&lt;/sup&gt;</td>
<td>24</td>
<td>0.050±0.003</td>
<td>138</td>
</tr>
<tr>
<td>Background</td>
<td>60</td>
<td>0.036±0.003</td>
<td>50</td>
</tr>
<tr>
<td>Colocalized neurons (%)</td>
<td>92.6</td>
<td></td>
<td>46.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Grain ratio: proportional grain area, i.e. grain area/scan area. For all measured brain regions, scan area/cell or background area = 153.8 μm<sup>2</sup>.

<sup>b</sup> Data represent the mean (+ S.E.M.) proportional grain area of Fos-positive neurons or background areas.

<sup>c</sup> Enk<sup>+</sup>: enkephalin mRNA-positive; Fos<sup>+</sup>: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.

<sup>d</sup> Neurons with grain ratios less than two times of the mean proportional grain area of the background were assigned to Fos<sup>+</sup> group.

** Significantly different from background area and Fos-positive neurons ( P<0.001 ).
Fig. 21. Bright-field views of brain sections from haloperidol-treated rats after \textit{in situ} hybridization with the Enk (A-C) or Dyn (D-F) probe. Most haloperidol-induced Fos-positive neurons in the striatum (A), and fewer in the NAc (B) and LSN (C) express Enk mRNA. Some haloperidol-induced Fos-positive neurons in the striatum (D), NAc (E) and LSN (F) contain Dyn mRNA.
(i) Clozapine-induced Fos colocalization.

The majority (61%) of Fos-positive neurons in the major island of Calleja also met the criterion for being dynorphinergic (Table 7; Fig. 20F). In addition, more than half of the clozapine-induced Fos positive neurons in the nucleus accumbens (53%) and lateral septal nucleus (59%) met this criterion (Table 7; Figs. 20D, E). In the medial prefrontal cortex about one-fifth (19%) of the Fos-positive neurons showed significant labeling with the $^{35}$S-labeled dynorphin probe.

(ii) Haloperidol-induced Fos colocalization.

A small population of haloperidol-induced Fos-positive neurons in the striatum and the lateral septum were labeled with the $^{35}$S-labeled Dyn probe: about one-fifth of the Fos-positive neurons in the striatum (20%) and lateral septal nucleus (18%) showed significant labeling for dynorphin mRNA (Table 8; Figs. 21D and 21F). The percentage of haloperidol-induced Fos-positive neurons that also met the criterion for being dynorphinergic was higher, reaching 38% (Table 8, Fig. 21E).

4. Discussion

a. D3 receptor mRNA distribution in antipsychotic-targeted neurons

In agreement with previous studies of D3 receptor mRNA distribution, the present study shows that D3 mRNA is expressed in the ICj, NAc, LSN and PFC [Bouthenet et al, 1991; Diaz et al, 1995; Giros et al, 1991; Landwehrmeyer et al, 1993b; Meador-Woodruff
Table 7. Proportion of clozapine-induced Fos-positive neurons that express dynorphin mRNA in the forebrain.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Prefrontal Cortex</th>
<th>Nucleus Accumbens</th>
<th>Lateral Septum</th>
<th>Major Island of Calleja</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of neurons</td>
<td>Grain ratio*</td>
<td>Number of neurons</td>
<td>Grain ratio*</td>
</tr>
<tr>
<td>Total</td>
<td>149</td>
<td>0.092±0.004**</td>
<td>298</td>
<td>0.129±0.006**</td>
</tr>
<tr>
<td>Dyn+/Fos+</td>
<td>29</td>
<td>0.092±0.004**</td>
<td>158</td>
<td>0.129±0.006**</td>
</tr>
<tr>
<td>Fos+</td>
<td>120</td>
<td>0.023±0.002</td>
<td>140</td>
<td>0.040±0.003</td>
</tr>
<tr>
<td>Background</td>
<td>30</td>
<td>0.021±0.002</td>
<td>50</td>
<td>0.037±0.003</td>
</tr>
<tr>
<td>Colocalized neurons (%)</td>
<td>19.4</td>
<td>53.0</td>
<td>58.7</td>
<td>61.2</td>
</tr>
</tbody>
</table>

* Grain ratio: proportional grain area, i.e. grain area/scan area. For the major island of Calleja, scan area/cell = 91.9 µm². For the other brain regions, scan area/cell or background area = 153.8 µm².

b Data represent the mean (± S.E.M.) proportional grain area of Fos-positive neurons or background areas.

c Dyn+: dynorphin mRNA-positive; Fos+: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.

d Neurons with grain ratios less than two times of the mean proportional grain area of the background were assigned to Fos+ group.

** Significantly different from background area and Fos+ neurons (P<0.001).
Table 8. Proportion of haloperidol-induced Fos-positive neurons that express dynorphin mRNA in the forebrain.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Striatum</th>
<th></th>
<th>Nucleus Accumbens</th>
<th></th>
<th>Lateral Septum</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of neurons</td>
<td>Grain ratio&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>Number of neurons</td>
<td>Grain ratio&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>Number of neurons</td>
<td>Grain ratio&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>315</td>
<td>456</td>
<td>246</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dyn&lt;sup&gt;c&lt;/sup&gt;/Fos&lt;sup&gt;c&lt;/sup&gt;</td>
<td>64</td>
<td>0.123±0.005**</td>
<td>175</td>
<td>0.132±0.008**</td>
<td>44</td>
<td>0.112±0.008**</td>
</tr>
<tr>
<td>Fos&lt;sup&gt;d&lt;/sup&gt;</td>
<td>251</td>
<td>0.026±0.002</td>
<td>281</td>
<td>0.034±0.002</td>
<td>202</td>
<td>0.028±0.002</td>
</tr>
<tr>
<td>Background</td>
<td>60</td>
<td>0.020±0.002</td>
<td>60</td>
<td>0.028±0.002</td>
<td>45</td>
<td>0.027±0.002</td>
</tr>
<tr>
<td>Colocalized neurons (%)</td>
<td>20.3</td>
<td>38.4</td>
<td>17.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Grain ratio: proportional grain area, i.e. grain area/scan area. For all measured brain regions, scan area/cell or background area = 153.8 µm².

<sup>b</sup> Data represent the mean (+ S.E.M.) proportional grain area of Fos-positive neurons or background areas.

<sup>c</sup> Dyn*: dynorphin mRNA-positive; Fos*: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.

<sup>d</sup> Neurons with grain ratios less than two times the mean proportional grain area of the background were assigned to Fos* group.

** Significantly different from background area and Fos* neurons ( P<0.001 ).
et al, 1996; Sokoloff et al, 1990]. The present results also confirm that most granule cells in the ICj express D3 receptor mRNA in high abundance (Fig. 15A), and indicate that a small population of neurons (about 30% of clozapine-induced Fos-positive neurons) in the PFC contain D3 receptor transcripts (Table 3). This is consistent with the report that both D3 mRNA and its shorter variant transcripts exist in the PFC [Giros et al, 1991]. Using antibodies against peptides of D3 receptors, Ariano and Sibley [1994] found D3-like immunoreactive neurons in the PFC. It is worth noting that there are differences of opinion regarding the abundance and the precise localization of D3 receptors as reflected by in situ hybridization. For example, in contrast to earlier studies, the oligonucleotide probes used in the present experiments did not indicate above-background levels of D3 mRNA in the NAc when the sections were viewed under low magnification (Fig. 14A and B). This difference is probably due to the use of $^{32}$P-labeled ribonucleic acid probes of much higher specific activity [Bouthenet et al, 1991; Diaz et al, 1995] than the oligonucleotide probes used here. Despite the absence of obvious labeling in the NAc at low magnification, at magnification above 400x, it was clear that significant numbers of neurons did contain above-background levels of D3 mRNA (Fig. 15B; Table 3), in accordance with earlier reports. Diaz and co-workers [1995] reported that D3 receptors were "almost exclusively expressed" in the ventromedial shell of the NAc and were "less widely expressed" in the core and the septal pole ("cone") of this nucleus. However, examination of relevant figures in Bouthenet et al. [1991, (Fig. 7C)] and in Landwehrmeyer et al. [1993b, (Fig. 1D)] indicate that D3 receptors are expressed in the septal pole. This is consistent with the finding that clozapine-induced c-fos expression is prominent in this region of the NAc [Robertson & Fibiger, 1992, (Figs. 4 and 5)] and that clozapine
increases Fos-like immunoreactivity in the majority of D3-expressing neurons in this nucleus (see below). The present experiments also indicated moderate levels of D3 mRNA expression in the caudal aspect of the LSN (Fig. 14A, B), an observation that has not been reported in previous in situ hybridization studies [Bouthenet et al, 1991; Diaz et al, 1995; Landwehrmeyer et al, 1993b], although PCR analysis has shown that a low level of D3 mRNA is expressed in the septum [Sokoloff et al, 1990]. The reasons for this discrepancy are not obvious. It is perhaps relevant, however, that labeling in the present study was observed primarily in the caudal half of the LSN and may for this reason have escaped detection in earlier studies. The presence of D3 mRNA in the LSN was confirmed when the brain sections were examined under higher power magnification as many neurons in this structure showed above-background labeling with the D3 probe (Fig. 13C; Table 3). Further studies on the precise distribution of D3 receptors are necessary, and immunohistochemical studies using antibodies against D3 receptor proteins may be helpful in this regard.

In the present experiments, nearly all (95%) of the granule cells in the ICjM in which clozapine increased Fos-like immunoreactivity expressed significant levels of D3 mRNA (Fig. 15A; Table 3). This would be expected inasmuch as this structure contains among the highest levels of D3 message in the CNS. The present experiments also show that the majority of clozapine-induced Fos-positive neurons in the NAc (69%) and LSN (73%) contain D3 receptor mRNA (Table 3). Pharmacological studies presented in Chapter II demonstrated that clozapine-induced c-fos expression in the limbic brain regions is blocked by D3 receptor agonists, suggesting that clozapine’s effects in the ICjM, NAc and LSN are due to its antagonist actions at D3 receptors. The co-localization of D3
receptor mRNA and Fos protein in limbic brain neurons following clozapine administration (20 mg/kg) is consistent with this hypothesis. In contrast, haloperidol (1 mg/kg) failed to increase c-fos expression in the ICjM, suggesting that haloperidol does not act on D3 receptors to affect c-fos. Haloperidol has an 18-fold higher affinity for D3 receptors than does clozapine [Sokoloff et al, 1990]. On a molar basis, at the doses used the animals received 23 times as much clozapine (6.1 x 10^5 moles) as haloperidol (2.7 x 10^7 moles). According to this calculation, haloperidol would have only slightly less activity (22 %) at D3 receptors than would clozapine and should have produced some increase in c-fos expression in D3-containing neurons. It did not ( Table 4). A number of possibilities may contribute to such a discrepancy. First, calculations based on in vitro receptor binding affinities ignore the possible presence of active metabolites of clozapine and haloperidol with different affinities for D2 and D3 receptors. Second, there may be different thresholds with respect to D2 and D3 receptor-mediated c-fos expression. Since haloperidol has 124-fold higher affinity for D2 receptors than does clozapine [Sokoloff et al, 1990], at the dose used haloperidol would be about 5 times as potent at D2 receptors than would clozapine, but would have only 78% of the activity of clozapine at D3 receptors. Third, haloperidol itself has 20-fold higher affinity for D2 than for D3 receptors, thus perhaps accounting for the preferential action of haloperidol on D2 receptors and the failure of haloperidol to increase c-fos expression in D3 receptor-expressing neurons. Finally, interactions between different DA receptor subtypes are probably involved in regulating c-fos expression in individual neurons. For example, co-administration of quinpirole and SKF38393 significantly enhances IEG expression in the DA-depleted striatum compared to that induced by SKF38393 alone [Keefe & Gerfen, 1995; Gerfen et
al, 1995]; this D1-D2 synergistic effect was attenuated by either D1-antagonist SCH23390 or D2-antagonist eficlopride. These data suggest that a D1-D2 interaction is involved in regulating IEG induction in DA-depleted striatum. As discussed later, the present experiments suggest that clozapine enhances c-fos induction in D3-expressing neurons which may co-express either D1 or D2 receptors, while haloperidol induces c-fos expression in D2-containing or D1/D2 co-containing neurons. This raises the possibility that different patterns of D3, D2 and D1 receptor-interaction, which may be related to different affinities of clozapine and haloperidol for these DA receptor subtypes, may be involved in the induction of Fos produced by these drugs. Therefore, the different effects of clozapine and haloperidol may not be due to the interactions of these drugs with an individual DA receptor subtype; rather, the different ratios of D3 to D1 and/or D2 receptor blockade of these drugs may permit clozapine but not haloperidol to induce c-fos expression in D3-expressing neurons.

The identification of three distinct D2-like subtypes (i.e. D2, D3 and D4 receptors) raised the possibility that these different subtypes may be responsible for the different effects of antipsychotic drugs, including both therapeutic effects and unwanted side effects. Due to the complexity of the DA system it has been difficult to establish the role(s) of each receptor subtype in vivo. D2 receptors are ubiquitously expressed in all major DA-input brain areas. The abundant expression of D2 receptors in the striatum, which controls motor function, suggests that the activity of striatal D2 receptors may mediate the EPS of neuroleptics. The selective expression of D3 receptors in limbic brain regions, which are known to be involved in the control of cognitive, emotional and reward processes, has led to speculation that D3 receptors may be involved in the therapeutic effect of antipsychotics
The present experiments indicate that D3 receptors mediate clozapine- but not haloperidol-induced c-fos expression in the forebrain and that D2 receptor mechanisms are involved in the effects of haloperidol (as discussed later). These findings are consistent with the view that different receptor mechanisms mediate the effects of clozapine and haloperidol. The present experiments also suggest that by differentially blocking D3 or D2 receptors, clozapine and haloperidol produce different patterns of neuronal activity which may be related to the different clinical profiles of these drugs.

b. Phenotype of clozapine-induced Fos-positive neurons

In the ICjM, clozapine-induced Fos-positive neurons are colocalized with Dyn mRNA (60%), but not Enk mRNA (Fig. 20F, C; Tables 5 and 7). Previous studies have shown that neurons in the ICj contain SP [Beckstead & Kersey, 1985; Gerfen & Young, 1988; Harlan et al, 1989]. It has also been demonstrated that neurons in the ICj express D1 [Fremeau, Jr. et al, 1991; Mengod et al, 1991], but not D2 receptors [Landwehrmeyer et al, 1993b]. Since most granule cells in the ICjM express D3 receptor mRNA (Table 3), at least some of these cells probably co-express D1 and D3 receptors and utilize Dyn/SP as neurotransmitters.

In the ICj, granule cells appear as undifferentiated neurons with a poorly developed dendritic tree and a short unramified axon that does not leave the island [Meyer et al, 1989]. The ICj receives DA, GABA, Enk, CCK and LHRH inputs [Fallon et al, 1983]. Efferents of the ICC seem to arise from medium- and large-sized cell populations [Fallon, 1983; Meyer et al, 1989]. In addition to SP and Dyn, the ICj may also use nitric oxide (NO) as neurotransmitter. For instance, NADPH-diaphorase histochemistry for NO
synthase (NOS) has revealed intense staining of the ICj [Vincent & Kimura]. Further, high NOS mRNA expression has been found in this structure [Sugaya & McKinney, 1994]. Therefore, it is likely that clozapine may also induce c-fos expression in NO neurons in the ICj. While the physiological significance of the ICj remains unknown, the reciprocal connections between the ICj and other brain areas including septum, NAc, ventral pallidum, SN, VTA, amygdala, piriform cortex, hypothalamus, etc., suggest that clozapine-induced neuronal activity in the ICj may be involved in the regulation of limbic- and motor-related functions.

In the NAc, clozapine-induced Fos-positive neurons include Enk- (40%) and Dyn-expressing (53%) neurons (Fig. 20A, D; Tables 5 and 7). Since the majority of the Fos-positive neurons in the NAc express D3 receptor mRNA (Table 3), the present findings are consistent with the recent report that D3 receptors in the NAc are mainly expressed in medium spiny neurons [Diaz et al, 1995]. Because the majority of Enk neurons co-express NT [Sugimoto & Mizuno, 1987], the present experiment is in agreement with the report that about 60% of D3 mRNA-expressing neurons in the ventromedial shell are NT neurons [Diaz et al, 1995]. Also, the present finding that some of the Fos-positive neurons are dynorphinergic is consistent with the observation that more than half of D3-expressing neurons in the NAc are retrogradely-labeled accumbonigral neurons (Ariano, personal communication; Ariano & Sibley, 1994).

As discussed earlier, striatal medium spiny neurons contain two subtypes: (1) striatopallidal enkephalinergic/neurotensinergic-neurons which express D2 receptors and (2) striatonigral Dyn/SP-containing neurons which express D1 receptors. Neurons in the NAc project to ventral pallidum and regions in the midbrain occupied by mesencephalic
DA neurons [Zahm & Heimer, 1990; Zahm & Brog, 1992; Nauta et al, 1978; Nauta & Domesick, 1984]. Since the cytoarchitecture, subcortical connections and neurotransmitters of the NAc have much in common with other parts of the striatum [Heimer, 1978; Heimer & Wilson, 1975; Beckstead & Kersey, 1985; Walaas, 1981; Young et al, 1986; Gerfen & Young, 1988; Gerfen et al, 1990; Le Moine et al, 1990; Meredith et al, 1992; Zahm, 1992; Zahm & Heimer, 1990], it is possible that medium spiny neurons in the NAc may similarly be classified into two subgroups, i.e. accumbopallidal neurons which co-express Enk/NT and D2 receptors and accumbomesencephalic (i.e. accumboven- tral tegmental and accumbonigral) neurons which contain Dyn/SP and D1 receptors. However, some evidence suggests that D1, D2 and D3 receptors might be co-expressed in different combinations in different populations of medium spiny neurons in the NAc. For instance, the majority of D3 receptor-expressing neurons in the NAc are NT neurons which have been found to co-express Enk and D2 receptors [Diaz et al, 1995; Le Moine et al, 1990; Sugimoto & Mizuno, 1987]. Likewise, the present findings show that some of the Enk-expressing neurons in the NAc contain D3 receptor mRNA. These observations suggest that at least some D3-containing neurons in this structure may co-express D2 receptors. On the other hand, recent studies have demonstrated that 80% of retrogradely-labeled striatonigral/accumbonigral neurons express D1-like receptors, while 65% express D3-like receptors [Ariano & Sibley, 1994; Ariano, unpublished observations]. This suggests that overlap may occur in the expression of D1 and D3 receptors in this population of medium spiny neurons. In support of this hypothesis, the present experiments demonstrate that some dynorphinergic neurons in the NAc express D3 receptors. Taken together, these findings suggest that D1 and D3 receptors are co-
expressed in some accumbomesencephalic neurons. This raises the possibility that clozapine increases \textit{c-fos} expression in both D3/D2- and D3/D1-coexpressing neurons in the NAc, while haloperidol induces \textit{c-fos} expression in different populations of accumbal neurons (see discussion below). This may provide a cellular mechanism to support the observation that clozapine and haloperidol produce distinct patterns of Fos-like immunoreactivity in the NAc [Robertson & Fibiger, 1992]. The definition of accumbal neurons which express either a single DA receptor subtype or co-express different subtypes remains to be determined.

The NAc forms part of the anterior cingulate circuit which is involved in integrating functions in emotional behavior and memory [Alexander et al, 1986]. The present findings suggest that clozapine may regulate the function of the limbic circuit as reflected by increased \textit{c-fos} expression in accumbopallidal Enk/NT neurons. In addition, the NAc also sends a prominent output to the ventral tegmentum [Nauta et al, 1978] and forms symmetric synaptic contacts on nigrostriatal neurons [Somogyi et al, 1981]. This monosynaptic pathway may provide a way in which information passing through networks in the limbic striatum could influence the dopaminergic modulation of information passing through networks in the more dorsal, sensorimotor striatum. By targeting both Enk/NT and Dyn/SP neurons in the NAc, clozapine may produce balanced opposition of accumbopallidal and accumbomesencephalic outputs. This may provide a mechanism for modulating brain functions concerned with both motivation and motor behaviour.

In the LSN, clozapine-induced Fos-positive neurons are colocalized with either Enk (32%) or Dyn (59%) mRNA (Fig. 20B, E; Tables 5 and 7). This is consistent with previous \textit{in situ} and immunohistochemical studies which showed that neurons in the LSN
are enkephalinergic [Kivipelto & Panula, 1986] and dynorphinergic [Young et al, 1986; Neal & Newman, 1989]. The LSN, as well as the other part of the septal area, has long been considered an important component of the limbic system. Anatomical studies have demonstrated that the LSN is a major recipient of hippocampal efferents [McLardy, 1955a; 1955b; Nauta, 1956; Defrance et al, 1971; Siegel et al, 1975; Ino et al, 1987]; it in turn sends projections to the cingulate and infralimbic cortices, ventral pallidum, olfactory tubercle, NAc and hippocampus [Staiger & Nurnberger, 1991]. Various experimental approaches have provided evidence which supports an important role of the LSN in emotion [Thomas, 1988; Yadin et al, 1993]. Although it is not known which brain structures are innervated by Enk and Dyn neurons in the LSN, it is likely, as viewed above, that modified neuronal activities in these neurons produced by clozapine influence limbic system functions.

**c. Phenotype of haloperidol-induced Fos-positive neurons**

A previous study suggested that most haloperidol-induced Fos immunoreactive striatal neurons are enkephalinergic [Robertson et al, 1992]. Consistent with this hypothesis, the present experiments demonstrate that 93% of haloperidol-induced Fos-positive neurons in the striatum express Enk mRNA (Fig. 21A; Table 7), while a small portion of haloperidol-induced Fos-positive neurons (20%) in the striatum contain Dyn mRNA (Fig. 21D; Table 8). Since 15-20% of striatal neurons have been reported to contain both SP and Enk and to co-express D1 and D2 receptors [Penny et al, 1986; Gerfen & Young, 1988; Gerfen, 1992], these striatal Dyn neurons, in which haloperidol increases Fos immunoreactivity, may belong to the population of neurons which co-express Enk and
Dyn/SP. Haloperidol increases Fos immunoreactivity in retrogradely labeled striatopallidal but not striatonigral neurons [Robertson et al, 1992]. Consistent with the view that D2 receptors mediate haloperidol-induced $c-fos$ expression in the striatum, the present findings suggest that haloperidol increases $c-fos$ expression in D2-expressing and D1/D2 co-expressing striatopallidal neurons.

The striatum has connections predominantly with somatosensory and motor areas of the cortex and is thought to be involved in regulation of basal ganglia motor output [Alexander & Crutcher, 1990]. Balanced opposition in the cortically controlled activity of striatopallidal and striatonigral pathways has been suggested to underlie the complex manner in which the basal ganglia affect behavior [Gerfen, 1992]. For example, elevation of activity in the striatopallidal pathway resulting from striatal DA deafferentation has been proposed to underlie bradykinesia in Parkinson’s disease [Albin et al, 1989; Mitchell et al, 1989]. Likewise, the EPS of haloperidol and other typical neuroleptics are thought to reflect D2 receptor blockade in the striatum [Seeman, 1980]. Previous studies have shown that haloperidol and other neuroleptics with high EPS liability induce NT/N gene expression in the dorsolateral striatum [Merchant et al, 1992; Merchant & Dorsa, 1993]. Also, it has been demonstrated that haloperidol-induced $c-fos$ regulates the increase of NT/N transcripts in the striatum [Merchant, 1994; Merchant & Miller, 1994]. These data, together with the fact that centrally administered NT produces pharmacological effects similar to those of typical neuroleptics [Nemeroff, 1980; Emson et al, 1985; Levant et al, 1990; Deutch & Zahm, 1992], suggest that haloperidol-induced $c-fos$ expression produces an up-regulation of NT expression in striatopallidal neurons. This unbalanced
striatopallidal activity may contribute to functional changes which mediate the EPS of haloperidol.

In contrast to its effect in the striatum, haloperidol induces \textit{c-fos} expression in both Enk (46\%) and Dyn (38\%) neurons in the NAc (Fig. 21B, E; Tables 6 and 8). Because most haloperidol-induced Fos-positive neurons do not express D3 receptor mRNA (Fig. 15E, F; Table 5), these Enk and Dyn neurons in which haloperidol increases Fos immunoreactivity are different than those targeted by clozapine. A previous study showed that about 50\% of haloperidol-induced Fos-positive neurons in the NAc are retrogradely-labeled accumbopallidal neurons [Robertson & Jian, 1995]. In agreement with this report, the present findings suggest that 46\% of haloperidol-induced Fos-positive neurons in the NAc are Enk (perhaps D2-expressing) neurons. However, the present experiments also demonstrate that 38\% of haloperidol-induced Fos-positive neurons in the NAc are dynorphinergic, in contrast to a previous study which showed that only 5-8\% of the Fos-positive neurons were retrogradely-labeled accumbomesencephalic neurons [Robertson & Jian, 1995]. This discrepancy may be due to the different methods used in the two studies, which may give different sensitivities in detecting accumbomesencephalic Dyn/SP neurons. Nevertheless, to the extent that Dyn and D1 receptors are colocalized in the NAc, the finding that haloperidol increases Fos immunoreactivity in Dyn-expressing neurons suggests that haloperidol may indirectly affect the activity of D1-expressing neurons. It is also possible that some of these Dyn neurons are D1/D2 co-expressing neurons. The target genes regulated by haloperidol-induced Fos induction in the NAc are uncertain since \textit{c-fos} antisense oligonucleotide does not attenuate haloperidol-induced NT/N gene expression in this region [Merchant, 1994].
In the LSN, a small portion of haloperidol-induced Fos-positive neurons are colocalized with either Enk (29%) or Dyn (18%) (Fig. 21D, F; Tables 7 and 8). Choline acetyltransferase (ChAT)- and glutamic acid decarboxylase (GAD)-positive neurons have been observed in the LSN [Kimura et al., 1990; Kohler & Chan-Palay, 1983; Mugnaini & Oertel, 1985]. Interestingly, both GAD- and ChAT-positive cell bodies are present in the ventral subdivision of the LSN [Kohler & Chan-Palay, 1983; Kimura et al., 1990], a region where \( c-fos \) expression is induced by haloperidol or clozapine. Therefore, it is possible that haloperidol may induce \( c-fos \) expression in GABAergic or cholinergic neurons in the LSN. As in other brain structures discussed, neurons targeted by haloperidol in the LSN are different populations of neurons from those targeted by clozapine, which do not express D3 receptor mRNA (Table 4). The connections between the LSN and other limbic brain areas suggest that haloperidol-induced neuronal activity in the LSN may be related to its antipsychotic effect.

In summary, the present findings suggest that DA D3 receptors mediate the effects of clozapine, but not haloperidol, on \( c-fos \) expression in the forebrain. These data support the view that different subtypes of D2-like receptors are responsible for the different effects of the two classes of antipsychotic drugs. Also the present experiments indicate that striatopallidal/accumbopallidal Enk neurons and striatonigral/accumbo-mesencephalic Dyn neurons are targeted by clozapine and haloperidol in different patterns. The regulation of balanced opposition of these pathways may account for the therapeutic effects of antipsychotics, while elevation of neuronal activity in the striatopallidal Enk neurons evoked by haloperidol may be responsible for the neuronal changes underlying the EPS of this typical neuroleptic drug.
CHAPTER IV. GENERAL DISCUSSION

The experiments presented in Chapters II and III have investigated receptor mechanisms mediating clozapine-induced c-fos expression in the forebrain and characterized the phenotypes of neurons targeted by clozapine and haloperidol. Using Fos immunostaining as a pharmacological activity indicator, this thesis has examined neurotransmitter systems involved in the effects of clozapine and haloperidol, including muscarinic, serotonergic, noradrenergic and dopaminergic systems. Further studies with oligonucleotide probes for D3 receptor, Enk and Dyn mRNAs have demonstrated that clozapine and haloperidol target D3-, Enk- and Dyn-expressing neurons in a different manner. The relevance of these findings with respect to the significance of different antipsychotic drug-induced neuronal activities in different brain regions are assessed below. Methodological considerations and further studies are also discussed.

1. Receptor and Cellular Mechanisms of Clozapine and Haloperidol

a. Striatum

Experiments in Chapter II demonstrated that the muscarinic antagonist scopolamine attenuated haloperidol-induced Fos immunoreactivity in the striatum but not in the NAc. These results suggest that a muscarinic component is involved in haloperidol’s effect in the striatum. This raises the possibility that antimuscarinic actions of clozapine may contribute to the failure of this drug to produce Fos induction in this structure. In situ
hybridization with oligonucleotide probes for Enk and Dyn mRNAs demonstrated that most haloperidol-induced Fos-positive neurons in the striatum expressed Enk mRNA (93%), while only a small portion express Dyn mRNA (20%) (Chapter III). These results suggest that haloperidol modulates the activity of the striatopallidal pathway by increasing c-fos expression in D2-expressing and D1/D2 co-expressing neurons in the striatum. While these data are consistent with the view that D2 receptors mediate haloperidol’s effect in the forebrain, the failure of quinpirole to block haloperidol-induced Fos immunoreactivity in the striatum and other brain regions suggests that other experimental approaches are necessary to address further the “D2 hypothesis” of haloperidol’s effect in the brain.

At a dose that reduces the cataleptic effect of haloperidol, scopolamine attenuates haloperidol-induced Fos immunoreactivity in the striatum. Clozapine produces neither EPS nor Fos induction in the striatum. Taken together, these findings suggest that haloperidol-induced striatal c-fos expression is relevant to the EPS of this drug. Balanced opposition in the activity of striatonigral and striatopallidal pathways has been suggested to underlie the manner in which the basal ganglia affect behavior [Gerfen, 1992]. Together with the finding that haloperidol-induced c-fos regulates the transcription of the downstream target NT/N gene [Merchant, 1994; Merchant & Miller, 1994], these data suggest that haloperidol-induced c-fos expression leads to an unbalanced up-regulation of NT expression in striatopallidal Enk/NT neurons. This in turn may contribute to the EPS produced by haloperidol.
b. NAc, LSN and ICj

To determine whether D3 receptors contribute to clozapine-induced c-fos expression in limbic brain regions such as the NAc, LSN and ICjM, the D3 receptor preferring agonists 7-OHDPAT and quinpirole were administered in combination with either clozapine or haloperidol (Chapter II). Both 7-OHDPAT and quinpirole markedly reduced clozapine-induced Fos immunoreactivity in the NAc, LSN and ICjM. These results suggest that D3 receptor mechanisms may be involved in clozapine’s effects in these limbic brain regions. In situ hybridization with an oligonucleotide probe for D3 receptor mRNA confirmed that clozapine-induced Fos-positive neurons in the NAc (69%), LSN (73%) and ICjM (95%) express D3 receptor mRNA (chapter III). In contrast, haloperidol-induced Fos-positive neurons rarely expressed D3 receptor mRNA in any brain region (Chapter III). Taken together, these results suggest that clozapine-induced c-fos expression in the NAc, LSN and ICjM are due to antagonist actions of this drug at D3 receptors.

Studies with oligonucleotide probes for Enk and Dyn mRNA indicated that clozapine increased c-fos expression in both Enk- and Dyn-containing neurons in the NAc (Enk 40%; Dyn 53%) and LSN (Enk 32%; Dyn 59%) (Chapter III). Haloperidol also increased c-fos expression in Enk and Dyn neurons, albeit in a different pattern (NAc Enk 46%, Dyn 36%; LSN Enk 29%, Dyn 18%) (Chapter III). The fact that the two antipsychotics differ in terms of their targeting of D3-expressing neurons in these limbic structures raises the possibility that clozapine increases c-fos expression in D3/D1 co-expressing and D3/D2 co-expressing neurons while haloperidol targets different populations of the enkephalinergic and dynorphinergic neurons (i.e. D2-expressing and/or
D2/D1 co-expressing neurons) (Fig. 22). These findings indicate that clozapine and haloperidol induce c-fos expression via different receptor mechanisms in different populations of neurons in limbic brain regions.

As discussed above, the NAc plays an important role in the brain limbic circuit which is involved in integrating emotional and memory functions. Clozapine and haloperidol may regulate activity in the limbic system in part by increasing c-fos expression: by targeting Enk/NT and Dyn/SP neurons in the NAc, these antipsychotic drugs may produce balanced opposition of accumbopallidal and accumbomesencephalic outputs which may represent the mechanisms by which they modulate emotional and motoric behaviors. Likewise, the modification of neuronal activity by clozapine and haloperidol in the LSN may also be related to the antipsychotic effects of these drugs. However, the target gene(s) regulated by clozapine- and haloperidol-induced c-fos expression in the NAc and LSN remain unknown. Further investigation of the downstream target genes will help us to understand the molecular substrates of antipsychotic drug actions.

Clozapine-induced c-fos expression in the ICj is unique. The therapeutic significance of this effect needs to be determined. Since the ICj contains among the highest levels of D3 receptors in both human and rat brain [Landwehrmeyer at al, 1993a; Sokoloff et al, 1990], neuronal activity in this structure may provide a pharmacological marker to examine the effect of novel D3 receptor agonists/antagonists.
Fig. 22. Schematic diagram of phenotypes of clozapine- and haloperidol-induced Fos-positive neurons in the PFC, striatum, NAc, LSN and ICjM. It is assumed that Dyn-containing neurons express D1 receptors and Enk/NT-containing neurons express D2 receptors in these forebrain areas. According to this hypothesis, clozapine- and haloperidol-induced Fos-positive neurons may be classified into different subpopulations. The phenotype of clozapine-induced Fos-positive neurons in the PFC is unknown, although these neurons may express D4 receptors. In the ICjM, clozapine increases c-fos expression in D3/D1 co-expressing cells that contain Dyn. In the NAc, clozapine induce c-fos expression in both D3/D1 co-expressing neurons that contain Dyn and D3/D2 co-expressing neurons that contain Enk/NT. In the LSN, clozapine increases c-fos expression in D3-expressing neurons that contain Enk and/or Dyn. In contrast, haloperidol-induced Fos-positive neurons in the striatum and NAc are thought to be D2-expression neurons that contain Enk and D2/D1 co-expressing neurons that co-store Enk and Dyn. Also, haloperidol may increase c-fos expression in D2-expressing neurons in the LSN.
The receptor mechanisms that mediate clozapine-induced *c-fos* expression in the PFC have proven difficult to unravel. In the present study, scopolamine either alone or in combination with haloperidol did not mimic clozapine-induced Fos immunoreactivity in the PFC, suggesting that antimuscarinic properties of clozapine do not contribute to its effect in the PFC (Chapter II). Neurotoxic lesion studies showed that 5,7-DHT MFB lesions and 6-OHDA DNB lesions produced extensive depletions of 5-HT and NA, respectively, in the forebrain. However, neither type of lesion affected clozapine-induced *c-fos* expression in the PFC and other brain regions (Chapter II). These data suggest that neither serotonergic nor noradrenergic mechanisms are involved in this action of clozapine. Consistent with this hypothesis, ritanserin either alone or in combination with haloperidol failed to mimic clozapine-induced *c-fos* expression in the PFC, suggesting that antagonist actions at 5-HT2 receptors do not contribute to this effect of clozapine (Chapter II). 7-OHDPAT has about 100- and 1000-fold higher affinity for the D3 receptor than for D2 and D4 receptors, respectively. The failure of 7-OHDPAT to attenuate clozapine-induced Fos immunoreactivity in the PFC suggests that D3 receptor mechanisms are not involved in clozapine’s effect in the PFC (Chapter II), despite the fact that a small portion (30%) of clozapine-induced Fos-positive neurons in the PFC colocalize with D3 receptor mRNA (Chapter III). However, quinpirole, which has approximately equal affinity for D3 and D4 receptors and about 100-fold lower affinity for D2 receptors, produced a small but significant decrease in the number of clozapine-induced Fos-positive neurons in the PFC (Chapter II). Given the different affinities of quinpirole and 7-OHDPAT for D2, D3 and D4 receptors, the present findings suggest that antagonist actions at D4 receptors may
partly contribute to clozapine-induced $c$-$fos$ expression in the PFC (Fig. 22). The present data also indicate that the primary mechanisms by which clozapine increases $c$-$fos$ expression in the PFC remain to be determined.

2. Future Studies and Methodological Considerations

As discussed above, D4 receptors may partly contribute to clozapine’s unique effect in the PFC. Probably due to its low expression levels in the brain, no systematic *in situ* hybridization studies for D4 receptor mRNA in rat brain have been published to date. A recent study has reported the localization of D4 receptors in primate brain by using a subtype-specific antibody against the D4 receptor [Mrzljak et al, 1996]. This raises the possibility that in future studies, Fos immunohistochemistry may be combined with D4 receptor antibody staining to determine whether clozapine increases Fos induction in D4 receptor-expressing neurons in the PFC of rats. This will provide an anatomical basis for the involvement of D4 receptors in clozapine-induced $c$-$fos$ expression in this brain structure. In addition, specific D4 receptor agonists and antagonists are required to determine clozapine’s effect on $c$-$fos$ expression in the PFC.

The possible involvement of glutamatergic and/or GABAergic systems in clozapine’s effects in the PFC are only beginning to be explored. PCP, an NMDA-glutamate receptor antagonist, produces psychosis that mimics negative as well as positive symptoms of schizophrenia [Westbrook & Jahr, 1989; Javitt & Zukin, 1991]. This has led to the speculation that an endogenous deficiency of NMDA receptor-mediated neurotransmission may play a role in schizophrenia [Javitt & Zukin, 1991]. The NMDA
receptor is composed of a cation-selective channel and receptor sites for glutamate, glycine and polyamines [Leeson & Iversen, 1994]. Glutamate (Glu) agonists maintain the channel in an open state and glycine and polyamines act synergistically with Glu [Leeson & Iversen, 1994]. PCP, however, antagonizes the Glu agonist effect and blocks the channel [Westbrook & Jahr, 1989; Javitt & Zukin, 1991]. While the therapeutic possibilities inherent in a facilitation of the NMDA receptor-complex remain to be determined, several studies have shown that administration of glycine both reverses PCP-induced behaviors in rodents [Toth & Lajtha, 1986] and produces significant improvement in negative symptoms in schizophrenic patients [Javitt et al, 1994; Zylberman et al, 1995]. These data suggest that glycine potentiates NMDA receptor-mediated neurotransmission in the brain. In addition, it has been proposed that there may be neuropathological changes in GABAergic systems in the cingulate gyrus of schizophrenics [Benes et al, 1992]. Increased GABA\textsubscript{A} receptor binding has been found in the cingulate cortex of schizophrenics [Benes et al, 1992], suggesting a deficiency in GABA neurotransmission. Moreover, bretazenil, a partial benzodiazepine agonist, produces a significant antipsychotic effect [Merz et al, 1988]. These data raise the possibility that glutamatergic and GABAergic systems may be involved in clozapine’s effects in the brain. Further investigation of glutamatergic and GABAergic mechanisms in clozapine-induced \textit{c-fos} expression in the PFC will facilitate our understanding of atypical drug actions.

Methodologically, Fos immunostaining has proved to be a useful marker of neuronal activity in the brain. It has been suggested that Fos immunohistochemistry can be used as a surrogate for 2-deoxyglucose (2-DG) metabolic mapping in the CNS [Morgan et
al, 1987; Sagar et al, 1988]. Several lines of evidence indicate that, for the purpose of this study, Fos immunostaining provides a better marker than does 2-DG for. First, it has been found that neocortical, hippocampal and thalamic glucose utilization was reduced but that there were no significant changes in the striatum, anterior cingulate and cerebellum in rats treated with haloperidol [McCulloch et al, 1982]. Although it is assumed that changes of metabolic rate in these brain regions may be parallel to the areas of drug action, the 2-DG method cannot distinguish between altered glucose metabolism of neurons intrinsic to a brain region from that of terminals of afferent fibers. In contrast, Fos immunohistochemistry stains the nuclei of activated cells, allowing the identification of specific neurons targeted by antipsychotic drugs. Second, Fos immunohistochemistry detects antipsychotic-induced neuronal activity in the striatum, NAc, LSN, PFC and ICjM, while the 2-DG method does not show metabolic changes in these brain regions [McCulloch et al, 1982]. Previous studies have shown that the patterns of 2-DG uptake and Fos immunostaining are not always coincident. For instance, Fos induction in the paraventricular nucleus is not associated with increased 2-DG uptake in water-deprived rats [Sagar et al, 1988]. Moreover, an increase in c-fos expression has been found in CA1 of the hippocampus following ischaemia, although 2-DG uptake is substantially decreased [Jorgensen et al, 1989]. It is thus suggested that induction of Fos requires the integration of extracellular stimuli and intracellular signal transduction pathways, which may or may not be associated with changes of metabolic activity [Morgan & Curran, 1989]. Third, PET studies have shown that haloperidol and clozapine produce similar patterns of glucose metabolism in patients [Holcomb et al, 1996; Potkin et al, 1994]. However, Fos immunostaining reveals different patterns of neuronal activity in the brain in response to
different antipsychotic drugs. Therefore, the regionally specific Fos induction can be used as a marker to investigate the receptor and molecular mechanisms of different antipsychotic drugs. Finally, since Fos immunostaining reflects activation of brain neurons with single-cell resolution, as demonstrated in the present experiments, these antipsychotic-activated cells (Fos-positive neurons) can be further characterized by location, receptor-expressing type, and neurotransmitter content (Chapter III). Since Fos immunohistochemistry permits a discrimination between pre- and post-synaptic elements in signal transduction pathways, it has been used in the present study as a pharmacological indicator to examine neurotransmitter systems involved in the effects of antipsychotic drugs (Chapter II).

Fos immunohistochemistry only labels some subsets of antipsychotic-activated neurons. For example, acute neuroleptics are known to increase the activity of mesencephalic DA neurons [Chiodo & Bunney, 1983], and yet these neurons are unresponsive with respect to Fos (unpublished observations). Furthermore, as discussed above, changes of glucose metabolism have been found in cortical, hippocampal and thalamic regions in rats treated with haloperidol [McCulloch et al, 1982], while Fos induction is not observed in these brain areas. Therefore, other approaches, perhaps including other IEGs, are need to investigate antipsychotic-targeted neurons which are Fos-negative. It is also worth noting that Fos induction maps neuronal activities evoked by acute treatment with antipsychotics. However, therapeutic effects are only evident after chronic administration of these drugs. Clearly, the acute Fos induction alone is not sufficient to examine chronic drug action.
Recent studies have demonstrated that long-term treatments can induce the so-called chronic or long-lasting AP-1 complex and Fos-related antigens (FRAs) that have much longer half-lives than do acutely induced FRAs [Pennypacker et al, 1995]. For example, chronic cocaine treatment induces a long-lasting increase in AP-1 binding in the rat NAc and striatum; the chronic AP-1 complex contains at least four FRAs, including FRA-1, FRA-2, FosB and ΔFosB [Hope et al, 1994b]. The time courses for the chronic FRAs indicate that they only appear following chronic but not acute cocaine treatment: Fos can be seen during the first 2-4 hr following acute cocaine, while FRAs, which are clustered around 41-46 kDa and 30-35 kDa [Cohen & Curran, 1988; Nakabeppu & Nathans, 1991; Dobrzanski et al, 1991; Nishina et al, 1990], can be observed between 4 and 12-18 hr after chronic cocaine [Hope et al, 1994b]. The induction of chronic FRAs and the AP-1 complex appears to be a general phenomenon found with other chronic treatments. For example, depletion of striatal DA by 6-OHDA lesions in the SN increases FRA expression, an effect that has been observed for up to three months after denervation [Dragunow et al, 1991]. Repeated apomorphine treatment following unilateral 6-OHDA lesions in the SN increases the expression of chronic FRAs and AP-1 binding activity in the striatum [Bronstein et al, 1994; Hope et al, 1994b]. Chronic treatment with the monoamine oxidase inhibitor tranylcypromine induces chronic FRAs and AP-1 binding activity in the frontal cortex, while chronic electroconvulsive shock (ECS) produces these long-lasting transcription factors in the cerebral cortex and hippocampus [Hope et al, 1994a; 1994b].
The regions where the chronic FRAs and AP-1 complex are induced are the same areas where acute Fos and AP-1 complex are evoked, suggesting a gradual change in the composition of the AP-1 complex with long-lasting FRAs replacing short-lasting acute Fos/FRAs [Bronstein et al, 1994; Hope et al, 1994a; 1994b]. Some evidence also indicates that chronic FRAs form dimers with a Jun-like protein, as do the acute Fos/FRAs [Kaminska et al, 1994; Pennypacker et al, 1992]. The different composition of the chronic versus acute AP-1 complexes may have functional consequences. For example, the AP-1 complexes induced by acute and chronic ECS have different affinities for the AP-1-like sites [Hope et al, 1994a]. This suggests that the acute and chronic AP-1 complexes may differentially regulate target genes. Therefore, in future studies it will be important to examine chronic FRAs and AP-1 complexes induced by chronically administered antipsychotic drugs.

Like many other chronic treatments, chronic antipsychotic drug treatments might induce long-lasting FRAs in the same brain regions where acute Fos/FRAs are induced. If this is the case, chronic FRAs could be expected to be induced in the PFC by chronic clozapine treatment and in the striatum after chronic haloperidol administration. The correlation between clinical effects of antipsychotics and chronic FRA induction in the PFC and other brain regions may be useful in understanding the sites of chronic antipsychotic drug action. Furthermore, it is speculated that the differences in chronic antipsychotic-induced FRA-immunoreactivity between the PFC and striatum will provide a useful means to predict the atypical potential of antipsychotic drugs. Such a difference, which is proposed here as the “PFC activity index”, may be defined as the value of the number of FRA-positive neurons in the PFC minus that in the striatum. It is predicted that
the PFC activity index would be positive for atypical compounds, but negative for typical neuroleptics following chronic drug treatment. This may be useful for discovering whether novel antipsychotics will have atypical potential or EPS liability.

Since chronic FRAs/AP-1 complexes may be more closely associated with the effect of chronic antipsychotic drug treatment than are acute FRAs, they would provide useful markers to examine neurotransmitter systems involved in drug actions. For example, using drug manipulations, antisense-knockout and *in situ* hybridization techniques in combination with immunohistochemistry for chronic FRAs or gel-shift for chronic AP-1 complexes, receptor mechanisms which mediate chronic antipsychotic drug actions can be determined.

In addition, chronic FRAs/AP-1 complex may regulate genes that are related to processes involved with chronic antipsychotic-induced neuronal plasticity. In future studies, mRNA differential display in combination with transgenic approaches can be used to investigate these target genes and their significance. This may provide insights into an understanding of the molecular basis of chronic antipsychotic drug action and provide important clues for the development of new antipsychotic drugs.
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