The Effects of Some Typical and Atypical Neuroleptics on Gene Regulation: Implications for the Treatment of Schizophrenia

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ABSTRACT

The cellular mechanisms by which antipsychotic medications (neuroleptics) produce their therapeutic effects in schizophrenia are largely unknown. Although neuroleptic efficacy is attributed to central dopamine D_2 and/or serotonin 5-HT_2 receptor antagonism, clinical improvements in schizophrenia are not seen until two or three weeks after daily neuroleptic administration. The mechanisms underlying the neuroleptic response must therefore occur downstream from initial receptor blockade and be a consequence of continuous, chronic neurotransmitter receptor blockade. The goal of the present study was to use neuroleptics with varing dopamine vs. serotonergic receptor blocking profiles to elucidate some of these intracellular post receptor mechanisms.

Since both the final steps of dopamine and serotonin synthesis require the enzyme aromatic L-amino acid decarboxylase (AADC), the effects of neuroleptics on AADC gene (mRNA) expression were examined in PC12 cells and compared to their effects on tyrosine hydroxylase (TH; the apparent rate limiting enzyme in the synthesis of dopamine) and *c-fos* (an early immediate gene [IEG] known to regulate tyrosine hydroxylase) gene expression. The neuroleptics examined did not significantly regulate AADC mRNA in PC12 cells, and only haloperidol regulated TH and *c-fos* mRNA.

Later *in vivo* studies in rats showed that acute neuroleptic administration increased *c-fos* mRNA in a number of brain regions, whereas the immunoreactivity of a related IEG (delta FosB) was increased upon chronic treatment. These studies and a subsequent dose response study demonstrated that upregulation of both *c-fos* mRNA and delta FosB immunoreactivity was most prominent in dopaminergic projection areas, including the striatum and nucleus accumbens.

Because it has been suggested that neuroleptic treatment might prevent neurodegeneration in schizophrenia, the effects of neuroleptics on the mRNA expression of neuroprotective target genes of delta FosB were examined both *in vitro* and *in vivo.* These genes included brain-derived neurotrophic factor (BDNF), the neuroprotective enzyme superoxide dismutase (SOD), and the low affinity nerve growth factor receptor (p75). While dopamine D_2 blockade unfavorably regulated BDNF and p75 mRNA, 5-HT_2 blockade either had no effect on or favorably regulated BDNF, SOD, and p75 mRNA.

Thus, although very little about the contribution of serotonergic blockade to the neuroleptic response was determined in the present study, dopaminergic blockade was found to regulate IEGs and several of their target genes. Future studies will be needed to understand the role of 5-HT_2 receptor blockade in the neuroleptic response.

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TABLE OF CONTENTS

The Effects of Some Typical and Atypical Neuroleptics on Gene Regulation: Implications for the Treatment of Schizophrenia

List of Tables

List of Figures

List of Abbreviations

°C	degrees Celsius
μ	mu (micro)
μCi	microcurie(s)
μg	microgram(s)
μL	microliter(s)
μm	micrometer(s)
μM	micromolar
3'	3 prime
5'	5 prime
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HT_{1a}	serotonin type 1a receptor
5-HT_{1c}	serotonin type 1c receptor
5-HT_{2}	serotonin type 2 receptor
5-HT_{2a}	serotonin type 2a receptor
A	adenine
A_{260}	absorption at 260nm
AADC	aromatic L-amino acid decarboxylase
ABC	avidin-biotin conjugated
AC	adenylate cyclase
ALS	amyotrophic lateral sclerosis
AMP	ampicillin
AMP+	ampicillin positive
AMP-	ampicillin negative
ANOVA	analysis of variance
AP-1	activator protein-1
ATP	adenosine triphosphate
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
C	cytosine
C/EBP	CCAAT enhancer binding protein
cAMP	3', 5' cyclic adenosine monophosphate
CCAAT	enhancer region on DNA
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CP	caudate putamen

cpm	counts per minute
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
cRNA	complementary ribonucleic acid
CSF	cerebrospinal fluid
CT	computerized tomography
CTP	cytidine triphosphate
D_1	dopamine type 1 receptor
D_2	dopamine type 2 receptor
D_4	dopamine type 4 receptor
DA	3,4-dihydroxyphenylethylamine (dopamine)
DAB	diaminobenzadine
DAG	diacylgycerol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DLStr	dorsolateral striatum
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DOPA	3,4-dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
dpm	disintegrations per minute
DSM IV	Diagnostic and Statistical Manual of Mental Disorders (4th ed.)
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E. Coli	Escherichia coli
ECT	electroconvulsive shock treatment
EDTA	ethylene diaminetetraacetic acid
EPS	extrapyramidal symptoms (side effects)
g	gram
G	guanine
GABA	gamma-aminobutyric acid
GDP	guanosine diphosphate
GITC	guanidinium isothiocyanate
GluR1	glutamate receptor subunit 1
GluR2	glutamate receptor subunit 2

GTP	guanosine triphosphate
H_2O	water
hr.	hour
HVA	homovanillic acid
i.p.	intraperitoneal
IEG	immediate-early gene
IP3	inositol triphosphate
IR	immunoreactivity
ISH	In situ hybridization
kDa	kilodalton
kg	kilogram
Km	Michaelis constant
LB	Luria-Bertani media
LC	locus coeruleus
L	liter(s)
LNGFR	low affinity nerve growth factor receptor
LSD	lysergic acid diethylamide
M	molar
mCi	millicurie(s)
mg	milligram
min.	minute(s)
mL	milliliter(s)
MLStr	mediolateral striatum
mm	millimeter
mM	millimolar
MOPS	morpholino propane sulfonic acid
MPAs	minor physical abnormalities
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
mV	millivolt
N	normal (concentration of ionizable groups)
n	number in a study or group
N-CAM	neural cell adhesion molecule
n.s.	not statistically significant
ng	nanogram
NGF	nerve growth factor
NIH	National Institutes of Health
nm	nanometer(s)
nM	nanomolar

NMDA	N-methyl-D-aspartate
NR2D	N-methyl-D-aspartate NR2D receptor subunit
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
NTP	nucleotide triphosphate
NuAcc	nucleus accumbens
OD	optical density
p	probability
p75	low affinity nerve growth factor receptor
PBS	phosphate-buffered saline
PBS-TX	phosphate-buffered saline with Triton-X 100
PC12	pheochromocytoma cells
PCP	phencyclidine
PCR	polymerase chain reaction
PFA	paraformaldehyde
PFC	prefrontal cortex
pH	negative log of the hydrogen ion concentration
PI	phosphadityl inositol
PK	protein kinase
PKA	c-AMP dependent protein kinase A
PKC	protein kinase C
PLC	phospholipase C
RE	restriction endonuclease
RMPI	Roswell Park Memorial Institute
RNA	ribonucleic acid
RNAse	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
RT- PCR	reverse transcriptase polymerase chain reaction
S.E.M.	standard error of the mean
SDS	sodium dodecyl sulfate
SN	substantia nigra
SOD	superoxide dismutase
SOD1	Cu^{2+}/Zn^{2+} dependent superoxide dismutase
SOD2	Mn^{2+} dependent superoxide dismutase
ss-phenol	saturated salt phenol
SSC	sodium chloride- sodium citrate buffer
SSPE	sodium chloride- sodium phosphate buffer
STE	sodium chloride- Tris- EDTA

T	thymine
TAE	tris-acetate/ EDTA buffer
Taq	Thermus aquaticus
TD	tardive dyskinesia
TE	tris- EDTA buffer
TEA	triethanolamine
TH	tyrosine hydroxylase
Tm	melting temperature of nucleotide duplexes
TNF	tumor necrosis factor
tris	tris(hydroxymethyl)aminomethane
Trk	tropomyosine related protein kinase receptor
TrkA	tropomyosine related protein kinase type A receptor
TrkB	tropomyosine related protein kinase type B receptor
TrkC	tropomyosine related protein kinase type C receptor
U	units
UTP	uridine triphosphate
UV	ultraviolet
VBR	ventricle-brain ratio
VTA	ventral tegmental nucleus

The Effects of Some Typical and Atypical Neuroleptics on Gene Regulation: Implications for the Treatment of Schizophrenia

1. INTRODUCTION

1.1. Schizophrenia

Schizophrenia is a serious mental disorder of thought and mood that is only partially understood in terms of both its causes and its treatment. It strikes approximately 1% of the population (Sartorious et al., 1972), and its most incapacitating forms often escape scientific understanding and remain resistant to medical intervention (Keefe et al., 1987). Victims of schizophrenia have been estimated to occupy 20% of all psychiatric hospital beds in the United States (Roberts, 1990) and 8% of all hospital beds in Canada (Statistics Canada, 1991), and often do not recover fully from their illnesses.

1.1.1 Definition and clinical presentation

The symptoms of schizophrenia are traditionally broken down into positive (delusions, hallucinations, disorganized/ bizarre behavior), negative (flattened affect, limited social adjustment), and cognitive (impaired attention, poverty of speech) symptoms (Andreasen et al., 1990; Andreasen, 1994). Vague psychiatric symptoms such as social withdrawal, changes in sleep patterns, and poor hygiene usually precede the appearance of symptoms that meet the DSM IV (1994) criteria for schizophrenia (Gross, 1997; Parnas, 1999). Patients often seek medical attention after a psychotic episode involving one or more positive symptoms (McGlashan and Johannessen, 1996). At this point in the illness, the patient has often experienced some of the negative and

cognitive symptoms of the disorder as well (Hafner, 1998; McGlashan, 1998a, b). Symptoms usually occur after the onset of adolescence (Hafner et al., 1993), with men experiencing an earlier onset of the disorder (late teens to mid twenties) than women (twenties to early thirties; Seeman, 1997).

1.1.2 Treatment and prognosis

Traditionally, the positive symptoms of schizophrenia usually respond well to neuroleptics, while the negative and cognitive symptoms have been difficult to treat (Crow, 1980; Andreasen et al, 1990). Prognosis varies, with approximately one third of patients responding well to treatment, one third having moderate symptom relief accompanied by relapses, and one third remaining resistant to treatment (Schellenberg et al., 1994). Older dopaminergic blocking neuroleptics such as haloperidol are efficacious in treating positive symptoms (Creese et al., 1976), but do not significantly ameliorate negative symptoms (Crow, 1985) and may in fact worsen them (Finzen, 1991; Palao et al., 1994). Traditional neuroleptics may also cause significant Parkinson-like motor side effects such as tremor, muscular rigidity, and akinesia (Van Putten, 1974; Sovner and DiMasico, 1978). In addition, long term treatment with typical neuroleptics can cause tardive dyskinesia (TD), a hyperkinetic disorder characterized by abnormal involuntary movements of the tongue and mouth (Clemens et al., 1976; Clyne and Juhl, 1976; Gunne and Barany, 1976; Stimmel, 1976; Burt et al., 1977). Newer neuroleptics such as risperidone, quetiapine, sertindole, ziprasidone, clozapine, and olanzapine, which block both dopaminergic and serotonergic receptors, not only have less severe motor side effects (Roose et al., 1988; Meco et al., 1989; Gerlach, 1991; Ichikawa and Meltzer, 1999), but also ameliorate some negative and cognitive symptoms (Bersani et al., 1986, 1990; Velligan and Miller, 1999; Remington and Kapur, 2000). Nevertheless, many people with schizophrenia never fully recover from the disorder and remain resistant to current treatments (Schellenberg et al., 1994).

1.2 Selected theories of cause and aetiology in schizophrenia

Although researchers have long searched for a unitary hypothesis of schizophrenia, current thought posits that schizophrenia is likely caused by a variety of environmental and biological factors. Some suggest that many different causes (genetic, environmental, etc.) converge on a common pathophysiology (Crow, 1980). However, most researchers presently agree that the clinical symptoms of schizophrenia represent a cluster of related disorders that are pathophysiologically heterogeneous (Carpenter and Kirkpatrick, 1988) involving a number of neural mechanisms, neurotransmitters, and brain regions (Tsuang et al., 1990; Lieberman and Koreen, 1993). The following is a brief review of some theories of cause and aetiology in schizophrenia.

1.2.1 Neurotransmitter and receptor theories

Dopamine (DA), serotonin (5-HT_2), glutamatergic, gamma-aminobutyric acid (GABA), noradrenaline, acetylcholine and several other neurotransmitters and their receptors have all been implicated in the pathophysiology of schizophrenia. Levels of these transmitters and/ or their receptors have either been found to be altered in schizophrenia or altered by neuroleptic treatment (reviewed by Duncan et al., 1999). Current neurotransmitter theories of schizophrenia and explanations surrounding the mechanisms of action of antipsychotics primarily involve dopamine and serotonin. Since both dopamine type 2 (D_2) and serotonin type 2 (5-HT_2) receptor antagonists have been successful in the treatment of some patients with schizophrenia, it follows that both transmitters are either implicated in the pathology of the disorder or can be altered to improve the symptoms of the disease (Huttunen, 1995). A brief description of the suspected roles of DA, 5-HT and glutamate in schizophrenia follows.

1.2.1.1 Dopamine

The dopamine hypothesis of schizophrenia was the primary theory directing schizophrenia research during the past 40 years (Lidow et al., 1998) and is still the most comprehensive theory of schizophrenia (Duncan et al., 1999). This hypothesis is primarily based on two findings (Carlsson, 1988; Civelli et al., 1993): That dopamine agonists such as 3,4-dihydroxyphenylalanine (L-DOPA) used to treat Parkinson's disease (Ehringer and Hornykiewicz, 1960; Birkmayer and Hornykiewicz, 1962; Hornykiewicz, 1966) often result in psychotic side effects (Crowley et al., 1978), and that most neuroleptics, such as chlorpromazine and haloperidol, block D_2 receptors and increase brain dopamine turnover (Carlsson and Lindquist, 1963) with a potency that parallels that of their antipsychotic effects (Seeman et al., 1975, 1976; Creese et al., 1976; Snyder, 1976).

Dopamine D_2 receptors are found in high concentrations in the caudate putamen, nucleus accumbens, and olfactory tubercles primarily as autoreceptors on presynaptic terminals (Maeno, 1982; Memo et al., 1983; Boyson et al., 1986; De Keyser et al., 1988). Findings of elevated striatal D_2 (Mita et al., 1986) and cortical D_4 (Seeman et al., 1993; Murray et al., 1995) receptors in schizophrenics would support a role of dopamine in schizophrenia. It is unclear, however, whether these receptor increases actually underlie the pathophysiology of the disorder or if they are due to normal compensatory mechanisms of the dopaminergic neuron aimed to counteract the dopamine blocking effects of antipsychotic drug treatment (Duncan et al., 1999). In addition, initial findings of D_2 (Sarkar et al., 1991; Catalano et al., 1992) and D_4 (DiBella et al., 1996) receptor gene poylmorphisms in schizophrenia have not been replicated (Su et al., 1993; Crawford et al., 1996; Ohara et al., 1998).

After more than forty years of investigation into the dopamine hypothesis of schizophrenia, there is still no direct evidence of altered neurotransmission in schizophrenia (Duncan et al., 1999). Since most typical D_2 receptor blockers do not ameliorate the negative and cognitive symptoms of schizophrenia (Crow, 1980; Andreasen et al, 1990; Gerlach, 1991), the biological correlates of schizophrenia are thought to involve more than one transmitter system or brain region (Samanin et al., 1978). This is supported by the development of highly beneficial antipsychotics such as clozapine, olanzapine, and risperidone. These compounds appear to act primarily at sites other than the D_2 receptor (Iversen, 1985; Meltzer and Nash, 1991; Niznik and Van Tol, 1992). Thus, the hypothesis of excess dopaminergic activity in schizophrenia cannot fully account for the disorder's etiology (Reynolds, 1992; DeLecuona et al., 1993).

In recent years, the dopamine hypothesis of schizophrenia has been modified to suggest that schizophrenia involves a regulatory imbalance, rather than an excess, of dopamine (Davis et al., 1991; Grace, 1991; Deutch, 1992). The positive symptoms of schizophrenia, which have typically responded well to classical D_2 receptor antagonists, are thought to involve hyperdopaminergic activity in mesolimbic structures of the brain, including projections from the ventral tegmental area (VTA) to the nucleus accumbens (NuAcc; Grace, 1991; Duncan et al., 1999). The negative symptoms of schizophrenia are thought to involve a relative deficit in dopaminergic activity in the mesocortical system, including the medial frontal cortex (mPFC), and the dopamine D_1 family of receptors (Volkow et al., 1987). In support of a role of hypodopaminergia in schizophrenia, Losonczy et al. (1987) found that a subgroup of schizophrenic patients showed clinical improvement in cognitive symptoms in response to dopamine agonists such as L-DOPA, methylphenidate, and dextroamphetamine. These compounds, however, worsen positive symptoms in many patients (Koreen et al., 1997; Szeszko et al., 1999), - further

illustrating the notion that schizophrenia is pathophysiologically heterogeneous and not likely to be understood in terms of a single transmitter system.

1.2.1.2 Serotonin

The hallucinogenic properties of the serotonin (5-HT) receptor antagonist lysergic acid diethylamide (LSD) first sparked interest in a serotonergic hypothesis of schizophrenia as early as the 1950s (Gaddum, 1953; Wolley and Shaw, 1954; Bleich et al., 1991). When it was found that other 5-HT antagonists lacked hallucinogenic properties and that LSD could also act as a 5-HT agonist (Van Praag, 1992), the serotonin hypothesis fell into disrepute (Iqbal and Van Praag, 1995). Interest in the serotonergic hypothesis was rekindled by the finding that newer neuroleptics such as clozapine, risperidone, and olanzapine block a greater proportion of serotonergic receptors (specifically, type 2a receptors) compared to dopaminergic receptors at clinically relevant doses (Meltzer, 1995).

High densities of serotonin type 2A ($5\text{-}HT_{2A}$) receptors are found in the prefrontal cortex (PFC), claustrum, and in platelets (Goodman, 1994). A role of serotonin in schizophrenia is supported by post mortem findings of elevated cortical $5\text{-}HT_{2a}$ receptors (Mita et al., 1986). These findings, however, may be confounded by the $5\text{-}HT_2$ blocking properties actions of neuroleptics, in that the $5\text{-}HT_{2a}$ receptor upregulation in schizophrenics may be a compensatory mechanism to counteract $5\text{-}HT_{2a}$ receptor blockade (Duncan et al., 1999). Initial findings of $5\text{-}HT_2$ receptor (Erdmann et al., 1996; Inayama et al., 1996) and the serotonin transporter (Dean et al., 1995, 1996; Naylor et al., 1996) allelic polymorphisms in schizophrenics have not been replicated (Ishigaki et al., 1996; Hawi et al., 1997; Verga et al., 1997; Stober et al., 1998; He et al., 1999). Some investigators have found increases in blood serotonin levels in schizophrenics (Todrick et al., 1960; Garelis et al., 1975), but others have not replicated these results (Feldstein et al., 1959; Halevy et al., 1965).

Although the role of serotonin in schizophrenia is unclear, it has been proposed that newer neuroleptics treat the negative symptoms of schizophrenia via their 5-HT_2 receptor antagonism (Gelders et al., 1989). Serotonin 5-HT_2 receptor antagonists, such as ritanserin, reduce negative symptoms in schizophrenia (Bersani et al., 1986; Ugedo et al., 1989; Kay and Sandyk, 1990) without the extrapyramidal side effects (ESP) often associated with D_2 receptor blockade. The reduction of negative symptoms by such 5-HT_2 blockers is thought to be due to the removal of serotonergic inhibition on VTA dopaminergic neurons which project to the PFC (Ugedo et al, 1989; Chen et al, 1992). This means that 5-HT_2 receptor blockade will increase dopaminergic transmission to the PFC where a hypodopaminergic state is hypothesized to exist in schizophrenia (Losonczy et al., 1987). Thus, the removal of serotonergic inhibition on mesocortical pathways by 5-HT_2 antagonists may increase the dopaminergic activity of the mesocortical pathways to reduce negative symptoms, which may in turn inhibit the dopaminergic activity of the mesolimbic system (and reduce positive symptoms).

Both D_2 and 5-HT_2 receptor antagonists have been successful in the treatment of symptoms in many patients with schizophrenia. Even if these transmitter pathways cannot be directly implicated in the disorder, it is well known that they can be altered to improve the symptoms of the disease in about two thirds of all patients (Andreasen and Black, 1997).

1.2.2.1 Glutamate

The discovery by Kim et al. (1980) of low glutamate in the cerebrospinal fluid (CSF) of schizophrenics first sparked interest in a glutamatergic hypothesis of the disorder (Olney and Farber, 1995). Although this particular finding was not consistently replicated (Perry, 1982), indirect evidence exists to support N-methyl-D-aspartate (NMDA) glutamate receptor hypofunction

(i.e., decreased function and efficiency) in schizophrenia. The cyclohexamine anesthetics phencyclidine (PCP) and ketamine are noncompetitive antagonists of the ionotrophic NMDA glutamate receptor, and produce behaviours similar to those of schizophrenia (Cohen et al., 1962; Javitt and Zutikin, 1991; Malhotra et al., 1997). These agents also exacerbate symptoms in those with schizophrenia (Ital et al., 1967), whereby ketamine will produce hallucinations and delusions mimicking those previously experienced by the patient (Lahti et al., 1995; Duncan et al., 1999). Since symptoms of schizophrenia are not usually seen before adolescence, it is also interesting that ketamine only produces psychosis in post-adolescent children and adults but not young children (Reich and Silvay, 1989), suggesting that neurodevelopment of glutamate and glutamate-related pathways during adolescence have a role in the development of schizophrenia.

Although cortical messenger ribonucleic acid (mRNA) levels of the NR2D subunit of the NMDA receptor have been reported to be increased in schizophrenia (Akbarian et al., 1996), while hippocampal glutamatergic GluR1 and GluR2 receptor subunit mRNA has been reported to be decreased (Harrison et al., 1991; Eastwood et al., 1995), these results may be confounded by neuroleptic treatment. Increased NR2D subunit mRNA could also be interpreted as a compensatory mechanism to combat NMDA receptor hypofunction. It has been hypothesized that cortical NMDA receptor hypofunction produces the positive symptoms and hyperdopaminergia of schizophrenia because it can increase dopaminergic neurotransmission from the VTA to the NuAcc via a removal of GABA-mediated inhibition of dopaminergic neurons (Freeman and Bunney, 1984; French et al., 1991). Interactions between dopamine and glutamate relevant to schizophrenia are, however, just beginning to be understood. Specifically, cortical dopamine/ glutamate interactions are thought to underlie several positive and negative symptoms of the disorder (Moghaddam et al., 1997; Sulzer et al., 1998; Moore

et al., 1999), especially those such as hallucinations, delusions, and thought disorders which are thought to be exacerbated by stress (Finlay and Zigmond, 1997). These neurotransmitter interactions are likely to be the focus of much future schizophrenia research.

1.2.2 Theories of neuropathology

Although several neuropathological findings have been reported in schizophrenia, few are specific to the disorder and even fewer are consistently replicated. Investigators have attributed the inconsistencies to the structural brain abnormalities of schizophrenia being subtle rather than large (Jeste and Lohr, 1989; Fukuzako et al., 1995), to the heterogeneity of the clinical presentations of the disorder (Tsuang et al., 1990), and to differences in methodology (Andreasen and Black, 1997).

Although not exclusive to schizophrenia, significant reductions in volume have been found in the hippocampus (Jeste and Lohr, 1989; Bogerts et al., 1990; DeLisi et al., 1997), the thalamus (Andreasen et al., 1990; Pakkenberg, 1990, 1992, 1993), and the prefrontal cortex (PFC; Rajkowska et al., 1994) accompanied by ventricular enlargement (Weinberger et al., 1979, 1982; Bogerts et al., 1985; Brown et al., 1986; Crow et al., 1989; Suddath et al., 1989; Dauphinais et al., 1990; Raz and Raz, 1990; DeGreef et al., 1992) in those with the disorder. These findings suggest that neuronal atrophy and/or cell loss occurs at some point previous to, during, or after the acute clinical phase of the disorder. Because post mortem analysis of schizophrenic tissue is usually performed after a long course of illness and/or drug treatment, it is difficult to ascertain if this tissue loss occurred predominantly during prenatal neurodevelopment, at the time of symptom onset, or throughout the course of the illness. Thus, it is unknown whether neurodevelopmental and/or neurodegenerative processes underlie the neuropathology of schizophrenia, or if reductions in brain volume are reflective of reduced cell numbers, atrophy of

schizophrenia are often pitted against neurodegenerative theories, it is likely that schizophrenia involves both processes, as outlined in the next two sections.

1.2.2.1 Neurodevelopmental theories

The neurodevelopmental hypothesis of schizophrenia is currently the most widely accepted theory of the pathogenesis of schizophrenia (Duncan et al., 1999). It poses that a genetic defect or environmental insult experienced by the fetus in the second trimester of pregnancy initiates the cascade of events leading the development of schizophrenia (Murray and Lewis, 1987; Weinberger, 1987; Bogerts, 1993; Jones and Cannon, 1998). The symptoms of schizophrenia are not often seen until adolescence, primarily because the neural pathways normally required to execute the brain functions that are abnormal in schizophrenia are not recruited until the development of executive cognitive function at puberty (Weinberger, 1987). The adolescent onset of schizophrenia may seem to contradict a neurodevelopmental hypothesis since one might expect a preterm insult to have immediately apparent behavioral and/or physical consequences. The finding that the dissociative anesthetic ketamine produces hallucinations only in post pubertal adolescent and adults, but not children (Reich and Silvay, 1989), suggests that the anatomical substrates for psychosis do not develop until puberty.

Several clinical findings in schizophrenics support these developmental theories. For example, neurological soft signs including motor and cognitive abnormalities are often found in children who go on to develop schizophrenia (Erlenmeyer-Kimling and Cornblatt, 1987; Green et al., 1989; Walker and Lewine, 1990; Done et al., 1994; Jones and Cannon, 1998) and other neurodevelopmental disorders such as autism (Goodman et al., 1994; Rodier et al., 1997). Schizophrenics also possess a greater number of minor physical

abnormalities than the general population (Lane et al., 1996, Trixler et al., 1997, Ismail et al., 1998; Bassett and Chow, 1999; Torrey, 1999) which include such physical anomalies as clinodactyly (adduction of the distal phalanx of the fifth digit, i.e., a bent baby finger) and minor abnormalities of the ears, mouth and eyes (Akabaliev and Sivkov, 1998). Although lacking in clinical significance, these minor physical abnormalities are thought to reflect subtle errors of developmental migration that correlate with neuro-developmental abnormalities (Buckley, 1998). Since the prominence of minor physical abnormalities in schizophrenia has been associated with the severity of negative symptoms and with lower premorbid functioning (Buckley et al., 1994; Waddington et al., 1999), it is possible that the degree of mismigration is also related to the severity of these symptoms.

Neuropathological findings in schizophrenia such as reduced brain volume and ventricular enlargement have traditionally been attributed to abnormal neurodevelopment during the second trimester of pregnancy (Bogerts, 1993; Harrison, 1995). Some researchers have found that these decreases in brain volume are stable and do not change throughout the illness (Fukuzako et al., 1995). Specific cytoarchitectural changes in the cingulate cortex (Benes et al., 1991), the entorhinal cortex (Arnold et al., 1991), the parahippocampal cortex (Jakob and Beckman, 1986), the frontal lobes (Akbarian et al., 1993), the hippocampus (Iritani et al., 1999), and alterations of prenatal forms of neural cell adhesion molecules ("NCAMs"; Vawter et al., 1998) that suggest neuronal mismigration and/or a failure of "inside out" migration during neuro-development in the second trimester of pregnancy, provide even more evidence of the neurodevelopmental basis to this disease. Some laboratories have, however, not replicated these findings of altered neuronal migration (Akbarian et al., 1996). Nevertheless, both animal models of schizophrenia which mimic such prenatal cytoarchitectural changes (Lipska et al., 1993) and NCAM knockout mice (Wood et al., 1998) consistently show the anatomical

and behavioural abnormalities that characterize the disorder and support a role of neurodevelopment in schizophrenia.

The most convincing evidence of a failure of inside-out migration during neurodevelopement in schizophrenia comes from studies of "Reeler" mice and the reelin gene (Gonzalez et al., 1997). In Reeler mice, the loss of the reelin protein causes a failure of "inside-out" migration in the cortex and produces a disorganized "outside-in" pattern of neuronal migration (Caviness and Sidman, 1973; Caviness and Rakic, 1978; Goffinet, 1979; Caviness, 1982) similar to that seen in the brains of some schizophrenics (Fatemi et al., 1999). The Reeler mouse shows some behavioral similarities to schizophrenia, such as an impaired prepulse inhibition of startle (Tueting et al., 1999). In addition, reelin mRNA is decreased in the prefrontal cortex, hippocampus, temporal cortices, caudate, and cerebellum of schizophrenics, while reelin protein is also decreased in the temporal cortex (Impagnatiello et al., 1998) in comparison to controls. Although some sort of neurodevelopmental insult is thought to underlie most forms of schizophrenia, the mechanism of action is still unknown, and is certainly not currently amenable to treatment.

1.2.2.2 Neurodegeneration in schizophrenia

As previously discussed, neurodevelopmental theories of schizophrenia posit that neurodevelopmental insults during the second trimester of pregnancy produce the disarrayed or "dysconnected" neuronal circuitry which underlie the disorder, and that overt symptoms emerge only when these disordered pathways are recruited at adolescence. While few investigators would deny that neurodevelopmental abnormalities underlie the disorder, many would argue that these neuronal changes are not static but progressively worsen throughout the course of the illness. There is much clinical and anatomical evidence to suggest that some aspects of schizophrenia involve an active neurodegenerative component which starts

prior to the onset of the symptoms and continues throughout the disorder (Woods, 1998). This includes the observations which are described below:

a. Symptoms of schizophrenia worsen over time (section 1.2.2.2.1).

b. Early intervention with certain neuroleptics improves long term outcome (section 1.2.2.2.2).

c. Some anatomical changes in schizophrenia are ongoing throughout the course of the illness (section 1.2.2.2.3).

1.2.2.2.1 Symptoms of schizophrenia worsen over time

Traditionally, the term "neurodevelopmental" has been used in the literature to describe events occurring in fetal and perinatal life and not those of adolescence. The neurodevelopmental hypothesis of schizo-phrenia as such therefore explains a good deal of the initial patho-physiology of the disorder, but does not explain why some symptoms continue to worsen and become more irreversible after key neuro-developmental periods (i.e., post adolescence; McGlashan, 1998a, b).

Although negative and cognitive symptoms often tend to progress as the patient ages, the positive symptoms of schizophrenia generally remain stable or regress with age (Waddington et al., 1997 a,b). It is thus thought that the negative and cognitive symptoms of the disorder may be characterized by a neurodegenerative process, which is supported by several findings. For example, cross-sectional studies have shown that negative but not positive symptoms significantly worsen with age and duration of the illness (Waddington et al., 1997a,b) in both younger (Peralta et al., 1995) and older (Arnold et al., 1995; Davidson et al., 1995) patients. McGlashan and Fenton (1992) found that negative, but not positive, symptoms were variable early in the course of the illness. Since this deterioration of negative symptoms occurs

long after initial neurodevelopmental periods, and since negative symptoms are particularly difficult to treat (Crow, 1980), it is possible that an ongoing neurodegenerative process accompanies and underlies the progressive worsening and seeming irreversibility of these symptoms.

Like the negative symptoms, cognitive symptoms have been found to worsen with time (Arnold et al., 1995; Delisi et al., 1995; Delisi, 1996; Davidson and McGlashan, 1997), especially in patients who do not have marked cognitive dysfunction at the beginning of such longitutindal studies (Harvey et al., 1995; 1996). Bilder et al. (1992) found that cognitive deterioration continued in young patients after the first episode of psychosis, and in a nineteen year longitudinal study it was found that the participants had a seven times greater chance of developing psychosis if cognitive abilities declined from childhood to adulthood (Kremen et al., 1998). Since cognitive decline is integral to many post pubertal neurodegenerative disorders such as Alzheimer's disease, Huntington's disease, and even Parkinson's disease (which also affect brain circuitry purported to be altered in schizophrenia), it would not be surprising if the cognitive deficits accompanying schizophrenia are also characterized by neurodegeneration.

1.2.2.2.2 Early intervention with neuroleptics improves long term outcome

Many clinicians attest that some of the symptoms of schizophrenia not only worsen over time (McGlashan and Fenton, 1993; DeQuardo, 1998) but that untreated schizophrenia can eventually develop into a more permanent (reviewed by Waddington et al., 1998a,b; 1997a,b; Fenton and McGlashan, 1994) and less treatable state (Waddington et al., 1991, 1995, 1997a,b, 1998, 1999; Wyatt et al., 1991a, b; 1995; 1997; Falloon, 1992; Loebel et al., 1992; ; McGlashan and Fenton, 1992, 1993; McGlashan, 1996a, b; McGlashan and Johannessen, 1996; McGorry, 1998). Studies addressing the abilities of neuroleptics to arrest progression of some symptoms of the disorder were done as early as 1968 at a time when the efficacy of neuroleptic treatment was

still in question (Wyatt, 1995). May et al. (1976a,b, 1981) found that patients who received antipsychotic treatment required fewer days of rehospitalization two years following the initial discharge. This was supported by later studies by McGlashan and Fenton (1992) and Fenton and McGlashan (1994) who found that negative symptoms worsened in schizophrenic patients over a course of five years and then became stable and more resistant to treatment. Davidson et al. (1995) reviewed eight years of outcome in schizophrenia and also found that a longer duration of untreated psychosis was associated with negative symptoms and poor outcome, and Rzewuska (1994) found that the duration of untreated psychosis during the first year of illness was a clear predictor of long term outcome in the disorder. Thus, there is clear evidence that symptoms of schizophrenia continue to worsen long after the onset of adolescence and the onset of the disorder.

1.2.2.2.3 Anatomical evidence of neurodegeneration in schizophrenia

Due to the absence in schizophrenia of markers of neurodegeneration such as gliosis (Nieto and Escobar, 1972; Stevens, 1982), neuro-degenerative theories of schizophrenia have been few. While one group has found evidence of gliosis in schizophrenia (Arnold et al., 1995; Arnold and Trojanowski, 1996) this has not been consistently replicated (Roberts et al., 1986; 1987; Bogerts et al., 1990; Falkai et al., 1999). The absence of gliosis does not, however, necessarily rule out a neuro-degenerative process. For example, apoptotic cells are cleared within 24 to 48 hours after death and do not leave traces of gross morphological damage (Cotman and Su, 1996). Schizophrenia has been said to be an absolute deterioration from previous levels of functioning (Miller, 1989), resulting in the irreversibility of symptoms. If neurodevelopmental pathological changes are already present before the onset of schizophrenia, yet as the disorder progresses symptoms become less reversible, it is quite possible that these symptoms become less treatable because they are in part caused by neurodegeneration such as neuronal atrophy or cell death which occurs during the course of the illness.

Anatomical and imaging studies of schizophrenia support a role of neurodegeneration in the disorder (DeLisi et al., 1995; Coyle, 1996; Nair et al., 1997). For example, many authors have reported that ventricular enlargement progresses over the course of the illness (Woods et al., 1990; DeLisi et al., 1995, 1997). While progressive ventricular enlargement is thought to play a role in other psychiatric disorders, it is likely that it is the most severe in disorders which are difficult to treat, including schizophrenia and advanced manic depression.

A progressive reduction in size of several brain structures has also been found in schizophrenia (Delisi et al., 1995). Using MRI, Jacobsen et al. (1998) found that there was a progressive decrease in the size of temporal lobe structures over the course of the illness in childhood schizophrenia, and DeLisi (1997) also found progressive hippocampal atrophy over the course of the illness, regardless of the age of onset. Superior temporal gyrus (O'Donnell et al., 1995) and frontal lobe (Turetsky et al., 1995) volumes have been found to decrease with age and duration of the illness, and Waddington et al. (1991) found that the progressive ventricular enlargement accompanying schizophrenia correlated with increasing age and the later prominence of cortical atrophy.

There are many clinical correlates to anatomical findings in schizophrenia suggesting neurodegeneration. Not only do imaging and neuropsychological studies suggest that ongoing neuronal atrophy accompanies schizophrenia (Turner et al., 1986; Waddington et al., 1991; Delisi et al, 1997), but several investigators have found that such atrophy is associated with poor outcome (Woods et al., 1990; Woods and Yurgelun-Todd, 1991). Delisi (1996) found that ventricular enlargement occurred throughout the course of the illness and correlated with symptom severity. Frecksa et al. (1994) found that patients

with a predominance of negative and cognitive symptoms showed a greater increase in ventricular size on computerized tomography (CT) scans in comparison to controls. Arnold et al. (1995) found that "patients with marked cognitive deterioration over time showed more gliosis restricted to the subiculum and orbitofrontal cortex than did otherwise similar patients without such cognitive impairment", suggesting that there are specific subtypes of schizophrenia which are more likely to be characterized by neurodegeneration.

1.2.2.3 Theories of neurodegeneration in schizophrenia

The mechanisms of neurodegeneration in schizophrenia are unknown. It is possible that neurodegeneration during the active phase of psychosis is facilitated by several factors including the underlying neurodevelopmental pathology, select environmental insults at adolescence, and the biological process of psychosis itself. There are many types of neurodegeneration, some of which include cell death (necrosis, gliosis, apoptosis), neuronal atrophy (withdrawal of axons, dendritic shortening), and cell membrane damage (oxidation). Most of these types of neurodegeneration have been implicated in schizophrenia, as will be discussed below.

1.2.2.3.1 Cell death as intrinsic to schizophrenia

Although some investigators have reported evidence of gliosis in schizophrenia (Arnorld et al., 1995), gliosis does not seem to play a primary role in the disorder. Some authors (Margolis et al., 1995; Olney and Farber, 1995; Coyle, 1996) have hypothesized that programmed cell death (apoptosis) may be intrinsic to schizophrenia, but since apoptotic cells are cleared within 24 to 48 hours of the initiation of cell death (Vincent, 1995; Benjamins and Nedelkoska, 1996; Cotman and Su, 1996), subtle ongoing apoptosis in schizophrenia would be almost impossible to detect by current methods. Other investigators have found that apoptosis occurs in projection sites of lesioned ventral hippocampus in rat models of schizophrenia (Khaing et al., 1999; Ashe, 2000), suggesting that damage to one brain region can cause cell death in its

projection neurons (such as those affected in schizophrenia). Cell counts of entire brain regions in schizophrenia suggest some degree of cell loss (see Bogerts et al., 1983; Benes et al., 1986; Pakkenberg, 1990, 1992, 1993). Unless mutant genes directly implicated in apoptosis (such as Bcl-2, p53, etc.) can be identified as altered in schizophrenia, investigators must focus on other signs of impending cell death (such as reduction of dendritic and axonal extensions) to support this hypothesis (see Cattsa and Catts, 2000 for a review on the potential role of p53 in schizophrenia).

1.2.2.3.2 Neuronal atrophy and cell death are due to excessive prepubertal synaptic pruning

According to Huttenlocher (1979), synaptic density peaks during childhood, declines 30 - 40% during adolescence, and stabilizes in adulthood. Specifically, cortical development during adolescence is characterized by the substantial reductions of synapses (Hoffman and McGlashan, 1997). Since large-scale neuronal losses do not normally occur during adolescence (Mani et al., 1986), synaptic elimination at puberty is suspected to limit and refine connectivity between neurons (Huttenlocher, 1979).

Feinberg (1982) was the first investigator to suggest that schizophrenia occurs due to excessive pruning and synaptic elimination during adolescence, and that symptoms of schizophrenia do not appear until puberty due to the redundancy of synapses until pruning during adolescence. Many studies suggest that excessive synaptic pruning during adolescence is associated with schizophrenia (Hoffman and Dobscha, 1989; Keshavan et al., 1994; Garey et al., 1995; Glantz and Lewis, 1995; Margolis et al., 1995; Olney and Farber, 1995; Selemon et al., 1995; Keshavan, 1999). Not only would this theory explain a number of anatomical and clinical findings in schizophrenia, but it would also explain increased cell packing and decreased neuropil in the schizophrenic cortex (Selemon et al., 1995; see figure 1.1). As reviewed by

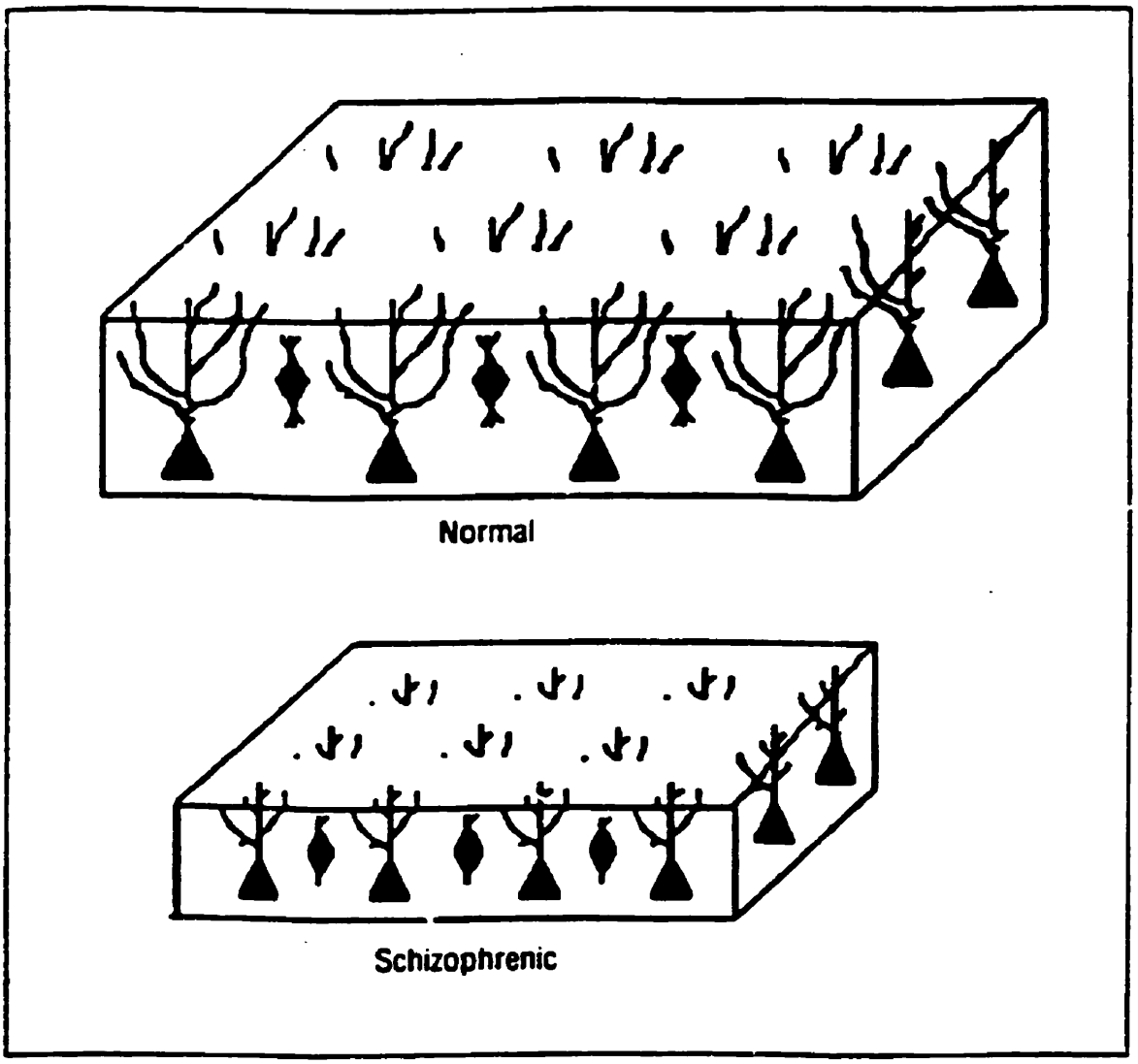

Figure 1.1. Schematic diagram of normal and schizophrenic cortices reproduced from Selemon et al., 1995. Neurons are smaller and neuropil is reduced in the schizophrenic coxtex. Although the relative number of neurons in the schizophrenic vs. control corticies appear to be "increased" per square unit of measure, it is more likely that there is either no change or is a reduction in cell numbers in the entire cortical area.

Waddington et al. (1997a, b), the brain regions where normal neuronal atrophy is seen are the same places where changes in schizophrenia are seen, and some have suggested that neurotrophin deficits could contribute to such a process (Bayer and Falkai, 1997). Thus, excessive synaptic pruning at adolescence might not only underlie neuronal atrophy in schizophrenia but may set up a compromised neural circuitry in the disorder which is more susceptible to neuronally damaging insults such as stress.

1.2.2.3.3 Neuronal atrophy and cell death are due to the toxic process of psychosis

The theory that neuronal atrophy and cell death in schizophrenia are due to the toxic processes of psychosis is quite similar to the hypothesis of excessive synaptic pruning. This hypothesis does not, however, propose that schizophrenia itself is characterized by abnormal synaptic pruning, but that the neuronal atrophy and cell death seen in the disorder are either caused by other factors extrinsic to the disorder such as stress (Nuechterlein et al., 1992) and drug abuse (Kril et al., 1997) or are epiphenomenal (Harrison, 1995) to the disorder (a result of psychosis). Synaptic pruning at adolescence may otherwise be normal. In other words, the additional reduction of neuropil in schizophrenia is not intrinsic to the disease process per say, but occurs either because stress will cause further neuronal damage on an already compromised neuronal circuitry (caused initially by neurodevelopmental abnormalities) and/ or because the process of psychosis itself (especially the positive symptoms) is excitotoxic to the brain and thus worsens negative symptoms by causing cell death and/ or atrophy in neurons previously made vulnerable by neurodevelopmental abnormalities. This hypothesis is supported by findings of increased adult forms of serum (Lyons et al., 1988) and CSF (Polkorak et al., 1995) NCAM, as this form of NCAM "has been found to be shed from tissues damaged by an ongoing disease process" (Waddington et al., 1997a, b).

1.2.2.4 Summary

On the whole, anatomical findings in schizophrenia imply that in addition to a probable neurodevelopmental defect in the disorder, there is a neurodegenerative component associated with symptom onset (Coyle, 1996; Delisi, 1997) that is positively affected by neuroleptic treatment (May et al., 1976a,b, 1981; McGlashen and Fenton, 1992; Fenton and McGlashen, 1994). Anatomical and clinical findings suggest ongoing neurodegeneration in schizophrenia. Although the neurodevelopmental hypothesis in part explains the initial pathophysiology of schizophrenia, it does not explain why these symptoms continue to worsen and become more irreversible at key neurodevelopmental periods such as adolescence. This not only suggests progressive neurodegeneration in schizophrenia, but that some neuroleptics may in part arrest this process.

The neurodegenerative hypothesis of schizophrenia can be summarized as follows: schizophrenia begins prenatally and progresses until reaching a critical threshold, typically in the second or third decade of life. Progressive brain volume loss is maximal in the first two decades and slows down with age. The tissue loss does not involve persistent gliosis, and damaged neurons may be removed by excessive neuronal apoptosis. Most likely, normal pruning at adolescence reduces connectivity in the pre-schizophrenic brain to levels which are now insufficient for normal neurotransmission, and additional neuronal atrophy and or cell death due to stress (Uno et al., 1989; Watanabe et al., 1992; Stein-Behrens et al., 1994) may cause further damage an already compromised circuitry. Increasing evidence that early intervention with neuroleptics can improve long term outcome demands that the molecular mechanism(s), by which neuroleptics could do this be elucidated, and is one of the primary foci of the present study. Thus, the apparent superior clinical efficacy of these neuroleptics may be due in part to their neuroprotective capabilities and their abilities to prevent further neurodegeneration in schizophrenia.

1.3 Treatment of schizophrenia

Although neuroleptic treatments for schizophrenia continue to improve, it is difficult to design efficacious medications for a disorder which is so poorly understood. Also, a neuroleptic which treats the majority of symptoms in the majority of patients is yet to be discovered (reviewed by Ayd, 1991; Lehmann and Ban, 1997).

1.3.1 History

The first efficacious treatment of the disorder was described by Sen and Bose in (1931), who reported treating insanity with the plant *Rauwolfia serpentina* (as cited in Bhatara et al., 1997). It is known today that there are several active alkaloids in this plant which produce similar antipsychotic effects. The use of this particular alkaloid, which is now known as reserpine (Giachetti and Shore, 1978; Guo, 1996), fell out of favor as other antipsychotics became available (Ayd, 1991). In 1951, Laborit and Huguenard administered the phenothiazine derivative chlorpromazine to presurgical patients as an anesthetic (Shen, 1999). Observations by Delay et al. (1952) of the mood stabilizing effects of chlorpromazine the led them to systematically test its effects in schizophrenics (Delay et al., 1952; Ayd, 1991). They found that while chlorpromazine could reduce psychotic and delusional symptoms, it did not affect "deficiency" (negative) symptoms (Delay et al., 1952).

The next landmark in the treatment of schizophrenia came with the synthesis of the butyrophenone haloperidol in 1958 by Bert Hermans of Janssen Pharmaceutica (Ayd, 1991; reviewed by Granger, 1999). Intended as an analgesic, Paul Janssen nevertheless arranged for psychiatrists to test haloperidol on patients before animal studies were complete (Ayd, 1991). Its subsequent clinical success precipitated the further development of many classes of neuroleptics including the azaphenothiazines, thioxanthines, benzoquinolizines, diphenylbutylpiperidines, and other butyrophenones and

phenothiazines (Lehmann and Ban, 1997), as well as the dibenzodiazepine derivative clozapine, which was first used in clinical practice in 1972 (Hector, 1998).

With the exception of clozapine, these neuroleptics all produced Parkinson-like motor extrapyramidal side effects (EPS), a side effect that, up until the 1970's, was taken as a mark of neuroleptic efficacy (Guo, 1996). The discovery that these neuroleptics blocked D_2 receptors with a potency that paralleled that of their antipsychotic effects (Creese et al, 1976; Seeman et al, 1976; Snyder, 1976), their ability to produce EPS, and the observations that the DA agonist amphetamine casued psychosis (Crowley et al., 1968; Espelin and Done, 1968) led researchers to form the initial dopamine hypothesis of schizophrenia. Clinical observations, however, that clozapine was efficacious in treating schizophrenia without producing EPS brought into question the necessity of EPS to predict the potency of neuroleptics (Stille and Hippius, 1971; Burki et al., 1973; Matz et al., 1974) and had researchers asking if the DA hypothesis could fully account for the symptoms, pathophysiology, and treatment of the disorder (Burki et al., 1975; Alpert and Friedhoff, 1980). Clozapine was thus called an "atypical" neuroleptic because it did not produce EPS as did the other "typical" neuroleptics of the 1970's (Lehmann and Ban, 1997).

1.3.2 The concept of typicality vs. atypicality

Although clozapine could treat the negative symptoms of schizophrenia, it carried with it a small risk of the fatal blood disorder agranulocytosis (Idanpaan-Heikkila et al., 1975; Baldessarini and Frankenburg, 1991). In addition, in some patients, clozapine did not completely ameliorate the positive symptoms of the disorder, suggesting that some D_2 blockade was still necessary for the treatment of positive symptoms. On the other hand, typical neuroleptics like haloperidol did not completely treat the negative symptoms of

the disorder (Strauss and Carpenter, 1977). They also produced motor side effects that not only reduced compliance (Van Putten, 1974; Sovner and DiMascio, 1978) but were sometimes, as in the case of tardive dyskinesia, irreversible (Cadet et al., 1986; Cadet and Lohr, 1989; Cadet and Kahler, 1994; Rotrosen et al., 1996). In the 1980's, pharmaceutical companies competed to produce a neuroleptic that would treat the positive, negative, and cognitive symptoms of schizophrenia without producing EPS or causing fatal blood dyscrasias, and the distinction between typical vs. atypical became the most popular way to characterize neuroleptics and guide their development. Because it was found that clozapine blocked 5-HT_2 receptors to a much greater extent than D_2 receptors, pharmaceutical companies aimed to design atypical neuroleptics with this receptor blocking profile. This led to several definitions of typical and atypical neuroleptics, as are described below (Lehmann and Ban, 1997):

a. Neuroleptics which cause EPS are called "typical", whereas neuroleptics which produce minimal EPS are called "atypical" (Fog, 1967; Matz et al., 1974; Mercugliano et al., 1992). "Typical" refers to all the classical neuroleptics which have the therapeutic efficacy and side effect profile of haloperidol, while neuroleptics producing a clinical profile similar to clozapine's would be called "atypical" (Lehmann and Ban, 1997). It wasn't until the late 1980's that other "atypical" neuroleptics (such as risperidone) became available for clinical use.

b. Neuroleptics which strongly block D_2 receptors in nigrostriatal (mesolimbic) pathways and weakly in the nucleus accumbens (mesocortex) are called "typical", whereas neuroleptics which weakly block nigrostriatal D_2 receptors and strongly block D_2 receptors in the nucleus accumbens are called "atypical" (Bunney et al., 1991).

c. Neuroleptics which block only dopamine D_2 receptors are called typical, whereas neuroleptics which block both dopamine D_2 and serotonergic 5-HT_2 receptors are called atypical (Meltzer, 1993).

d. Neuroleptics which ameliorate only the positive symptoms of schizophrenia are called typical, whereas neuroleptics which ameliorate positive, negative, and cognitive symptoms are called atypical. Currently, this is the most widely accepted distinction of typical vs. atypical neuroleptics, as it does not rely on the receptor blocking properties of the neuroleptic to predict its clinical response or its effect on dopaminergic systems (for example, remoxipride strongly blocks D2 receptors, but has an "atypical" therapeutic and side effect profile).

1.3.3 Typical neuroleptics used in the present study

1.3.3.1 Haloperidol

Haloperidol, a butyrophenone derivative (Janssen and Niemegeers, 1959; Divry et al., 1960), is one of the most commonly used "typical" neuroleptics in the treatment of schizophrenia. It strongly blocks nigrostriatal dopaminergic neurons (Snyder et al., 1973; Seeman et al., 1975), causes significant EPS (Divry et al., 1960), weakly blocks serotonergic receptors (Schotte et al., 1993), and either does not affect or worsens negative and cognitive symptoms (Crow, 1980, Van Putten, 1987). At therapeutic doses, it primarily has affinity for D_2 receptors, followed by $alpha_1$ adrenergic, D_1, and 5-HT_2 receptors (Schotte et al., 1996). Haloperidol is often still used as the first line treatment for the floridly psychotic patient who visits the emergency room for the first time. In light of the development of newer neuroleptics with more favorable side effect profiles, haloperidol is used less often, even in the treatment of first episode patients.

Along with a significant risk of producing EPS, haloperidol is highly sedative and may cause hyperprolactinemia. In addition, long term haloperidol

treatment may produce tardive dyskinesia (TD), a potentially irreversible motor disorder characterized by involuntary movements of the face, tongue, trunk, and extremities (Clemens et al., 1976; Clyne and Juhl, 1976; Gunne and Barany, 1976; Stimmel, 1976; Burt et al., 1977). Although the mechanism by which haloperidol causes tardive dyskinesia is unknown, many researchers suggest that haloperidol-like neuroleptics cause cell death in the affected motor regions (Cadet et al., 1986; Cadet and Lohr, 1989; Lohr et al., 1990; Lohr, 1991, 1992), perhaps due to increased free radical formation caused by neuroleptic induced increases in dopamine turnover (Stein and Wise, 1971; Graham et al., 1978; Matsumoto et al., 1983). This is supported by the finding of a toxic metabolite of haloperidol (Fang and Gorrod, 1991; Fang and Yu, 1997), increased iron in post-mortem brains of those with TD (Hunter et al., 1968; Campbell et al., 1985), the discovery of a reduced free radical scavenging enzyme in patients with neuroleptic induced TD (Yamada et al., 1997), and the improvement of TD following treatment with the antioxidant vitamin E (Lohr et al., 1987, 1988; Elkashef et al., 1990; Schmidt et al., 1991; Egan et al., 1992; Shriqui et al., 1992, Adler et al., 1992; Peet et al., 1993). Nevertheless, haloperidol remains an effective treatment for those schizophrenics who have predominantly positive symptoms.

1.3.4 Atypical neuroleptics used in the present study

The atypical neuroleptics chosen for the present study were primarily selected based on their receptor binding profiles. Since efficacious neuroleptics bind to D_2 and/ or 5-HT_2 receptors, three of the neuroleptics used (remoxipride, risperidone, ritanserin) were chosen based on their differential blockade of these receptors (D_2, D_2/ 5-HT_2, and 5-HT_2 receptors respectively). However, their degree of clinical use, availability for laboratory testing, and chemical structures were also considered for their inclusion in the planned experiments. Clozapine and haloperidol were chosen due to their substantial clinical use and for their different clinical indications in

schizophrenia, while olanzapine was included in later studies as a comparison to clozapine when it became available for laboratory use.

1.3.4.1 Risperidone

Risperidone is a benzisoxazole derivative which blocks both 5-HT_2 and D_2 receptors at clinically effective doses (Huang et al., 1993). It was the first successful new atypical antipsychotic since clozapine and a significant improvement over previous neuroleptics (Leysen et al., 1988). Not only can it treat both positive and negative symptoms of the disorder, but it produces less EPS and does not cause agranulocytosis (Claus et al., 1992; Chouinard et al., 1993). It is currently considered by many physicians to be first line treatment for schizophrenia, and some clinicians report that risperidone can even correct pre-existing motor abnormalities (Claus et al., 1992; Chouinard et al., 1993).

Risperidone binds most strongly to 5-HT_{2A} receptors, followed by noradrenergic alpha$_1$, D_2, histaminergic, serotonergic type 1A (5-HT_{1A}), noradrenergic alpha$_2$, and D_1 receptors (Megens et al., 1994; Leysen et al., 1992; Schotte et al., 1989). Risperidone and its active metabolite 9-hydroxyrisperidone, are equally potent receptor blockers (He et al., 1995). It has a narrow therapeutic window, as dose determines its 5-HT_2 to D_2 receptor blocking ratio in vivo (Janssen et al., 1988; Ereshefsky et al., 1993). At therapeutic levels, risperidone produces 50% 5-HT_2/ 20% D_2 blockade at low doses to >80% 5-HT_2/ >80% D_2 blockade at higher doses, roughly corresponding to 1-6 mg/day (Schotte et al., 1989; reviewed by Grant and Fitton, 1994). Lower doses lack efficacy, and higher doses produce EPS comparable to those caused by haloperidol (Claus et al., 1992, Chouinard et al., 1993). The narrow therapeutic window and dose-dependent variations in the relative 5-HT_2 to D_2 blocking ratio of risperidone make it an excellent candidate to study the molecular mechanisms underlying therapeutic efficacy vs. side effects.

1.3.4.2 Ritanserin

Ritanserin was designed as a pure 5-HT_2 receptor blocker a few years before the synthesis of risperidone (Leysen et al., 1985) and was used in conjunction with haloperidol to treat the negative symptoms of schizophrenia (Bersani et al., 1986; Ugedo et al., 1989; Kay and Sandyk, 1990). Its ability to reduce haloperidol-induced EPS led to the hypothesis that, at least in extrapyramidal motor systems, 5-HT_2 receptor blockade could counteract the effects of DA receptor blockade. Currently, it has limited clinical use due to availability of other compounds which better treat the whole spectrum of schizophrenic symptoms. It is still nonetheless a valuable research tool to determine the role of serotonin in the action of neuroleptics (Reyntijens et al., 1986).

1.3.4.3 Clozapine

Developed in the 1960's, the dibenzodiazepine derivative clozapine was the only neuroleptic able to treat the negative symptoms of schizophrenia until the advent of risperidone. It has a unique receptor binding profile, with a strong affinity for 5-HT_2, histaminergic, and noradrenergic alpha1 receptors.

Clozapine also binds to 5-HT_{1C}, D_2, noradrenergic alpha$_2$, cholinergic muscarinic, and 5-HT_{1A} receptors at higher doses, and blocks D_4 receptors with a potency 5 times greater than D_2 receptors (Kapur et al., 1999). Clozapine reduces both the positive and negative symptoms of schizophrenia (Maj et al., 1974; Meltzer, 1993) without inducing the EPS associated with traditional D_2 blockers, presumably in part due to its strong 5-HT_{2A} receptor antagonism (Bleich et al., 1988). Although it is arguably still the most potent neuroleptic for the refractory, negative symptoms of schizophrenia (Small et al., 1987; Kane et al., 1988), it is often used only as a last resort due to its potential to cause agranulocytosis and the consequent weekly blood monitoring required.

1.3.4.4 Olanzapine

Olanzapine, a thienobenzodiazepine, was introduced clinically in 1997. Structurally similar to clozapine except for a few minor substitutions, it was designed to have a clinical efficacy similar to clozapine's without causing agranulocytosis (Fulton and Goa, 1997). Unlike any of the other neuroleptics tested in the present study, its' strongest affinity is for muscarinic receptors, followed by 5-HT_{2A}, histaminergic, D_2, D_1, and noradrenergic $alpha_1$ and $alpha_2$ receptors (Bymaster et al., 1996; Schotte et al., 1996). It has greater D_2 receptor blockade than clozapine and can cause EPS, although like clozapine it can in some cases reverse particular motor dysfunctions (Littrell et al., 1998; O'Brien and Barber, 1998). It treats positive symptoms with the same efficacy as haloperidol and treats negative symptoms with an improved efficacy (Beasley et al., 1997). Although no direct comparisons have been made, many clinicians agree that clozapine is still more efficacious than olanzapine in treating the refractory negative symptoms of schizophrenia (Sanders and Mossman, 1999).

1.3.4.5 Remoxipride

Remoxipride, a benzamide derivative, was released in 1990. It is clinically equivalent to haloperidol in its ability to treat primarily the positive, but not the negative, symptoms of schizophrenia (Awad et al., 1997; Lapierre et al., 1999). Although it is a relatively selective D_2 blocker (Ögren et al., 1984), it produces EPS only at high doses, the reason for which is unknown (den Boer and Westenberg, 1990; Holm et al., 1993). Remoxipride is no longer used clinically because it causes blood dyscrasias such as aplastic anemia (Karlsson, 1993; Kerwin, 1993; Laidlaw et al., 1993; Philpott et al., 1993). Nevertheless, it remains a useful research tool to study the dopaminergic mechanisms underlying neuroleptic efficacy that are separate from the dopaminergic mechanism thought to underlie EPS.

1.4 Candidate genes potentially regulated by neuroleptics

Efficacious neuroleptics alter both dopaminergic and serotonergic neurotransmission. If dopaminergic and/or serotonergic receptor blockade were exclusively responsible for treating schizophrenia, one would expect to see clinical improvement almost immediately following neuroleptic administration (Pickar, 1988; Pickar and Breier, 1989). As clinical improvement in schizophrenia is not seen until two or three weeks after initiating chronic neuroleptic administration, the neural mechanisms by which neuroleptics produce their therapeutic effects must occur downstream from the receptor (Duman et al., 1994). Although these mechanisms are presently poorly understood, it is thought that the neuroleptic response involves a cascade of molecular events starting at the receptor and culminating at the nucleus of the neurons that produce long term changes in the expression of target genes (Hyman and Nestler, 1993, 1996; see Figure 1.2).

1.4.1 Signal transduction

Signal transduction is the term used to describe how cells take an extracellular message and convert it into the appropriate intracellular response (Charney et al., 1999). Such responses might include activation or inhibition of ion channels, neurotransmitter synthesis and release, or protein synthesis (reviewed by Stahl, 1999a-d). Chronic neuroleptic treatment is now known to produce long term changes in signal transduction by increasing or decreasing the synthesis of several target genes, including enzymes, receptors, and growth associated proteins (Hyman and Nestler, 1993), although their actual role in the neuroleptic response remains unknown.

Although neuroleptics have been shown to upregulate the gene expression of several proteins relevant to schizophrenia such as the dopamine D_2 receptor, the intracellular events leading up to such neuroleptic-induced changes are unknown (Duncan et al., 1999) and alterations in the target genes specific to the clinical response of the neuroleprics have not been identified.

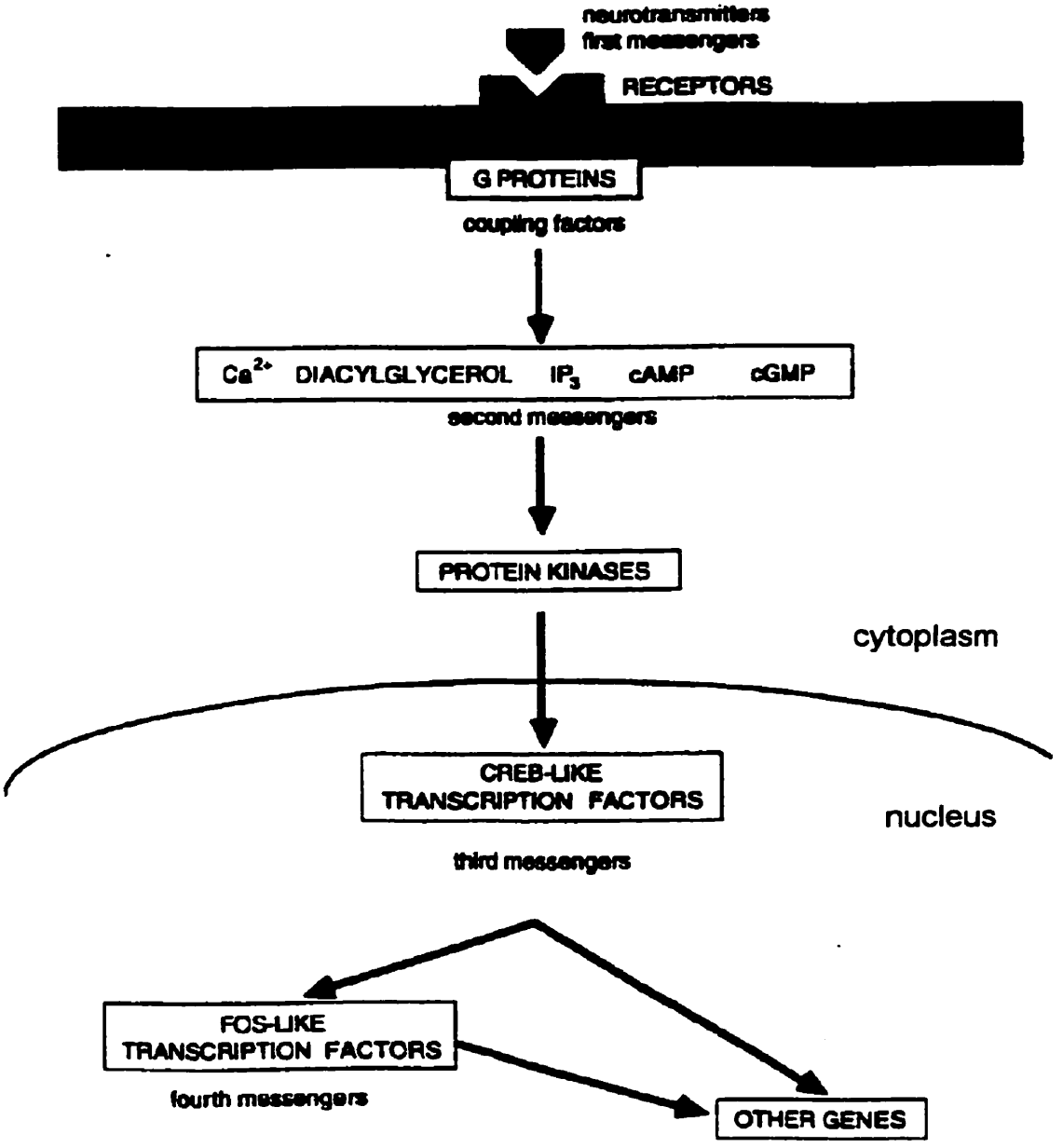

Figure 1.2: Schematic diagram depicting the intracellular signalling pathway for first, second, third and fourth messengers. Taken from Hyman and Nestler, 1993 (The Molecular Foundations of Psychiatry. Washington, DC: American Psychiatric Press).

The elucidation of long term changes in signal transduction and the regulation of target genes by chronic neuroleptic treatment is therefore the current focus of much neuroleptic research (Hyman and Nestler, 1993; 1996).

1.4.1.1 D_2 and 5-HT_2 receptor-mediated signal transduction

The neurotransmitters and peptides which bind to extracellular receptors are often referred to as the "first messengers" of the signaling cascade (Giacobini, 1976). With respect to neurotransmitter/ receptor initiated signal transduction, the extracellular message is detected at the nuclear level only after a number of sequential intracellular events take place. Although blockade of D_2 and 5-HT_2 receptors produce opposite effects on intracellular signaling cascades, they utilize similar signaling machinery (Charney et al., 1999).

Immediately coupled to D_2 and 5-HT_2 receptors are intracellular coupling factors called G-proteins (Battaglia et al., 1984; Titeler et al., 1984; Neve et al., 1989; Senogles et al., 1990). G-proteins are so named due to their ability to bind the nucleotides guanosine triphosphate (GTP), and guanosine diphosphate (GDP) (Hyman and Nestler, 1993). The trimeric G-protein does not bind to the receptor until it is occupied by its neurotransmitter, at which point the GDP-bound alpha subunit of the G-protein is phosphorylated. This in turn causes the displacement of GDP by GTP, which then causes the alpha subunit to dissociate from the rest of the G-protein (Cassel and Selinger, 1978; Cote et al., 1980). This subunit can then either directly mediate ion channels or membrane- bound enzymes such as adenylate cyclase (AC) which converts adenosine triphosphate (ATP) to the second messenger cyclic adenosine monophosphate (cAMP), and phospholipase C (PLC) which converts phosphatidylinositol (PI) to the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG) (Arad et al., 1984; Schramm and Selinger, 1984; Gonzales and Crews, 1985).

1.4.1.1.1 Specific signaling pathways of dopamine

The D_2 receptor is linked to a Gi/Go alpha subunit of its G-protein, meaning that the binding of DA to this receptor will either cause an influx of potassium (K+) or an inhibition of adenylate cyclase (AC; Neve et al., 1989; Senogles et al., 1990). Neuroleptic-induced antagonism of this receptor will therefore inhibit K+ influx or stimulate AC. In general, neuroleptic-induced activation of AC has been studied in much greater detail than neuroleptic induced inhibition of K+ channels (Ahn et al., 1976, Smith et al., 1988; Rodriguez-Sanchez et al., 1994). The activation of AC and the subsequent increase of intracellular cAMP then causes activation of the phosphorylating enzyme cAMP dependent protein kinase (cAMP dependent PK), which dissociates the inhibitory regulatory element from the catalytic subunit PKA (Gnegy et al., 1976).

Not only can PKA phosphorylate and therefore alter the function of a number of intracellular proteins, but it can phosphorylate a third messenger called the cAMP response element binding protein (CREB; Montminy and Bilezikjian, 1987). The CREB protein sits unphosphorylated on the cAMP responsive element on the promoter region of cAMP responsive genes and remains inactive until phosphorylated by PKA (Sun and Maurer, 1995). Once phosphorylated, CREB can increase the transcription of its target genes (Gonzalez et al., 1989). Example of genes containing CRE's and known to be subject to increased synthesis by CREB include tyrosine hydroxylase (Kim et al., 1993; Lazaroff et al., 1995; Ghee et al., 1998) and the early immediate gene (or "fourth messenger") *c-fos* (Sassone-Corsi et al., 1988).

1.4.1.1.2 Specific signaling pathways of serotonin

5-HT_{2A} receptors are linked to a Gq alpha subunit of its G-protein, which means that the binding of serotonin to this receptor will stimulate PLC activity (Roth and Chuang, 1987). Neuroleptic-induced antagonism of this receptor will therefore inhibit additional PLC activity, which in the long run will reduce

the release of Ca^{2+} stores and inhibit the activation of PKA (see Dwivedi and Pandey, 1999). Although blockade of 5-HT_2 receptors would not be expected to cause increases in fourth messengers such as *c-fos*, the inhibition of serotonergic neurotransmission may have effects on downstream neurons in which increases in such messengers may be observed (Hyman and Nestler, 1993).

1.4.1.1.3 Fourth messengers: transcription factors/ immediate early genes

Transcription factors are nuclear proteins which bind specific regulatory DNA sequences on target genes to either enhance or inhibit transcription (Curran et al., 1988). Immediate early genes (IEG's) are transcription factors which are induced rapidly and transiently upon stimulation and are often used to trace neuronal activity in the brain (Morgan et al., 1987; Morgan and Curran, 1989; 1991). They are induced by a variety of pharmacological, biological, and behavioural stimuli. Since the induction of a particular IEG may increase or decrease the rate of transcription of various other genes, a factor that changes levels of brain IEG's may in part exert its function by changing the amount of a particular target gene that is transcribed (Hyman and Nestler, 1993).

1.4.1.2 *c-fos*

1.4.1.2.1 Structure and function

C-fos is an early immediate gene which due to its ability to be induced by a wide variety of stimuli is often used as a marker of neuronal activity (Dragunow and Fuall, 1989). It is rapidly induced throughout the CNS by a number of stimuli, including seizures (Morgan et al., 1987), morphine (Chang et al., 1988), caffeine (Nakajima et al., 1989), cocaine (Brown et al., 1992), amphetamine (Nguyen et al., 1992), stress (Duncan et al., 1993), fear (Beck and Fibiger, 1995), and nicotine (Paglusi et al., 1996). Its transcription can be activated within minutes (Greenberg and Ziff, 1984; Greenberg et al., 1985). Its mRNA reaches its highest levels about 30 to 45 minutes following

stimulation, with peak protein levels occurring about two hours following stimulation (Muller et al., 1984). It appears that increases in levels of the *c-fos* gene product (the protein) are primarily regulated at the transcriptional level (Greenberg et al., 1986), meaning that its mRNA must first be increased in order to elevate its protein levels.

The *c-fos* gene encodes a 380 amino acid 55 kDA protein (Fos) which after phosphorylation has an apparent molecular weight of 62 kDA (Curran et al., 1984). After dimerizing with other proteins in its "leucine zipper" family (Fos, Jun, JunB, JunD; Rauscher et al., 1988; Fugii et al., 1991), Fos binds to the activator protein-1 (AP-1) binding site in the enhancer region of DNA (Chiu et al., 1988; Curran and Franza, 1988; Morgan and Curran, 1991) and usually facilitates the transcription of its target genes [note: Fos/JunB dimers have been shown in some cases to inhibit transcription (Chiu et al., 1989; Schutte et al., 1989)]. It has a CRE (Sassone-Corsi et al., 1990), is phosphorylated by cAMP dependent PK and calcium (Ca^{2+})/calmodulin dependent PK (Curran et al., 1987) and inhibits its own transcription when phosphorylated (Schonthal et al., 1991). Examples of genes containing AP-1 sites in their promoter regions and known to be subject to increased synthesis by Fos include proenkephalin (Uhl et al., 1988), neurotensin/neuromedian N (Kislauskis and Dobner, 1990), and tyrosine hydroxylase (Icard-Liepkalns et al., 1992).

1.4.1.2.2 Regulation by neuroleptics

Interest in the role of *c-fos* in the neuroleptic response first began when it was found that a single intraperitoneal (i.p.) injection of haloperidol dose-dependently increased striatal *c-fos* mRNA 30 minutes to one hour after the injection (Dragunow et al., 1990; Miller, 1990). This increase could be reversed by a D_2 agonist (Miller, 1990). Given that haloperidol's propensity to cause EPS is linked to its blockade of striatal D_2 receptors, many investigators proposed that not only could striatal *c-fos* activation be used to predict the EPS liability of neuroleptics, but that neuroleptic-induced *c-fos* activation in

other brain regions might be used to help elucidate other neural substrates underlying neuroleptic efficacies and side effect profiles. In addition, it was thought that, being a transcription factor, the Fos gene might play a role in the long term regulation of target genes which contribute to the neuroleptic therapeutic response.

Over the last ten years, a large number of investigators have examined the regulation of the Fos family of proteins by neuroleptics (Deutch, 1992; Robertson and Fibiger, 1992; Dilts et al., 1993; Merchant and Dorsa, 1993, Fibiger, 1994; Fink-Jensen and Kristensen, 1994; Robertson et al., 1994; Merchant et al., 1994; Deutch et al., 1995; Deutch and Duman, 1996; Semba et al., 1996; Nomikos et al., 1997; Arnt and Skarsfeldt, 1998; and many others). The most interesting findings were that as hypothesized, regionally specific induction of *c-fos* was caused by typical vs. atypical neuroleptics. Whereas typical neuroleptics such as haloperidol increased c-fos mRNA and/or the Fos protein in the striatum but not the mPFC, the opposite was true for atypical neuroleptics such as clozapine that had superior efficacy in treating negative and cognitive symptoms (Robertson and Fibiger, 1992). In addition, it was found that all neuroleptics increased *c-fos* mRNA in the nucleus accumbens, suggesting that activation of this structure might underlie neuroleptic efficacy. Later studies also demonstrated that both typical and atypical neuroleptics increased *c-fos* mRNA in the lateral septal nucleus (Robertson et al., 1994).

1.4.1.3 Delta FosB

1.4.1.3.1 Structure and function

Delta FosB is a member of the Fos family of transcription factors (Zerial et al., 1989). It is a 35 kDA protein that is a splice variant of the 338 amino acid FosB gene which encodes both the FosB and delta FosB gene (Mumberg et al., 1991, Nakabeppu and Nathans, 1991). The delta FosB protein lacks the last 101 amino acids found in the C-terminus of FosB, but the proteins are

otherwise identical in structure and have similar binding characteristics (Nakabeppu and Nathans, 1991). Like *c-fos*, delta FosB preferably dimerizes to the Jun family of transcription factors and binds to AP-1 sites in the promoter region of target genes (Zerial et al., 1989). Unlike *c-fos*, delta FosB dimers are not strong activators of transcription and may actually repress transcription by competitively inhibiting the binding of transcription activating Fos/Jun dimers (Mumberg et al., 1991; Nakabeppu and Nathans, 1991). In addition, as opposed to *c-fos*, apparent increases (accumulation) of delta FosB seem to be primarily regulated by phosphorylation while mRNA levels of delta FosB are activated rapidly and transiently (Chen et al., 1997).

1.4.1.3.2 Regulation by neuroleptics

Delta FosB has been shown to have not short, but long induction kinetics, meaning that it is not rapidly increased in the short term but when increased remains elevated for long periods of time. While delta FosB has been shown to be upregulated for periods of greater than 3 months after the experimental manipulation in certain experimental paradigms (Doucet et al., 1996), increases in *c-fos* have been shown to be desensitized by chronic treatments, including chronic haloperidol and clozapine treatment (Merchant et al., 1995; Sebens et al., 1995). Recent studies have shown that chronic (but not acute) haloperidol and clozapine administration increases delta FosB in regions similar to, although not identical with, those where *c-fos* mRNA upregulation is seen upon acute administration (Hiroi and Graybiel, 1996; Vahid-Anisari et al., 1996). Since the therapeutic effects of neuroleptics are not seen until after several weeks of chronic drug administration (Pickar, 1988; Pickar and Breier, 1989; Stern et al., 1993), it is of interest that unlike *c-fos*, neuroleptic induced changes in delta FosB follow a time pattern similar to the emergence of the therapeutic efficacy of the drug.

1.4.2 Target genes I: synthetic enzymes

1.4.2.1 Tyrosine hydroxylase (TH)

1.4.2.1.1 Regulation and function

The enzyme tyrosine hydroxylase (TH) (EC 1.14.16.2) hydroxylates the *meta* position of tyrosine to form the precursor of DA, 3,4-dihydroxy-L-phenylalanine (L-DOPA). It is considered to be the rate limiting enzyme in the synthesis of the catecholamines (Spector et al., 1965) DA, noradrenaline, and adrenaline (Levitt et al., 1965). It is found in catecholamine-synthesizing cells in the central and peripheral nervous systems (Elfvin et al., 1975; Pickel et al., 1975a, b), and TH mRNA is found in regions such as the substantia nigra, ventral tegmental area, and the locus coeruleus (Han et al., 1987), and its protein is found in projection neurons of these areas (such as the striatum, nucleus accumbens, and cortex/thalamus respectively) as well as in adrenal glands and chromafin tissue (Pickel et al., 1975a,b).

TH is a heavily regulated enzyme. It is activated by phosphorylation by either PKA (Lovenberg and Bruckwick, 1975), Ca^{2+}/calmodulin- dependent PKII (Vulliet et al., 1984), or PKC (Albert et al., 1984) at specific serine residues. It is subject to long term regulation, such as its synthesis being increased by nerve stimulation (Thoenen et al, 1969) and cold stress (Gordon et al, 1966). The enzyme is inhibited by its end product (Ikeda et al, 1966; Nagatsu et al., 1964), is inactivated by cAMP- dependent phosphorylation activation (Vrana and Roskoski, 1983) and can autophosphorylate. It has an AP-1 site (Icard-Liepkalns et al., 1992) and a CRE (Kim et al., 1993; Lazaroff et al., 1995; Ghee et al., 1998) site on its promoter and is regulated by Fos (Icard-Liepkalns et al., 1992).

1.4.2.1.2 TH in schizophrenia

Although the DA hypothesis of schizophrenia is no longer considered to be sufficient to explain schizophrenia, TH is still suspected to play a role in the disorder. Many recent investigations of polymorphisms of the TH gene in

schizophrenia have examined 1st intron polymorphisms, microsatellite repeats, allele/genotype frequencies, and amino acid substitutions. For example, two groups have found that polymorphisms of the 1st intron of the TH gene are related to differences in catecholamine turnover (Thipaut et al., 1997; Wei et al., 1997). Microsatellite DNA tetra repeats (TCAT*)n in the first intron have been found to be associated with altered excitability of noradrenergic neurons in schizophrenia, and have been found in unaffected relatives of patients with schizophrenia (Meloni et al., 1995). Not only have others failed to replicate these results (Ishiguro, 1998; Jonsson et al., 1998), but the finding of altered satellite DNA in non-affected relatives suggests that disruptions of the TH gene are not sufficient to cause schizophrenia, or that TH just happens to be linked to the causative genes. Changes in allele and genotype frequencies have not been replicated (Jonsson et al., 1996), nor has an amino acid substitution at valine 486 for methionine (Kunugi et al., 1998) been replicated (Ishiguro et al., 1998).

Changes in TH enzyme activity and mRNA/ protein levels in schizophrenia are not clear. Higher TH activity has been observed in the caudate putamen (CP) of schizophrenics (Toru et al., 1982), but this was not found by Crow et al. (1979). While Parkinsonian brains have low levels of TH mRNA in the substantia nigra as compared to controls, schizophrenic brains do not show these changes, although some schizophrenics have been found to have lower TH mRNA relative to DOPA decarboxylase mRNA levels (Ichinose et al., 1994). Even if there were consistent findings of altered TH in schizophrenia, post mortem analyses of TH protein and mRNA levels may be confounded by neuroleptic treatment (see below), and the schizophrenic subjects in the study of Ichinose et al. (1994) had been treated with neuroleptics.

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*Nucleotide bases of DNA: T= thymine, C=cytosine, A=adenine, G=guanine

1.4.2.1.3 Regulation by neuroleptics

Acute administration of neuroleptic drugs such as haloperidol has been found to increase TH enzyme activity (Zivkovic and Guidotti, 1974; Zivkovic et al, 1974), while chronic neuroleptic treatment can decrease it (Lerner et al, 1977). Remoxipride (5-125 μmol/kg i.p.) increases striatal DA turnover (as indicated by increased 3,4-dihydroxyphenylacetic acid [DOPAC] and homovanillic acid [HVA]) and increases the rate of striatal DA disappearance following inhibition of TH (Magnusson and Fowler, 1989).

Acute haloperidol and spiperone have been shown to upregulate TH mRNA in mouse striatum (Cho et al., 1997). Cottingham et al. (1990; 1991), however, found no changes in TH mRNA in the substantia nigra (SN), VTA, or locus coeruleus (LC) upon acute and chronic haloperidol administration (3 or 19 days). In addition, Buckland et al. (1992; 1993) found no effect of 0, 1, 2, 4, 8, 16, or 32 day haloperidol, loxapine, sulpiride, or clozapine treatment on whole brain TH mRNA. Levinson et al. (1998) found that haloperidol withdrawal downregulates nigral TH IR increasingly over time, demonstrating that continued neuroleptic administration is required to maintain induction of TH IR. This, along with the mRNA data, suggests that DA receptor blocking neuroleptics may increase transcription of TH mRNA, that this may translate to protein, and that withdrawal of neuroleptics stops this upregulation. It is interesting that chronic antidepressant treatment has a differential effect on TH, whereby it downregulates TH mRNA (Nestler et al., 1990) and alters different aspects of monoamine synthesis (Moret and Briley, 1992).

1.4.2.2 Aromatic L-amino decarboxylase (AADC)

1.4.2.2.1 Regulation and function

Both DA and 5-HT synthesis require the enzyme aromatic L-amino acid decarboxylase (AADC) (EC4.1.1.28) for decarboxylation of the amino acids L-DOPA and 5-hydroxytryptophan (5-HTP; Lovenberg et al., 1962; Christenson

et al., 1972). AADC is found throughout the central and peripheral nervous systems in catecholamine- and serotonin-containing neurons (Hökfelt et al., 1973). AADC mRNA is located in those brain areas that contain dopaminergic and serotonergic cell bodies (Coge et al., 1990; Tisson et al., 1991; Eaton et al., 1992, 1993) such as the SN, CP, LC, raphe nuclei, brain stem, the nuclei of the hypothalamus, the limbic system, as well as in the circumventricular organs and the pituitary gland (Black and Geen, 1975; Saavedra, 1977). Both AADC mRNA (Li et al., 1992) and protein (Juorio et al., 1993) have been found in glial cells, where AADC is proposed to decarboxylate stray L- amino acids (Berry et al., 1996).

Since the brain has relatively high activity of AADC and the concentrations of L-DOPA and 5-HTP are lower than the Michaelis Menten Constant (Km) of the enzyme, it has been assumed that the enzyme is not rate limiting, at least with respect to the synthesis of DA and 5-HT (Dairman et al., 1971; Bowsher and Henry, 1986). This assumption has been supported by the apparent absence of DA or 5-HT decreases following AADC inhibition (Brodie et al, 1962). It is likely, however, that AADC is the rate limiting factor in the treatment of Parkinson's disease with L-DOPA (Gjedde et al., 1993). The finding that brain AADC is rate limiting for the synthesis of the trace amines such as phenylethylamine and tryptamine (Lovenberg et al., 1962; Boulton, 1976) and is regulated by DA receptors (Zhu et al., 1992; 1993; 1994 Hadjiconstantinou et al., 1993) suggests that AADC is a regulated enzyme and that its role in monoamine synthesis may be more important than once believed (re-viewed by Zhu and Juorio, 1995; Berry et al., 1996; Fisher et al., 2000).

Dopamine D_2 receptor blockade has been shown to increase AADC activity (Rossetti et al., 1990; Zhu et al., 1992), while stimulation of this receptor has the opposite effect (Hadjiconstantinou et al., 1993, Zhu et al., 1993). There are many other molecular mechanisms by which AADC is

regulated (for review, see Berry et al., 1996), and its primary protein structure has phosphorylation sites for both PKA and PKC (Kemp and Pearson, 1990) which are a part of the signal transduction pathways for dopamine and serotonin respectively.

1.4.2.2.2 AADC in schizophrenia

AADC is widely distributed in many brain regions, several of which have been implicated in the pathophysiology of schizophrenia. Since the therapeutic actions of neuroleptics are attributed to their D_2 and/or 5-HT_2 receptor blockade, and since AADC is the final enzyme in the synthetic pathway of both of these neurotransmitters, it would not be surprising if AADC played a role in the pathophysiology of schizophrenia. No known allele polymorphisms of AADC have, however, been identified in schizophrenia, and no post mortem changes in AADC mRNA have been found in schizophrenic brains (Ichinose et al., 1994), although two allele variants have been found in bipolar disorder (Borglum et al., 1999). Although imaging studies have revealed increased AADC in the brains of living schizophrenic patients, these findings may be confounded by neuroleptic administration (Reith et al., 1994). Theories of the possible role of AADC in schizophrenia have rested primarily on the effects of various psychoactive drugs on AADC mRNA, as discussed below.

1.4.2.2.3 Regulation by neuroleptics

An elevated expression of AADC mRNA after neuroleptic expression has been found in many paradigms, suggesting that the alteration of its message by neuroleptics may contribute to the therapeutic mechanism of these drugs. Buckland et al. (1992) found that acute and chronic haloperidol or loxapine significantly increased AADC but not TH mRNA over time (1, 2, 4, 8, 16, 32 days) in whole brain homogenates of rats (sacrificed 4 hrs after last injection, injected twice a day) to 240% and 180% of control levels at 32 days. Sulpiride

(Buckland et al., 1992) and clozapine (30 mg/kg/day) were without effect over the 32 day injection period, demonstrating that only some neuroleptics can induce AADC mRNA.

Activity of AADC is also altered by dopaminergic drugs. Zhu et al. (1993) found that *cis*-flupenthixol and remoxipride increased striatal AADC activity significantly above controls, while bromocriptine (10mg/kg), but not (-)-quinpirole or d-amphetamine, decreased AADC activity in comparison to controls. These findings together suggest that downregulation of AADC mRNA by neuroleptics might cause a compensatory increase in AADC enzyme activity. Cho et al. (1997) found that haloperidol and spiperone upregulated AADC protein levels and increased enzyme activity, but not AADC mRNA levels in the mouse striatum, suggesting that Buckland et al.'s findings of neuroleptic induced upregulation of AADC mRNA occurs in brain regions other than the striatum. Therefore, regardless of the relative dopaminergic vs. serotonergic blockade of a particular neuroleptic, it is possible that a shared mechanism of action of neuroleptics is the upregulation of AADC mRNA.

Interestingly, Buckland et al. (1996) found that chronic administration of the dopamine agonist amphetamine and the GABA agonist vigabatrin downregulated AADC mRNA increasingly over 8, 16, and 32 days in the hippocampus, olfactory tubercles, hypothalamus, striatum, cerebellum, cortex, NuAcc, and PFC, while cocaine (40 and 50 mg/kg/day) was without effect. This led Buckland et al. (1996) to hypothesize that factors which cause psychosis decrease AADC mRNA, while those that ameliorate psychosis increase AADC mRNA. Buckland et al. (1997), however, found that 32 day PCP or LSD administration upregulated AADC mRNA in the striatum, nucleus accumbens, hippocampus, and cerebellum between 50 and 150% over controls, demonstrating that psychosis-inducing agents can either upregulate or downregulate AADC mRNA.

1.4.3Target genes II: Selected factors affecting neuroprotection

Neuroanatomical studies of schizophrenia suggest that progressive neuropathological changes (such as neuronal atrophy and/or cell death) occur over the lifetime course of the disease (DeLisi et al., 1995; Coyle, 1996; Nair et al., 1997). Early intervention with atypical neuroleptics has been shown to prevent progression of at least some symptoms, although the mechanisms by which neuroleptics may do this remain unknown (Waddington et al., 1991, 1995, 1997a,b, 1998a,b, 1999; Wyatt et al., 1991a,b; Falloon, 1992; Loebel et al., 1992; McGlashan and Fenton, 1992, 1993; Fenton and McGlashan, 1994; McGlashan and Johannessen, 1996; McGlashan, 1996a, b; McGorry, 1998).

There are at least three mechanisms by which neuroleptics may prevent further neurodegeneration in schizophrenia. The potential mechanisms of interest in the present study and selected factors which may be involved in neuroprotection are as follows:

a. Protection against cellular oxidation: superoxide dismutase (SOD1; section 1.4.3.1)

b. Protection against apoptosis: low affinity nerve growth factor receptor (p75; section 1.4.3.2)

c. Protection against neuronal atrophy: brain derived neurotrophic factor (BDNF, section 1.4.3.3)

1.4.3.1 Superoxide dismutase (SOD)

1.4.3.1.1 Regulation and function

A possible target gene of neuroleptic regulation includes the Cu^{2+}/Zn^{2+} dependent superoxide dismutase (SOD1, E.C.1.15.1.6), a 32 kDA homodimeric metalloenzyme which reduces cellular oxidative stress and neuronal damage via the inactivation of oxygen free radicals (Hartz and

Deutsch, 1972). SOD is a ubiquitous enzyme in all aerobic cells, but has been found in greater amounts in large pyramidal neurons of the neocortex and hippocampus (Delacourte et al., 1988; Ceballos et al., 1991). There are three isozymes of SOD, including the Cu^{2+}/Zn^{2+} form (SOD1; which exists in the cytosol; McCord and Fridovich, 1969), the Mn^{2+} dependent form (SOD2; mitochondrial; Marklund, 1982), and an extracellular form (Weisiger and Fridovich, 1973). Although there is no known AP-1 site on the SOD1 promoter, the major activator for transcription of SOD1 is the CCAAT enhancer binding protein C/EBP alpha (Kim et al., 1997; Seo et al., 1997).

SOD reduces reactive oxygen species through the dismutation of superoxide anion into molecular oxygen and hydrogen peroxide (McCord and Fridovich, 1969; Furuta et al., 1995). A primary target for damage by free radicals is the fatty acids which are in cell membranes. In neurons, where there are high levels of oxygen consumption, cell membranes are particularly susceptible to lipid peroxidation (Furuta et al., 1995). Numerous factors which are neuroprotective upregulate SOD1 activity and/ or transcription, such as neurotrophins (Wengenack et al., 1997), L-deprenyl (Li et al., 1998) and ginsenosides (Kim et al., 1996). SOD1 is upregulated by the neurotrophin brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF; Mattson et al., 1995), and *in vivo* studies have demonstrated that upregulation of this enzyme is neuroprotective in ischemia (Wengenack et al., 1997) and glutamate neurotoxicity (Furukawa et al., 1997). Mutant, dysfunctional SOD1 has been associated with motor neuron degeneration resembling amyotrophic lateral sclerosis (ALS; Wengenack et al., 1997). Reduced SOD1 activity has been found in ALS (Kong and Xu, 1998) and point mutations in SOD1 have been found in patients with the familial form of this disorder (Deng et al., 1993; Rosen et al., 1993). Lifespan is reduced in SOD1 knockout mice (Schwartz and Coyle, 1998), suggesting that this enzyme plays a significant role in the aging process as well.

1.4.3.1.2 SOD in schizophrenia

Oxidative stress is long thought by many to be involved in the pathology of schizophrenia (Lohr, 1991; Cadet and Kahler, 1994; Goodman, 1994; Mahadik and Gouda, 1996; Mahadik and Mukherjee, 1996; Mahadik and Scheffer, 1996; Mukherjee et al., 1996; Reddy and Yao, 1996; Baez et al., 1997; Smythies, 1997) and tardive dyskinesia (Lohr et al., 1990; Yamada et al., 1997). There have been findings of increased superoxide anion production by neutrophils from the blood of schizophrenic patients (Melamed et al., 1998), and Mukherjee et al. (1996) found that impaired antioxidant systems including reduced SOD activity were present at the onset of psychosis. Reduction of SOD1 activity has also been observed to play a role in dopamine- mediated neurotoxicity (Greenlund et al., 1995) which may be of significance in the hyperdopaminergia of schizophrenia.

1.4.3.1.3 Regulation by neuroleptics

SOD1 is a ubiquitous enzyme and is widely distributed in the central nervous system (CNS), including many regions purported to be atrophied in schizophrenia such as the hippocampus and cerebral cortex (Lohr et al., 1990; Selemon et al., 1995). It is therefore conceivable that upregulation of the gene expression of this enzyme by neuroleptics could prevent further free radical induced neurotoxicity/ neurodegeneration in schizophrenia. It is likely, however, that neuroleptics which induce TD (such as strong D2 blocking neuroleptics like haloperidol) have an opposite effect on SOD1 from neuroleptics such as clozapine which appear to reverse TD (Delecluse et al., 1998; Gao et al., 1998; Karp et al., 1999). Haloperidol decreases SOD1 enzyme activity, and SOD1 activity is decreased in tardive dyskinesia in the erythrocytes of haloperidol treated patients (Yamada et al., 1997). Long term treatment with the typical neuroleptic chlorpromazine, but not the mood stabilizer lithium, decreases SOD1 activity in the brain, liver and erythrocytes of rats (Abdalla and Bechara, 1994), further suggesting that reduced SOD1 activity is associated with D_2 receptor blockade. Haloperidol has been

specifically shown to create reactive oxygen species (ROS; Sagara, 1998; Yao et al., 1998), while Behl et al., (1996) have demonstrated that oxidative stress-resistant cells are protected against haloperidol-induced neurotoxicity.

1.4.3.2 The low affinity nerve growth factor receptor (p75)

1.4.3.2.1 Regulation and function

A second candidate gene includes the low affinity nerve growth factor receptor (p75), a 75 kDA transmembrane cytokine receptor-like protein which constitutively induces neural cell death in the absence of its primary ligand, nerve growth factor (NGF) (Thoenen and Barde, 1980; Levi-Montalcini, 1987; Greene and Kaplan, 1995; Kaplan and Miller, 1997; Muller and Clos, 1997). The expression of p75 receptor mRNA in adults is primarily limited to the medial septal nucleus and the nucleus of Broca's diagonal band (Vazquez and Ebendal, 1991).

The p75 receptor has been nicknamed the "death" receptor. While cultured rat pheochromocytoma (PC12) cells normally do not need NGF to survive, treatment of such cultures with NGF causes the induction of the p75 receptor. Induction of p75 is thought to make these neurons "dependent" on NGF, because if the NGF is withdrawn, the PC12 cells will die. This is supported by the finding that both the rate and extent of PC12 cell death following NGF withdrawal is determined by the degree of p75 expression (Barrett and Georgiou, 1996). In addition, both the antisense blockade of its mRNA (Barrett and Georgiou, 1996) and the inhibition of its function by antibodies (Rabizadeh et al., 1993; Farde et al., 1996) have been associated with reduced cell death in PC12 cells. Conversely, the p75 gene has an AP1 site on its promoter as well as many others, but is more likely regulated by *c-jun* than *c-fos*. Its primary transcription factor is *Zif268/Egr-1* (Nikam et al., 1995).

1.4.3.2.2 Structure of neurotrophins and their receptors

The p75 receptor will bind all members of the neurotrophin family with an equal affinity (Lipton and Kalil, 1995); these include the prototypical NGF, brain-derived neurotrophic factor (BDNF), and the more recently discovered neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Snider and Johnson, 1989; Snider, 1994). All members of the neurotrophin family are 12 kDa basic proteins that share significant structural homologies (Hofer et al., 1990; Maisonpierre et al., 1991a), including several similar amino acid sequences surrounding the neurotrophins' six conserved cysteine residues and a 48% amino acid homology between the three neurotrophins (Maisonpierre et al., 1991b). Neurotrophins are produced by the cleavage of larger precursor protein with the mature protein, corresponding to the C-terminal half of the precursor (Darling et al., 1983), but all are products of separate genes (Maisonpierre et al., 1991a). Regardless, mature forms, tissue distribution and neuronal specificities of the neurotrophins are conserved across mammals (Maisonpierre et al., 1991a).

Although each neurotrophin preferentially binds to its receptor as a homodimer, there is some overlap in receptor binding, and NT-3/ BDNF heterodimers (Philo et al., 1994) do bind to both TrkB and TrkC receptors in vitro (Arakawa et al., 1994). Though the function of p75 is unclear, it is thought that in addition to playing a direct role in cell death, it may enhance dimerization of the neurotrophins (see Carter and Lewin, 1997 for review and illustration) and post receptor neurotrophin signaling (Davies et al., 1993; Verdi et al., 1994).

1.4.3.2.3 p75 in schizophrenia

There are no reports of alterations of p75 in schizophrenia. It is highly possible, however, that p75-positive neurons and their targets are altered in the disease. As previously discussed, the expression of p75 receptor mRNA in adults is primarily limited to the medial septal nucleus

and the nucleus of Broca's diagonal band (Vazquez and Ebendal, 1991). These nuclei of the basal forebrain contain cholinergic neurons which project to the hippocampus and cerebral cortex (Ebendal, 1992), two areas which are found to be reduced in volume in schizophrenia. The atrophy of these neurons would result in decreased input to the hippocampus and cortex, which could exacerbate, temporarily or permanently, the positive, negative, and cognitive symptoms of schizophrenia, and/ or contribute to the degeneration and death of target neurons. Interestingly, the expression of p75 in cholinergic neurons is associated with Alzheimer's disease (Woolf et al., 1989) and ß-amyloid toxicity (Rabizadeh and Bredesen, 1994), demonstrating that the alteration of p75 can play a role in the deterioration of cognition.

1.4.3.2.4 Regulation by neuroleptics

Little research has been done on the effects of neuroleptics on p75. Pollmacher et al. (1996; 1997) found that chronic treatment with clozapine, but not with haloperidol, increased the plasma level of p75 as well as that of tumor necrosis factor-alpha (TNF-alpha), soluble TNF receptors p55 and sIL-2r. (Note: These studies were done to determine if clozapine-induced fever was associated with these measures; but the increases were independent of prior or concurrent medication and also occured in patients who did not experience clozapine-induced fever). The authors concluded that "haloperidol (but not clozapine) is unlikely to confound the results of studies investigating disease-related alterations in the levels of a broad range of cytokines and soluble cytokine receptors in schizophrenia". In the aforementioned study, it was undetermined how plasma p75 levels correlated with the effects of the neuroleptics on schizophrenic symptomotology.

1.4.3.3 Brain derived neurotrophic factor (BDNF)

1.4.3.3.1 Distribution of BDNF and its receptor (TrkB) in the CNS

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors (Barde, 1989). In vivo, three isoforms of TrkB are

generated by differential splicing - one having full function and two having truncated receptors lacking kinases (Mocchetti and Wrathall, 1995). BDNF is the most abundant neurotrophin in the brain, with highest levels in hippocampus, cerebral cortex, and forebrain cholinergic synaptic targets (Philips et al., 1990; Maisonpierre et al., 1991b; Marty et al., 1996). Distribution of BDNF is more widespread in the brain than that of other neurotrophins, and high levels of BDNF mRNA and protein have been observed in neurons of the hippocampus, including pyramidal, hilar, and granule cells of the dentate gyrus, and in scattered cells in the amygdala, and in the cingulate, parietal, and entorhinal corticies (Hofer et al., 1990; Maisonpierre et al., 1991a). TrkB mRNA is widely distributed, and is found in high quantities in the olfactory system, cerebral cortex, hippocampal formation, amygdala, and cerebellar cortex (Masana et al., 1993) in the adult rat brain. Interestingly, it is often these brain regions that are the most vulnerable to insults such as ischemia, glutamate toxicity, and glucose deprivation and are altered in schizophrenia (Mattson et al., 1995; Selemon et al., 1995).

1.4.3.3.2 Function

BDNF is not only involved in the survival of adult neurons, but is also involved in embryonic growth and development in the CNS. Tissue culture studies have helped establish the role of BNDF and its receptor in embryonic development (Barbacid, 1994; Ernfors et al., 1994; Snider, 1994; Eide et al., 1996). For example, disruption of BDNF or trkB genes produces a loss of sensory neurons in mice (Snider, 1994). BDNF promotes neurite extension of rat cerebellar cells in culture, suggesting that it is important in the maturation and maintenance of differentiated cells (Hughes et al., 1993) BDNF has also been shown to enhance NMDA receptor maturation in cultured mouse cerebellar granule cells (Allsopp et al., 1995).

There is clear evidence that growth factors such as BDNF increase survival and help protect neurons from ischemic (Beck et al., 1994; Tsukahara et al.,

1994), glutamatergic (Mattson et al., 1995), and hypoglycemic (Mattson et al., 1993) insults. Specifically, via stimulation of SOD1 activity, BDNF prevents neurons from the above listed neuronal insults (Mattson et al., 1995). In summary, these findings indicate that BDNF is necessary to neurons throughout the life cycle.

BDNF has an AP-1 site on its promoter (Hayes et al., 1997) and there is some evidence to suggest that BDNF can be induced as an early immediate gene. Hughes et al. (1993) found that hippocampal focal brain injury transiently increases BDNF mRNA but that this increase was not blocked by the protein synthesis inhibitor cycloheximide. BDNF immunoreactivity is also seen in the cytoplasm of cholinergic neurons where the mRNA has not been detected, suggesting that some BDNF enters the nucleus to regulate transcription. Genes with BDNF binding sites have not been identified. However, it is possible that BDNF influences the transcription of other neuroprotective genes via stabilization of a common transcription factor/ DNA binding domain complex, by removing/ adding inhibition of transcription starts, or simply by binding to the genome itself. Marsh et al. (1993) have demonstrated that BDNF administration to cultured hippocampal neurons will stimulate auto-phosphorylation of TrkB within five minutes, allowing rapid phosphorylation of effector proteins, which suggests that BDNF can quickly alter protein conformation and function.

1.4.3.3.3 BDNF in schizophrenia and regulation by neuroleptics

Neurotrophins such as BDNF are involved in the creation, maintenance, and growth of neuritic processes and thus have the ability to regulate synaptic connectivity. Inadequate trophin supply will lead to either neuronal atrophy, shrunken processes, or in extreme cases, cell death and will thus disrupt neurotransmission. Interestingly, BDNF is decreased by factors correlated with the onset of first episode psychosis such as stress (Smith et al., 1995) and estrogen withdrawal / menopause (Singh et al., 1995), and is increased by

factors associated with the clinical treatment of schizophrenia (such as 5-HT_2 receptor blockade and ECT treatment; Vaidya et al., 1997; Nibuya et al., 1995). In addition, reduced hippocampal BDNF mRNA (Brouha et al., 1996) and an allele variant of the BDNF gene (Vicente et al., 1996) in schizophrenia suggests that disruptions of BDNF may play a role in the etiology of this disorder by compromising neuroplasticity or altering normal neurotransmission. In addition, given the role of BDNF in the normal maturation of the brain, disruptions of this neurotrophin could also underlie some of the neurodevelopmental abnormalities of schizophrenia as well as many other aspects of the disease. Thus, BDNF is a prime candidate on which to study the effects of neuroleptics.

1.5 Present study

Schizophrenia is a severely dehabilitating mental disease that is poorly understood in terms of its causes, etiology, and treatment. While newer "atypical" neuroleptics generally cause fewer side effects and successfully alleviate symptoms in a greater number of patients than did previous treatments, all antipsychotics still carry a high risk of causing significant adverse side effects, and approximately one quarter of all patients do not find any symptomatic relief with today's neuroleptics. Moreover, the neurodevelopmental abnormalities which are thought in part to underlie the disorder are not currently amenable to pharmacological correction. At the present, the greatest hope for improving the treatment of schizophrenia is through improving the efficacy of neuroleptics.

Although it is known that neuroleptics antagonize a wide variety of neurotransmitter receptors (such as dopaminergic, serotonergic, adrenergic, muscarinic, and histaminergic receptors), it is not known why neuroleptics must be administered for at least two to three weeks before a therapeutic

effect is observed. This delay in action suggests that the molecular mechanism underlying the therapeutic efficacy of neuroleptics lies beyond the receptor (Hyman and Nestler, 1993; 1996). We (and many others) thus propose that neuroleptics exert their effects by altering the gene (mRNA) expression of key proteins downstream from the receptor. Such mechanisms likely include long term changes in gene regulation in the affected neurons and in the amount of protein expressed by these genes (Duman et al., 1994; 1997). Thus, the central goal of this project is to try to elucidate the effects of neuroleptics on the mRNA expression of three selected groups of candidate genes, which include:

1. Early immediate genes (genes that are rapidly transcribed and translated upon activation) including *c-fos* and delta FosB.

2. Target genes of the early immediate gene family including:

a. enzymes in the synthetic pathways of the neurotransmitters dopamine and/ or serotonin, including aromatic L-amino acid decarboxylase and tyrosine hydroxylase.

b. genes involved in the survival of neurons, including the neuroprotective enzyme superoxide dismutase, the low affinity nerve growth factor receptor, and the most abundant growth factor in the brain, brain derived neurotrophic factor.

The neuroleptics used in the present study were chosen in order to have a wide representation of neuroleptics with primarily D_2 blocking activity, mixed D_2/ 5-HT_2 receptor blocking activity, selective 5-HT_2 blocking activity, and prototypical typical and atypical neuroleptics and included the following:

1. Remoxipride (a dopamine D_2 blocker)
2. Risperidone (a D_2/ serotonin 5-HT_2 receptor blocker)
3. Ritanserin (a 5-HT_2 blocker)
4 Haloperidol (a strong D_2/ moderate noradrenergic receptor blocker)
5. Clozapine (a 5-HT_2/ D_4 receptor blocker)

Figure 1.3 outlines the flow and direction of the present study. As previously discussed, transcription factors such as *c-fos* and delta FosB which are known to be upregulated by neuroleptic treatment may possibly regulate the long term gene expression of either or both of the two sets of indicated target genes.

1.6 Hypotheses

Both dopaminergic and serotonergic pathways have been implicated in the pathophysiology of schizophrenia, and current research suggests that efficacious neuroleptics reduce the symptoms of schizophrenia, at least in part, via DA_2 and/or 5-HT_2 receptor antagonism. Since current neuroleptics pharmacologically manipulate dopaminergic and serotonergic systems, and since dopaminergic vs. serotonergic blockade produces some similar, but many different, effects clinically, the following three hypotheses with respect to each factor chosen for examination are proposed:

1. Neuroleptics will alter the gene expression of proteins downstream from the receptor.

2. Neuroleptics will upregulate *c-fos* mRNA and delta FosB immunoreactivity upon either acute or chronic administration.

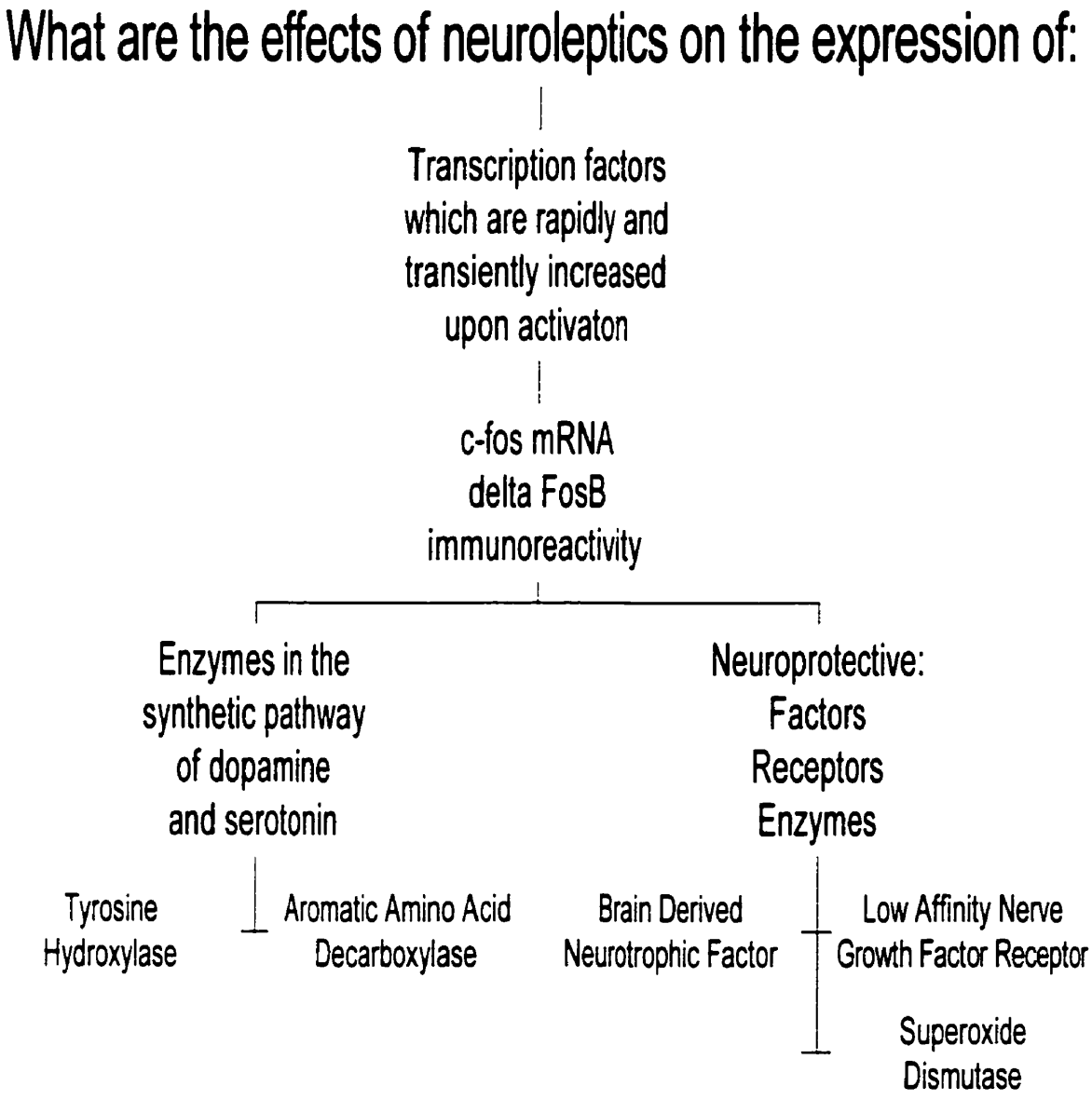

Figure 1.3. Flow and direction of present study. Transcription factors such as c-fos and delta FosB are increased upon neuronal stimulation and can regulate the gene expression of many target genes. These genes include a number of candidate genes thought to be involved in the neuroleptic response, as illustrated in the above diagram.

3. Target genes of the Fos family of transcription factors (such as AADC, TH, SOD1, p75, and BDNF) will also be altered by neuroleptic treatment.

Specifically, we hypothesize:

a. That all neuroleptics will upregulate TH and AADC mRNA in a similar manner in PC12 cells.

b. That atypical, but not typical, neuroleptics will regulate protective genes in a positive manner.

c. Early immediate gene expression will be associated with the regulation of these target mRNAs.

2. MATERIALS AND METHODS

For list of chemicals used and company addresses, please see Appendix A.

2.1 Cell culture: The PC12 rat pheochromocytoma cell line

2.1.1 History and development

PC12 cells were derived from epinephrine- and norepinephrine- producing chromaffin cells from adrenal medullary pheochromocytoma tumors (Greene and Tischler, 1976, 1982). As tumor cells, PC12 cells continually replicate and provide a self propagating system in which to study catecholamine synthesis, release, and metabolism. The PC12 rat pheochromocytoma cell line was developed in the 1970's by Greene and Tischler (1976). When it was discovered that the neurotrophin nerve growth factor (NGF) could stimulate neurite outgrowth, these investigators determined the culture conditions necessary to propagate these cells in order to further study neuronal differentiation (Greene and Tischler, 1976). PC12 cells contain all the machinery required for the synthesis of dopamine and norepinephrine including TH, AADC, and dopamine ß-hydroxylase (Itoh and Ohmori, 1973; Chalfie and Perlman, 1976; Greene and Tischler, 1976). Undifferentiated PC12 cells have D_2 (Greene and Tischler, 1976), 5-HT_2 (Humblot et al., 1997) and p75 (Loeb et al., 1991) receptors and express both *c-fos* and SOD1. Currently, PC 12 cells are widely used as a model for the study of catecholamines as well neuronal differentiation and cell death (reviewed by Greene and Tischler, 1982; Stepfanis et al., 1997).

2.1.2 Preparation of cell culture materials

All manipulations were performed using sterile technique and performed in a class II laminar flow hood. Nanopure water was used in all solutions and buffers and was prepared by the experimenter with the use of a Barnstead RO Pure and Nanopure II system with an organic cartridge. This process removes organic compounds and ionic particles. The plates and flasks used to grow PC12 monolayers were collagen coated to enhance adhesion to the inner cell culture walls. Collagen was diluted 10X with sterile, autoclaved nanopure water and poured as a thin layer onto the inner flask surface. The flasks were capped and left overnight. The following day, the remaining collagen was poured out of the flasks and reused twice before discarding. To coat six-well plates, 1 mL of 10X diluted collagen was placed in each well and the wells were covered for at least 4 hours. After removing the excess collagen, the plates were left open to dry for 2 hours in the hood under ultraviolet (UV) light to kill any bacterial growth in the wells of the plates. The plates were covered and sealed with parafilm until use.

2.1.3 Preparation of culture and conditions

The PC12 cell line was obtained from American Type Culture Collection (Rockville, Maryland) and cultured in RPMI 1640 medium (developed at Roswell Park Memorial Institute by Moore et al., 1966a,b) containing 5% fetal calf serum and 10% horse serum, 0.03% glutamine plus 100 units/mL penicillin and 100 µg/mL streptomycin as described in protocols provided by supplier. One (1)mL 2M NaOH was added to 1L of medium after the addition of the serum to raise the pH of the medium back up to approximately 8.0. Stock solutions of penicillin (5% in sterile nanopure H_20), streptomycin (5% in sterile nanopure H_20), and 3% glutamine were made in advance and filtered with a 0.2µ Nalgene Millipore filter. Aliquots were stored at -20°C until use.

Cells were thawed from -70°C in 8-10 mL of medium in a 37°C water bath. Incubations were carried out at 37°C in 95% humidified air with 5% CO_2. The

culture medium was initially changed after 3 days, and the old medium was discarded. To ensure the discarding of dead cells (which do not stick to the collagen coated walls) the flask was gently shaken before removing the old media. Viable cells adhering to the collagen were then haversted by forcefully pipetting at once approximately 20 mL of medium against the flask surface for approximately one minute. Using a 10 mL syringe and a 22.5 gauge needle, cell clumps were broken up into individual cells by pulling the mixture up into the syringe and quickly forcing the cells out through the needle against a new, sterile tissue culture plate. This was repeated 10 times. The cell suspension was then divided between three new collagen- coated flasks, and 20 mL of flesh medium was added to each flask. The cells were allowed to grow for three days before the medium was changed. The medium was subsequently changed twice a week by removing the old media and adding 20-24 mL of fresh media.

2.1.4 Plating procedure

To plate cells, the cells were removed from the 20 mL flasks and were separated through a 22.5 gauge needle as previously described. When placed into 1200 mL of serum-free medium, the cells from about ten 20 mL flasks provided enough cells to reach a density of 200,000 to 500,000 cells/mL. Four (4)mL of medium was then plated into each well of the 6-well poly-l-lysine coated plates. After a three day incubation period, (which allowed for the newly plated cells to grow and adhere to the flask) the medium was carefully removed and replaced with 3 mL of serum-free RPMI medium.

2.2 Animals

2.2.1 Handling and housing

Male Wistar rats (215-260 g, Charles River Canada, Montréal, Québec) were individually housed under a 12:12-hour light:dark cycle with food and water *ad libitum*. All procedures involving animals were performed in

accordance with the Canadian Council on Animal Care guidelines and were approved by the University of Saskatchewan Animal Care Committee.

All animals were allowed 3 days to settle after being transported to the animal housing facility before being exposed to contact with the experimenter. Animals were handled for 4 days to allow them to become accustomed to the future injection protocol. Animals were also transported to the injection room and handled once daily for 3 days prior to receiving actual injections. The animals were allowed one hour to settle after being moved to the injection room.

2.3 Preparation and administration of drugs

All drugs were freshly prepared as needed for the experiments. For chronic animal experiments, fresh drug solutions were prepared as follows every 3 to 4 days and were stored in a refrigerator at 4°C until use. The following drugs were used in both cell culture and/or animal experiments: remoxipride, risperidone, ritanserin, haloperidol, clozapine, and olanzapine (see Appendix A for source of drugs).

2.3.1 Cell culture

All drugs were dissolved in 1 mL Aristar grade ethanol and sterile filtered through a 0.2µ Nalgene microfilter. This solution was then serially diluted in sterile 0.9% NaCl 4 times by a factor of 10 (dilutions = 1:10, 1:100, 1:1000, 1:10000). Final drug concentrations were calculated using the following formula:

$$1\text{M drug concentration} = \frac{\text{molecular weight of drug X culture well volume}}{\text{volume of drug added}}$$

$$1\mu\text{M drug concentration} = \frac{\text{molecular weight of drug X 3000 } \mu\text{L/ 25 } \mu\text{L}}{1{,}000{,}000}$$

The cells that had been plated in 6-well plates were treated 3 days later with the various neuroleptics. Drug concentrations for cell culture experiments were chosen based on those reported in the literature for similar studies. The neuroleptics were administered to cells using a 25 µL repeat Eppendorf pipette. Cells were harvested at time points ranging for 0.5 hours to 48 hours at doses ranging from 0.01 µM to 250 µM.

2.3.2 Animal experiments

All drugs were dissolved in 0.8% glacial acetic acid in 5% glucose (5mg glucose in 100mL sterile nanopure H_20). Due to solubility problems, clozapine was made up in 1.5 times the volume of the other neuroleptics and was therefore injected as 50% more volume. Final drug doses were calculated as mg/kg. Neuroleptics were given in amounts approximately four times greater than the mg/kg human dose and were similar to neuroleptic doses reported in the literature traditionally in animal studies.

For acute injection experiments, at least 3 animals per group received a single i.p. injection of either vehicle (0.8% glacial acetic acid in 5% glucose), remoxipride, risperidone, ritanserin, haloperidol, or clozapine at various doses corresponding to a range of high, moderate, and low clinical doses. For chronic and chronic challenge injections, three animals per group received daily i.p. injections of either vehicle (0.8% glacial acetic acid in 5% glucose, remoxipride (3 mg/kg), risperidone (1 mg/kg), ritanserin (2 mg/kg), haloperidol (1 mg/kg) or clozapine (20 mg/kg) for 20 days (olanazapine was only included in later studies when it became available in the last 3 years for laboratory study), with challenge animals receiving an additional injection 30 minutes before sacrifice. In the dose-response study, 3 animals per group received daily i.p. injections of either vehicle (0.8% glacial acetic acid in 5% glucose), risperidone (4.11, 0.67, or 0.067 mg/kg), or haloperidol (1 mg/kg) for 20 days. Animals were sacrificed and perfused as described below. The injection

volume ranged from 250-350uL of solution, depending on the weight of the animal (mg/kg).

2.4 Tissue preparation for analysis of mRNA and protein

All reagents used for the analysis of mRNA were of molecular biology grade to ensure a ribonuclease RNAse-free environment. Nanopure water treated with the RNAse inhibitor diethylpyrocarbonate (DEPC: 1.16mg/ml) was used in all experiments. DEPC-treated water was made by dissolving 4 mL DEPC in 4L nanopure H_2O and autoclaving it for thirty minutes with the caps loosened.

For analysis of mRNA in PC12 cells, the medium was removed from the plate after each time point and replaced with 1.25 mL GITC buffer (4 M guanidinium isothiocyanate, 5 mM sodium citrate, 0.5% N-lauroylsarcosine, 0.1M ß-mercaptoethanol, adjusted to a pH of 5.5 with sodium acetate to pH=6.0). Plates were stored at -20°C until the total cellular and tissue RNA was extracted.

Animals required for analysis of brain tissue mRNA only were sacrificed by stunning and decapitation. For northern blot analysis, brain regions were dissected out over ice, were placed in plastic 15mL Falcon centrifuge tubes and stored on dry ice. Three ml GITC buffer was then added to each tube. Regions for disection were defined as in Horn et al. (1974). Samples were homogenized in the GITC buffer and stored at -70°C until the extraction of RNA.

The brains to be used for *in situ* hybridization were quickly rinsed in saline, quick frozen in a dry ice/ isopentane slurry at -50°C and stored at -70°C until sectioning. Animals required for analysis of both mRNA and protein were sacrificed by anesthesia with Nembutal (50 mg/kg, i.p.), were transcardially

perfused with 0.1M Na-K phosphate buffer (PBS; pH 7.4) and the brains were then fixed by perfusion with 4% paraformaldehyde (PFA) in 0.1M PBS. Brains were post-fixed in a 4% PFA solution at 4°C for 4 hours, cryoprotected in 30% sucrose (30mg sucrose in 100 mL sterile DEPC-treated H_2O) at 4°C for at least 2 days (or until the brains had sunk to the bottom of their container), quick frozen in a dry ice/ isopentane slurry at -50°C and stored at -70°C until sectioning.

2.5 Preparation of templates and probes for northern and *in situ* hybridization

2.5.1 Templates prepared by PCR *(c-fos)*

2.5.1.1 Principles of PCR

The polymerase chain reaction (PCR) allows the amplification (i.e., the rapid, exponential increase) of a specific, short strand of DNA which, when "amplified", can be used for a variety of applications. The reaction simply requires that a DNA template, buffer, the appropriate primers, and a polymerase lacking 3' to 5' exonuclease activity be added at the beginning of the reaction and cycled through temperatures sufficient for DNA denaturation (94-95°C), primer annealing (37-72°C), and primer extension (72°C) (Saiki et al., 1988). Unlike older DNA amplification methods, PCR allows the amplification of a single molecule of DNA template without its introduction into Escherichia (E.) coli (Davis et al., 1994). In addition, whereas polymerases previously used in such amplifications were destroyed by temperatures required for denaturation, use of the thermostable Taq polymerase (from the hot spring residing bacteria *Thermus aquaticus*) allowed repeated amplification without having to add new polymerase after each denaturation step. Since PCR can amplify as little one DNA molecule, the presence of only a single DNA species is crucial for purity of amplification.

2.5.1.2 Preparation of *c-fos* probe

Two 48 base oligonucleotide primers (designed by Dr. Xin-Min Li, Dept. Psychiatry, University of Saskatchewan) based on the rat *c-fos* complementary DNA (cDNA) were synthesized on a PCR-MATE Applied Biosystems 391 DNA synthesizer. Primer-1 was 5' GGG ATT TAG GTG ACA CTA TAG AAC TTA AAT GCT TTT ATT GAC AAT GAC AAT GTA 3' and primer-2 was 5' CTG TAA TAC GAC TCA CTA TAG GGT GGT CTG AAT GTT CTG ACA TTA ACA 3'. Synthesis and further amplification was performed for 30 cycles (denaturation: 94°C for 2 minutes, annealing: 65°C for 1 minute, extension: 72°C for 1 minute) using a DNA thermal cycler (Perkin Elmer/Cetus) and Perkin Elmer/Cetus GeneAmp PCR Core Reagents. The reaction mix included 2.5 U/100 µL AmpliTaq DNA polymerase; 200 µM each for dATP, dCTP, dGTP, and dTTP; 1x PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.3)), 1.0 -4.0 mM $MgCl_2$, 5ng/ 100 µL template (rat c-fos cDNA), and 0.2- 1.0 µM each of primer 1 and primer 2 in a total volume of 100 µL.

2.5.2 Templates derived from plasmids (general procedure)

2.5.2.1 Preparation of media

The media (LB, or Luria-Bertani media) used to grow untransformed, transformed and transfected *Escherichia coli (E. coli)* contained 20g bacto-tryptone,10g bacto-yeast, and 20g NaCl made up to 2000 ml with nanopure water. The pH was adjusted to 7.4 with 1M NaOH. To make agar plates, 15g agar was added to half of the mixture. The medium was then autoclaved, left to cool to room temperature, and had ampicillin (AMP) added to a portion of it. Stock solutions (1000x) of AMP were made by adding 50 mg AMP per 1 mL H_20 and filtering the solution with a 0.2µ Nalgene Millipore filter. Aliquots were stored at -20°C until use.

2.5.2.2 Transformation of bacteria

The *E. Coli* strain JM109 was streaked onto antibiotic-free LB plates and left overnight to incubate in a dry chamber at 37°C. The following day, one

colony was selected to grow in 40 mL of antibiotic-free LB liquid media in a flask in a shaking water bath at 37°C until the rate of growth of the colony reached log phase kinetics. This was determined by measuring the optical density (OD) of the bacterial solution at 600nm until it reached an OD of 0.3 to 0.5. This process usually took between 3 to 7 hours.

The bacterial colony was centrifuged at room temperature at 1500g for approximately 5 minutes until a pellet became visible. The excess fluid was discarded and the cells were resuspended in 20 mL of 50 mM $CaCl_2$ in order to make the cells competent for transfection. After sitting on ice for 30 minutes the cells were spun as previously for 5 minutes at 4°C. The cells were resuspended in 4 mL $CaCl_2$ and stored at 4°C until use.

Dry plasmid DNA conferring probe sequences for various genes was mixed in nanopure H_2O at a concentration of 1-2µg per 50µL (for details of the plasmid vectors used and the restriction cuts, please see sections 2.5.3 and 2.5.4). Plasmid DNA was mixed with competent cells in ratio of 1:2000. The mixture was stored on ice for 30 minutes and then heat shocked at 42°C for 2 minutes. One (1)mL of LB medium (without antibiotic) was added and mixed in a shaking water bath at 37°C for 45 minutes to allow for the expression of the antibiotic resistance gene for AMP contained within the newly inserted plasmid.

2.5.2.3 Plating of cells and selection of culture

The bacteria were then grown on both ampicillin negative (AMP-) and ampicillin positive (AMP+) agar (inverted) plates overnight at 37°C. Only bacteria containing the plasmid would be resistant to the ampicillin, and therefore only transformed bacteria could grow on the AMP+ plates. Wild type (untransformed) bacteria were grown on AMP+ plates as a negative control. To ensure that the colony selected for the plasmid miniprep consisted of

transformed bacteria, a single colony that was not touching or growing on top of any other colonies was selected from the AMP+ plate.

2.5.2.4 Amplification and preparation of plasmid DNA

To amplify the plasmid of interest, a single colony of bacteria transfected with the probe coding AMP-resistant plasmid was grown in 100 mL liquid AMP+ LB media overnight at 37°C in a shaking water bath (Maniatis et al., 1982). This was then centrifuged at 2000Xg, the supernatant was discarded, and the pellet was resuspended in 450 µL of glucose buffer (25 mM Tris [tris {hydroxymethyl} aminomethane], pH 8.0; 50 mM glucose, 10 mM EDTA [ethylene diaminetetraacetic acid]). This was followed by the addition of 1.2 mL 0.2M NaOH with 1% sodium dodecyl sulfate (SDS) to lyze the bacterial cell membrane and free the plasmid DNA. The solution was centrifuged for 10 minutes after the addition of 900 µL ice cold potassium acetate solution (29.4g potassium acetate, 11.5 mL glacial acetic acid to 100 mL H2O). The supernatant was saved and the precipitate discarded. After adding 1.5 mL isopropanol to the supernatant, the mixture was stored at -20°C for at least 15 minutes and was then centrifuged for 15 minutes at 12,000g. The pellet was resuspended in 400 µL of Tris/EDTA buffer (TE) buffer (10 mM Tris pH 7.4; 0.1 mM EDTA pH 8.0), and 40µL 3M sodium acetate.

2.5.2.5 Purification of DNA

A phenol/ chloroform extraction was used to partially purify the plasmid DNA. Volumes in a ratio of 25:24:1 of saturated salt (ss)-phenol: chloroform: isoamyl alcohol were mixed and vortexed, followed by centrifugation. The phenol was buffered to prevent DNA damage from oxidized materials in the phenol. During the purification, proteins denature and collect in the lower organic (phenol) phase, while nucleic acids remain in the aqueous phase.

The top aqueous layer was saved followed by the addition of approximately 2.5 volumes of 95% ethanol to the top aqueous layer and the mixture was

stored at -20°C overnight. After being centrifuged for 10 minutes at 4°C, the supernatant was discarded, excess salt was removed from the remaining pellet with 80% ethanol, and the mixture was recentrifuged for 2 minutes. The pellet was left to airdry overnight and was resuspended in 50µLTE buffer the following morning.

To further purify the plasmid DNA and remove *E. coli* DNA, the total DNA was run on a 0.8% agarose gel in 100 mL 1X TAE (Tris acetate/ EDTA) buffer (1.6 M Tris base, 0.8M sodium acetate, 40 mM EDTA, adjusted to pH 7.2 with acetic acid) and 10 mg/ml of ethidium bromide (an intercalating agent). The gel loading solution contained (in 10 mL) 5 mL glycerol, 250 µL 40X TAE buffer, 1 mL saturated bromophenol blue, 1 mL10% suspension xylene cyanol, and 2.75 mL H_2O. The part of the gel containing the plasmid DNA was cut out and purified using a Geneclean II kit (Promega). Purity was confirmed by mini-gel electrophoresis.

2.5.2.6 Restriction digest of plasmid DNA

Restriction endonucleases (RE's) are enzymes found in bacteria that, by recognizing and cutting specific sequences in double-stranded DNA, protect the bacteria from foreign DNA (Zielinski et al., 1999). Most RE's cut at recognition sequences 6 to 8 base pairs long, many of the recognition sequences being palindromes (a sequence which is mirrored on both strands in the 5' --> 3' direction). Molecular biologists have presently identified over 600 "type II" RE's which, unlike other types of RE's, do not require adenosine triphosphate (ATP) for cleavage and cleave close to or at the recognition sequence (Davis et al., 1994). These RE's are commercially available for applications including the analysis of chromosome polymorphisms and the recombination of DNA (Maniatis et al., 1982).

To run an analytical digest, 1µL (0.2 to 10U) of the appropriate RE(s), 1µL (0.1 to 0.5 µg) of the plasmid to be cut, 1µL of the appropriate buffer, and 7µL

H_2O were incubated at 37°C for 1 hour. After stopping the reaction by incubation at 65°C for 2 minutes, the sample plus an equal volume of loading buffer was run for 1 hour on a 1% agarose gel at 75 mV against a DNA marker to confirm probe length and that the plasmid had been cut in the proper location.

To determine the concentration of plasmid DNA, 4 µL of the solution containing the plasmid DNA was added to 1mL H_20, was mixed, and added to a crystal cuvette for measurement under UV light at 260 nM on a Beckman Du 460 spectrophotometer and the concentration calculated using the following equation:

Concentration of Nucleic Acid (DNA or RNA) in µg/µL=

Absorption at 260 nm (A260) X extinction coefficient X *dilution factor

*Dilution factor =

$$\frac{\text{Total volume of solute in cuvette (mL)}}{\text{Volume of nucleic acid measured (µL)}}$$

Concentration of DNA (µg/µl)=

$$\frac{\text{Absorption at 260 nm (A260) X 50 µg/ mL X 1mL}}{\text{4 µL}}$$

The extinction coefficient of DNA is 50 µg/mL, 40 µglml for RNA and 30 µg/µL for oligonucleotides. Extinction coefficients correspond to nucleotide concentrations that produce an optical density of 1.0 at A260 (Davis et al., 1994).

2.5.3 Labeling cDNA probes: TH, AADC, SOD, p75, cyclophilin

The cDNA probes were radiolabled by random primer synthesis with [α-^{32}P] dCTP as described by Fainberg and Vogelstein (1983) using a random primer

kit from Boehringer Manheim. Briefly, the probe template (50 ng/μL) single stranded or double stranded DNA was denatured at 95°C for at least 5 minutes and quickly placed on ice to prevent renaturation. Once denatured, single stranded DNA acts as the template to synthesize the radiolabeled probe. The reaction mixture contained 2 μL of random hexonucleotide primers (0.5 M Tris-HCl, 1M $MgCl_2$), 1 mM dithiothreitol (DTT), 2 mg/mL bovine serum albumin, hexanucleotides @ 62.5 A260 units/ml (pH 7.20, 1 μL (2U) Klenow fragment, 5 μL ^{32}P dCTP (50 μCi) and 3 μL dNTP's (dATP, dGTP, dTTP) each at a concentration of 0.5 mM, and was incubated with 25 ng denatured template DNA made up to 19 μL with sterile redistilled H_20 for at least 1 hour at 37°C. The reaction was stopped with 10 μL 0.01M EDTA followed by the addition of 130 μL STE buffer (NaCl-Tris-EDTA Na_2 buffer, pH 7.4). The probe was column purified with Bio-Spin 30 chromatography columns according to the manufacturer's instructions. Salmon sperm DNA (1 mg/mL) which had been heat denatured was added to block non-specific polar groups on the membrane and was added in an amount to bring the solution up to 500μL. The mixture was then alkali denatured with 0.1 volumes 3N NaOH for 5 minutes, followed by the addition of 0.05 volumes 1M Tris-HCl, pH 7.2 and 0.1 volumes 3N HCl.

SOD1 cDNA was kindly provided by Dr. J. T. Coyle (Harvard Medical School, Boston, Massachusetts, USA). AADC and TH cDNA were obtained from rat adrenal gland total RNA by RT-PCR (Li et al., 1992). The p75 cDNA probe (from rat complementary RNA [cRNA]) was from Dr. Ron Lindsay (Regeneron Pharmaceuticals, Terrytown, New York, USA) and the cyclophilin probe was from Dr. Barry Kosofsky (Harvard Medical School, Boston, Massachusetts). SOD1, cyclophilin, and AADC cDNA were placed in PGEM-3 inserted into JM109 E.Coli. SOD1 cDNA was cut with Pst1, cyclophilin was cut with BamH1, and the AADC cDAN was cut with EcoR1 and Pst1.

2.5.4 Labeling cRNA probes: *c-fos*, BDNF

The cRNA probes were radiolabelled with either [α -^{32}P] or [α -^{35}S] using a transcription kit from Boehringer Manheim. Briefly, the template coding for probe sequences was first inserted into a plasmid between two promoter regions which code for the start site of transcription of selected DNA-dependent RNA polymerases, including SP6, T7, and T3 RNA polymerases (SP6 polymerase is from the Salmonella bacteriophage SP6, while T7 and T3 polymerases are from the E. Coli bacteriophages T3 and T7). The plasmid was cut with the appropriate restriction enzyme at one of the promoter regions to define the 3' end of the transcription unit for the synthesis of the antisense probe. In vitro transcription was started with 0.5 µg/µl template/cut plasmid, 2 µL 10X buffer, 1 µL of 1:50 CTP, 3µL NTP (ATP, GTP, UTP) each at a concentration of 10µM, and the mixture was incubated at 37°C for at least 2 hours. The RNA polymerase was heat denatured (65°C) for 15 minutes and 1µl (10 U) deoxyribonuclease (DNase) was incubated with the mixture for one hour at 37°C to digest the template cDNA. After the addition of 10µl 0.1 EDTA and 65 µl STE buffer, the probes were column purified with Bio-Spin 30 Chromatography Culumns according to the manufacturer's instructions. Unincorporated nucleotides are retained in the column while the longer, synthesized transcripts pass through the column and are collected for use in either the northern or in situ hybridizations.

To measure the specific activity of [^{35}S] labeled probes, 1 µl of each purified probe was added to 10 ml of scintillation fluid and counted as counts per minute (cpm)/µg on a Beckman LS 5000TD scintillation counter. To be used for *in situ* hybridization experiments, probes needed to label between 5 X 10^5 to 2 X 10^6 cpm/ml (% incorporation could be calculated by spectrophotometry).

The probe for *c-fos* (antisense SP6) routinely labeled at counts lower than the other RNA probes, so two variations in the procedure were performed to increase the specific activity of the probe. While double labeling with [^{35}S] CTP

and ATP did not seem to increase specific activity, reduction of the amount of cold CTP (1:100 vs. 1:50) did seem to increase specific activity by a factor of 1.5 times.

The BDNF cRNA probe (from rat cRNA) was the gift of Dr. Ron Lindsay (Regeneron Pharmaceuticals, Terrytown, New York, USA). The BDNF probe template (1127 base pairs) was inserted in the Bluescript SK- from Lamba Zap II, with the mRNA sequence starting at 14 nucleotides after an ECOR-1 linker and ending at 1108 base pairs before the ECOR-1 linker between promoter regions for T7 and T3 (Maisonpierre et al., 1991a,b). The antisense probe to detect mRNA was generated using a T7 promoter. The transcription region was defined by cutting the inserted plasmid just before the start site of the T3 promoter with the BAM HI restriction endonuclease. To generate a template for a sense strand, the plasmid was cut just before the T7 promoter with the Xho I restriction endonuclease. Both endonucleases are non-cutters of the BDNF coding region and cut at only one site in the polycloning region of the plasmid. The *c-fos* probe was not placed in a plasmid, but linked to an SP6 promoter region for probe synthesis and was used immediately after its symthesis by PCR.

2.6 Estimation of mRNAs by northern hybridization

The northern hybridization is one of the most useful methods available to estimate the amount of RNA in target cells and tissues. The amount of mRNA present of a particular gene in a given tissue can provide useful information about the conditions in a cell that can increase or decrease its availability to serve as the template for the protein it encodes. Although the northern blot hybridization cannot provide information about the cellular location of a particular mRNA, it can be used to quickly estimate if a particular treatment will have an effect on a target mRNA is a particular tissue.

2.6.1 Isolation of mRNA

Total cellular RNA was prepared from treated cells by extraction in GITC buffer and collected by ultracentrifugation (21 hours, 32,000 revolutions per minute [rpm], Beckman) under vacuum through 5.7 M cesium chloride (CsCl: 95.97 g CsCl, 0.83 mL 3M sodium acetate, 2000 mL DEPC-treated H_2O). After being filtered through a 0.8 μM Millipore filter, the CsCl solution was transferred into 2L bottles, treated with 1mL DEPC, left overnight and placed in a shaker bath in a fume hood at 60°C to get rid of the DEPC.

The RNA was chloroform-extracted, ethanol-precipitated, resuspended in DEPC treated water, and stored at -70°C until use. RNA was measured spectrophotometrically by absorbance at 260 nm. The purity of RNA was checked by the ratio of the UV absorbance at 260nm/ 280nm which was about 1.8. Ethidium bromide staining of the sample trackings indicated that the samples were not degraded.

The total RNA was denatured at 65°C for 15 minutes in MOPS (3-(N-morpholino) propanesulfonic acid) buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0) containing 50% formamide and 2.2M formaldehyde to keep the RNA denatured. Total RNA was separated by electrophoresis in a 1.0% agarose gel containing MOPS buffer and 2.2M formaldehyde. Following electrophoresis, a picture of the 18s and 28s RNA bands were taken to ensure standardization of gel loading (see below). The RNA was transferred to nylon membranes and the membranes were cross-linked in a UV Stratalinker 2400. Membranes were visualized under UV light to ensure adequacy of transfer.

2.6.2 Principles of hybridization

Hybridization techniques rely on the ability of a labeled single stranded DNA or RNA probe to form selective homo- (DNA/DNA, RNA/RNA) or heteroduplexes (RNA/DNA) with a target DNA or mRNA, and on the creation

of conditions which remove excess or background labeled nucleotides and allow the desired duplex to remain for detection. RNA/RNA hybrids are the most stable, followed by RNA/DNA and then DNA/DNA interactions. This means that RNA probes will bind more tightly to mRNA than will DNA probes during the detection of mRNA by northern hybridization and will thus require more stringent wash conditions to remove excess probe.

Other factors affect the stability of duplexes. The "melting temperature", or Tm, of such nucleotide duplexes is defined as the temperature at which half of the nucleotides in a duplex melt or lose their pair bonding capacity. Since GC pairs have three hydrogen bonds, while AT(/Uracil) bonds only have two, duplexes with a higher GC content have a higher Tm, and thus need a higher temperature wash to lower the stability of and thus "melt" nucleotide duplexes. The average Tm is 85°C, and northern hybridization wash conditions are typically 20°C lower than the Tm. In addition, the higher the degree of complementarity between duplexes, the more stable the duplex. This factor allows the specific probe/ target mRNA hybrid to remain while non-specific duplexes can be washed out of the membrane.

Salts such as NaCl stabilize nucleotide duplexes by interacting with negatively charged phosphate groups on the nucleotide backbone. At room temperature, DNA will completely denature in water, but will remain double stranded at a salt concentration of 2.0 to 6.0 M. A 0.15 M salt solution will lower the Tm by 20°C. The lower the salt concentration of the wash conditions and the higher the "stringency" (the ability to disrupt nucleotide duplexes) of the wash, the more nucleotide/probe interactions are disrupted (nonspecific duplexes are washed out first). Formamide disrupts hydrogen bonds and thus can be added if high temperature and low salt concentrations fail to sufficiently remove undesired probe and excess nucleotides from the membrane. Thus, wash conditions can be altered based on the composition of the particular probe used.

2.6.3 Procedure

Filters were prehybridized at 60°C for 2 hours with prehybridization solution containing 10% dextransulfate, 5X SSPE [(sodium chloride/ sodium phosphate buffer; 5 X Denhardt's solution (0.1 % Bovine Serum Albumin (BSA), 0.1 % Ficoll, 0.1 % polyvinylpyrrolidone)], 0.5% SDS, and denatured salmon sperm DNA (200 µg/ml). Hybridization was performed at 60° C for 18 hours. After hybridization, membranes were washed at room temperature twice in 2 X SSPE-0.1 % SDS, once in 0.1 X SSPE-0.1% SDS at 60°C, and once in 0.1 X SSPE-0.1% SDS at 60°C. The membranes were washed and exposed to X-Omat AR film with intensifying screens at -70°C to obtain autoradiograms. The autoradiograms were scanned with a computerized densitometer (Du 640, Beckman) for quantitative estimations, and the signals were adjusted according to the signals of rehybridization with a cyclophilin probe.

2.6.4 Variations of wash conditions

Both TH and AADC probes required a higher stringency wash. Incubations and washes were performed at 65°C, and longer wash times at 0.1x SSPE were employed to acquire membranes with low enough counts for autoradiography. The *c-fos* RNA probe required a low stringency wash at 60°C with reduced wash times at the lower salt concentrations. The SOD and p75 probes required wash conditions similar to but slightly less stringent than that of AADC and TH. The BDNF probe required incubation at 68°C with long washes with low salt concentrations at anywhere between 68 and 72°C.

2.6.5 Autoradiography and exposure times

After washing, membranes were exposed to X-Omat AR Kodak film with intensifying screens, at -70°C to obtain autoradiograms. Exposure time for the development of measurable autoradiographs ranged from 0.5 hours to 10 days. In general, each probe had an optimal development time, with TH

autoradiographs requiring the shortest exposure times, and *c-fos* autoradiographs requiring the longest exposure times.

2.6.6 Standardization of gel loading

There are two methods commonly employed to ensure that the amounts of total RNA run on an electrophoretic gel are equal to one another: intensity of 18s/ 28s bands, and hybridization to housekeeping genes such as ß-actin and cyclophilin. When intercalated with ethidium bromide, 18s and 28s structural RNAs can be visualized in a gel under UV light. If the amount of total RNA loaded into each lane of the gel is equal, the intensity of the 18s and 28s bands under UV light should be equal. If they are not equal, band intensities can be measured and loading can be standardized in comparison to the controls. The disadvantage of this procedure is that it does not take into account the possibility of uneven transfer of RNA to the membranes during the transblot procedure. Membranes can be visualized under UV light and the image transferred to a computer-based image analysis program, but such images are not always sufficient for such analysis.

Housekeeping genes are those genes whose protein product is constitutively expressed and is resistant to induction of its production by intracellular/ and or extracellular inputs. Hybridization of a housekeeping gene such as ß-actin or cyclophilin to the same membrane probed for the mRNA to be measured, accounts for the possibility of uneven transfer and is even a better control if hybridized to the membrane at the same time as the other probe, which is only possible if the mRNA species migrate at a far enough distance from the housekeeping gene being measured. In practice, it is probably safe to use a housekeeping gene if it is first established that the experimental manipulation does not regulate the housekeeping gene used, which can in part be established by a comparison to 18 and 28s intensities. If the experimental condition regulates the housekeeping gene, it would be

necessary to find a different housekeeping gene and/ or standardize to the 18 and 28s bands.

In the present study, efforts were made to standardize all membranes to cyclophilin mRNA. If hybridizations to cyclophylin were faulty or if treatments altered cyclophilin mRNA, standardizations to the 18s and 28s were employed.

2.6.7 Quantification of Autoradiographs

2.6.7.1 Quantification program and preparations of linearity standards

Autoradiographs from membranes were scanned with an Acer Acer310P scanner and analyzed using an IBM compatible version of NIH Image (Scion Image). Since film can become "maxed out" or saturated, it is possible to be unable to record further radioactive signal. Film actually has three ranges of linearity and it is ideal to measure film densities in the middle and most dynamic range.

When choosing a radioisotope for a particular application, one must consider the degree of resolution desired and the amount of time one is willing to wait for a result. If one wants quick results and the resolution of the film image obtained is not a primary concern (such as in a northern blot), a high beta-emitting radioisotope such as ^{32}P is a good choice. If resolution is important, as it would be in an *in situ* hybridization, a radioisotope with a lower emission energy such as ^{35}S is neccessary to ensure a more detailed film image; although some films may need up to 3 week exposure periods to produce a quantifiable image.

There are several methods available to ensure the linearity of autoradiographic signals, including the use of commercial ^{3}H-polymer standards (Geary et al., 1985), but ^{3}H autoradiography has the disadvantage of requiring weeks to obtain quantifiable radiographic signals due to the relatively low emission energy of ^{3}H (Nazarali et al., 1989). For northern blot

analyses where the strong beta emitter ^{32}P is often used as the radiolabel, simple standards can be prepared by applying differing concentrations of ^{32}P to nylon membranes and including these membranes in the film cassette with the probed membranes of interest. Standardization of autoradiography with ^{35}S *in situ* hybridization (ISH) requires the use of brain paste standards or commercially available calibrated ^{14}C or ^{125}I- polymer standards (Davenport and Hall, 1998). In the present study, ^{35}S brain paste standards were made by the present investigator as outlined in section 2.8.4.1.

2.6.7.2 Positive controls

Since one of the mRNAs probed for in the present study *(c-fos)* is usually undetectable in control conditions, positive controls were employed. Male Wistar rats were injected with 10 mg/kg kainic acid and were observed for 2 to 6 hours for signs of seizure using Racine's rating scale (Racine and Burnham, 1984). In addition, at the onset of the present study, it was known that acute haloperidol treatment (1 mg/kg, animals sacrificed 30 to 45 minutes after a single i.p. injection) would significantly increase striatal *c-fos* mRNA over controls. If an ISH did not produce positive results with the kainate- and haloperidol-treated animals, it could be concluded that the ISH did not work. Conversely, if these positive controls yielded significant *c-fos* mRNA labeling but changes were not observed in the other sections, we could be certain that the negative results (i.e., the absence of signal) were true lack of gene induction and not merely "false negatives".

2.7 Preparation of tissue for *in situ* hybridization analysis

2.7.1 Poly-L-lysine coating of slides

Melvin Freed microscope slides were placed in slide racks (19 per rack) and cleaned in 6N HCl for 1/2 hour. The slides were rinsed in tap water for approximately one hour and left to soak in nanowater overnight. The slides were dried at 60°C overnight, allowed to cool to room temperature, and

soaked in a 10 fold dilution of poly-L-lysine in DEPC water for 1/2 hour. After being dried at 60°C overnight, the slides were allowed to cool, were dipped in poly-L-lysine again for one half hour, and dried overnight. Precautions were taken to ensure an RNAse-free environment. Slides were stored at 4°C up to one month until use. The poly-L-solution was stored at 4°C and reused once before being discarded.

2.7.2 Sectioning

The cryostat temperature was kept at -18°C or lower to prevent the activation of RNAses (especially in fresh frozen tissue). The slides being used were also kept at this temperature in the cryostat. Brains to be sectioned were taken from the -70°C freezer and left covered in the cryostat chamber for about 2 hours to equilibrate to the chamber temperature. Coronal sections for ISH were cut at either 12 or 20 µm. Sections from different experimental conditions were put on each slide, and the relative position of each experimental group was rotated to ensure that samples from one experimental group did not always end up on the same position on the slide (this control is particularly important for emulsion analysis, since the top section on the slide spends less time in the emulsion than the bottom slide). The slides were kept covered in the chamber and sections were cut as quickly as possible to prevent drying of tissue. Sections were stored at -20°C until immunohistochemistry, *in situ* hybridization, and/or prehybridization (for fresh frozen tissue).

2.7.3 Prehybridization

Sections from fresh frozen tissue must be prehybridized with paraformaldehyde in order to preserve tissue and prevent the breakdown of RNA. However, fixation time must be limited to that time which provides good tissue morphology but still allows an acceptable signal-to- noise ratio after in situ hybridization. Sections were carefully warmed to room temperature and placed in 4% paraformaldehyde (PFA) for 5 minutes, and washed in 1x PBS

(phosphate buffered saline; 0.01M Na_2HPO_4, 0.01M NaH_2PO_4, and 0.26 M NaCl pH= 7.4 in DEPC-treated water) for 2 and then 3 minutes. In order to block polar and charged groups on the sections (which would cause non-specific binding of probe), the sections were placed in 1X (0.1M) triethanolamine-HCl (TEA; NaCl, 0.1M triethanolamine, adjusted to pH 8.0 with NaOH) for 10 minutes, 1X TAE/ 0.25% acetic anhydride for 10 minutes, 2X SSC for 5 minutes, 70% ethanol for 1 minute, 80% ethanol for 1 minute, 95% ethanol for 2 minutes, and 100% ethanol for 1 minute The lipids were then extracted from the sections in chloroform 2X 5 minutes, 100% ethanol for 1 minute, and 95% ethanol for 1 minute. Slides were allowed to dry on the bench at room temperature for at least an hour before being sealed in airtight slide boxes and stored at -20°C.

2.8 Estimation of mRNA by *in situ* hybridization

In situ hybridization is often used to localize results found by northern blot analysis in the brain. The principles of nucleic acid binding for *in situ* hybridization are similar to those for northern hybridizations (see section 2.6.6).

2.8.1 General procedure

Special care must be taken to prevent endogenous RNAse enzymes in the brain tissue from degrading the mRNAs we wished to measure. Sections were removed from storage at -20°C and allowed 1.5 hours to warm to room temperature (to prevent tissue damage from frost). The hybridization buffer for *in situ* hybridization contained 0.6M NaCl, 0.08M Tris buffer pH 7.5, 0.004M EDTA, 0.1% sodium pyrophosphate, 10% dextran sulfate, 0.2% SDS, 0.02% heparin NaCl, and 50% ultraformamide, which was made in advance and stored in aliquots at -20°C until use. This was added 10:1 to the ^{35}S radiolabelled RNA probe (1.0-3.0 X 10^6 cpm), was boiled for 5 to 10 minutes, placed on ice, and had 100 mM DTT added to prevent oxidation. Approximately 40 to 45 µL of the hybridization solution was added to each

slide. The slides were coverslipped, placed in a polystyrene box containing 2 pieces of Whatman filter paper soaked with 2X SSC/ 50% formamide (2X SSC made from 20X SSC stock: 3M NaCl, 0.3M sodium citrate, adjusted to pH 7.0 with 1M HCl), and incubated overnight at 50°C for at least 18 hours.

The following day, the sections were soaked for 10 minutes in 2X SSC buffer at room temperature, and the coverslips were then carefully removed. Sections were washed for 3X 5 minutes in 2XSSC buffer, treated with 10 mg/mL RNAse in 500 mL RNAse buffer (0.3M NaCl, 0.01M Tris buffer pH=8, and 0.005M EDTA pH=8) for 30 minutes, washed in RNAse buffer for 30 minutes, and washed in 2XSSC for 5 minutes. Depending on the radioactivity left on the slide, the sections were washed at varying degrees of time, temperature and SSC concentrations. A series of trials were performed to determine the appropriate wash conditions. For BDNF, sections were washed in 2X SSC at 60°C for 30 minutes and 0.2X SSC at 60°C for 30 minutes. For *c-fos*, sections were washed in 2X SSC at 50°C for 15 minutes and 0.2X SSC at 50°C for 10 minutes. Sections were dried in ascending ethanol concentrations (50, 70, 90, 95% ethanol/ 0.3M ammonium acetate) and allowed to air dry for at least one hour prior to exposure to film and/or emulsion.

2.8.2 Variations in procedures

Due to the initial weak signal obtained from tissue probed with the ^{35}S-radiolabelled *c-fos* probe, a competition assay was performed to determine if the appropriate probe concentration was being used. When it was verified that the probe concentration being used was appropriate, other variations in the procedure were undertaken to increase the *c-fos* hybridization signal. The substitution of ^{32}P for ^{35}S allowed for a stronger signal. In some experiments, the section thickness was increased from 12 to 20 µM which also increased the signal. Conditions were otherwise varied in a manner similar to that of the northern hybridizations. *C-fos* probes required incubation at 50°C, less RNAse

A treatment and fewer, shorter washes than did BDNF (incubation at 54°C, high RNAse A treatment, longer post hybridization washes). Longer incubation times (up to 24 hours) provided stronger signals on autoradiographs.

2.8.3 Autoradiography and exposure times

Exposure time for the development of measurable autoradiographs ranged from 1.5 days to 2 weeks. In general, each probe had an optimal development time. BDNF autoradiographs produced clear, strong signals in as little as 1.5 hours, while *c-fos*-generated autoradiographs took up to 2 weeks for development and had significant background. After development times of two weeks, background often became too dark for adequate measurement.

2.8.4 Quantitative analysis

2.8.4.1 Preparation of brain paste standards

Standards which have been calibrated to brain paste standards are commercially available (Nazarali et al., 1989). Although ^{14}C commercial standards are available for calibration of ^{35}S autoradiographic signals, ^{35}S brain paste standards were made by the present investigator for calibration and were verified to be linear on a Molecular Dynamics Phosphoimager System. Briefly, brain paste standards were prepared by weighing out approximately 150 mg rat brain which had been ground into a paste. After choosing a desired concentration (dpm/mg) of ^{35}S to be present in each of 14 brain pastes (which included dilutions in concentrations from 50, 100, 200 dpm/mg to 409,600 dpm/mg), these dilutions were added in equal volumes to the pastes and mixed well. The pastes were frozen in Eppendorf tubes, removed and mounted for sectioning while frozen, and cut at the same tissue section thickness (12 μM) as the tissue sections being analyzed for *in situ* hybridization autoradiography, with one section from each of the 14 brain pastes being present on each slide. A brain paste slide was included with

slides being exposed to film for quantification to ensure that the autoradiographs were in a linear range.

2.8.4.2 Positive controls

Positive controls with kainic acid-treated and haloperidol-treated animals were employed as described section in 2.6.7.2.

2.8.4.3 Imaging system

In situ hybridization data were analyzed using an Image 1 system (Universal Imaging Corporation) and NIH image. Two density measurements were made in each region measured on each side of the section. Sections from different drug conditions but that were similar anatomically were represented in the analysis at least in triplicate. Gray scale values were calibrated by ^{35}S brain paste standards on Image 1 and the data was converted to disintegrations per minute (dpm)/mm^2.

2.8.5 Photographic emulsion

Photographic emulsion is indicated when either cellular localization of signal is required or the structure containing the desired signal is too small to measure by film autoradiography. Kodak NTB2 photographic emulsion was diluted 1:1 with nanopure water in a dark room one day previous to the procedure (this allows bubbles to rise out of the dilution) and stored at 4°C in tinfoil-wrapped 50mL Falcon tubes in a radioactivity-free refrigerator. The following day, the water bath was warmed to 42°C and the emulsion was squeezed out into a copland jar in the waterbath. Slides to be dipped were placed in peel-aways. A test slide was first dipped to ensure that there were no bubbles. The slides were dipped for 3 seconds and allowed to dry for at least 2 hours. Test slides for early, middle, and late development times were also prepared. The slides were then placed in light-tight boxes with desiccant and stored at 4°C until development. After development (2 minutes in Kodak developer, 30 seconds in water, 3 minutes in Kodak fixer prepared according

to the manufacturer's instructions) the sections were counterstained with cresyl violet.

2.9 Immunohistochemistry

2.9.1 Procedure

Immunostaining was performed as described by Hiroi and Graybiel (1996) with a polyclonal antiserum against FosB/ delta FosB (Santa Cruz Biotechnology, Santa Cruz, CA; kindly donated by Dr. X. Zhang) on free floating sections at 1:1500. Sections were stained by the avidin-biotin (ABC) technique. Sections were rinsed for 10 minutes in 0.01 M phosphate buffered saline (PBS; 0.9% NaCl, 0.1M KH_2PO_4, 0.1M Na_2HPO_4) containing 0.2% Triton X-100 (PBS-TX), were treated for 10 minutes with 3.0 % H_2O_2 in PBS-TX to prevent endogenous peroxidase activity, were blocked for 3 hours in 5% normal goat serum in PBS, and were incubated with the primary antisera (anti-rabbit) at 4°C for 15 hours in 0.01M PBS, 0.8% Triton X-100, and 1% normal serum.

The appropriate concentration of the primary antibody was determined using two sets of concentration gradients on both the tissue of interest and a positive control tissue (kainate-treated animals). The first gradient included ratios of primary antibody: PBS of 1:100, 1:500, 1:1000, 1:5000, 1:10000. The best concentration from this trial along with two concentrations below and two concentrations above were then employed to find the best primary concentration in this range. Too low a concentration obviously produces a weak signal, while a too high concentration is wasteful and may cause the primary to compete with itself for binding.

2.9.2 Variations in procedure

Variations in TritonX-100 concentrations were also employed. Triton solubilizes cell membranes and enhances the ability of the primary antibody to bind to its target protein. If the Triton concentration was too low,

immunoreactivity was significantly reduced. Although tissue morphology is disrupted by Triton, there was not much difference in tissue preservation at 0.2 vs. 0.8% TritonX-100. In addition, various incubation times were employed to determine the best incubation period for the primary antibody. Longer incubation times disrupted tissue morphology regardless of triton concentrations, but produced better immunoreactivity. Thus, an 0.8% Triton solution and the shortest incubation times possible were used due to its ability to improve primary antibody binding. Sections were washed 3X 10 minutes in PBS-TX and then incubated with the goat-anti-rabbit secondary antiserum (1:500, Vector) for 1 hour (in PBS-TX, 1% normal serum). The concentration of the secondary antibody and incubations times were determined in a manner similar to that of the primary antibody.

The sections were again washed in PBS-TX and subsequently incubated with the avidin-biotin-peroxidase complex (1:150, Vectastain Kit) for 45 minutes in PBS. Longer incubations in the tertiary produced excessive background staining. Horseradish peroxidase conjugated with biotin was developed with diaminobenzidine (DAB) in the presence of H_2O_2 (0.001%) in Tris-HCl buffer (0.1M). The sections were washed 1X 10 minutes Tris-HCl, 1X 10 minutes tap water, and were mounted on slides using gelatin-chrom-alum. Sections were then dried through ascending alcohols, cleared with xylene and coverslipped.

Control sections receiving no primary antibody and no secondary antibody were included to ensure that there are no non-specific interactions of DAB with the tissue. A secondary antibody and tertiary only control ensured that there was no non-specific binding of the secondary that could be mistaken for primary antibody binding, and indicated whether or not tissue blocking was sufficient (blocking must be sufficient to block non-specific binding but low enough to permit the binding of the primary antibody). It was also discovered

through a number of experiments that the immunohistochemistry worked much better on free floating sections vs. those mounted on poly-L-lysine coated slides. The reason for this is unknown, but might have been due to a reduced opportunity for primary antibody penetrance on mounted sections.

2.9.3 Quantitation

The number of FosB/ delta FosB-positive neurons in the medial prefrontal cortex (400 X 600 μm, mPFC), dorsolateral striatum (DLStr), mediolateral striatum (MLStr), and nucleus accumbens (NuAcc) was quantified by image analysis using Northern Eclipse software (Empix Imaging, Inc.). Areas counted were chosen as defined by Robertson et al. (1994). Total area counted for the mPFC and NuAcc was 400 X 600 μm^2, while total area for both striatal regions was 800 X 1200 μm^2. Two measurements on each section and its replicate were performed. In order to confirm that staining was nuclear, several sections were checked to ensure that the staining being counted disappeared in and out of focus through the tissue.

2.10 Statistical analysis

All results were analyzed by one or two way analysis of variance performed using the CLR ANOVA program (Clearlake Research, Houston, TX, U.S.A.). In the presence of significant F values, individual comparisons between means were made using Newman-Keuls test.

3. RESULTS

3.1 The effects of neuroleptic administration on *c-fos* and monoamine synthetic enzymes (TH and AADC) mRNA in PC12 cell culture

3.1.1 The effects of remoxipride, risperidone, ritanserin, haloperidol and clozapine on TH and AADC mRNA after 0.5, 1, 2, 6, 12, 24, and 48 hours in culture, as determined by northern blot analysis in PC12 cells

Initial experiments examined the effects of remoxipride, risperidone, and ritanserin administration to PC12 cells at 1, 6, and 24 hours at concentrations of 0.1, 1.0, and 10.0 (2.0 for ritanserin) μM on TH and AADC mRNA. No statistical differences were found, so further studies were performed using a wider range of doses and time points. Doses ranged from 0.01 to 250 μM and time points ranged from 0.5 to 48 hours. Since haloperidol and clozapine are considered clinically to be prototypical "typical" and "atypical" neuroleptics respectively, they were included in subsequent studies as a comparison to the three other neuroleptics and were examined at a greater number of time points*. The following neuroleptics were used at the following doses:

Remoxipride:	0.01, 0.1, 1.0, 10.0, 100.0 μM
Risperidone:	0.01, 0.1, 1.0, 10.0, 100.0 μM
Ritanserin:	0.01, 0.1, 1.0, 2.0, 10.0 μM
Haloperidol:	0.01, 0.1, 1.0, 10.0, 100.0 μM
Clozapine:	0.25, 2.5, 25.0, 250.0 μM

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*Note: Olanzapine was only used in later PC12 studies involving SOD and p75 mRNA.

Data for these experiments are seen in figures 3.1 to 3.10. All doses and time points presented in these figures represent an n of 3 or greater.

3.1.2 The effects of remoxipride, ritanserin and clozapine on TH and AADC mRNA at 1, 6, and 24 hours

Figures 3.1 to 3.6 illustrate that incubation of PC12 cell cultures with neither remoxipride, ritanserin, nor clozapine had any statistically significant effect on AADC and TH mRNA. Incubation of PC12 cells with 0.1, 1.0, or 10.0 μM of remoxipride for 1, 6 and 24 hours produced no effects of dose [$F(3,24) = 1.17$, $p = 0.34$] or time [$F(2,24) = 0.11$, $p = 0.098$] nor were there any interactions of dose and time [$F(6,24) = 0.51$, $p = 0.79$] on AADC mRNA, as disclosed by a two way ANOVA (Figure 3.1). Figure 3.2 demonstrates that there were no effects of remoxipride on TH mRNA at these doses [$F(3,24) = 1.16$, $p = 0.35$] and time points [$F(2,24) = 0.11$, $p = 0.90$], nor were there any interactions of time and dose [$F(6,24) = 0.66$, $p = 0.68$] as revealed by a two way ANOVA.

Likewise, incubation of PC12 cells with 0.1, 1.0, and 2.0 μM of ritanserin produced no effect of dose [$F(3,24) = 1.27$, $p = 0.31$] or time [$F(2,24) = 0.05$, $p = 0.96$] on AADC mRNA nor was there any interaction of dose and time [$F(6,24) = 0.26$, $p = 0.95$] on AADC mRNA, as disclosed by a two way ANOVA (Figure 3.3). Figure 3.4 demonstrates that there were no effects of ritanserin on TH mRNA at these doses [$F(3,24) = 0.50$, $p = 0.68$] and time points [$F(2,24) = 0.20$, $p = 0.83$],], nor were there any interactions of time and dose [$F(6,24) = 0.28$, $p = 0.94$], as revealed by a two way ANOVA.

Although an initial trend was observed for clozapine to upregulate AADC and especially TH mRNA at 1 hour, none of these results were statistically significant (see Figures 3.5 and 3.6). Incubation of PC12 cells with 2.5 or

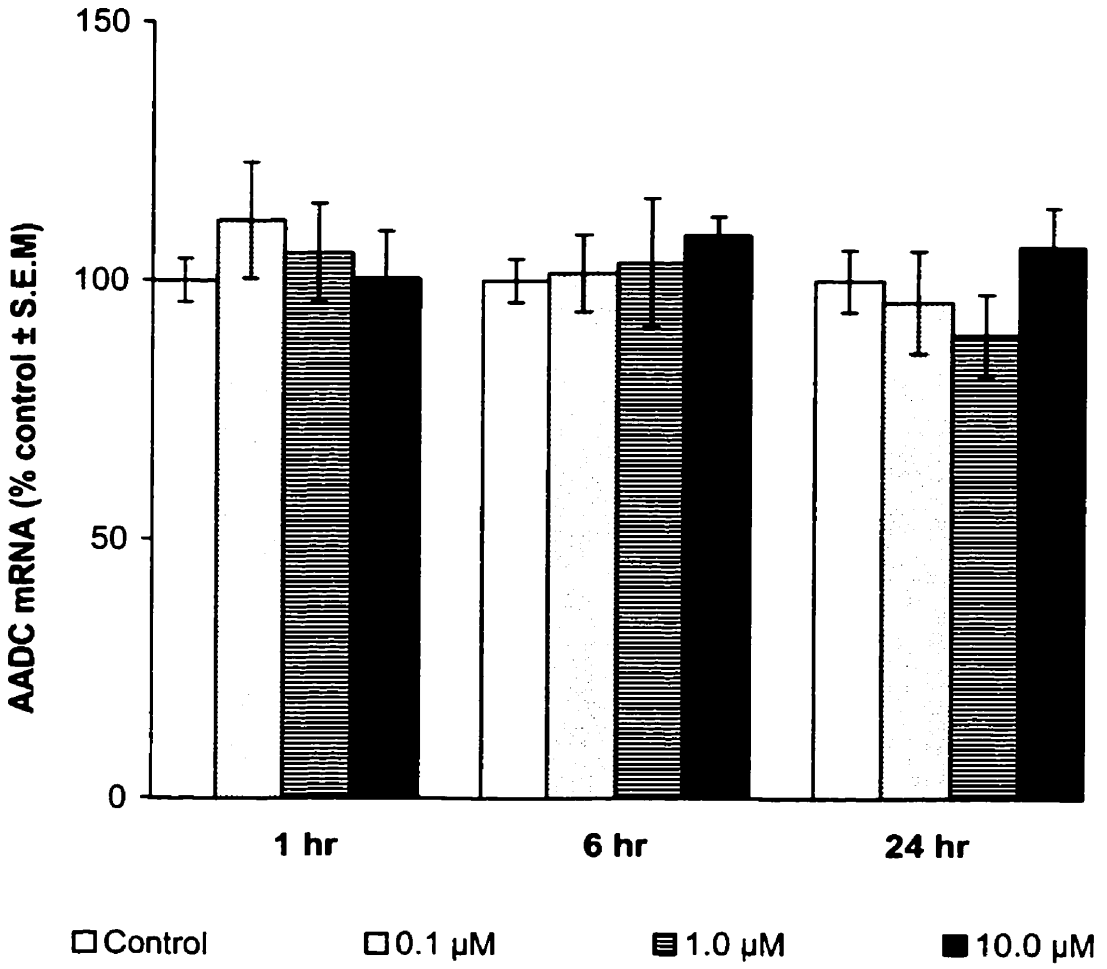

Figure 3.1. The effects of remoxipride on AADC mRNA in PC12 cells as determined by northern blot analysis. PC12 cells were treated with 0.1, 1.0, or 10.0 µM remoxipride and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

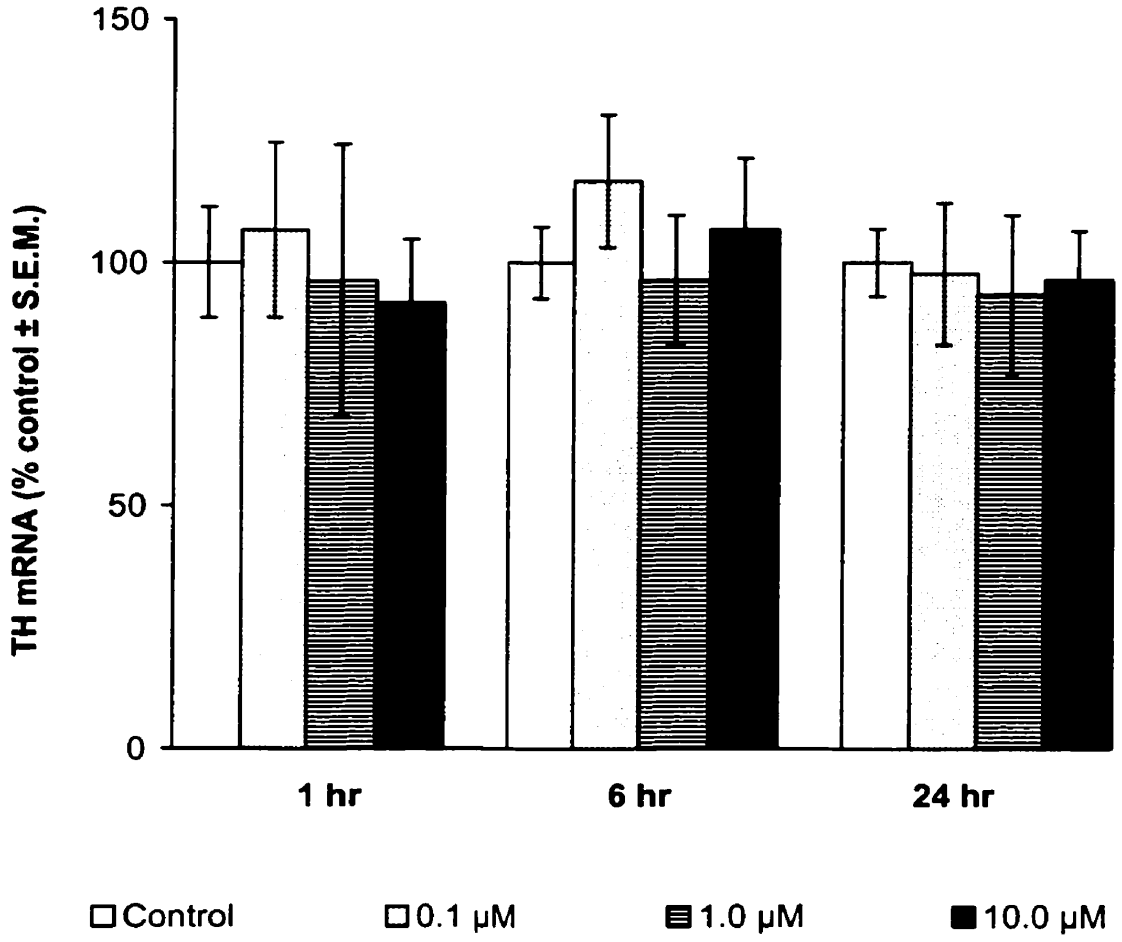

Figure 3.2. The effects of remoxipride on TH mRNA in PC12 cells as determined by northern blot analysis. PC12 cells were treated with 0.1, 1.0, or 10.0 μM remoxipride and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

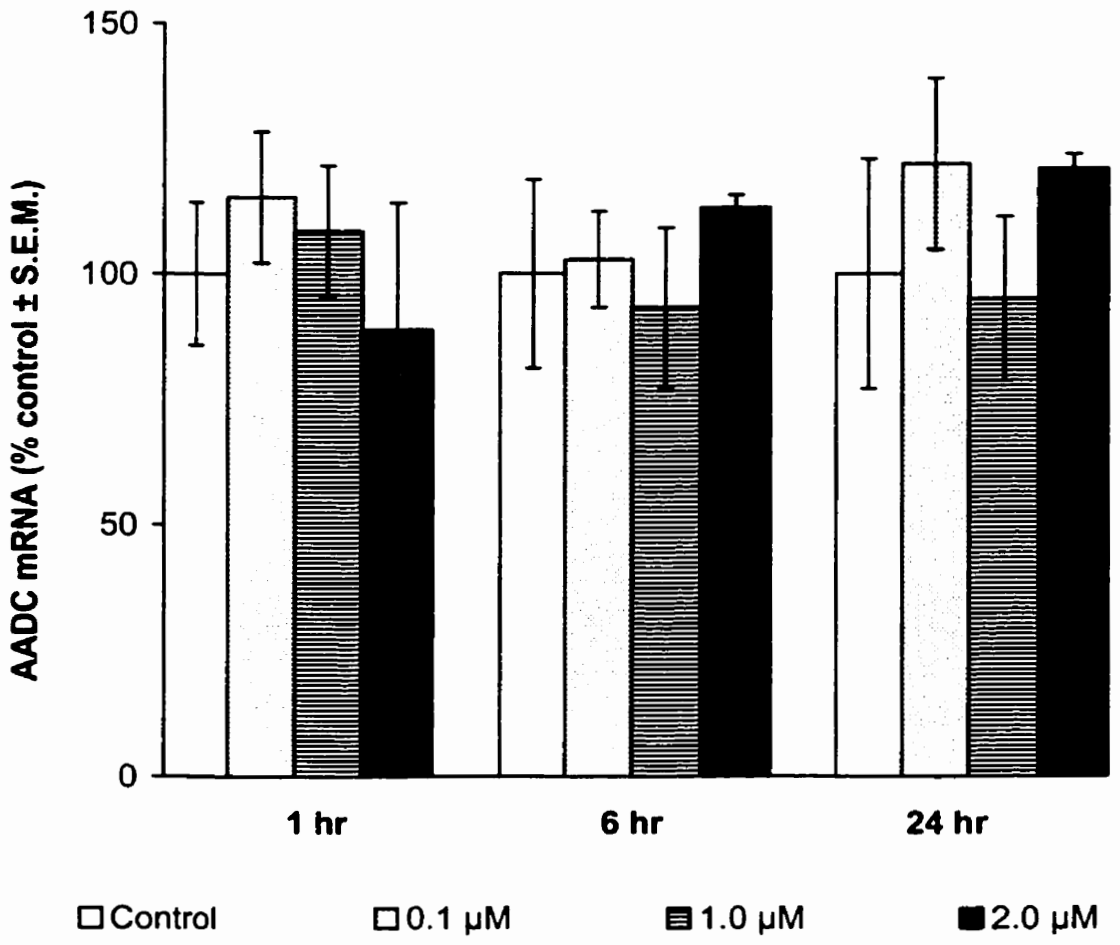

Figure 3.3. The effects of ritanserin on AADC mRNA in PC12 cells as determined by northern blot analysis. PC12 cells were treated with 0.1, 1.0, or 2.0 µM ritanserin and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

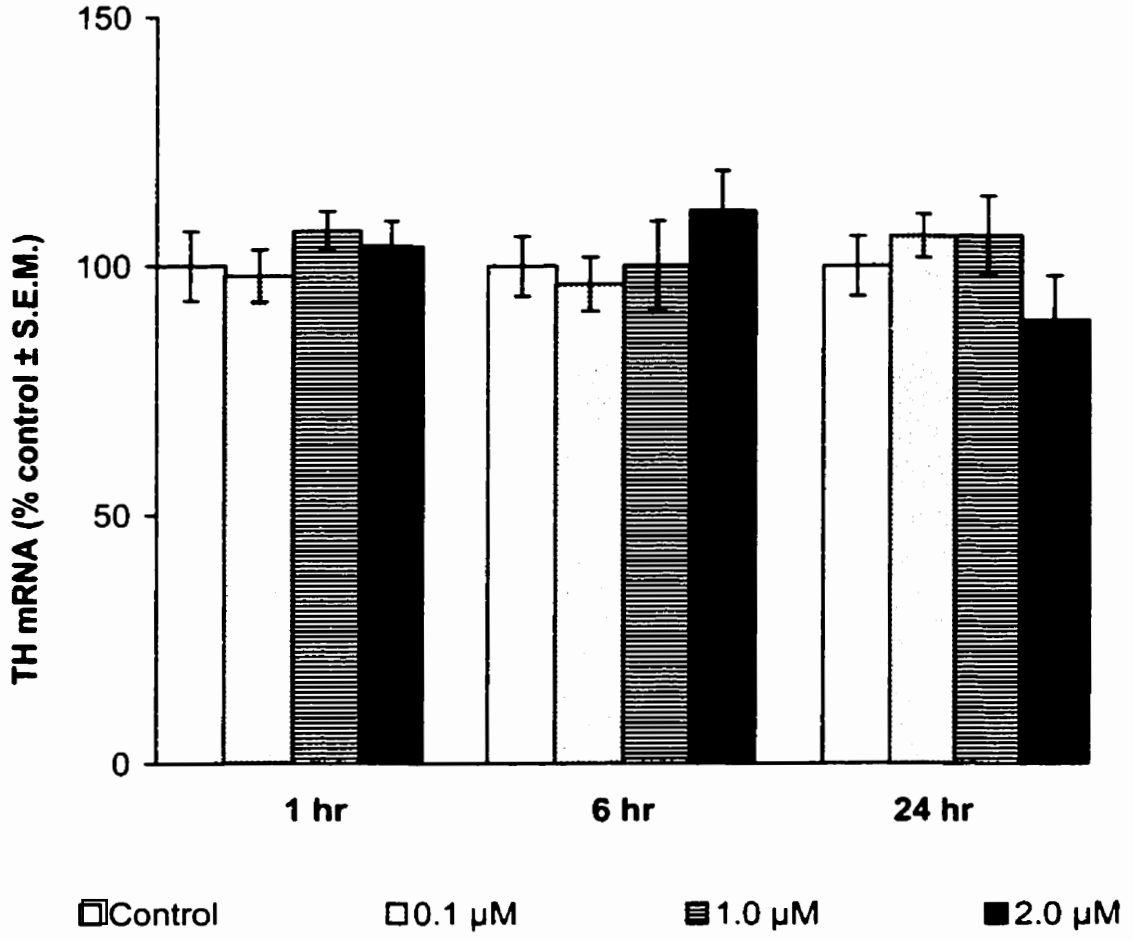

Figure 3.4. The effects of ritanserin on TH mRNA in PC12 cells as determined by northern blot analysis. PC12 cells were treated with 0.1, 1.0, or 2.0 μM ritanserin and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

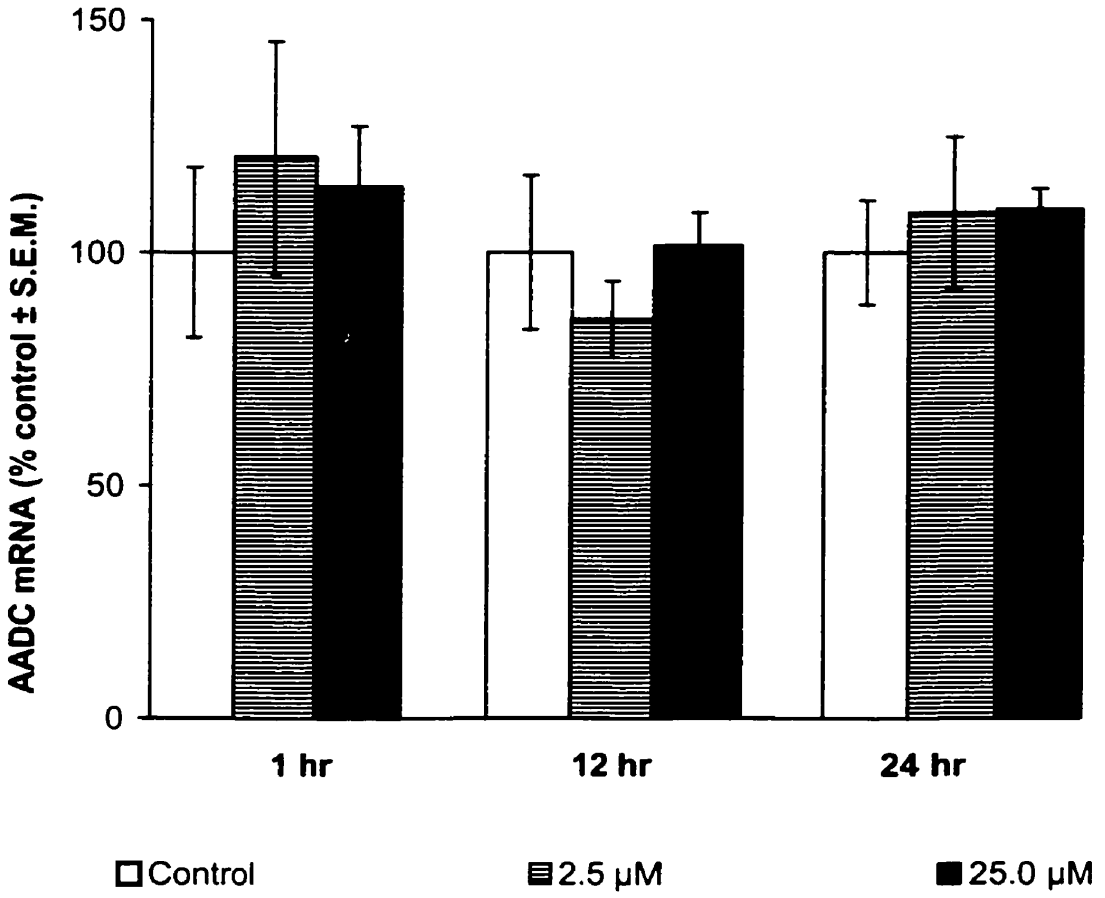

Figure 3.5. The effects of clozapine on AADC mRNA in PC12 cells as determined by northern blot analysis. PC12 cells were treated with 2.5 or 25.0 µM clozapine and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

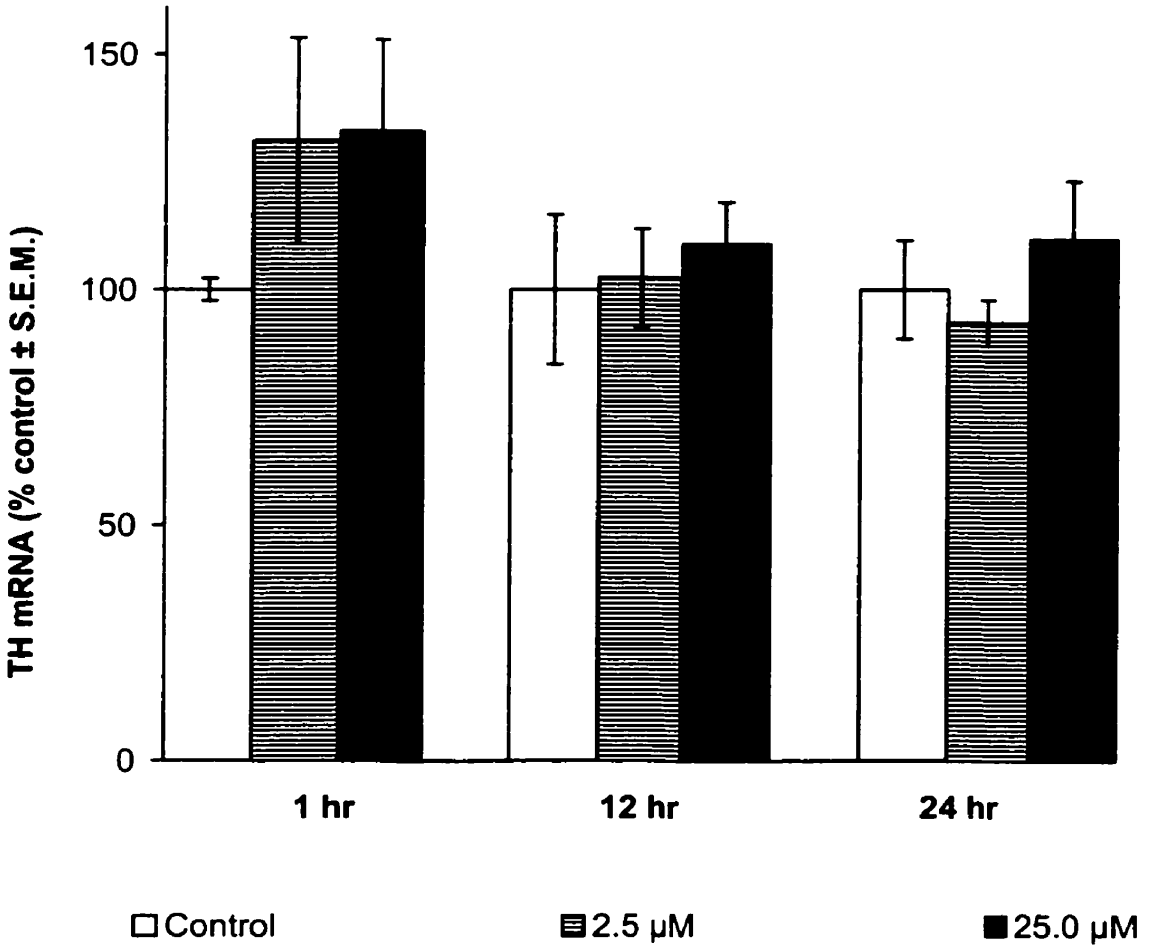

Figure 3.6. The effects of clozapine on TH mRNA in PC12 cells as determined by northern blot analysis. PC12 cells were treated with 2.5 or 25.0 μM clozapine and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

25.0 μM clozapine produced no effect of dose [$F(2,18) = 0.27$, $p = 0.77$] or time [$F(2,18) = 1.60$, $p = 0.23$], nor was there an interaction of dose and time [$F(4,18) = 0.42$, $p = 0.79$] on AADC mRNA, as disclosed by a two way ANOVA (Figure 3.5). Figure 3.6 demonstrates that there were no effects of clozapine on TH at these doses [$F(2,18) = 0.19$, $p = 0.83$] and time points [$F(2,18) = 2.81$, $p = 0.09$], nor were there any interactions of time and dose mRNA [$F(4,18) = 0.71$, $p = 0.59$], as revealed by a two way ANOVA.

To ensure that an effect of clozapine on AADC and TH mRNA did not occur between 1 and 12 hours, a subsequent experiment was performed which included a 2 hour time point. One and two way ANOVAs in subsequent experiments demonstrated that AADC and TH mRNA levels returned to control levels by 2 hours (data not shown).

3.1.3 The effects of risperidone on AADC and TH mRNA at 1, 6, and 24 hours

Figures 3.7 and 3.8 illustrate that incubation of PC12 cell cultures for 1 hour with risperidone significantly upregulates AADC and TH mRNA, but that this effect is attenuated by 6 and 24 hours respectively. Although incubation of PC12 cells with 0.1, 1.0, or 10.0 μM risperidone produced no effect of dose [$F(3,24) = 2.42$, $p = 0.09$] on AADC mRNA, there was an effect of time [$F(2,24) = 7.31$, $p = 0.003$], as disclosed by a two way ANOVA (Figure 3.7), whereby 10 μM risperidone upregulated AADC mRNA at one hour to 135% of control values (post hoc by Newman Keuls, $p<0.05$). There were no interactions of dose and time [$F(6,24) = 1.02$, $p = 0.44$] as revealed by the two way ANOVA.

Figure 3.8 demonstrates that incubation of PC12 cell cultures with 1 or 10 μM risperidone produced significant increases in the levels of TH mRNA that were observed at 1 hour. Although incubation of PC12 cells with 0.1, 1.0, or 10.0 μM risperidone produced no effect of time [$F(2,24) = 2.99$, $p = 0.07$] on

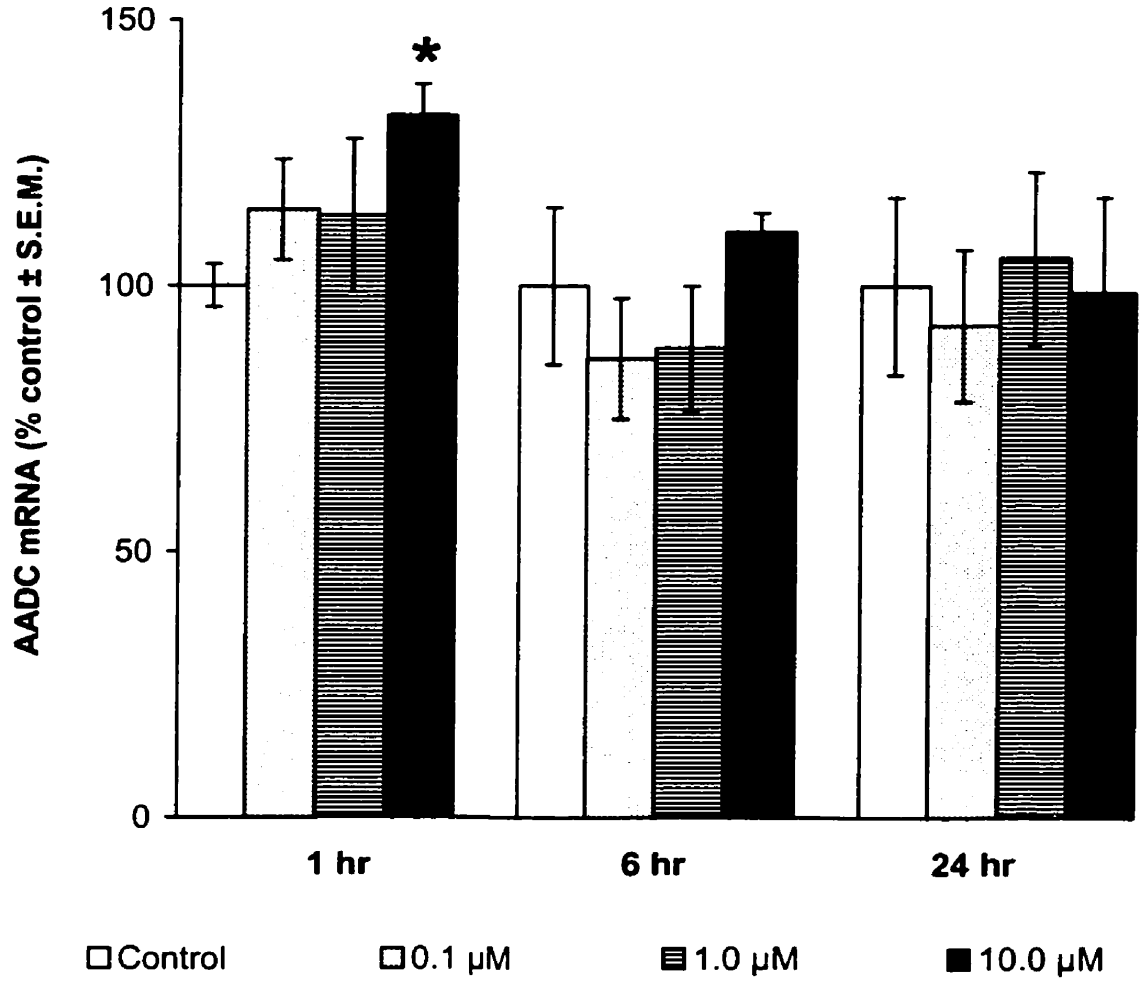

Figure 3.7. The effects of risperidone on AADC mRNA in PC12 cells as determined by northern blot analysis. PC12 cells were treated with 0.1, 1.0, or 10.0 µM risperidone and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

* significantly different from controls, $p<0.05$.

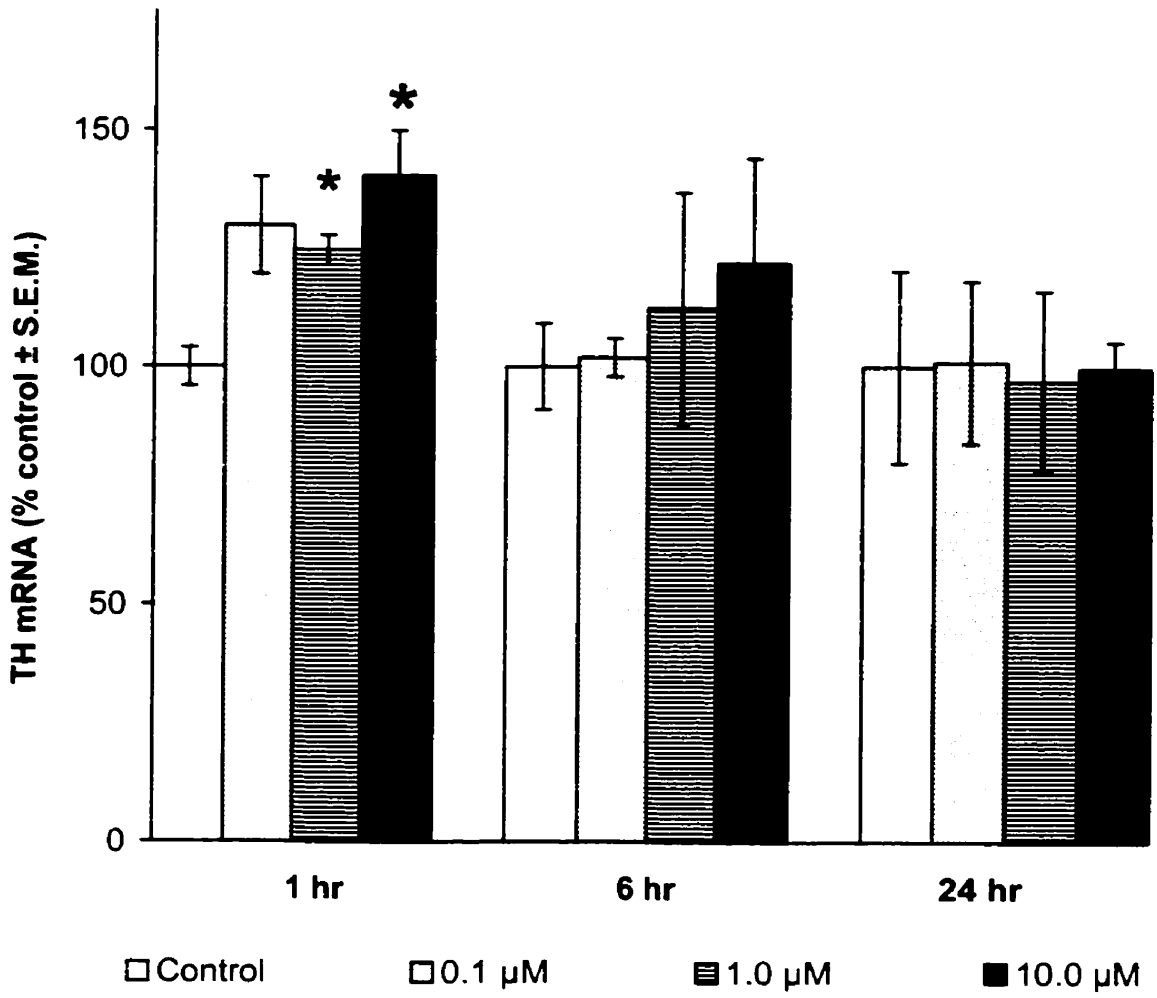

Figure 3.8. The effects of risperidone on TH mRNA in PC12 cells as determined by northern blot analysis. PC12 cells were treated with 0.1, 1.0, or 10.0 μM risperidone and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

*significantly different from controls, $p<0.05$.

TH mRNA, there was an effect of dose [F(3,24) = 5.33, p = 0.006] on TH mRNA as disclosed by a two way ANOVA (Figure 3.8), whereby 1.0 and 10.0 μM risperidone significantly upregulated TH mRNA at one hour to 124% and 141% respectively of control values (post hoc by Newman Keuls, $p<0.05$). No interaction of dose and time [F(6,24) = 1.96, p = 0.11] was observed.

3.1.4 The effects of haloperidol on AADC and TH mRNA at 1, 2, 12, and 24 hours in PC12 cells

Figures 3.9 and 3.10 demonstrated that while incubation of PC12 cell cultures with 1.0 or 10 μM of haloperidol produced no significant increases in AADC mRNA, significant increases in the levels of TH mRNA were observed at 2 and 12 hours of incubation. Figure 3.9 shows that incubation of PC12 cells with 1.0 or 10.0 μM haloperidol produced no effect of dose [F(2,24) = 0.22, p = 0.80] or time [F(3,24) = 1.41, p = 0.27] on AADC mRNA, nor was there an interaction of dose and time [F(6,24) = 0.63, p = 0.70], as disclosed by a two way ANOVA. Although statistically nonsignificant, the apparent increase in AADC mRNA at 2 hours seems to be followed by a compensatory decrease at 12 hours (Figure 3.9).

Incubation of PC12 cell cultures with 1 or 10 μM of haloperidol produced significant increases in the levels of TH mRNA that were observed at 2 and 12 hour time points (Figure 3.10). A two way ANOVA disclosed an effect of dose [F(2,24) = 6.51, p = 0.006, an effect of time [F(3,24) = 12.14, p = 0.00006], and an interaction of dose and time [F(6,24) = 3.04, $p < 0.03$], whereby 1.0 and 10.0 μM risperidone upregulated TH mRNA at 2 hours to 134% and 132% respectively of controls, and at 12 hours to 211% and 222% respectively of control values (post hoc by Newman Keuls, $p<0.05$). Levels of TH mRNA returned to control levels by 24 hours.

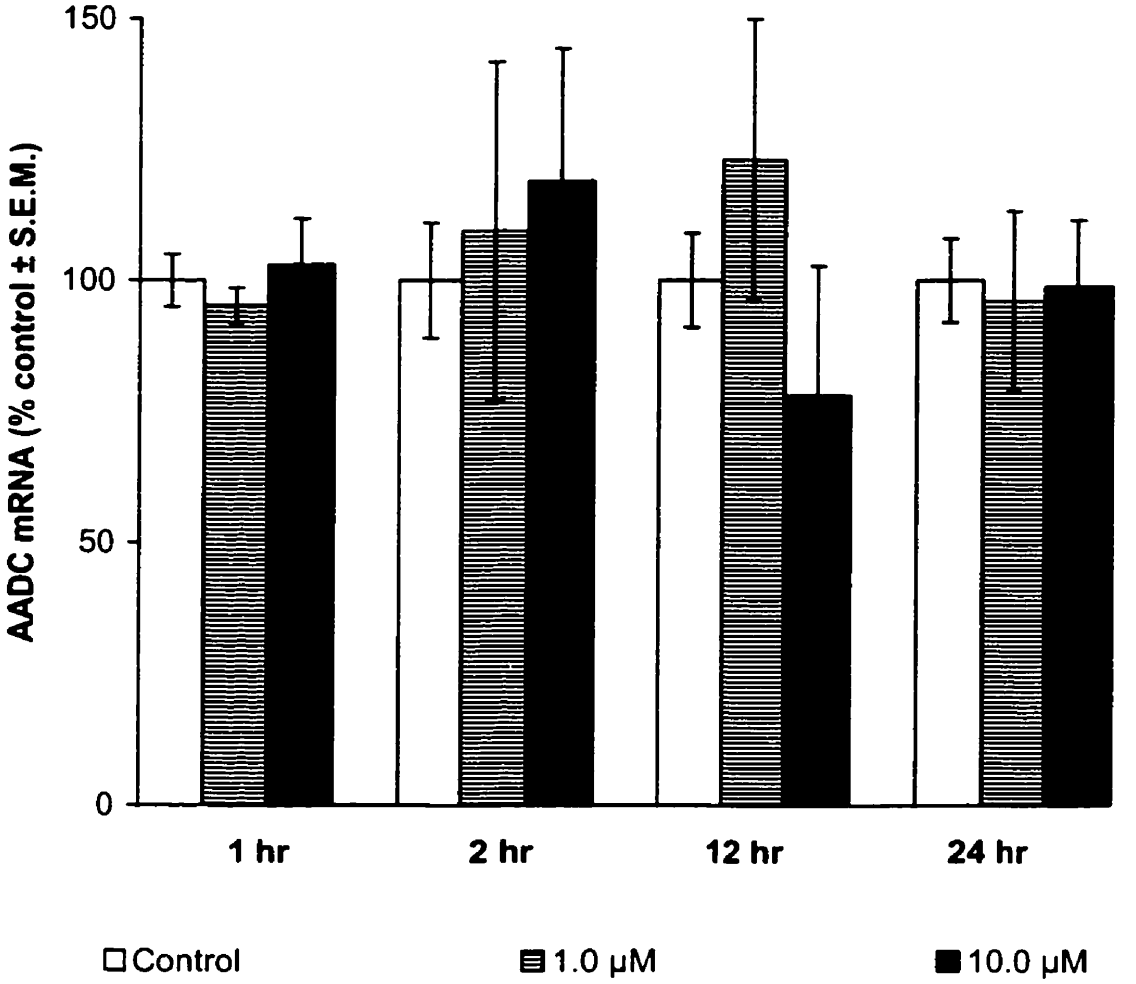

Figure 3.9. The effects of haloperidol on AADC mRNA in PC12 cells as determined by northern blot analysis. PC12 cells were treated with 1.0 or 10.0 µM haloperidol and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

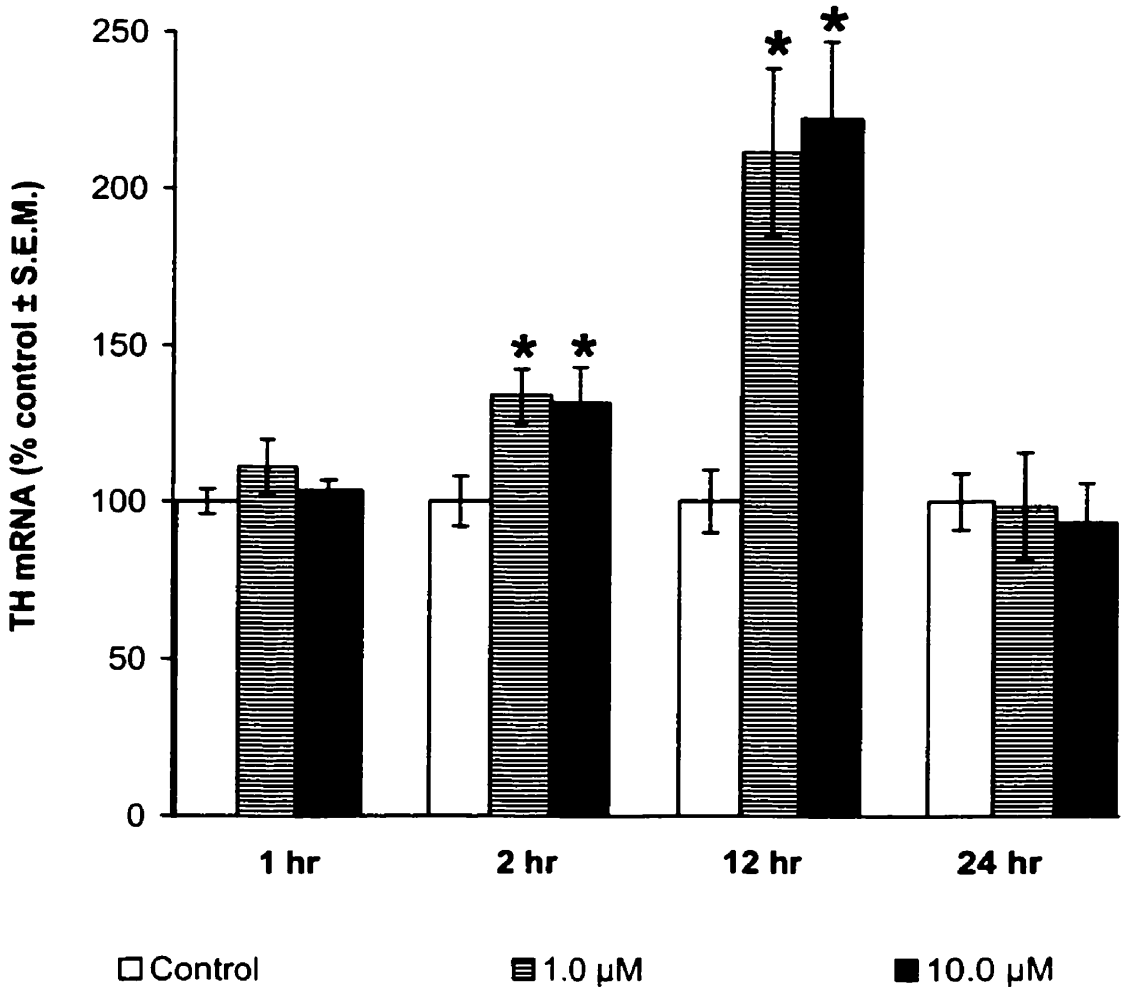

Figure 3.10. The effects of haloperidol on TH mRNA in PC12 cells as determined by northern blot analysis. PC12 cells were treated with 1.0 or 10.0 µM haloperidol and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

* significantly different from controls, $p<0.05$.

Since these results did not support the hypothesis that the neuroleptics used in the present study would regulate AADC mRNA, no further studies of the effects of these neuroleptics on TH and AADC mRNA were performed in PC12 cells or in animal studies.

3.1.5 The effects of remoxipride, risperidone, ritanserin, haloperidol and clozapine on *c-fos* mRNA at 0.5, 1.0, and 2.0 hours as determined by northern blot analysis in PC12 cells

Figure 3.11 shows representative autoradiographs of the effects of the five neuroleptics on AADC, TH, and *c-fos* mRNA. Although a clear trend was observed for a high dose of haloperidol (100 μM) to upregulate *c-fos* (and TH) mRNA but not AADC mRNA, no effects of the other neuroleptics on *c-fos* mRNA were observed. Interestingly, any trends of neuroleptic -induced increases in *c-fos* mRNA appeared to be paralleled by changes in TH but not AADC mRNA. Due to the low expression of *c-fos,* these data were not statistically analyzed.

3.2 The effects of neuroleptic administration on c-fos mRNA and delta FosB

The effects of acute, chronic, and chronic/challenge neuroleptic administration on *c-fos* mRNA were examined *in vivo* with the previously mentioned neuroleptics at various doses in the striatum, hippocampus, nucleus accumbens, and frontal cortex by northern blot, film, and emulsion analysis, as described below. In summary, strong D_2 receptor blocking neuroleptics, including haloperidol and risperidone, upregulated striatal *c-fos* mRNA, while the other neuroleptics tested did not. Risperidone, clozapine, and haloperidol appeared to increase *c-fos* mRNA in the nucleus accumbens, while none of the neuroleptics tested changed *c-fos* mRNA in the frontal cortex or hippocampus.

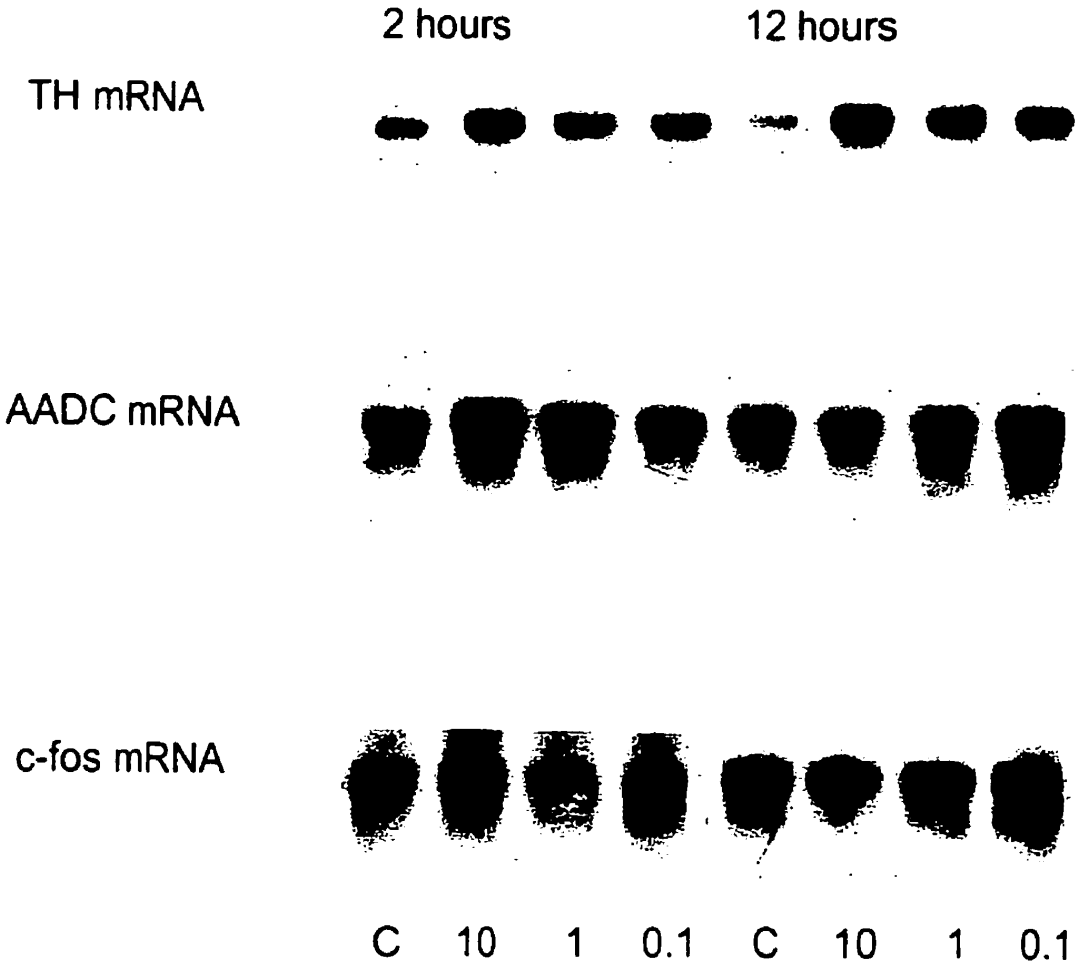

Figure 3.11. Representative autoradiographs of the effects haloperidol on TH, AADC, and c-fos mRNA in PC12 cells. Only TH mRNA continues to be upregulated at 12 hours.

3.2.1 Northern blot analysis of the effects of acute moderate and high dose neuroleptic administration on *c-fos* mRNA

The effects of the acute, single dose administration of some neuroleptics on the expression of *c-fos* mRNA were examined in the rat striatum, hippocampus, nucleus accumbens and frontal cortex by northern blot analysis. The rats were sacrificed 45 minutes following the i.p. administration of remoxipride (3 mg/kg), risperidone (3 mg/kg), ritanserin (3 mg/kg), haloperidol (3 mg/kg), clozapine (30 mg/kg) or an equal volume of vehicle. Haloperidol produced a significant increase in the striatal expression of *c-fos* mRNA that was 200% of the values of the vehicle control group, as disclosed by one way ANOVA [$F(5,12) = 7.97$, $p < 0.00162$] (Fig. 3.12), but no changes were observed after administration of any of the other drugs. Risperidone did show a strong tendency to upregulate striatal *c-fos* mRNA, but statistical significance was not reached on the post-hoc Newman Keuls test.

The neuroleptics produced no changes in *c-fos* mRNA in the hippocampus, as disclosed by one way ANOVA [$F(5,12) = 2.34$, $p < 0.106$]. Although risperidone and haloperidol seemed to upregulate hippocampal *c-fos* mRNA (see Figure 3.12), statistical significance was not reached due to high variation. For the nucleus accumbens, samples from three animals (n=1) was pooled in order to have enough sample with which to run a northern blot - thus classical statistics could not be run on this sample. These values were used as positive controls to compare the present data to that found in the literature, which states that both haloperidol and clozapine upregulate *c-fos* mRNA in the nucleus accumbens. All five neuroleptics tested increased *c-fos* mRNA in the nucleus accumbens over controls, with the largest increases being for haloperidol (240% of control levels), clozapine (204% of control levels), and risperidone (135% of control levels).

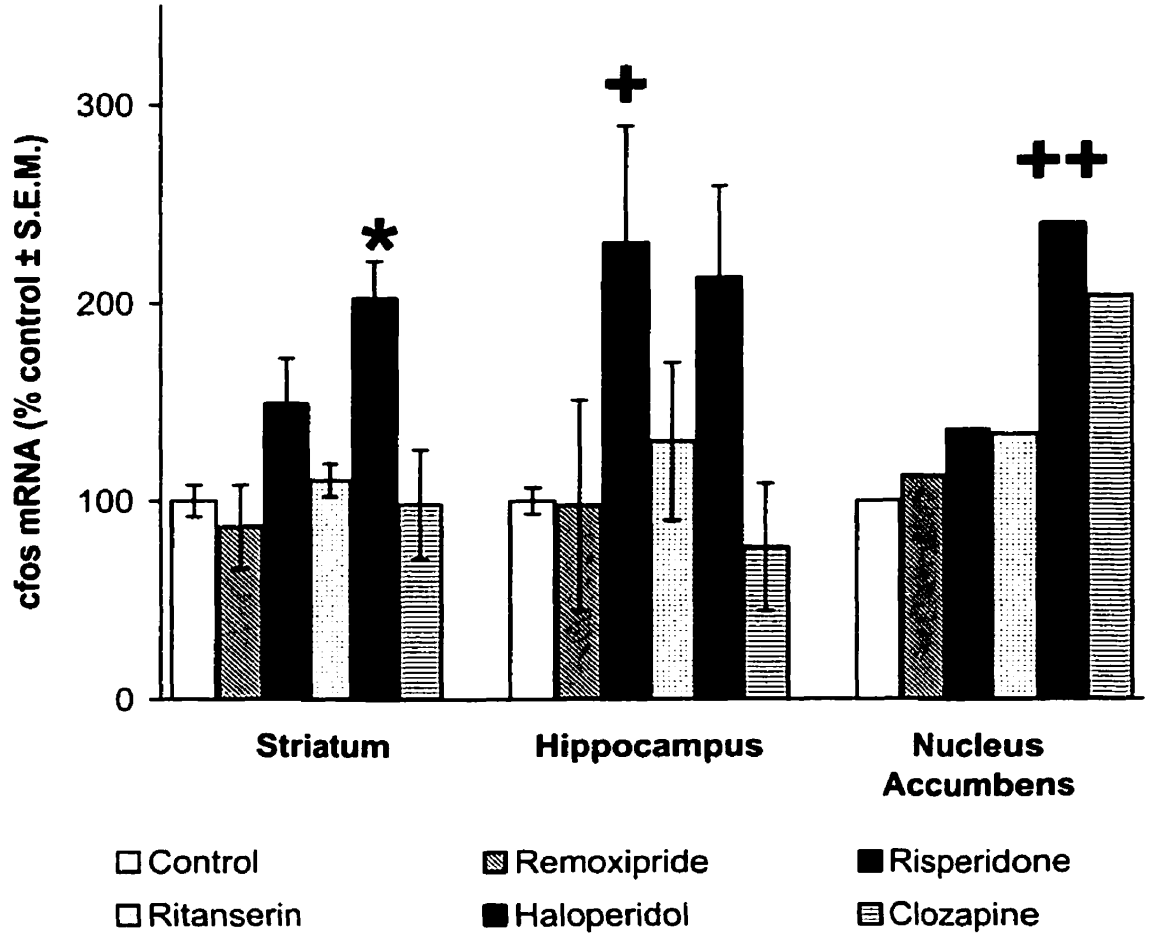

Figure 3.12. The effects of acute high dose neuroleptic administration on c-fos mRNA in rat striatum, nucleus accumbens, and hippocampus by northern blot analysis. Northern blot data for c-fos mRNA. Animals were sacrificed 45 minutes after i.p. injections (acute, high dose condition). Doses were 3 mg/kg for all drugs except clozapine (30 mg/kg). For striatum and hippocampus, n=3. Results are expressed as percent control ± S.E.M.

* significantly different from controls, $p<0.05$.
+ Statistical significance not reached due to high variation.
++ For the nucleus accumbens, n=1, pooled from three animals in order to have enough sample with which to run a northern blot - thus classical statistics could not be run on this sample. Values were used as positive controls to compare other data to the literature.

In a repeat study, Figure 3.13 shows the differential effects of striatal and hippocampal *c-fos* mRNA by risperidone, haloperidol, and clozapine. A one way ANOVA and subsequent post-hoc analysis by Newman Keuls revealed that both haloperidol ($p<0.0001$) and risperidone ($p<0.05$) significantly upregulated striatal *c-fos* mRNA to 519% and 292% of the control values, respectively [$F(3,8) = 20.67$, $p = 0.0004$]. In addition, the upregulation by haloperidol of *c-fos* mRNA was significantly greater than that by risperidone ($p<0.01$). Clozapine did not significantly upregulate striatal *c-fos* mRNA. No significant effects of any of the neuroleptics tested were seen on hippocampal *c-fos* mRNA [$F(3,8) = 0.43$, $p=0.74$]. These results were similar to those illustrated in Figure 3.12.

Figure 3.14 shows the differential effects of the acute administration of a moderate dose of risperidone (1mg/kg), haloperidol (1mg/kg), and clozapine (15 mg/kg) on striatal and frontal cortex *c-fos* mRNA. A one way ANOVA and post-hoc Newman Keuls tests revealed that both haloperidol ($p<0.0001$) and risperidone ($p<0.05$) significantly upregulated striatal *c-fos* mRNA to 486% and 292% of the control values respectively [$F(3,8) = 20.67$, $p = 0.0004$] while clozapine was without effects on these parameters. Statistical significance is observed for risperidone due to reduced variability. No significant effects of any of the neuroleptics on c-fos mRNA were seen the frontal cortex [$F(3,12) = 0.18$, $p = 0.91$].

Figure 3.15 contains pictures of the autoradiographs for the data in Figure 3.14 and shows the increased intensity of *c-fos* but not cyclophilin mRNA by haloperidol and risperidone in the striatum but not the frontal cortex The intensity of striatal *c-fos* mRNA is unchanged for clozapine, and none of the neuroleptics tested upregulated cyclophilin mRNA in either brain region. *C-fos* data was normalized to cyclophilin mRNA on each nitrocellulose membrane (i.e., *c-fos* and cyclophilin mRNA are measured from the same membrane to ensure that RNA was loaded equally for each of the twelve lanes).

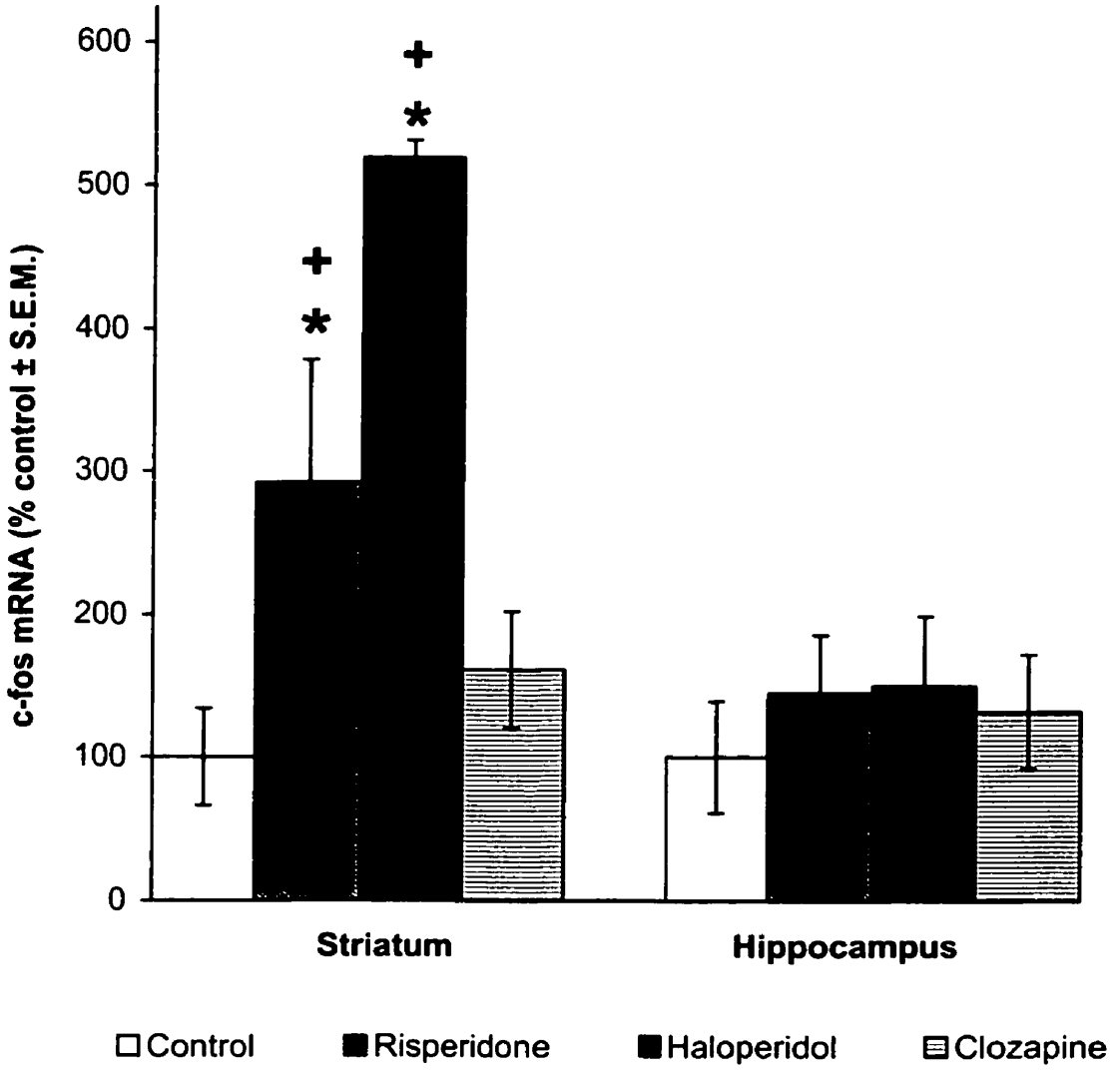

Figure 3.13 The effects of high dose neuroleptics on c-fos mRNA in rat striatum and hippocampus (repeat study) as determined by northern blot analysis. Animals (n=3) were sacrificed 45 minutes after i.p. injections (acute, high dose condition). Doses were 3 mg/kg for all drugs except clozapine (30 mg/kg). Results are expressed as percent control ± S.E.M.

* significantly different from controls, $p<0.001$.
+ haloperidol significantly different from risperidone, $p<0.05$.

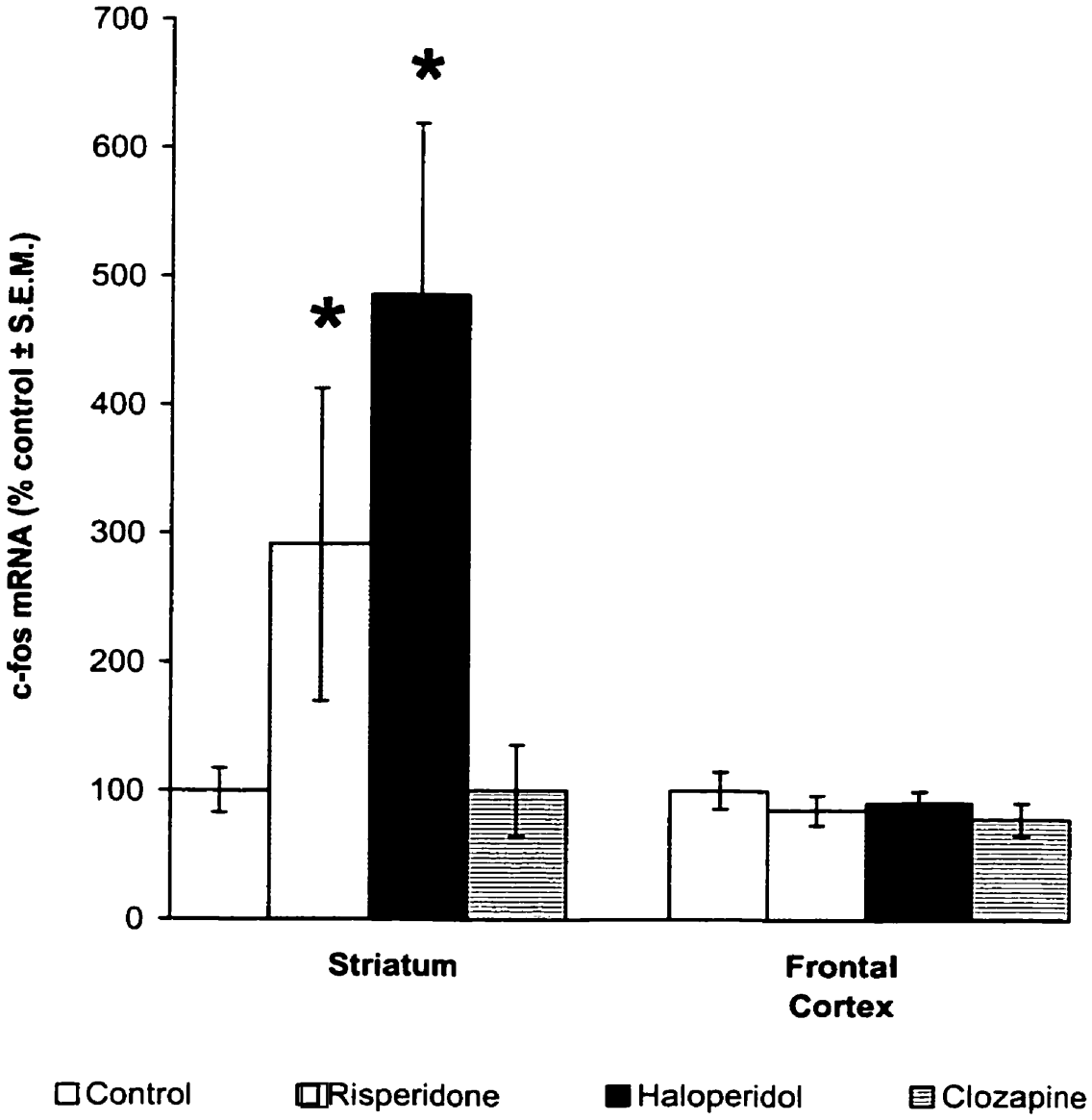

Figure 3.14 The effects of acute moderate dose neuroleptics on c-fos mRNA in the striatum and frontal cortex as determined by northern blot analysis. Animals (n=4) were sacrificed 45 minutes after i.p. injections. Doses were 1 mg/kg for all drugs except clozapine (15 mg/kg). Results are expressed as percent control ± S.E.M.

* significantly different from controls, $p<0.05$.

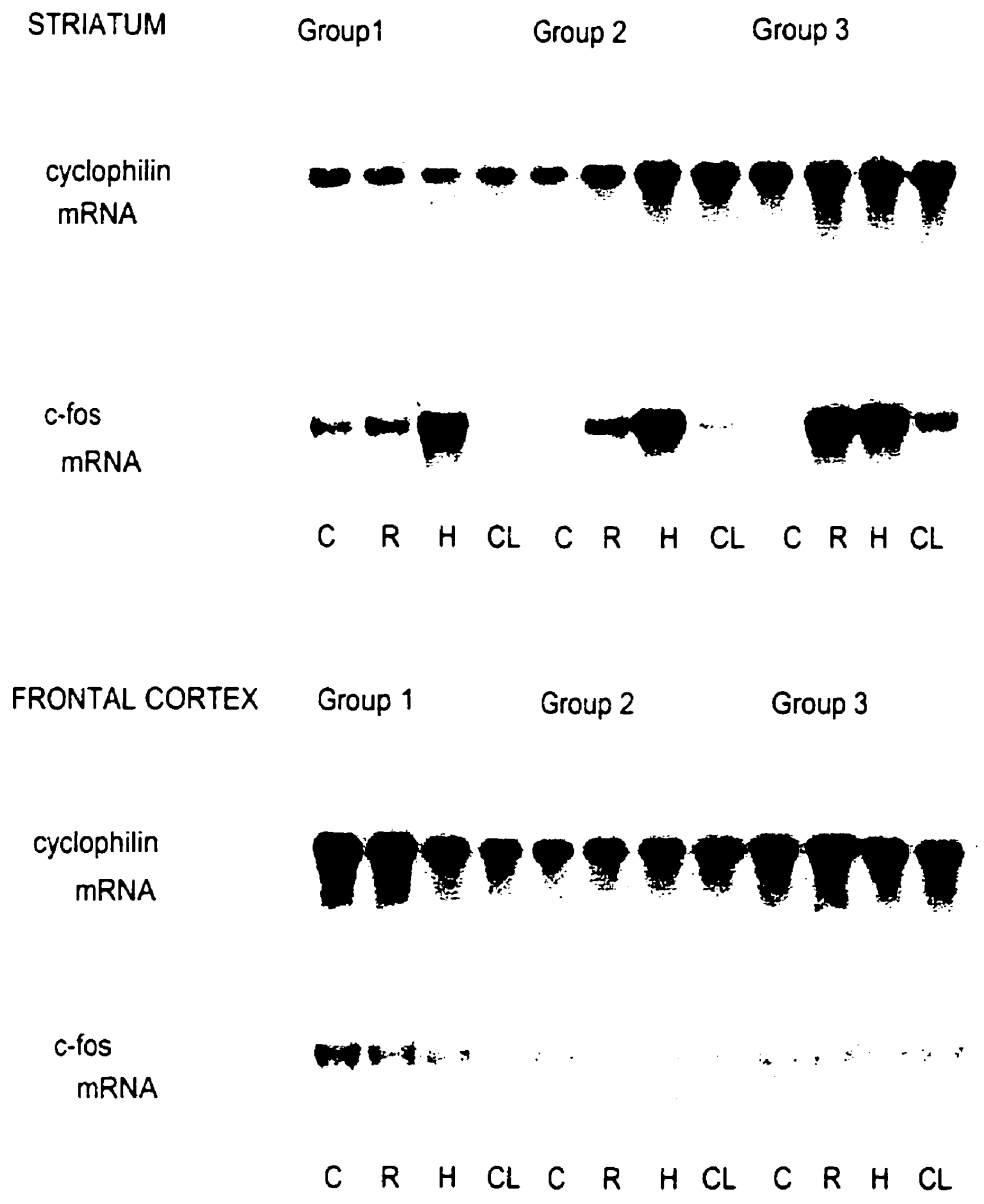

Figure 3.15. Autoradiographs of c-fos mRNA in striatum and frontal cortex by northern blot analysis. Animals received acute injections of either vehicle (C), 1 mg/kg risperidone (R), 1 mg/kg haloperidol (H), or 15 mg/kg clozapine (C) and were sacrificed 45 minutes following the injection. C-fos mRNA is upregulated by haloperidol and risperidone but not clozapine, and the effect is specific to the striatum (not seen in frontal cortex).

3.2.2 Dose response study to determine the effects of acute neuroleptic administration on hippocampal c-fos mRNA

Figure 3.16 shows the results of a dose-response study for the effects of neuroleptics on hippocampal *c-fos* mRNA. This dose-response study was performed to determine if trends observed in the hippocampus in initial studies could be clarified (see figure 3.12). Animals (n=3) received i.p. injections of risperidone (0.5, 1.0, and 1.5 mg/kg), haloperidol (0.5, 1.0, and 1.5 mg/kg), or clozapine (10, 20, and 30 mg/kg) and were sacrificed 45 minutes after i.p. injections. No significant differences were disclosed by one way ANOVA for any of the drugs tested [risperidone, $F(3,8) = 0.22$, $p=0.88$; haloperidol, $F(3,8) = 0.21$, $p=0.89$; clozapine, $F(3,8) = 0.59$, $p=0.64$].

A high dose of clozapine (30 mg/kg) significantly downregulated hippocampal cyclophilin mRNA in comparison to controls and the other two doses ($p=0.05$, see figure 3.17), the statistical significance of which even held in a one way ANOVA including data for haloperidol and risperidone [$F(11,36) = 2.26$; $p<0.002$; post-hoc by Newman Keuls) to 51% of control values. This trend was also observed in PC12 cells treated with high dose clozapine (250 μM). Individually, neither haloperidol [$F(3,8) = 0.48$, $p=0.70$] nor risperidone [$F(3,8) = 0.22$, $p=0.70$] significantly altered hippocampal cyclophilin mRNA. This effect is not seen in the striatum or cortex (data not shown). High doses of clozapine also tended to downregulate cyclophilin mRNA in PC12 cells, further suggesting the robustness of this finding.

3.2.3 The effects of acute neuroleptic administration on striatal *c-fos* mRNA as determined by in situ hybridization

In initial acute experiments, three animals per group received a single i.p. injection of either vehicle (0.8% glacial acetic acid in 5% glucose), remoxipride (3 mg/kg), risperidone (3 mg/kg), ritanserin (2 mg/kg), haloperidol (3 mg/kg) or

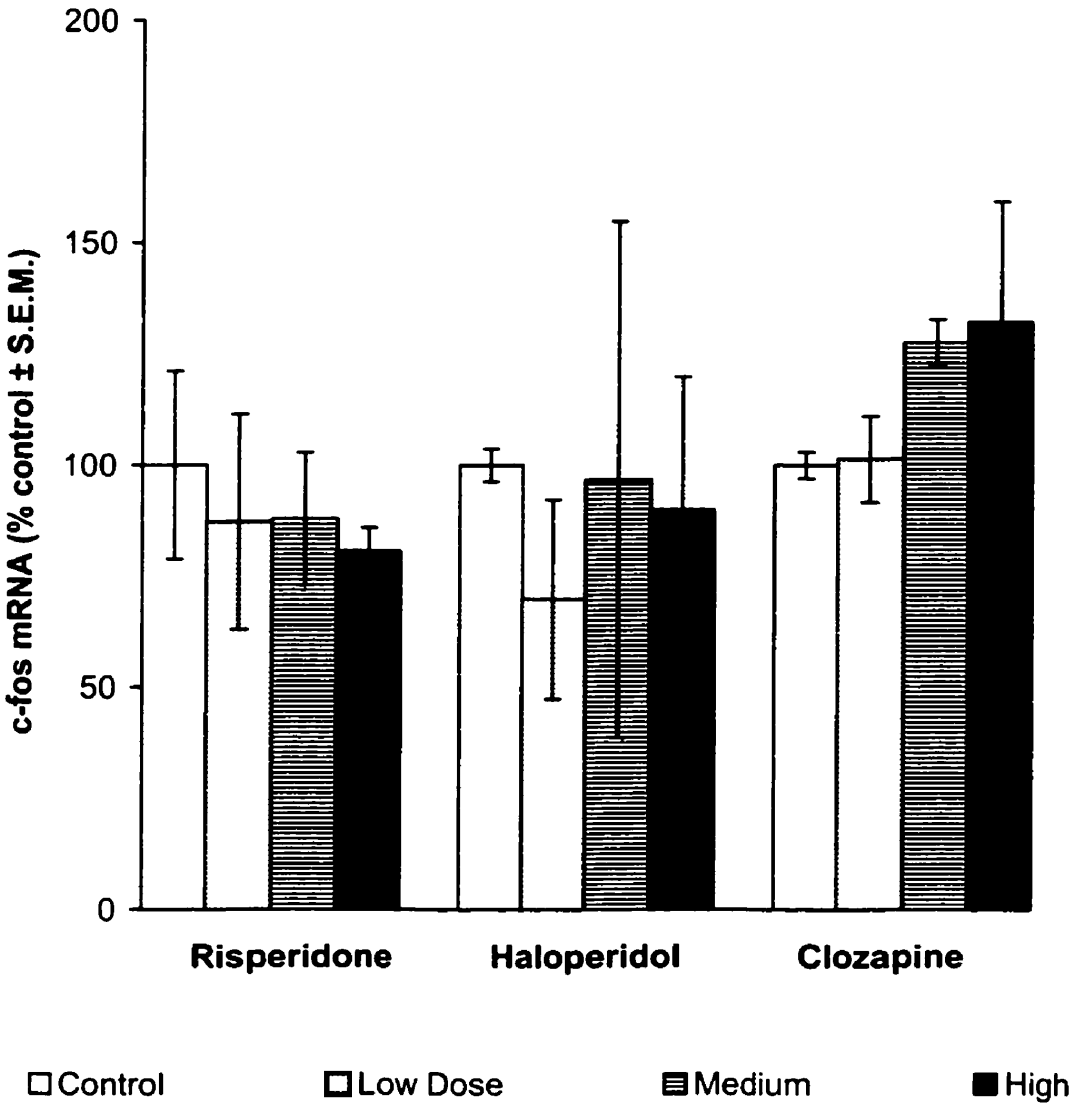

Figure 3.16 Dose-response study for the effects of neuroleptics on c-fos mRNA in the hippocampus as determined by northern blot analysis. Animals (n=3) were sacrificed 45 minutes after I.P. injections Doses were 1.5, 1.0, and 0.5 mg/kg for risperidone and haloperidol. Doses were 10, 20, and 30 mg/kg for clozapine. Results are expressed as percent control ± S.E.M.

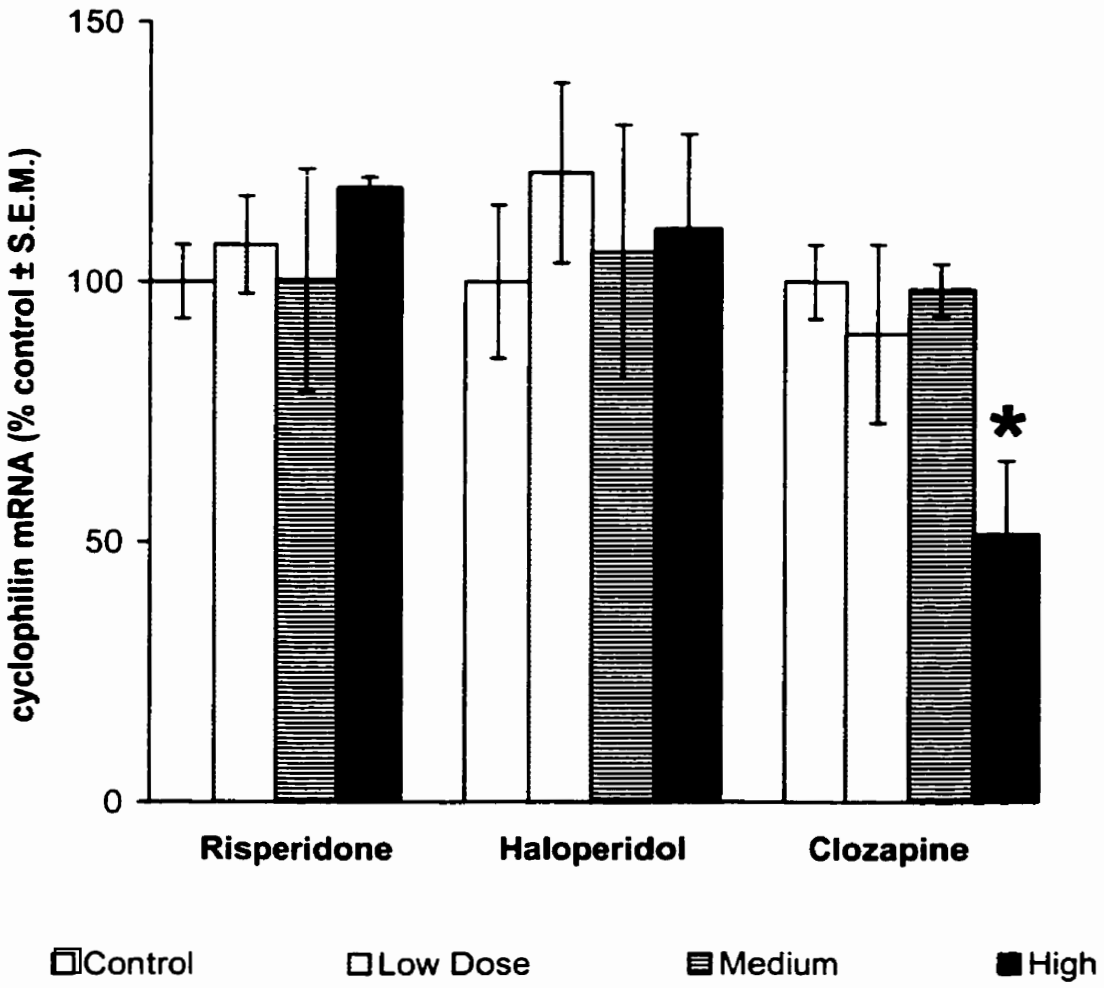

Figure 3.17 High dose clozapine (30 mg/kg) significantly downregulates hippocampal cyclophilin mRNA as determined by northern blot analysis. Animals (n=3) were sacrificed 45 minutes after I.P. injections. Doses were 1.5, 1.0, and 0.5 mg/kg for risperidone and haloperidol. Doses were 10, 20, and 30 mg/kg for clozapine. Results are expressed aspercent control ± S.E.M.

* significantly different from controls; $p<0.05$.

clozapine (30 mg/kg), and were sacrificed 45 minutes after the injection. Lower doses were used in subsequent experiments. In these experiments, animals received a single i.p. injection of either vehicle (0.8% glacial acetic acid in 5% glucose), risperidone (1 mg/kg), haloperidol (1 mg/kg) or clozapine (20 mg/kg) and were sacrificed at either 30 or 45 minutes after the injection.

The autoradiographs shown in Figure 3.18a illustrate an in situ hybridization time course study done to determine the time where maximal *c-fos* stimulation would occur in the striatum upon the administration of acute haloperidol. Although many investigators doing similar studies used a 45 minute time point to examine haloperidol- induced *c-fos* mRNA in the striatum, future studies required that we determined the time point for the strongest induction of *c-fos*. The results in Figure 3.18a demonstrate that while *c-fos* mRNA expression by haloperidol is strong at both 30 and 45 minutes, the strongest response was observed at 30 minutes. In a separate study (n=3), this was confirmed for both haloperidol and risperidone (data not shown). None of the other neuroleptics tested showed an induction of *c-fos* mRNA in the striatum (data not shown). The absence of an induction of *c-fos* was confirmed by the inclusion of positive controls in the in situ hybridization (animals receiving a single i.p. injection of 1 mg/kg haloperidol and sacrificed 30 minutes later; and rats that have kainic acid-induced seizures, see section 2.6.7.2 for details).

Although some initial in situ hybridization studies had suggested that all the neuroleptic treatments induced *c-fos* mRNA in various thalamic nuclei, it was later determined that the effect could not be separated out from the large induction of *c-fos* mRNA that was demonstrated by the controls (this problem has been observed by other investigators as was confirmed by a personal discussion with an associate of Dr. A.Y. Deutch, Yale School of Medicine). Thus, these data were not statistically analyzed. The in situ hybridization studies also demonstrated that acute neuroleptic administration does not induce *c-fos* mRNA in the hippocampus or prefrontal cortex, and these data

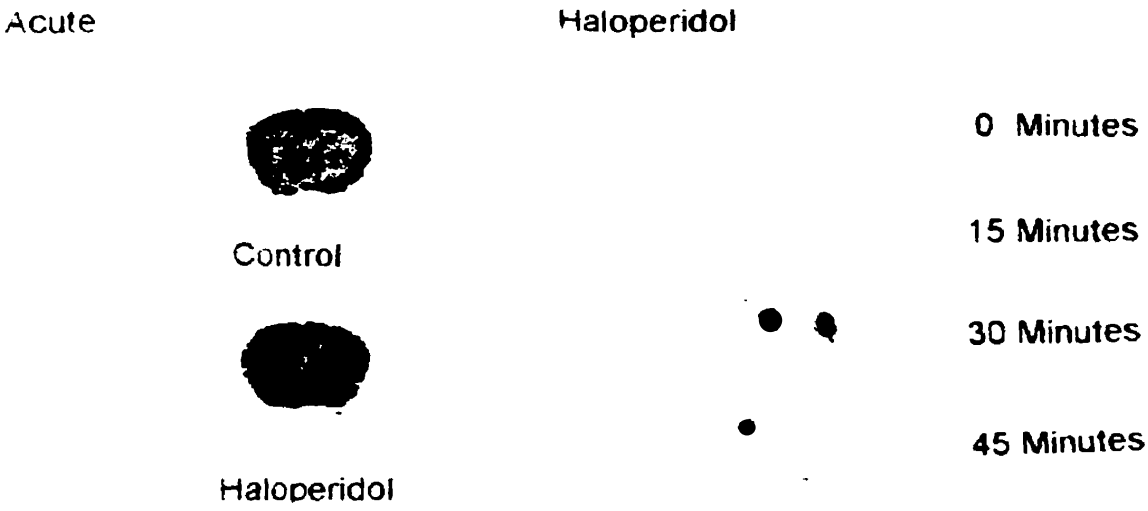

Figure 3.18a. In situ hybridization autoradiographs of c-fos mRNA: Time course for haloperidol.

were not statistically analyzed due to the low levels of *c-fos* mRNA expression in these areas. The absence of response was confirmed by including positive controls (sections from animals that had seizures) that showed strong *c-fos* induction in both the hippocampus and prefrontal cortex.

3.2.4 The effects of chronic and chronic/challenge neuroleptic administration on striatal *c-fos* mRNA by *in situ* hybridization analysis

For chronic experiments, three animals per group received daily i.p. injections for 19 days of either vehicle (0.8% glacial acetic acid in 5% glucose), remoxipride (3 mg/kg), risperidone (1 mg/kg), ritanserin (2 mg/kg), haloperidol (1 mg/kg) or clozapine (20 mg/kg), and were sacrificed the next day. For chronic/challenge experiments, animals received the same doses and injections for 19 days, plus they also received an injection on the next day 30 minutes before being sacrificed. The chronic/challenge group was included in the study to determine if the acute administration of neuroleptics such as haloperidol would still induce striatal *c-fos* mRNA after being chronically injected for 19 days.

The autoradiographs in Figure 3.18b illustrate that there was absolutely no induction of striatal *c-fos* mRNA by either injection paradigm. The absence of an induction of *c-fos* was confirmed by the inclusion of the haloperidol and kainic acid positive controls in the procedure, and the in situ hybridizations were repeated several times. In addition, no induction of hippocampal or fronto-cortical *c-fos* mRNA was observed in either injection paradigms (hippocampal data not shown). To further confirm that there was no induction of *c-fos* mRNA, c-fos labeled sections were dipped in photographic emulsion and allowed to develop for 6 months. No radioactivity above background was detected in any of the aforementioned areas except in the control sections (see Figure 3.19).

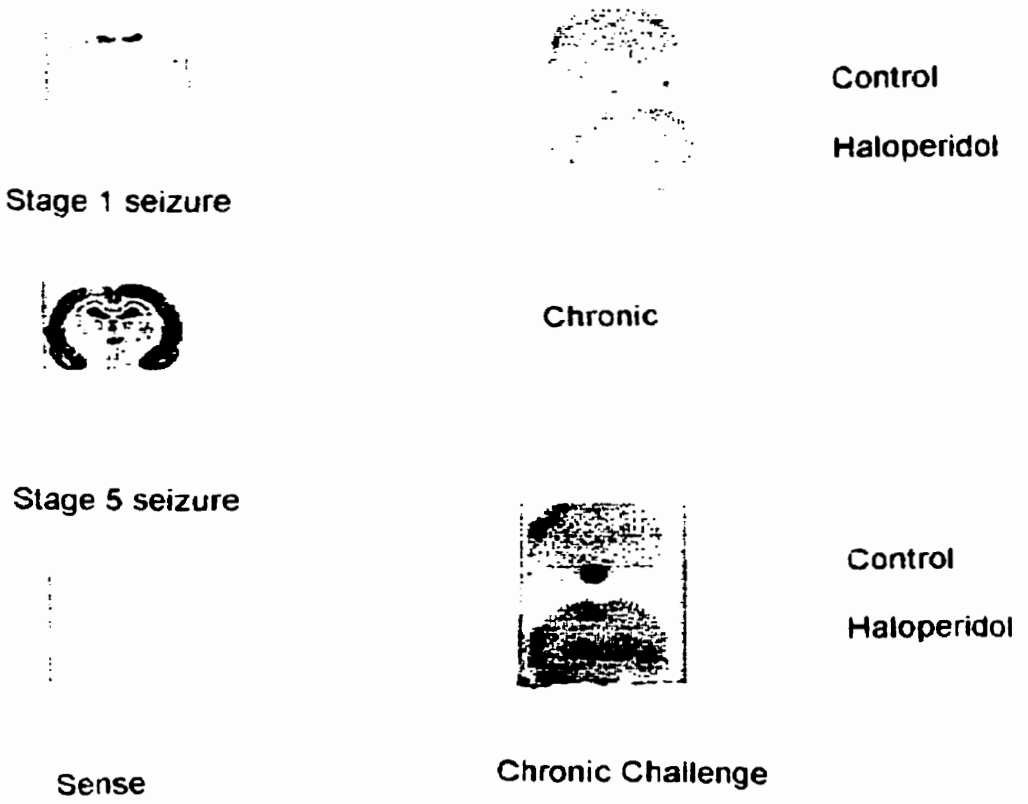

Figure 3.18b. In situ hybridization autoradiographs of c-fos mRNA: Chronic and chronic challenge injections

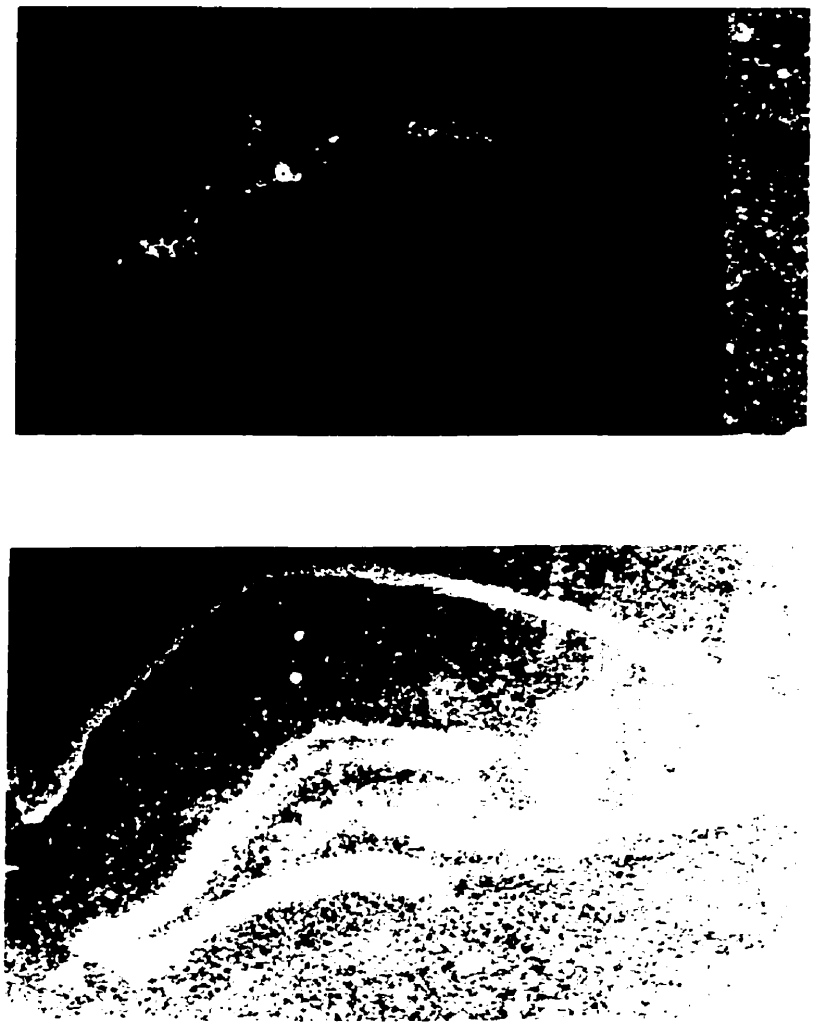

Figure 3.19. *In situ* hybridization emulsion analysis of c-fos mRNA in seized rats: No effects of chronic or chronic challenge neuroleptic administration. In control section (top photo), the background is blocked out in black in order to highlight where c-fos mRNA would be seen in the dentate gyrus if there was induction of its mRNA. The second photo shows induction of BDNF mRNA in the dentate gyrus of an animal that has experienced a stage 5 seizure.

3.2.5 Dose response studies of neuroleptic-induced delta FosB immunoreactivity (IR) in the striatum, nucleus accumbens, and medial prefrontal cortex

Figure 3.20 depicts the areas analyzed for delta FosB immunoreactivity (see methods for further details). Measurements of delta FosB immunoreactivity (IR) as opposed to delta FosB mRNA were taken in chronically treated rats on the advice of Dr. Eric Nestler, (Yale University, Dept. of Psychiatry) that the mRNA of this gene was not upregulated by neuroleptics. In addition, the RNA probes were too small to use in our in situ hybridization procedure.

In general, haloperidol and risperidone increased the number of delta FosB positive neurons in striatal regions, while clozapine did not. Risperidone, but not clozapine or haloperidol increased delta FosB IR in the medial prefrontal cortex, and although there was a trend for all of the neuroleptics tested to increase delta FosB IR in the nucleus accumbens, only the increases induced by risperidone were statistically significant.

Since the commercially available delta FosB antibody used in the present study recognizes both FosB and delta FosB, a control study was performed to determine that the changes detected by the delta FosB antibody were due to changes in delta FosB only. Antibodies to FosB and FosB/ delta FosB were obtained from Dr. Y. Nakabeppu (Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan) and immunoreactivity detected in samples known to be positive for the Santa Cruz antibody were tested with both these antibodies. Induction of immunoreactivity was observed with the FosB/ delta FosB but not FosB only antibody, demonstrating that the immunoreactivity seen with the Santa Cruz delta FosB antibody was due to induction of delta FosB alone. This is not surprising, since FosB immunoreactivity does not appear to be subject to induction (Robertson et al., 1994).

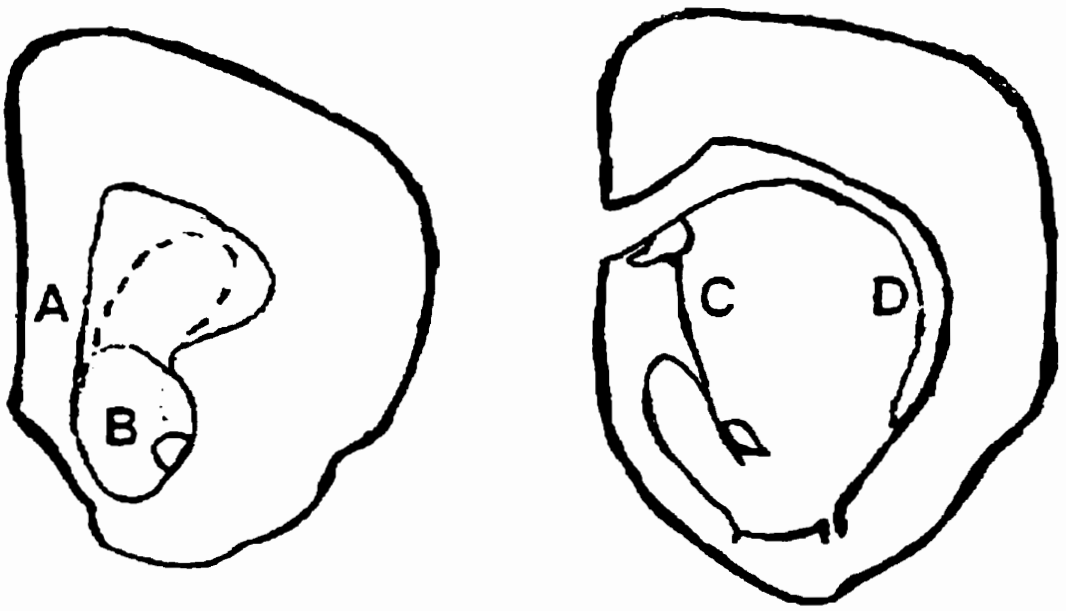

Figure 3.20. Regions analyzed for delta FosB immunohistochemistry. Modified from camera lucida drawings of respesentative sections form Roberston et al. (1994) used to count delta Fos immunoreactivity. A= medial prefrontal cortex, B= nucleus accumbus (measured slightly more posteriorly than the prefrontal cortex, as indicted by broken lines), C= mediolateral striatum, D= dorsolateral striatum.

(Note: Sections are derived from the Paxinos and Watson Rat Brain Atlas (2^{nd} edition, 1986. The first diagram is a representation of a coronal section from plate 9, the second diagram being derived from plate 22 as defined in the atlas).

Table 3.1 reports the effects of chronic neuroleptic administration on delta FosB immunoreactivity in the DLStr, MLStr, mPFC and the NuAcc. The data were analyzed in two separate groups, each with their own control (which is why there are two different values for the control condition). The data in Table 3.1 show that delta FosB induction in the DLStr is the greatest for haloperidol and the high dose of risperidone, as disclosed by ANOVA [$F(4,10) = 8.803$, $p=0.0029$] and a subsequent post-hoc analysis by Newman Keuls ($p<0.05$). In the MLStr, however, the highest dose of risperidone produced the greatest increase in delta FosB IR [$F(4,10) = 4.848$, $p=0.0218$; $p<0.01$), followed by haloperidol and a moderate dose of risperidone to a lesser extent ($p<0.05$), as revealed by ANOVA and a subsequent test by Newman Keuls. Only the high and medium doses of risperidone significantly increased the number of delta FosB positive neurons in the mPFC [$F(4,10) = 3.78$, $p=0.046$; $p<0.05$), and nucleus accumbens [$F(4,10) = 22.2$, $p=0.0001$; $p<0.01$ for high dose, $p<0.05$ for medium dose risperidone], although haloperidol also tended to increase delta FosB immunoreactivity in these areas somewhat. Clozapine did not significantly upregulate delta FosB in any of the areas studied. No effects of injection stress on delta FosB were observed in comparison to non-injected control animals.

Figure 3.21 illustrates some of the areas where delta FosB was upregulated by chronic haloperidol treatment, including the caudate putamen.

3.3 The effects of neuroleptics on protective factors

Nylon membranes prepared for the analysis of the effects of neuroleptic treatment on TH, AADC, and c-fos mRNA in PC12 cells were reused to examine the effects of neuroleptic treatment on p75 and SOD mRNA. Newer cell experiments included olanzapine, and these membranes were also probed for p75 and SOD mRNA. These olanzapine membranes were not probed for TH, AADC, or *c-fos* mRNA since it was concluded that any such data would not add to the previous results of these experiments.

Table 3.1. The effects of chronic neuroleptic administration on delta FosB immunoreactivity in the dorsolateral striatum (DLStr), mediolateral striatum (MLStr), the medial prefrontal cortex (mPFC) and the nucleus accumbens (Nuacc).

	DLStr	MLStr	NuAcc	mPFC
Control 1	15 ± 17	15 ± 4	35 ±12	25 ± 7
Haloperidol	137* ± 46	86* ± 27	53 ± 9	42 ± 19
Risperidone High	148* ± 36	111* ± 7	144** ± 6	69* ± 16
Risperidone Medium	81* ± 8	79* ± 7	81* ± 15	66* ± 9
Risperidone Low	17 ± 4	14 ± 3	23 ± 6	39 ± 9
Control 2	73 ± 13	52 ± 12	67 ± 16	35 ± 10
Clozapine High	112 ± 50	78 ± 37	84 ± 22	37 ± 15
Clozapine Medium	85 ± 27	48 ± 8	81 ± 19	41 ± 8
Clozapine Low	86 ± 19	49 ± 13	64 ± 7	33 ± 6
Non-Injected Control	69 ± 14	50 ± 17	61 ± 7	32 ± 4

All values expressed as the number of immunoreactive nuclei $\pm$ S.E.M. Data analyzed by ANOVA, post-hoc by Newman Keuls.

* significantly different from controls, $p<0.05$.

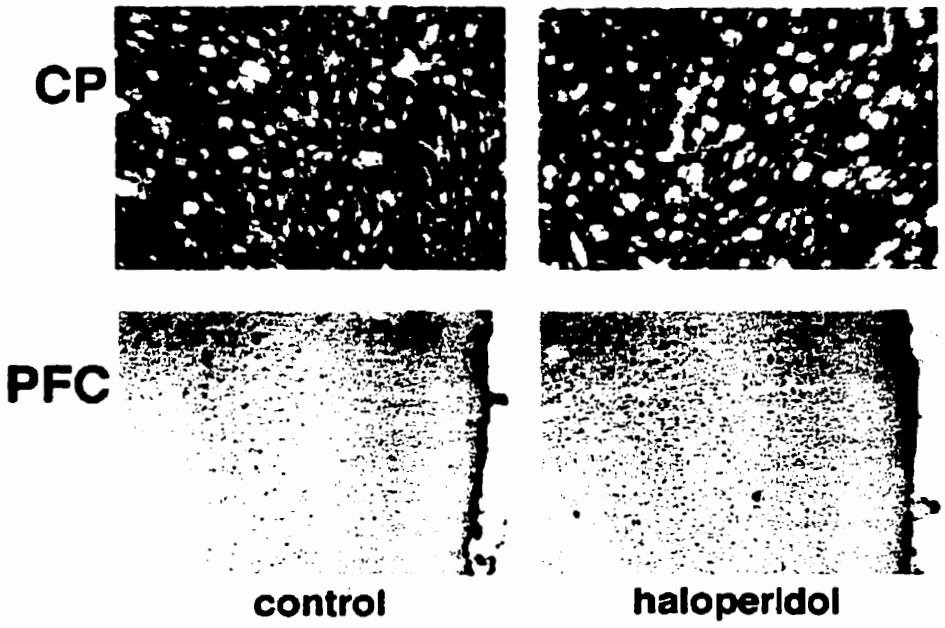

Figure 3.21. Delta FosB immunoreactivity in the striatum (caudate putamen, CP) and prefrontal cortex (PFC). Large increases in the number of delta FosB positive neurons (small black dots) are seen in the CP of haloperidol treated vs. control animals. A slight induction of delta FosB immunoreactivity can also be seen in the cortical layers of the PFC in haloperidol treated animals.

3.3.1 The effects of remoxipride, risperidone, ritanserin, haloperidol, clozapine, and olanzapine on p75 at 12, 24, and 48 hours as determined by northern blot analysis in PC12 cells

No effects on p75 mRNA were seen for remoxipride or ritanserin (data not shown). Comparisons were made between the three other drugs (risperidone, haloperidol, clozapine) by ANOVA at each of the time points studied.

Figure 3.22 demonstrates that albeit not significantly, at 12 hours, haloperidol (1.0 μM and 10.0 μM) upregulated p75 mRNA to 166% and 134% of control levels, while clozapine (2.5 and 25.0 μM) downregulated p75 mRNA expression 78%, and 71% of control values respectively as disclosed by a one way ANOVA [$F(8,18) = 0.43$, $p = 0.89$]. These data may not have reached significance since two data points only had an n of two (sample lost during tissue processing). Figure 3.23, however, demonstrates that these doses of haloperidol (1.0 μM and 10.0 μM) downregulated p75 mRNA at 24 hours to 63% and 58% of control levels, and similarly downregulated p75 mRNA to 68% and 56% of control levels at 48 hours as revealed by a one way ANOVA [$F(8,18) = 3.57$, $p = 0.012$]. Figure 3.24 demonstrates that clozapine (2.5 and 25.0 μM)) downregulated p75 mRNA at 24 hours to 68% and 56% of control levels, and similarily downregulated p75 mRNA to 65% and 68% of control levels at 48 hours as revealed by a one way ANOVA [$F(8,18) = 5.42$, $p = 0.001$]. Risperidone did not appear to regulate p75 mRNA (data not shown).

Figure 3.25 demonstrates that incubation with (at concentrations of 10 or 100 μM) reduced p75 gene expression as disclosed by two way ANOVA ($F_{2,12} = 17.17$, $p < 0.0003$). No effect of time ($F_{2,12} = 2.56$, $p < 0.135$) or interaction between time and olanzapine treatment ($F_{2,12} = 2.49$, $p < 0.125$) were observed. One way ANOVA disclosed the effect of the olanzapine treatment ($F_{2,6} = 16.84$, $p < 0.004$). Significant reductions in p75 gene

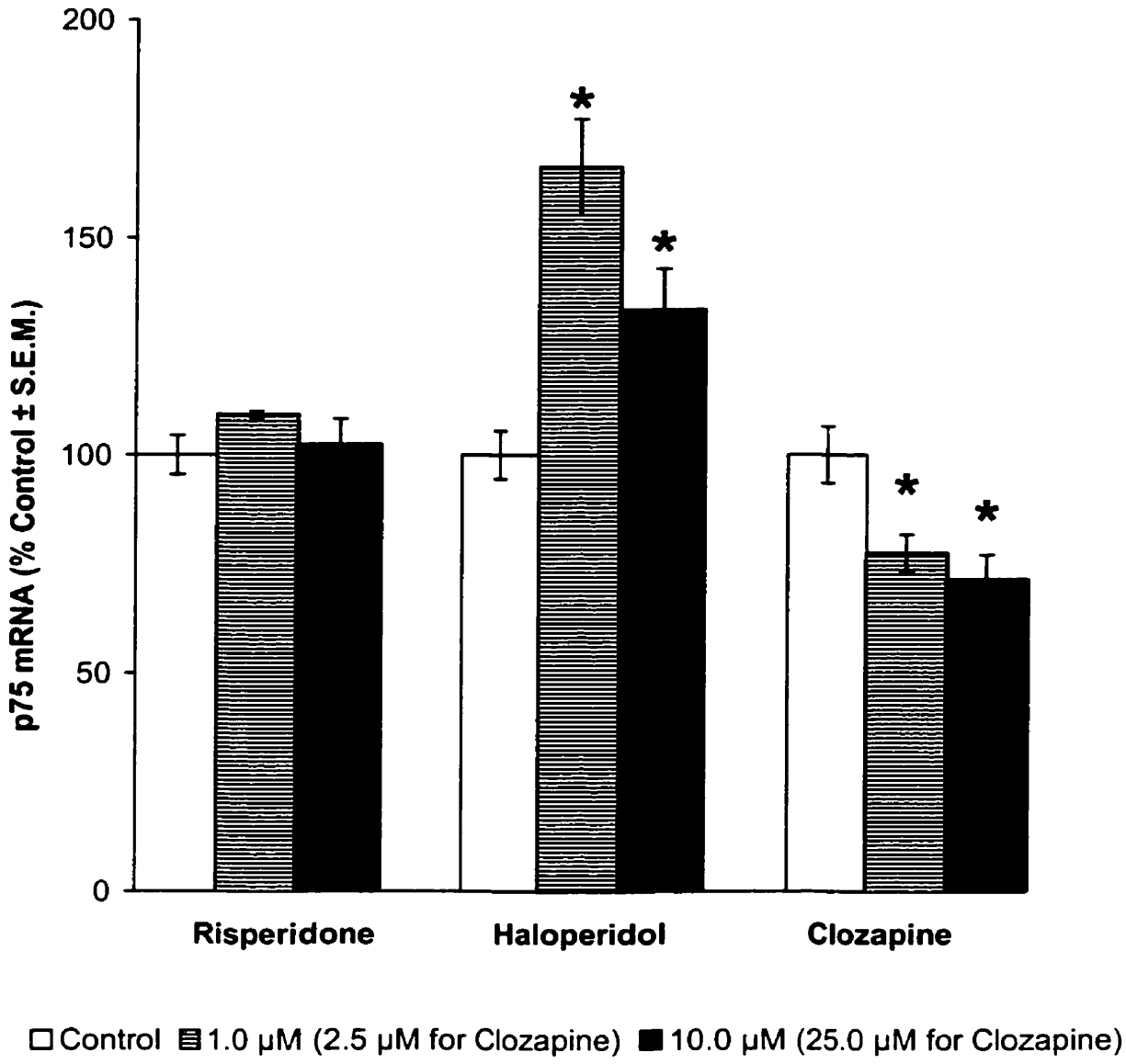

Figure 3.22. The effects of risperidone, haloperidol, and clozapine on p75 mRNA in PC12 cells at 12 hours. PC12 cells were treated with two doses of the aforementioned neuroleptics and harvested at 12 hours (n=3). Results are expressed as percent control ± S.E.M.

* significantly different from controls; $p<0.05$.

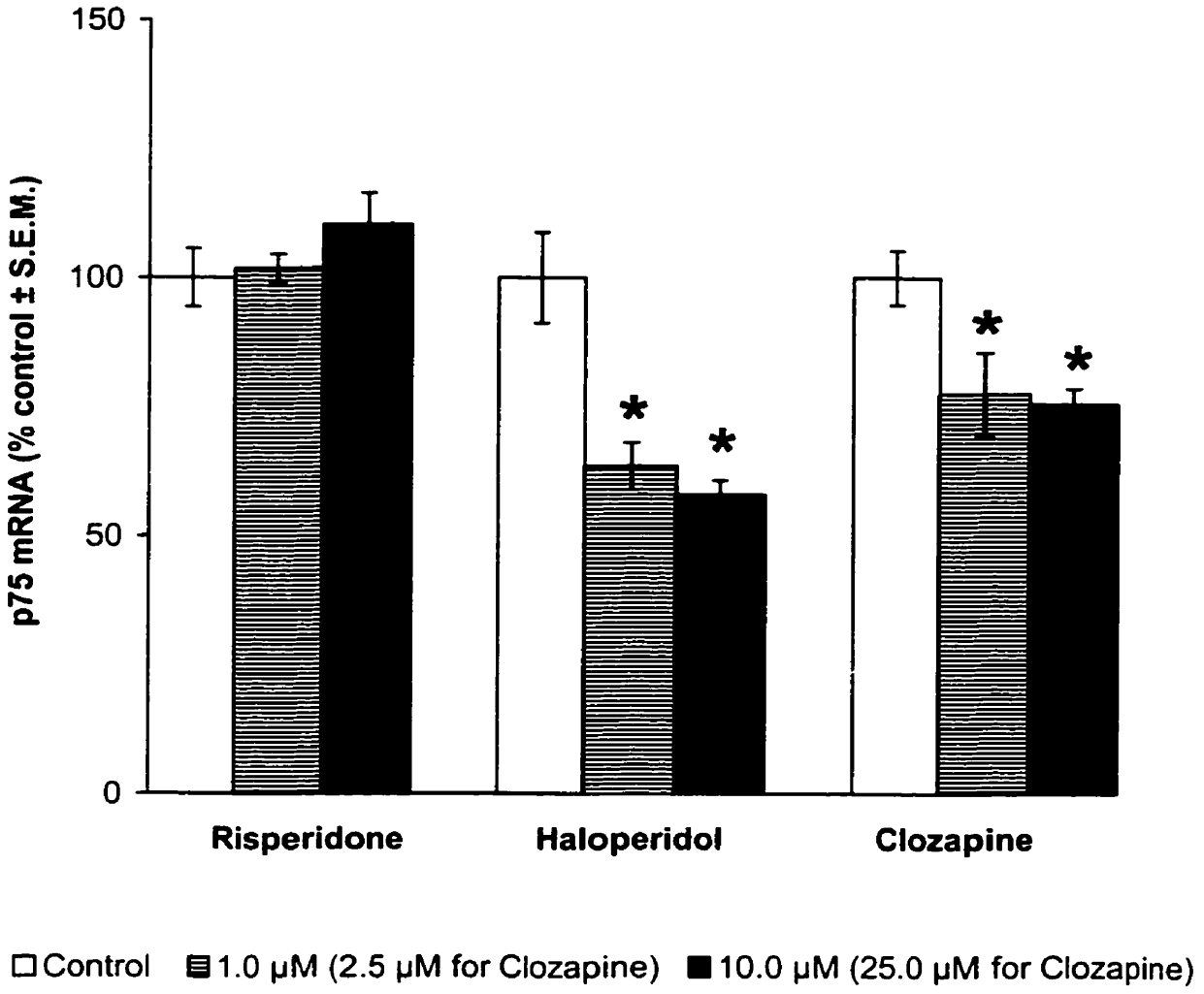

Figure 3.23. The effects of risperidone, haloperidol, and clozapine on p75 mRNA in PC12 cells at 24 hours. PC12 cells were treated with two doses of the aforementioned neuroleptics and harvested at 24 hours (n=3). Results are expressed as percent control ± S.E.M.

* significantly different from controls; $p<0.05$.

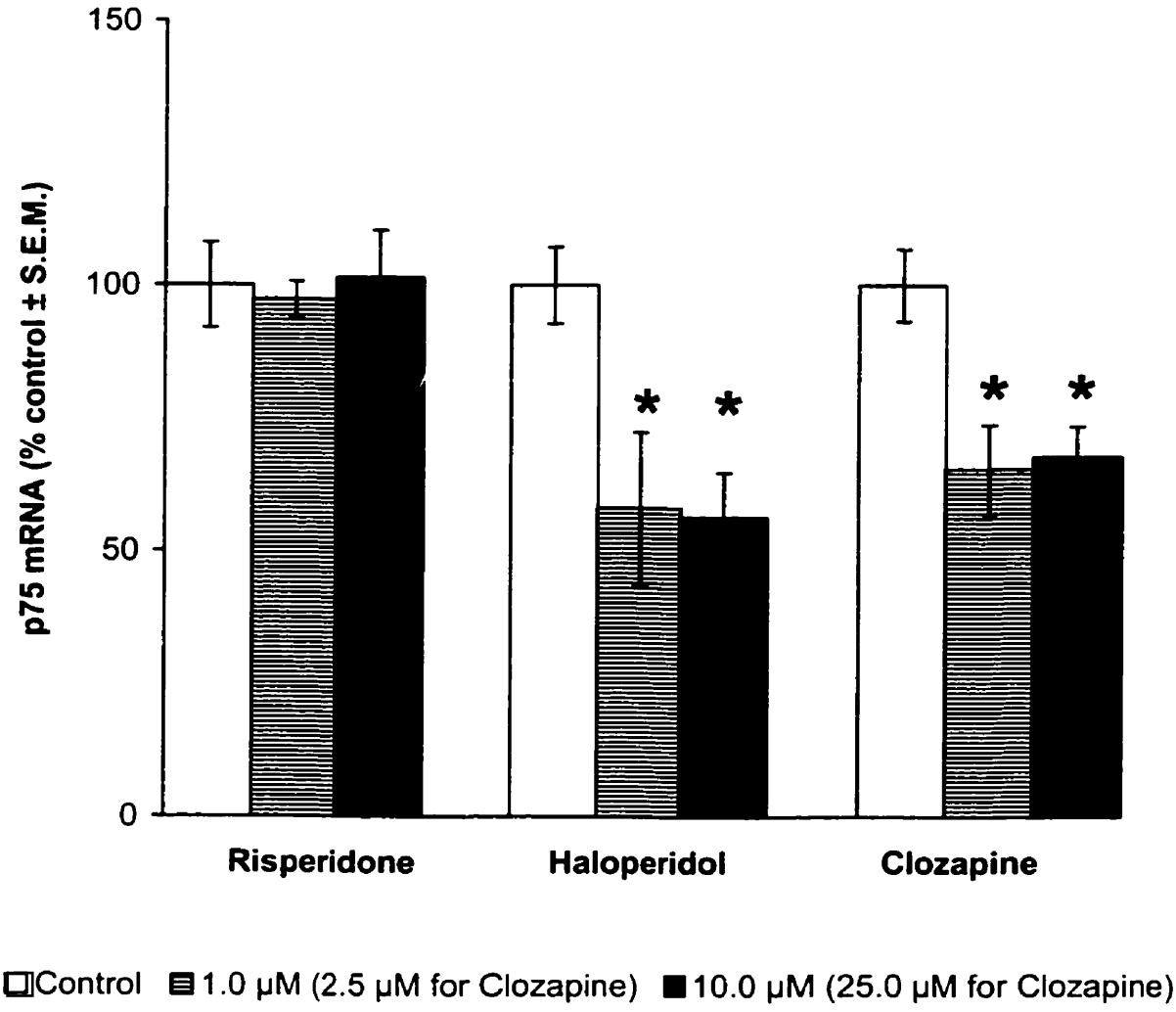

Figure 3.24. The effects of risperidone, haloperidol, and clozapine on p75 mRNA in PC12 cells at 48 hours . PC12 cells were treated with two doses of the aforementioned neuroleptics and harvested at 48 hours (n=3). Results are expressed as percent control ± S.E.M.

* significantly different from controls; $p<0.05$.

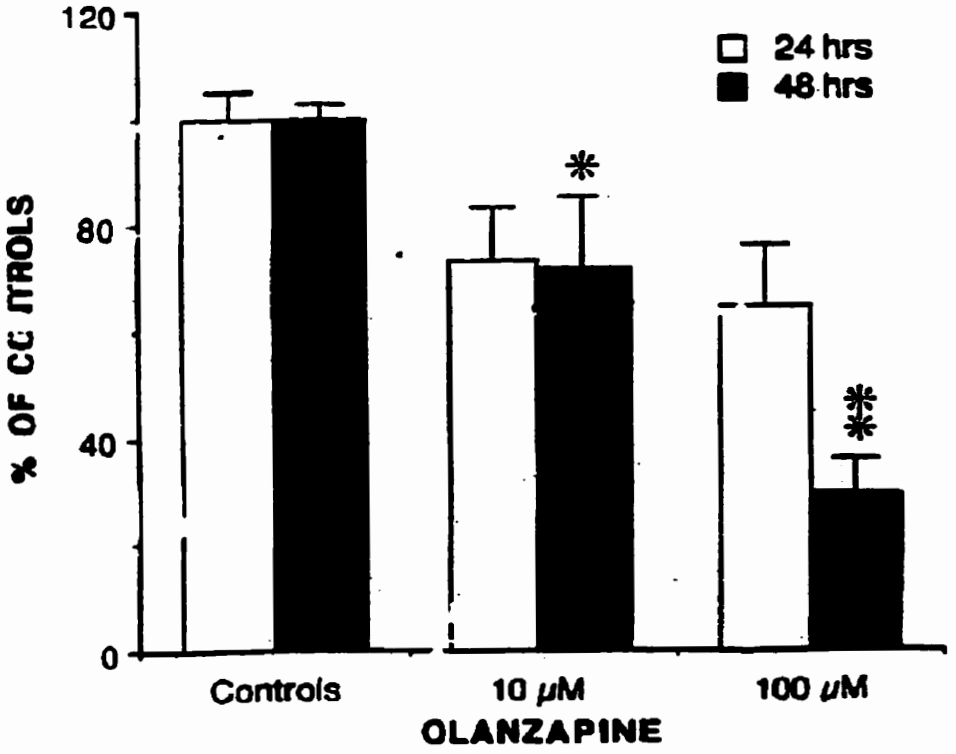

Figure 3.25. The effects of olanzapine on p75 mRNA at 24 and 48 hrs in PC12 cells. PC12 cells were treated with two doses of olanzapine and harvested at each time point, n=2, *p <0.05 **p<0.001. Results expressed as % control ± S.E.M.

expression were observed 48 hours after incubation with the 10 or 100 μM doses by 28 and 71 % of their respective controls.

3.3.2 The effects of remoxipride, risperidone, ritanserin, haloperidol, clozapine, and olanzapine on SOD mRNA at 12, 24, and 48 hours as determined by northern blot analysis in PC12 cells

Figure 3.26 demonstrates that risperidone (albeit at a high dose of 100 μM) upregulates SOD mRNA in PC12 cell cultures. Although incubation of PC12 cells with 1.0 or 10.0 μM risperidone produced no interaction of dose and time [$F(6,24) = 0.21$, $p = 0.78$] or an effect of time [$F(2,24) = 0.25$, $p = 0.78$] on SOD mRNA as disclosed by two way analysis of variance (Figure 3.26), there was an effect of dose [$F(3,24) = 9.67$, $p = 0.0002$]. Incubations of 100 μM risperidone increased SOD mRNA levels to 162%, 133%, and 151% of SOD mRNA levels in comparison to controls, 10.0 μM risperidone, and 1.0 μM risperidone respectively. The high dose of risperidone (100 μM) upregulated SOD mRNA at 12, 24, and 48 to 160%, 174%, and 168% respectively of control values.

Figure 3.27 demonstrates that no effects on SOD mRNA were observed at any time point for haloperidol [$F(8,27) = 0.42$, $p = 0.89$] by either a one or two way ANOVA. Figure 3.28 demonstrates that 25 μM clozapine increases SOD mRNA at 48 hours by a one way ANOVA ($p < 0.05$). Figure 3.29 demonstrates that incubation of PC12 cells with 10 or 100 μM of olanzapine for 24 or 48 hours increased SOD1 gene expression as disclosed by two way ANOVA ($F_{2,12} = 13.84$, $p < 0.0008$). No effect of time ($F_{2,12} = 1.10$, $p < 0.31$) or interaction between olanzapine treatment and time ($F_{2,12} = 0.29$, $p < 0.75$) was observed. One way ANOVA showed significant increases in SOD1 gene expression observed at 48 hours after treatment with 10 or 100 μM of olanzapine that reached 47 and 72 % above their

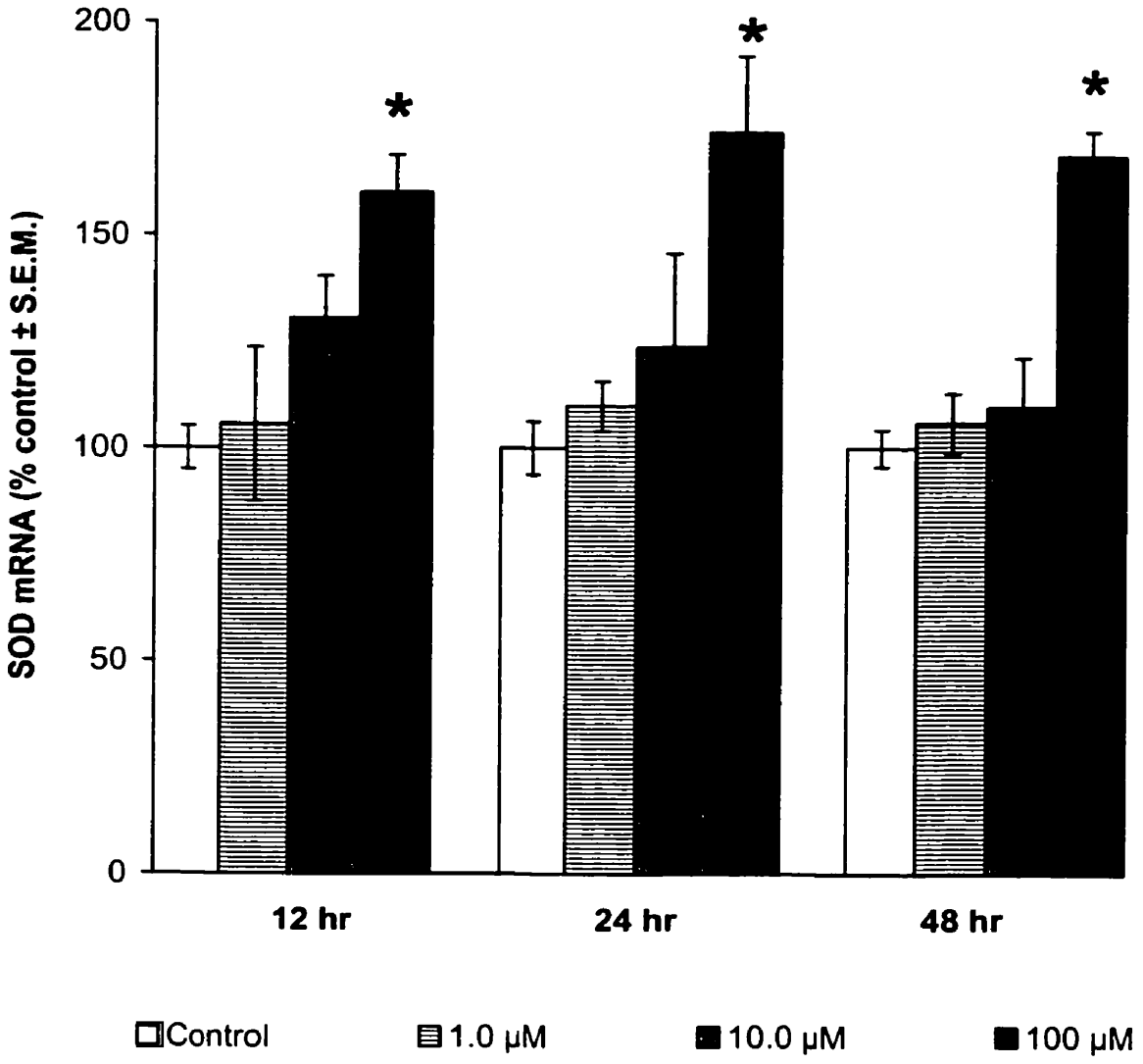

Figure 3.26. The effects of risperidone on SOD mRNA at 12, 24, and 48 hrs in PC12 cells. PC12 cells were treated with three doses of risperidone and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

* significantly different from controls; $p<0.05$.

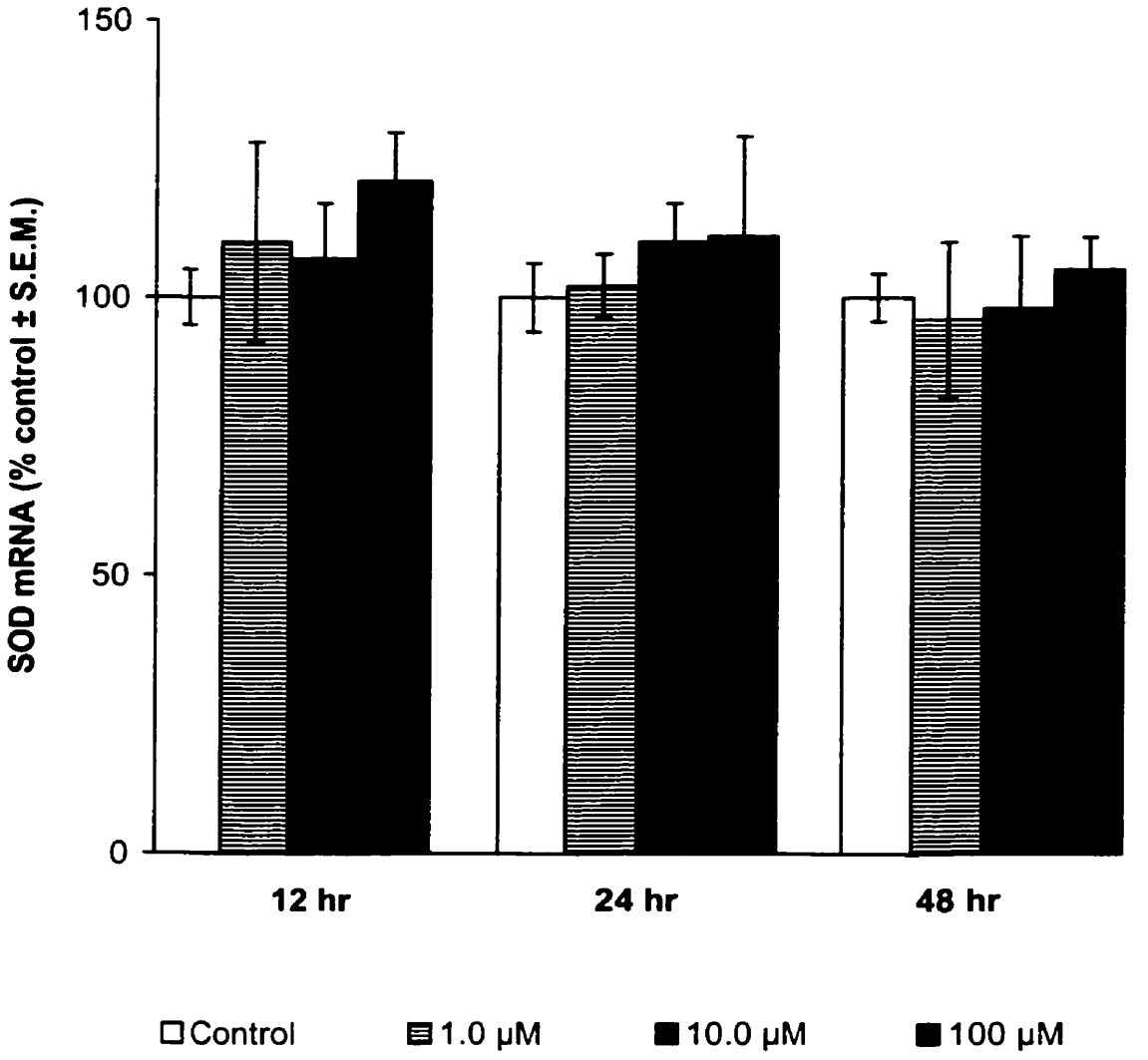

Figure 3.27. The effects of haloperidol on SOD mRNA at 12, 24, and 48 hrs in PC12 cells. PC12 cells were treated with three doses of haloperidol and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

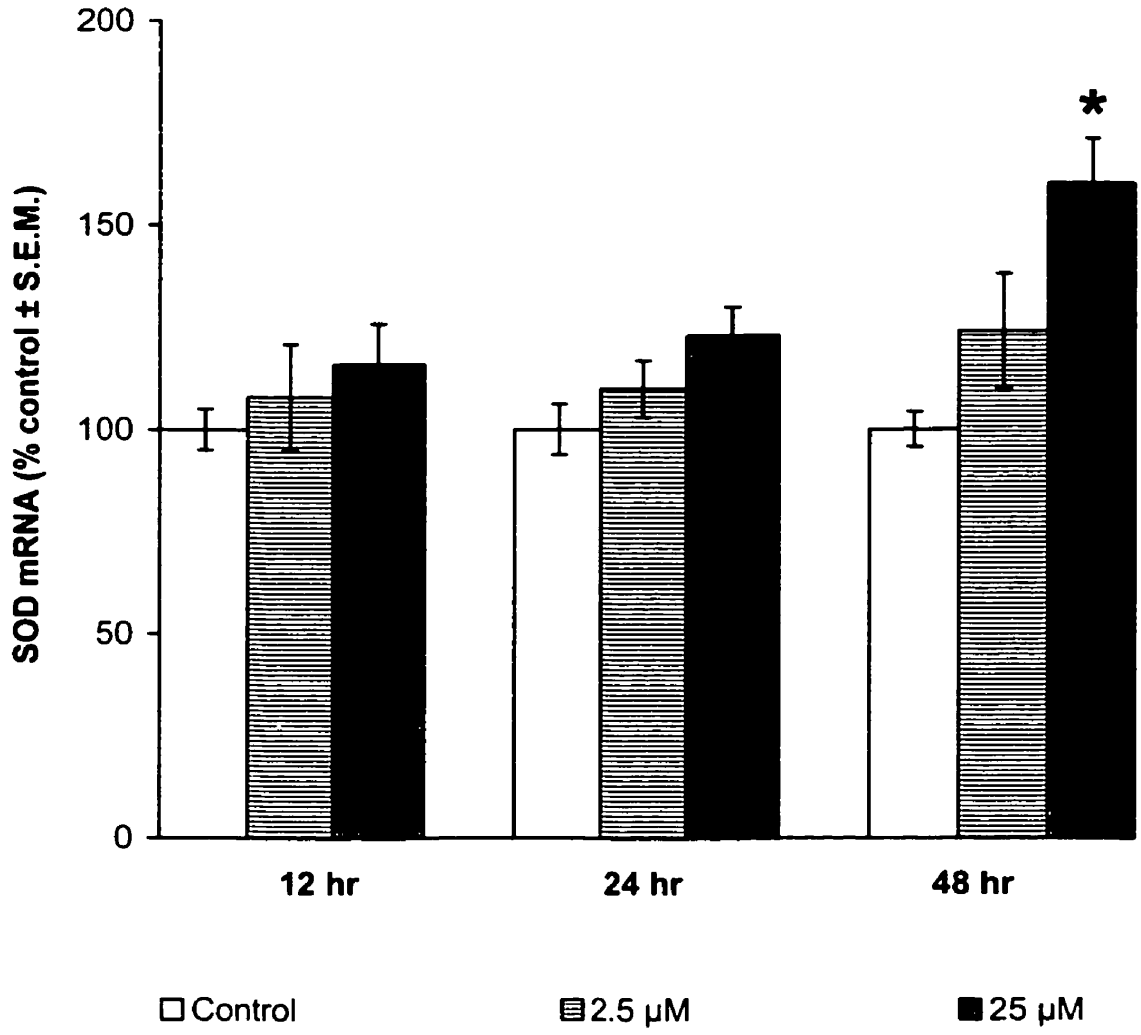

Figure 3.28. The effects of clozapine on SOD mRNA at 12, 24, and 48 hrs in PC12 cells. PC12 cells were treated with three doses of clozapine and harvested at each time point (n=3). Results are expressed as percent control ± S.E.M.

* significantly different from controls; $p<0.05$.

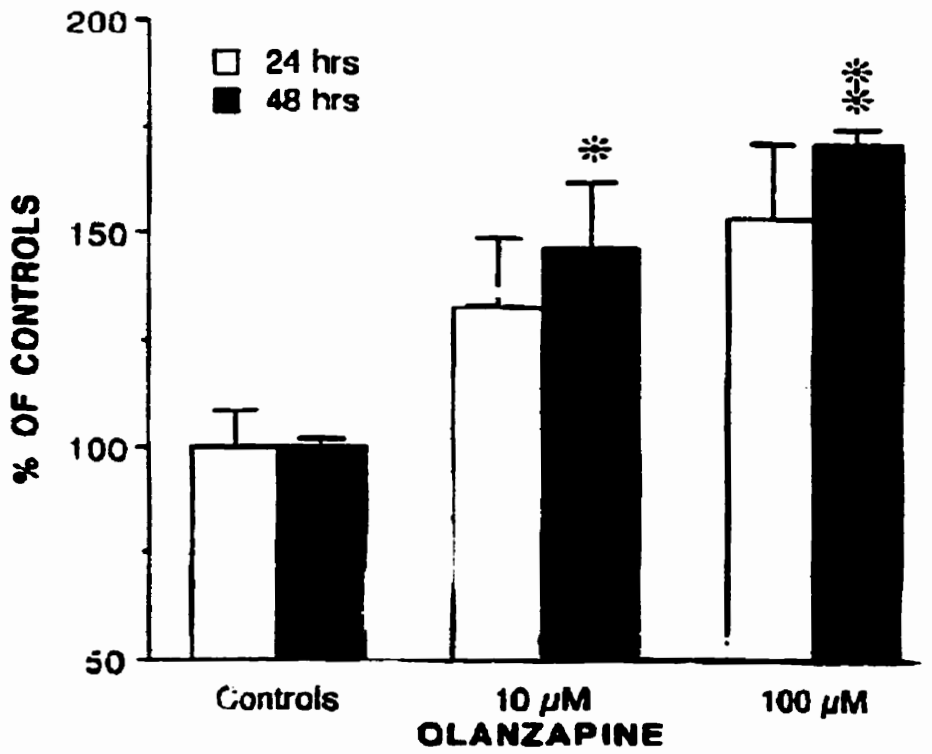

Figure 3.29. The effects of olanzapine on SOD mRNA at 24 and 48 hrs in PC12 cells. PC12 cells were treated with two doses of olanzapine and harvested at each time point, n=2, *p <0.05 **p<0.001. Results expressed as % control ± S.E.M.

corresponding control levels. Figure 3.30 shows representative autoradiographs of the induction of p75 and SOD mRNA for clozapine.

3.3.3 The effects of chronic and acute neuroleptic treatment on hippocampal and fronto-cortical BDNF mRNA

Figure 3.31 demonstrates that acute (45 minute) administration of risperidone, haloperidol, or clozapine did not alter either hippocampal or fronto-cortical BDNF mRNA [$F(5,12)= 0.42$, $p=0.53$], nor were there any trends to do so.

The effects of the chronic administration of remoxipride, risperidone, ritanserin, haloperidol, and clozapine on hippocampal and mPFC BDNF mRNA are shown in Figure 3.32 and reveal some interesting trends. In hippocampal regions, while haloperidol and high doses of atypical neuroleptics downregulated BDNF mRNA, the 5-HT2 blocker ritanserin and lower doses of atypical neuroleptics upregulated BDNF mRNA. Specifically, for chronic injections, in situ hybridization was used to confirm the preliminary results achieved by northern blot analysis (it was deemed that the work that went into injecting rats for 20 days was better directed to in situ hybridization rather than northern blot analysis).

An ANOVA revealed that although no statistical differences existed between the control and drug treatment groups, dentate gyrus BDNF mRNA levels were significantly lower in haloperidol vs. risperidone treated rats (Newman Keuls). Haloperidol significantly downregulates, while ritanserin upregulates hippocampal BDNF mRNA ($p < 0.05$). Haloperidol reduced BDNF mRNA to 67% and 76% of control levels in the dentate and CA1 region respectively, while ritanserin increased CA1 BDNF mRNA to 124% of control levels. Risperidone and clozapine either did not significantly alter hippocampal BDNF mRNA, or slightly upregulated it. Clozapine significantly upregulated BNDF mRNA in the mPFC ($p < 0.01$). F Values: Dentate, $F(5,12) = 3.299$,

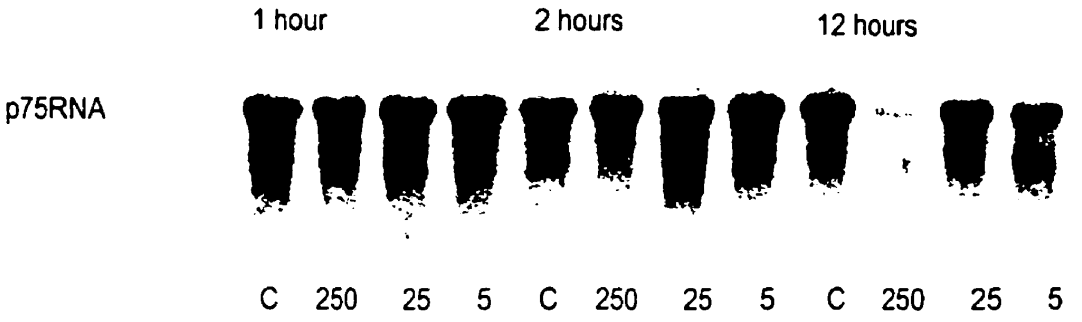

Figure 3.30. Represenative northern blot autoradiographs for the effects of clozapine on p75 mRNA. Clozapine downregulates p75 mRNA at not one or two hours of incubation, but after 12 hours of incubation at all three doses used.

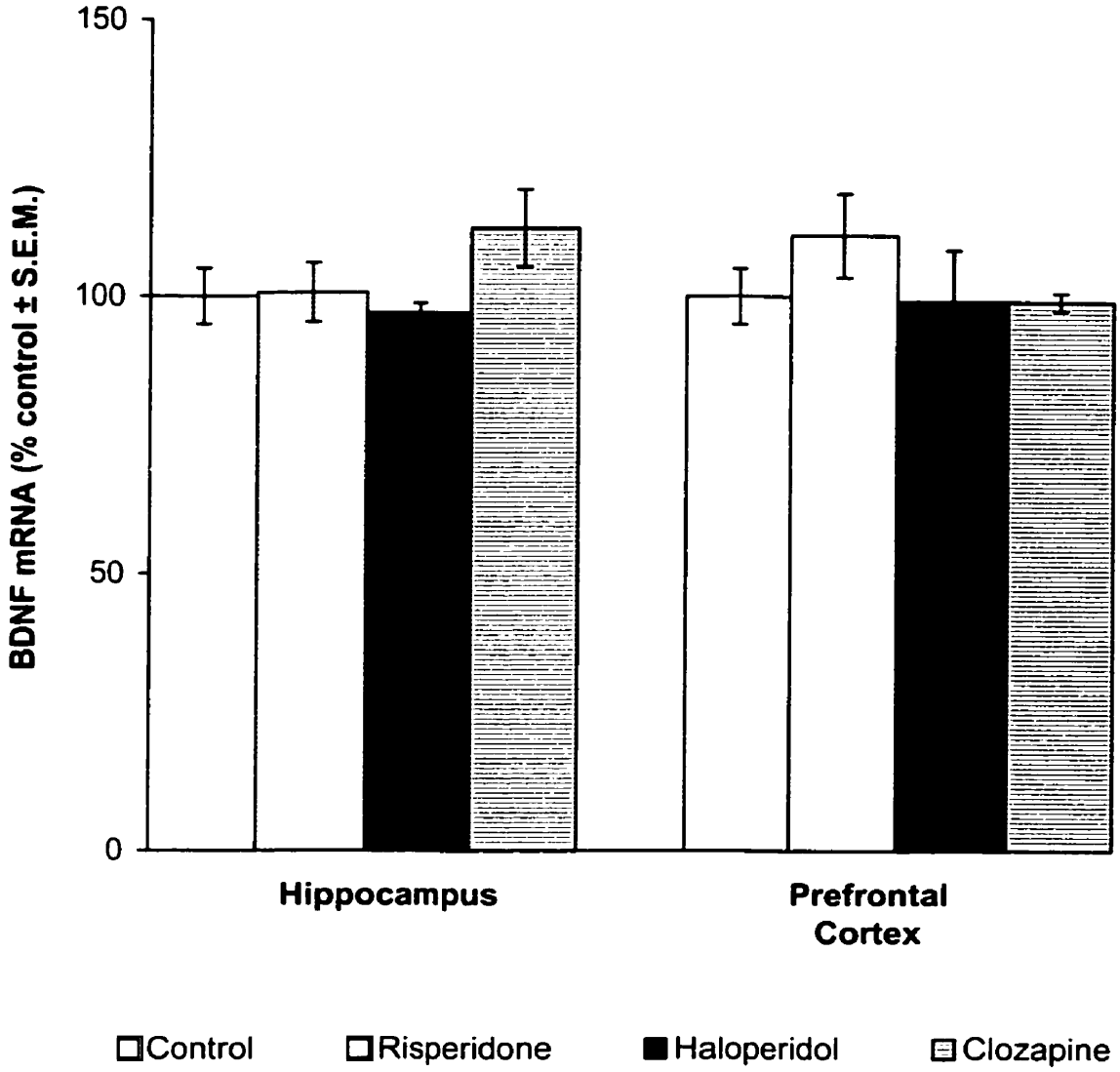

Figure 3.31. The effects of acute neuroleptics administration on hippocampal and prefrontal cortical BDNF mRNA by northern blot analysis. Animals were sacrificed 45 minutes after i.p. injections. Doses were 1mg/kg for all drugs except clozapine (15 mg/kg). Results are expressed as percent control ± S.E.M.

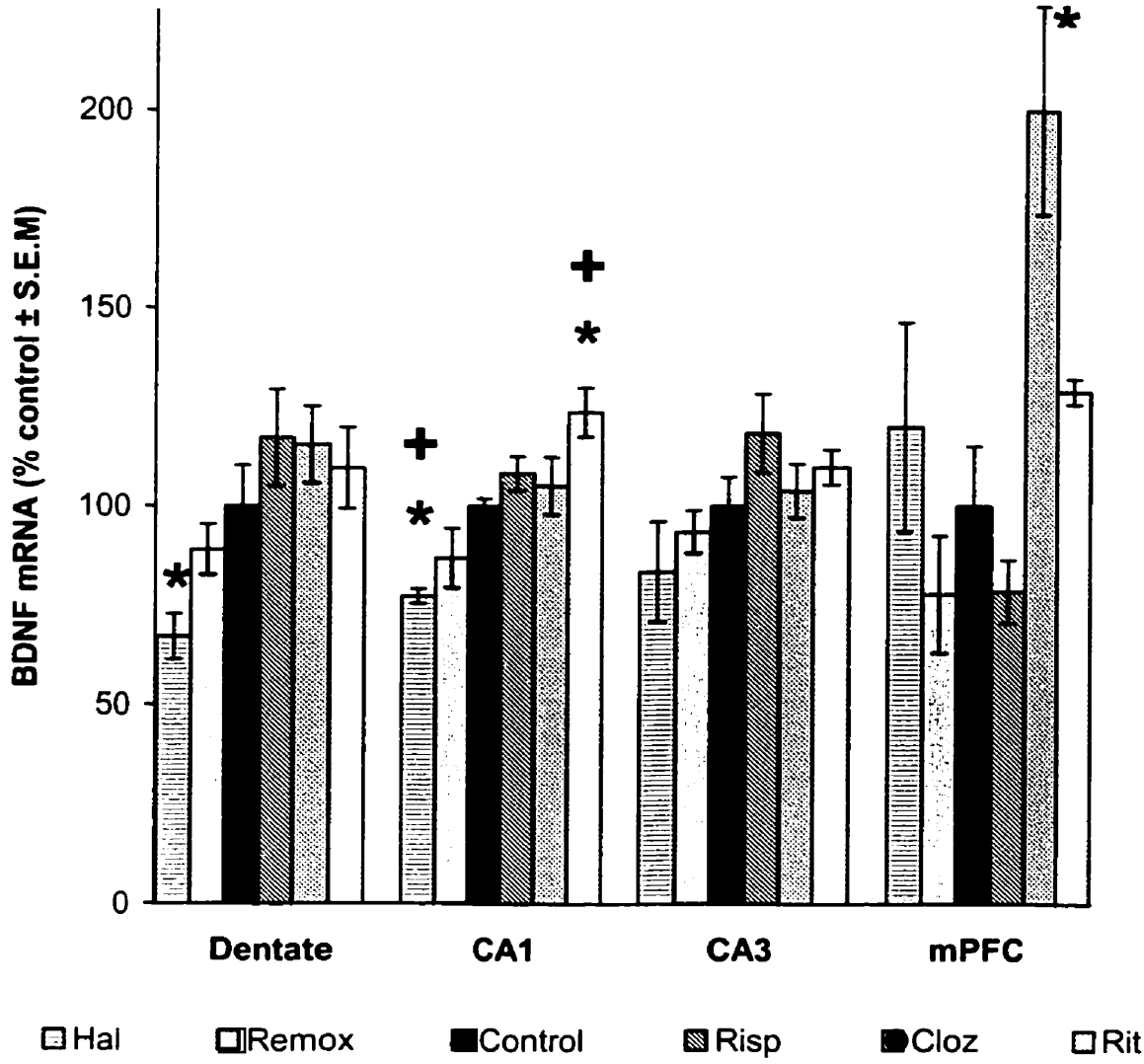

Figure 3.32. The effects of chronic neuroleptic administration on hippocampal and mPFC BDNF mRNA as determined by northern blot analysis. Animals received i.p. injections for 19 days and were sacrificed 18 hours after the last injection. Results are expressed as percent control ± S.E.M.

* significantly different from controls; $p<0.05$
+ haloperidol significantly different from risperidone, $p<0.05$.

$p <.$ 0.042; CA1, $F(5,12) = 6.969$, $p < 0.0029$; CA3, $F(5,12) = 2.240$, $p < 0.1172$); mPFC, $F(5,15) = 8.76$, $p < 0.001$).

3.3.4 Dose response studies of the effects of neuroleptics on hippocampal BDNF mRNA

Figures 3.33 and 3.34 show the results of a dose response study with neuroleptics. Rats were treated with either vehicle, haloperidol (1 mg/kg i.p.), risperidone (0.066, 0.67, 4.11 mg/kg i.p.), or clozapine (1.3 mg/kg, 9.0 mg/kg, 30.0 mg/kg i.p.) daily for 20 days (n=3). These doses were chosen on the basis of ex-vivo receptor occupancies in the rat brain (Schotte et al., 1993). An additional group of rats receiving no injections were used to control for injection stress. Haloperidol significantly decreased hippocampal BDNF mRNA ($p < 0.05$) to 64% and 74 of controls in the dentate and CA1 respectively. While higher doses of risperidone tended to downregulate BDNF mRNA (75% of control levels), lower doses of risperidone and clozapine tended to upregulate BDNF mRNA only in the CA1 region (dopaminergic blockade seems to downregulate, while serotonergic blockade tends to upregulate hippocampal BDNF mRNA). Statistical significance for many of these findings might be expected to be attained with an increased n. Representative autoradiographs are shown in Figures 3.35 and 3.36.

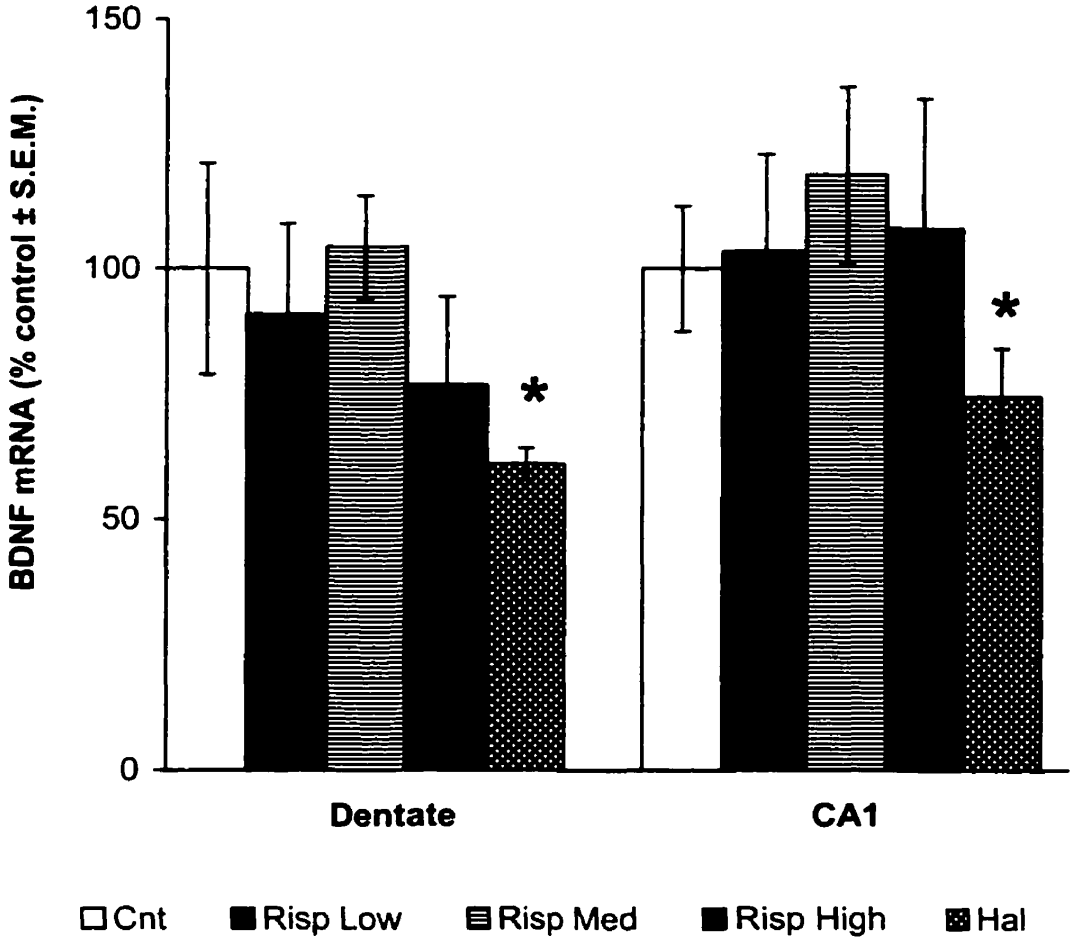

Figure 3.33. Dose-response study for the effects of chronic risperidone and haloperidol administration on hippocampal BDNF mRNA by *in situ* hybridization (note: dose-response is for risperidone only, only one dose of haloperidol was used in this experiment). Animals were injected with vehicle, haloperidol (1 mg/kg i.p.) or risperidone (0.066, 0.67, 4.11 mg/kg i.p.) daily for twenty days. Data analyzed by ANOVA, post-hoc by Newman Keuls (n=3).). Results are expressed as percent control ± S.E.M.

* significantly different from controls; $p<0.05$.

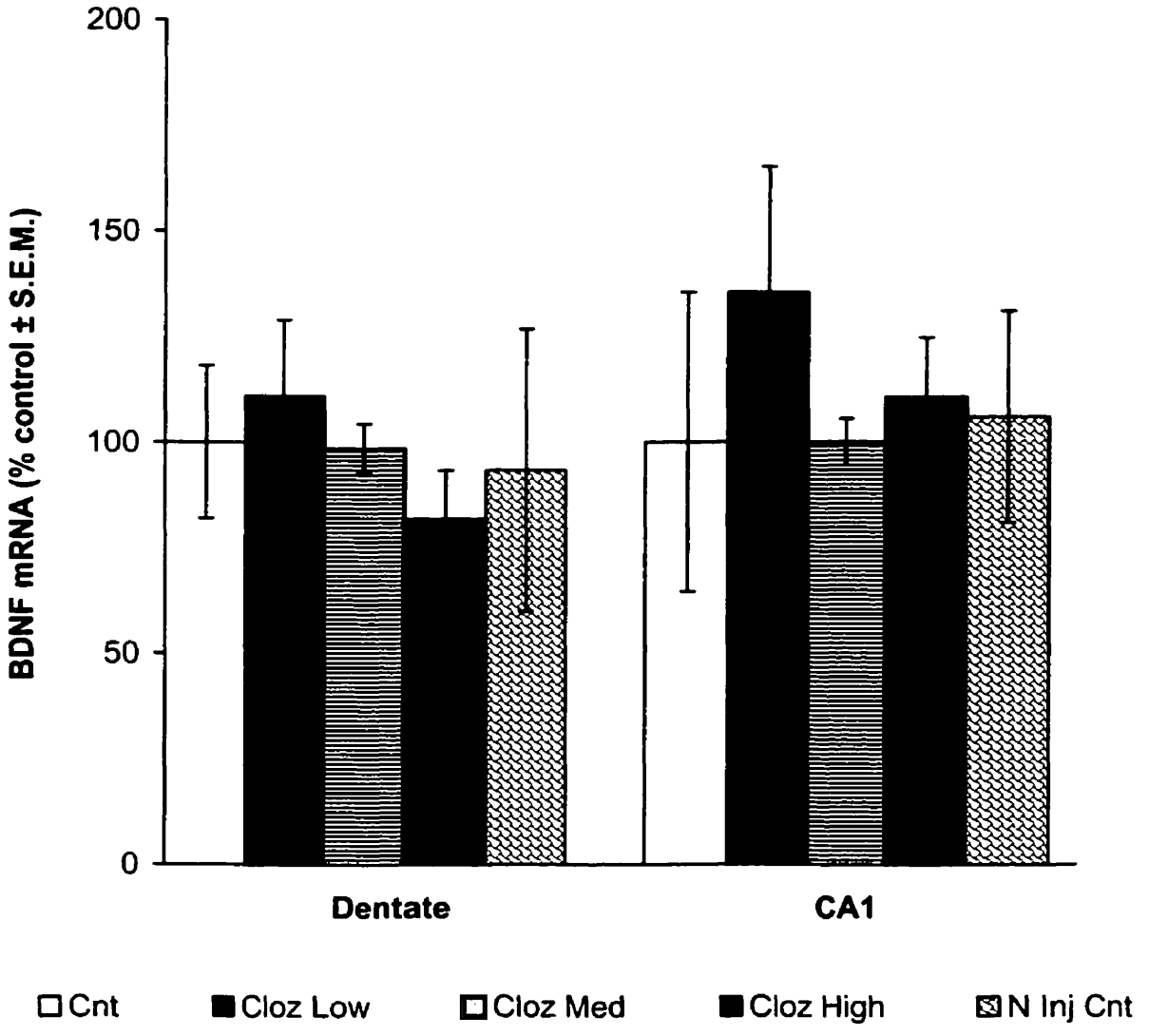

Figure 3.34. Dose-response study for the effects of chronic clozapine administration and non-injected controls on hippocampal BDNF mRNA by in situ hybridization. Animals were injected with vehicle, clozapine (1.3, 9.0, or 30.0 mg/kg i.p.) daily for twenty days or received no injection. Data analyzed by ANOVA, post-hoc by Newman Keuls (n=3). Results are expressed as percent control ± S.E.M.

* significantly different from controls; $p<0.05$.

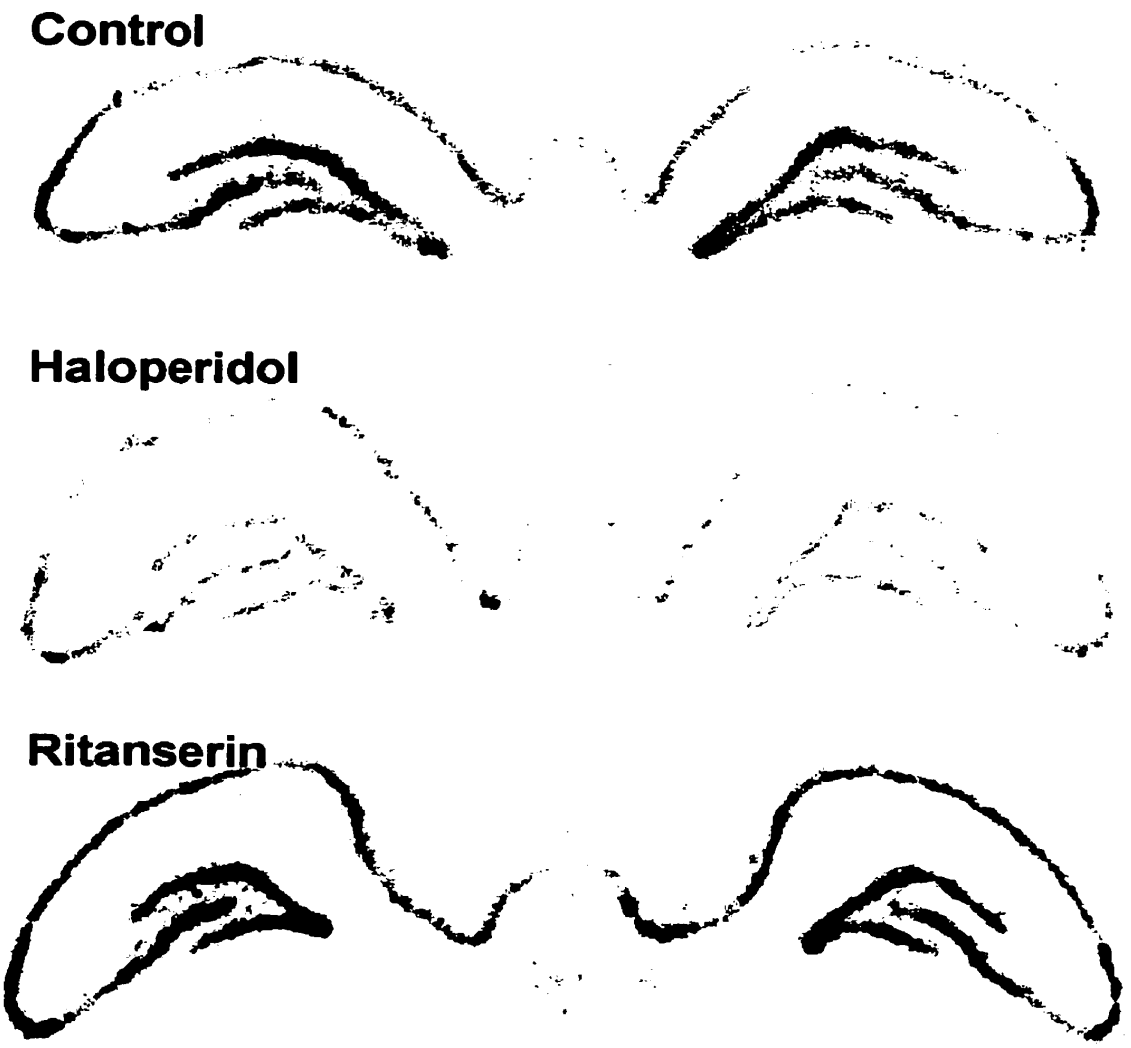

Figure 3.35. Autoradiographs of hippocampal BDNF mRNA. Preliminary study, n=3. Haloperidol significantly downregulated, while ritanserin upregulated, hippocampal BDNF mRNA as determined by *in situ* hydridization autoradiography.

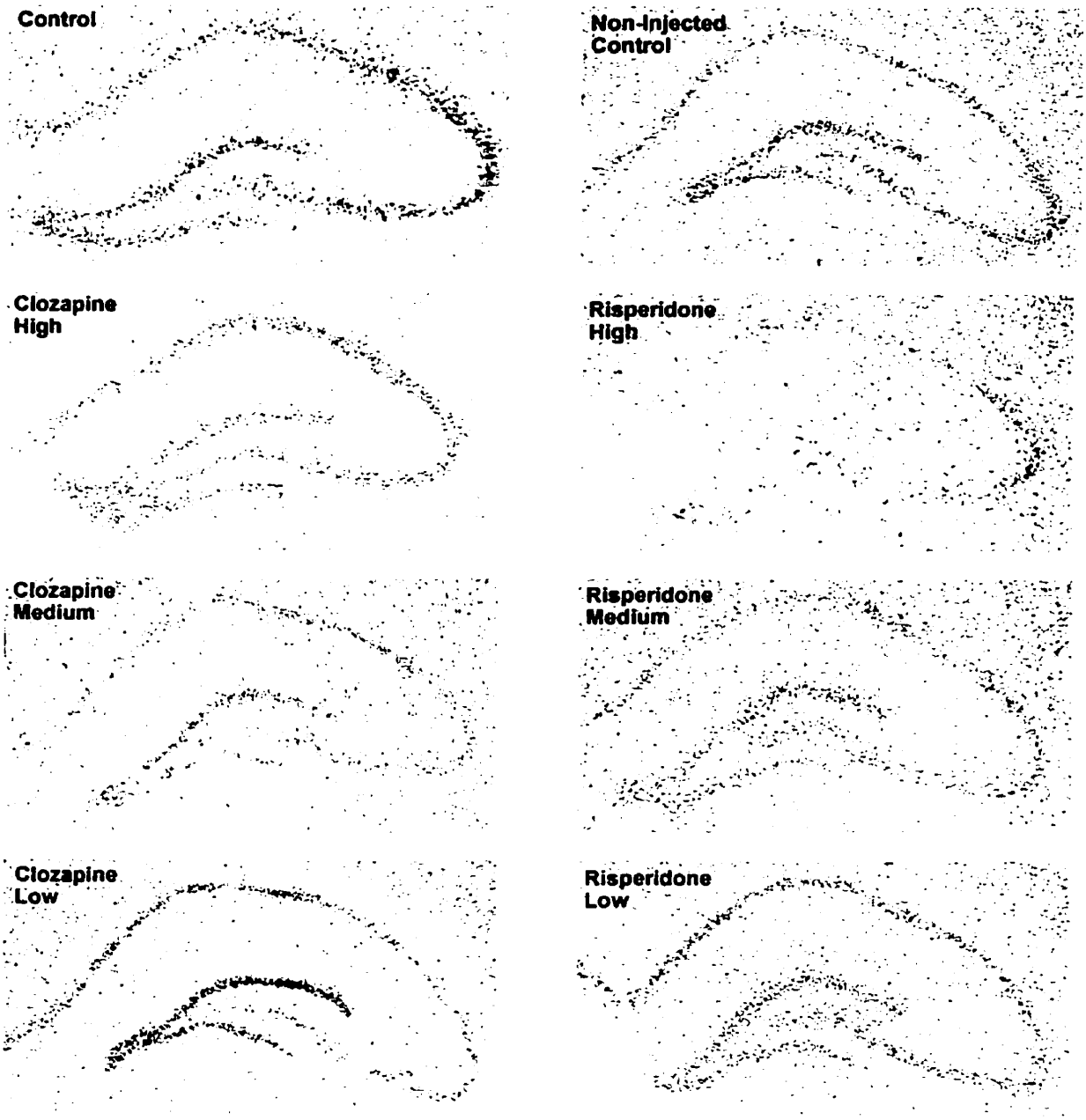

Figure 3.36. Autoradiographs of hippocampal BDNF mRNA in dose-response study. No significant differences as determined by *in situ* hybridization film autoradiography are observed between the controls and the non-injected controls. High dose risperidone tends to downregulate hippocampal BDNF mRNA, while low dose clozapine may upregulate BDNF mRNA in the dentate.

4. DISCUSSION

Since both dopamine D_2 and serotonin 5-HT_2 receptor blockers ameliorate symptoms of schizophrenia (Bersani et al., 1986; 1990; Roose et al., 1988; Meco et al., 1989; Huttunen, 1995), it was hypothesized in the present study that examining the differential effects of D_2, 5-HT_2, and D_2/5-HT_2 receptor antagonists on various biochemical parameters and brain regions would contribute to our understanding of their varied efficacies and side effect profiles. It was found that the neuroleptics used in the present study did alter the gene expression of various target proteins in vitro and in vivo upon acute and chronic administration. In addition, neuroleptics with different therapeutic efficacies and side effect profiles activated gene expression in different brain regions.

4.1 Neuroleptics do not upregulate TH, AADC, or c-fos mRNA as hypothesized in PC12 cells

4.1.1 Regulation of TH mRNA in PC12 cells

Since D_2 blockers have been shown to upregulate TH mRNA in vivo (Cho et al., 1997), it was expected in the present study that haloperidol, risperidone, and remoxipride would also upregulate TH mRNA in PC12 cells. It was found that two of the neuroleptics used which could strongly block D_2 receptors did upregulate TH mRNA in vitro; risperidone (Figure 3.7) at one hour, and haloperidol (Figure 3.9) at 2 and 12 hours respectively. Since ritanserin and clozapine primarily act on serotonergic receptors, it was not surprising that these two neuroleptics did not upregulate TH mRNA (Figures 3.3, 3.5).

While these findings suggest that D_2 blockade could be responsible for the upregulation of TH mRNA, additional results obtained suggest that other factors may be involved in this upregulation. Both risperidone and haloperidol block a number of neurotransmitter receptors other than D2 receptors (Leysen et al., 1992; Huang et al., 1993; Schotte et al., 1993; 1996; Megans et al., 1994). In addition, remoxipride did not upregulate TH mRNA and was the most selective D2 blocker used in the present investigation (Wadworth and Heel, 1990). Although risperidone significantly upregulated TH mRNA at two doses (10 and 1.0 μM) at one hour (Figure 3.7), the significance of this finding is limited due to the short duration (< 6 hours) of the response. Given that Buckland et al. (1992, 1993) did not find that either acute or chronic neuroleptic administration altered whole brain TH mRNA, the results in the present study may not be all that surprising.

The finding that neither remoxipride nor ritanserin altered TH mRNA suggests that neither D_2 or 5-HT_2 blockade alone is sufficient to regulate the gene expression of this enzyme. Therefore, the upregulation of TH mRNA by haloperidol and risperidone is not dependent on either D_2 or 5-HT_2 blockade alone. The finding that clozapine does not regulate TH mRNA suggests that the combination of D_2 and 5-HT_2 blockade is not sufficient to induce TH mRNA. Thus, a receptor that is blocked by haloperidol and risperidone, but not the other neuroleptics tested might be involved in the induction of TH mRNA.

An alternative explanation for the effects of these neuroleptics on TH mRNA might be regulation of sigma receptors. These receptors are found on a number of cells in the CNS, and are found on PC12 cells (Yang et al., 1989; Morio et al., 1994). Although their function is poorly understood, sigma receptors are regulated by haloperidol and risperidone but not by ritanserin, remoxipride, or clozapine (Clissold et al., 1992; Nakata et al., 1999). Since

remoxipride is quite specific for D_2 receptors, especially in comparison to other neuroleptics (Ögren et al., 1983; Wadworth and Heel, 1990), but has little effect on sigma receptors in comparison to haloperidol and risperidone (Ericson and Ross, 1992; Lang et al., 1994; Okuyama, 1999), it is possible that remoxipride's failure to induce TH mRNA is due to its relative lack of sigma activity. In support of a role of sigma receptors in the regulation of TH, drugs active at sigma receptors have been found to increase rat striatal TH enzyme activity (Weiser et al., 1995).

4.1.2 Regulation of AADC mRNA in PC12 cells

As both D_2 and 5-HT_2 receptor blockers ameliorate the symptoms of schizophrenia (Bersani et al., 1986, 1990; Roose et al., 1988; Meco et al., 1989; Huttunen, 1995) and since AADC is required for the synthesis of both DA and 5-HT (Lovenberg et al., 1962; Christenson et al., 1972), it was hypothesized in the present study that all neuroleptics would regulate AADC mRNA in a similar manner. Although the five aforementioned neuroleptics were used at a wide range of doses and time points, few changes in AADC mRNA levels were observed. Remoxipride, ritanserin, haloperidol and clozapine did not significantly regulate AADC mRNA in PC12 cells (Figures 3.2, 3.4, 3.6, 3.10), and risperidone significantly upregulated AADC mRNA only at the one hour time point. Taken with the TH mRNA results of this study, it would appear that in PC12 cells, neuroleptics have little if any effect on the gene expression of either TH or AADC mRNA.

4.1.3 Regulation of *c-fos* mRNA in PC12 cells

It was hypothesized that D_2 blocking neuroleptics would upregulate *c-fos* mRNA in PC12 cells. The data are difficult to analyze due to the poor quality of the autoradiographs, so the results were assessed to identify any obvious trends. Interestingly, any trends of neuroleptic-induced increases in *c-fos* mRNA were paralleled by changes in TH but not AADC. It appeared that the haloperidol-induced changes in TH mRNA were preceded and paralleled by

changes in *c-fos* mRNA at 1, 2, and 12 hours (data not shown; see Figure 3.11 for representative autoradiographs). Esteve et al. (1995) confirmed that haloperidol (albeit at high doses of 100 μM) upregulated *c-fos* mRNA at 0.5, 1, and 2 hours in PC12 cells. The present study also demonstrated that haloperidol upregulated *c-fos* mRNA at 100 μM (data not shown, see representative autoradiographs in Figure 3.11), but these results must be treated with caution due to the potential toxicity of the high dose of haloperidol used (i.e., at this dose, an increased number of the PC12 cells in the well plates became detached from the collagen-coated surface, indicating that more cells were dying during the experiment at this dose).

It is not surprising that it was observed in the present investigation that the upregulation of *c-fos* mRNA preceded that of TH mRNA (Ozaki et al., 1998; Yukimasa et al., 1999), since the Fos protein is known to upregulate TH mRNA (Icard-Liephalns et al., 1992). One investigator found, in a PC12 cell culture study of the effects of haloperidol administration on *c-fos* mRNA, that the upregulation of *c-fos* mRNA was accompanied by a Ca^{2+} dependent increase in AP1 binding activities (Esteve et al., 1995), suggesting that the Fos protein could be binding to the AP1 site on the TH promoter (Icard-Liepkalns et al., 1992). In the case of risperidone, it is quite possible that any *c-fos* mRNA inducing effects caused by its blockade of D_2 receptors would rapidly be counteracted by its blockade of 5-HT_2 receptors, since inhibition of 5-HT_2 receptors induction by 5-HT_2 receptor blockers such as ketanserin and mesulergine has been shown to block 5-HT_2 induction of *c-fos* (Humblot et al., 1997).

Since the AADC promoter was not well characterized at the time of our investigations, it was not known whether or not its promoter region contained an active AP-1 site. To date, an active AP-1 site has not been identified on the AADC promoter (Raynal et al., 1998). Thus, it is not surprising that changes in AADC mRNA do not parallel changes in *c-fos* mRNA. As AADC protein has

phosphorylation sites for both protein kinases A and C which are part of the signaling pathways for DA and 5-HT respectively (Kemp and Pearson, 1990), it is possible D_2 and 5-HT_2 receptor blockade might regulate AADC at the post translational and not the transcriptional level, or that AADC transcriptional regulation involves other early immediate genes.

4.1.4 Summary of *in vitro* results

In summary, four general conclusions can be made from the investigations with the PC12 cell culture:

a. The neuroleptics examined do not significantly regulate AADC mRNA in PC12 cells.

b. Only haloperidol significantly regulated TH mRNA.

c. The induction by haloperidol of *c-fos* mRNA precedes that of TH mRNA.

d. The D_2 vs. 5-HT_2 distinction is not meaningful for neuroleptic regulation of TH and AADC mRNA in PC12 cells, and such regulation is more likely to involve other receptors such as the sigma receptor.

Since the working hypothesis of the present study (that neuroleptics would regulate AADC and TH mRNA in a similar manner) was not supported by the experiments, future in vivo studies were limited to examining the effects of neuroleptics on AADC mRNA in situ. Preliminary northern blot and in situ hybridization experiments demonstrated that although AADC mRNA could be detected in the substantia nigra, this mRNA was difficult to detect in the cortex or hippocampus due to its relatively low expression levels in the area. As the neuroleptic induced increases in AADC mRNA found by Buckland's group (1993) involved studies that were performed with whole brain homogenates, it

may be that the changes found by this group might occur in the glia that in order to be detected require a tissue sample as large as the entire brain.

4.2 Regulation of *c-fos* mRNA and delta FosB immunoreactivity in vivo by neuroleptics does not follow expected patterns

4.2.1 Regulation of *c-fos* mRNA by acute neuroleptic treatment

As previously discussed, there is little understanding of the molecular mechanisms underlying the therapeutic response and side effect profile of neuroleptics, and no common mechanisms of action have been identified to date.

Since the Fos protein was known to be induced by a variety of behavioral and psychoactive stimuli (Morgan et al., 1987; Brown et al., 1992; Duncan et al., 1993), and transcriptionally regulated by a number of genes (Uhl et al., 1988; Kislauskis et al., 1990), investigators reasoned that perhaps the differential clinical effects of neuroleptics could be mapped by examining early immediate gene regulation in various brain regions thought to be involved in the clinical efficacies and side effect profiles of neuroleptics (Robertson and Fibiger, 1992). The knowledge that the upregulation of such a protein (i.e., a transcription factor) could alter the expression of other genes made it an attractive candidate to not only track the neuronal substrates activated by neuroleptics, but to help elucidate the target genes involved in the neuroleptic response.

Earlier animal studies demonstrated that acute haloperidol administration significantly upregulated *c-fos* mRNA in the striatum (Dragunow et al., 1990; Miller, 1990; Robertson and Fibiger, 1992). The functional validity of these findings at the time was significant because it provided one of the first reliable markers of neuronal activity which correlated with the effects of haloperidol in the striatum and the production of EPS. At the time the present studies were

initiated, researchers had established that the typical neuroleptic haloperidol and the atypical neuroleptic clozapine increased *c-fos* mRNA and/or the Fos protein in differing brain regions (Robertson and Fibiger, 1992). Therefore, the hypothesis was made that the differing mechanisms of action of typical vs. atypical neuroleptics could be localized to specific brain regions and that drug design could use the *c-fos* response as a marker for therapeutic efficacy and side effect profiles (Robertson and Fibiger, 1992; Merchant and Dorsa, 1993). No other neuroleptics besides haloperidol and clozapine at that time had been examined for their effects on *c-fos* mRNA, so this was measured in the present study for a number of neuroleptics with a wide range of DA to 5-HT blocking properties.

4.2.1.1 Regulation of *c-fos* mRNA in the striatum

It was found in the present study that the acute administration (45 minutes) of haloperidol and higher doses of risperidone increased *c-fos* mRNA in the striatum, while remoxipride, ritanserin, and clozapine were without effects in this brain region (Figures 3.12 to 3.16). This was confirmed in later studies by both northern blot and *in situ* hybridization studies. Studies conducted in other laboratories at this time also demonstrated that all typical neuroleptics examined with a high propensity to cause EPS (such as haloperidol) increased *c-fos* expression in the dorsal lateral striatum, whereas atypical neuroleptics including risperidone, ritanserin and clozapine did not induce any or as great an increase in *c-fos* mRNA in this region (Robertson et al., 1994; Deutch et al., 1995; Deutch and Duman, 1996).

4.2.1.2 Regulation of *c-fos* mRNA in the medial prefrontal cortex and nucleus accumbens

Some investigators had suggested that clozapine was not only unique in its ability to treat refractory schizophrenia and the negative symptoms of the disorder, but that it alone would elevate *c-fos* mRNA in the medial prefrontal cortex (mPFC) (Merchant et al., 1996). Robertson et al., (1994) however,

found that a number of atypical neuroleptics in addition to clozapine (such as remoxipride and fluperapine, but not risperidone) elevated Fos-like immunoreactivity in this region. The present experiments showed no induction of *c-fos* mRNA by clozapine, remoxipride, or any of the other neuroleptics tested in this brain region (Figures 3.18 and 3.19), and other investigators (Hiroi and Graybiel, 1996) have failed to replicate these findings of Robertson et al. (1994) in the mPFC.

These investigators also found that both typical and atypical neuroleptics increased *c-fos* mRNA in the nucleus accumbens (NuAcc) (Robertson et al., 1994). This is in agreement with the results of the present study which found that all the neuroleptics tested elevated *c-fos* mRNA in the NuAcc (see figure 3.12). The shared therapeutic molecular mechanism of many neuroleptics was thus thought perhaps to involve the modulation of neuronal activity in the NuAcc, while neuroleptic induced EPS may be related to c-fos stimulation in the striatum (Robertson and Fibiger, 1992).

4.2.2 Regulation of *c-fos* mRNA by chronic neuroleptic treatment

As it turned out, the mechanisms underlying the neuroleptic induction of *c-fos* were more complex than it initially appeared. Other studies demonstrated that the initial neuroleptic-induced increases in *c-fos* mRNA were attenuated in rats upon chronic neuroleptic administration and it was found that the *c-fos* induction produced by many types of acute stimuli were also attenuated upon long term treatment (Merchant and Dorsa, 1993; Merchant and Miller, 1994; Merchant, 1994; Merchant et al., 1996). This finding was difficult to explain with respect to the early immediate gene hypothesis of neuroleptic treatment, since the therapeutic effects of neuroleptics were not observed until two or three weeks of continuous administration. One would conceivably want to identify an intracellular factor which is altered by neuroleptic treatment at the same time course at which therapeutic efficacies and side effects were observed in the clinic.

In order to determine if the neuroleptic-induced induction of striatal *c-fos* mRNA was truly attenuated or was reduced but not absent followinf repeated exposure, rats received either chronic neuroleptic injections for 19 days and were sacrificed on Day 20 (18 hours after the last injection) or received 19 days of injections, followed by a further injection on day 20 given 30 minutes before sacrifice. Time course studies for the effects of acute neuroleptic administration on striatal *c-fos* mRNA were also performed to determine the time point at which maximal induction of striatal *c-fos* mRNA by haloperidol was observed. It was hypothesized that long term changes in *c-fos* mRNA might be not be observed in chronic paradigms because maximal induction of *c-fos* mRNA is seen 30 to 45 minutes following an injection, while *c-fos* mRNA was measured in many chronic paradigms at time points much greater than this 30 to 45 minute time range.

The results obtained by *in situ* hybridization and emulsion autoradiography did not support the working hypothesis of the present study. Not only were there were no changes in striatal *c-fos* mRNA with chronic neuroleptic treatment, but the haloperidol treatment lost its ability (produced tolerance) to upregulate striatal *c-fos* mRNA when administered 45 minutes before sacrifice after it had been injected chronically for 19 days previous to the last injection. The validity of these experiments was confirmed by including tissue samples from a group of rats treated with haloperidol and another group of rats that had been treated with kainic acid (which causes some of the rats to develop seizures) which showed intense activation of *c-fos* expression in the brain. Findings of the absence of *c-fos* induction with chronic haloperidol treatment were confirmed in these in situ hybridizations. No other brain regions showed *c-fos* induction in these injection paradigms in our studies.

Many of the findings of the present study have been confirmed by others in the literature (Merchant et al., 1994; Merchant et al., 1996). Specifically,

Merchant et al. (1994) found that both chronic clozapine and chronic haloperidol administration to rats did not alter *c-fos* mRNA in the dorsolateral striatum, while 28 day saline injections followed by an acute (1 hour before sacrifice) injection of haloperidol resulted in an approximately 150% increase in *c-fos* mRNA over 28 day saline-injected controls. While chronic haloperidol administration followed by an acute haloperidol injection was also found by these investigators to significantly increase *c-fos* mRNA above control levels, the increase was no more than 10% of controls. Thus, while changes in c-fos mRNA immunoreactivity may identify the neural structures which are activated by neuroleptics, it is not likely that the upregulation of *c-fos* mRNA parallels the time frame in which neuroleptics begin to exert their side effects and therapeutic efficacies.

4.2.3 Regulation of delta FosB by neuroleptics

The gene expression of early immediate gene delta FosB was the next target upon which to examine the effects of neuroleptics because unlike *c-fos*, it had been shown to be upregulated upon chronic stimulation in a number of experimental paradigms (Jian et al., 1993; Kelz et al., 1999; Nestler et al., 1999). This "atypical" early immediate gene is not maximally upregulated upon acute stimulation, but instead accumulates over time with increasing phosphorylation (Chen et al., 1997). Thus, if the *c-fos* and delta FosB responses are related, it is possible that one of the targets of *c-fos* is the upregulation of phosphorylating enzymes such as the protein kinases which phosphorylate proteins and contribute to their cellular longevity (Doucet et al., 1996; Chen et al., 1997; Nestler et al., 1999).

It was the hypothesis of the present study that chronic neuroleptic administration would upregulate delta FosB in the same brain regions where *c-fos* mRNA is elevated by acute neuroleptic treatment. Vahid-Ansari et al. (1996) have demonstrated that chronic haloperidol and clozapine

administration increases delta FosB in regions similar to those where c-fos mRNA upregulation is seen in the acute state. It was found in the present study that haloperidol and risperidone did increase the number of delta FosB immunoreactive neurons in the striatum (Table 3.1, Figure 3.21). It is interesting that whereas haloperidol and only high doses of risperidone increased delta FosB IR in the dorsolateral striatum (DLStr), haloperidol and both high and moderate doses of risperidone increased delta FosB immunoreactivity in the mediolateral striatum (MLStr). Since moderate doses of risperidone clinically alleviate symptoms of schizophrenia but do not necessarily cause EPS (Leysen et al., 1988), these findings support the hypothesis that the motor side effects of neuroleptics are caused in the DLStr (which receives projections from the substantia nigra, SN), but that the amelioration of positive symptoms in part is related to activity of the MLStr (which receives projections from the ventral tegmental area, VTA: Grace, 1991).

However, the present investigations demonstrated that while delta FosB immunoreactivity followed expected patterns in the DLStr and MLStr, we (and others) were unable to replicate findings of clozapine- induced delta FosB IR in the medial prefrontal cortex (mPFC) (Table 3.1, Figure 3.21). In fact, induction was significant for risperidone, and it seemed that there was some induction of delta FosB immunoreactivity in the mPFC. Differences in results could reflect different criteria applied to counting methods, as one could either count changes in the number of IR positive neurons (which was done in the present study), or the intensity of already delta FosB positive neurons could be counted.

Based on the findings of the present study, an alternative hypothesis for the regulation of delta FosB (and *c-fos*) can be proposed (Atkins et al., 1999). The results of the present study demonstrated that the in vitro and in vivo regulation of both *c-fos* mRNA and delta FosB were only associated with dopaminergic

(and/ or sigma) receptor blocking neuroleptics and did so only at doses which would produce significant D_2 or sigma receptor binding (Schotte et al., 1993; 1996). On the other hand, serotonin receptor blocking neuroleptics did not seem to alter the expression of either early immediate gene, which is not surprising given that serotonergic agonism (and not 5-HT2 receptor blockade) increases *c-fos* mRNA (Humblot et al., 1997).

Although the clozapine-induced induction of *c-fos* mRNA in the mPFC is short lived, its induction may be indicative of the activation of pathways other than those induced by the blockade of D_2 receptor containing pathways. Long term changes in early immediate gene expression in the mPFC for clozapine may either not exist or may translate into the regulation of an entirely different set of early immediate genes. It is quite intriguing, therefore, that a statistically significant increase in mPFC BDNF mRNA upon acute injection of clozapine was found in the present study. Nevertheless, induction of delta FosB IR by chronic neuroleptic administration may provide researchers with a useful map to unravel the molecular mechanisms of antipsychotic drugs. Future studies would do well to elucidate the target proteins of delta FosB.

4.3 Neuroleptics demonstrate neuroprotective potential by positively regulating genes involved in neuroprotection and neurodegeneration

As previously discussed, neuroanatomical studies of schizophrenia suggest that progressive neuropathological changes (such as neuronal atrophy and/or cell death) occur over the lifetime course of the disease (DeLisi, 1997). Early intervention with atypical neuroleptics has been shown to prevent progression of at least some symptoms, although the mechanisms by which neuroleptics may do this remain unknown.

Not only is it concluded that untreated schizophrenia progresses into a less treatable state, but it has been proposed that long term improvement of outcome in schizophrenia can occur throughout the entire course of neuroleptic

treatment (Edwards et al., 1999; Ende et al., 2000). It is unknown, however, if these changes require continuous neuroleptic treatment or, if the neuropathology will return to its initial state after the discontinuation of medication. If schizophrenia is characterized by ongoing neuronal atrophy, it is possible that the atrophied neurons can be induced to rebuild and strengthen their processes by the appropriate treatment, and could go on to form strengthened synaptic connections. The present investigator would argue that the findings of the current studies support this notion. It is even possible that some neuroprotective genes are target genes of delta FosB (Nikam et al., 1995; Hayes et al., 1997), and it was of interest to determine if any of the following neuroprotective factors were regulated in a manner similar to delta FosB.

4.3.1 Superoxide dismutase (SOD1) and the low affinity nerve growth factor receptor p75

In the present study, PC12 cells were used to determine the effects of a number neuroleptics on the gene expression of superoxide dismutase (SOD) and the low affinity nerve growth factor receptor (p75). As previously discussed, the free radical scavenging enzyme SOD1 is widely distributed in the CNS, including many regions reported to be atrophied in schizophrenia, such as the hippocampus and cerebral cortex (Lohr et al., 1990; Selemon et al., 1995) . It is therefore conceivable that upregulation of this enzyme by neuroleptics could prevent further free radical induced neurotoxicity/ neurodegeneration in schizophrenia. The p75 or low affinity nerve growth factor receptor, which binds all neurotrophins with an equal affinity, must be occupied with neurotrophin in order for its "host" neuron to survive. Its mRNA expression in adults is primarily limited to the medial septal nucleus and the nucleus of Broca's diagonal band (Vazquez and Ebendal, 1991). These nuclei of the basal forebrain contain cholinergic neurons which project to the hippocampus and cerebral cortex (Ebendal, 1992). The atrophy of these neurons would result in decreased input to the hippocampus and cortex, which

could either exacerbate symptoms of schizophrenia, or contribute to the degeneration and death of target neurons. Interestingly, the increased expression of p75 in cholinergic neurons is associated with Alzheimer's disease (Woolf et al., 1989) and ß-amyloid toxicity (Rabizadeh et al., 1994).

While no effects on SOD mRNA in PC12 cells were observed for remoxipride, ritanserin, or haloperidol; clozapine, olanzapine and risperidone did upregulate this neuroprotective enzyme. No effects on SOD or p75 mRNA were seen for remoxipride or ritanserin, suggesting that neither the D_2 nor the 5-HT_2 receptor are specifically involved in the SOD and/ or p75 response. However, at 12 hours, haloperidol seemed to upregulate, while clozapine seemed to downregulate p75 mRNA (Figure 3.26), although these trends did not reach statistical significance. At 24 and 48 hours, both haloperidol and clozapine downregulated p75 mRNA. This differential regulation of p75 mRNA by haloperidol vs. clozapine is quite interesting, given that haloperidol has been found to be neurotoxic in many experimental paradigms (Fang and Gorrod, 1991; Fang and Yu, 1997) and that elevated p75 receptors could possibly facilitate cell death. It is possible that if haloperidol is neurotoxic, the initial upregulation of p75 by haloperidol may reflect its propensity to increase cell death, and the ability of the PC12 cells to attenuate the upregulation of p75 at a later time point may reflect a compensation of the initial upregulation. These results suggest that since atypical neuroleptics such as clozapine and risperidone more favorably regulate genes involved in neuroprotection and neurodegeneration, they would be more likely than haloperidol to reduce any clinical signs of pathology due to neurodegeneration in schizophrenia. Further studies at longer time points would be necessary to confirm this hypothesis.

After becoming available for laboratory investigation approximately two years ago, olanzapine was included in later PC12 studies. The PC12 cultures were treated with olanzapine for a longer time than the other neuroleptics used in order to observe its effects at lower doses over a longer period of time. The

results showed that olanzapine increases SOD and decreases p75 gene expression at concentrations of 10 and 100 μM after 48 hours of incubation in PC12 cultures. It is interesting that olanzapine, clozapine, and risperidone significantly upregulated SOD mRNA, while olanzapine, haloperidol and clozapine (but not risperidone) significantly downregulated p75 mRNA. It is difficult to interpret the meaning of the differential stimulation by neuroleptics in this paradigm, but it may involve differences in receptor binding profiles. The role of cell death and perhaps the p75 receptor in schizophrenia is supported by the finding that there is altered death of neurons in neuronal cultures from adults with schizophrenia (Féron et al., 1999).

PC12 cells have been widely used as a model for the study of catecholamine synthesis, release, and metabolism; and neuronal differentiation and cell death (reviewed by Greene and Tischler, 1982). The present investigation shows for the first time that some neuroleptics increase SOD1 and decrease p75 gene expression in PC12 cells. The results support the hypothesis that neuroleptics could protect neurons by upregulating the expression of genes coding for a neuroprotective enzyme (SOD1) and downregulating the expression of neurodegenerative genes that code for a receptor (p75) associated with programmed cell death. Since, as suggested by several investigators, one of the mechanisms by which stress-induced cell damage and/ or death is via oxidative damage, then stress experienced prior to the onset of schizophrenia may produce greater cell damage in a system that is already neurodevelopmentally compromised. Since stress increases glucocorticoid secretion and glucocorticoids downregulate SOD1 activity (Cvijic et al., 1995), SOD might protect against such damage.

4.3.2 BDNF mRNA

It was the hypothesis of the present study that disruptions of BDNF may play a role in the etiology of schizophrenia by compromising neuroplasticity or altering normal neurotransmission. and that neuroleptics may in part restore

the balance of this neurotrophin. As previously mentioned, BDNF is the most abundant neurotrophin in the brain (Marty et al., 1996; Maisonpierre et al., 1991b; Philips et al., 1990). Not only is it decreased in the hippocampus of persons with schizophrenia (Brouha et al., 1996) but an allele variant of this gene has been found in persons with schizophrenia (Vahid-Anisari et al., 1996). BDNF is decreased by factors associated with the exacerbation of schizophrenia such as stress (Smith et al., 1995) and estrogen withdrawal (Singh et al., 1995), and is elevated by treatments for schizophrenia, including 5-HT_{2A} receptor antagonists and ECT treatment (Vaidya et al., 1997).

It was initially expected in the present study that atypical neuroleptics would upregulate hippocampal BDNF mRNA, while typical neuroleptics would not regulate this neurotrophin. In addition, it was hypothesized that chronic, but not acute, neuroleptic treatment would upregulate BDNF mRNA. While the present study demonstrated that chronic, but not acute, neuroleptic administration altered BDNF mRNA (see Figures 3.31 and 3.32), it was not in the hypothesized pattern. Chronic haloperidol administration siginifcantly downregulated hippocampal BDNF mRNA in the dentate gyrus and CA1 region, while ritanserin significantly upregulated BDNF mRNA in the CA1. Although the atypical neuroleptics clozapine and risperidone modestly upregulated hippocampal BDNF mRNA, this effect did not reach statistical significance; the other atypical neuroleptic, remoxipride, tended to downregulate hippocampal BDNF mRNA.

These findings led to the revision of the initial working hypothesis and a subsequent dose-response study to elucidate the effects of varying the D_2 to 5-HT_2 blocking ratio of atypical neuroleptics. It was expected that the atypical neuroleptics risperidone and clozapine would upregulate hippocampal BDNF mRNA at lower doses where their DA D_2 blocking potencies did not outweigh their 5-HT_2 receptor blocking properties. This hypothesis was based on the

aforementioned findings that the D_2 blockers haloperidol and remoxipride downregulated, while the 5-HT_2 blocker ritanserin upregulated BDNF mRNA.

The dose-response study demonstrated that while the lower doses of risperidone and clozapine did not produce statistically significant increases in BDNF mRNA levels over controls, they showed a consistent trend to upregulate this neurotrophin in the hippocampus, while the single dose of haloperidol used consistently decreased BDNF mRNA throughout the hippocampus (see Figures 3.33-3.36). Conversely, high doses of clozapine, and especially risperidone, caused a reduction of BDNF mRNA which almost reached statistical significance by ANOVA ($p< 0.07$). These findings have been supported by other investigators, including Khaing et al. (1999) and Molteni et al. (1999). If regulation of BDNF mRNA by atypical neuroleptics is truly indicative of the neuroprotection they can afford, more (a higher dose) is not necessarily better.

In conclusion, the present experiments showed that when the dose of atypical neuroleptics such as risperidone and clozapine theoretically reaches a 50% D_2 receptor occupancy (as estimated from neuroleptic ex vivo receptor occupancies determined by Schotte et al., 1993), the effects D_2 receptor inhibition overrides any effect that 5-HT_2 receptor blockade might have on the elevation of hippocampal BDNF mRNA and will cause downregulation of BDNF mRNA that approaches that of haloperidol. In addition, the results of the present study would suggest that it is unlikely that any neuroleptic which blocks only D_2 receptors will significantly upregulate BDNF mRNA levels beyond controls. It is difficult to ascertain whether or not these findings have clinical relevance, but it is of interest that both the clinical use of haloperidol and the reduction of hippocampal BDNF mRNA in BDNF-deficient mice are associated with memory loss, decreased learning, and decreased motor activity (Neeper et al., 1996; Ma et al., 1998; Beuzen et al., 1999).

Since 5-HT_{2A} blockers can block stress-induced decreases in BDNF mRNA (Vaidya et al., 1997), this may be quite important in the function of atypical neuroleptics - especially since stress has been associated with the exacerbation of schizophrenia and itself can cause atrophy of neurons (Smith et al., 1995). It is conceivable that in a person predisposed to schizophrenia, impaired neuronal circuitry is more vulnerable to deficiencies in neurotrophin levels which could be caused by stress (Smith et al., 1995), estrogen withdrawal (Singh et al., 1995), or other insults, and early intervention with a 5-HT_2 receptor-blocking neuroleptic could at least prevent trophin decreases induced by these events. Stress (Magarinos et al., 1996) and glucocorticoids (Sapolsky et al., 1985), both of which have been implicated in the etiology and exacerbation of schizophrenia, cause dendritic atrophy (Sapolsky et al., 1990) and reduction of BDNF and NT3 in the hippocampus (Smith et al., 1995), Thus, it would be of considerable value to study the effects of typical and atypical neuroleptics on BDNF mRNA in models of "onset" of schizophrenia by determining if atypical neuroleptics have the ability to block decreases in BDNF mRNA during these critical periods associated with first episode onset of schizophrenia.

4.3.3 Summary of the effects of neuroleptics on neuroprotective and neurodegenerative factors

Many clinical studies have shown that some of the symptoms of schizophrenia not only worsen over time, but that untreated schizophrenia can eventually develop into a more permanent and less treatable state (Wyatt et al., 1995). This would imply that in addition to a probable neurodevelopmental defect in schizophrenia, there is a neurodegenerative component (such as neuronal atrophy or cell death) to the disorder associated with symptom onset (Coyle et al., 1996), and it is most likely that the developmental and degenerative components significantly interact and are not mutually exclusive. Increasing evidence that the early treatment of schizophrenics with

neuroleptics can improve long term outcome demands that the molecular mechanism(s) by which neuroleptics do this be elucidated, as we have attempted to do in the present study.

Neuroanatomically, it is likely that neurodegeneration does occur long before the observation of overt clinical symptoms, but the exact time is difficult to determine. Ventricular enlargement suggesting neuronal atrophy in schizophrenia has been known for years (Weinberger et al., 1979), but it is unknown if this neuronal atrophy is static or ongoing. According to Woods et al. (Woods et al., 1996, Woods, 1998), any increase in extracerebral CSF caused by an earlier non-progressive lesion would tend to be masked by subsequent "outward growth", as brain volume more than triples between birth and five years (Wood et al., 1998). This would suggest that ventricular enlargements observed in schizophrenia must result from a diffuse lesion resulting in a loss of brain tissue and a reduction in its volume that occurs after maximal brain volume expansion. Since ventricles can enlarge with both early and late tissue volume loss, cross-sectional studies (which only look at the ratio of lateral ventricular volume to total brain tissue volume) can detect tissue loss but cannot distinguish between early and later time of occurrence. Only volume measurements that include extracerebral CSF volumes can determine whether or not there has been later volume loss. Findings by Nopoulos et al. (1995) that patients with schizophrenia had less brain tissue, more extracerebral CSF, and more ventricular CSF suggest that the patients' brain volume loss occurred in the time period after maximum brain volume expansion but before onset of overt illness.

Presently, there are ongoing studies attempting to demonstrate the abilities of neuroleptics to arrest some symptoms of schizpohrenia (Edwards et al., 1999). This is a difficult theory to test for a number of reasons, including the anatomical ones described above. Because the prodromal symptoms of schizophrenia are not particularly specific to the disorder, clinicians who

hypothesize that neuroleptics arrest the progression of some symptoms of schizophrenia must wait until the first clinical signs of schizophrenia are observed. Most people with schizophrenia have already experienced at least one or more psychotic episodes before seeking medical treatment (Wyatt et al., 1991a,b). Although the course of schizophrenia varies between patients, a short prodromal phase of general psychopathology (one to two years) generally precedes the onset of the illness, characterized by a non-specific signs of psychopathology such as social withdrawal, poor hygiene, and changes in sleep patterns. Some researchers would argue that this is the point where individuals at risk must be targeted for treatment (Parnas, 1999).

As stated by Waddington et al. (1997a): "One fundamental issue is the extent to which the new antipsychotics might shift materially the risk benefit balance towards intervention, not just at the earliest possible stage following the onset of psychosis but at a yet earlier, 'prodromal' phase of the disorder where there is a considerably greater likelihood of 'treating' behavioural disturbances that prove not to be the harbingers of psychotic illness". McGlashan (1998a,b) states that the urgency of treatment, however, comes from the belief that "it is likely these deficit processes begin some time before the first manifest signs and symptoms of illness in the prodromal phase and are time limited, diminishing in activity at or shortly after onset in the majority of cases" and that "currently these processes appear irreversible, but anecdotal experience with treatment in the very early phase of schizophrenia suggests that brain plasticity may be retained and that efforts at preventing deterioration should become a focus of active scientific clinical research". Since it is thought that this treatment must occur early in the active phase of the illness to prevent progression, it is this time point of neurodegeneration in schizophrenia that researchers should target, which makes the identification of vulnerability markers imperative (Matsuoka et al., 1999). Further studies are required to determine if such progression is specific to schizophrenia, or if it also characterizes other psychiatric disorders.

There are obvious ethical problems with testing such a theory (Falloon, 1992) since it is not acceptable to delay treatment in order to determine if patients will have additional long term brain damage. Mahadik and Scheffer (1996) and Mahadik and Gouda (1996b) propose that if schizophrenia is characterized by neurodegenerative processes involving oxidative stress, pretreatment in individuals with a high risk to develop schizophrenia may include the addition of vitamin E to their diets. In addition, it is not wise to initiate treatment with antipsychotics whose mechanisms of action are not fully understood unless it has been consistently demonstrated that these drugs will arrest some sort of neurodegenerative process.

Usually, negative and cognitive symptoms increase over time, while positive symptoms can lessen in severity (Ende et al., 2000). Not only do these findings suggest that some symptoms progress over the course of the illness, but they further demonstrate the heterogeneity of this disease. This would be supported by clinical observations that the degree, ratio, and type of positive, negative, and cognitive symptoms varies greatly among patients. Since the ratio of positive to negative symptoms varies among patients, people with a predominance of negative and cognitive symptoms may be at greater risk for further deterioration. Since cognitive decline is integral to many post pubertal neurodegenerative disorders such as Alzheimer's disease, Huntington's disease, and even Parkinson's disease (which also affect brain circuitry purported to be altered in schizophrenia), it would not be surprising if the cognitive deficits accompanying schizophrenia are also characterized by neurodegeneration. Arnold et al. (1995) found that "patients with marked cognitive deterioration over time showed more gliosis restricted to the subiculum and orbitofrontal cortex than did otherwise similar patients without such cognitive impairment", suggesting that there are specific subtypes of schizophrenia which are more likely to be characterized by neurodegeneration.

4.4 Limitations of present research

There were several limitations in the present studies, some of which included the use of tissue culture. Although cell culture is an excellent means by which to analyse the effects of drugs at a cellular level, it does not provide information about what treatments do in vivo. In addition, the animals used in these studies do not have the brain abnormalities present in schizophrenia, making it possible that the biocheminical substrates regulated by neuroleptics might only become apparent in the schizophrenic neuroanatomy. It is probable that massive upregulation of BDNF by neuroleptics would not be desirable, as increased seizure activity is associated with elevated BDNF mRNA and protein (Ernfors et al., 1991; Rocamora et al., 1992; Nawa et al., 1995; Salin et al., 1995), while decreased seizure suseptibilities are seen in BDNF-deficient, mutant mice (Kokaia et al., 1995). In addition, a low n (ranging between 3 and 4 for each culture condition or animal group tested) was used for the majority of the studies. A higher n may have led to more statistically significant results, and therefore the ability to draw more definite conclusions.

4.5 Suggestions for future research

Given the role of stress, estrogen, and NMDA receptor fuction (Mohn et al., 1994; Deutsch et al., 1997; Woolley; 1998) in both schizophrenia and the regulation of BDNF, it would be of interest to further examine these factors in the neuroleptic response. It is still unknown if neuroleptics could protect against stress-induced or estrogen withdrawal- induced decreases in BDNF mRNA. Studies need to be done to confirm that: a) stress- or estrogen withdrawal-induced decreases in BDNF mRNA cause neuronal damage or death, b) neuroleptics can prevent stress- or estrogen-induced decreases in BDNF mRNA, and that this action specifically leads to neuroprotection. Future studies will test the ability of neuroleptics to prevent stress-induced decreases in BDNF mRNA and additional markers of neuroprotection.

4.6 General conclusions

The causes, etiology, and treatment of schizophrenia are poorly understood. The relative D_2 to 5-HT_2 blocking ratios of neuroleptics seem to predict neuroleptic differences in efficacy and side effect profiles, but we found in our studies that this was not always the best way to characterize the molecular effects of typical vs. atypical neuroleptics.

As no common mechanism of action of all neuroleptics has been found to date, we had hoped that we would elucidate some mechanisms of action of neuroleptics that were both characteristic of all neuroleptics and that differentiated among the various classes of neuroleptics. We generally found that early immediate gene regulation correlated with D_2 blocking activity, and that the neuroprotective properties of neuroleptics were associated with doses that produced lesser D_2 blockade. We found out very little about the contribution of serotonergic blockade in the neuroleptic response at the molecular level, except to say that perhaps 5-HT_2 blockade upregulates BDNF. Future studies will be needed to truly understand the role of 5-HT_2 receptor blockade in the neuroleptic response.

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Appendix A: List of Chemicals and Reagents

Abbott Laboratories (Montreal, Quebec, Canada)
- sodium pentobarbital

Aldrich Chemical Company (Milwaukee, Wisconsin, USA)
- D.P.X. mounting medium

American Type Culture Collection (Rockville, Maryland, USA)
- PC12 cells

Amersham Phamacica Biotech (Baie d'Urfe, Quebec, Canada)
- ACS (Aqueous counting scintillant)
- dextran sulfate
- Hybond N Nylon paper

Astra Pharmaceutical Inc. (Mississauga, Ontario, Canada)
- remoxipride HCl

Baker (J.T.) Chemical Co. (Phillipsburg, NJ)
- CaCl2
- Triton X-100

Beckman Instruments (Mississauga, Ontario, Canada)
- polyallomer tubes

BDH (Toronto, Ontario, Canada)
- aristar alcohol
- chloral hydrate
- chloroform
- ethidum bromide
- formamide
- gelatin
- hydrogen peroxide
- isopentane
- potassium phosphate (monobasic)
- sodium chloride
- sodium hydroxide
- sucrose
- toluene
- ultraformamide
- xylene

Becton Dickinson Canada (Missisauga, Ontario, Canada)

- Falcon® 6 well plates

Falcon® cell culture flasks

Bio/can Scientific (Missisauga, Ontario, Canada)
glass milk
Gene Clean II® Kit

BIORAD (Missisauga, Ontario, Canada)
Biospin-30 chromatography columns

Boehringer Manhiem Canada (Laval, Quebec, Canada)
Hexanucleotide Mixture
Random Primed DNA Labelling Kit
SP6/T7 Transcription Kit

Charles River Canada (Montreal, Quebec)
Male Wistar rats

Colorado Serum Co. (Denver, Colorado, USA)
normal goat serum

Difco Laboratories (Detroit, Michigan, USA)
bacto-tryptone
bacto-yeast extract

Eastman Kodak (Rochester, New York, USA)
developer
fixer
NTB2 emulsion
octyl sodium sulfate

Eli Lilly
olanzapine

FISHER Scientific Co. (Nepean, Ontario, Canada)
chromium potassium sulfate
Melvin Freed microscope slides
Permount (metioned in thesis?)
potassium acetate
potassium chloride
Premium glass cover slips
sodium azide

FISHER/ Promega (Madison, Wiconsin, USA)
restriction endonucleases
T3 RNA polymerase
x-gal

Gibco BRL/ Life Technologies (Burlington, Ontario, Canada)
- agarose (ultra-pure)
- CsCl
- GITC (guanidium isothiocyanate)
- phenol

ICN Biomedicals, Inc. (Costa Mesa, California, USA)
- ampicillin
- DEPC (diethylpyrocarbonate)
- GITC (guanidium isothiocyanate)
- iodoacetamide
- N-ethyl Maleimide
- penicillin G, sodium salt
- streptomycin sulfate
- TEA (triethanolamine HCl)

International Biotechnologies (New Haven, Connecticut)
- ammonium acetate
- DTT (dithiothreitol)
- Tris HCl

Janssen Pharmaceutica
- haloperidol
- risperidone
- ritanserin

Mandel / New England Nuclear (NEN; Boston, MA, USA)
- [^{32}P] CTP
- [^{32}P] dCTP
- [^{35}S] CTP
- Reflection autoradiograph film NEF 496

Nalgene
- 0.2µ and 0.8µ Millipore filters

Novartis
- clozapine

PDI Bioscience
- Protienase K

Perkin-Elmer
- AmpliTaq
- DNTP's
- PCR buffer

PIERCE (Rockford, IL, USA)

Pharmacia Biotech. *(Uppsala, Sweden?)*
- DNAse I
- Nucleoside triphosphates (ATP, CTP, GTP, UTP)
- RNase A

Research Biochemicals Inc. (RBI, Natick, MA, USA)
- dextromethorphanhydrobromide

Santa Cruz
- FosB/ delta FosB antibody

Sigma Chemical Co. (St. Louis, MO, USA)
- 2-mercaptoethanol
- acetic anhydride
- agar
- ammonium acetate
- bovine serum albumin (BSA)
- bromophenol blue
- citric acid (trisodium salt dihydrate)
- collagen solution type I
- cresyl violet acetate
- DAB (3,3'-diaminobenzidine tetrahydrochloride)
- dextran sulfate
- dithiotreitol (DTT)
- EDTA
- fetal calf serum
- Ficoll
- formamide
- glucose
- glutamine
- heparin sulfate (sodium salt)
- horse serum
- kainic acid (2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine)
- MgCl2
- MOPS (3-(N-morpholino) propanesulfonic acid)
- NaCl
- NaOH
- N-lauryl-sarcoside
- paraformaldehyde (PFA)
- poly-l-lysine
- polyvinyl pyrrolidone
- potassium chloride
- potassium phosphate (monobasic)

salmon sperm DNA
sodium acetate
sodium phosphate
speridine trihydrochloride
sucrose
Tris
Trizma (tris base)

Vector Laboratories
ABC Vectastain Elite kit
avidin-biotin peroxidase complex
goat-anti rabbit 2° antiserum