Regulation of Gene Expression in the Basal Ganglia

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SUMMARY

Using in situ hybridisation histochemistry we have demonstrated that :

1. Enkephalin gene expression is modulated by the dopaminergic system in the rat striatum. Intraperitoneal administration of the typical neuroleptic drugs haloperidol (1mg/kg) and fluphenazine (3mg/kg) significantly increase levels of proenkephalin mRNA in the caudal striatum compared to saline treated controls, although the atypical neuroleptic drug clozapine (3mg/kg) has no effect on the levels of proenkephalin mRNA in the rat. Methiothepin (10mg/kg), an antipsychotic with the ability to antagonise both serotonin and dopamine receptors can also induce proenkephalin mRNA levels in rat striatum. This induction is observed twenty-four hours after the drugs are given.

2. Tachykinin gene expression is modulated by the dopaminergic system in the rat striatum. Intraperitoneal administration of the typical neuroleptic drugs haloperidol (1mg/kg) and fluphenazine (3mg/kg) significantly reduce the levels of preprotachykinin mRNA in the rat striatum, compared to saline treated controls. This effect is observed twenty-four hours after drug administration. The atypical neuroleptic drug clozapine (3mg/kg) and the antipsychotic methiothepin (10mg/kg) have no significant effect on the levels of preprotachykinin mRNA in the rat striatum.

3. Somatostatin gene expression in the rat striatum is significantly increased by low doses of the atypical neuroleptic drug clozapine (3mg/kg) and by the antipsychotic drug methiothepin (10mg/kg), after acute administration. Acute treatment with typical neuroleptics have no effect in the rat striatum. Similarly, acute treatment

with clozapine (3mg/kg) and methiothepin (10mg/kg) significantly increase the levels of preprosomatostatin mRNA in the rat nucleus accumbens. Chronic treatment with clozapine (6mg/kg) also significantly increases preprosomatostatin mRNA levels in the rat striatum and in the olfactory tubercle.

4. Neuropeptide Y gene expression in the rat striatum is not changed significantly following acute neuroleptic drug treatment.

5. Chronic treatment with the neuroleptics fluphenazine (6mg/kg) and clozapine (6mg/kg) significantly increases the levels of D1 dopamine receptor mRNA in the rat rostral striatum. Chronic treatment with the above has no effect on the levels of 5-HT6 receptor mRNA in the rat striatum.

6. Typical neuroleptics including haloperidol (1mg/kg) and fluphenazine (3mg/kg) can significantly induce the immediate early genes c-fos, zif/268, and jun-B in the rat striatum after 30-45 minutes. Haloperidol (1mg/kg), but not fluphenazine (3mg/kg), significantly induces jun-D mRNA in the rat striatum. Haloperidol (1mg/kg) can significantly increase c-fos and jun-B mRNA expression in the rat nucleus accumbens, while fluphenazine (3mg/kg) can significantly induce zif/268 and jun-B mRNA expression in the rat nucleus accumbens. The atypical neuroleptic, clozapine (3mg/kg) had no significant effects on the expression of c-fos, zif/268, jun-B, and jun-D in either the rat striatum or accumbens regions. However, the immediate early, gene brain factor-1 (BF-1), was significantly induced in the rat caudal striatum twenty-four hours after clozapine (3mg/kg) treatment. RHS2 gene expression was not affected by clozapine (3mg/kg) after twenty-four hours.

7. Striatal neurones in culture have a similar phenotype to striatal neurones in vivo. Cultured striatal neurones express the genes that encode enkephalins, substance P, somatostatin, and neuropeptide Y proteins similar to the in vivo situation, but not dynorphins. A small percentage of striatal neurones in culture are NADPH-positive and acetylcholinesterase-positive similar to the in vivo situation.

8. Dopamine (5μM) and SKF38393 (1μM), a D1/D5 receptor agonist, can significantly induce c-fos and zif/268 mRNA in cultured striatal neurones, compared to vehicle treated cultures. Maximal induction occurs after 45 minutes. This induction is blocked by SCH23390 (1μM), but not by eticlopride (1μM). The SKF38393-induced expression of both c-fos and zif/268 mRNA can be significantly reduced by pretreatment with the non-selective kinase inhibitor H7 (50μM) and with the selective protein kinase A inhibitor, KT5720 (2μM). The phorbol ester, 12-myristate-13-acetate (10^{-7}M) can significantly induce both c-fos and zif/268 mRNA after 45 minutes. The protein kinase C inhibitor, calphostin C (1μm), can significantly block the phorbol ester- induced expression of both c-fos mRNA and zif/268 mRNA. Calphostin C (1μM) can also significantly reduce the SKF38393- induced expression of c-fos mRNA, but is unable to block the SKF38393- induced expression of zif/268 mRNA. Quinpirole (1μM), a D2-like receptor agonist, can significantly induce c-fos mRNA in striatal cultures compared to control. The SKF38393- induced expression of both c-fos and zif/268 mRNA can be significantly reduced by coadministration of SKF38393 with quinpirole (1μM).

9. SKF38393 (1nM-5μM) can induce c-fos mRNA and zif/268 mRNA in a dose-dependent manner in striatal neurone cultures. Pretreatment with antisense oligonucleotides directed towards the D1 dopamine receptor and the D5 dopamine

receptor at a concentration of 1μM can significantly shift the dose response curves representing c-fos induction and zif/268 mRNA induction by SKF38393, in a downward direction. However, pretreatment with the D1 sense oligonucleotide (1μM) has no effect on the dose response curve representing c-fos induction by SKF38393 (1nm-5μM). Although the D1 sense oligonucleotide caused a significant downward shift of the curve representing zif/268 induction by SKF38393 (1nM-5μM).

10. Serotonin (5-HT) (1nM-5μM) can significantly induce both zif/268 and c-fos mRNA in a dose-dependent manner in striatal neurone cultures. Pretreatment with ondansetron (200nM), a 5-HT3 receptor antagonist, can block the 5-HT-induced expression of zif/268 mRNA , and to a lesser extent c-fos mRNA. Pretreatment with the 5-HT1/2 receptor antagonists cyproheptadine (500nM), methiothepin (200nM), mesulergine (30-100nM) and spiperone (30-100nM) can block the 5-HT- induced expression of both zif/268 and c-fos mRNA. The 5-HT2C receptor agonist m-CPP (1nM-5μM) had no effect on the expression of zif/268 or c-fos mRNA. Similarly 8-OH-DPAT (1nM-5μM), the 5-HT1A receptor agonist, had no significant effects on either zif/268 or c-fos expression in striatal cultures. Administration of the 5-HT2A receptor agonist ± DOI (1nM-5μM) had no significant effect on the expression of zif/268 and c-fos mRNA in cultured striatal neurons.

Abbreviations

AchE	Acetylcholinesterase
GABA	γ-amino butyric acid
NPY	Neuropeptide Y
-ir	Immunoreactive
met-Enk	methionine enkephalin
cAMP	cyclic adenosine monophosphate
MPTP	1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine
NMDA	N-methyl-D-aspartate
PKA	protein kinase A
PKC	protein kinase C
DAG	Diacylglycerol
IEG	Immediate early gene
IP3	Inositol triphosphate
CREB	cAMP-response element binding protein
mRNA	messenger ribonucleic acid
EMEM	Earle's modified eagles medium
HBSS	Hank's balanced salts solution
NSE	neurone specific enolase
GFAP	glial fibrillary acidic protein
NGS	normal goat serum
PBS	phosphate buffered saline

Chapter 1

INTRODUCTION

1.1 Anatomical Organisation of the Basal Ganglia

The basic circuitry of the basal ganglia is classically known as being composed of multiple intrinsic loops through which most of the key structures of the system are reciprocally linked. As early as the 1970's it was proposed that the structures within the basal ganglia functioned as integrative centres, chanelling information from different origins. The basal ganglia form a forebrain system that affects signals from a large part of the neocortex and focusses the output of this integrated information to target regions of the frontal lobe and brainstem involved in aspects of motor planning and motor memory.

The basal ganglia comprise a number of structures including the striatum, which is the largest structure in the basal ganglia, the substantia nigra, the globus pallidus, the subthalamic nucleus and the entopeduncular nucleus. The striatum, which itself comprises caudate nucleus, putamen and ventral striatum, receives an excitatory innervation from the substantia nigra pars compacta and dorsal raphe nucleus that is primarily dopaminergic and serotoninergic respectively (Graybiel and Ragsdale., 1978), and also an excitatory glutamatergic innervation from specific cortical areas (Spencer., 1976; Divac et al., 1977). In contrast, the striatum projects massively only to the globus pallidus, the substantia nigra pars reticulata and to the entopeduncular nucleus.

Immunocytochemical studies have revealed an inner complexity to the structure of the striatum. Biochemically specialised compartments were first detected in sections stained for acetylcholinesterase (AchE) activity (Graybiel and Ragsdale., 1978). Small AchE-poor regions referred to as striosomes or "patches" were embedded in an AchE-rich tissue of the striatum, which was

later referred to as the extrastriosomal matrix. In time, it was found that there were differences in the connections of striosomes and matrix compartments to the internal circuit within the basal ganglia, thus making functional differences between the striatal compartments likely. The matrix compartment recieves the striatal inputs most directly related to sensorimotor processing. By contrast, striosomal compartments tends to recieve inputs from neural structures, affiliated with the limbic system (Donoghue and Herkenham., 1986). Similarly the efferent projections from different striatal compartments show functional organisation. This mosaic pattern is presumably important for efficient and selective information processing by allowing a specific chemical environment to exist.

It has previously been suggested that the rat basal ganglia have a similar structural make-up to the human basal ganglia, and the organisation of connections within the rat basal ganglia have been shown to be remarkably similar to those in the human basal ganglia. Therefore the rat has been used in recent years as an adequate model for experimental research on basal ganglia function (Parent et al., 1991).

1.2 Neurotransmitters and Neuropeptides in the Basal Ganglia

The basal ganglia contain a remarkable diversity of neuroactive substances, organised into functional subsystems that have unique developmental histories and vulnerabilities in neurodegenerative diseases.

Approximately 95% of striatal neurons in vivo are thought to be GABA-ergic projection neurons. Coexistence of GABA with neuropeptides in

various combinations in the principle circuits and interneurons of the basal ganglia, allows different combinations of cortical inputs, when projected into the basal ganglia, to be channeled not only into different output pathways, but also into different chemical environments.

In vivo, GABA-ergic projection neurons have been demonstrated to contain either enkephalin peptides or dynorphin and tachykinin peptides (Bolam et al., 1985; Graybiel., 1990; Besson et al., 1990). The remainder comprise possibly three populations of interneurons releasing acetylcholine, or GABA and neuropeptide Y (NPY), or somatostatin, NPY and nitric oxide (Bolam et al., 1984; Phelps et al., 1985; Smith and Parent., 1986; Graybiel, 1990; Vuillet et al., 1990). Different neuronal populations have been demonstrated to project to different areas of the basal ganglia. It has been shown previously, with the use of retrograde tracing techniques, that the majority of neurons expressing enkephalins project to the globus pallidus and few to the substantia nigra. In contrast, neurons expressing dynorphin and substance P primarily project to the substantia nigra, with only small projections to the globus pallidus (Graybiel, 1986; Besson et al., 1990). It has also been reported that there are differences in the abundance of mRNAs encoding neuropeptides contained in the same population of cells. For example, protachykinin mRNA appears to be considerably more abundant than prodynorphin mRNA (Young et al., 1986), although both are located in striatoentopeduncular and striatonigral projection neurons. Similarly, somatostatin mRNA appears to be much more abundant than neuropeptide Y mRNA (Morris, 1989), although they are coexisting in the same cells. Protachykinin mRNA and prodynorphin mRNA show some indication of being preferentially located in the "patch" compartment of the striatum (Gerfen and Young, 1988; Morris et al., 1989).

In vitro, there is only limited evidence to suggest what classes of neurons exist in the striatum. However, extracts of primary striatal cultures have been shown to contain immunoreactive (ir) somatostatin and substance P

(Kessler, 1986) and proenkephalin mRNA (Schwartz and Simantov, 1988; Vilijn et al., 1988), while analysis at the cellular level has shown the presence of GABA (Messer, 1981; Weiss et al., 1986; Surmeier et al., 1988), along with ir-leu-enkephalin (Surmeier et al., 1988) which could arise from the expression of either the prodynorphin gene or the proenkephalin gene and ir-somatostatin (Williams et al., 1991). Of these, only GABA, ir-leu-enkephalin and ir-somatostatin have been localised to neuronal cells rather than glia (Surmeier et al., 1988; Williams et al., 1991). Therefore, it remains unclear to what extent the other neuroactive substances detected in culture extracts are derived from non-neuronal cells.

1.3 Characterisation of Dopamine receptors

1.3.1 Molecular Biology

The dopaminergic system relies on the interaction of dopamine with several receptors. Dopamine can either inhibit or excite neurons (Bloom et al., 1965; McLennan and York., 1976). In 1979, Kebabian and Calne characterised two classes of dopamine receptor namely the D1 and D2 receptors. The concept of two distinct types of dopamine receptors mediating these two electrical effects (Cools and Van Rossum., 1976) was strengthened when it was found that the inhibition or excitation by dopamine could be selectively blocked (Struyker Boudier et al., 1974; Heiss and Hoyer., 1974). These two receptors were found to exert their biological actions by coupling to, and activating

different G-protein complexes. The D1 dopamine receptor interacts with G_s complexes resulting in the activation of adenylyl cyclase and an increase in intracellular cyclic AMP levels (Kebabian and Greengard., 1971). The D2 dopamine receptor interacts with G_i complexes to inhibit cyclic AMP production and thus decrease intracellular levels (De Camilli et al., 1979).

Over the past decade the introduction of new molecular biological techniques have enabled the dopaminergic receptors to be cloned. The D2 receptor was originally cloned using a low stringency screening approach of rat complementary DNA libraries (Bunzow et al., 1988). However the polymerase chain reaction approach was used originally to clone the D1 receptor from rat striatum (Zhou et al., 1990; Sunahara et al., 1990) and from mouse neuroblastoma cells (Monsma et al., 1990). The low stringency approach to cloning the D1 receptor also proved to be successful (Dearry et al., 1990).

Molecular cloning of G-protein linked receptors advanced rapidly with three further members of the dopamine receptor family being cloned, namely the D3, the D4 and the D5 dopamine receptors. The D3 receptor was originally identified using a DNA fragment of the D2 receptor as a probe under low stringency hybridisation conditions (Sokoloff et al., 1990; Giros., 1990). When expressed in eukaryotic cells this receptor was shown to bind D2, but not D1 ligands. By analysing mRNA's of a human cell line with D2 receptor complementary DNA (cDNA) probes, another D2-related mRNA was detected. The corresponding cDNA and gene analysis lead to the characterisation of the D4 receptor (Van Tol et al., 1991). The D4 receptor when expressed in COS-7 cells, binds D2 antagonists with a pharmacological profile distinct from, but reminicent of that of the D2 receptor. The G-protein coupled D4 receptor has also been demonstrated to be coupled to adenylate cyclase in the mouse retina (Cohen et al., 1992).

Finally the D1 dopamine receptor clone was used as a hybridisation probe to identify D1-related genes. A human D5 and a rat D1B receptor were

subsequently characterised (Zhou et al., 1990; Sunahara et al., 1991; Grandy et al., 1991; Tiberi et al., 1991; Weinshank et al., 1991). The genes encoding for D5 and D1B receptors are intronless, unlike the genes encoding for the D2, D3 and D4 receptors, which are known to contain introns. The D5 and D1B display the same pharmacological profile, similar to that of the D1 receptor and are able to stimulate adenylyl cyclase activity.

1.3.2 Dopamine Receptor Ontogenesis

The recent development of molecular biology techniques such as in situ hybridisation (Le Moine et al., 1991) and Northern blot analysis (Jaber et al., 1992) have been used to study the localisation and regulation of dopamine receptor gene expression in rat and human brain. Using in situ hybridisation , it has recently been shown that the mRNA for the two isoforms of the dopamine D2 receptor, are detectable from gestational day 14 (G14) in the rat striatal primordium. At day 16 , D2 receptor mRNA was present in the lateral part of the striatum and in the germinal ventricular zone lining the lateral ventricle. At day 18, D2 receptor mRNA was found in neurons of the caudate-putamen, the nucleus accumbens, the olfactory tubercle and the subependymal zone lining the lateral ventricle. The labelled cells were shown to have a neuroblastic and immature aspect before birth. After birth the topography and aspect of labelled cells was similar to the one observed in the adult animals (Guennoun and Bloch, 1991). A more sensitive method of detection known as reverse transcriptase polymerase chain reaction has recently been used to show D2 mRNA prenatally (Mack et al., 1991). This method has been used to detect D1,

D1B and the D3 mRNA. The D1 receptor mRNA was detectable at gestational day 11 (G11) in the rat forebrain. The hybridisation signal remained relatively faint until G18 when it took on a more intense signal (Cadoret et al., 1993). By gestational day 19, the D1 receptor mRNA was detectable by in situ hybridisation methodology (Guennoun and Bloch, 1992). The presence of D3 receptor mRNA was clearly detectable from G14 to G20 (with a maximum at G15), even though weak signals were observed as early as G11 (Cadoret et al., 1993). The rat D1B receptor mRNA was detectable from gestational day 12, but the signal remained weak until G18-20 (Cadoret et al., 1993). At present, the ontogenesis of the D4 receptor is not fully characterised.

The presence of dopaminergic markers has been reported at very early stages of brain development. Indeed, tyrosine hydroxylase and dopamine have been detected in the rat striatum at G14 (Foster et al., 1987) before the full maturation of the dopaminergic system. It has also been suggested that the development of dopaminergic receptors is dependent on the development of the presynaptic nerve terminals containing the transmitters (Deskin et al., 1981). Increasing evidence has shown that the dopamine receptors can be functional at very early stages of development. In avian retina embryonic cells in vitro, it has been demonstrated that stimulation of early dopamine receptors greatly inhibits the motility of the neuronal growth cone via a functional D1-type system (Lankford et al., 1988). The D2 receptor has previously been shown to be modulated by guanylnucleotides as early as gestational day 15 (G15), suggesting that these receptors were already functionally coupled to a regulatory G-protein (Sales et al., 1989). Furthermore, presynaptic D2 receptors appear to be functional as soon as dopamine release from striatal slices can be detected (De Vries et al., 1992). Therefore the evidence to date suggests that the chemical environment (i.e. neurotransmitters) acts as a regulator of neuronal growth during development (Lankford et al., 1988; Mattson , 1988).

1.3.3 Tissue Distribution of Dopamine Receptors

The distribution of the different dopamine receptor subtypes have revealed some important differences that may underlie functional differences. Since tissue distribution of the different dopamine receptors overlap in the central nervous system some selectivity may be obtained quantitatively rather than qualitatively. It has been shown using blotting analysis that the D3, D4 and D5 mRNAs are one to two orders of magnitude lower in abundance than D1 or D2 mRNAs (Sokoloff et al., 1990; Van Tol et al., 1991; Tiberi et al., 1991). The anatomical distribution of both D1 and D2 subtypes is such that mRNA for both subtypes exists in high levels in the caudate-putamen, nucleus accumbens and the olfactory tubercle (Meador-Woodruff et al., 1991). However, high levels of D2 mRNA, but not D1 mRNA are present in the substantia nigra and ventral tegmental area, whereas conversely the amygdala contains predominantly D1 mRNA. The distribution of D5 receptor mRNA was first reported to overlap the D1 mRNA (Sunahara et al., 1991), but recent studies have subsequently conflicted with this view. It has since been found that D5 mRNA is present only in the hippocampus, hypothalamus and the parafasicular nucleus of the thalamus (Tiberi et al., 1991; Meador-Woodruff et al., 1992).

The newly cloned D2-like receptors, the D3 and the D4 subtypes have also been shown to have some unique tissue distribution. The distribution of D3 receptor gene transcripts in rat brain has been shown to be markedly different from that of the D2 receptor gene transcripts. A weak hybridisation signal has been observed in restricted parts of the striatum, although D3 receptor mRNA was found to be highly expressed in the olfactory tubercle-island of Calleja complex, the bed nucleus of stria terminalis and in the nucleus accumbens (Sokoloff et al., 1990; Levesque et al., 1992; Sokoloff et al., 1992).

The expression of the D4 mRNA was shown to be relatively high in the monkey frontal cortex, midbrain area, amygdala and medulla, but lower levels were detected in the basal ganglia (Van Tol et al., 1991). In the rat, significant levels of expression were found in the hypothalamus, thalamus, olfactory bulb and frontal cortex. However, 20-fold higher levels of D4 mRNA were observed in the cardiovascular system (O' Malley et al., 1991). The D4 dopamine receptor transcripts have also been observed in the mouse retina (Cohen et al., 1992).

1.3.4 Cellular Localisation of Dopamine Receptors

The cellular localisation of dopamine receptors is currently a matter of much controversy. At present there are two schools of thought concerning this issue as some research groups support segregation of D1 and D2 dopamine receptors in the striatum (Gerfen et al., 1990; Le Moine et al., 1990; Gerfen, 1992; Le Moine et al., 1992), whilst other groups hold to the idea that D1 and D2 dopamine receptors occur on the same cell (Seeman et al., 1989; Bertorello et al., 1990; Piomelli et al., 1991; Surmeier et al., 1993).

Research supporting segregation of D1 and D2 dopamine receptors in the striatum is based on studies performed in intact rats, and thus the segregation is thought not to be a consequence of denervation of dopaminergic nerves. Stimulation of D1 and D2 receptors has been shown to preferentially affect the function of striatonigral and striatopallidal neurons, respectively, in the intact striatum. Treatment with D2 antagonists rapidly induces expression

of the immediate early gene c-fos specifically in striatopallidal neurons (Dragunow et al., 1990; Robertson et al., 1992). Conversely, indirect dopamine receptor agonists result in D1-mediated changes in the mRNA and protein levels of immediate early genes (Cole et al., 1992; Beretta et al., 1992; Cenci et al., 1992; Steiner et al., 1993) predominantly in striatonigral neurons. The effects of dopamine depletion on striatal neurons are also thought to be consistent with differential localisation of D1 and D2 receptors on striatonigral and striatopallidal neurons. Enkephalin mRNA and protein, which are predominantly localised to striatopallidal neurons (Gerfen and Young, 1988), are elevated by this depletion (Young et al., 1986; Gerfen et al., 1990; Li et al., 1990; Gerfen et al., 1991; Engber et al., 1992). Conversely in striatonigral neurons, mRNA and peptide levels of substance P and dynorphin are decreased (Young et al., 1986; Gerfen et al.., 1990; Li et al., 1990; Gerfen et al., 1991). The levels of D1 and D2 receptor mRNA, together with receptor binding, are decreased and increased respectively (LaHoste et al., 1991., Joyce, 1991; Blunt et al., 1992). The lesion-induced alterations of peptide and D1 receptor mRNAs in striatonigral neurons are reversed by treatment with D1-selective agonists, whereas the alterations in peptide and D2 receptor mRNAs in striatopallidal neurons are reversed by D2 receptor agonists (Gerfen et al., 1990; Engber et al., 1992). In situ hybridisation studies have also demonstrated that D1 and D2 receptors are expressed preferentially in one of two populations of striatal efferent neurons, substance P-containing and enkephalin-containing neurons respectively, with little or no overlap of expression (Le Moine et al., 1990; Gerfen et al., 1990; Le Moine et al., 1991).

Alternatively the hypothesis for colocalisation of dopamine receptors is also strongly supported. On the basis of sensitive RNA detection techniques, current recordings obtained from single cells in vitro (Surmeier et al., 1992) and immunohistochemical data (Ariano et al., 1992), Surmeier and colleagues have suggested that D1, D2 and D3 receptors are largely colocalised in most

striatal efferent neurons, especially striatonigral neurons. Using immunohistochemical techniques, the D2 receptor staining pattern in both striatonigral and striatopallidal neurons has been observed by several research groups (Ariano et al., 1993; Boundy et al., 1993). Other immunocytochemical work relevant to the issue includes localisation of DARPP-32 which is strongly correlated with D1 receptor binding in both striatonigral and striatopallidal neurons. Recently, other signalling elements in the D1 receptor pathway have been found in both striatonigral and striatopallidal neurons. It has been shown that most medium spiny neurons are immunoreactive for a particular G-protein α-subunit that mediates D1 receptor activation of adenylate cyclase in the neostriatum (Herve et al., 1993). Some of the in vitro research supporting colocalisation has been argued against, because of the possible alterations in cellular environment affecting mRNA levels. However there has been much research performed in vivo supporting the convergence theory, including peptide expression assays (Haverstick et al., 1989; Morris and Hunt., 1991; Angulo, 1992) and electrophysiological studies (Hu et al., 1990). Ultimately, further research and more sensitive techniques are necessary in order to answer the question of dopamine receptor localisation.

1.4 Drug Pharmacology of Dopamine Receptors

1.4.1 D1-Like Receptors

The sensitivities of the cloned D1 and D5 receptors to antagonist drugs are very similar and are also generally the same as those D1 receptors in native tissue (Seeman and Van Tol, 1994). It has been previously shown, with the use of binding studies in COS-7 cells, that the compound SCH23390 is a very potent antagonist, producing Ki values of 0.35nM and 0.30nM at D1 and D5

receptors respectively. Fluphenazine was shown to have some antagonistic properties at the D1 and D5 receptors, although much less than SCH23390 (Sunahara et al., 1991).

For dopamine receptor agonists, however, it has proved difficult to obtain accurate comparisons of the agonist sensitivities of the cloned receptors with those in native tissues. This is because dopamine receptors in native tissues can readily adopt either a high-affinity state or low-affinity state for an agonist, while cultured cells vary in their ability to reveal the high affinity state. COS-7 cells, for example, do not have sufficient or appropriate G-protein subunits to allow the high affinity state of a receptor to exist. Agonist drugs such as SKF38393, R-fenoldopam, and (-) apomorphine have a very high affinity for the D1-like receptors. However, an important consistent difference between the D1 and the D5 receptors is that dopamine itself is about ten times more potent at the D5 receptor (Sunahara et al., 1991; Seeman and Van Tol, 1994).

1.4.2 D2-Like Receptors

Accurate values for many of the agonist potencies at D2, D3, and D4 receptors are not currently available. Nevertheless, some important selectivities are clearly emerging. For example, bromocriptine is about two orders of magnitude more potent at D2 and D3 receptors than at D4 receptors (Seeman and Van Tol, 1993). Moreover, 7-hydroxy-DPAT is approximately tenfold more potent at D3 receptors than at D2 receptors (Seeman and Van Tol, 1993). Quinpirole also has a relatively high affinity for all D2-like receptors (Van Tol et al., 1991; Seeman and Van Tol, 1994).

In the case of antagonists acting at D2-like receptors, haloperidol and fluphenazine have relatively high affinity for all D2-like receptors. Clozapine has been shown to be one to ten orders of magnitude more potent at D4 receptors than at D2 or D3 receptors (Van Tol et al., 1991; Seeman and Van Tol, 1994). With the use of comparative binding studies, the density of D4 receptors in the human striatum has been shown to be approximately 10% of the total population of the D2-like receptors (Seeman et al., 1993). Methiothepin has also been shown to block dopaminergic receptors (Enjalbert et al., 1978; Lloyd and Bartholini, 1974). A small displacement of ^{3}H-methiothepin was observed following treatment with high concentrations of haloperidol, a dopaminergic receptor antagonist, suggesting that part of bound ^{3}H-methiothepin is also associated with dopaminergic receptors (Nelson et al., 1979).

1.5 Basal Ganglia Dysfunction- The Clinical Implications

1.5.1 Parkinson's Disease

Parkinson's disease was first characterised by James Parkinson in 1817 as a late onset disease manifested as tremor, ridgitity, postural abnormalities and bradykinesia. By 1960, Ehringer and Hornykiewicz reported that these abnormalities were accompanied by a neurochemical change taking the form of a loss of dopamine in the corpus striatum. In the early eighties Parkinson's disease was referred to as a neurodegenerative disease characterised by the loss of dopaminergic neurons from the pigmented substantia nigra (Forno, 1982).

A considerable amount of research has been focussed on basal ganglia function. Animal studies, together with human postmortem analysis have provided a much greater understanding of the pathophysiological basis for Parkinson's disease. The basic observation of decreased striatal dopamine in patients with Parkinson's disease was mimicked in the laboratory with chemicals such as reserpine, the neurotoxins 6-hydroxydopamine, which causes a permanent lesion of dopaminergic projections to the striatum (Ungerstedt , 1971) resulting in severe neurological impairment in rats reminiscent of several symptoms of Parkinson's disease (Hornykiewicz and Kish, 1987), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administered in primates which produces classic Parkinsonian-like symptoms thought to be due to the build up of a toxic metabolite (Langston, 1985). Further work revealed that dopamine depletion in rat brain via lesions increased dopamine turnover in the remaining terminals as a result of neuroplastic changes in the striatum (Zigmond et al., 1984). Moreover, recent evidence indicates that this neurochemical response is associated with the maintenance of the extracellular dopamine concentration at near normal levels despite the loss of dopamine terminals (Robinson and Wishaw, 1988; Zhang et al., 1988). Disruption of dopaminergic transmission with the use of dopamine antagonist drugs such as spiroperidol in rats previously lesioned, has been shown to result in significantly impaired behaviour patterns, but no effects were observed on intact rats given the same dose (Heffner et al., 1977). An increase in the spontaneous firing rates of striatal cells have also been associated with dopamine depletion, suggesting that the dopamine neurons exert an inhibitory influence on striatal activity (Orr et al., 1986).

A standard treatment for Parkinson's disease sufferers involves administration of levodopa, which is taken up by nerve terminals where it is decarboxylated to dopamine. The dopamine then activates receptors in the region of the depleted striatum and substantia nigra (Kandel and Schwartz,

1985; Gilman et al., 1985). Several studies have also demonstrated that dopaminergic D2 receptor agonists such as apomorphine can produce locomotion and stereotyped behaviour in lesioned animals when administered at dose levels which were shown to be ineffective in normal rats (Hollister et al., 1974; Kelly et al., 1975). This lead to the suggestion that dopamine receptors became "supersensitive" after lesioning (Hollister et al., 1975). The indirect dopamine receptor agonist amphetamine is also known to enhance locomotor activity at low doses, whilst producing repetitive stereotyped behaviour at high doses. These two aspects of animal behaviour have been attributed to separate neural systems, such that dopamine in the caudate is essential for stereotyped behaviour and dopamine in the nucleus accumbens septi is linked to an enhanced locomotor activity following amphetamine administration, after a 6-hydroxydopamine lesion (Kelly et al., 1975). Amphetamine has been shown to cause the release of dopamine from the caudate-putamen (Ungerstedt, 1971; Voigtlander and Moore, 1973).

Dopamine-containing neurons of the substantia nigra pars compacta are known to release dopamine from both the terminals in the striatum and from the dendrites in the substantia nigra pars reticulata (Cheramy et al., 1981). When dopamine neurons degenerate as in Parkinson's disease, or are destroyed by neurotoxins, the D2 dopamine receptors in the substantia nigra pars compacta disappear because the D2 receptors are almost entirely confined to dopamine neurons (Quik et al., 1979; Murrin et al., 1979). However, D1 receptor density in the substantia nigra pars reticulata, which is as high as the D1 receptor density in the striatum (Boysen et al., 1986), is unaffected by such dopamine neurotoxin lesions because the D1 receptors are presynaptic and are located on the terminals of descending striatonigral fibres (Savasta et al., 1986). Supersensitivity of dopamine D1 receptors has been demonstrated in the absence of dopamine stimulation (Savasta et al., 1986). Electrophysiological studies have also suggested that D1 dopamine receptor stimulation decreases

cell firing rates in the substantia nigra pars reticulata, presumably by increasing release of the inhibitory transmitter GABA from the terminals of the striatonigral fibres (Weick and Walters, 1987). This has also been demonstrated in nigral slice studies in vitro (Aceves et al., 1991). It has since been suggested that a D1-D2 synergy exists, in which dopamine plays a central role as both a regulator and effector (Robertson, 1992).

A reduction in peptide levels in Parkinsonian basal ganglia has also been reported (Agid and Javoy-Agid, 1985). A decrease in methionine-enkephalin (met-Enk) and leucine-enkephalin has been observed in the putamen and in both segments of the globus pallidus (Taquet et al., 1983, 1985) indicating that these neuronal systems are altered in the disease. The level of the neuropeptide substance P has also been shown to decrease in the lateral globus pallidus in Parkinson's disease patients (Mauborgne et al., 1983).

The interaction between dopamine and other neurotransmitters in the basal ganglia, in particular the dopamine-glutamate interaction, has been implicated in neurotoxicity at the nigrostriatal dopamine terminals. Stimulation of excitatory amino acid receptors of the N-methyl-D-aspartate (NMDA) subtype has been shown to enhance dopamine release (Snell and Johnson, 1986; Clow and Jhamandas, 1989) and synthesis (Arias-Montano et al., 1992) within the striatum. In the dopamine-depleted striatum, an elevation of NMDA receptor binding was shown and was found to be greatest in the dorsal caudate and putamen, regions that were found to have extensive loss of dopamine transporter sites (Weihmuller et al., 1992). Similar findings of a relationship between reduced striatal dopamine content and increased binding to NMDA receptors have been reported in the 6-hydroxydopamine treated rat model of Parkinson's disease (Samuel et al., 1990).

It has therefore been proposed that a decreased dopamine activity in the striatum of Parkinson's disease patients will, through direct and indirect basal ganglia pathways, release the substantia nigra pars reticulata and

entopeduncular neurons from their inhibitory controls, due to a lack of dopaminergic excitation from the substantia nigra pars compacta. Consequently thalamic targets of the substantia nigra pars reticulata and entopeduncular nucleus will be inhibited, leading to a diminished thalamocortical activation. This reduced cortical activation is thought to result in a decreased activity of striatocortical neurons and thus reduced striatal glutamate release. Thus the overall effect of dopamine loss in the striatum is inhibition of cortically initiated movement, to cause akinesia, hypokinesia, and bradykinesia. As a consequence, upregulation of NMDA receptors within striatal target neurons may occur as a compensatory mechanism (Weihmuller et al., 1992). In coordination with this result, chronic NMDA receptor antagonism can lead to increased receptor sensitivity (Norman et al., 1990).

1.5.2 Schizophrenia

The discovery of neuroleptics in 1952 (Delay et al., 1952) initiated research seeking a possible biological basis for schizophrenia. Various hypotheses have been proposed and are currently under investigation.

The dopamine hypothesis of schizophrenia proposes that dopamine pathways are overactive in the disease state (Van Rossum, 1967; Matthysse, 1973). Much of the evidence for this hypothesis is based on the fact that neuroleptic drugs block dopaminergic D2 receptors (Seeman et al., 1975; Creese et al., 1976). This action of neuroleptics is thought to account for neuroleptic-induced Parkinsonism and hyperprolactinaemia. Additional evidence for a dopaminergic basis for schizophrenia comes from the exacerbating effects of dopamine-mimetic drugs (Seeman, 1987; Lieberman, 1987) and the elevation of dopamine D2 receptors in the post-mortem brains taken from schizophrenic patients (Cross et al., 1981; Joyce et al., 1988;

Reynolds et al., 1988; Seeman and Niznik, 1990). The density of D1 dopamine receptors remains normal in schizophrenia (Seeman et al., 1987; Seeman and Niznik, 1990).

A different approach to understanding the pathophysiology of schizophrenia is provided by use of phencyclidine, which can be used to induce a syndrome of both negative and positive schizophreniform symptoms. The behavioural effects of phencyclidine are due at least in part, to its ability to block glutamate function via the NMDA receptor (Javitt and Zukin, 1991). Reported alterations in glutamatergic synaptic markers, particularly of frontal cortex in schizophrenic patients (Nishikawa et al., 1983; Deakin et al., 1989) may provide neurochemical evidence for a dysfunction of this transmitter system. Hence it has been suggested that drugs effective in alleviating the symptoms of schizophrenia that do not respond to classical antipsychotics may do so by interaction with glutamate systems. Recent evidence has emerged showing human cortical pyramidal neurons to be enriched with 5-HT1A receptors (Bowen et al., 1992). This provides a potential receptor mechanism for the modulation of cortical glutamatergic neurons, raising the possibility that an interaction with 5-HT1A receptors may contribute to the restoration of normal glutamatergic function after neuroleptic drug administration.

1.6 Second Messenger Pathways induced by Dopamine Receptor Stimulation

Cori and co-workers made the initial discovery that protein phophorylation was essential for all forms of signal transduction (Cori and Cori., 1945). In the mid 1950's Sutherland and his associates contributed to this finding by discovering that cyclic AMP was the "second messenger" involved in the transmission of effects of noradrenaline in the liver, and later demonstrated

that cyclic AMP caused the transfer of phosphate groups between key proteins (Robison and Sutherland., 1971).

The ability of dopamine receptors to induce different second messenger pathways has thus far been studied in D1 and D2 receptors (Vallar et al., 1990). The D1 and D2 dopamine receptors induce two types of signal transduction pathway, one obligatory and several cell specific. In D1 or D2 receptors the obligatory pathway is detected in every cellular environment and represents stimulation or inhibition of adenylyl cyclase, respectively. However, dopamine can also induce additional and sometimes different signal transduction pathways depending on the cell type.

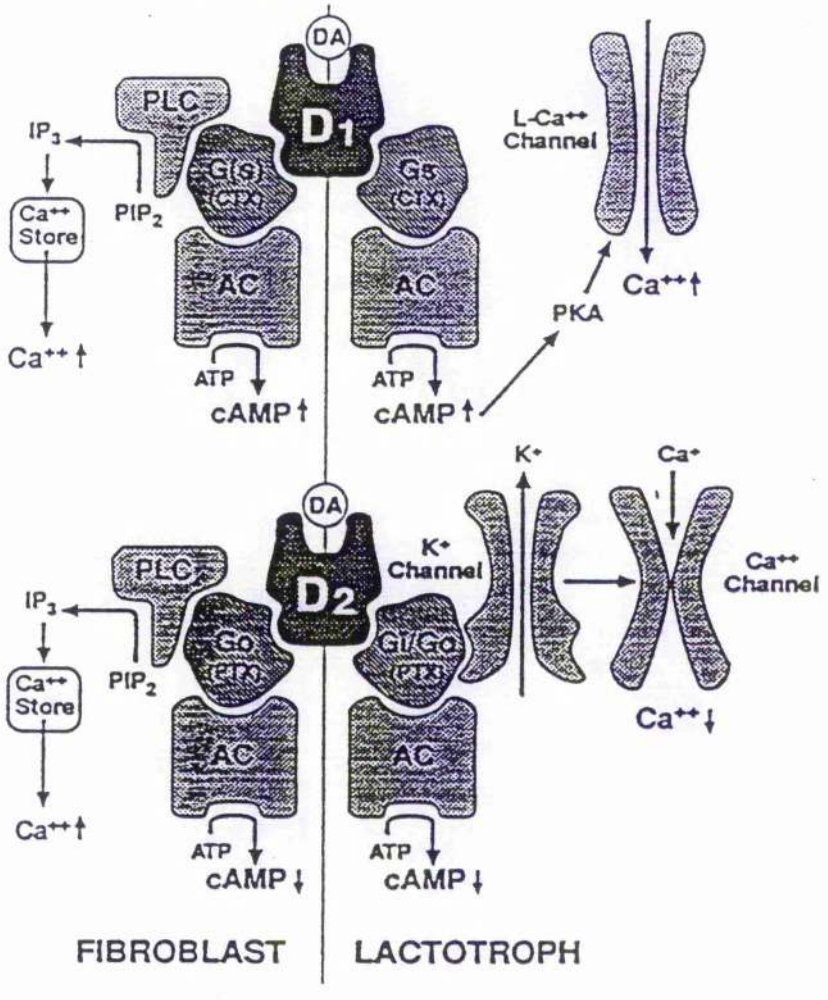


Figure 1 : Signalling pathways of the D1 and D2 dopamine receptors in the mouse fibroblast Ltk-and the rat somatomammotroph GH4Cl cells. DA represents dopamine, CTX and PTX means cholera toxin- and pertussis toxin-sensitive, respectively. G = G protein; PLC = phospholipase C; AC = adenylyl cyclase; PIP2 = phosphoinositol bisphosphate; IP3 = inositol triphosphate. The direction of arrows indicates whether the second messenger increases or decreases.

In every cell studied, stimulation of the D1 dopamine receptor increases cyclic AMP production. In addition, in GH4C1 cells, the D1 receptor potentiates activation of L-type voltage-dependent calcium channels in a cyclic dependent manner (Civelli et al, 1993) The D1 receptor has also been associated with an increased hydrolysis of phopholipids and calcium mobilization in xenopus oocytes (Mahan et al., 1990). The D2 receptor, while inhibiting adenylyl cyclase activity, does not affect phosphoinositide hydrolysis in GH4C1 cells and causes a decrease in intracellular free calcium mediated by a hyperpolarizing effect, mostly due to activation of potassium channels. (Figure 1) However, in Ltk-cells, activation of the D2 receptor leads to an increase in intracellular free calcium partly due to release of calcium ions from intracellular stores following the rapid stimulation of phosphoinositide hydrolysis and , in part, to influx from extracellular medium (Gatti et al., 1991). In addition, in chinese hamster ovary (CHO) cells, D2 receptors have been shown to mediate the potentiation of arachidonic acid release by a mechanism that involves protein kinase C, and is independent of adenylyl cyclase inhibition (Kanterman et al., 1990).

Activation of kinase enzymes prior to protein phosphorylation and the resultant physiological effect has proved to be an important target for the understanding of receptor mediated events. It has become clear that a great many different protein kinases exist (Hunter, 1987). These kinases can be broadly divided into two large groups, the protein seryl or threonyl kinases and the protein tyrosyl kinases, based on their specificities towards the different amino acids in the protein (Krebs, 1985). A given kinase may phosphorylate a number of different proteins, however it usually recognises a short sequence of amino acids referred to as a 'consensus sequence'. This was first demonstrated by work on the cyclic AMP-dependent protein kinase (PKA) (Kemp et al., 1975; Humble et al., 1975; Kemp et al., 1976). Using synthetic peptides the consensus substrate sequence for PKA was defined (

Kemp et al., 1977). It was concluded that PKA preferentially phosphorylated a

serine located at the second position after a pair of arginines as in the following sequence X-Arg-Arg-X-Ser-X (X= neutral amino acid).

1.7 Cyclic AMP-dependent Protein Kinase (PKA)

Cyclic AMP-dependent protein kinase (PKA) is an enzymatically inactive tetramer composed of two regulatory (R) and two catalytic (C) subunits. PKA undergoes dissociation when the enzyme is activated by cyclic AMP (Brostrom et al., 1971; Gill and Garren, 1971; Kumon et al., 1972; Tao et al., 1970). The following equation for the activation reaction was composed after the quaternary structure of the enzyme had been determined (Beavo et al., 1975; Rosen et al., 1975) and the extent of cyclic AMP binding clarified (Corbin et al., 1978).

$$R_2C_2 + 4cAMP = R_2(cAMP)_4 + 2C$$

The regulatory subunits exist in dimeric form which is retained after dissociation, whereas the catalytic subunits exist as monomers. Each R subunit binds two molecules of cyclic AMP.

In the brain PKA is broadly distributed in specific areas (Cadd and Mc Knight., 1989). It plays an important role in neuronal regulation over different time frames. Protein kinase A acutely modulates the activity or function of neuron -specific enzymes (Zigmond et al., 1989) and/or channel proteins (Li et al., 1992; Sculptoreanu et al., 1993) by catalysing the phosphorylation of these proteins. In such instances, evoked PKA activity produces rapid changes in neuronal function via direct phosphorylation of existing protein molecules. Alternatively, translocation of the active catalytic subunit of PKA into the nucleus and subsequent phosphorylation of nuclear proteins, including transcriptional activators, gives rise to slower long term changes in neuronal

function. In such instances PKA regulates de novo synthesis of mRNA leading to new protein synthesis.

In many cyclic AMP-responsive genes an octomer sequence motif known as the cyclic AMP (cAMP) response element (CRE) resides in the 5' upstream region and mediates cAMP responsiveness (Roesler et al., 1989; Goodman et al., 1990). PKA regulates the transcriptional activity of the CRE by catalysing the phosphorylation of transcription factors. One such transcription factor is the CRE-binding protein CREB (Hoeffler et al., 1988; Gonzalez et al., 1989). After phosphorylation by PKA CREB can activate transcription of CRE-containing genes (Gonzalez and Montminy., 1989; Lee et al., 1990).

1.8 Protein Kinase C (PKC)

In the nervous system, activation of protein kinase C (PKC) has been related to enhancement of neurotransmitter release (Malenka et al., 1986; Malenka et al., 1987), control of growth differentiation (Burgess et al., 1986; Spinelli and Ishii, 1983) and modification of neuronal plasticity (Routenberg, 1985).

PKC consists of a family of closely related proteins highly concentrated in the central nervous system. This protein kinase is present in a wide variety of tissues. Recent reports indicate that PKC's are heterogenously distributed in the brain and that neurons contain the highest levels of PKC (Huang, 1987; Brandt et al., 1987). Molecular cloning has established the existence of several PKC isoforms, illustrating the diversity of this enzyme family. PKC's can be divided into two major groups, group A and group B, based on their sequence similarity at several coding regions. Group A PKC-catalysed phosphorylation

has been shown to be dependent on calcium and phosphatidylserine for its activation (Takai et al., 1979; Kaibuchi et al., 1981).

Protein kinase C can be activated by a naturally occurring product diacylglycerol (DAG), an intermediate in the biosynthesis and degradation of glycerolipids. DAG is thought to increase the affinity of the enzyme for calcium and phospholipid (Takai et al., 1979; Kishimoto et al., 1980). Tumour-promoting phorbol esters have also been shown to stimulate activity of PKC's in vitro (Blumberg, 1980; Chiang et al., 1981). It is currently the idea that DAG may be derived from the hydrolysis of a small pool of membrane phosphatidylinositol 4, 5-bisphosphate (PIP_2) by phosphatidylinositol-specific phospholipase C, resulting in a rise in inositol triphosphate (IP_3) and consequently intracellular calcium and diacylglycerol (Berridge, 1987). This signalling pathway has been linked to the actions of many neurotransmitters. Another potential source of DAG is derived through hydrolysis of phosphatidylcholine (Chan et al., 1989).

1.9 Immediate Early Genes

Immediate early genes (IEGs) were initially characterised in non-neuronal cells while studying growth factor responsive-genes. This research lead to the discovery of a unique class of genes whose transcription can be activated within minutes. The c-fos and c-myc protooncogenes were among the first IEGs to be identified (Kelly et al., 1983; Greenberg and Ziff, 1984). Many IEGs have since been identified in the brain and can be divided into distinct families based on their structural similarities. Among these families is the fos family whose members are closely related to c-fos (e.g. fos-B, Zerial et al., 1989) or the jun family, whose members are closely related to c-jun (e.g. jun-B, Ryder et al.,

1988) and jun-D (Ryder et al., 1988) or the homeobox POU-domain containing proteins (e.g. RHS2, Le Moine and Scott Young, 1992) or the homeobox fork head gene, BF-1 (Mathis et al., 1992). However, some IEGs are structurally unique, as for example zif/268, a zinc finger containing protein (Millbrandt, 1987). IEGs have a number of characteristics that makes them different from late response genes. Their expression tends to be low in quiescent cells, but is rapidly induced at the transcriptional level within minutes of extracellular stimulation. This transcriptional induction is transient and independent of new protein synthesis. Although, the subsequent shut-off of transcription requires new protein synthesis.

The IEG is currently thought to function as a transcription regulatory factor, that may act as a molecular switch coordinating changes in gene expression (Sheng and Greenberg, 1990). Direct evidence for a regulatory function came from the findings that many IEGs were found to encode transcription factors. This has been best established for c-fos and c-jun, both of which were initially identified as protooncogenes (Van Beveren et al., 1983; Maki et al., 1987). Recently it has been demonstrated that the products of these IEGs can interact with each other to form a heterodimeric transcription factor complex (Halzonetis et al., 1988; Kouzarides and Ziff, 1988; Nakabeppu et al., 1988; Rauscher et al., 1988). This interaction was later shown to occur through a conserved dimerisation domain referred to as a 'leucine zipper' that is present in both fos and jun proteins (Landschultz et al., 1988; Kouzarides and Ziff., 1988; Bos et al., 1989; Gentz et al., 1989; O'Shea et al., 1989). The leucine zipper domain was shown to have an α- helical structure (Landschultz et al., 1988). Adjacent to the leucine zipper, a highly basic stretch of amino acids were identified and shown to be required for the interaction of the fos/jun heterodimer with DNA (Kouzarides and Ziff., 1988; Gentz et al., 1989; Neuberg et al., 1989; Turner and Tjian, 1989). The heterodimer was demonstrated to bind with high affinity to the DNA consensus sequence -

TGACTCA- (reviewed by Curran and Franza, 1988). The sequence -TGACTCA- was initially identified as a phorbol ester-inducible promoter element and the binding site for a transcription factor activity termed AP-1 (Angel et al., 1987; Lee et al., 1987). Fos and jun have since been shown to be major components of the AP-1 site (Bohmann et al., 1987; Rauscher et al., 1988). Different combinations of fos/jun and jun/jun dimers have been demonstrated to bind to the AP-1 site in vitro (Nakabeppu et al., 1988; Cohen et al., 1989). There is also some evidence of functional specificity for some IEGs. It has been shown in vitro that c-fos/c-jun complexes activate transcription of genes containing AP-1 sites, while c-fos/jun-B complexes appear, under some circumstances, to repress transcription (Chiu et al., 1989; Schutte et al., 1989).

The mRNA encoding the immediate early genes Jun-D and c-jun is present constitutively in striatal neurons, and the corresponding proteins are likely to be available to form complexes with c-fos once it is synthesised. The mRNA encoding jun-B is not normally detectable in striatal neurons. Zif/268, the zinc finger containing immediate early gene protein, was shown to bind to a different DNA consensus sequence, -GCGGGGGGCG-. The DNA binding domain encoded by the homeobox genes, RHS2 and BF-1 is relatively large in comparison to other gene families studied. The POU-domain for RHS2 spans 145 amino acids (Le Moine and Scott Young, 1992) and for BF-1, the DNA binding sequence spans 56 amino acids (Tao and Lai, 1992).

1.10 Mechanisms of Induction of Immediate Early Genes

IEGs have been shown to be induced by a wide variety of physiological stimuli, including electrical excitation, neurotransmitters and growth factors (Greenberg et al., 1985, 1986; Morgan and Curran, 1986; Bartel et al., 1989). Studies have shown that many of the stimuli used to induce IEGs have a common feature in that they lead to or substitute for, calcium influx through voltage sensitive calcium channels (Greenberg et al., 1986; Morgan and Curran, 1986).

The molecular basis of IEG regulation by trans-synaptic stimulation has been studied primarily in PC12 cells. Transcriptional activation of c-fos as a result of calcium influx involves a specific site on the gene termed a calcium response element (CaRE) (Sheng et al.,1988). This site was later shown to be indistinguishable from the cAMP response element (CRE) and that cAMP and calcium signals converge on this element in vivo (Sheng et al., 1990). It has been demonstrated that both calcium and cAMP stimulation of PC12 cells result in the rapid phosphorylation of transcription factor CREB, the nuclear protein that binds specifically to the calcium/CRE (Gonzales and Montminy, 1989; Sheng et al., 1990). In response to cAMP, CREB is phosphorylated at a specific residue (Ser-133) known to be recognised by cyclic AMP dependent protein kinase (PKA) and to be important for activation of CREB as a transcription factor in vitro and in vivo (Yamamoto et al., 1988; Gonzales and Montminy, 1989). This phosphorylation is thought to activate CREB via an allosteric mechanism (Yamamoto et al., 1990), although it is still incompletely understood. CREB is phosphorylated at the identical Ser-133 position in response to calcium stimulation (Sheng et al., 1990). However evidence suggests that this phosphorylation is mediated by a calcium regulated protein kinase (Sheng et al.,

1990). The phosphorylated CREB then is thought to interact with the basic transcriptional machinery to stimulate gene transcription.

Genes containing calcium/CRE's include not only IEGs like c-fos and zif/268 (Christy et al., 1988; Changelian et al., 1989), but also a group of neuropeptide genes including somatostatin (Montminy et al., 1986; Montminy and Bilezikjian, 1987), and proenkephalin (Comb et al., 1986).

While calcium and cAMP signalling pathways are likely to mediate gene activation by many different neurotransmitters in the central nervous system, another intracellular signalling mechanism has been shown to be involved in the regulation of neuronal IEG expression. The activation of the IEG, c-fos, by the protein kinase C pathway has been demonstrated to be mediated by a distinct upstream regulatory element known as the serum response element (Gilman et al., 1986; Prywes and Roeder, 1986; Treisman, 1986; Greenberg et al., 1987; Sheng et al., 1988; Gilman, 1988). The serum response element is a specific binding site for a nuclear protein termed the serum response factor (Gilman et al., 1986; Prywes and Roeder, 1986; Treisman, 1986; Greenberg et al., 1987). This interaction plays a crucial role in c-fos activation by a diverse set of stimuli, including phorbol esters (Sheng et al., 1988).

1.11 Neuroleptics

Neuroleptics of various chemical classes have been used for more than 35 years for the treatment of psychiatric disorders (Leysen and Niemegeers, 1985). Neuroleptic drugs have been divided into two major classes, namely 'typical' neuroleptics, such as haloperidol and fluphenazine, and 'atypical' neuroleptics such as clozapine and fluperlapine. It is now well accepted that blockade of dopamine D2 receptors plays a major role in their antipsychotic

action (Leysen and Niemegeers, 1985; Meltzer et al., 1989; Richelson, 1984; Seeman, 1980). However treatment with higher dosages of 'typical' neuroleptics has been shown to provoke adverse reactions such as extrapyramidal side effects following acute treatment and tardive dyskinesia after prolonged chronic treatment. Mesolimbic and mesocortical dopamine D2 receptors were suggested to play a role in the antipsychotic effects of the drugs whereas extensive occupancy of striatal D2 receptors was thought to be responsible for extrapyramidal side effects (Crow et al., 1977; Stevens, 1973; Wilk et al., 1975). The atypical neuroleptic, clozapine, has been shown to produce much less acute extrapyramidal side effects than typical neuroleptic drugs (Matz et al., 1974). There have been no reliable reports that clozapine produces or exacerbates tardive dyskinesia, although it can block the symptoms of tardive dyskinesia (Meltzer and Luchins, 1984). In two relatively recent clinical trials, clozapine was shown to be a more effective antipsychotic drug than the typical neuroleptic chlorpromazine (Claghorn et al., 1987; Kane et al., 1988). Meltzer (1989) suggested that the clinical advantages of the use of clozapine may be due to a biological effect of the drug on neurotransmitter systems. They later demonstrated that atypical antipsychotic drugs could be distinguished from typical antipsychotic drugs on the basis of lower D2 and higher 5-HT2 pKi values.

More recently it has been suggested that potent serotoninergic 5-HT2 receptor antagonism could alleviate negative symptoms in schizophrenia. Moreover, 5-HT2 antagonists were shown to reduce neuroleptic-induced catalepsy in rats (Balsara et al., 1979; Hicks, 1990), to increase neuroleptic-induced dopamine turnover (Saller et al., 1990), to cause a slight, indirect activation of midbrain dopaminergic neurons (Ugedo et al., 1989), restoring phasic rather than tonic activity (Svensson et al., 1989), and to reduce neuroleptic-induced extrapyramidal side effects (Reyntjens et al., 1986;

Bersani et al., 1990). Therefore it has been suggested that a critical balance between 5-HT2 and dopamine D2 receptor antagonism is essential for the beneficial effects of antipsychotic drugs (Meltzer et al., 1989).

Clozapine, the prototype for atypical neuroleptics, shows higher affinity for 5-HT2 than for D2 receptors. However, it also shows a potent histamine H1 receptor antagonism, which results in severe sedation. In addition, clozapine binds to 5-HT1C receptors, to α1- adrenergic and to muscarinic receptors with 5-10 times higher affinity than to D2 receptors. Therefore these additional properties may play a role in both the therapeutic and resultant side effects of clozapine treatment (Grohmann et al., 1989). The relatively higher affinity of clozapine for D4 receptors than for D2 receptors was proposed as a possible underlying mechanism for the particular clinical properties of this compound (Van Tol et al., 1991). Interest has consequently focussed on the ability of neuroleptic drugs to cause long-lasting alterations in basal ganglia function, by changing the pattern of gene expression in striatal neurons.

1.12 Long term effects on late response genes in the basal ganglia

In 1978, Hong and colleagues demonstrated that either chronic blockade of dopamine receptors by neuroleptic drugs or lesioning the nigrostriatal pathway could increase levels of enkephalin peptides in the striatum. Consistent with this, in situ hybridisation studies later revealed changes in mRNA levels of proenkephalin after chronic treatment with dopamine receptor antagonists. Chronic treatment with haloperidol, the relatively non-selective dopaminergic receptor antagonist, dramatically increased the levels of proenkephalin mRNA in the rat striatum (Sabol et al., 1983; Tang et al., 1983; Angulo et al., 1986; Sivam et al., 1986; Romano et al., 1987; Morris et al., 1988). In situ hybridisation

studies have also shown that unilateral nigral lesions induce a dramatic ipsilateral increase in striatal proenkephalin mRNA levels (Angulo et al., 1986; Young et al., 1986; Morris et al., 1989; Cadet et al., 1991), suggesting that the nigrostriatal pathway exerts a tonic inhibition on the activity of the striatal enkephalin-containing cells. The cells of the ventral tegmental area were demonstrated to exert a similar inhibition on the activity of the enkephalin cells of the nucleus accumbens (Morris et al., 1989). More recently, the elevation in striatal proenkephalin mRNA levels following a nigrostriatal lesion, has been positively correlated with an increase in rotation rate after subcutaneous administration of amphetamine (Cadet et al., 1991).

Conversely, studies have shown that the repeated administration of dopamine receptor antagonist drugs can decrease the concentration of substance P in the substantia nigra (Hong et al., 1978; Hanson et al., 1981; Le Douarin et al., 1983; Oblin et al., 1984; Sudzak and Gianutsos, 1984). Further evidence has demonstrated that the levels of protachykinin mRNA, the precursor protein for substance P, are decreased in the rat striatum after chronic treatment with the antipsychotic drug, haloperidol (Bannon et al., 1986). From in situ hybridisation studies, the levels of striatal protachykinin mRNA has been shown to be decreased following a nigral lesion, suggesting a tonic dopaminergic facilitation of the activity of the tachykinin cells (Young et al., 1986). Recent evidence suggests that the release of substance P is decreased in the ipsilateral substantia nigra after dopaminergic denervation of the striatum (Lindefors et al., 1989).

In animal models of Parkinson's disease, met-Enk levels are increased in the striatum (Engber et al., 1991; Dacko and Schneider, 1993), while substance P has been shown to be either unaltered (Sivam et al., 1987) or decreased (Lindefors et al., 1989). Ceballos and colleagues have more recently suggested that the population of neurons co-localising met-enkephalin and substance P can adapt according to the extent of degeneration in the substantia nigra in Parkinson's disease patients. Patients with greater than 80% loss of dopamine in

the caudate show an increase in both met-enkephalin and substance P levels. However, in patients with approximately 50% loss of dopamine content in the caudate, levels of both peptides were markedly reduced by 80%.

A decrease in somatostatin mRNA has been observed in the striatum of rats treated with haloperidol, suggesting that dopamine may be involved in the regulation of somatostatin mRNA levels (Salin et al., 19). Haloperidol decreases preprosomatostatin mRNA levels not only in the caudoputamen, but also in the nucleus accumbens and in the frontal cortex. Therefore this suggests a widespread influence of dopamine on somatostatin neurons in target areas of the mesencephalon. This is compatible with previous findings showing a decrease in immunoreactive somatostatin measured by radioimmunoassay in the striatum and in the nucleus accumbens after chronic haloperidol treatment (Beal and Martin, 1984; Radke et al., 1988). It was suggested that this effect may be related to the blockade of a stimulatory action of dopamine on somatostatinergic neurons mediated through dopamine D2 receptors, since haloperidol was shown to preferentially block D2 receptors (Saller and Salama, 1986). However, it has since been shown that the region within the striatum, in which haloperidol significantly affects the levels of preprosomatostatin mRNA crresponds to an area of relatively low D2 dopaminergic binding sites (Dubois and Scatton, 1985; Boysen et al., 1986). This implies that other mechanisms may be involved in the effect of haloperidol on preprosomatostatin mRNA levels in this brain region. Evidence to support this further came from the observation that lesions of the nigrostriatal dopaminergic neurons, which markedly reduce dopamine levels in the striatum do not affect the number of neurons expressing somatostatin-like immunoreactivity in the striatum ipsilateral to the lesion (Salin et al., 1990).

The mRNA levels of the neuropeptide prodynorphin were not greatly affected by chronic neuroleptic drug treatment (Morris et al., 1988). A similar lack of effect on expression of prodynorphin mRNA was observed following nigral lesions (Young et al., 1986; Morris et al., 1989).

Saller and colleagues (1989) found that chronic treatment with the atypical neuroleptic clozapine increased the intensity of preprosomatostatin mRNA in neurons, but not the number of neurons expressing the gene, in the nucleus accumbens. No effect was observed with clozapine in the striatum. The hypothesis that dopamine exerts opposite effects on the levels of preprosomatostatin mRNA in the striatum through an action on D1 and D2 dopamine receptors was thought to be compatible with this lack of effect in the striatum. However, it has been suggested that the blockade of other neurotransmitter receptors such as muscarinic, 5-HT2, and noradrenergic receptors by clozapine (Kelley and Miller, 1975; Peroutka and Snyder, 1980) may be responsible for the different effects of clozapine and haloperidol on somatostatinergic neurons in the striatum (Saller et al., 1990). This evidence substantiates the present suggestion for a differential effect of some antipsychotic drugs in the nigrostriatal and mesolimbic system (Chiado, 1988). The idea of a different mechanism of action of atypical and typical antipsychotic drugs has gained further support from the finding that suggests that clozapine, but not haloperidol, increases the levels of glutamic acid decarboxylase mRNA, the enzyme involved in the synthesis of GABA, in neurons of the globus pallidus (external pallidum), implying a differential effect of these antipsychotic drugs on one of the major output systems of the basal ganglia (Mercugliano et al., 1989).

Dopaminergic receptor antagonists of D1 and D2 receptors have also been demonstrated to affect in opposite directions, the levels of immunoreactive neuropeptide Y, a peptide colocalised with somatostatin in striatal neurons (Sandell et al., 1986; Smith and Parent, 1986). Chronic treatment with SCH23390, which preferentially blocks D1 dopamine receptors, increases the number of neurons expressing neuropeptide Y-like immunoreactivity in the striatum. The dopaminergic D2 receptor antagonists, haloperidol and sulpiride were shown to have an opposite effect (Kerkerian et al., 1988). Similarly at the cellular level it was shown that neuropeptide Y mRNA could be clearly increased

in the rat striatum following chronic administration of the D1/D5 receptor antagonist SCH23390 for three days (Morris, 1989).

1.13 Effect of Antipsychotic Drug Treatment on Immediate Early Gene Expression

The antipsychotic drugs are effective in the treatment of severe psychiatric disorders. Although many exert serious side effects and have limitations in efficacy which in turn has spurred a search for better compounds and a greater understanding of the pharmacology of antipsychotic drugs. The full therapeutic effect of antipsychotic drugs generally takes several weeks to emerge. Therefore it has been postulated that their interaction with neurotransmitter receptors are merely the initial step in their actions, and that the therapeutic effects result from adaptive processes that may occur in response to repetitive or chronic receptor occupancy by the drugs. A likely candidate mechanism for such drug induced neural plasticity is receptor -mediated regulation of neuronal gene expression. It is currently the idea that the induction of cellular IEGs may be a critical signal transduction step in neural plasticity induced by neuroleptic drugs, with the protein products of IEGs functioning to activate or repress genes that encode proteins involved in the differentiated functions of target neurons.

Recent evidence has shown that the dopaminergic system in brain is capable of responding in an adaptive manner to neuroleptic drug exposure, by inducing a number of dopamine D2 receptors (Seeman, 1980). This neurotransmitter system has also been shown to participate in the induction of certain IEGs. The dopamine precursor L-DOPA has previously been demonstrated to activate the IEG c-fos in the rat striatum ipsilateral to a 6-hydroxydopamine lesion of the substantia nigra via stimulation of dopamine D1 receptors (Robertson et al.,

1989). In agreement with this, systemic administration of D1 receptor agonists, in particular SKF38393, have been shown to induce c-fos and zif/268 mRNA in striatal neurones of rat brain after lesioning (Robertson et al., 1990; Cole et al., 1992). In contrast, haloperidol, a relatively non-selective dopamine receptor antagonist together with selective dopamine D2 receptor antagonists such as YM 09151, eticlopride and (+) butaclamol have been shown to induce c-fos protein in rat striatal neurons (Miller, 1990; Deutch et al., 1992; Sirinathsinghji et al., 1994) and in the nucleus accumbens (Dragunow et al., 1990; Deutch et al., 1992), but not in other brain regions. Similarly, Nguyen and colleagues (1992) have shown that both c-fos and zif/268 can be induced in the caudate-putamen at the mRNA level by systemic administration of haloperidol or amphetamine. However the atypical neuroleptic, clozapine, was unable to induce c-fos mRNA in the caudate-putamen, but was able to induce the expression of c-fos in the nucleus accumbens. A similar induction of fos immunoreactivity has been demonstrated in the shell but not the core region of the nucleus accumbens in the rat after acute systemic clozapine treatment (Deutch et al., 1992). Whilst the selective D1 dopamine receptor antagonist, SCH23390 was demonstrated to have no effect on the levels of fos protein (Dragunow et al., 1990). A similar lack of effect on the expression of c-fos mRNA in rat striatum has been observed following systemic administration of the selective D2 receptor agonist LY 171555, but this compound was able to reverse completely the haloperidol induced expression of c-fos mRNA (Miller, 1990). It was later shown that a 6-hydroxydopamine lesion of the substantia nigra alone could lead to a long lasting expression of fos related antigens in striatal neurons ipsilateral to the side of the lesion (Dragunow et al., 1991). At present there is circumstantial evidence linking the haloperidol induction of c-fos to the subsequent increase in proenkephalin mRNA (Sonnenberg et al., 1989; Donovan et al., 1992), but this has been questioned by recent results suggesting that it is not the fos protein that interacts with the proenkephalin enhancer, but it is a cAMP-responsive element

binding protein that appears to play a major interactive role (Konradi et al., 1993).

A number of psychomimetic drugs including D-amphetamine and cocaine, with markedly different pharmacologic and clinical properties, have also been reported to activate striatal c-fos expression and a clear drug-specific pattern of gene activation has been shown in neurons of the sensory-motor and limbic striatum (Robertson et al., 1989; Graybiel et al., 1990; Nguyen et al., 1992). The mechanism of induction of c-fos is thought to involve the stimulation of D1 dopamine receptors (Robertson et al., 1989; Graybiel et al., 1990). Therefore, both PKA and calcium or PKC signalling may underlie the induction of c-fos, as D1 receptors are positively coupled to adenylate cyclase (Stoof and Kebabian., 1981) and to inositol phospholipid (Mahan et al., 1990) signalling pathways. It has previously been shown in vitro that the IEG c-fos can be induced through interaction of nuclear proteins with cAMP-responsive regulatory elements (CREs) present in the gene (Greenberg et al., 1985) and some PKC effects have been reported as well (Goodman, 1990).

It is known that c-fos can form dimers with members of the jun family of IEGs in order to effect target gene transcription. The mRNAs encoding two members of this family, c-jun and jun-D, are present constitutively in striatal neurons and the corresponding proteins are likely to be available to form complexes with c-fos when it is synthesised. The mRNA encoding another IEG belonging to this family, jun-B, is not normally detectable in striatal neurons. However there is some evidence for the induction of jun-B after acute administration of cocaine (Hope et al., 1992), amphetamine and apomorphine (Cole et al., 1992). These drugs are known to have agonist properties at a number of receptors, including dopamine receptors.

As yet there is still no firm evidence linking the induction of any immediate early gene with any effect on a late-reponse gene. However, there has been some speculation on a potential link between the induction of fos and jun

proteins and enkephalin mRNA expression (Sonnenberg et al., 1989) Although, contrary to this suggestion, more recent research implies that it is not the fos protein that interacts with the enkephalin enhancer (Konradi et al., 1993).

1.14 Characterisation of Serotonin Receptor Subtypes

Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic amine neurotransmitter that is known to be involved in the homeostatic regulation of the fundamental physiological functions such as sleep, temperature regulation, learning and memory and pain. Disruptions in the serotoninergic system may be a critical factor in many pathological disorders affecting society today, including anxiety, depression, schizophrenia, eating disorders and obsessive compulsive disorder (Lopez-Ibor, 1988). A centralisation of the serotoninergic system in the raphe area of the central nervous system, together with its diverse projections to all brain areas confirms the potential of serotonin to be a homeostatic regulator.

The transduction of serotoninergic signals across the neuronal membrane is mediated by a diversity of receptor subtypes that in mammals appear to fall into seven distinct classes, designated 5-HT1 to 5-HT7, on the basis of their affinities for serotonin or sequence similarities. With the exception of the 5-HT3 receptor which is a ligand gated ion channel, all of the other members of the serotonin receptor family belong to the large class of receptors that are linked to their effector function via guanine-nucleotide binding proteins with the putative seven transmembrane domain structure characteristic of many receptors (Hoyer and Schoeffter, 1991). Within receptor classes, a number of serotonin receptor subtypes have been identified. The functional significance, if any, in terms of the physiological role of many of these newly cloned receptors is as yet to be identified and therefore is of great importance in understanding the functions of serotonin in the brain.

The 5-HT1-like subcategory has been further subdivided into four different subtypes (Hartig et al., 1992), which have recently been cloned, namely the 5-HT1A (Kobilka et al., 1987; Fargin et al., 1988), the 5-HT1B (Voigt et al., 1991; Branchek et al., 1991), the 5-HT1D (Hamblin and Metcalf, 1991) in rat,

the 5-HT1D in the canine (Zgombick et al., 1991), the 5-HT1Db (Demchyshyn et al., 1992) in human and the 5-HT1E (Hartig et al., 1992) in the rat. All members of the 5-HT1 subgroup are characterised by their high affinities for serotonin (Ki < 100nM) and their ability to inhibit cellular cAMP production (De Vivo and Mayaani, 1990). Distinction between the receptors in this group has been made on the basis of the affinities for a series of drugs showing some subtype selectivity e.g. 8-hydroxy DPAT is a selective agonist for the 5-HT1A receptor (Gozlan et al., 1983) and rawolscine a selective agonist for the 5-HT1D receptor (Frazer et al., 1990). 5-HT1A receptors have been widely studied and agonist compounds acting at this receptor site have been shown to possess anxiolytic (Traber and Glasser, 1987; Gleeson et al., 1989; Brocco et al., 1990), antidepressive (Cervo et al., 1988; Glasser, 1988; Glennon, 1990), antihypertensive (Hartog and Wouters, 1988; Mandal et al., 1989; Stubbs et al., 1991) and possibly antipsychotic (Ahlenius, 1989) and neuroprotective (Bielenberg and Burkhardt, 1990; Bode-Greuel et al., 1990) properties. Indeed the 5-HT1A partial agonists buspirone, ipsapirone and gepirone have been shown to exert anxiolytic and antidepressive effects in humans (Glennon, 1990; Robinson et al., 1989; Traber and Glasser, 1987). The 5-HT1B receptor was distinguished from the 5-HT 1A subtype with the use of the drug spiperone, as 5-HT1A sites have high affinity for spiperone, while conversely the 5-HT1B site has a low affinity for spiperone (Pedigo et al., 1981). A similar distribution of 5-HT1A receptors has been observed in many different animal species (Marcinkiewicz et al., 1984; Waeber et al., 1989). The highest density of 5-HT1A sites was observed in the limbic system including the hippocampus, septum, amygdala and cortical limbic areas. This high concentration in the limbic system is thought to be consistent with their involvement in mood and anxiety. The recently cloned 5-HT1B receptor subtype has been shown to have high affinity binding of ^{3}H-5-HT with a different pharmacological profile to the 5-HT1A subtype. In situ hybridisation has revealed that the 5-HT1B mRNA is

located within cells of the dorsal and median raphe nuclei suggesting an autoreceptor function, in the CA1 region of the hippocampus, in the striatum, cortex and in the cerebellum. The human mRNA encoding the 5-HT1B receptor has been shown to be most abundant in the striatum (Jin et al., 1992). Both the 5-HT1B and the 5-HT1D receptor subtypes are defined on both pharmacological and biochemical grounds by their ability to inhibit adenylate cyclase activity (Schoeffter et al., 1990). The 5-HT1B receptor has been suggested to be the evolutionary antecedent of the 5-HT1D receptor which is present in bovine and human brain in high concentrations in the substantia nigra and basal ganglia (Heuring and Peroutka, 1987). The 5-HT1E receptor mRNA has been located on the terminal fields of some serotoninergic neurons. Characterisation of this receptor subtype is still necessary.

Another subclass of 5-HT receptors is the 5-HT2 subtype. At present there are three structurally similar subtypes referred to as 5-HT2A, 5-HT2B (formerly the 5-HT2F), and 5-HT2C (formerly the 5-HT1C) (Pritchett et al., 1988) whose activation results in phospholipase C-mediated phosphatidylinositol lipid hydrolysis (Conn and Sanders-Bush, 1986; Conn et al., 1986) which liberates the second messengers diacylglycerol and inositol triphosphate. Diacylglycerol activates the enzyme protein kinase C, whereas inositol triphosphate increases the intracellular free calcium concentration (Jaken, 1989; Irvine, 1989). Both increases in intracellular calcium and activation of protein kinase C have been observed in response to activation of 5-HT2A (Pritchett et al., 1988; Berg et al., 1994) and 5-HT2C receptors (Lutz et al., 1993; Boddeke et al., 1993). In addition to coupling to phospholipase C- mediated phosphatidylinositol lipid hydrolysis, 5-HT2A and 5-HT2C receptors have been shown to mediate phospholipase A2-mediated arachidonic acid release (Felder et al., 1990). Long term exposure to agonists (Dillon-Carter and Chuang, 1989; Sanders-Bush and Breeding, 1990; Ivins and Molinoff, 1991) or antagonists (Blackshear et al., 1983; Sanders-Bush and Breeding, 1988) have also been demonstrated to down

regulate these receptors. The newly cloned 5-HT2B (formerly 5-HT2F) receptor (Foguet et al., 1992; Kursar et al., 1992; Loric et al., 1992) has recently been characterised. The 5-HT2B receptor subtype was shown to stimulate production of inositol 1,4,5-trisphosphate in transformed cells and has a unique pharmacological profile consistent with the evidence that this receptor subtype mediates contractions in the rat stomach fundus (Wainscott et al., 1993).

The distribution of 5-HT2 receptor subtypes shows some overlap, although there are some differences. Both the 5-HT2A and 5-HT2C subtype mRNA have been shown to be present at intermediate levels in the caudate-putamen, accumbens nucleus and substantia nigra pars compacta. However, their distribution in the caudate-putamen is quite different. The 5-HT2C receptor mRNA is in addition present in the basal nucleus of meynert, stria terminalis, entopeduncular nucleus and subthalamic nucleus and was found to be most predominant in the amygdala. While the 5-HT2A transcripts were restricted to only a few nuclei in these areas (Pompeiano et al., 1993). It has very recently been shown that there are important fundamental differences in the signal transduction systems for 5-HT2A and 5-HT2C subtypes. In transfected CHO cells activation of 5-HT2C, but not 5-HT2A receptors reduces receptor mediated inhibition of adenylate cyclase activity. Furthermore, this effect of the 5-HT2C subtype is independent of activation of PKC and an increase in intracellular calcium concentration (Berg et al., 1994). Attempts to visualise transcripts for the third member of the 5-HT2 receptor family, the 5-HT2B (5-HT2F) with probes and PCR analysis has so far failed in rat brain (Foguet et al., 1992).

The 5-HT3 receptor is a ligand-gated ion channel which when activated causes a fast depolarising response. Kilpatrick and colleagues (1987) were the first to identify 5-HT3-binding sites in the brain. More recently, Maricq and colleagues have isolated a cDNA clone encoding a 5-HT3 receptor from NCB-20 cells (Maricq et al., 1991). The mRNA encoding this receptor is identified in

brain, spinal cord and in the heart. The receptor protein is located mainly in the dorsal hindbrain, nuclei of the cranial nerves, and the area postrema (Palacios et al., 1990).

A 5-HT4 receptor subtype is also known to exist, however it is the least well characterised of all the 5-HT receptors. The 5-HT4 receptor has been detected in the CNS by measuring 5-HT stimulation of cyclic AMP formation either in intact neurons in culture (Dumuis et al., 1988, 1989) or in isolated neuronal membranes (Bockaert et al., 1989, 1990), or in the gastrointestinal tract by monitoring 5-HT-mediated changes in contractile responses of the guinea pig ileum (Clarke et al., 1989; Craig and Clarke, 1990). This receptor has also been characterised in radioligand binding studies (Clarke et al., 1989), but has not yet been cloned.

A fifth member of the 5-HT receptor family has been isolated from mouse brain (Plassat et al., 1992). This 5-HT receptor is referred to as the 5-HT5A, as a 5-HT5B subtype has recently been sequenced (Matthes et al., 1993). 5-HT5 receptors are known to have very high affinity for ergot compounds such as lysergic acid diethylamide and methysergide, but have relatively low affinity for 5-HT. The 5-HT5A subtype was found to be expressed predominantly in the cerebellum, cerebral cortex, hippocampus and olfactory bulb. While the 5-HT5B receptor mRNA was expressed predominantly in the medial habenula and in the CA1 field of the hippocampus and raphe nuclei.

A recent report has described the cloning of another structurally and pharmacologically distinct 5-HT receptor from rat brain, namely the 5-HT6 receptor. This receptor subtype has been shown to exhibit high affinity for a number of antipsychotic and tricyclic antidepressant drugs, including clozapine, amoxapine and amytriptyline (Monsma et al., 1993). In cells transfected with this receptor, serotonin elicits a potent stimulation of adenylyl cyclase activity which is blocked by antipsychotic and antidepressant drugs (Sebben et al., 1994). This receptor subtype has been localised in motor limbic and cortical regions of the

brain, with the highest levels present in the striatum, a potentially important site of action of antipsychotic drugs with regard to both extrapyramidal sysmptoms and perhaps cognitive functioning. (Monsma et al., 1993). A similar 5-HT receptor subtype has recently been isolated in mouse neuroblastoma cells (Unsworth and Molinoff, 1993).

Most recently a 5-HT7 receptor subtype has been proposed (Shen et al., 1993). The serotoninergic 5-HT7 receptor has since been cloned and shown to be positively coupled to adenylate cyclase when transfected into a chinese hamster ovary cell line (Tsou et al., 1994). Similar to the 5-HT6 receptor, this receptor subtype has high affinity for antipsychotic drugs such as clozapine. The 5-HT7 receptor has not yet been fully characterised, however, both the 5-HT6 and 5-HT7 receptor subtypes are known to pharmacologically resemble the 5-HT2 receptor (Monsma et al., 1993; Shen et al., 1993).

1.15 Drug Pharmacology of Serotonin receptors

Early on in the studies on neuroleptic drugs, evidence was obtained demonstrating that the serotoninergic system could influence the action of antipsychotic drugs (Kostowski et al., 1972) and some neuroleptic drugs could increase 5-HT turnover, perhaps by blockade of 5-HT receptors (Leysen et al., 1978). With the discovery of specific agonists and antagonists for serotonin receptor subtypes, a potential role of particular clinical importance was evident, since the binding affinites of many atypical antipsychotics such as clozapine, melperone and amperozide (Meltzer et al., 1989; Christensson et al., 1990) are particularly high for certain serotonin receptor subtypes.

In vitro, the pKi values of typical neuroleptic drugs for 5-HT2-binding sites tends to vary over a wide range, with a number being quite potent, such as

methiothepin and spiperone (pKi = 9.4) (Meltzer et al., 1989). Both fluphenazine (pKi = 8.6) and haloperidol (pKi = 7.7) have some affinity for the 5-HT2A receptor subtype (Meltzer et al., 1989; Audinot et al., 1993). Haloperidol has a much lower affinity for the 5-HT2C serotonin receptor subtype (Audinot et al., 1993). Previous studies have also demonstrated that haloperidol has only a weak affinity for the 5-HT1A, 5-HT1B, 5-HT1D and 5-HT3 receptors (Meltzer et al., 1989). Therefore it has been suggested that haloperidol would not be expected to achieve effective blockade at clinically relevant doses at 5-HT1D, 5-HT2C, or 5-HT3- binding sites in humans with the possible exception of weak 5-HT2A receptor blockade, because the clinical dose is based on its potent D2 receptor antagonism (pKi = 9.0) (Meltzer and Nash, 1991).

Many atypical antipsychotic drugs have been found to have a relatively high affinity for 5-HT2-binding sites in vitro as compared to the typical drugs relative to their respective affinities for D2 dopamine receptors (Meltzer et al., 1989). Clozapine has been shown to have high affinity for both the 5-HT2A (pKi = 8.3, Meltzer et al., 1989; pKi = 8.3, Roth et al., 1991; pKi = 7.6, Audinot et al., 1993) and the 5-HT2C receptor (pKi = 8.14, Roth et al., 1991; pKi = 8.1, Audinot et al., 1993). As yet, it is not clear whether atypical drugs, like clozapine, are superior in treating schizophrenia compared with typical antipsychotic drugs, and whether they also do not produce tardive dyskinesia. It is likely that at clinically effective doses of clozapine, which correlate with its affinity for the D2 receptor (Seeman, 1981), clozapine produces effective occupancy of the 5-HT2A and 5-HT2C receptors because it has a significantly higher affinity for these receptors than for the D2 receptor. Clozapine has also been reported to have submicromolar affinity for 5-HT1A receptors in human brain tissue (Mason and Reynolds, 1992) and a moderate affinity for the 5-HT3 receptor (Watling et al., 1990).

There is a possibility that, because of pharmacokinetic and pharmacodynamic reasons, in vitro affinities may not predict in vivo occupancies of specific drugs. Active metabolites may rapidly dissociate from a receptor site or there may be local differences in drug metabolism due to differences in brain cytochrome P450 enzymes may cause significant differences between in vivo occupancy and in vitro affinities (Meltzer and Nash, 1991). Meltzer et al. (1989) have previously demonstarted that some drugs such as chlopromazine, are significantly more effective in blocking D2 receptors in vivo than would be predicted from their in vitro affinities.

The highly selective D1-like receptor antagonist, SCH23390, has also been reported to interact with the central 5-HT system (Bischoff et al., 1988). Similar to selective 5-HT2 receptor blockers, SCH23390 was shown to inhibit [^{3}H]-spiperone binding in the rat frontal cortex, in vivo. In vitro, SCH23390 was shown to inhibit [^{3}H]-ketanserin binding to 5-HT2 sites. Biochemical parameters linked to dopamine and 5-HT were not changed, except in the striatum where SCH23390 increased homovanilic acid levels. SCH23390 was shown to have only a weak affinity for 5-HT1B and 5-HT1A receptors.

In general terms, the following research work was performed in order to understand further the role of the immediate early gene in striatal functioning, which at present is unclear. We have attempted to investigate how major transmitter systems, in particular the dopaminergic and serotoninergic systems, have the ability to activate certain immediate early genes known to be present in the striatum. With the use of a number of selective drugs, novel molecular biological approaches and an in vitro 'model' of the striatum, a wide spectrum of possible receptor mechanisms will be investigated. The ultimate aim of which will be to isolate a receptor mechanism and second messenger pathway prior to the induction of an immediate early gene under investigation.

Since a number of disease states including Parkinson's disease and schizophrenia have been identified, and in turn linked to a dysfunction of the basal ganglia, we were particularly interested in the neuropeptide systems in the striatum which are known to be vulnerable in many neurological diseases. Neuroleptic drugs are the conventional treatment for schizophrenia, therefore to understand how neuroleptics effect transmitter / neuropeptide systems in the striatum may provide an important insight into certain diseases. Recently it has been proposed that the activation of immediate early genes may be connected to a change in the expression of a late response gene (for example, a gene encoding a neuroepeptide). Therefore, to aid in the understanding of this potential relationship, we have also investigated how neuroleptics effect a number of immediate early genes. Hopefully this research will provide useful information on the complex functioning of the basal ganglia.

Chapter 2

METHODS

2.1 Oligonucleotide Probes

Oligonucleotide probes of unique sequence were synthesised on an Applied Biosystems DNA synthesiser. The probe sequences were as follows: c-fos (45-mer), complementary to nucleotides spanning amino acids 1-15 (Curran et al, 1987) ; Jun-B (60-mer), complementary to nucleotides spanning the last 20 amino acids of the predicted protein (Ryder et al, 1988) ; Jun-D (60-mer), complementary to the last 20 amino acids of the predicted protein (Ryder et al, 1989) ; zif/268 (45-mer), complementary to nucleotides spanning amino acids 2-16 (Millbrandt, 1987) ; preproenkephalin (45-mer),complementary to bases 388-432 of the predicted gene sequence (Howells et al, 1984; Yoshikawa et al, 1984) ; prodynorphin (45-mer), complementary to bases 862-906 of the predicted gene sequence (Civelli et al, 1985; Morris et al, 1986) ; proneuropeptide Y (45-mer), complementary to bases 1624-1668 of the predicted gene sequence (Larhammar et al, 1987) ; preprosomatostatin (45-mer) , complementary to the sequence encoding for amino acids 78-92 of the predicted protein (Montminy et al, 1984) ; preprotachykinin (36-mer), complementary to bases 172-207 of the predicted gene sequence (Krause et al, 1987) ; BF-1 (45-mer), complementary to bases 445-489 (Tao and Lai, 1992) ; RHS2 (45-mer), complementary to bases 35-79 (Le Moine et al, 1992) ; D1 dopamine receptor (45-mer), complementary to bases 142-186 (Monsma et al, 1990) ; D2 dopamine receptor (45-mer), complementary to bases 640-684 (Bunzow et al , 1988), and the 5-HT6 receptor (45-mer), complementary to bases 702-746 (Monsma et al, 1992); Tubulin T26 (45-mer) complementary to part of the 3' non-coding region:

GGGAAACAGCATAGAAGCATCGATGCCTGC AGCTAGTGCT GGAGC

(Miller et al., 1987); D1 dopamine receptor antisense complementary to bases 1-15 (Sunahara et al., 1990) ; D5 dopamine receptor antisense complementary

to bases 1-15 (Sunahara et al., 1991) and a D1 sense. A sense "message" probe for preprosomatostatin and Microtubule-associated protein (MAP2) were also synthesised.

2.2 In situ Hybridisation on cryostat sections

In situ hybridisation was performed according to the method described previously (Morris et al, 1986, Wisden et al., 1991. Wisden and Morris, 1994). Frozen coronal brain sections (20μm) were cut using a cryostat (Reichart-Jung) at the level of the rostral and medial striatum (taken 1.6mm anterior and 0.4mm posterior to bregma). The sections were mounted onto sterile fridge-cooled slides (BDH) previously baked in aluminium foil at 180°C and coated in a 0.01% solution of poly-L-lysine hydrobromide made up in Diethylpyrocarbonate (DEPC)-treated water. The sections were then dried at room temperature. Following this, the sections were fixed in an ice cold 4% (weight/volume) solution of depolymerized paraformaldehyde freshly prepared in 1x phosphate-buffered saline (1xPBS) [10 x PBS is 1.3M sodium chloride, 70mM di-sodium hydrogen orthophosphate, 30mM sodium di-hydrogen orthophosphate made up in sterile water.] for 5 minutes. The sections were then transferred into 1xPBS for 1-2 minutes and then dehydrated in graded ethanol solutions (i.e. 70%, 95%, and 100%) for several minutes.

0.6pmol oligonucleotide probes (45-mers) were 3' -end labelled with 10pmol of the isotope 5-[α- ^{35}S]dATP (NEN, specific activity 1300 Ci/mmol), i.e. using a 33:1 molar ratio of isotope to oligonucleotide and with terminal deoxynucleotidyl transferase (TdT, Pharmacia). The specific activity of the probes was in the region of 1.0 x 10^9 dpm/μg.

For a 12.5μl reaction volume, in a sterile eppendorff tube mix together:

4.0μl DEPC-water

2.5μl 5 x reaction buffer. Reaction buffer (5x) contains 1M potassium cacodylate, 125mM Tris/HCl, 1.25mg ml-1 bovine serum albumin, pH 6.6, at 25^{o}C.

2.0μl of oligonucleotide at concentration of 0.3pmol μl^{-1} for a 45-mer , 0.3pmol is equivalent to 5 ng μl^{-1})

2.8μl [α-^{35}S]dATP (1300 Ci/mmol)

1.2μl TdT .

The reagents were mixed very carefully by pipette. Then the mixture was incubated for 1-3 hours in a water bath at 30-35^{o}C. The reaction was then inhibited by adding 40μl DEPC-treated water. A Sephadex G-25 spin column was then prepared using a 1ml syringe plugged at the tip with glass wool. The syringe was filled with Sephadex G-25 solution (i.e. a 1:1 solution of sephadex G-25 powder and Tris/ EDTA buffer[10mM Tris, 1mM EDTA] and spun for 1 minute to obtain a column. Then 50μl of the reaction mixture was added to the Sephadex G-25 spin column and spun at 2000 rpm for 1 minute in a MSE MINOR centrifuge, to remove unincorporated nucleotides. After collecting the eluate in a sterile eppendorff tube, 2 μl of the eluate was analysed by liquid scintillation counting. The counts would be expected to be in the range 300 000 to 800 000 d.p.m. 2μl^{-1}. Then 2 μl of a 1M solution of dithiothreitol (DDT) made up in sterile water was added to the tube. This is known to preserve the probe from oxidation. The probe was stored at -20^{o}C until use.

Sections were removed from 100% ethanol and allowed to air dry. Following this, the sections were hybridised with the selected probe at 42^{o}C overnight. In a sterile eppendorff tube, the radiolabelled probe was diluted 1:100/1:200 in a hybridisation buffer containing 50% deionised formamide, 0.02% poly adenylic acid,10% 4x standard saline citrate (4x SSC) [1x SSC= 0.15M sodium chloride, 0.015 M sodium citrate, pH= 7.0], 5% 0.5M sodium phosphate, pH 7.0, 1% 0.1M sodium pyrophosphate and 10% dextran sulphate made up in sterile water. After adding 2μl of DTT to the mixture and vortexing

thoroughly, 120μl of probe/hybridisation buffer was applied to each slide. The slides were gently covered with a parafilm coverslip. Any air bubbles present were removed with blunt ended forceps. A small piece of tissue paper was saturated with 4 x SSC and placed alongside the slides in a sealed petri dish to maintian humidity. The slides were then incubated overnight at 42^{o}C.

After overnight incubation slides were transferred into coplin jars containing 250ml of prewarmed 1 x SSC at 55^{o}C. The parafilm was gently peeled off and discarded. The slides were washed twice in 1 x SSC at 55^{o}C for approximately 30 minutes per wash with constant agitation. Following this, the slides were transferred into 0.1 x SSC at room temperature for 15 minutes, and then briefly dehydrated through 70% ethanol, 95% ethanol and 100% ethanol, before being allowed to air dry.

The sections were then exposed to autoradiographic film (Kodak XAR-5) at room temperature. Dry slides were attached to paper with scotch tape and exposed to autoradiographic film for 3 days to 2 weeks depending on the probes used and the efficiency of their tailing. Under safe light conditions films were developed for 1 minute in D19 (Kodak), rinsed in water for 30 seconds, fixed in Unifix fixative (Kodak) for 4 minutes and finally rinsed in water for 20 minutes. Once the films had been developed , the slides were dipped in Ilford K5 liquid photographic emulsion. Slides were dipped under safe light in a filtered 1:1 solution of emulsion : distilled water containing 0.5% glycerol prewarmed to 45^{o}C. Slides were allowed to dry before being transferred to light-tight slide boxes containing fresh silica gel. The boxes were stored in the fridge at 4^{o}C until time of development. As a general rule slides were stored for 5 X the exposure time on film.

Exposed boxes were allowed to come to room temperature. Under safe light conditions the slides were then transferred into glass racks prior to immersion in 250ml D19 developer (Kodak) at 19^{o}C for 2 minutes. After developing, slides were immersed in water for 30 seconds and then transferred

into 250ml of a freshly prepared solution of 30% sodium thiosulphate for 4 minutes. The slides were then transferred into water for at least 30 minutes in order to wash off excess fixative. Sections were then counterstained in either a 0.2% solution of neutral red for 2-5 minutes or a 0.25% solution of cresyl violet for 5 minutes and dehydrated in serial solutions of ethanol for several minutes. Finally sections were transferred into histoclear for several minutes. Excess histoclear was allowed to drain off , following which the slides were mounted with glass coverslips (Chance) and DPX mounting medium.

2.3 In situ hybridisation of cell cultures

The method used for in situ hybridisation of cell cultures was essentially identical to that described for in situ hybridisation of cryostat sections. However we found it necessary to dilute 'hot' probes to 1:400 with hybridisation buffer to reduce the risk of background labelling to a minimum.

Autoradiographic films and slides were developed as previously described. Cultures were counterstained with a 0.2% solution of neutral red for 1 minute or with a 0.25% solution of cresyl violet for 1-5 minutes before dehydration and coverslipping.

2.4 Controls for in situ hybridisation

For in situ hybridisation using synthetic oligonucleotides, two types of control experiment were employed.

1. Competition hybridisations - where sections/cultures were hybridised with a 25 -fold excess of the unlabelled oligonucleotide probe.

2. Sections/cultures were hybridised with a 'sense' oligonucleotide probe having the same length, base composition and specific activity as a region on the messenger RNA (mRNA).

2.5 RNA Extraction

The brains from six wistar rats (250-300g) were removed and tissue from several brain regions including the cerebellum, hippocampus, striatum, cortex and olfactory bulb was carefully dissected out and rapidly frozen on dry ice. The brain tissue was weighed individually and stored at -80°C in sterile plastic tubes until extraction.

Total RNA was extracted using the guanidinium/ hot phenol method previously described by Chomzynski and Sacchi in 1987. Under sterile conditions, tissue of predetermined weight was put into a 50ml plastic tube containing 5ml/g guanidinium isothiocyanate solution (guanidinium isothiocyanate solution = 100g guanidinium thiocyanate powder, 5% 1M Tris, pH 7.6, and 2% 0.5M EDTA made up to 94.84mls in DEPC-treated water. Heat solution to 60°C, sterile filter, and add 21.2mls 20% sarcosyl and 2.1mls β- mercaptoethanol such that the total volume = 212mls) and a few drops of antifoam. Using a polytron homogeniser at setting 7, tissue was homogenised for a few minutes. An equal volume (5ml/g) of phenol preheated to 60°C, was then added and the tissue allowed to stand in a water bath at 60°C. Following this, 2.5ml/g preheated salt solution, solution III, was added at 60°C (solution III = 100mM sodium acetate, pH 5.2, 10mM Tris/ hydrochloric acid, pH 7.4, and 1mM EDTA). Then 5ml/g chloroform, maintained at room temperature, was added to the tissue and the tube shaken vigorously for 5-10 minutes whilst in the water bath at 60°C. The tissue was then cooled on ice and centrifuged at

4000rpm for 10 minutes at room temperature. The aqueous phase was removed to another tube and re-extracted with phenol/chloroform (volume ratio = 3:1). Following this, the aqueous layer was removed to another tube and re-extracted four times with phenol/chloroform (volume ratio = 1:1) and finally extracted with chloroform alone (volume ratio = 1:1). Then 2.5 volumes of ethanol was added to precipitate the RNA. At this stage strands of RNA were visibly forming in the ethanol. After overnight storage at -20°C an RNA pellet was formed. The tube was spun for 10 minutes at 4000rpm at room temperature, and then the pellet resuspended in 50μl DEPC-treated water and diluted 1:100. The optical density at 260/280 was measured, and 10μl aliquots stored at -80°C until use.

2.6 Northern Blot Analysis

The method for northern blotting was performed according to the method previously described by Wisden et al., in 1988. Firstly a formaldehyde gel was prepared in a total volume of 150mls by heating together 1.64g agarose powder with 96.4mls DEPC-treated water in a conical flask previously baked to 180°C. Once melted, the gel was allowed to cool slightly. Following which, 23.6mls formaldehyde was added together with 30mls DEPC-treated 5 X Running buffer (5 X Running buffer = 200mM morpholinopropanesulphonic acid (MOPS), pH 7.0, 50mM sodium acetate, pH 7.0, and 5mM EDTA, the solution appears yellow after autoclaving) in a fume hood. Meanwhile the sides of an ethanol cleaned perspex gel holder were taped securely with scotch tape. The comb was attached to one end approximately 2.0mm from the base. The gel holder was then positioned in a horizontal gel electrophoresis apparatus (BIO-RAD) previously sterilised with ethanol. Warm gel was slowly

poured into the holder to ensure no bubble formation and allowed to set. Once the gel had set, the tape and the comb were removed. A 1 x MOPS solution was added to the apparatus and the gel was then pre-run at 65 volts for 1 hour. During this time brain tissue RNA samples were prepared by adding together:

10μl aliquots of p(A) + RNA

4.6μl 5 X Running buffer

3.5μl formaldehyde

10μl formamide.

The sample was vortexed and left for 15 minutes at 55^{o}C in order to denature the RNA. The sample was then chilled on ice. 4μl loading buffer (loading buffer = 50% glycerol in water, 1mM EDTA, 0.4% bromophenol blue, and 0.4% xylene cyanole made up in DEPC-treated water) was added and then the samples were allowed to run for 4 hours at 120 volts or until the bromophenol dye had migrated three-fourths down the gel. A standard DNA sample was run alongside the tissue samples as a marker. Then the gel was removed from the gel holder and placed onto clingfilm. After cutting the standard DNA sample away from the gel, it was immersed in a ethidium bromide solution (1μg/ml) for 15 minutes and visualised under ultra violet light. The gel was then photographed on an ultraviolet transilluminator, before being blotted to record marker positions. The gel to be blotted was then transferred directly onto nylon membrane. After soaking approximately ten pieces of Whatman 3MM filter paper approximately 23 x 50cm, in 10 X SSC, the filter paper was placed onto a tray. Carefully the gel was then placed upside down (i.e. sample well openings down) on top of the filter papers. Following this, a piece of hybond filter paper (nylon membrane) was cut to fit the gel and placed on top and smoothed out carefully. Then, 2 x 3MM wet filters (Whatman) were packed on top followed by a box of tissues. Finally a weight was placed over the gel to ensure good transfer. The gel was then left overnight to allow the transfer process to take place. By capillary action, the 10 X SSC would be expected to

soak up the tissues and in the process, the single strand RNA in the gel would be transferred to the underside of the nylon membrane. The RNA was then crosslinked by ultraviolet irradiation onto the nylon membrane. The blot could then be stored in the freezer at -80°C until use.

Northern blots were hybridised with oligonucleotide probes previously 3'- end labelled with the enzyme TdT and the isotope α^{32}P-dATP (6 000 Ci/mmol, Dupont) at 42°C overnight. The probes were labelled to a specific activity of 1.0×10^{10} dpm/μg. The method of probe labelling was identical to the method previously described under in situ hybridisation with ^{35}S- labelled probes, however the quantity of components within the reaction mixture were adjusted according to the radioisotope, such that the reaction mixture contained:

2.5μl 5 X buffer

1.25μl oligonucleotide

1.55μl DEPC-treated water

6.0μl ^{32}P- dATP

1.2μl TdT.

When using the isotope α^{32}P-dATP, a high energy β- particle emitter, all procedures should be shielded by perspex to avoid radiation contamination. Blots were prehybridised with 10ml hybridisation buffer for 1 hour at 40°C. Following this, the blot was hybridised with a 3'- end labelled probe overnight at 42°C in a heat-sealed plastic bag. The following day, the blot was washed in 1 X SSC at 55°C for 1 hour, then in 1 X SSC for 15 minutes at room temperature, and finally briefly rinsed in 0.1 X SSC at room temperature. The blot was then covered with cling film and smoothed. Finally the northern blot was exposed to Kodak XAR-5 autoradiographic film at -80°C with an intensifying screen for approximately 2-5 days. Development of the film was as previously described. The blot was then washed in 0.5 X Denhardt's solution, 25mM Tris-HCl, pH 7.5, and 0.1% sodium dodecyl sulphate for 60 minutes at

90-95°C, followed by a wash in distilled water at 65°C for 15 minutes. The nylon membrane was then reusable for hybridisation with another ^{32}P-labelled probe.

2.7 Primary Neuronal Culture

Striatal neurons were cultured using a modification of the methods of Thayer et al (1986), Surmeier et al (1988) and Vilijn et al (1988). Timed pregnant Wistar rats (200-300g) were killed with an overdose of anaesthetic injected intraperitonealy (Sagatal) on gestational day 17 (E17). Embryos were removed from the uterus, decapitated and placed in ice cold calcium-free Earle's Modified Eagles Medium (EMEM). Following removal of the brains from the crania, the dorsal striata of each embryo was dissected out in cold EMEM using the optic chiasm as the posterior marker (Misgeld and Dietzell, 1988). From each dissecting session the tissue was pooled from ten embryos (on average). The tissue was then minced and washed three times in cold EMEM. Cells were dissociated in 10ml of a 0.67% trypsin solution made up in sterile saline, at 37°C for 45 minutes. The trypsin was inactivated with 1.5mls heat-inactivated foetal calf serum (FCS). Following trypsinisation the cells were washed three times in cold EMEM and spun at 500g in a DAMON/IEC centrifuge for 2 minutes per wash. The cells were then resuspended in Dulbecco's Modified Eagles medium (DMEM) supplemented with 20% FCS (volume/volume), and penicillin-streptomycin solution (100 units penicillin, 100μg/ml streptomycin). The tissue was triturated in supplemented DMEM using a pasteur pipette and cells were seeded at a density of approximately 0.15 x 10^6 cells/cm^2 as monolayer cultures onto either sterile glass coverslips in 24-well plastic culture plates (Nunc) or onto 8-well multichamber glass slides

(Nunc) previously coated with poly-D-lysine (4μg/ml) for 24 hours, rinsed in EMEM and then coated with laminin (6μg/ml) for 6 hours. The cultures were grown in a humidified incubator supplied with 95% oxygen and 5% carbon dioxide. After 24 hours the medium was changed to DMEM supplemented with 10% horse serum, and on day 5 the medium was replaced with DMEM/10% horse serum containing 20μM cytosine arabinoside in order to control proliferation of glial and other non-neuronal cells. On day 9 the cytosine arabinoside was omitted. The culture medium was changed every 4 days thereafter in the 24-well culture plates used for immunocytochemistry. However for the cells seeded onto multichamber slides to be used for drug stimulation, the medium was changed to DMEM supplemented with 'Ultroser G' (Gibco, BRL), and penicillin-streptomycin solution. This was necessary to obtain serum-free conditions. Cells were generally used 12-20 days after seeding. Prior to use, cells were fixed in a 4% buffered paraformaldehyde solution.

2.8 Immunohistochemistry

Prior to immunohistochemistry, cultures were fixed in an ice cold 4% solution of buffered formalin in 0.1M phosphate-buffered saline (PBS). The cell culture wells were rinsed three times in 0.1M PBS for 10 minutes and then incubated in 0.1M PBS containing 20% normal goat serum (NGS), at room temperature for 60 minutes. The cultures were then incubated overnight with specific antibodies against neurone specific enolase (NSE), met-enkephalin (met-ENK), and glial fibrillary acidic protein (GFAP) , at dilutions of 1: 20 000, 1: 60 000, and 1: 40 000 respectively, in 50mM PBS with 1% NGS, 1.5% sodium chloride, and 0.5% Triton X-100 added. After rinsing in PBS three

times for 5 minutes per rinse, the cultures were incubated with biotinylated secondary antibody (Vector) for 60 minutes, rinsed in 0.1M PBS twice for 10 minutes, and then incubated with ABC-conjugated horse radish peroxidase (ABC Elite kit, Vector) for 60 minutes in accordance with manufacturers instructions. The peroxidase was visualised with 0.1% diaminobenzidine made up in 0.1M phosphate buffer, pH 7.2, containing 0.02% hydrogen peroxide and 0.05% nickel chloride. 50μl of filtered DAB chromogen was added to each well. A blue/grey reaction product was visible 2-15 minutes after the addition of DAB chromogen.

2.9 NADPH-Diaphorase Staining

NADPH-diaphorase histochemistry was performed according to the method of Valtschanoff et al (1992). Tissue culture slides or slide- mounted sections (50μm) of rat brain were fixed by immersion in 4% paraformaldehyde solution in 0.1M phosphate buffer, pH 7.2. Slides were preincubated in 0.1M phosphate buffer containing 0.25% Triton X-100 for 5-10 minutes and then transferred to a freshly prepared buffer-Triton solution containing 0.5 mg/ml β- NADPH and 0.2mg/ml nitro blue tetrazolium in a light-tight container. The slides were incubated in this solution at 37^{o}C for 1-2 hours. After incubation the slides were rinsed in phosphate buffer, dehydrated in serial solutions of alcohol and finally coverslipped with DPX mounting medium.

2.10 Acetylcholinesterase Staining

Adult rat striatal sections and cultured striatal neurones were collected on slides as previously described. The slides were then washed four times in distilled water for 3 minutes per wash and incubated in a modified 'Koelle' medium containing 500μg/ml copper sulphate, 750μg/ml glycine, 1.15mg/ml acetylthiocholine iodide, and 0.05mg/ml ethopropazine, in sodium acetate buffer, pH 5.0. The slides were incubated in modified 'Koelle' medium for approximately three hours at 37^{o}C. After incubation the slides were washed four times, 3 minutes per wash, in distilled water and then immersed in an ammonium sulphide solution made up in 0.5M acetic acid, for 60 seconds. Following this, the slides were washed four times in distilled water for 3 minutes each wash. The stain was then enhanced with a 0.25% silver nitrate solution for 30 seconds followed by four rinses of 3 minutes each in distilled water. Finally slides were dehydrated in 70%, 95%, and 100% ethanol respectively for 4 minutes each and coverslipped in DPX mounting medium.

2.11 Drugs and Compounds Used

Tris[hydroxymethyl] aminomethane, dimethyl sulphoxide, N,N-dimethyl formamide, ethylenediaminetetraacetic acid, (3-[N-Morpholino]propanesulfonic acid) MOPS, cyproheptadine hydrochloride, cresyl violet acetate, poly-L-lysine hydrobromide, poly-D-lysine hydrobromide, 3-hydroxytyramine (dopamine), 5-hydroxytryptamine, fluphenazine dihydrochloride, haloperidol, nickel chloride hexahydrate, nitro blue tetrazolium, hydrogen peroxide, cytosine β-D-arabinoside, DL- Dithiothreitol, diethylpyrocarbonate, acetylthiocholine iodide, ethopropazine, phorbol-12-myristate-13-acetate, polyadenylic acid potassium salt, β- nicotinamide adenine dinucleotide phosphate tetrasodium salt, glycerol, β- mercaptoethanol, diaminobenzidine, triton X-100, phorbol-12 myristate-13-acetate (Sigma). Methiothepin mesylate, mesulergine hydrochloride, (±) -1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), S(-)-eticlopride hydrochloride, (±) SKF-38393, R(+) - SCH-23390 hydrochloride, spiperone hydrochloride, (±) -8-hydroxy-2- (di-n-propylamino)-tetralin hydrobromide, 1-(3-chlorophenyl)piperazine 2-hydrochloride, (-)-quinpirole hydrochloride (RBI). Sodium chloride, sodium dihydrogen orthophosphate, disodium hydrogen orthphosphate, sodium thiosulphate, sodium citrate, DPX mountant, sodium acetate, sodium hydroxide, copper sulphate (BDH). Silica gel 4-7 mesh (Fisons). Antifoam A, polyethylene glycol, guanidinium thiocyanate, sodium N-lauroylsarcosinate, formamide, formaldehyde (Fluka). Ammonium sulphide solution, glycine, paraformaldehyde (Aldrich Chemical Co. Ltd.). Ultra pure DNA Grade Agaroses, sodium dodecyl sulphate, xylene cyanole, bromophenol blue, ethidium bromide tablets (BIO-RAD). Clozapine (Sandoz). Ondansetron (Glaxo). Sephadex G-25 medium, dextran sulphate sodium salt, terminal deoxynucleotidyl transferase (Pharmacia). Terminal deoxynucleotidyl transferase buffer (Boehringer-Mannheim). [α- ^{35}S]dATP,

^{32}P-dATP (NEN, Dupont). PGEM marker (Promega) Acetic acid, hydrochloric acid, chloroform (Prolab). Histoclear (National Diagnostics). Ethyl alcohol (Hayman Ltd.). Silver nitrate (Johnson Matthey Materials Technology U.K.). Dulbecco's Modified Eagle's medium, Earle's Modified Eagle's medium, Dulbecco's Modified Eagle's medium nutrient mixture - F12, heat-inactivated foetal calf serum, Hank's Balanced salts solution without calcium and magnesium, Ultroser G, trypsin solution (2.5%), trypsin-EDTA solution (0.25%), mouse laminin, penicillin-streptomycin solution, horse serum, normal goat serum (Gibco, BRL). Neurone specific enolase, met-enkephalin, glial fibrillary acidic protein (Affiniti). 8-well multichamber slides (Nunc).

All drug compounds used for in vivo experiments were dissolved in 0.9% physiological saline, with the exceptions of haloperidol which was dissolved in 0.2% acetic acid , fluphenazine dihydrochloride, and clozapine which were dissolved in 0.5% dimethylsulphoxide.

All drug compounds used for in vitro experiments were dissolved in DEPC-treated water, with the exceptions of haloperidol which was dissolved in 0.2% acetic acid, fluphenazine dihydrochloride and clozapine which were dissolved in 0.5% dimethylsulphoxide, KT5720, calphostin C, and phorbol-12-myristate-13 acetate which were dissolved in dimethylsulphoxide. The stock drug solutions were then diluted in Hank's Balanced Salts Solution, to obtain the final concentration.

2.12 'In vivo' Experimental Design

2.12.1 Acute Drug Treatment

Four groups of male Wistar rats (180-250g) were treated acutely with vehicle (isotonic saline, n=5), haloperidol (1mg/kg, n=3), fluphenazine (3mg/kg, n=3; 6mg/kg, n=3), clozapine (3mg/kg, n=5). Drugs were delivered by intraperitoneal injection in volumes of 1ml/kg of body weight at staggered 5 minute time intervals. After 30 minutes the animals were killed by stunning and decapitation, the brains rapidly removed and placed onto dry ice. The brains were then processed for the detection of mRNA encoding the transcription factors c-fos, zif/268, jun-B and jun-D using in situ hybridisation histochemistry.

Five groups of male wistar rats (180-250g) were treated for a 24 hour time period with vehicle (physiological saline, n= 5), haloperidol (1mg/kg, n= 3), fluphenazine (3mg/kg, n= 3), clozapine (3mg/kg, n= 6), and methiothepin (10mg/kg, n= 3). Drugs were injected intraperitonealy (1ml/kg of body weight) at staggered time intervals. After 24 hours, the animals were killed by stunning and decapitation and their brains processed for the detection of mRNA encoding a specific region of the preproenkephalin, preproneuropeptide Y, preprosomatostatin, preprotachykinin, RHS2 and BF-1 peptides, using in situ hybridisation histochemistry.

2.12.2 Chronic Drug Treatment

Three groups of male Wistar rats (250-280g) were chronically treated for 5 days with vehicle (osmotic mini pump containing vehicle, n= 4), clozapine (3mg/kg, n= 4), and fluphenazine (3mg/kg, n= 4). The drugs were administered

via osmotic mini pumps (Alzet). Freshly prepared drug solutions were carefully injected into the osmotic mini pumps according to the manufacturers instructions. The animals were anaesthetised with equethesin (4ml/kg) and the osmotic mini pumps inserted subcutaneously into the hind limb region. Drug release was moderated by the pump to 1μl/ hour. Animals were weighed on the second, third and fifth day after insertion of the osmotic mini pumps and checked regularly for any symptomatic effects of the drugs. After five days the animals were killed by stunning and decapitation, and their brains rapidly processed for the detection of mRNAs encoding preprosomatostatin, and also the dopaminergic D1 receptor and the serotoninergic 5-HT6 receptor.

2.12.3 Time Course Experiments

Male Wistar rats (180-250g) were treated with vehicle (n= 2 per time point) or either fluphenazine (6mg/kg) or clozapine (3mg/kg) for the following time periods: 15 minutes (n= 3), 30 minutes (n= 3), 45 minutes (n= 3), 90 minutes (n= 3) and 3 hours (n= 3). Both drugs were delivered by intraperitoneal injection in volumes of 1ml/kg. After the specified times, the animals were killed by stunning and decapitation, and their brains rapidly frozen on dry ice. The brains were then processed for the detection of mRNA encoding the transcription factors c-fos, jun-B, and zif/268, and the late response genes proenkephalin and preprosomatostatin.

2.13 'In vitro' Experimental Design

All striatal neurone cultures were treated with drugs for 45 minutes at 37°C. The drugs were delivered directly to the bathing medium in 20μl volumes, making the assumption that the total volume bathing the cells was 0.4ml. After 45 minutes the cells were fixed in a 4% buffered paraformaldehyde solution, rinsed in 1 X PBS, dehydrated in ethanol and processed for in situ hybridisation as previously described.

2.13.1 Time Course Experiment In Vitro

Striatal neurone cultures were treated with haloperidol (final bath concentration 2μM) administered to each well of an 8-well multichamber slide for the following time intervals: 90 minutes (n= 3), 60 minutes (n= 3), 45 minutes (n= 3), 30 minutes (n= 3), 20 minutes (n= 3), 10 minutes (n= 3), 5 minutes (n= 3), and at time zero (n= 3). The slides were then fixed and processed for the detection of mRNA encoding the transcription factors zif/268, jun-B and c-fos.

2.13.2 Treatment with Agonist Compounds

Striatal neurone cultures were treated with vehicle (Hank's Balanced Salts Solution), the dopaminergic agonists SKF 38393 (final bath concentration 1nM-5μM), dopamine (final bath concentration 1nM-5μM), and quinpirole (final bath concentration 1μM), the serotoninergic agonists 8-hydroxy-DPAT (final bath concentration 1nM-5μM), mCPP (final bath concentration 1nM-5μM), ±-DOI (final bath concentration 1nM-5μM) and the protein kinase C

stimulating drug phorbol-12- myristate-13-acetate (final bath concentration 10^{-7}M) at 37°C. After 45 minutes the cells were fixed and processed for the detection of c-fos and zif/268 mRNA using in situ hybridisation.

2.13.3 Treatment with Antagonist Compounds

Striatal neurone cultures were pretreated for 30 minutes with the dopaminergic antagonist compounds SCH 23390 (final bath concentration 1μM), eticlopride (final bath concentration 1μM), the serotoninergic antagonist compounds cyproheptadine (final bath concentration 500nM), ondansetron (final bath concentration 200nM), methiothepin (final bath concentration 200nM), mesulergine (final bath concentration 30, 100nM), clozapine (final bath concentration 100nM), and spiperone (final bath concentration 30,100nM) prior to the addition of SKF 38393 (final bath concentration 1nM-5μM) or 5-hydroxytryptamine (final bath concentration 1nM-5μM) respectively. Similarly cultures were pretreated for 30 minutes with the protein kinase A inhibitor, KT5720 (final bath concentration 2μM) and the protein kinase C inhibitor, calphostin C (final bath concentration 1μM) prior to the addition of SKF 38393 (final bath concentration 1μM). Cultures treated with calphostin C were left under conventional room lighting for the first five minutes of the incubation period in order to activate this compound (Bruns et al., 1991). After 45 minutes the cells were fixed and processed for the detection of c-fos and zif/268 mRNA using in situ hybridisation.

2.14 Dopamine Receptor Antisense Experiment

Striatal neurone cultures were treated for 2 days at 37°C with 15-mer antisense oligonucleotides complementary to the D1 dopamine receptor and to the D5 dopamine receptor. The antisense oligonucleotides were delivered in a volume of 0.5μl, such that the final bath concentration was 1μM. As an appropriate control for specificity of effects of these oligonucleotides, cultures were also treated with a D1 sense oligonucleotide, identified as having the same sequence as a region of the D1 receptor mRNA, administered at a concentration of 1μM. After 2 days, the cultures were acutely treated with SKF 38393 (1nM-5μM) for 45 minutes, at 37°C . Slides were fixed and then processed for the detection of both c-fos and zif/268 mRNA using in situ hybridisation.

2.15 Image Analysis and Statistics Used

To quantitate any drug induced changes in the levels of mRNA for the transcription factors c-fos, zif/268, jun-B, jun-D, RHS2, BF-1, for the neuropeptides preproenkephalin, preprotachykinin, preprosomatostatin, proneuropeptide Y, and for the 5-HT6, and dopamine D1 receptors, initially densitometry of film images was done using the MCID SCIR image analysis system. Bilateral measurements of mean relative optical density (R.O.D.) were taken from brain sections on autoradiographic film, in the regions of the striatum and nucleus accumbens from two sections per animal. For cell culture analysis, four measurements of mean R.O.D. were taken from each well of an 8-well multichamber slide exposed to film. For a higher cellular resolution quantification the density of silver grains were measured after dipping slides in liquid photographic emulsion using a Macintosh image analysis system ("image

v1.52" software [NIH]). For a single culture well, a total of fifteen measurements of mean number of pixels occupied by silver grains per unit area, were taken from three fields of view.

Following this, analysis of variance ANOVA, (one-way unstacked or two-way stacked) was performed on the data for each drug treatment (Minitab). The Dunnett's post-hoc test was then used to assess statistical significance of any drug induced changes between individual groups in all experiments unless stated otherwise. However, for the in vivo experimental work on immediate early genes it was necessary to use the more stringent statistical test, the Mann-Whitney U test. Two-way ANOVA, general linear model (Minitab), was used to analyse in vitro drug dose response curves in the presence and absence of antagonist compounds.

Chapter 3

Regulation of Late Response Gene Expression in vivo

INTRODUCTION

3.0 Neuroleptic Modulation of Late Response Genes In Vivo

It has been known for some time that neuroleptics act by blocking dopamine receptors and are clinically effective in the treatment of a number of diseases which are thought to originate from a dopaminergic dysfunction in the central nervous system. Disease states such as schizophrenia and Parkinson's disease are known to be manifestations arising from a dysfunction of the dopaminergic system within the brain. Blockade of dopamine receptors in the striatum by neuroleptic drugs such as haloperidol have since been shown to cause an elevation in the levels of preproenkephalin mRNA and its corresponding protein product (Hong et al., 1978; Sabol et al., 1983; Tang et al., 1983; Angulo et al., 1986; Sivam et al., 1986; Romano et al., 1987; Morris et al., 1988). Conversely, neuroleptic drug treatment has been shown to reduce the levels of the brain neuropeptide substance P in the striatum (Bannon et al., 1986).

However, most of this research has been performed under conditions of chronic drug administration, probably due to the fact that neuroleptic drug treatment is clinically effective only after prolonged administration. At present there is virtually no information on the acute effects of neuroleptic drugs on late response genes in the region of the striatum. With the rapid progression of research into immediate early genes and the recent findings that some typical neuroleptic drugs can elevate the levels of certain immediate early genes including c-fos, jun-B and zif/286 in the striatum (Nguyen et al., 1992), the acute effects of neuroleptics may provide a greater understanding of the sequence of events following dopamine receptor blockade.

The atypical neuroleptics such as clozapine, which are known to produce less extrapyramidal side effects (Ayd, 1961; Claghorn et al., 1983),

and are therefore an improved treatment for certain disease states, have been shown to exert different effects on certain late response genes in comparison to typical neuroleptics drugs. For example the typical neuroleptic, haloperidol, is known to decrease the levels of preprosomatostatin mRNA in the striatum and accumbens (Salin et al., 1989). Whereas the atypical neuroleptic, clozapine, has been shown to increase the levels of somatostatin mRNA in the nucleus accumbens (Saller et al., 1989). This differential regulation of gene expression by atypical compared to typical neuroleptics, may contribute to the different clinical profile of drugs such as clozapine. It is known that clozapine is an anomoloy, in that it has a much higher affinity for the dopamine D 4 receptor than the typical neuroleptic drugs (Van Tol et al., 1991). Therefore it is of great interest to understand further the complete profile of clozapine in terms of its effect on late response genes and the possible receptor mechanisms involved in the actions of this class of drugs.

RESULTS

Short-term Regulation of Proenkephalin Gene Expression in vivo

3.1 Early time course of Fluphenazine on Proenkephalin mRNA expression

Following intraperitoneal administration of fluphenazine (6mg/kg) to male wistar rats (n= 3), the levels of proenkephalin mRNA were not significantly changed 15, 30, 45, 90, and 180 minutes after drug administration, compared to saline treated control animals (n= 2 per treatment group) injected at the corresponding time points. It can be seen that the mRNA levels for the enkephalin gene in the rostral striatum [Figure3.1.1] were not significantly affected by fluphenazine (6mg/kg) at time periods up to three hours. A similar lack of effect was observed in the caudal striatum after treatment with fluphenazine (6mg/kg) compared to saline treated control animals [Figure 3.1.2].

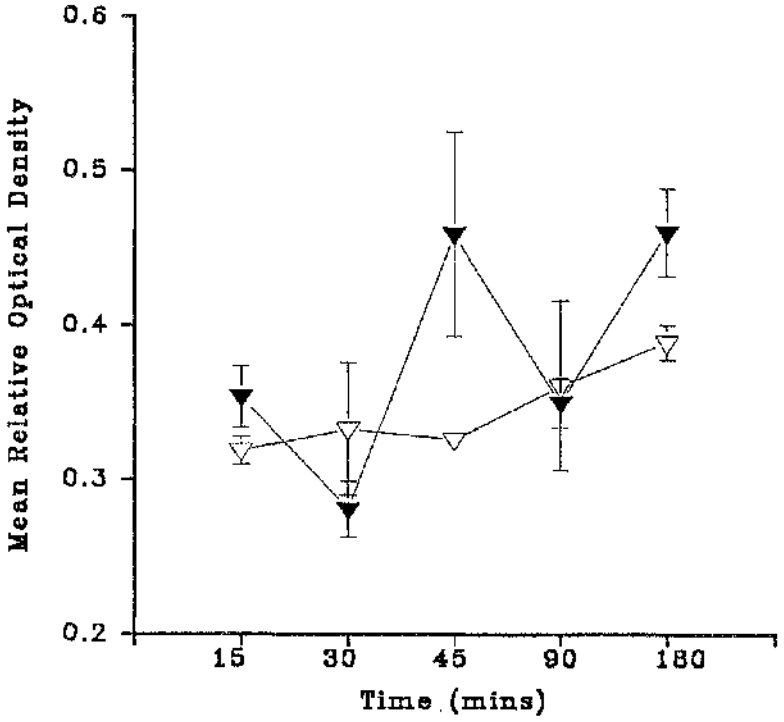
3.2 Effect of acute Antipsychotic drug treatment on Proenkephalin mRNA levels in the caudal striatum 24 hours later

Chronic treatment with neuroleptics is well known to cause an increase in the

levels of the enkephalin gene in the region of the striatum (Sabol et al., 1983; Tang et al., 1983; Angulo et al., 1986; Sivam et al., 1986; Romano et al., 1987; Morris et al., 1988). Therefore we were interested in the acute effects of neuroleptic drug treatments on the expression of the enkephalin gene after 24 hours.

Figure 3.1.1 : Effect of fluphenazine on preproenkephalin mRNA levels in the rat rostral striatum. Fluphenazine (6mg/kg, n= 3, closed triangles) or vehicle (0.9% saline, n= 2, open triangles) were injected i.p. and the animals killed after the specified time intervals i.e.- 15, 30, 45, 90, and 180 minutes, and their brains processed for in situ hybridisation. Results, obtained from film autoradiographs, are expressed as mean relative optical density ± s.e.m.

Figure 3.1.2 : Effect of fluphenazine (6mg/kg) on preproenkephalin mRNA levels in rat caudal striatum. Fluphenazine (6mg/kg; n= 3, closed triangles) or vehicle (0.9%; n= 2, open triangles) were injected i.p., the animals killed after the specified time intervals i.e- 15, 30, 45, 90 and 180 minutes, and their brains processed for in situ hybridisation. Results, obtained from film autoradiographs, are expressed as mean relative optical density ± s.e.m.


0.6
0.5
0.4
0.3
0.2
Mean Relative Optical Density
15
30
45
90
180
Time (mins)

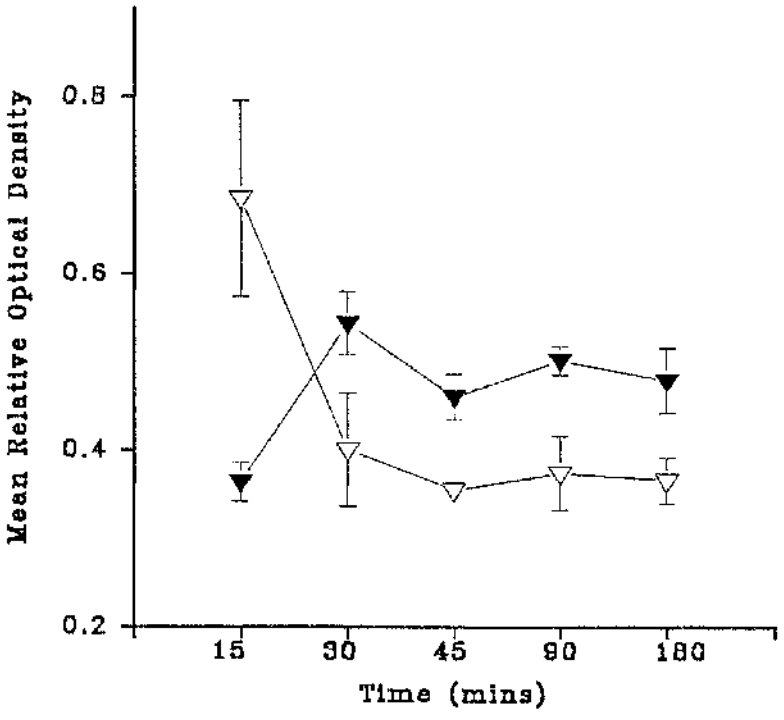
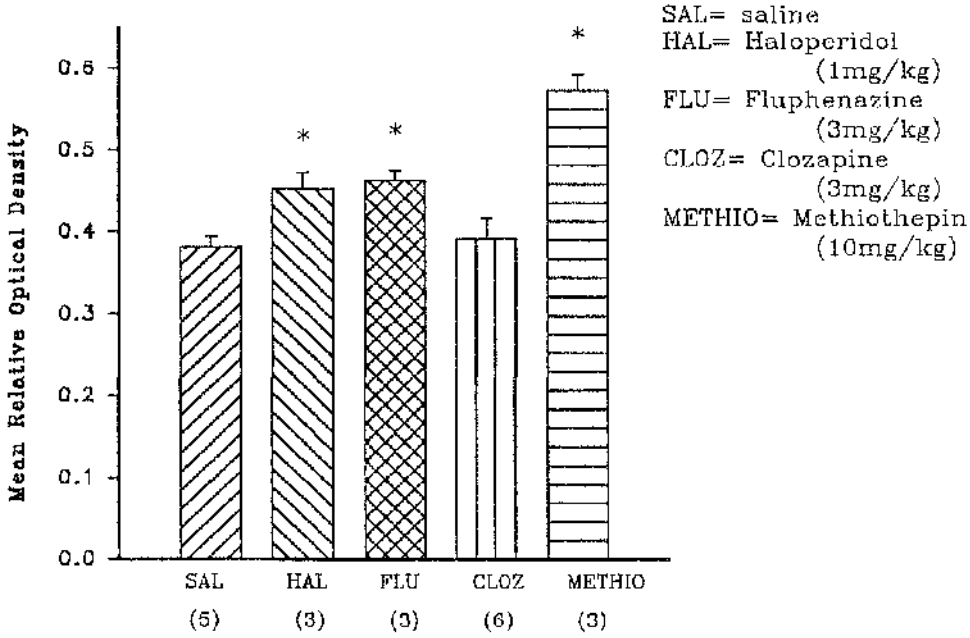

0.8
0.6
0.4
0.2
Mean Relative Optical Density
15
30
45
90
180
Time (mins)

Figure 3.2.1 : Effect of acute neuroleptic drug treatment on the expression of proenkephalin mRNA in the rat caudal striatum. Animals were injected i.p. with vehicle (0.9% saline), haloperidol (1mg/kg), fluphenazine (3mg/kg), clozapine (3mg/kg), or with methiothepin (10mg/kg). After 24 hours the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m.; the number of animals per group is shown in parentheses. Statistical significance was determined by one way ANOVA followed by post-hoc Dunnett's test. $p < 0.05$* relative to saline group.


Mean Relative Optical Density
0.0
0.1
0.2
0.3
0.4
0.5
0.6
*
*
*
SAL
HAL
FLU
CLOZ
METHIO
(5)
(3)
(3)
(6)
(3)
SAL= saline
HAL= Haloperidol (1mg/kg)
FLU= Fluphenazine (3mg/kg)
CLOZ= Clozapine (3mg/kg)
METHIO= Methiothepin (10mg/kg)

In saline treated animals a moderate hybridisation signal for preproenkephalin was observed in the caudal striatum. Typical neuroleptic drug treatments, together with methiothepin were demonstrated to significantly increase the levels of proenkephalin mRNA in the caudal striatum [$F(4, 15) = 14.40$, $p = 0.001$]. Following intraperitoneal administration of the typical neuroleptic haloperidol (1mg/kg, n= 3) a significant induction in the levels of preproenkephalin mRNA were seen ($p < 0.05$) [Figure 3.2.1]. A similar increase was observed following treatment with fluphenazine (3mg/kg, n= 3), and this induction was shown to be significantly different from saline treated control rats (n= 5), such that $p < 0.05$ [Figure 3.2.1]. The atypical neuroleptic clozapine (n= 6) had no effect on the expression of the enkephalin gene. However, following administration of the non-selective 5-HT1/2 receptor antagonist, methiothepin (10mg/kg, n= 3), also having a relatively high affinity for the dopamine D2 receptor, the levels of preproenkephalin were significantly induced such that $p < 0.05$ [Figure 3.2.1].

Short-term regulation of preprotachykinin gene expression in the rat striatum

3.3 Effect of acute drug treatment on the expression of preprotachkyinin mRNA levels in the rat striatum 24 hours later

It has previously been reported that chronic treatment with typical neuroleptics can decrease the levels of preprotachykinin mRNA in the rat striatum (Bannon et al., 1986). In this study we have investigated the acute effects of typical and atypical neuroleptic drugs on preprotachykinin gene expression.

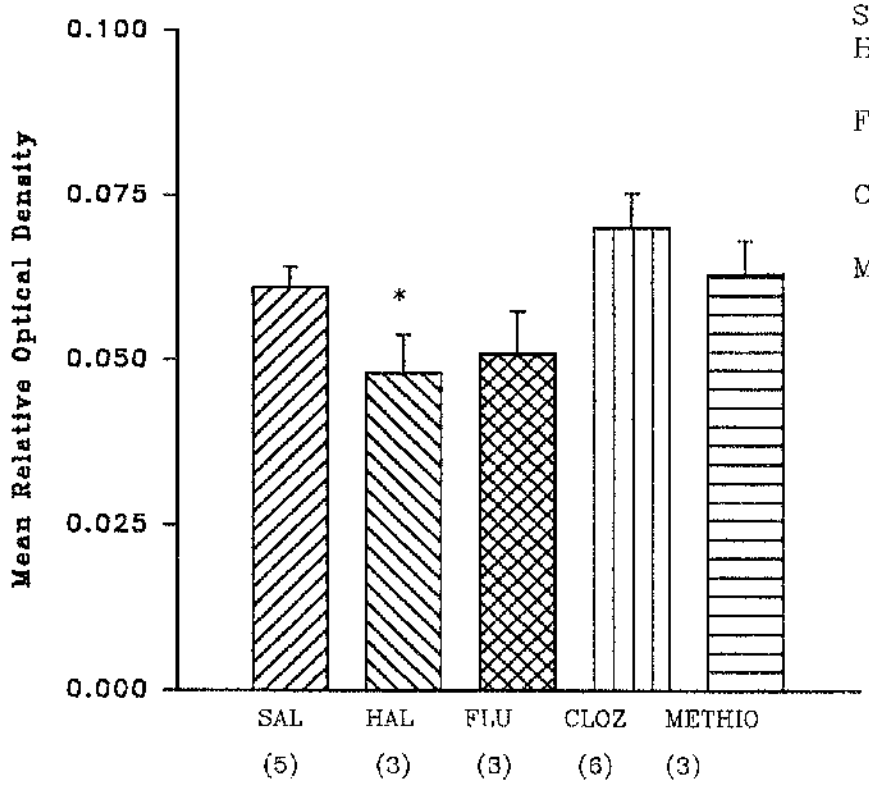
Following intraperitoneal injections of saline (n= 5), or haloperidol (1mg/kg; n= 3), or fluphenazine (3mg/kg; n= 3), or clozapine (3mg/kg; n= 6), or methiothepin (10mg/kg; n= 3), the levels of preprotachykinin mRNA were measured in the rat striatum 24 hours after drug administration.

Analysis of variance revealed that the typical neuroleptics had an effect on the level of preprotachykinin mRNA in both the rostral [$F(4, 15) = 6.92$, $p < 0.001$] and caudal [$F(4, 15) = 3.82$, $p < 0.05$] striatum. Haloperidol (1mg/kg, n= 3) caused a reduction in the levels of preprotachykinin mRNA in the rostral striatum, compared to saline treated control animals. This decrease was shown to be significantly different from the levels expressed in saline treated controls ($p < 0.05$) [Figure 3.3.1]. Fluphenazine (3mg/kg, n= 3) had a tendency to decrease preprotachykinin mRNA levels in the rostral striatum, although this decrease was not found to be significant. The atypical neuroleptic clozapine (3mg/kg, n= 6) or methiothepin (10mg/kg, n= 3) had no effect on preprotachykinin mRNA levels after 24 hours in the rostral striatum. [Figure 3.3.1]

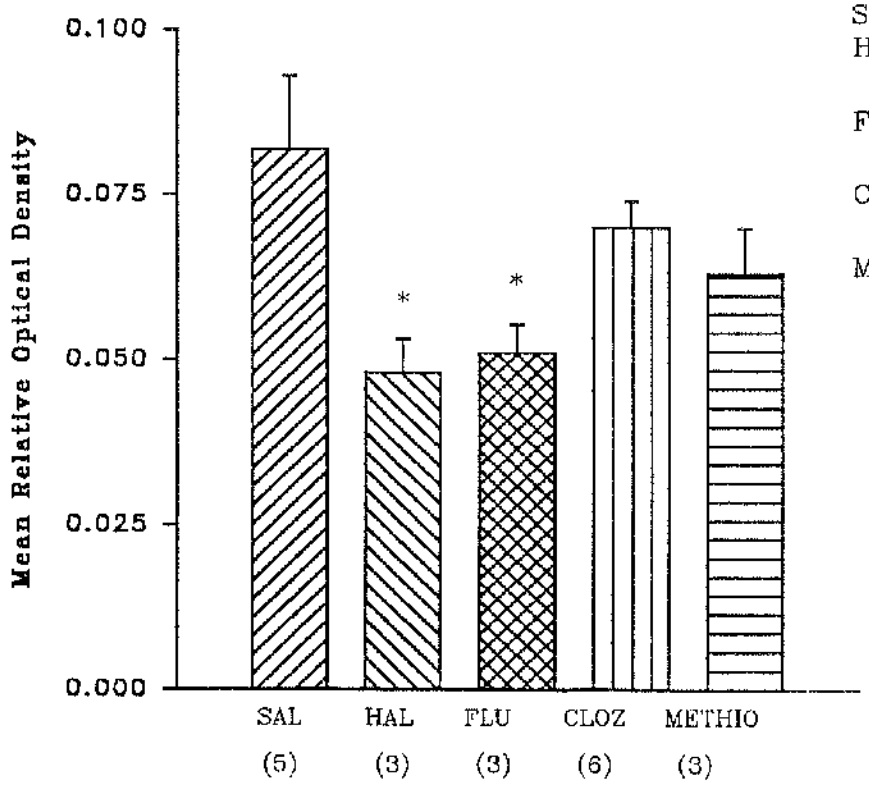
A similar significant reduction in the levels of preprotachykinin mRNA compared to saline treated controls, was observed in the caudal striatum 24 hours after treatment with haloperidol (1mg/kg, n= 3) ($p < 0.05$) [Figure 3.3.2]. Fluphenazine (3mg/kg, n= 3), also caused a significant decrease in preprotachykinin mRNA levels in the caudal striatum, such that $p < 0.05$ [Figure 3.3.2]. However, clozapine (3mg/kg, n= 6) and methiothepin (10mg/kg, n= 3) had no effects on the levels of preprotachykinin mRNA expressed in the rat caudal striatum. [Figure 3.3.2].

Figure 3.3.1 : Effect of acute neuroleptic drug treatment on the expression of preprotachykinin mRNA in the rat rostral striatum. Animals were injected i.p. with vehicle (0.9% saline), or haloperidol (1mg/kg), or fluphenazine (3mg/kg), or clozapine (3mg/kg) or with methiothepin (10mg/kg). After 24 hours the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m.; the number of animals per group is shown in parentheses. Statistical significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test. $p < 0.05$* relative to saline group.

Figure 3.3.2 : Effect of acute neuroleptic drug treatment on the expression of preprotachykinin mRNA in the rat caudal striatum. Animals were injected i.p. with vehicle (0.9% saline), or haloperidol (1mg/kg), or fluphenazine (3mg/kg), or clozapine (3mg/kg) or with methiothepin (10mg/kg). After 24 hours the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m.; the number of animals per group is shown in parentheses. Statistical significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test. $p < 0.05$* relative to saline group.



SAL= saline
HAL= Haloperidol (1mg/kg)
FLU= Fluphenazine (3mg/kg)
CLOZ= Clozapine (3mg/kg)
METHIO= Methiothepin (10mg/kg)



SAL= saline
HAL= Haloperidol (1mg/kg)
FLU= Fluphenazine (3mg/kg)
CLOZ= Clozapine (3mg/kg)
METHIO= Methiothepin (10mg/kg)

Short-term regulation of preprosomatostatin gene expression in the rat striatum

3.4 Effect of acute drug treatment on the levels of preprosomatostatin mRNA in the caudal striatum and in the nucleus accumbens 24 hours later

Previous studies have suggested that high doses of clozapine can cause an induction of preprosomatostatin mRNA in the rat nucleus accumbens (Salin et al, 1989). Therefore in this study we have investigated the effect of acute (24 hour) treatment with low doses of clozapine, together with two typical neuroleptics haloperidol and fluphenazine, on the expression of preprosomatostatin mRNA.

The levels of preprosomatostatin mRNA in the caudal striatum were not changed by the administration of haloperidol (1mg/kg, n= 3) or fluphenazine (3mg/kg, n= 3) compared to saline treated control animals (n= 5). However, following treatment with clozapine (3mg/kg, n= 6) or methiothepin (10mg/kg, n= 3), an increase in the level of preprosomatostatin mRNA was observed in the region of the caudal striatum. This induction was shown to be significantly different from saline treated control animals [$F(4, 15) = 9.46$, $p = 0.01$, $p < 0.05$] [Figures 3.4.1]. The effect of clozapine (3mg/kg, n= 6) on the expression of preprosomatostatin in the caudal striatum was significantly different from control animals, such that $p < 0.05$ [Figure 3.4.1]. This acute effect of clozapine can also be seen in figure 3.4.2, as an increase in the number of silver grains over cell bodies in the striatum expressing the somatostatin gene. Treatment with methiothepin (10mg/kg, n= 3), caused a greater increase in the levels of preprosomatostatin mRNA in the rat caudal striatum than did

clozapine, and this increase was significantly different from control animals ($p< 0.01$) [Figure 3.4.1].

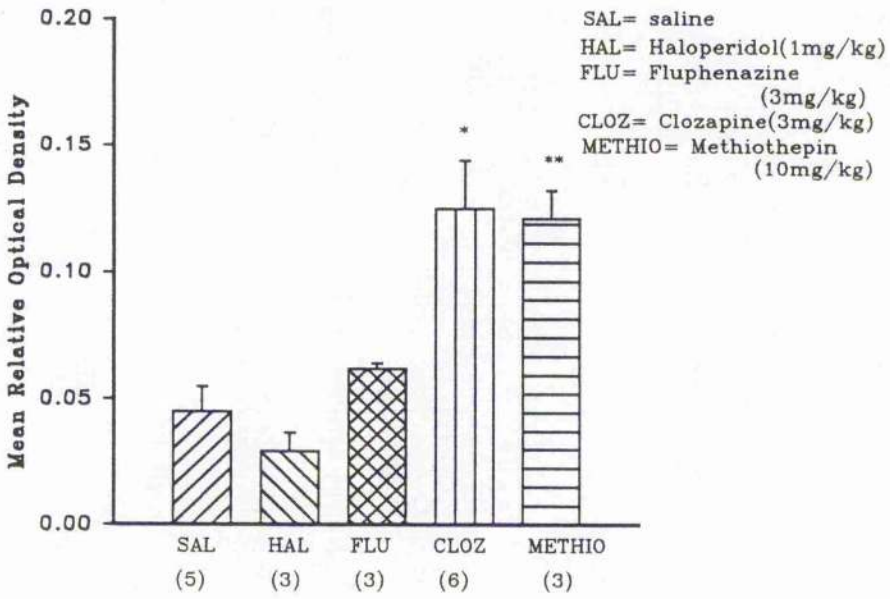
A similar pattern of induction was observed in the rat nucleus accumbens. Analysis of variance revealed that the drugs under investigation caused an induction in somatostatin gene expression after 24 hours [$F(2, 9) = 7.97$, $p= 0.006$, $p< 0.01$]. Clozapine (3mg/kg, n= 6) caused an increase in the expression of preprosomatostatin mRNA in the core region of the accumbens. This increase was significantly different from the levels expressed in saline treated rats ($p< 0.01$). [Table 1]. Similarly administration of methiothepin (10mg/kg, n= 3) resulted in an induction of preprosomatostatin mRNA in the core region 24 hours after administration ($p< 0.01$) [Table 1].

Drug Treatment	Nucleus Accumbens	
Saline	11.10 ± 0.82	
Clozapine	22.63 ± 2.60	**
Methiothepin	18.15 ± 1.50	**

Table 1: The expression of preprosomatostatin mRNA in the rat nucleus accumbens following a 24 hour treatment with vehicle (physiological saline), clozapine (3mg/kg, n= 6), or with methiothepin (10mg/kg, n= 3). Results, obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by one-way ANOVA, followed by *post-hoc* Dunetts t-test. $p< 0.01$ **

Figure 3.4.1 : Effect of acute neuroleptic drug treatment on the expression of preprosomatostatin mRNA in the rat caudal striatum. Animals were injected i.p. with vehicle (0.9% saline), haloperidol (1mg/kg), fluphenazine (3mg/kg), clozapine (3mg/kg), or with methiothepin (10mg/kg). After 24 hours the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m.; the number of animals per group is shown in parentheses. Statistical significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test. $p < 0.05$* $p < 0.01$** relative to saline group.

Figure 3.4.2 : Effect of acute treatment with clozapine on the expression of preprosomatostatin mRNA in the rat striatum. Photomicrographs show the distribution of silver grains over cell bodies in the caudal striatum. On the left, the animal was injected i.p with vehicle (0.9% saline) (A) and on the right, with clozapine (3mg/kg) (B) Animals were killed after 24 hours, following which their brains were processed for in situ hybridisation. Scale bar represents 25μm.


Mean Relative Optical Density
0.20
0.15
0.10
0.05
0.00
SAL= saline
HAL= Haloperidol(1mg/kg)
FLU= Fluphenazine (3mg/kg)
CLOZ= Clozapine(3mg/kg)
METHIO= Methiothepin (10mg/kg)
*
**
SAL (5)
HAL (3)
FLU (3)
CLOZ (6)
METHIO (3)

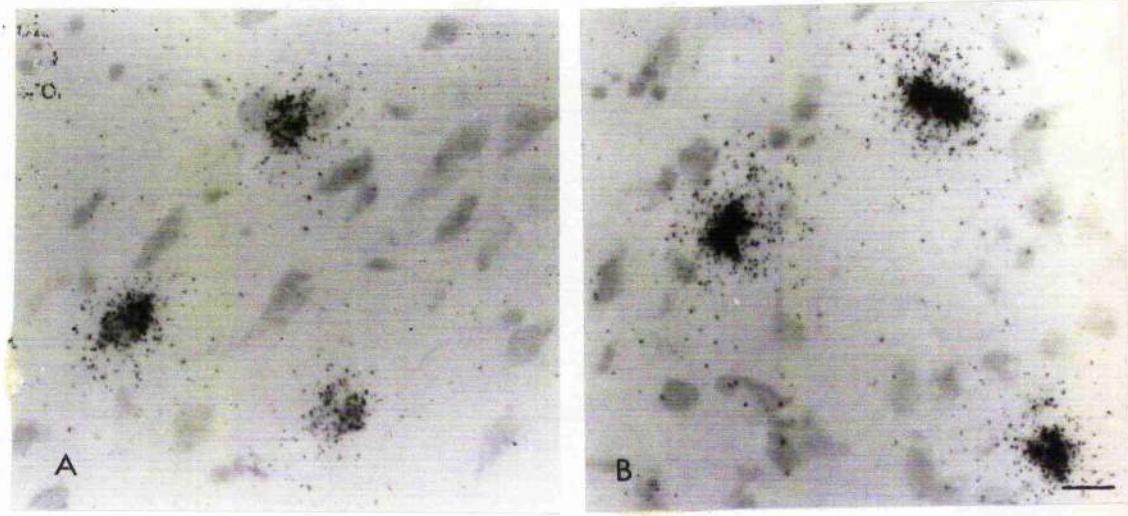
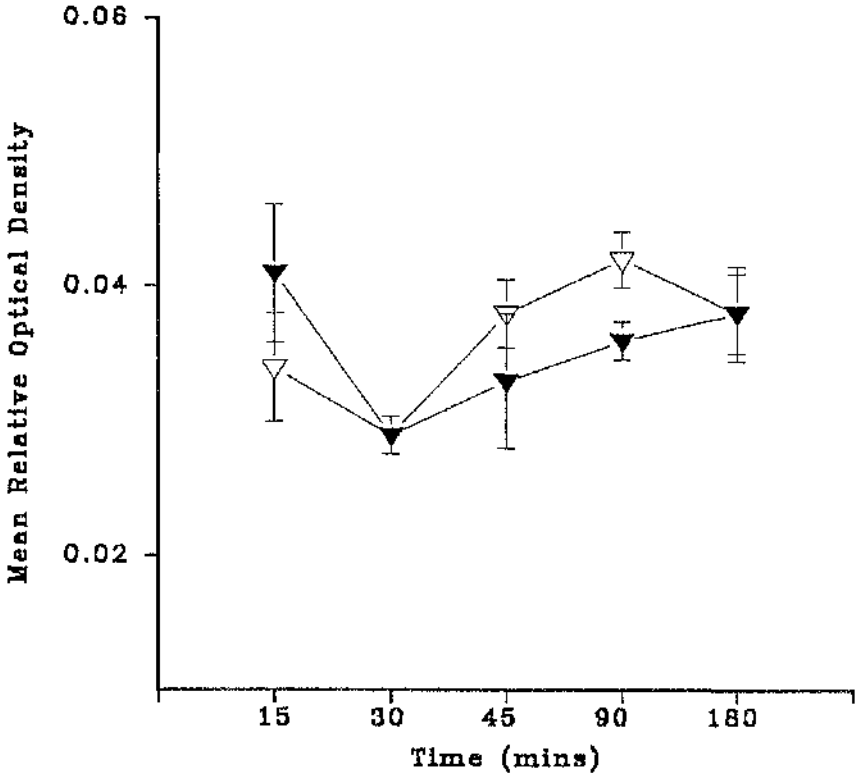
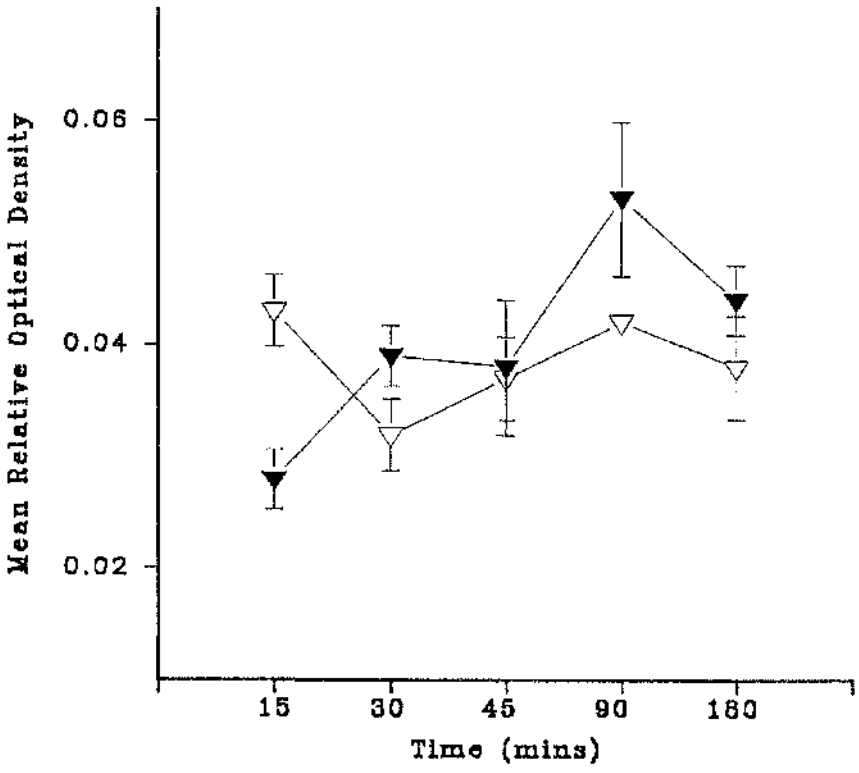

A
B

Figure 3.5.1 : Time course effect of clozapine on somatostatin mRNA levels in the rat rostral striatum. Clozapine (3mg/kg; n= 3, closed triangles) or vehicle (0.9% saline; n= 2, open triangles) were injected i.p., the animals killed after the specified time intervals i.e.- 15, 30, 45, 90, and 180 minutes, and their brains processed for in situ hybridisation. Results, obtained from film autoradiographs, are expressed as mean relatove optical density ± s.e.m.

Figure 3.5.2 : Time course effect of clozapine on somatostatin mRNA levels in the rat caudal striatum. Clozapine (3mg/kg; n= 3, closed triangles) or vehicle (0.9% saline; n= 2, open triangles) were injected i.p., the animals were killed after the specified time intervals i.e.- 15, 30, 45, 90, and 180 minutes, and their brains processed for in situ hybridisation. Results, obtained from film autoradiographs, are expressed as mean relative optical density ± s.e.m.


0.06
0.04
0.02
Mean Relative Optical Density
15
30
45
90
180
Time (mins)


0.06
0.04
0.02
Mean Relative Optical Density
15
30
45
90
180
Time (mins)

3.5 The early time course of the clozapine effect on the expression of preprosomatostatin mRNA in the rat striatum

The levels of preprosomatostatin mRNA following treatment with saline (n= 2) or clozapine (3mg/kg; n= 3) were not significantly different after the following time points: 15, 30, 45, 90, 180 minutes, in either the rostral striatum [Figure 3.5.1] or caudal striatum [Figure 3.5.2] in the rat.

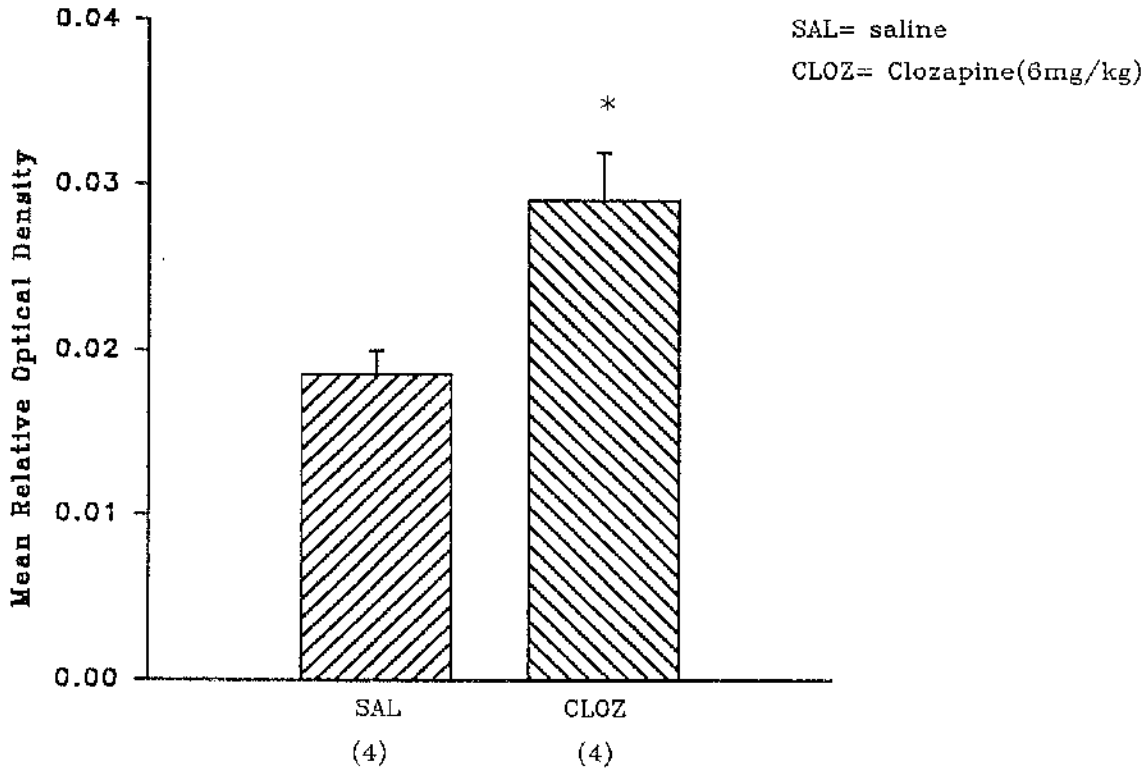
Long-term regulation of somatostatin gene expression in the rat striatum and nucleus accumbens

3.6 Effect of low doses of clozapine given chronically, on the expression of preprosomatostatin mRNA in the rat striatum and olfactory tubercle

Animals were treated with either saline (n= 4) or clozapine (6mg/kg /day, n= 4), administered via an osmotic mini pump for 5 consecutive days, following which the levels of preprosomatostatin mRNA were measured by in situ hybridisation. Moderate levels of preprosomatostatin mRNA were expressed in saline treated animals, in a small population of cells known to express the somatostatin gene in both the rostral and caudal striatum. Following chronic treatment with clozapine (6mg/kg, n= 4), an increase in the level of preprosomatostatin mRNA was observed in both the rostral and caudal striatum. The induction of preprosomatostatin mRNA in the rostral region was shown to be significantly different from the levels expressed in saline treated rats [$F(1, 6) = 7.92$, $p=0.022$, $p< 0.05$] [Figure 3.6.1]. A similar, and highly

Figure 3.6.1 : Effect of chronic treatment with clozapine on the expression of preprosomatostatin mRNA in the rat rostral striatum. Clozapine (6mg/kg) or vehicle (0.9% saline) was administered via an osmotic mini pump for 5 days. Following this, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m. The number of animals per group are shown in parentheses. Significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test. $p < 0.05$* relative to saline group.

Figure 3.6.2 : Effect of chronic treatment with clozapine on the expression of preprosomatostatin mRNA in the rat caudal striatum. Clozapine (6mg/kg) or vehicle (0.9% saline) was administered via an osmotic mini pump for 5 days. Following this, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m. The number of animals per group are shown in parentheses. Significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test. $p < 0.01$** relative to saline group.


0.04
0.03
0.02
0.01
0.00
Mean Relative Optical Density
*
SAL
CLOZ
(4)
(4)
SAL= saline
CLOZ= Clozapine(6mg/kg)

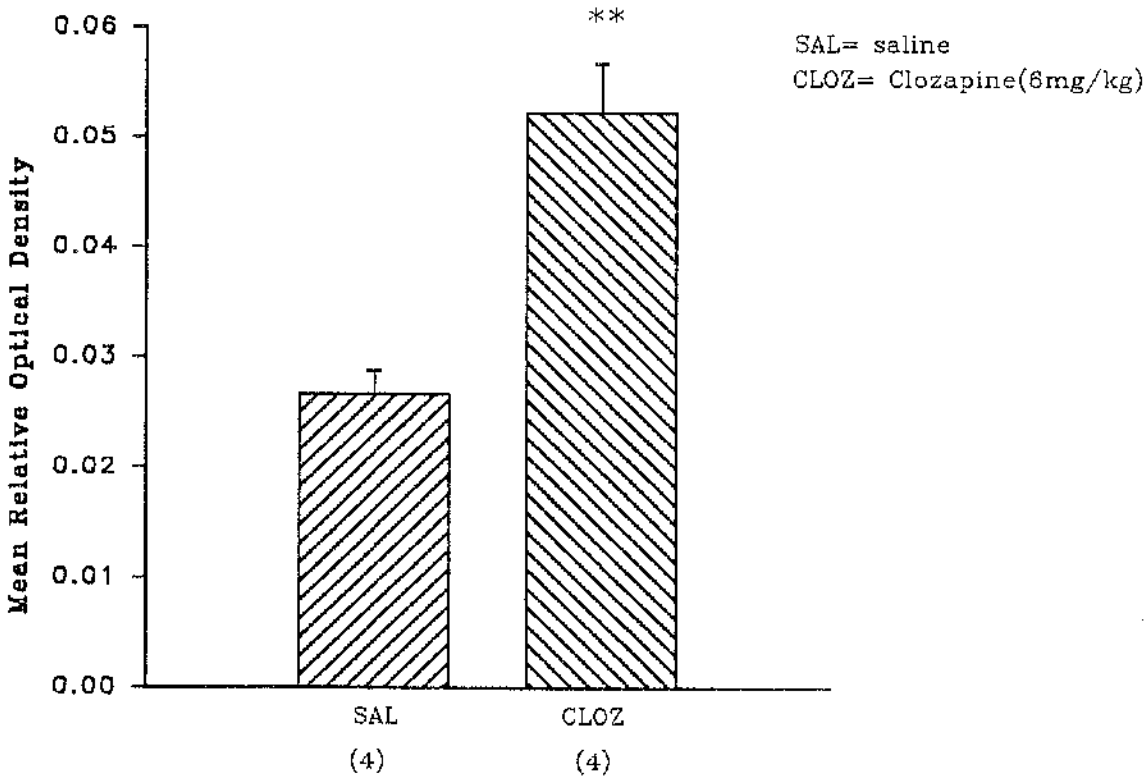
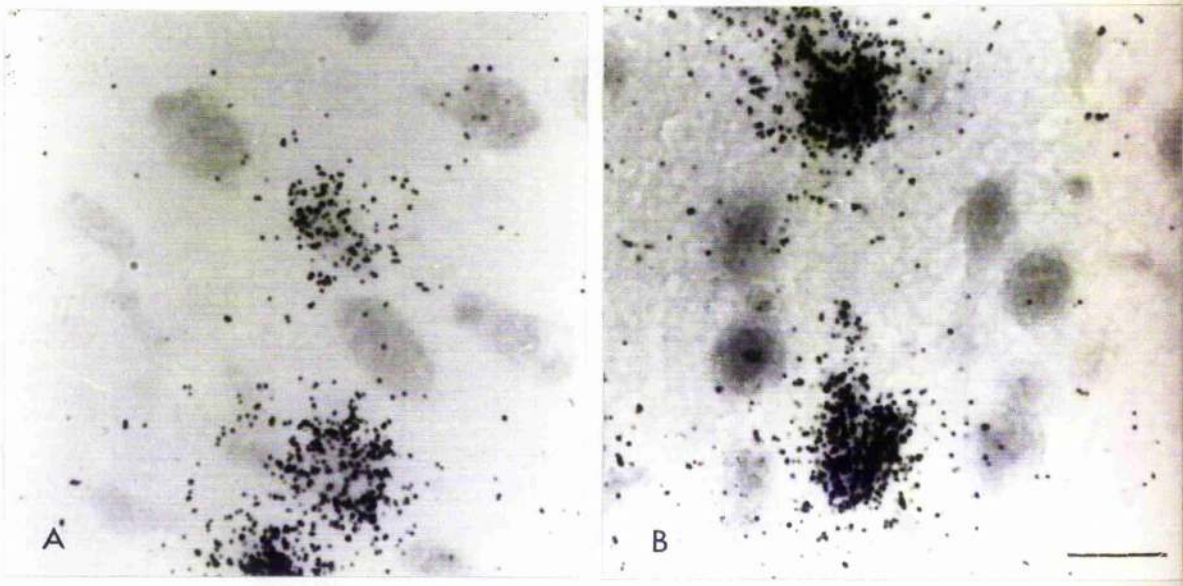
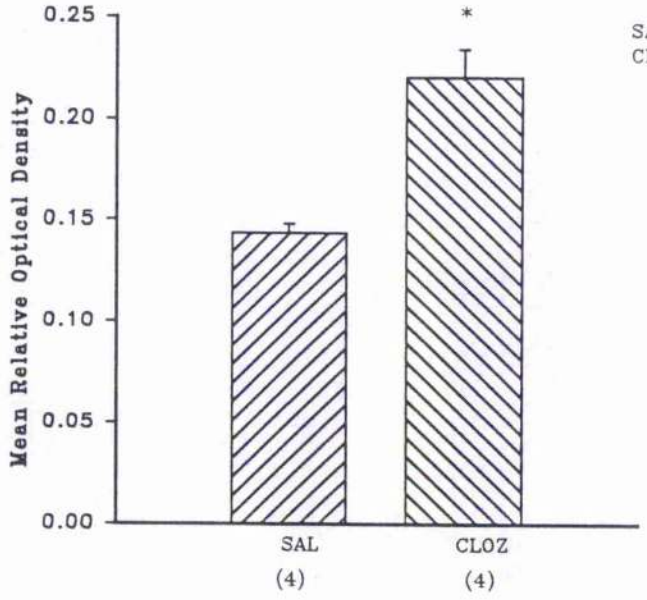

0.06
0.05
0.04
0.03
0.02
0.01
0.00
Mean Relative Optical Density
**
SAL
CLOZ
(4)
(4)
SAL= saline
CLOZ= Clozapine(6mg/kg)

Figure 3.6.3 : Effect of chronic treatment with clozapine on the expression of preprosomatostatin mRNA in the caudal striatum of the rat. Photomicrographs show the distribution of silver grains over striatal cell bodies that express the somatostatin gene. On the left, is an animal chronically treated with saline (A), and on the right , an animal chronically treated with clozapine (6mg/kg), via osmotic mini pump for 5 days (B). $p<0.01$**. Scale bar represents 25μm.

Figure 3.6.4 : Effect of chronic treatment with clozapine on the expression of preprosomatostatin mRNA in the rat olfactory tubercle. Clozapine (6mg/kg) or vehicle (0.9% saline) were administered via osmotic mini pumps for 5 days. Following this, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m. The number of animals per group are shown in parentheses. Significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test. $p < 0.05$* relative to saline group.





SAL= saline
CLOZ= Clozapine(6mg/kg)

significant increase in the levels of preprosomatostatin mRNA compared to the saline treated controls, was also observed in the caudal regions of the rat striatum [$F(1,6) = 20.24$, $p = 0.005$, $p < 0.01$] [Figures 3.6.2, 3.6.3], following low doses of clozapine.

In the olfactory tubercle, a similar induction of the somatostatin gene was observed after chronic treatment with clozapine (6mg/kg, n= 4), and this induction was shown to be significantly different from saline treated control animals [$F(1,6) = 19.28$, $p = 0.019$, $p < 0.05$] [Figure 3.6.4].

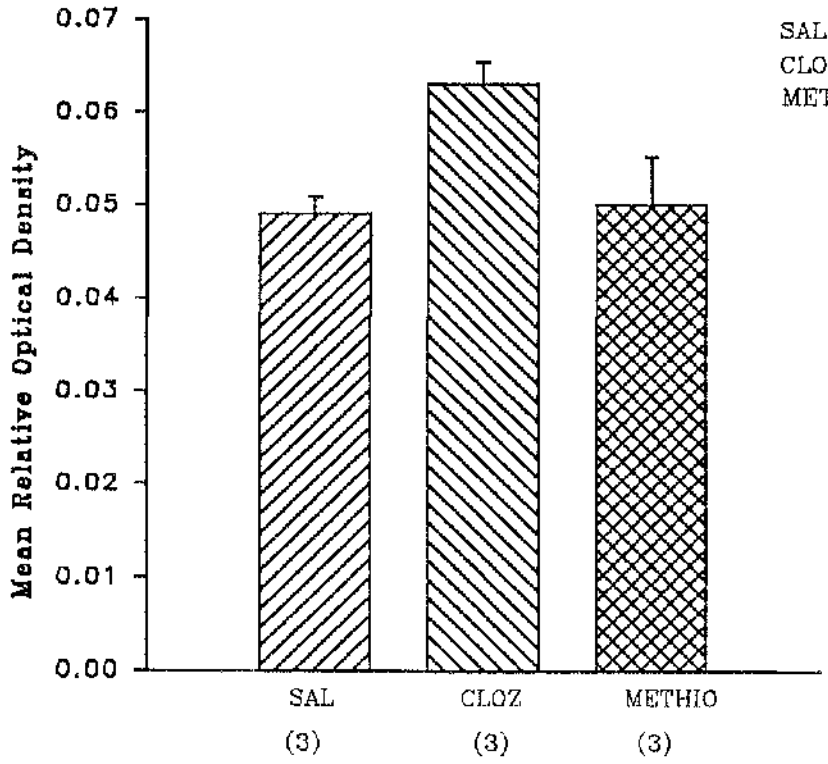
Short-term regulation of proneuropeptide Y gene expression in the rat striatum

3.7 Effect of acute drug treatment on the expression of proneuropeptide Y mRNA levels in the rat striatum 24 hours later.

Following intraperitoneal injection of saline (n= 3), or clozapine (3mg/kg, n= 3), or methiothepin (10mg/kg, n= 3), proneuropeptide Y was measured in the regions of the rostral and caudal striatum 24 hours after drug administration. No significant change in the level of proneuropeptide Y mRNA was seen in either the rostral [Figure 3.7.1] or in the caudal [Figure 3.7.2] regions of the rat striatum, compared to saline treated control animals.

Figure 3.7.1 : Effect of acute drug treatment on the expression of proneuropeptide Y mRNA in the rat rostral striatum. Clozapine (3mg/kg, n= 3) , methiothepin (10mg/kg, n= 3) or vehicle (0.9% saline, n= 3) were injected i.p. After 24 hours, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from film autoradiographs, are expressed as mean relative optical density + s.e.m.

Figure 3.7.2 : Effect of acute drug treatment on the expression of proneuropeptide Y mRNA in the rat caudal striatum. Clozapine (3mg/kg, n= 3), methiothepin (10mg/kg,n= 3) or vehicle (0.9% saline, n= 3) were injected i.p. After 24 hours the animals were killed and their brains processed for in situ hybridisation. Results, obtained from film autoradiographs, are expressed as mean relative optical density + s.e.m.


Mean Relative Optical Density
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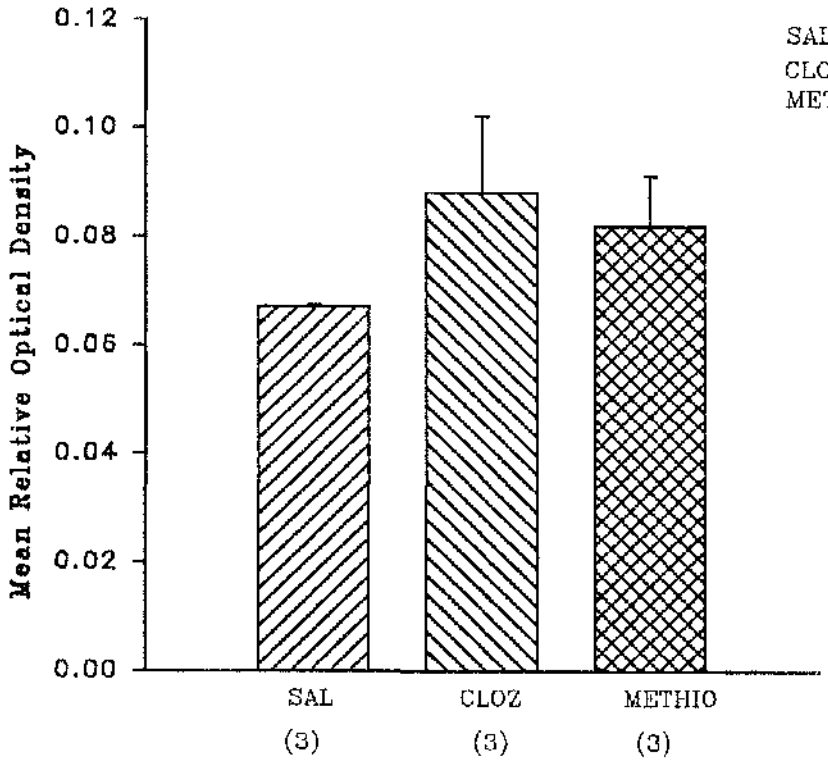
0.05
0.04
0.03
0.02
0.01
0.00
SAL
CLOZ
METHIO
(3)
(3)
(3)
SAL= saline
CLOZ= Clozapine(3mg/kg)
METHIO= Methiothepin
(10mg/kg)


Mean Relative Optical Density
0.12
0.10
0.08
0.06
0.04
0.02
0.00
SAL
CLOZ
METHIO
(3)
(3)
(3)
SAL= saline
CLOZ= Clozapine(3mg/kg)
METHIO= Methiothepin
(10mg/kg)

Long-term effects of neuroleptics on receptors

3.8 Effect of chronic neuroleptic drug administration on the expression of the dopamine D1 receptor mRNA in the rat striatum

The expression of the D1 dopamine receptor in the rat striatum is known to be homogenously distributed throughout the striatum.

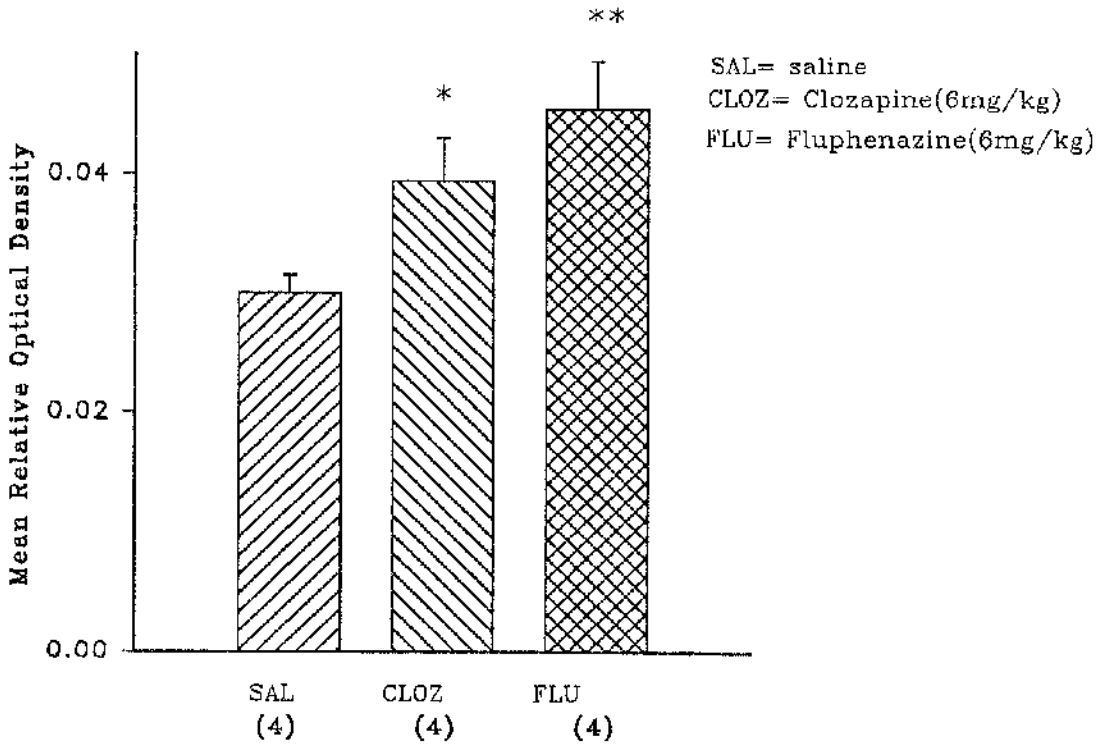
Animals were treated chronically with saline (n= 4), clozapine (6mg/kg, n= 4) or with fluphenazine (6mg/kg, n= 4) for 5 days, following which the levels of D1 receptor mRNA were measured as previously described.

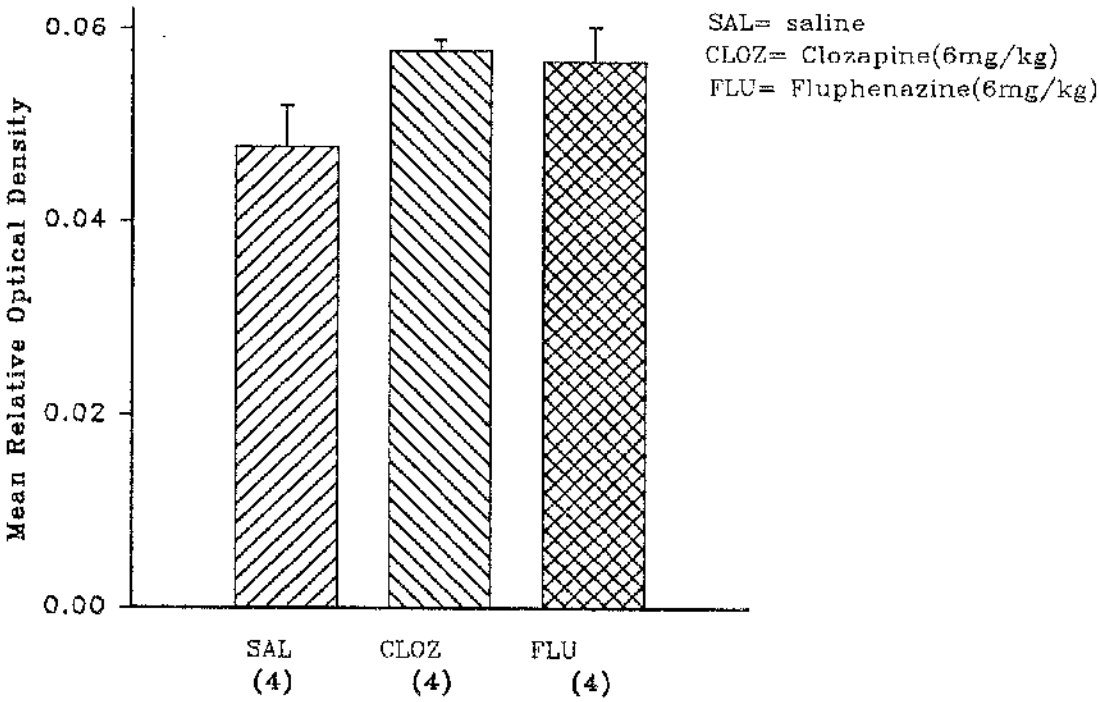
It was found that both clozapine (6mg/kg, n= 4) and fluphenazine (6mg/kg, n= 4) when given chronically could increase the expression of D1 mRNA in the rostral striatum of the rat, $F(2, 9) = 5.84$, $p= 0.01$, $p< 0.01$ [Figure 3.8.1]. The increase in D1 mRNA after clozapine treatment was significantly different from control animals, such that $p< 0.05$. Similarly chronic treatment with fluphenazine (6mg/kg, n= 4) caused an increase in D1 mRNA in the rostral striatum. This increase was greater than the induction due to clozapine, such that $p< 0.01$ [Figure 3.8.1].

In caudal regions , both clozapine (6mg/kg, n= 4) and fluphenazine (6mg/kg, n= 4) had a tendency to increase the levels of D1 mRNA expressed in the striatum. Although statistical analysis revealed no significant differences when compared to the control animals [Figure 3.8.2].

Figure 3.8.1 : Effect of chronic neuroleptic drug treatment on the expression of dopamine D1 receptor mRNA in the rat rostral striatum. Clozapine (6mg/kg), or fluphenazine (6mg/kg), or vehicle (0.9% saline) were administered via osmotic mini pumps for 5 days. Following this, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m. The number of animals per group are shown in parentheses. Significance was determined by one-way ANOVA, followed post-hoc Dunnett's test. *$p < 0.05$, **$p < 0.01$ relative to saline group.

Figure 3.8.2 : Effect of chronic neuroleptic drug treatment on the expression of dopamine D1 receptor mRNA in the rat caudal striatum. Clozapine (6mg/kg), or fluphenazine (6mg/kg), or vehicle (0.9% saline) were administered via osmotic mini pumps for 5 days. Following this, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m. The number of animals per group are shown in parentheses. Significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test.


**
*
Mean Relative Optical Density
0.04
0.02
0.00
SAL
(4)
CLOZ
(4)
FLU
(4)
SAL= saline
CLOZ= Clozapine(6mg/kg)
FLU= Fluphenazine(6mg/kg)


Mean Relative Optical Density
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0.04
0.02
0.00
SAL
(4)
CLOZ
(4)
FLU
(4)
SAL= saline
CLOZ= Clozapine(6mg/kg)
FLU= Fluphenazine(6mg/kg)

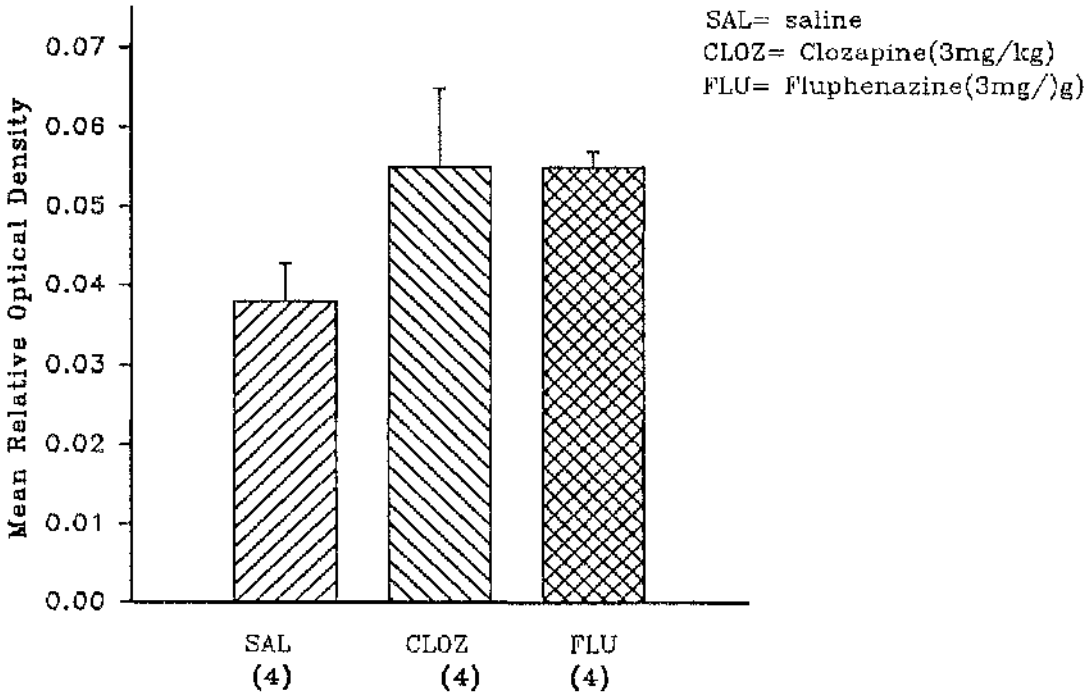
3.9 Effect of chronic neuroleptic drug administration on the levels of 5-HT6 receptor mRNA in the rat striatum

The recently cloned 5-HT6 receptor is known to be expressed in the rat striatum and have a high affinity for certain anitpsychotic drugs. Therefore we have investigated if chronic administration of the neuroleptic drugs clozapine (6mg/kg, n= 4) or fluphenazine (6mg/kg, n= 4) have any effect on the levels of 5-HT6 receptor mRNA compared to saline treated control animals.

However, it was found that low doses of clozapine (6mg/kg) or fluphenazine (6mg/kg) had no effect on the levels of 5-HT6 mRNA in either the rostral [Figure 3.9.1] or the caudal regions in the rat striatum [Figure 3.9.2], such that the levels of 5-HT6 mRNA were not significantly different from vehicle treated animals.

Figure 3.9.1 : Effect of chronic neuroleptic drug treatment on the expression of serotoninergic 5-HT6 receptor mRNA in the rat rostral striatum. Clozapine (6mg/kg), or fluphenazine (6mg/kg), or vehicle (0.9% saline) were administered via osmotic mini pumps for 5 days. Following this, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m. The number of animals per group are shown in parentheses.

Figure 3.9.2 : Effect of chronic neuroleptic drug treatment on the expression of serotoninergic 5-HT6 receptor mRNA in the rat caudal striatum. Clozapine (6mg/kg), or fluphenazine (6mg/kg), or vehicle (0.9% saline) were administered via osmotic mini pumps for 5 days. Following this, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m. The number of animals per group are shown in parentheses.


Mean Relative Optical Density
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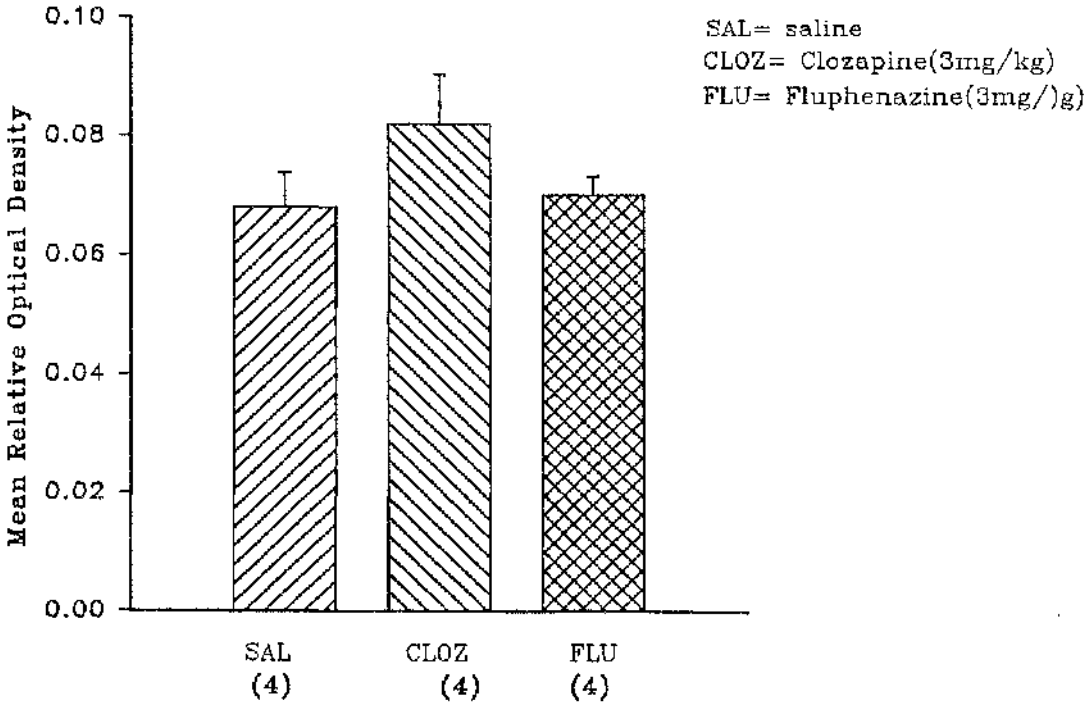
0.05
0.04
0.03
0.02
0.01
0.00
SAL
(4)
CLOZ
(4)
FLU
(4)
SAL= saline
CLOZ= Clozapine(3mg/kg)
FLU= Fluphenazine(3mg/)g)


Mean Relative Optical Density
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0.08
0.06
0.04
0.02
0.00
SAL
(4)
CLOZ
(4)
FLU
(4)
SAL= saline
CLOZ= Clozapine(3mg/kg)
FLU= Fluphenazine(3mg/)g)

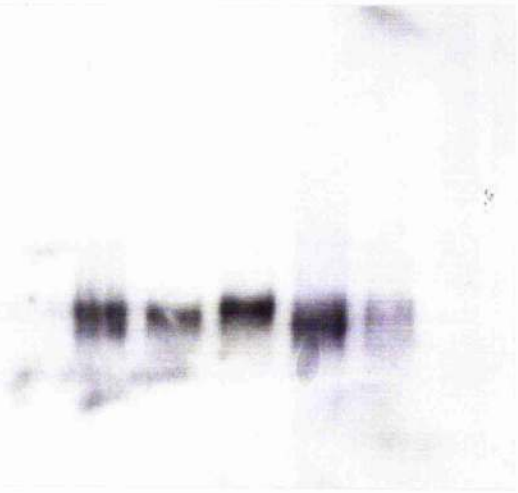
3.10 Northern blot hybridisation analysis of rat RNA

After total poly (A) + RNA (8μg/ sample) from each brain region was denatured, electrophoresed on a 1% agarose/ formaldehyde gel and transferred to a nylon membrane, the blot was hybridised with a ^{32}P- labelled tubulin T26 probe, as described under methods. The tubulin isoform T26 is a cytoskeletal protein present in all brain tissue and therefore was used as an appropriate control for RNA content in brain tissue samples. The blot was exposed to film for forty-eight hours at -80°C. As shown in the autoradiograms of figure 3.20, the rat T26 probe hybridised with a single major band of total poly (A) + RNA [Figure 3.10.1, panel a] of length 1800 nucleotides from all brain regions tested (i.e.- striatum, hippocampus, cortex, olfactory bulb and cerebellum), but not from rat liver. As can be seen from the autoradiogram, the intensity of labelling was very similar in all regions after incubation with a tubulin T26 mRNA probe. In accordance with previous findings, the size of band is consistent with that described for rat tubulin T26 in the brain (Miller et al., 1987). Any other bands appearing on the blot were considered to represent degraded material.

To assess the sensitivity and specificity of the rat preproenkephalin probe, the northern blot of total poly (A) + RNA was hybridised with a ^{32}P-labelled rat preproenkephalin mRNA probe (45-mer, previously described). After a thirty-six hour exposure to film at -80°C, a single band of length approximately 1500 nucleotides was observed in the region of the striatum, but not in the liver, a tissue essentially devoid of proenkephalin peptides [Figure 3.10.1, panel b]. This size is consistent with that previously determined for bovine and rat striatal preproenkephalin mRNA (Dandekar and Sabol, 1982; Tang et al., 1983). The pattern of expression of preproenkephalin mRNA on

Figure 3.10.1 : Northern blot analysis of (A) tubulin T26 mRNA and (B) preproenkephalin mRNA from various regions of the rat brain. Total poly(A)+ RNA (8μg/sample) taken from five brain regions [striatum (ST), hippocampus (H), cortex (CTX), olfactory bulb (OB), and cerebellum (CB) and also from the liver (L)] was denatured and electrophoresed on a 1% agarose, formaldehyde gel, transferred to a nylon membrane and hybridised with either (A) a ^{32}P-labelled tubulin T26 probe (previously described) or with (B) a ^{32}P-labelled preproenkephalin probe (previously described) as described under methods.

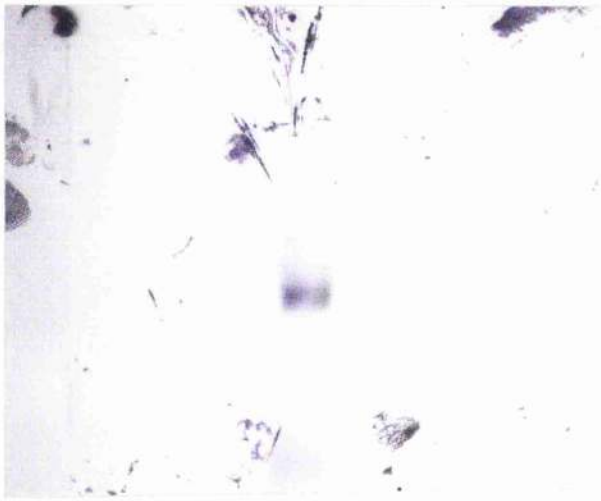
A



1·8 Kb

K CB H ST CTX OB

B



1·5 Kb

the northern blot appeared relatively free from non-specific hybridisation, and showed greatest abundance in the rat striatum, with virtually no signal in other brain regions. However, with a longer time of exposure, it may be expected that a signal representing preproenkephalin mRNA may appear in the region of the cortex and cerebellum, but with a much lower intensity (Yoshikawa et al., 1984).

DISCUSSION

Short-term Regulation of Proenkephalin Gene Expression in vivo

These studies were undertaken to initially determine whether enkephalin gene expression is induced by certain antipsychotic drugs after their acute administration. It is well known information that antipsychotic drugs can induce proenkephalin mRNA levels when given chronically (Sabol et al., 1983; Tang et al., 1983; Angulo et al., 1986; Sivam et al., 1986; Romano et al., 1987; Morris et al., 1988). We have looked at short-term regulation of a number of late response genes, in an attempt to gain further understanding into the functional significance of the class of genes referred to as immediate early genes. By gaining information on the possible receptor mechanism involved in the induction of both late response genes and immediate early genes we have been able to contribute indirect information that may aid in understanding the relationship between late response genes and immediate early gene induction, a theory that currently remains controversial and not well understood.

3.11 Early time course of Fluphenazine on Proenkephalin mRNA expression

The drug fluphenazine was used in this study because of its profile as a typical neuroleptic. Clinical use of typical neuroleptic drugs is frequently associated with the appearance of undesirable side effects including extrapyramidal movement disorders, which further divide into a parkinsonian-like syndrome and tardive dyskinesia, dependent on the length of drug exposure (Baldessarini, 1985; Tarsy and Baldessarini, 1986; Meltzer, 1991). These unwanted extrapyramidal behavioural side effects are thought to be a

consequence of extensive blockade of dopaminergic D2-like receptors in the nigrostriatal (A9) dopamine system (Baldessarini, 1979; Klawans et al., 1980). Fluphenazine is reported to have a marginal selectivity for the D2-like dopamine receptors (Saller et al., 1987), over the D4 dopamine receptor (Van Tol et al., 1991) with a moderate potency at dopamine D1-like receptors (Christensen et al., 1984), which was an important reason for selecting this drug for the following studies.

The results obtained in this study show that acute injection of the typical neuroleptic drug fluphenazine has no effect on the levels of proenkephalin mRNA in the rat striatum up to three hours after the initial injection, when compared to control animals. As in other studies, changes in the mRNA level have been correlated to changes in protein levels (Shiomi et al., 1986; O'Connell et al., 1987; Adler et al., 1988). It may be assumed that fluphenazine does not affect enkephalin protein levels in either the rostral or caudal region of the rat striatum up to three hours after administration. The dosage used would be expected to bind to greater than 50% of D2 dopamine receptors in vivo according to the reported affinity of fluphenazine for D2 dopamine receptors (Van Tol et al., 1991). As the number of animals per group were particularly small, this may have introduced a greater error, and thus masked any small changes in proenkephalin gene expression that may have occurred, at any one time point. In a previous report it has been suggested that a single acute injection of haloperidol can induce proenkephalin mRNA in some animals by fifteen minutes, and in all animals tested by forty-five minutes (Konradi et al., 1993). Similarly the mRNA levels of other peptidergic transmitters, for example, neurotensin, can be affected by acute treatment with dopaminergic D2 receptor antagonists (Augood et al., 1991). The lack of an induction of proenkephalin mRNA after acute treatment with fluphenazine may reflect a slower drug action of this typical neuroleptic. A longer time period

may be necessary for fluphenazine to penetrate the blood-brain barrier and thus have an effect on the levels of proenkphalin mRNA.

3.12 The effect of Antipsychotic drug treatment on Proenkephalin mRNA levels twenty-four hours after administration

In this study the effect of the typical neuroleptics haloperidol and fluphenazine, which have relatively high affinities for D2-like dopamine receptors and moderate potency at D1-like dopamine receptors (Christensen et al., 1984), were compared with the atypical neuroleptic clozapine, which has been demonstrated to show some selectivity for D4 dopamine receptors (Van Tol et al., 1991) and with the 5-HT1/2 receptor antagonist drug methiothepin, also known to bind to dopamine receptors, on the expression of proenkephalin mRNA in the rat striatum twenty-four hours after drug administration.

From the results obtained it was observed that the typical neuroleptics haloperidol and fluphenazine increased the expression of proenkephalin mRNA in the rat striatum after twenty four hours. These antipsychotic drugs are known to induce enkephalin mRNA and enkephalin protein after chronic administration (Hong et al., 1978; Sabol et al., 1983; Tang et al., 1983; Angulo et al., 1986; Sivam et al., 1986; Romano et al., 1987; Morris et al., 1988). At present it is generally accepted that the clinical therapeutic effects of these antipsychotic drugs are mediated through blockade of dopamine D2-like receptors (Creese et al, 1976; Seeman et al., 1976; Baldessarini, 1985). The primary evidence for this hypothesis is two-fold. Firstly, most of the available antipsychotic drugs are dopamine D2-like receptor antagonists (Carlsson, 1978; Peroutka and Snyder, 1980; Baldessarini, 1985). Evidence for this comes from an increased dopamine turnover and antagonism of dopamine D2-like

agonist effects, during acute administration, and an increase in D2-like dopamine receptor density following chronic administration (Carlsson, 1978; Muller and Seeman, 1978; Baldessarini, 1985; Wilmot and Szczepanik, 1989). Secondly it has been shown that there is a strong correlation between the relative potencies of antipsychotic agents in competing for dopamine D2 sites in vitro and the doses of antipsychotic drugs used in the clinical treatment of psychoses (Peroutka and Snyder, 1980; Seeman, 1980; Cohen and Lipinski, 1981). However, it should be noted that the drugs and doses used would distinguish poorly between D2, D3 and D4 dopamine receptor subtypes. Furthermore, the antipsychotic activity of neuroleptic drugs may, in fact, be related to the interaction of neuroleptics with other transmitter systems in the brain. At clinically effective doses, neuroleptics interact with a high degree of affinity with the α-adrenergic, histamine, muscarinic acetylcholine and serotonin receptors (Peroutka and Snyder, 1980).

A similar induction of proenkephalin mRNA was observed twenty-four hours after treatment with the non-selective 5-HT1/2 receptor antagonist methiothepin. Recently, it has been suggested that potent serotonin 5-HT2 receptor antagonism could alleviate negative symptoms in schizophrenia. Moreover, 5-HT2 antagonists have been shown to reduce neuroleptic-induced catalepsy in rats (Balsara et al., 1979; Hicks, 1990), to increase neuroleptic-induced dopamine turnover (Saller et al., 1990), to cause a slight, indirect activation of midbrain dopaminergic neurons (Ugedo et al., 1989), restoring phasic rather than tonic activity, and to reduce neuroleptic-induced extrapyramidal side effects (Reyntjens et al., 1986; Bersani et al., 1990). As methiothepin has previously been shown to possess the ability to block dopaminergic receptors in binding studies (Lloyd and Bartholini, 1974; Enjalbert et al., 1978; Nelson et al., 1979; Christensen, 1985), it is likely that the methiothepin-induced increase in proenkephalin mRNA levels in the rat striatum may be due its actions at dopamine D2-like receptors in vivo.

Although the possibility still remains that a 5-HT receptor blockade may also be contributing to this effect. Both haloperidol and fluphenazine are known to possess 5-HT2 receptor binding properties, but with much lower affinity than methiothepin (Meltzer et al., 1989).

The atypical neuroleptic drug clozapine, which is known to be associated with a low incidence of extrapyramidal side effects, a property that as yet has not been attributed to any one physiological effect of the drug, was shown to have no effect on proenkephalin mRNA levels twenty-four hours after administration. Previous studies have revealed that chronic treatment with clozapine does not increase the density of dopamine D1 or dopamine D2 receptors (Seeger et al., 1982; Severson et al., 1984; Lee and Tang, 1984; Rupniak et al., 1984; Cohen and Lipinski, 1986). It has been suggested that the atypical antipsychotic agents exert preferential effects on the mesolimbic (A10) dopamine system in vivo (Borison et al., 1983; Borison and Diamond, 1983).

These results are the first observations indicating that striatal proenkephalin mRNA is induced after acute treatment, not only with certain typical neuroleptics, but also with the 5-HT1/2 receptor antagonist compound, methiothepin, and not with the atypical neuroleptic drug clozapine. It may be suggested that the extrapyramidal side-effects associated with typical neuroleptic drug treatment could be partially attributed to the rapid induction of the enkephalin gene in the striatum shortly after drug administration and the subsequent sustained elevation with chronic use. This would be consistent with evidence that, in rodents with dopamine lesions, the degree of extrapyramidal motor deficit is related to the magnitude of the change in proenkephalin gene expression (Cadet et al., 1992). The induction of proenkephalin mRNA following acute typical neuroleptic drug treatment is most likely due to blockade of D2 or D3 dopamine receptor subtypes in vivo, rather than the D4 dopamine subtype, as clozapine, having some selectivity for the D4 receptor over the D2 or D3 receptor subtypes (Van Tol et al., 1991), was ineffective.

Although in contrast to this, a previous study has demonstrated a decrease in proenkephalin gene expression after chronic treatment with high doses of clozapine (Mercugliano and Cheeselet, 1991). It must also be considered that 5-HT receptor blockade may contribute to this effect, since the non-selective 5-HT receptor antagonist methiothepin, together with both the typical neuroleptics fluphenazine and haloperidol, share the ability to block 5-HT2 receptors.

Short-term Regulation of Preprotachykinin mRNA Expression in vivo

3.13 The effect of Antipsychotic drug treatments on the levels of Preprotachykinin mRNA in the rat striatum twenty-four hours after administration

In this study the typical neuroleptic haloperidol clearly reduced the level of preprotachykinin mRNA in the rat rostral striatum after twenty-four hours, whilst both haloperidol and fluphenazine were shown to decrease preprotachykinin mRNA levels in the rat caudal striatum after acute treatment.

These results are in agreement with previous studies demonstrating that the level of protachykinin mRNA, the mRNA encoding for substance P, is reduced in the rat striatum after chronic treatment with the antipsychotic drug haloperidol (Hong et al., 1983; Bannon et al., 1986). This is the first observation for an acute effect of these neuroleptics on preprotachykinin mRNA levels in vivo. There was no significant change in preprotachykinin mRNA levels in the striatum, twenty-four hours after administration of the atypical neuroleptic, clozapine. This is in agreement with previous reports

indicating that the atypical neuroleptic clozapine does not alter preprotachykinin mRNA in the rat striatum following chronic treatment (Bannon et al., 1987; Shibata et al., 1990). The dose of clozapine used here would be expected to be blocking 80% of α-1 receptors, approximately 65% 5-HT2 receptors, with relatively little blockade of D2 dopamine receptors (Schotte et al., 1993). Since clozapine has a decreased propensity for inducing extrapyramidal side-effects, it is conceivable that decreased tachykinin gene expression in the basal ganglia is somehow related to the production of these adverse effects. It is known that chronic administration of most antipsychotic drugs causes a depolarisation inactivation of dopamine neurons innervating the rat striatum. This results in a decreased number of spontaneously active dopamine cells and a diminished release of striatal dopamine (Chiodo, 1988). It is the decrease in dopamine neurotransmission that apparently causes decreased tachykinin biosynthesis similar to that seen after experimental dopamine cell lesioning or in post-mortem material from Parkinson's disease patients (Mauborgne et al., 1983; Tenovno et al., 1984; Young et al., 1986; Clevens and Beal, 1989). In contrast, atypical antipsychotic drugs elicit neither depolarisation inactivation of dopamine cells innervating the striatum nor decreased tachykinin biosynthesis. However, both typical and atypical antipsychotic drugs induce depolarisation inactivation of those dopamine neurons innervating primarily limbic targets (Chiodo, 1988), leading to speculation that decreased limbic dopamine influence is the common mechanism of antipsychotic drug action. Although various typical and atypical antipsychotic agents may differ somewhat in their affinities for various dopamine receptor subtypes, some other property or properties of atypical drugs (such as antiadrenergic or antiserotoninergic effects) may be responsible not only for the differing propensities for induction of adverse side effects, but also for additional therapeutic effects (Chiodo, 1988; Meltzer, 1988). The dose of clozapine used in this study was chosen with the knowledge that clozapine

has a particularly high affinity for the D4 dopamine receptor compared to most typical neuroleptics (Van Tol et al., 1991). Doses of clozapine of 10mg/kg and above have been shown to bind to striatal D2 binding sites in rats (Saller et al., 1987; Audinot et al., 1993). It is therefore likely that the lower dose of 3mg/kg used here is preferentially blocking D4 sites in vivo rather than D2 sites. Therefore, it appears unlikely that the D4 receptor is involved in mediating the decrease in preprotachykinin mRNA in the rat striatum, following acute treatment with either haloperidol or fluphenazine.

Similarly, the antipsychotic methiothepin did not alter the levels of preprotachykinin mRNA in this study. As it is most likely that the typical neuroleptic agents studied caused a reduction in preprotachykinin gene expression through a mechanism involving blockade of dopamine receptors, a possible explanation for the lack of effect with methiothepin may be that the affinity of methiothepin for the dopamine D1 and D2 receptors is not sufficiently strong to decrease preprotachykinin gene expression in the striatum of the rat after acute administration. Another possible explanation could be that combined 5-HT antagonism together with dopamine receptor blockade is preventing the reduction of preprotachykinin mRNA. Haloperidol and fluphenazine also possess 5-HT receptor antagonistic properties, but perhaps the blockade of dopamine neurotransmission by these typical neuroleptics overrides the serotonin receptor blockade.

Short-term Regulation of Preprosomatostatin gene expression in vivo

3.14 The effect of Antipsychotic drug treatments on the level of Preprosomatostatin mRNA twenty-four hours after administration

In this study the typical neuroleptic drugs haloperidol and fluphenazine did not significantly alter the levels of preprosomatostatin mRNA in the caudal striatum or in the nucleus accumbens of the rat, twenty-four hours after administration. However, it has previously been reported that haloperidol can decrease somatostatin gene expression in the striatum, nucleus accumbens and in the frontal cortex of the rat after chronic treatment (Beal and Martin, 1984; Radke et al., 1988; Salin et al., 1989). It has also been suggested that dopamine may be involved in the regulation of somatostatin mRNA levels after chronic treatment (Salin et al., 1989). The lack of effect with haloperidol or fluphenazine after twenty-four hours may reflect the time course necessary to affect the levels of this brain neuropeptide. It seems most likely that the decrease in somatostatin gene expression in the rat striatal and accumbens regions, observed after chronic treatment with the typical neuroleptic agent haloperidol arises after a prolonged time period, and thus may be associated with the appearance of extrapyramidal side effects, which also appear after prolonged drug use (Creese et al., 1976; Seeman et al., 1976; Baldessarini, 1985).

In contrast to this, a low dose of the atypical neuroleptic clozapine caused a significant increase in the levels of preprosomatostatin mRNA in both the caudal region of the striatum and in the nucleus accumbens of the rat after twenty-four hours. In chronic studies, it has previously been suggested that high doses of clozapine can cause an induction of preprosomatostain mRNA in the rat nucleus accumbens (Salin et al., 1989). These results provide the first evidence for a differential regulation of preprosomatostatin gene expression

after acute treatment with typical versus atypical antipschotic drugs. It appears that the time course for the atypical agent, clozapine, to induce somatostatin gene expression is relatively more rapid than the time course for haloperidol, the typical neuroleptic, to cause a reduction in somatostatin mRNA levels. The underlying mechanisms involved in the clozapine-induced expression of somatostatin may be due to blockade of dopamine receptors. The low dose of clozapine used in this study may reflect the possibility that the D4 dopamine receptor is involved in this effect, as clozapine has a ten-fold higher affinity for the D4 receptor than for the D2 or D3 dopamine receptors, previously demonstrated in in vitro binding studies (Van Tol et al., 1991). High doses of clozapine (greater than 10mg/kg) have been shown to bind to striatal D2 sites in rats (Saller et al., 1987; Audinot et al., 1993). The low dose of 3mg/kg used here, is preferentially blocking D4 sites in vivo rather than D2 sites. The atypical neuroleptic clozapine is also known to have relatively high affinity for some serotonin receptor subtypes, in particular the serotonin 5-HT2A and 5-HT2C receptors (Zifa and Fillion, 1992; Canton et al., 1993). The distribution of the receptor mRNA's encoding both the 5-HT2A and the 5-HT2C serotonin receptors in the rat, has previously been demonstrated. Intermediate levels of both subtypes have been shown in the caudate-putamen, nucleus accumbens and substantia nigra pars compacta. However, in the caudate-putamen their distributions are quite different. The 5-HT2A receptor is most abundant in the medial striatum, and the 5-HT2A receptor mRNA has been shown to increase in the mediolateral direction. Expression of the 5-HT2C receptor mRNA, present in the medial striatum has been shown to decrease in a mediolateral direction, and in addition, it was expressed in patches of higher density rather than an even distribution (Pompeiano et al., 1993). Thus the possibility that clozapine is acting via a serotoninergic mechanism to increase somatostatin gene expression in both the striatum and nucleus accumbens, after acute treatment, is not unlikely. However, both haloperidol and fluphenazine have been shown to have

some affinity for 5-HT2A/2C receptors, but with much less potency than clozapine (Canton et al., 1993). Also, it has recently been reported that intracerebroventricular injection of 5,7-dihydroxytryptamine, a selective serotonin neurotoxin, can increase preprosomatostatin mRNA levels in the rat striatum. From this, it has been suggested that serotonin has an inhibitory effect on the biosynthesis of somatostatin in the striatum (Bendotti et al., 1993).

Antipsychotic drugs which have strong affinities for 5-HT2 binding sites are known to be associated with a low incidence of extrapyramidal side effects (EPS) (Meltzer, 1988). The role of the 5-HT2A and 5-HT2C receptor sites are at present unclear. However, the antagonism of these sites could contribute to clozapine's low EPS profile. Clozapine has also been reported to antagonise the inhibitory effect of 5-HT3 receptor agonists on the firing of cortical receptors in the rat (Ashby et al., 1989). Blandina et al (1988) have shown that 5-HT3 receptor stimulation may facilitate striatal dopamine release. However, the extent to which the clozapine-induced release of striatal dopamine involves 5-HT3 receptor mechanisms is at present uncertain.

A similar induction of preprosomatostatin mRNA was seen in both the rat striatum and nucleus accumbens regions after treatment with the non-selective 5-HT1/2 receptor antagonist, methiothepin. This result further substantiates the possibility of a role for a 5-HT receptor subtype in this response. Methiothepin has previously been demonstrated to have a very high affinity for the 5-HT2A/2C receptors, as shown in binding studies (Leysen et al., 1982; Conn and Sanders-Bush, 1986). Since the atypical antipsychotic drug clozapine also has a high affinity for both the 5-HT2A and 5-HT2C, it could be hypothesised that the blockade of either or both of these serotonin receptor subtypes is a contributing factor to the increase in preprosomatostatin mRNA observed after acute administration of clozapine and methiothepin.

A short time course of the effect of low doses of clozapine on preprosomatostatin mRNA, in the rat striatum was then performed in order to

further address this drug-specific acute effect. However, we observed no significant changes in the levels of preprosomatostatin mRNA up to three hours after the drug was administered.

3.15 Effect of Chronic Antipsychotic treatment on Preprosomatostatin mRNA levels

In this study we have also demonstrated that low doses of clozapine can induce preprosomatostatin mRNA in the rostral and caudal regions of the rat striatum and in the olfactory tubercles, after chronic administration. Previous evidence demonstrating an induction of somatostatin gene expression in the accumbens, involved the administration of much higher doses of clozapine than the dose used here. The low dose of 3mg/kg clozapine may be reflecting a preferential D4 dopamine receptor blockade rather than D2 receptor blockade in order to increase the levels of preprosomatostatin mRNA. However, in previous studies the typical antipsychotic drug haloperidol has been shown to have an opposite effect on somatostatin mRNA levels. Chronic treatment with haloperidol was shown to decrease somatostatin mRNA levels in the striatum, nucleus accumbens and frontal cortex in the rat (Salin et al., 1989). This effect is thought to be the result of blockade of a stimulatory action of dopamine on somatostatinergic neurons mediated through dopamine D2-like receptors (Saller and Salama, 1986). Therefore, it seems unlikely that the effect of low doses of clozapine to increase somatostatin mRNA is mediated by blockade of dopaminergic transmission at D2 or D3 receptors. There is also evidence for an opposing action of typical versus atypical neuroleptics on muscarinic receptors in the striatum. The typical neuroleptic fluphenazine, has been shown to decrease muscarinic receptor density in the striatum, whereas in contrast, the atypical neuroleptic clozapine was shown to elevate muscarinic binding

densities in the striatum, cortex and in the hippocampus in the rat (Friedman et al., 1983). The effect of clozapine to induce somatostatin gene expression is likely to involve blockade of the D4 dopamine receptor or blockade of another neurotransmitter system in the brain. A possible candidate would be the serotoninergic system. As previously mentioned, clozapine is a very potent antagonist at certain serotonin receptor subtypes, in particular the 5-HT2A/2C subtypes. The effect of clozapine to induce somatostatin gene expression in the olfactory tubercles may also be mediated by a serotonin receptor blockade. It has been shown that the olfactory tubercles have intermediate levels of mRNA for both the 5-HT2A and 5-HT2C receptor subtypes in the rat (Pompeiano et al., 1993). Recently clozapine has also been demonstrated to have a relatively high affinity for the 5-HT6 and 5-HT7 serotonin receptor subtypes and it has been shown that the 5-HT6 receptor is expressed at high levels in the rat striatum (Monsma et al., 1993). Since a number of atypical neuroleptics have been shown to possess a high affinity for the 5-HT6 receptor, it has been suggested that this receptor subtype may be involved in mediating the antipsychotic actions of certain neuroleptic drugs (Monsma et al., 1993). In a recent report, fluphenazine has been demonstrated to have a high affinity for the cloned 5-HT6 subtype, with a Ki value of less than 20nM, although haloperidol had essentially no affinity for the 5-HT6 receptor (Roth et al., 1993). In addition to its known affinity for certain 5-HT receptor subtypes, clozapine is well known to have an antagonising action on many different receptors, including α-adrenergic receptors and muscarinic receptors, which must still be considered in this effect of the drug.

It has been hypothesised that a disruption of somatostatin gene regulation may have a role in a number of identified psychoses in man, such as schizophrenia, parkinsonism, Huntington's disease and alcohol dementia. It has been demonstrated that individuals with Parkinson's disease have lower levels of somatostatin-like immunoreactivity in lumbar spinal fluid than their control

counterparts (Cramer et al., 1989). Similarly a recent study has shown the reduction of somatostatin peptide in the neocortex of Parkinson's disease sufferers (Leake and Ferrier, 1993). A functional interaction between central nervous system somatostatin and dopamine systems has been implicated, as fluphenazine treatment has been shown to reduce cerebrospinal fluid somatostatin levels in patients with schizophrenia (Doran et al., 1989). Another study has demonstrated a decrease in somatostatin-like immunoreactivity in the cerebrospinal fluid of chronic schizophrenic patients with a cognitive impairment (Reinikainen et al., 1990). The development of schizophrenia has previously been suggested to involve autoimmune processes, such that blood serum samples taken from schizophrenic patients manifest a higher level of immune responsiveness to somatostatin, as compared to the control group (Rogaeva et al., 1990). A further level of complexity in the development of schizophrenia has been implicated in this study, since the increased immune responsiveness in blood serum to somatostatin was evident in patients suffering from malignant and paranoid schizophrenia, but was not evident in patients suffering from a slow-progressive form of schizophrenia (Rogaeva et al., 1990). This would suggest that different forms of the disease can differentially affect somatostatin levels. From this, it would appear that a number of physiological changes are necessary for the manifestation of schizophrenia, although the 'chemical balance' may be affected differently in different forms of the disease. In Huntington's chorea, a marked down-regulation of somatostatin receptors in the human striatum has been shown (Palacios et al., 1990). It has been postulated that the down-regulated somatostatin receptors may be localised on a population of neurons which are at risk in Huntington's chorea (Palacios et al., 1990). A reduction in cerebrospinal fluid somatostatin levels has also been demonstrated in alcoholic amnestic disorder or Korsakoff's psychosis (Martin et al., 1989) and in senile dementia of the Alzheimer's type (Leake and Ferrier, 1993). Therefore it would seem that a decrease in

somatostatin levels is one of the major physiological changes that takes place in a number of cognitive disorders.

Short-term Regulation of Neuropeptide Y gene expression in vivo

3.16 The Effect of Antipsychotic drug treatment on the levels of Proneuropeptide Y gene expression twenty-four hours after administration

NeuropeptideY, a peptide which shows many of the properties characterisitic of a neurotransmitter or neuromodulator is known to be highly abundant in mammalian brain. It has previously been detected in the same neurons as catecholamines and enkephalins (Hunt et al., 1981; Hokfelt et al., 1983; Everitt et al., 1984), and many neurons in the forebrain have been shown to contain both neuropeptide Y and somatostatin (Vincent and Johansson, 1983; Chan-Pallay, 1987). In the striatum a small population of neurons are known to express both neuropeptide Y and somatostatin (Graybiel, 1990; Vuillet et al., 1990). Therefore we were interested in the effect of acute treatment with the atypical neuroleptic clozapine and the serotonin receptor antagonist, methiothepin, on the levels of proneuropeptide Y mRNA in the striatum, as these drugs have previously been demonstrated to increase somatostatin mRNA levels in the striatum.

However, the results obtained indicate a lack of effect of both clozapine and methiothepin on proneuropeptide Y mRNA levels in either the rostral or caudal regions in the rat striatum. Due to the small number of animals per treatment group, any small changes in the level of neuropeptide mRNA may remain undetected despite a possible drug effect.

In previous studies selective D1 dopamine receptor blockade has been demonstrated to increase cellular levels of neuropeptide Y mRNA in the rat striatum (Morris, 1992). This is in contrast to the decrease in somatostatin mRNA levels that have been reported in the same population of neurons following chronic D2 receptor blockade (Weiss and Cheeselet, 1989; Salin et al., 1989). It has therefore been suggested that dopamine is tonically and simultaneously exerting an opposite control on neuropeptide Y and somatostatin gene expression in the striatum (Morris, 1992). In view of this, it may not be surprising that both clozapine and methiothepin had no effect on neuropeptide Y gene expression. If neuropeptide Y and somatostatin are differentially regulated within the same cell, it would perhaps have been more likely to see a decrease in proneuropeptide Y mRNA levels, although our results did not show this to occur. Similarly the neuropeptides dynorphin and preprotachykinin which are expressed in striatonigral neurons have previously been shown to be differentially regulated by dopamine in the same cell population (Gerfen et al., 1991).

3.17 The effect of chronic neuroleptic drug treatment on dopamine D1 receptor expression

It has long been known that chronic treatment with antipsychotic drugs can up-regulate dopamine receptor subtypes. The class of drugs known as 'typical neuroleptics' such as haloperidol, have previously been demonstrated to increase the mRNA encoding the D1A, the D2 and the D3 dopamine receptor subtypes (Buckland et al., 1992). The levels of mRNA encoding the D1B/D5 receptor in the rat have been reported to remain unchanged after chronic haloperidol treatment (Buckland et al., 1992). The results obtained from this study demonstrate that the typical neuroleptic fluphenazine, when

given chronically, can significantly increase D1A receptor mRNA levels in the rostral region , in the rat striatum. This is in accordance with previous reports suggesting that blockade of dopamine D1 receptors can increase D1 receptor mRNA (Creese et al., 1992). The effect of fluphenazine did not prove to be significant in the caudal region of the striatum, despite a tendency towards an increase in D1A mRNA expression.

With the 'atypical' neuroleptic drug clozapine an elevation of D1A receptor mRNA levels was also observed in the rostral striatum, although the increase was less than that seen with fluphenazine. The dose of clozapine used in this study would not be expected to be occupying more than 30-35% of D1 receptors in vivo, according to the demonstrated affinities of clozapine at D1 compared to D4 dopamine receptors (Van Tol et al., 1991). This therefore makes the observed induction D1 receptor mRNA in the striatum after chronic clozapine treatment relatively difficult to interpret. However, it is not impossible that only a small percentage of D1 receptors need to be occupied in order to increase D1 mRNA. In previous reports chronic treatment with high doses of clozapine has been shown to both increase (O' Dell et al., 1990) and have no effect on the density of dopamine D1A receptors (Seeger et al., 1982; Severson et al., 1984; Lee and Tang, 1984; Rupniak et al., 1984; Cohen and Lipinski, 1986; Buckland et al., 1993). Other workers have studied the effect of clozapine on D2 receptor mRNA and receptor numbers. Wilmot and Szczepanik, (1989) found no increase in D2 receptor numbers in any rat brain regions. Similarly Van Tol et al (1991) found no increase in D2 receptor mRNA levels in rat striatum following chronic treatment with clozapine. Although, chronic clozapine treatment has recently been associated with an increase in D3 receptor mRNA levels. Buckland et al., (1993) have demonstrated that high doses of 30mg/kg clozapine can increase D3 receptor mRNA levels by five-fold after four days of treatment. In addition, they also found that the levels of D3 receptor mRNA dropped back to basal levels after

thirty-two days of treatment. In the previous studies that suggest clozapine has no effect on the levels of D1A receptor mRNA, it is likely that monitoring of mRNA levels occurred after twenty days or more. It is possible that the increase in D1A receptor mRNA levels that we observed after five days of treatment has declined back to basal levels after three weeks of treatment, similar to that observed for the D3 receptor. Therefore it would be necessary to monitor the level of D1A receptor mRNA more closely, over a prolonged time period, in order to clarify this result.

3.18 The effect of chronic neuroleptic drug treatment on the expression of the 5-HT6 receptor mRNA

Clozapine has previously been reported to have a high affinity for the serotoninergic 5-HT6 receptor in binding studies (Monsma et al., 1993). Therefore we were interested to see the effects of clozapine and the typical neuroleptic fluphenazine on the levels of 5-HT6 receptor mRNA in the rat striatum, following chronic drug treatment.

However, in this study both clozapine and fluphenazine had little effect on the levels of 5-HT6 mRNA in vivo after five days of chronic treatment. This would suggest that the 5-HT6 receptor is not up-regulated by chronic blockade with both typical and atypical neuroleptic drugs. Also it may indicate that the serotoninergic control of this receptor subtype is low, in that there may be a low tonic activation of 5-HT6 receptors in the striatum.

Chapter 4

Regulation of Immediate Early Genes in vivo

INTRODUCTION

4.0 Neuroleptic Modulation of Immediate Early Genes In Vivo

At present, immediate early genes are thought to function by altering the rate of transcription of a target gene, by binding to a specific site on the late response gene. There has been a large amount of evidence gained to suggest that modulation of the level of dopaminergic activity can induce the immediate early genes c-fos and zif/268. Systemic treatment with dopaminergic agonists, in particular SKF38393, increases the levels of c-fos and zif/268 after a 6-hydroxydopamine-induced lesion (Robertson et al., 1990; Cole et al., 1992), whereas evidence also exists showing that they are induced in the rat striatum after administration of haloperidol, a dopaminergic D2 receptor antagonist (Dragunow et al., 1990; Nguyen et al., 1992). This induction of both c-fos and zif/268 by typical neuroleptics is likely to be mediated by the blockade of either D2 or D4 dopamine receptors, because drugs active on these receptors share the ability to increase the expression of these immediate early genes (Dragunow et al., 1990; Cole et al., 1992; Nguyen et al., 1992; Robertson and Fibiger, 1992). By using the neuroleptic drug clozapine, which has a ten fold higher affinity for the dopamine D4 receptor than for the D2 receptor, given at low doses , we have attempted to investigate further the nature of the receptor involved in these responses.

Currently there is very little evidence to demonstrate the hypothesis that immediate early genes may provide the link to changes in late response genes. However, it has been suggested that there is a link between the haloperidol induction of c-fos and the subsequent increase in the levels of preproenkephalin mRNA (Sonnenberg et al., 1989; Donovan et al., 1992). More recently this circumstantial evidence has been questioned by results obtained by Konradi and

colleagues (1993). At present there is no indication as to the mechanisms leading to a decrease in preprotachykinin gene expression.

It is known that c-fos forms dimers with members of the jun family of immediate early genes to affect target gene transcription. The mRNA encoding two members of this family, c-jun and jun-D, is present constitutively in striatal neurons, and the corresponding proteins are likely to be available to form complexes with c-fos once it is synthesised. The mRNA encoding another immediate early gene belonging to this family, jun-B, is not normally detectable in striatal neurons. However, jun-B is potentially of interest, because in primary rat embryo cells it has been linked to the suppression, rather than the enhancement of downstream gene transcription (Chiu et al., 1989; Schutte et al., 1989). We were therefore interested to determine whether jun-B and jun-D is induced in the striatum and nucleus accumbens, by neuroleptic administration.

The newly cloned POU-domain -containing gene, RHS2 and the HNF/3 fork head gene BF-1, have recently been shown to possess a transcription regulatory region. However, as yet there has been very little research done on these genes. As both gene have been shown to be present in the region of the striatum (Le Moine and Scott-Young, 1992; Tao and Lai, 1992), we have been interested in the effects of typical and atypical neuroleptic drug administration on the expression of these two genes in the rat striatum.

RESULTS

4.1 Time course of Fluphenazine effect on c-fos mRNA levels in the region of rostral and caudal striatum

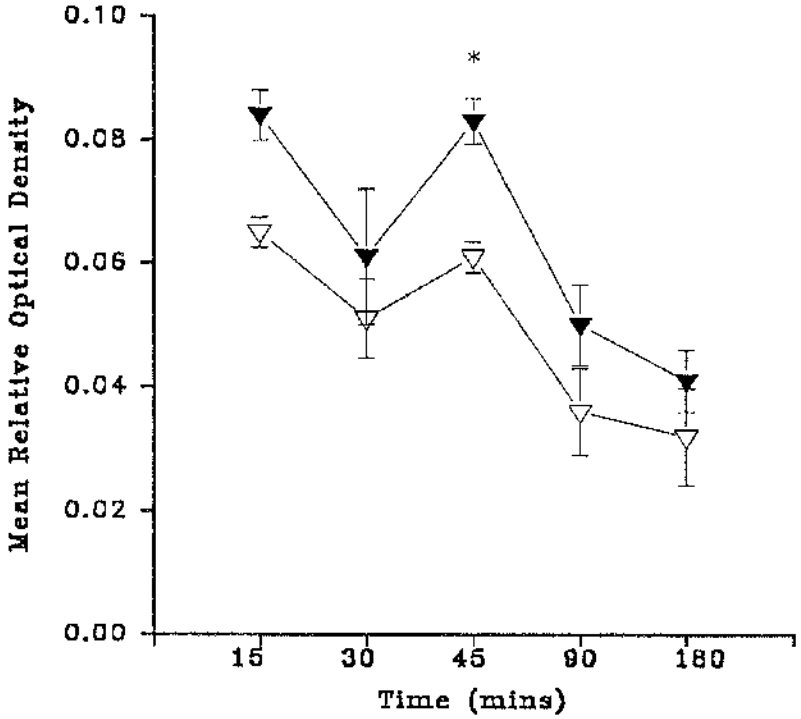
It is known that the immediate early gene c-fos can be induced by administration of the relatively non-selective, typical neuroleptic drug haloperidol (Nguyen et al ,1992). Therefore in this study we have investigated the effects over time of the typical neuroleptic drug fluphenazine (6mg/kg), having high affinity for, and being a potent antagonist of the D2 dopamine receptor.

Following intraperitoneal administration of fluphenazine (6mg/kg) to male wistar rats (n= 3), it was observed that the levels of c-fos mRNA were increased significantly in the rostral striatum 15 minutes [$F(4,20) = 7.60$, $p = 0.008$, $p < 0.01$] and 45 minutes, $p < 0.01$, after drug treatment compared to the animals treated at corresponding time points with physiological saline (n=2) [Figure 4.1.1]. After 45 minutes the levels of c-fos mRNA declined back to basal levels.

In the caudal striatum , a maximal and significant induction of c-fos mRNA levels after fluphenazine treatment occurred 45 minutes after drug administration. [Figure 4.1.2] The levels of c-fos mRNA were significantly different such that $F(4,20) = 8.67$, $p = 0.0067$, $p < 0.01$, from saline treated animals. A similar decline back to basal levels was observed after 45 minutes. The pattern of induction of c-fos mRNA was primarily observed in the dorsolateral striatum.

Figure 4.1.1 : Effect of fluphenazine (6mg/kg) on c-fos mRNA levels in the rat rostral striatum. Fluphenazine (6mg/kg; n= 3) or vehicle (0.9% saline; n= 2) was injected i.p. and the animals killed after the specified time intervals. Results, obtained from film autoradiographs are expressed as mean relative optical density ± s.e.m. The open triangle = saline treated groups, closed triangle = fluphenazine (6mg/kg) treated groups for the respective time periods. Significance was determined by one-way ANOVA, followed by the student's t-test. *$p < 0.05$.

Figure 4.1.2 : Effect of fluphenazine (6mg/kg) on c-fos mRNA levels in the rat caudal striatum. Fluphenazine (6mg/kg; n= 3) or vehicle (0.9% saline; n= 2) were injected i.p. and the animals killed after the specified time intervals. Results, obtained from film autoradiographs, are expressed as mean relative optical density ± s.e.m. The open triangles = saline treated groups, closed triangles = fluphenazine (6mg/kg) treated groups for the respective time periods. Significance was determined by one-way ANOVA, followed by the student's t-test. *$p < 0.05$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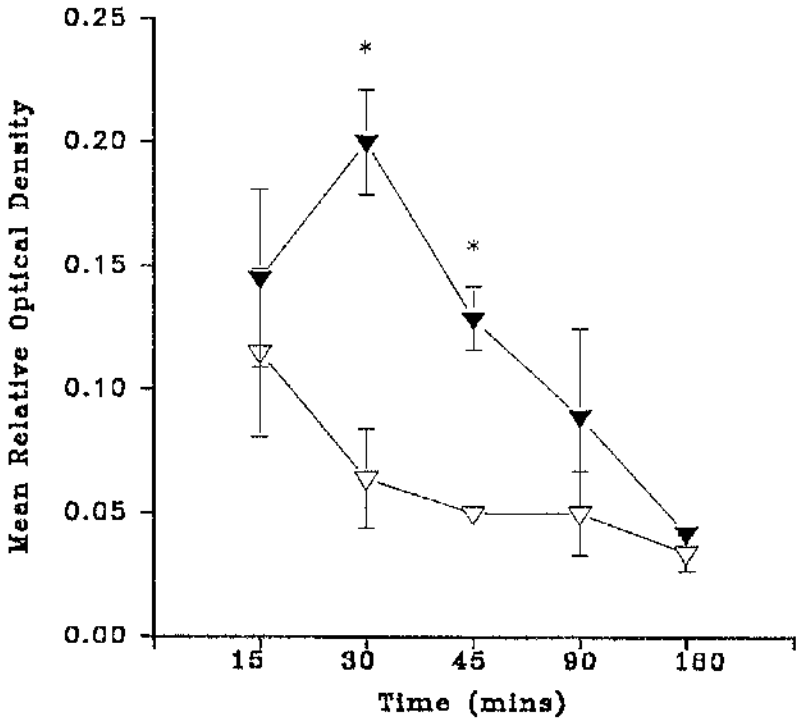
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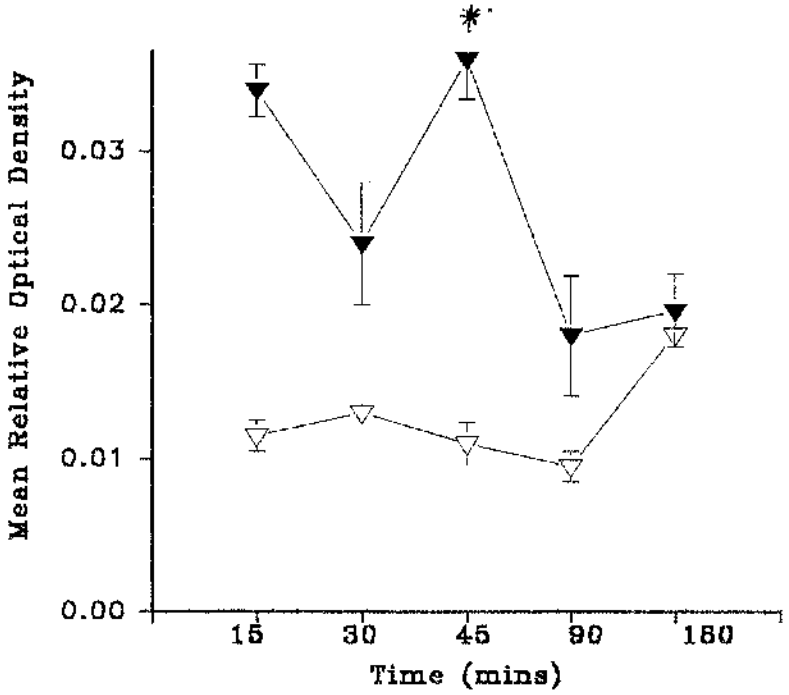
Time (mins)

Figure 4.2.1 : Effect of fluphenazine (6mg/kg) on zif/268 mRNA in the rat rostral striatum. Fluphenazine (6mg/kg; n= 3) or vehicle (0.9% saline; n= 2) were injected i.p. and the animals killed after the specified time intervals. Results, obtained from film autoradiographs, are expressed as mean relative optical density ± s.e.m. The open triangles = saline treated groups, closed triangles = fluphenazine (6mg/kg) treated groups for the respective time periods. Significance was determined by one-way ANOVA, followed by the student's t-test. * $p < 0.05$

Figure 4.2.2 : Effect of fluphenazine (6mg/kg) on zif/268 mRNA levels in the rat caudal striatum. Fluphenazine (6mg/kg; n= 3) or vehicle (0.9% saline; n= 2) were injected i.p. and the animals killed after the specified time intervals. Results, obtained from film autoradiographs, are expressed as mean relative optical density ± s.e.m. The open triangles = saline treated groups, closed triangles = fluphenazine (6mg/kg) treated groups for the respective time periods. Significance was determined by one-way ANOVA, followed by the student's t-test. * $p< 0.05$.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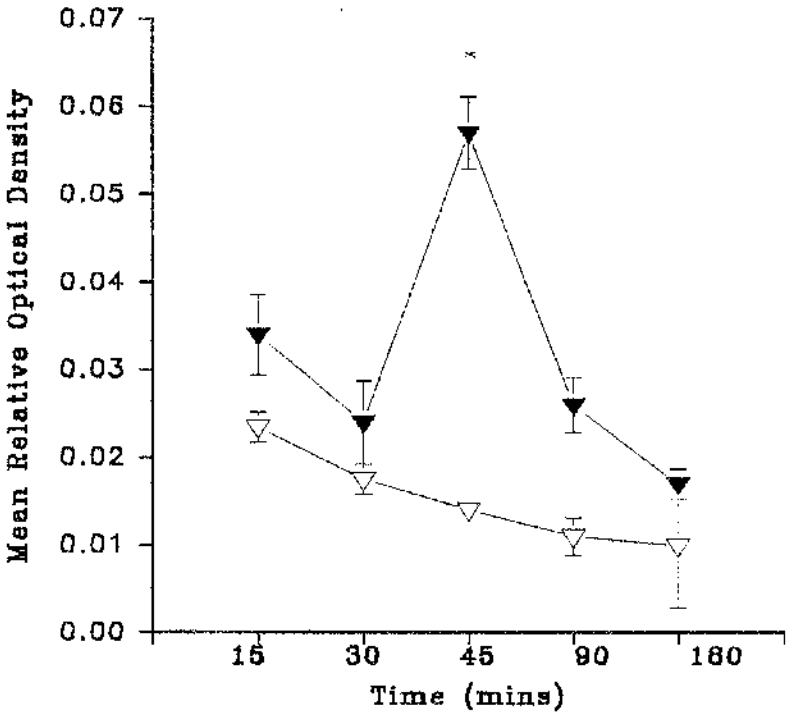
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Time (mins)

4.2 Time course of Fluphenazine effect on zif/268 mRNA levels in the regions of the rostral and caudal striatum

Zif/268, a zinc-finger containing protein, is also known to be induced by treatment with the typical neuroleptic haloperidol in the striatum (Nguyen et al, 1992). Therefore the time course effect on zif/268 mRNA induction by fluphenazine (6mg/kg, n= 3) was investigated.

The levels of zif/268 mRNA were significantly induced compared to saline treated animals, [$F(4,20) = 4.89$, $p = 0.04$, $p < 0.05$] after 45 minutes in the rostral striatum. [Figure 4.2.1]. However , in the caudal striatum it was observed that zif/268 mRNA levels were induced 30 minutes after fluphenazine (6mg/kg, n= 3) administration. This increase was significantly different from saline treated animals (n= 2) at the corresponding time point, such that $F(4,20) = 3.50$, $p = 0.047$, $p < 0.05$, DF= 1 [Figure 4.2.2]. Induction of zif/268 was significantly different from saline treated animals 45 minutes after drug administration, $p < 0.05$, following which, a gradual decline back to basal levels was observed. [Figure 4.2.2]. Again, the pattern of induction was primarily observed in the dorsolateral regions of the rat striatum.

4.3 Effect of acute neuroleptic drug administration on c-fos mRNA and zif/268 mRNA expression in the rat striatum and nucleus accumbens

Drug Treatment	Striatum	Nucleus accumbens
Saline	26.80±5.70	25.50±5.50
Haloperidol	144.00±19.00 *	101.70±24.50 *
Fluphenazine	60.60±21.10 *	18.50±2.10
Clozapine	23.70±6.30	20.70±3.70

Table 1: The expression of c-fos mRNA levels in the rat striatum and nucleus accumbens following treatment with vehicle (physiological saline), or the typical neuroleptics haloperidol (1mg/kg), and fluphenazine (3mg/kg) or the atypical neuroleptic clozapine (3mg/kg). Results, obtained from film autoradiographs, are expressed as mean relative optical density x 10^3 ± s.e.m., n=3-5. Significance was determined by one-way ANOVA, followed by the *post-hoc* Mann-Whitney U test. $p < 0.05$*

Drug treatment	Striatum	Nucleus accumbens
Saline	46.75±5.76	41.70±5.42
Haloperidol	152.30±13.30 *	95.30±14.90
Fluphenazine	203.70±42.10 *	152.00±36.30 *
Clozapine	64.30±15.70	43.30±17.80

Table 2: The expression of zif/268 mRNA levels in the rat striatum and nucleus accumbens following treatment with vehicle (physiological saline), or the typical neuroleptic drugs haloperidol (1mg/kg), and fluphenazine (3mg/kg), or the atypical neuroleptic clozapine (3mg/kg). Results, obtained from film autoradiographs, are expressed as mean relative optical density x 10^3 ± s.e.m., n=3-5. Significance was determined by one-way ANOVA, followed by the *post-hoc* Mann-Whitney U test. $p < 0.05$*

Animals were intraperitonealy injected with saline (n= 3), the typical neuroleptic drugs haloperidol (1mg/kg, n= 3) and fluphenazine (6mg/kg, n= 5)

and the atypical neuroleptic drug clozapine (3mg/kg; n= 3). After 30 minutes the levels of mRNA were measured using in situ hybridisation histochemistry. Prior to the use of the above drug concentrations, a lower dose of 1mg/kg was used for both fluphenazine and clozapine, however no induction of either c-fos or zif/268 mRNA was observed. The effects of a higher dose of 6mg/kg fluphenazine were also examined in rats, in the event that a higher dose may cause much more potent inductive effects. However, it was demonstrated that 6mg/kg fluphenazine caused a comparable increase in c-fos and zif/268 mRNA levels to the induction observed with the lower dose of 3mg/kg fluphenazine (data not shown).

The above tables demonstrate that low levels of both c-fos mRNA [Table 2] and zif/268 mRNA [Table 3] are observed following treatment with physiological saline, the basal zif/268 hybridisation signal being approximately double that for c-fos mRNA. in both the rat striatum and nucleus accumbens.

The levels of c-fos mRNA in the striatum 30 minutes after treatment with the typical neuroleptic haloperidol (1mg/kg) were significantly increased compared to animals treated with saline, such that $F = 50.56$, $p = 0.011$, $p < 0.05$ [Table 2]. Haloperidol had a tendency to increase c-fos mRNA expression in the nucleus accumbens, and the increase was significantly different from saline treated animals ($F = 15.58$, $p < 0.05$). Similarly fluphenazine (3mg/kg) significantly induced c-fos mRNA levels in the striatum ($F = 10.16$, $p < 0.05$), but not in the nucleus accumbens. Clozapine (3mg/kg) had no effect on the expression of c-fos in both the striatum and nucleus accumbens. [Table 2].

A similar pattern of induction was observed for zif/268 mRNA in the striatum. The increase in zif/268 mRNA levels after administration of haloperidol (1mg/kg) for 30 minutes was significantly different from control rats ($F = 42.63$, $p < 0.01$) [Table 3]. A tendency towards induction of zif/268 was observed in the nucleus accumbens following haloperidol (1mg/kg), although this increase failed to reach significance. Fluphenazine (3mg/kg) caused a

significant induction of zif/268 mRNA in both the striatum ($F=8.85$, $p< 0.05$) and nucleus accumbens ($F=7.94$, $p< 0.05$). Clozapine (3mg/kg) had no effect on the levels of zif/268 mRNA in both the striatum and the nucleus accumbens.[Table 3].

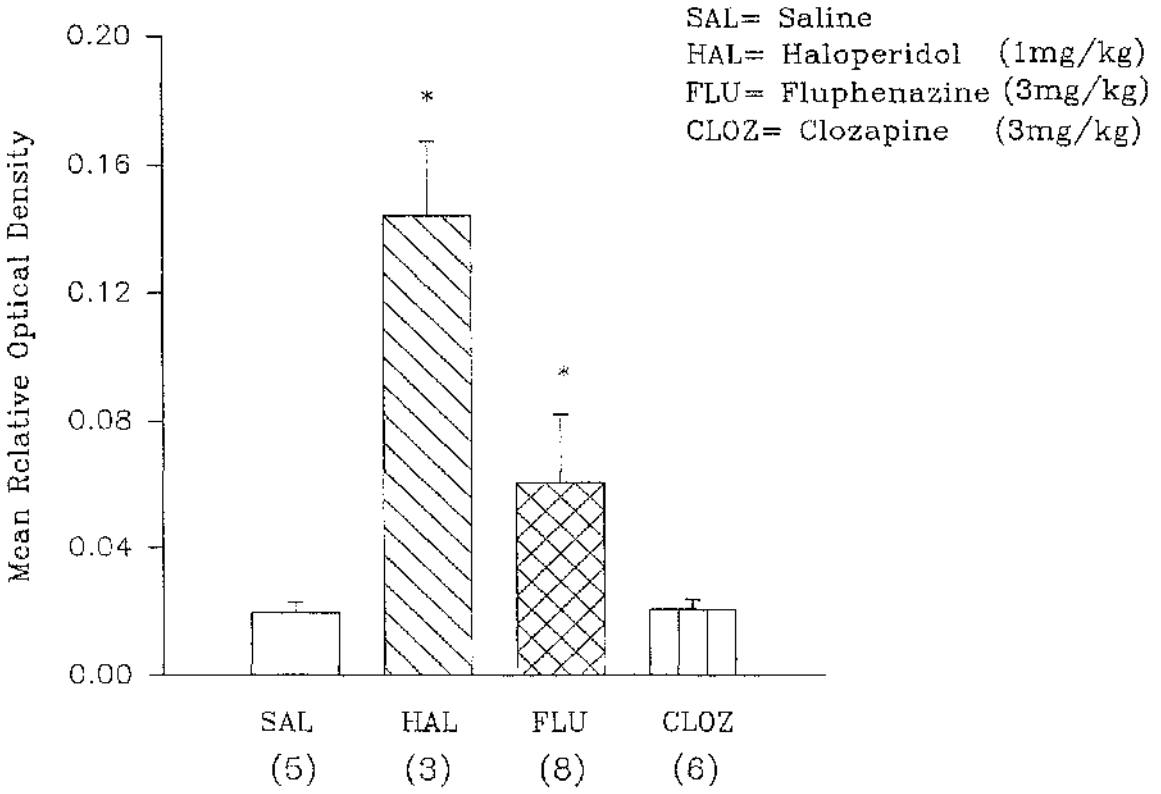
The expression of c-fos and zif/268 mRNA had a tendency towards slightly higher levels in the dorsolateral region of the striatum, although a general induction was observed throughout the striatum. The levels expressed in the nucleus accumbens were distributed homogeneously over core and shell regions.

4.4 The effect of acute treatment with neuroleptics on the expression of jun-B mRNA and jun-D mRNA in the rat striatum and nucleus accumbens

In saline treated animals a very low hybridisation signal for jun-B mRNA was observed in all brain regions, similar to the low levels of c-fos mRNA following saline treatment. Following administration of the typical neuroleptic haloperidol (1mg/kg; n= 3), a dramatic increase in the levels of jun-B mRNA was observed 30 minutes after drug treatment, in the rat striatum ($F=60.41$, $p< 0.05$).[Figure 4.4.1]. A clear induction of jun-B mRNA was also observed in the nucleus accumbens ($F=15.80$, $p< 0.05$), following haloperidol (1mg/kg). [Figure 4.4.2]. Similarly fluphenazine (3mg/kg, n= 8), a typical neuroleptic having a high affinity for the dopamine D2 receptor, was shown to cause a significant induction in the expression of jun-B mRNA levels in the rat striatum ($F=4.22$. $p< 0.05$) [Figure 4.4.1, 4.4.5], and in the nucleus accumbens ($F=35.15$, $p< 0.05$) [Figure 4.4.2]. The pattern of jun-B expression in the striatum was primarily in the dorsolateral regions, whilst in the nucleus accumbens the tendency was towards a homogeneous distribution of jun-B mRNA . The atypical neuroleptic clozapine (3mg/kg, n= 6) had no effect on the

Figure 4.4.1: Effect of acute administration of neuroleptic drugs on jun-B mRNA levels in the rat striatum. Haloperidol (1mg/kg), or fluphenazine (3mg/kg), or clozapine (3mg/kg), or vehicle (0.9% saline) were injected i.p. 30 minutes before sacrifice. Results, obtained from film autoradiographs, are expressed as mean relative optical density + s.e.m.; n=3-8. Significance was determined by ANOVA followed by the post-hoc Mann-Whitney U test. * $p< 0.05$ relative to saline group.

Figure 4.4.2: Effect of acute administration of neuroleptic drugs on jun-B mRNA levels in the rat nucleus accumbens. Haloperidol (1mg/kg), or fluphenazine (3mg/kg), or clozapine (3mg/kg), or vehicle (0.9% saline) were injected i.p. 30 minutes before sacrifice. Results, obtained from film autoradiographs, are expressed as mean relative optical density + s.e.m.; n=3-8. Significance was determined by ANOVA, followed by the post-hoc Mann-Whitney U test. *$p< 0.05$ relative to saline group.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HAL= Haloperidol (1mg/kg)
FLU= Fluphenazine (3mg/kg)
CLOZ= Clozapine (3mg/kg)
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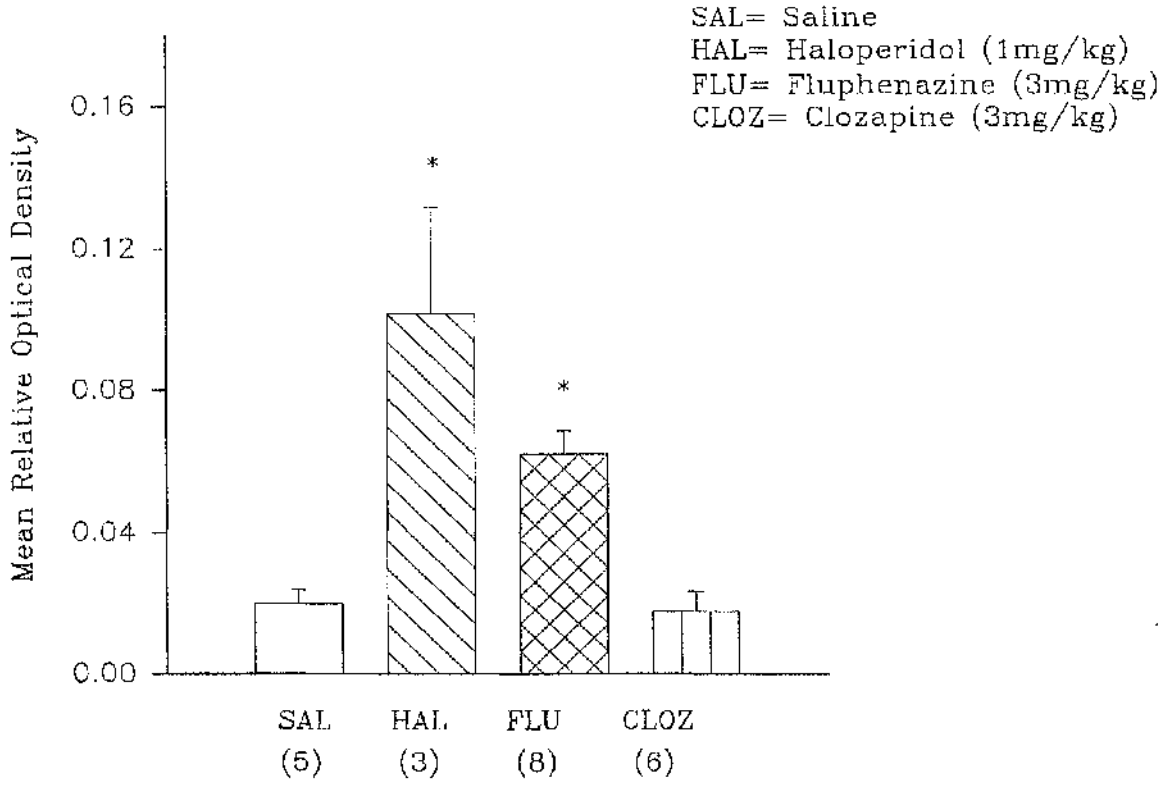
SAL= Saline
HAL= Haloperidol (1mg/kg)
FLU= Fluphenazine (3mg/kg)
CLOZ= Clozapine (3mg/kg)
Mean Relative Optical Density
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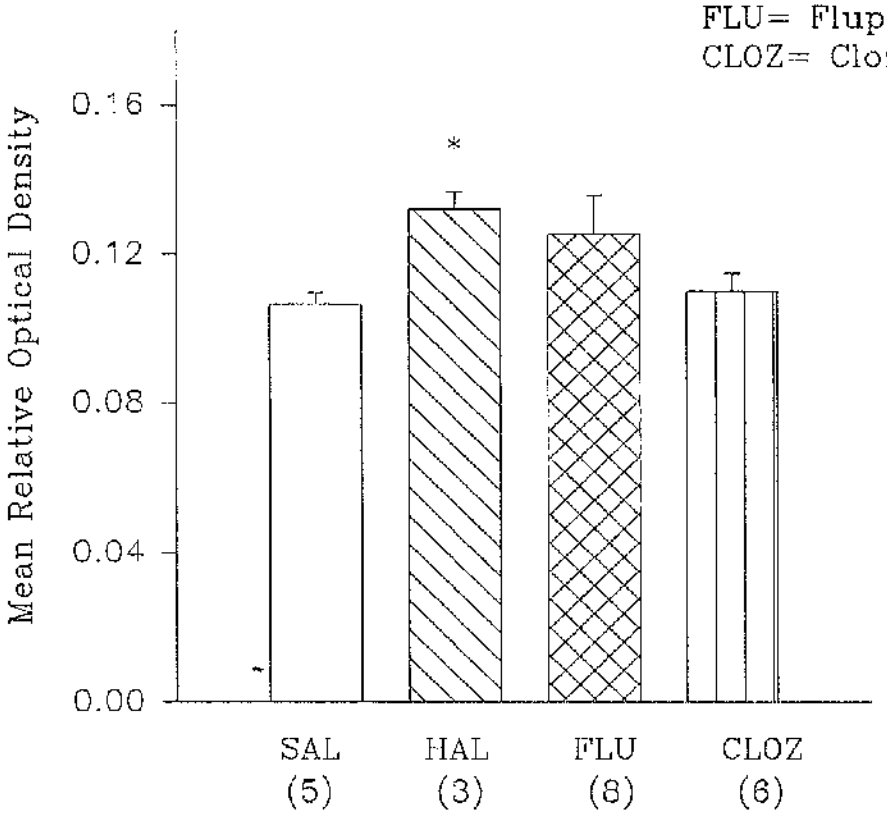
0.12
0.08
0.04
0.00
*
*
SAL
HAL
FLU
CLOZ
(5)
(3)
(8)
(6)

Figure 4.4.3: Effect of acute administration of neuroleptic drugs on jun-D mRNA levels in the rat striatum. Haloperidol (1mg/kg), or fluphenazine (3mg/kg), or clozapine (3mg/kg) or vehicle (0.9% saline) were injected i.p. 30 minutes before sacrifice. Results, obtained from film autoradiographs are expressed as mean relative optical density + s.e.m.; n=3-8. Significance was determined by ANOVA, followed by the post-hoc Mann-Whitney U test. * $p < 0.05$ relative to saline group.

Figure 4.4.4 : Effect of acute administration of neuroleptic drugs on jun-D mRNA levels in the rat nucleus accumbens. Haloperidol (1mg/kg), or fluphenazine (3mg/kg), or clozapine (3mg/kg), or vehicle (0.9% saline) were injected i.p. 30 minutes before sacrifice. Results, obtained from film autoradiographs, are expressed as mean relative optical density + s.e.m.; n=3-8. Significance was determined by ANOVA followed by the post-hoc Mann-Whitney U test. * $p < 0.05$ relative to saline group.

SAL= Saline
HAL= Haloperidol (1mg/kg)
FLU= Fluphenazine (3mg/kg)
CLOZ= Clozapine (3mg/kg)



SAL= Saline
HAL= Haloperidol (1mg/kg)
FLU= Fluphenazine (3mg/kg)
CLOZ= Clozapine (3mg/kg)

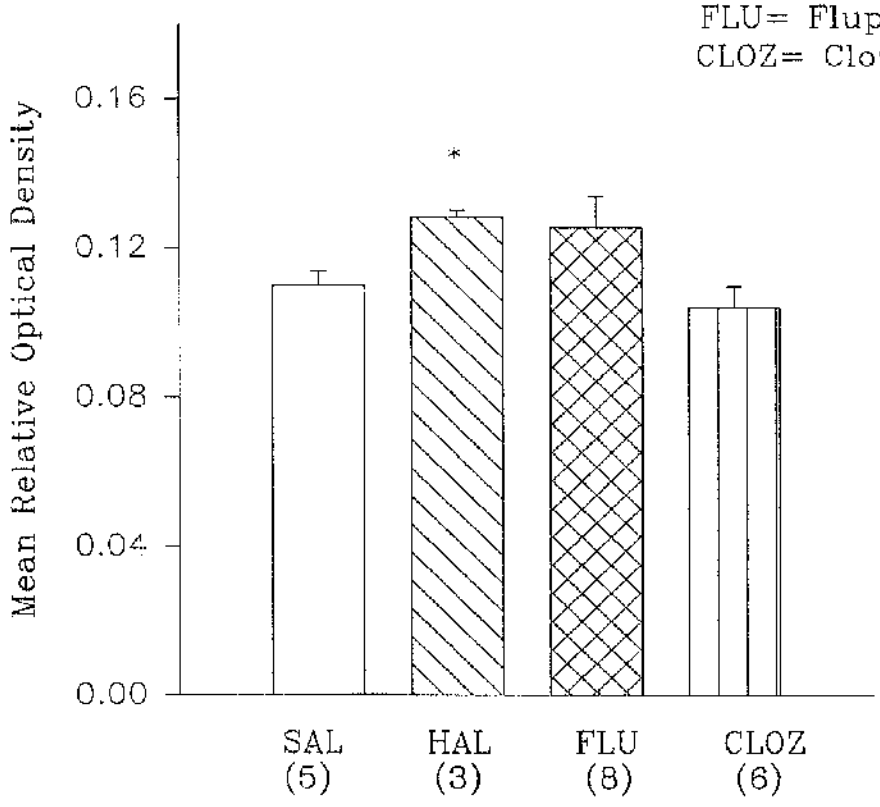
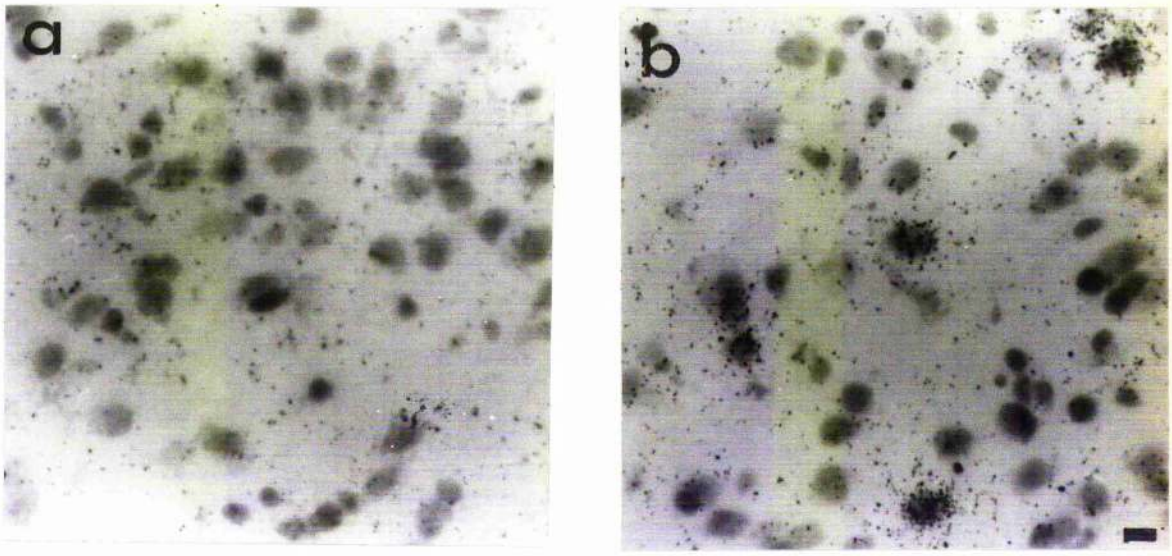
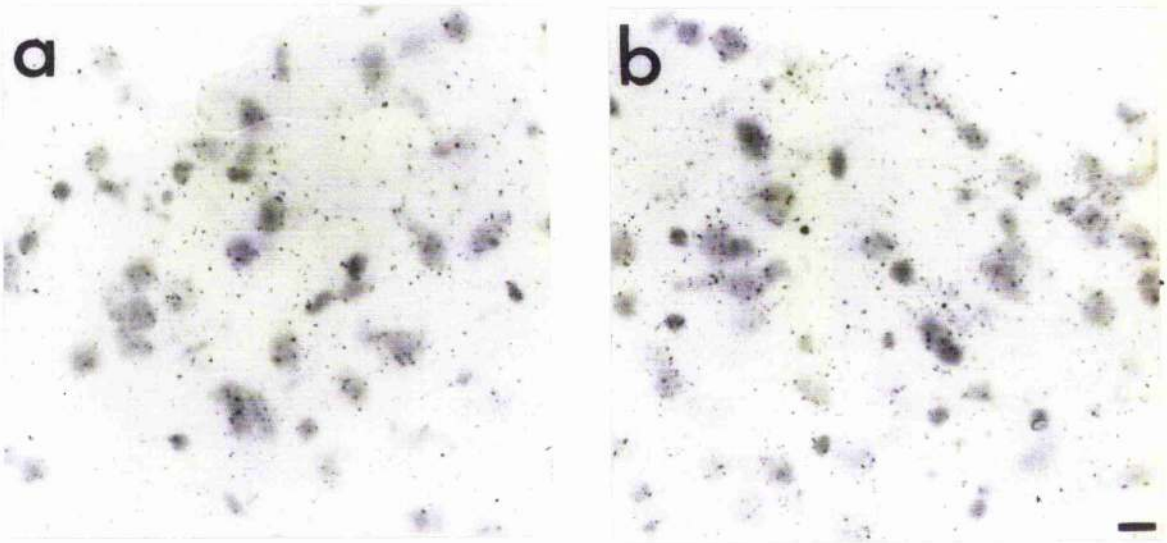


Figure 4.4.5 : Effect of fluphenazine on the expression of jun-B mRNA in the rat striatum. Photomicrographs show the distribution of silver grains over cell bodies in the caudal striatum. On the left, the animal was treated with vehicle (0.9% saline) (A), and on the right, with fluphenazine (3mg/kg), injected i.p. The animals were killed 30 minutes later. Scale bar represents 25μm.

Figure 4.4.6 : Effect of haloperidol on the expression of jun-D mRNA in the rat striatum. Photomicrographs show the distribution of silver grains over cell bodies in the caudal striatum. On the left, the animal was treated with vehicle (physiological saline) (A), and on the right , with haloperidol (1mg/kg) (B), administered i.p. The animals were killed 30 minutes later. Scale bar represents 25μm.


a
b


a
b

expression of jun-B mRNA levels in either the striatum or nucleus accumbens compared to saline treated control animals.

Another member of the jun family of immediate early genes, jun-D, was investigated. In comparison to the other IEGs under investigation, jun-D is anomolous in that it is basally expressed in much higher levels throughout the brain.The expression of jun-D mRNA was shown to be increased in the striatum 30 minutes after treatment with haloperidol (1mg/kg, n= 3) [Figure 4.4.3, 4.4.6], compared to saline treated control animals. This induction was shown to be statistically significant relative to control ($F=20.70$, $p< 0.05$). Likewise a similar increase in jun-D mRNA levels were seen in the nucleus accumbens after haloperidol (1mg/kg, n= 3) ($F=19.00$, $p< 0.05$) [Figure 4.4.4]. However fluphenazine (3mg/kg, n= 8) and clozapine (3mg/kg, n= 6) had no observed effect on the expression of jun-D mRNA in either the striatum or nucleus accumbens.

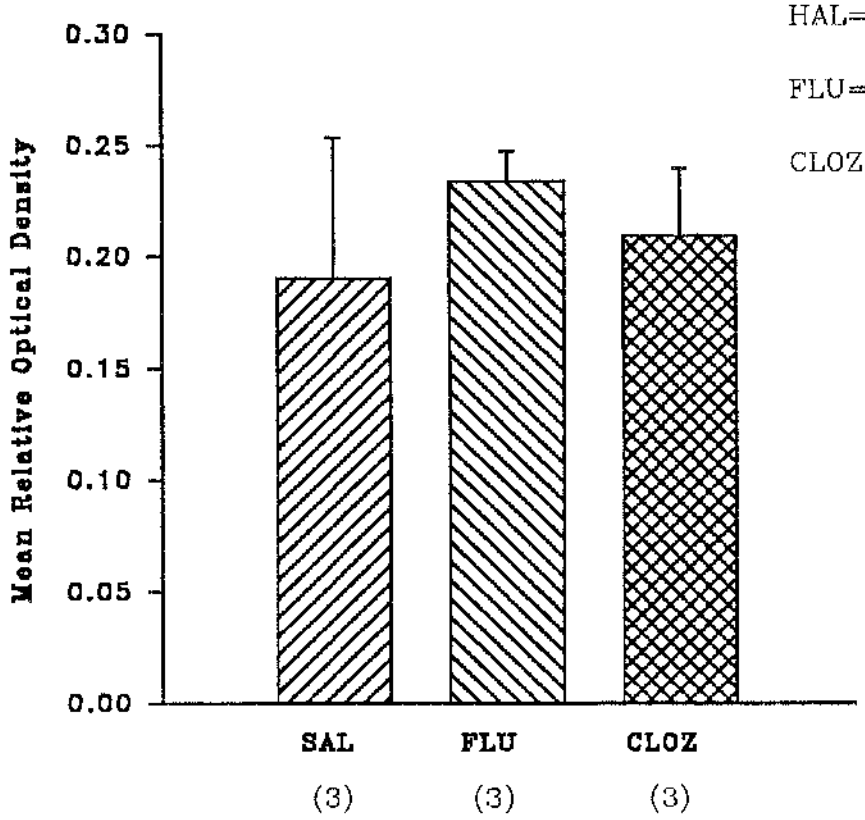
4.5 Effect of acute treatment with neuroleptics on the transcription factor genes BF-1 and RHS2.

The recently cloned homeobox genes BF-1 and RHS2 have been shown to be expressed at moderate levels in the striatum.(Tao and Lai, 1992; Le Moine and Young, 1992). Therefore, we have investigated the effect of neuroleptic drugs on the levels of expression of these genes in the rat striatum.

The hybridisation signal obtained for BF-1 and RHS2 gene expression was quite high in saline treated animals (n= 3). Thirty minutes after the administration of the typical neuroleptic drug fluphenazine (3mg/kg, n= 3) or the atypical neuroleptic clozapine (3mg/kg, n= 3), there was no significant

Figure 4.5.1 : Effect of neuroleptic drug treatment on the expression of BF-1 mRNA in the rat caudal striatum. Three animals per group were injected i.p. with vehicle (0.9% saline), fluphenazine (3mg/kg) or with clozapine (3mg/kg). After 30 minutes the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m. The number of animals per group is shown in parentheses.

Figure 4.5.2 : Effect of neuroleptic drug treatment on the expression of RHS2 mRNA in the rat caudal striatum. Three animals per group were injected i.p. with vehicle (0.9% saline), or fluphenazine (3mg/kg), or with clozapine (3mg/kg). After 30 minutes their brains were processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m. The number of animals per group is shown in parentheses.


SAL= Saline
HAL= Haloperidol (1mg/kg)
FLU= Fluphenazine (3mg/kg)
CLOZ= Clozapine (3mg/kg)
Mean Relative Optical Density
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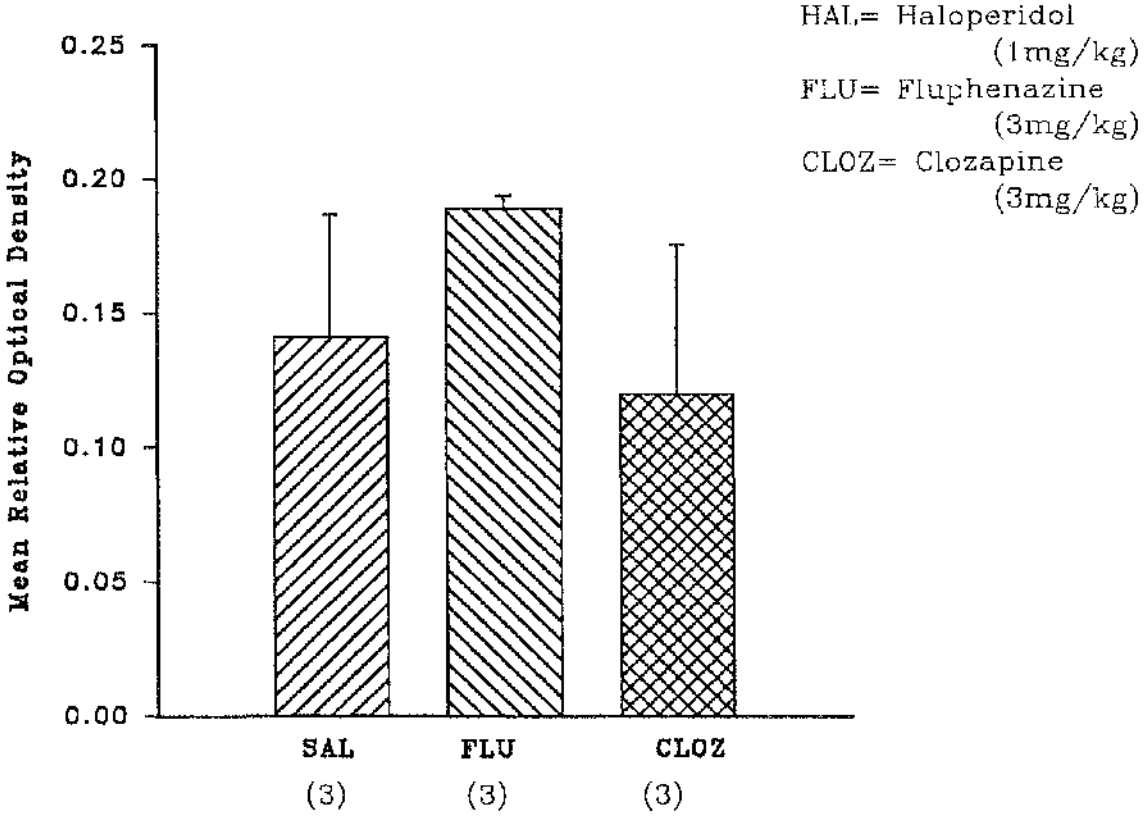
0.25
0.20
0.15
0.10
0.05
0.00
SAL
FLU
CLOZ
(3)
(3)
(3)


SAL= Saline
HAL= Haloperidol (1mg/kg)
FLU= Fluphenazine (3mg/kg)
CLOZ= Clozapine (3mg/kg)
Mean Relative Optical Density
0.25
0.20
0.15
0.10
0.05
0.00
SAL
FLU
CLOZ
(3)
(3)
(3)

change in the levels of expression of BF-1 mRNA [Figure 4.5.1] or RHS2 mRNA [Figure 4.5.2] compared to saline treated control animals.

4.6 Effect of acute neuroleptic drug treatment on the expression of BF-1 mRNA in the rat striatum 24 hours later.

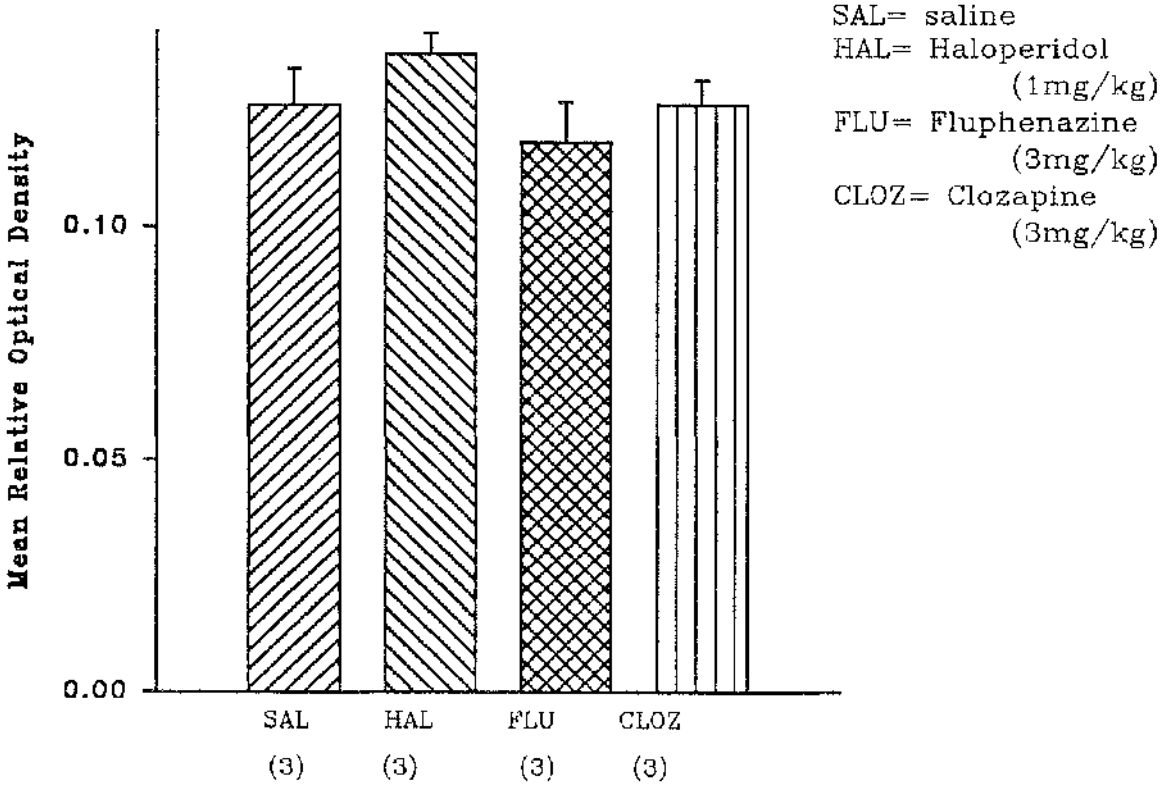
The levels of BF-1 mRNA expressed in saline treated animals were generally distributed in relatively high levels throughout the brain, particularly in the forebrain regions. Following treatment with haloperidol (1mg/kg, n= 3), fluphenazine (3mg/kg, n= 3), or clozapine (3mg/kg, n= 3), the levels of BF-1 mRNA in the rostral striatum were unchanged compared to the levels expressed in saline treated control animals. [Figure 4.6.1]. However, in the rat caudal striatum, it was observed that intraperitoneal injection of clozapine (3mg/kg, n= 3) caused an increase in the levels of BF-1 mRNA after 24 hours. This induction by clozapine was shown to be significantly different from saline treated animals [$F(3,8) = 4.93$, $p = 0.038$, $p < 0.05$] [Figure 4.6.2]. Haloperidol (1mg/kg, n= 3) and fluphenazine (3mg/kg, n= 3) had no effect on BF-1 mRNA levels in the caudal striatum 24 hours after drug administration. [Figure 4.6.2].

4.7 Effect of acute neuroleptic drug treatment on the expression of the POU-domain containing gene RHS2 in the rat striatum

Low levels of RHS2 mRNA were expressed in both the rostral and caudal striatum, in saline treated rats. Treatment with haloperidol (1mg/kg, n= 3), fluphenazine (3mg/kg, n= 3) or clozapine (3mg/kg, n= 3) had no significant effect on the levels of RHS2 mRNA in the rostral striatum of the rat [Figure 4.7.1]. Similarly in the rat caudal striatum, treatment with haloperidol (1mg/kg, n= 3), fluphenazine (3mg/kg, n= 3) or with clozapine (3mg/kg, n= 3) caused no

Figure 4.6.1 : Effect of acute neuroleptic drug treatment on the expression of BF-1 mRNA in the rat rostral striatum. Animals were injected i.p. with vehicle (0.9% saline), or haloperidol (1mg/kg), or fluphenazine (3mg/kg), or with clozapine (3mg/kg). After 24 hours, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m.; the number of animals per group is shown in parentheses.

Figure 4.6.2: Effect of acute neuroleptic drug treatment on the expression of BF-1 mRNA in the rat caudal striatum. Animals were injected i.p. with vehicle (0.9% saline), or haloperidol (1mg/kg), or fluphenazine (3mg/kg), or with clozapine (3mg/kg). After 24 hours, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m.; the number of animals per group is shown in parentheses. Statistical significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test. $p < 0.05$* relative to saline group.


Mean Relative Optical Density
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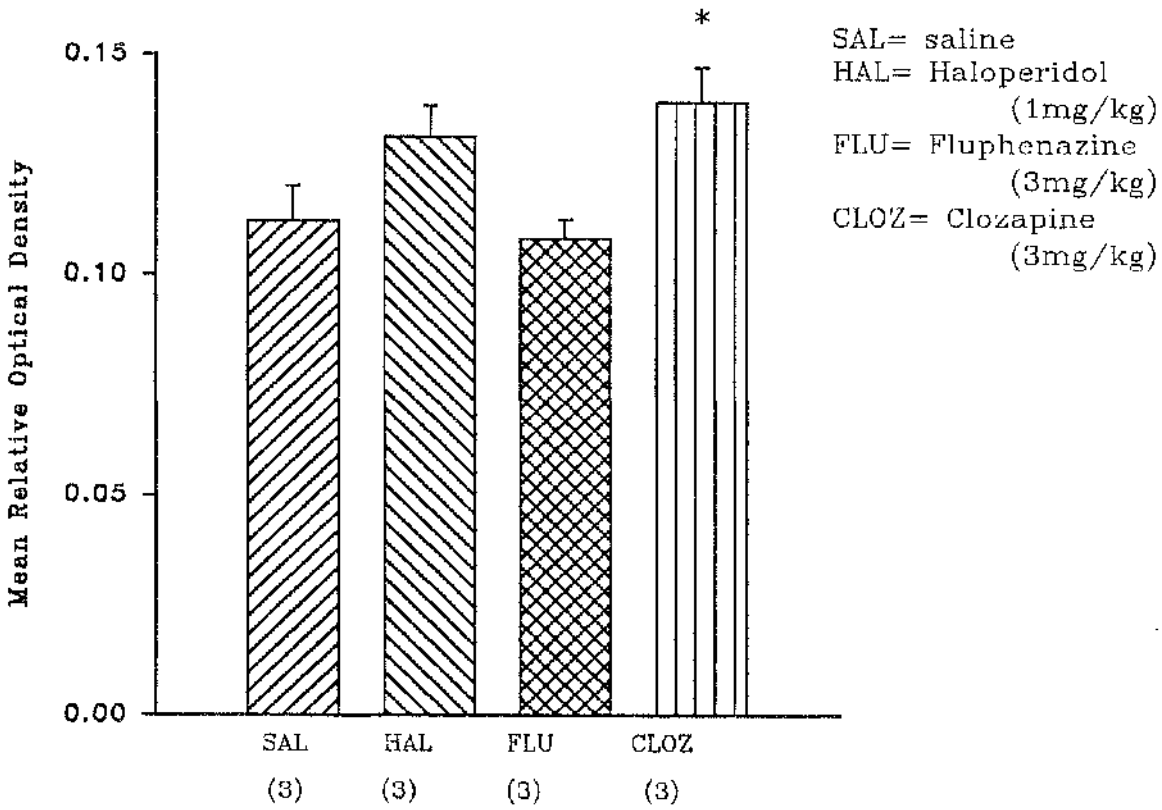
0.05
0.00
SAL
HAL
FLU
CLOZ
(3)
(3)
(3)
(3)
SAL= saline
HAL= Haloperidol
(1mg/kg)
FLU= Fluphenazine
(3mg/kg)
CLOZ= Clozapine
(3mg/kg)


*
Mean Relative Optical Density
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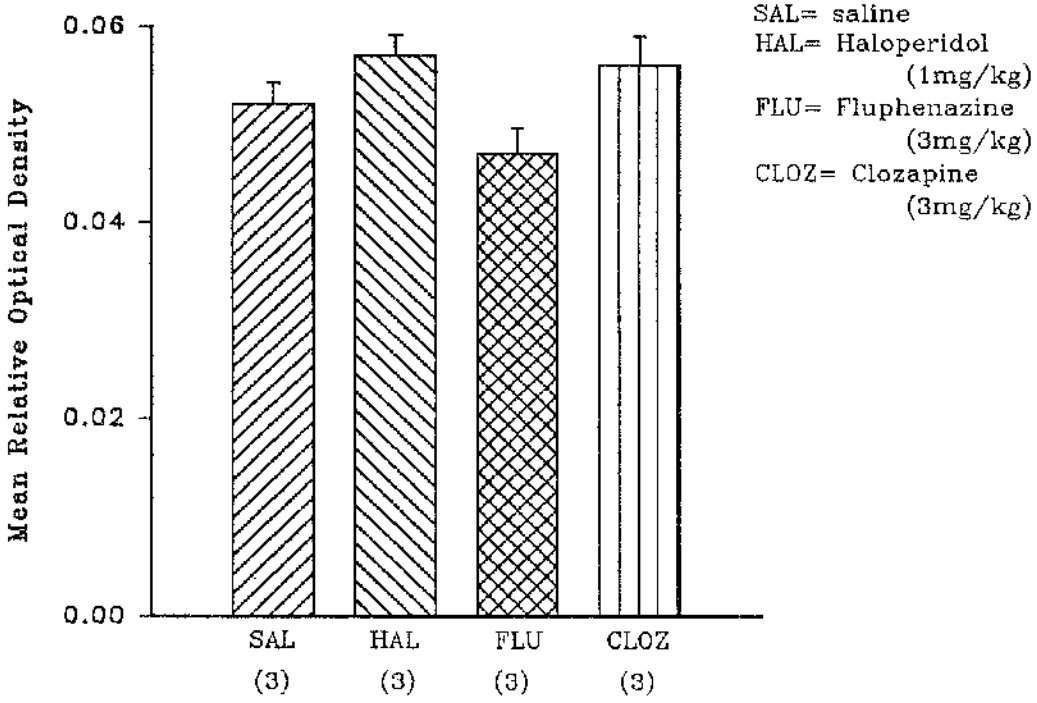
0.10
0.05
0.00
SAL
HAL
FLU
CLOZ
(3)
(3)
(3)
(3)
SAL= saline
HAL= Haloperidol
(1mg/kg)
FLU= Fluphenazine
(3mg/kg)
CLOZ= Clozapine
(3mg/kg)

Figure 4.7.1 : Effect of acute neuroleptic drug treatment on the expression of RHS2 mRNA in the rat rostral striatum. Animals were injected i.p. with vehicle (0.9% saline), or haloperidol (1mg/kg), or fluphenazine (3mg/kg), or with clozapine (3mg/kg). After 24 hours, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m.; the number of animals per group is shown in parentheses.

Figure 4.7.2 : Effect of acute neuroleptic drug treatment on the expression of RHS2 mRNA in the rat caudal striatum. Animals were injected i.p. with vehicle (0.9% saline), or haloperidol (1mg/kg), or fluphenazine (3mg/kg), or with clozapine (3mg/kg). After 24 hours, the animals were killed and their brains processed for in situ hybridisation. Results, obtained from autoradiographic film, are expressed as mean relative optical density + s.e.m.; the number of animals per group is shown in parentheses.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0.00
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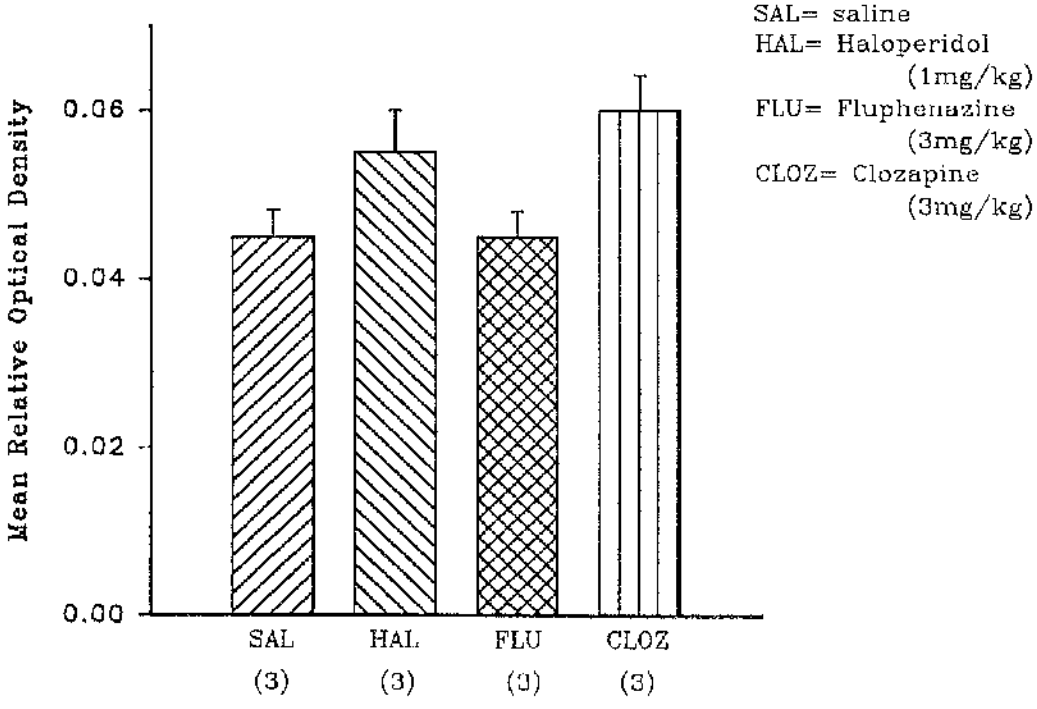
(3)
HAL
(3)
FLU
(3)
CLOZ
(3)
SAL= saline
HAL= Haloperidol
(1mg/kg)
FLU= Fluphenazine
(3mg/kg)
CLOZ= Clozapine
(3mg/kg)


Mean Relative Optical Density
0.06
0.04
0.02
0.00
SAL
(3)
HAL
(3)
FLU
(3)
CLOZ
(3)
SAL= saline
HAL= Haloperidol
(1mg/kg)
FLU= Fluphenazine
(3mg/kg)
CLOZ= Clozapine
(3mg/kg)

significant changes in the levels of RHS2 mRNA compared to saline treated animals in the rat [Figure 4.7.2].

DISCUSSION

Regulation of Immediate Early Genes in vivo

4.8 Time Course of fluphenazine effect on c-fos and zif/268 mRNA levels

The typical neuroleptic drug fluphenazine, having a high affinity for dopaminergic D2 receptors, was shown to maximally induce c-fos mRNA levels in the rat striatum forty-five minutes after the drug was given, and the levels were reduced back to basal levels after three hours. This finding is similar to previous reports that suggest a peak induction of c-fos can occur from thirty to sixty minutes after an acute treatment with psychostimulants or neuroleptic drugs (Robertson et al., 1989; Cole et al., 1992; Nguyen et al., 1992; Bhat et al., 1992). Our results also showed a significant increase in c-fos mRNA after fifteen minutes in the rostral region of the rat striatum, but not in the caudal regions. In a former study, zif/268 mRNA levels have been shown to be significantly increased after fifteen minutes (Cole et al., 1992), and therefore the observed induction of c-fos mRNA fifteen minutes after fluphenazine treatment is not unlikely. It may be that the drug fluphenazine is absorbed more quickly, and thus an induction of c-fos gene expression is occurs more rapidly. Differences in the rate at which drugs are absorbed across the blood-brain barrier are likely to reflect small differences in peak expression time.

The immediate early gene zif/268 was also demonstrated to be induced maximally in the caudal region of the striatum after thirty minutes, and the levels declined back to basal levels after three hours. In the rostral regions of the rat striatum maximal zif/268 expression was observed after forty-five minutes which then decreased back to basal levels over a three hours time period. In previous reports it has been shown that zif/268 is induced maximally by thirty to forty-five minutes after neuroleptics (Cole et al., 1992; Nguyen et

al., 1992), although an induction can be measured as early as fifteen minutes (Cole et al., 1992). Our results are therefore consistent with previous studies (Cole et al., 1992; Nguyen et al., 1992; Bhat et al., 1993).

These studies were undertaken in order to reproduce the current evidence suggesting that 'typical' neuroleptics can induce both c-fos and zif/268 gene expression in the rat striatum and accumbens regions. In addition we were interested in the effects of certain 'typical' and 'atypical' antipsychotic drugs on the expression of two members of the jun family of immediate early genes, namely jun-B and jun-D, and to elucidate the receptor mechanisms that precede the induction of these immediate early genes.

4.9 Effect of acute Typical Neuroleptic drug treatment on c-fos and zif/268 gene expression in the rat striatum and nucleus accumbens

The results of this study show that acute injections of the typical antipsychotic drugs haloperidol and fluphenazine can cause the induction of a number of immediate early genes including c-fos and zif/268 in the striatal region of the rat brain. A considerable induction of zif/268 mRNA, and to a lesser extent c-fos mRNA, was observed in the striatum. The induction of both c-fos and zif/268 mRNA by these typical antipsychotics followed a dorsolateral pattern. In addition, haloperidol was also shown to induce c-fos mRNA levels homogeneously in the nucleus accumbens. This is in agreement with previous reports suggesting that haloperidol can induce fos protein in both the core and shell regions, as well as in the striatum (Deutch et al., 1992; Robertson and Fibiger, 1992). However, the level of c-fos mRNA was considerably less in the accumbens region after fluphenazine, compared with the levels seen in the striatum after fluphenazine administration. Merchant and Dorsa (1993),

observed no induction of c-fos mRNA in the nucleus accumbens after administration of typical antipsychotic agents. Conversely, zif/268 mRNA was dramatically induced by fluphenazine in the nucleus accumbens, the effect being quantitatively greater than that of haloperidol. It is clear from this that the immediate early genes under investigation have different induction patterns after typical neuroleptic drug administration. This may reflect stimulation of different cell populations within the caudate-putamen. The inductive effect of haloperidol on both c-fos and zif/268 is consistent with previous reports demonstrating an increase in the expression of these immediate early genes by haloperidol in the caudate-putamen (Dragunow et al., 1990; Miller, 1990; Nguyen et al., 1992; Robertson et al., 1992).

As many antipsychotic drugs are known to be potent dopamine receptor antagonists, it has been suggested that the induction of certain immediate early genes such as c-fos and zif/268 may be mediated by blockade of dopamine neurotransmission.

More recent evidence also indicates that the "late response gene" dynorphin acting through kappa opioid receptors can inhibit the cocaine-induced expression of c-fos, mediated by stimulation of D1 receptors, in striatonigral neurons (Steiner and Gerfen, 1995). This suggests that the striatal dynorphin system acts to inhibit the dopamine input to striatonigral neurons, implying that endogenous feedback mechanisms can control the level of the immediate early gene, c-fos, that is expressed. This reveals a further complexity to cellular events, and in the future may provide an insight into the manifestation of certain disease states.

4.10 Effect of acute Typical Neuroleptic drug treatments on jun-B and jun-D gene expression in the rat striatum and nucleus accumbens

We report here for the first time that haloperidol can dramatically induce the expression of jun-B mRNA in the rat striatum and nucleus accumbens. The level of expression of jun-B mRNA in the striatum was similar to the levels expressed for c-fos mRNA and zif/268 mRNA after administration of haloperidol, compared with the very low levels expressed in saline-treated control animals. The large proportion of cells positively labelled for jun-B in the striatum suggest that jun-B is induced primarily in the medium-sized spiny neurons, because this cell type is known to represent 95% of striatal neurons (Graybiel et al., 1986).The induction due to haloperidol followed a rostral-caudal decline that matches the proposed rostral-caudal decline in the density of D2 dopamine receptors in the striatum (Boysen et al., 1986). Therefore, this evidence suggests that the induction of jun-B is predominantly modulated by D2 dopamine receptors.

Whereas this is the first demonstration that jun-B is induced by neuroleptic treatment, there is some evidence for the induction of jun-B after acute administration of cocaine (Hope et al., 1992), amphetamine, and apomorphine (Cole et al., 1992). It is well known that these drugs have agonist properties at a number of receptors, including dopamine receptors. Therefore our evidence suggests that the expression of jun-B is regulated according to the level of dopamine activity.

This study has also revealed another member of the jun family of immediate early genes to be induced by typical neuroleptic drug administration. After haloperidol, a small but significant induction of jun-D mRNA was observed in both the striatum and nucleus accumbens of the rat. High basal

levels of jun-D mRNA exist throughout the brain, which makes this immediate early gene different from jun-B. Similar to jun-D, high levels of basal expression of c-jun are also present in certain brain areas. This is the first evidence that the level of expression of the jun-D gene can be modulated by neurotransmitter activity. The induction of jun-D after haloperidol administration suggests that jun-D, as well as jun-B, may be interacting with fos proteins to regulate the expression of downstream genes. In a manner similar to jun-B, expression of jun-D mRNA followed a dorsolateral pattern of distribution in the striatum and a homogeneous distribution in the nucleus accumbens after haloperidol administration.

The effect of fluphenazine, also having a typical antipsychotic drug profile, was clearly very similar to that of haloperidol. Fluphenazine, given at relatively high doses was shown to induce jun-B mRNA. A dramatic induction of jun-B expression was observed in the striatum and a similar level of induction was observed for jun-B mRNA in the nucleus accumbens after fluphenazine. As typical neuroleptics are potent dopamine receptor antagonists in vivo, this further suggests that blockade of dopamine transmission may be involved in mediating this effect.

4.11 Effect of the Atypical neuroleptic, clozapine on the expression on the immediate early genes c-fos, zif/268, jun-B and jun-D and the possible receptor mechanism(s) mediating immediate early gene induction by antipsychotic drugs

The results obtained demonstrate that low doses of clozapine have no effect on the expression of c-fos, zif/268, jun-B, or jun-D mRNA levels in either the rat striatum or nucleus accumbens after acute administration.

Previous reports have demonstrated that higher doses of the atypical antipsychotic clozapine can induce fos-immunoreactivity in the shell region of the nucleus accumbens, but not in the striatum (Deutch et al., 1992; Robertson

and Fibiger, 1992). It is currently thought that the accumbens is associated with the mesolimbic system and is one of the prime targets for neuroleptic drugs. Behaviuoral studies have shown that chronic clozapine treatment selectively enhances apomorphine-induced locomotor activity, behaviour associated with dopamine nesolimbic activation. Whereas, treatment with haloperidol significantly enhanced apomorphine-induced chewing and sniffing stereotypies, thought to be associated with dopamine nigrostriatal activation (Seeger et al., 1982). The extrapyramidal side effects associated with many typical neuroleptic drug treatments may be attributed to their activity on motor systems within the caudate-putamen. Therefore, the pattern of expression of c-fos and other immediate early genes may provide an important insight into the regional mechanisms of atypical antipsychotics. In this study low doses of the atypical antipsychotic drug clozapine had no effect on the levels of expression of c-fos, zif/268, jun-B and jun-D mRNA in either the striatum or nucleus accumbens of the rat brain. It has previously been shown through in vitro binding studies that fluphenazine has a greater affinity for the D2 dopamine receptor than the D4 dopamine receptor (Van Tol et al., 1991). Clozapine, however, has a ten-fold greater affinity for the D4 site compared with the D2 site (Van Tol et al., 1991). Clozapine has been shown to bind appreciably to striatal D2 sites in rats only at doses of 10mg/kg and above (Saller et al., 1987; Audinot et al., 1993). It is therefore likely that the lower dose of 3mg/kg used in these acute experiments is preferentially blocking D4 sites in vivo rather than D2 sites. This is supported by the evidence that a dose of 3mg/kg clozapine blocks a substantial proportion of the serotonin 5-HT2 receptors in vivo (Canton et al., 1993). Indeed clozapine has a similar affinity for D4 and 5-HT2 sites (Van Tol et al., 1991; Zifa and Fillion, 1992), implying that this dose will produce D4 antagonism in vivo. At higher doses of clozapine (10-20mg/kg), an induction of zif/268 has been reported in the caudate-putamen (Nguyen et al., 1992).This therefore suggests that at higher doses, clozapine is acting at D2 receptors to

produce this effect. As clozapine had no effect on the immediate early genes under investigation, this would imply that the increased expression observed after typical neuroleptic drug administration is most likely due to a D2 dopamine receptor blockade rather than a D4 effect.

D1 dopamine receptor blockade may be considered to have negligible contribution to the effect on immediate early gene expression by any of the neuroleptics used in these studies. It has been shown that low doses of fluphenazine occupy primarily D2 dopamine receptors in vivo (Saller et al., 1987). Dragunow et al. (1990), have demonstrated previously that the specific D1 dopamine receptor antagonist SCH23390 did not induce c-fos protein in striatal neurons. Therefore, it is likely that the effect of haloperidol, fluphenazine or clozapine on D1 or D5 dopamine receptors does not contribute to the clear induction of the immediate early genes c-fos, zif/268, jun-B, and jun-D seen after drug administration. However, the affinity of fluphenazine for D1 receptors is relatively high; therefore, it may be considered possible that the induction of jun-B mRNA after fluphenazine treatment is reduced in comparison with the induction observed after haloperidol, due to the ability of fluphenazine to antagonize D1 receptors.

Despite their high affinity for dopamine receptors, neuroleptics also have considerable affinity for many serotonin receptors. From both in vivo and in vitro binding studies, clozapine is known to be a potent antagonist at serotonin 5-HT2 and 5-HT1C receptors (Zifa and Fillion, 1992; Canton et al., 1993). It is likely that clozapine administered at a dose of 3mg/kg would be blocking approximately 50% of 5-HT2/1C receptors (Canton et al., 1993). Likewise the typical neuroleptics haloperidol and fluphenazine were shown to have some antagonist binding properties at 5-HT2 receptors, but with much lower affinity than clozapine. The relative potencies of these drugs, together with a lack of effect of clozapine at a dose likely to be blocking 5-HT2/1C receptors in vivo, would therefore suggest that 5-HT receptors are not

involved in the effects reported here. Nevertheless, the consequences of neuroleptics having simultaneous effects on multiple transmitter systems must be taken into consideration.

A number of mechanisms have been proposed that could explain the increased expression of many immediate early genes by typical neuroleptic drug administration. One possible interpretation of these results is that haloperidol and fluphenazine produce their effects by increasing the release of dopamine from nigral-striatal terminals, which then acts on D1 dopamine receptors to induce immediate early genes. Related to this idea is the more likely possibility that D2 dopamine antagonists induce immediate early gene mRNA in striatal neurons by allowing unopposed D1 receptor stimulation. After a 6-hydroxydopamine lesion, the specific D1 dopamine receptor agonist SKF 38393 has been shown to increase c-fos in striatal neurons of the rat brain (Robertson et al., 1990). The link between D1 receptor activation and the induction of immediate early genes, has been further supported by Cole et al. (1992), who have demonstrated that administration of SKF 38393, but not the specific D2 receptor agonist LY171,555, causes an induction of c-fos, zif/268 and jun-B in the caudate-putamen after a 6-hydroxydopamine lesion, and this can be abolished by pretreatment with SCH 23390. Recent evidence also suggests that SCH 23390 decreases the mRNA levels of zif/268 in the adult intact striatum of the mouse (Mailleux et al., 1992). Therefore the current evidence implies strongly that the induction of a number of immediate early genes including c-fos, zif/268 and jun-B may be the result of a stimulation of the D1 dopamine receptor. Previous reports have also demonstrated that destruction of mesotelencephalic dopaminergic neurons with 6-hydroxydopamine abolishes the increase in fos expression in the striatum and nucleus accumbens produced by haloperidol (Robertson and Fibiger, 1992). This provides further support for the induction of c-fos after D1 dopamine receptor activation. However, it should be noted that SCH23390 does not

block the induction of c-fos by D2 receptor antagonism (Robertson et al., 1992; Merchant and Dorsa, 1993). Therefore the precise mechanisms operating remain unclear.

Our results suggest that neuroleptic drug administration induce a pattern of immediate early gene induction more complex than has been appreciated until now. This includes the induction, at the mRNA level, of two members of the jun family of leucine zipper proteins, jun-B and jun-D. The results also suggest that these effects occur in response to blockade of D2 rather than D4 receptors. It would appear that a fine balance exists in vivo between D1 and D2 receptor stimulation. When the balance is disturbed, as in psychotic disorders, the expression of a number of immediate early genes may be affected. As yet, the consequences of these transient changes on the expression of downstream genes is not fully understood. However, a link has been suggested between c-fos and proenkephalin gene regulation (Sonnenberg et al., 1989). Although, more recently, this idea has been argued against, as it has been implied that it is not the fos protein that interacts with the enkephalin enhancer (Konradi et al., 1993). As the protein CREB has also been shown to act as a transcription factor, this may be a more likely candidate.

4.12 Effect of acute neuroleptic drug treatment on the expression of BF-1 and RHS2 transcription factor genes

Our results show that the expression of the recently cloned transcription factor genes RHS2 and brain factor-1 (BF-1) were not affected thirty minutes after treatment with either the typical neuroleptic drug fluphenazine, or the atypical neuroleptic drug clozapine, compared to saline treated controls. However, the expression of BF-1 mRNA after twenty -four hours was significantly induced by low doses of the atypical neuroleptic clozapine in the

medial striatum, but not by fluphenazine. There was a tendency towards an induction of RHS2 mRNA in the striatum after clozapine treatment, although the levels failed to reach significance. These results provide the first evidence that neuroleptics can induce BF-1 gene expression, at the mRNA level, in the adult rat brain.

In the developing mammalian brain, three separate regions known as the forebrain, midbrain and hindbrain are initially formed from the rostral part of the neural tube. The most rostral forebrain region, the telencephalon, gives rise to the structures of the cerebral hemispheres, which include the cerebral cortex and basal ganglia. Previous studies have demonstrated that the expression of BF-1, a member of the HNF-3/fork head gene family, is restricted to the telencephalon region in the developing rat brain (Tao and Lai, 1992). Therefore it has been suggested that BF-1 may have a role as a transcriptional regulator in the complex signalling system that coordinates the precise temporal and spatial patterns of diiferential gene expression in multicellular organisms. Northern blotting analysis has shown that the BF-1 gene is expressed in the cortex, olfactory bulb, hippocampus and caudate-putamen in the adult rat brain, the levels of expression being four-fold lower than in the fetal rat brain (Tao and Lai, 1992). More recently, the identification of a gene in human fetal tissue having high homology at the nucleotide level to brain factor-1 in the rat, has been shown to be expressed in the telencephalon, with strong expression being observed in the dentate gyrus and hippocampus. The expression of human BF-1, also referred to as HFK1, was also found to be restricted to neuronal cells in the telencephalon (Murphy et al., 1994). The basal expression of BF-1 mRNA in the adult rat striatum showed that a large proportion of cells were positively labelled for BF-1, which suggests that BF-1 is expressed primarily in the mediun-spiny neurons, because this cell type has previously been demonstrated to represent 95% of striatal neurons (Graybiel et al., 1986). Therefore, it may

be suggested that the induction of BF-1 mRNA seen after clozapine treatment, is primarily in the medium-spiny neuronal cell poulation within the striatum.

The low dose of clozapine used in this study may reflect a mechanism involving either a dopamine D4 receptor stimulation or a 5-HT receptor subtype stimulation, which has previously been discussed in earlier studies using the same dosage of clozapine. Although clozapine is known to have a high affinity for many receptor types, including adrenergic, histaminergic and muscarinic receptors and therefore a variety of different receptors may be contributing to the observed induction of BF-1 after low doses of clozapine.

This is the first evidence that the a member of the HNF-3/ fork head gene family, brain factor-1, can be modulated by neuroleptic drug administration. Since the atypical neuroleptic, clozapine, but not the typical neuroleptic fluphenazine was able to induce BF-1 mRNA in the striatum, this may reflect a previously undiscovered role for BF-1 in the control of antipsychotic disorders, and a possible contributory factor in the lack of extrapyramidal side effects observed clinically after clozapine treatment. However, the potential target genes, whose level of expression is dependent on BF-1 induction, remains to be identified.

Chapter 5

Striatal Neuronal Cultures

INTRODUCTION

5.0 Striatal Neurone Morphology

As our understanding of the molecular mechanisms regulating cellular activity has grown, the information that can be obtained from in vivo experiments has become more difficult to interpret, and the importance of in vitro systems has consequently increased. The intercellular and subcellular processes which coordinate and control the activity of neurones in particular brain regions such as the striatum can frquently only be studied in tissue maintained in vitro, where reagents can be applied in known concentrations, directly onto the cells. This has been emphasised by the recent identification of novel dopamine and serotonin receptors which are expressed at relatively high levels in striatal tissue (Sunahara et al., 1991; Van Tol et al., 1991; Monsma et al., 1993). The absence of selective ligands for these receptors means that their role in regulating the activity of striatal neurons can essentially only be studied in vitro, where the drugs can be applied in precise concentrations, and hence relative selectivity can be calculated exactly.

It has been clear for some years that striatal neurons, dissociated from embryonic rat brain and maintained under cell culture conditions, can survive well in vitro for a number of weeks (Messer, 1981; Kessler, 1986; Weiss et al., 1986; Surmeier et al., 1988). These primary striatal neuron cultures have proved to be a useful system for electrophysiological and second messenger studies, looking at the effects of stimulation of receptors for dopamine, GABA, glutamate and growth factors (Murphy et al., 1987; Dumuis et al., 1988; Dubinsky, 1989; Misgeld and Dietzel, 1989; Sproson and Woodruff, 1990; Marin et al., 1992; Simpson and Morris, 1994). However, there is evidence that dopamine is likely to have different effects on the different classes of neuron in the striatum in vivo (Morris et al., 1988; Le Moine et al., 1991; Pollack and

Wooten, 1992). It is clearly of considerable importance to know which of the neuronal types normally present in the striatum are also present in the primary cultures. It may be that some classes of neuron do not survive at all, and that the intracellular effects that can be studied are those occuring in only one or two different neuronal types. Conversely, all casses of neuronal types may be present in vitro, in which case these primary cultures may provide a suitable model for studing the regulation of activityof the whole range of striatal neurons.

Approximately 95% of striatal neurons in vivo are thought to be GABA-ergic projection neurons additionally containing either enkephalin peptides or dynorphin and tachykinin peptides (Bolam et al., 1985; Graybiel, 1990; Besson et al., 1990). The remainder comprise possibly three populations of interneurons releasing acetylcholine, or GABA and neuropeptide Y, or somatostatin, neuropeptide Y and nitric oxide (Bolam et al., 1984; Phelps et al., 1985; Graybiel, 1990; Vuillet et al., 1990). Despite the importance of a comprehensive phenotypical characterisation of striatal neurons in culture, there is to date only limited evidence as to what classes of neurons are present in vitro. Extracts of primary striatal cultures have previously been shown to contain immunoreactive somatostatin and substance P (Kessler, 1986), and proenkephalin mRNA (Schwartz and Simantov, 1988; Vilijn et al., 1988), while analysis at the cellular level has shown the presence of GABA (Messer, 1981; Weiss et al., 1986; Surmeier et al., 1988), along with immunoreactive leu-enkephalin (Surmeier et al., 1988), which could arise from either the expression of the prodynorphin gene, or the proenkephalin gene, and immunoreactive somatostatin (Williams et al., 1991). Of these, only GABA, immunoreactive leu-enkephalin and immunoreactive somatostatin have been localised to neurons rather than glia (Surmeier et al., 1988; Williams et al., 1991). Striatal glia in culture are known to express the enkephalin gene at high levels (Vilijn et

al., 1988), so it remains an open question to what extent the other neuroactive substances detected in culture extracts are derived from non-neuronal cells.

Therefore, before striatal neurons in culture can be used as a model for the in vivo situation, it is important to characterise the cell types that are present in vitro.

RESULTS

5.1 Characterisation of rat striatal neuronal cultures

In striatal neurone cultures grown in DMEM, a high proportion of cells were identified as astrocytes on the basis of their large, flat morphology and their characteristic immunoreactivity for glial fibrillary acidic protein (GFAP), revealing large unstained nuclei, as shown in figure 5.1.1. To reduce astrocytic contamination of the cultures, a range of different concentrations of cytosine arabinoside was added to the culture medium. It was found that cytosine arabinoside (20μM), optimally reduced astrocytic contamination of the cultures to less than 5% of the total cell population without dramatically affecting the numbers of neurones. Cytosine arabinoside treatment was therefore included in all subsequent experiments. Neuronal cells were clearly distinguishable from astrocytes on the basis of their morphology. However, their identity was confirmed by using neurone-specific enolase (NSE)- immunostaining, together with in situ hybridisation with an oligonucleotide probe specific for microtubule-associated protein 2 (MAP 2) mRNA, found only in neuronal cell types. A variety of neuronal morphologies could be identified in neurones stained with the anti-NSE antiserum, including bipolar cells with oval somata, multipolar cells with oval somata, and cells with triangular somata giving rise to large numbers of dendritic processes. [Figure 5.1.2] revealing the diversity of cell types within the striatum. Following in situ hybridisation with an oligonucleotide probe for MAP 2 mRNA, neuronal cells counterstained with cresyl violet, can be seen to express MAP 2 message, compared to the complete absence of MAP 2 mRNA following hybridisation of cultures with a MAP 2 sense oligonucleotide probe (not shown).

A large number of cells in culture, approximately 30-40%, were stained using the met-enkephalin antiserum [Figure 5.1.3], characteristically staining the cell soma. Cells from each of the morphological groups were identified as met-enkephalin immunoreactive neurons. In accordance with this result, a relatively high proportion of neurones in culture (30-40%), were found to contain proenkephalin (Penk) mRNA, when assayed by in situ hybridisation histochemistry [Figure 5.1.4]. After hybridisation with a 25-fold excess of unlabelled Penk oligonucleotide included in the hybridisation buffer, no labelling was observed, confirming the specificity of the hybrisation reaction. The cells containing immunoreactive-met-enkephalin or those expressing Penk mRNA were generally of medium size (12-20μm diameter). In comparison, cortical cultures grown under the same conditions contained no met-enkephalin immunoreactive neurons (not shown).

A smaller number of neurones were also found to express preprotachykinin (PPT) mRNA [Figure 5.1.5], although the hybridisation signal was much weaker than with the Penk probe. No specific signal could be detected with an oligonucleotide probe specific for prodynorphin mRNA at any time point from 7 DIV to 21 DIV.

Following hybridisation of striatal cultures with the somatostatin antisense probe, only a small proportion of neurones were labelled, but the intensity of the hybridisation signal was very strong. [Figure 5.1.6]. Cells labelled with the somatostatin probe were generally larger (15-25 μm), than those labelled for Penk or PPT. Again, the hybridisation signal was completely removed by the inclusion of a 25-fold excess of unlabelled somatostatin oligonucleotide in the hybridisation reaction. No specific signal was seen when sense probes were used under identical conditions [Figure 5.1.7]. The NPY probe was expressed in the same number of cells as the somatostatin probe, although the hybridisation signal was considerably weaker than that observed for the somatostatin probe [Figure 5.1.8], and similarly a 25-fold excess of

unlabelled probe when added to the hybridisation reaction, abolished the hybridisation signal.

A very small number of striatal neurons in culture also stained positively for acetylcholinesterase [Figure 5.1.9]. These cells tended to be larger than average (20-25μm diameter) and stained very darkly for acetylcholinesterase. Characteristically, these cells gave rise to a number of heavily-stained processes that appeared to make contact with many neighbouring cells.

A relatively small percentage (< 5%) of cultured striatal neurons were demonstrated to contain NADPH-diaphorase activity [Figure 5.1.10]. These cell types generally had large triangular or oval somata (20-25μm diameter), and appeared similar in terms of their gross morphology to the neurones exhibiting NADPH-diaphorase activity in sections of adult rat striatum [Figure 5.1.11]. The cultured NADPH-diaphorase-positive neurones gave rise to long processes with axon-like morphology, with an extensive plexus of connections with neighbouring cells. These "axons" were characterised by the presence of pronounced variscosity-like swellings along their length, which loosely resembles the appearance of the diaphorase-positive fibres in adult rat striatal tissue.

Figure 5.1.1 : Primary striatal cultures ,in the absence of cytosine arabinoside, processed for immunocytochemistry using antisera against glial fibrillary acidic protein (GFAP). The stained cells are large and flat with unstained nuclei. Scale bar represents 25μm.

Figure 5.1.2: Primary striatal cultures processed for immunocytochemistry using antisera against neurone specific enolase (NSE). Groups of neurones show strong intensity staining for NSE, including a variety of different morphological types (bipolar, multipolar, triangular). Scale bar represents 25μm.

Figure 5.1.3 : Primary rat striatal cultures processed for immunocytochemistry using antisera against met-enkephalin. The cell bodies of neurones in culture stained positively for met-enkephalin. The characteristics of enkephalin positive neurones tended to be those with oval somata and a bipolar morphology. Scale bar represents 25μm.

Figure 5.1.4 : Cultured striatal neurones following hybridisation with a probe specific for proenkephalin (Penk) mRNA.. Exposure time 2 weeks. Photomicrographs show cultured striatal neurones expressing the Penk gene, as seen by the distribution of silver grains over certain cell bodies. Approximately 40-50% of cells were labelled positively with the Penk probe. Scale bar represents 25μm.

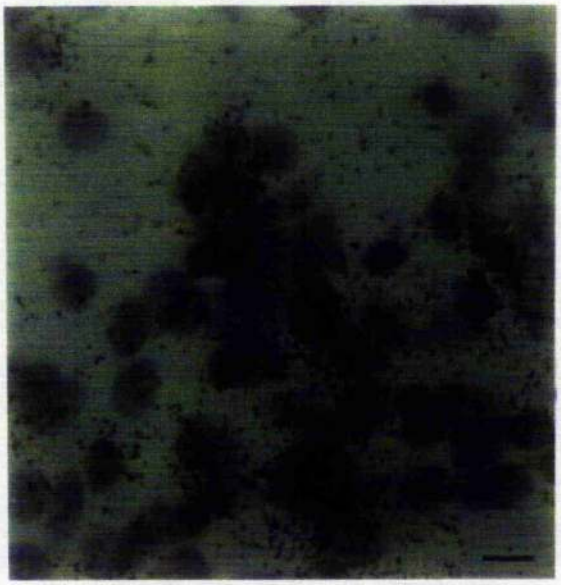


Figure 5.1.5 : Cultured rat striatal neurones following hybridisation with a radiolabelled probe specific for preprotachykinin (PPT) mRNA. Exposure time approximately 6 days. Photomicrographs show striatal neurones counterstained in cresyl violet. Approximately 25-35% of cells were labelled positively for PPT mRNA. Scale bar represents 25μm.

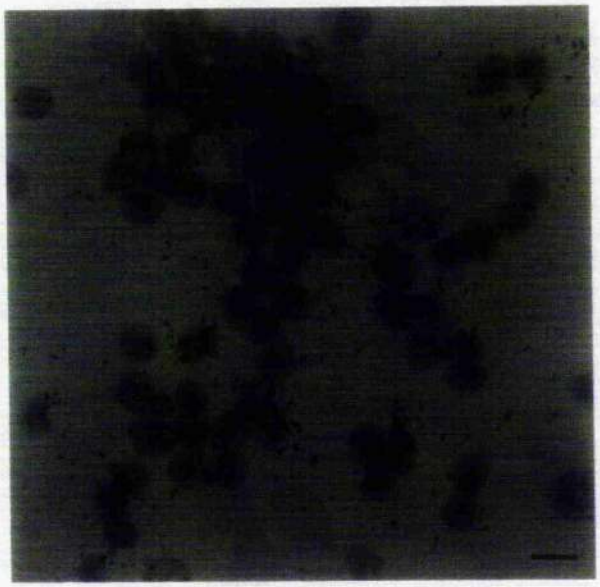
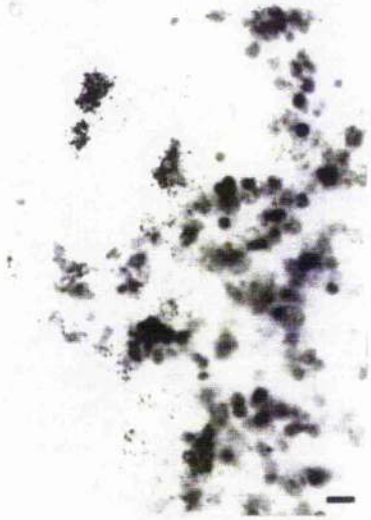


Figure 5.1.6 : Cultured rat striatal neurones following hybridisation with a radiolabelled probe specific for preprosomatostatin mRNA. Exposure time 4 days. Photomicrographs show striatal neurones counterstained in cresyl violet. A relatively small percentage of cells in culture were labelled positively for preprosomatostatin mRNA, as shown by an increase in the density of silver grains over certain cell bodies. Characteristically, the positively labelled cells were of larger size. Scale bar represents 25μm.

Figure 5.1.7 : Cultured rat striatal neurones following hybridisation with a radiolabelled somatostatin 'sense' probe. Exposure time 5 days. Photomicrographs show striatal neurones counterstained in cresyl violet. Scale bar represents 25μm.



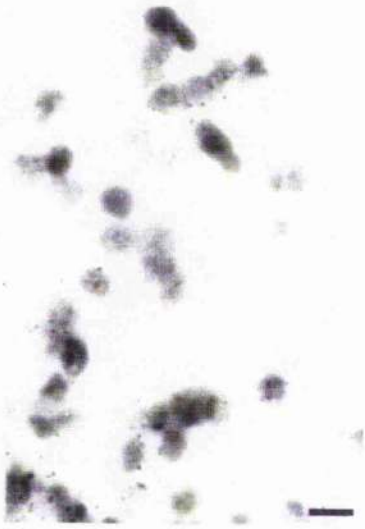


Figure 5.1.8 : Cultured rat striatal neurones following hybridisation with a radiolabelled probe specific for proneuropeptide Y mRNA. Exposure time 5 days. Photomicrographs show striatal neurones counterstained in cresyl violet. A small percentage of cells were labelled positively for proneuropeptide Y mRNA, as shown by an increase in the number of silver grains over certain cell bodies. However the intensity of labelling was generally much lower than for preprosomatostatin mRNA. Scale bar represents 25μm.

Figure 5.1.9 : Primary rat striatal neurone cultures processed for acetylcholinesterase staining. A small percentage of cells stained positively for acetylcholinesterase in culture, observed as a brown stain. Again both cell soma and dendrites were stained intensely and characteristically the labelled cells were much larger with many dendritic processes. Scale bar represents 25μm.

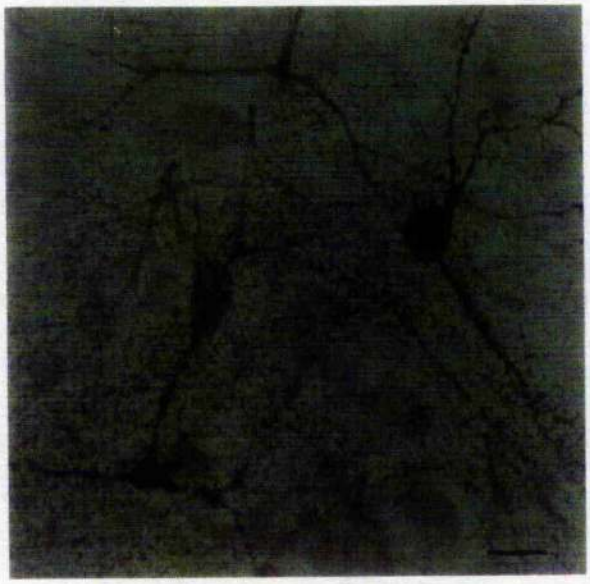
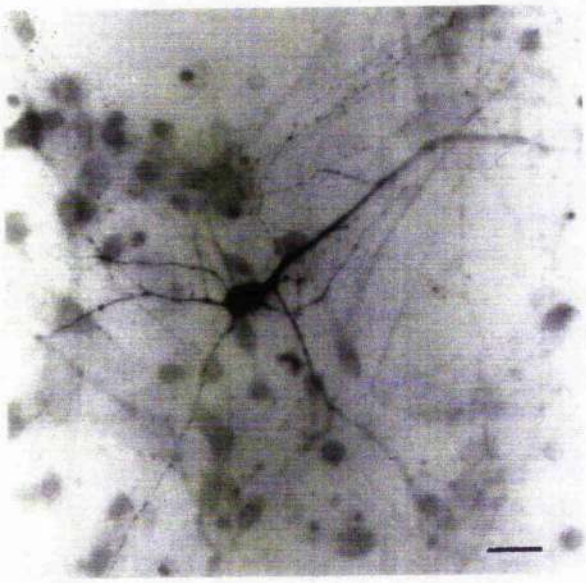
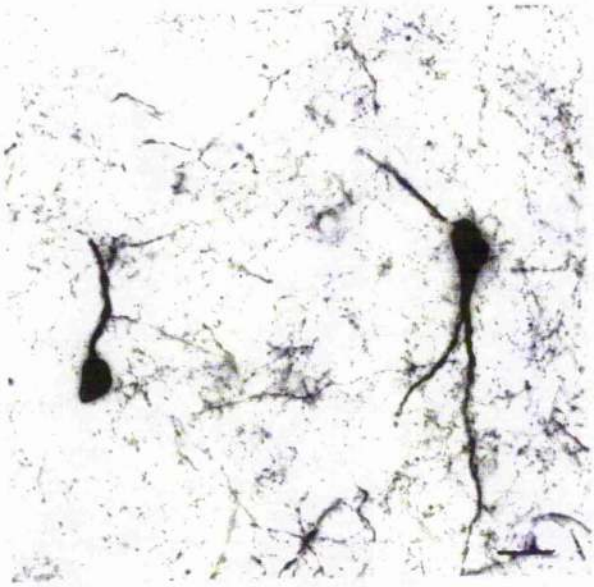


Figure 5.1.10 : Primary rat striatal neurone cultures processed for NADPH-diaphorase staining. Cultures were incubated with phosphate buffer containing NADPH and nitro blue tetrazolium for 1 hour at 37°C. A small percentage of cells in culture stained positively for NADPH-diaphorase, which can be seen here as a blue stain. Both the cell soma and dendrites stained positively. The population of cells that were NADPH-diaphorase positive tended to be large with numerous dendritic processes. Scale bar represents 25μm.

Figure 5.1.11 : Adult rat cryostat sections of caudal striatum processed for NADPH-diaphorase staining. Sections were incubated with phosphate buffer containing NADPH and nitro blue tetrazolium for 1 hour at 37°C. A small percentage of cells in adult rat sections stained positively for NADPH-diaphorase, seen here as a blue stain. Both cell soma and dendrites stained positively. Scale bar represents 25μm.





DISCUSSION

5.2 Phenotypic characterisation of rat striatal neurones in primary culture

Striatal neurones in primary culture have already proven to be a useful model system for studying the molecular events associated with striatal function. The use of primary cultures may be of particular importance with the rapid discovery of newly cloned receptors, many of which are known to be expressed in striatal tissue, such as the newly cloned dopaminergic D4 receptor (Van Tol et al., 1991) and serotoninergic 5-HT6 receptor (Monsma et al., 1993). In this study we have therefore examined primary striatal cultures for the presence of the various neurochemical phenotypes which are found in the rat striatum in vivo. The use of in situ hybridisation, rather than immunohistochemistry, allows the unequivocal identification of the pattern of gene expression in these cultured neurons.

The specificity of the hybridisation protocol is suggested in various pieces of evidence. We have shown repeatedly that in normal tissue, our hybridisation sequences allow the specific localization of the mRNA sequences to which the probes are complementary (Morris, 1989; Morris et al., 1990; Sirinathsinghji et al., 1990; Morris and Hunt, 1991; Morris, 1992). The high stringency conditions we employ prevent the hybridisation of the probes to any related sequences in the tissue. The lack of any signal in striatal cultures when sense probes are used, and the observation that the hybridisation signal with the antisense probes can be inhibited competitively by a 25-fold excess of the unlabelled oligonuleotide, provide further evidence that the hybridisation signals that we detect represent the authentic complementary mRNA.

Previous work has established that a high proportion of cultured striatal neurons are GABA-ergic (Messer, 1981; Weiss et al., 1986; Surmeier et al.,

1988). This is consistent with the in vivo situation. The majority of these GABA-ergic neurons in vivo are thought to be projection neurons, where GABA coexists with either dynorphins and tachykinins, or enkephalins (Besson et al., 1990). It could therefore be predicted that the cultures should contain a high percentage of neurons expressing the preproenkephalin, preprotachykinin and prodynorphin genes.

In primary striatal neuron cultures, it was observed that many neurons stained positively with an antiserum raised against met-enkephalin and there was negligible cross-reactivity to any of the peptide products of the prodynorphin (Proenkephalin B) gene. Since these cells closely resemble those demonstrated by Surmeier et al., (1988), to contain immunoreactive-leu-enkephalin, this suggests that these neurons express the proenkephalin rather than the prodynorphin gene. This was confirmed by in situ hybridisation analysis using a proenkephalin oligonucleotide. These results indicate that a high proportion of cells in these cultures express the proenkephalin phenotype.

An exact determination of the percentage of neurons expressing a particular phenotype in vitro is impossible. Although many neurons remain spread evenly over the substrate after seeding, other neurons migrate at an early stage to form regions where the neurons are clumped together, (Schwartz and Simantov, 1988; Surmeier et al., 1988), and accurate counting cannot be performed. However, a rough estimate of the proportion of neurons containing either proenkephalin mRNA or immunoreactive-met-enkephalin in culture suggests that enkephalinergic cells are the predominant cell type in vitro, as in vivo, although the proportion is rather lower than the 60% of striatal neurons that are known to be enkephalinergic in vivo. On the basis of the number of cells involved, it is likely that these cultured neurons expressing the proenkephalin phenotype are identical to the GABA-ergic cells already identified in primary culture (Messer, 1981; Weiss et al., 1986; Surmeier et al., 1988).

A considerable number of neurons in culture were also found to expresss preprotachykinin mRNA, although the proportion appears to be slightly lower than the proportion which are enkephalinergic. Considering the impossibility of making exact determinations of the percentage of labelled neurons in primary striatal culture, this would be approximately consistent with the reports that roughly 40-50% of striatal neurons in vivo contain immunoreactive substance P (Besson et al., 1990). It therefore seems clear that neuronal rather than non-neuronal cells are the source of immunoreactive-substance P detected in extracts of primary striatal cultures (Kessler, 1986).

In situ hybridisation is not a truly quantitative technique, in the sense that the kinetics and equilibrium position of the hybridisation reaction are unknown for each oligonucleotide. The absolute levels of a particular mRNA species cannot be determined, and so it is not possible to compare the abundance of different mRNA species, unless it is assumed that the hybridisation reaction has an identical efficiency for each probe/mRNA combination. This assumption is likely to be valid, but a formal proof would be essentially impossible. If this assumption is made, then the results suggest that the neuronal levels of preprotachykinin in cultured striatal neurons are markedly lower than the levels of preproenkephalin mRNA. This appears to be the situation in vivo (Young et al., 1986; Sirinathsinghji et al., 1990), although the relative difference between the hybridisation signals with the two probes is probably greater in the cultures. Removal of the dopaminergic innervation of the striatum in vivo increases the levels of proenkephalin mRNA and decreases the levels of protachykinin mRNA (Young et al., 1986; Morris et al., 1989). This potentially exaggerated difference (based on the above assumption) between the apparent levels of proenkephalin mRNA and preprotachykinin mRNA in culture may, if correct, be due to the absence of dopamine in the environment of the cultured neurons.

We found that no striatal neurons in culture contained detectable levels of prodynorphin mRNA. This is in agreement with an earlier study analysing extracts of primary cultures (Vilijn et al., 1988). This is interesting, when it is considered that striatal tachykinins and dynorphins are thought to be located predominantly in the same population of neurons (Besson et al., 1990). The presence of neurons expressing the preprotachykinin phenotype in vitro suggests that this class of neurons is able to survive under these conditions. It may be that some developmental signal, for example the secretion of a particular growth factor, is required for the appearance of the prodynorphin phenotype in the cells, and that this signal is not present in our culture medium. Alternatively, prodynorphin mRNA may be expressed, but at levels below the limit of sensitivity of the hybridisation reaction. However, this is unlikely, given that the protocol is highly sensitive (Morris, 1992).

The medium aspiny neurons which represent only a few percent of the total number of neurons in the normal striatum, are thought to contain somatostatin, neuropeptide Y and NADPH-diaphorase (Vincent and Johansson, 1983; Smith and Parent, 1986; Graybiel, 1990). We report here that neurons expressing the somatostatin gene are present in primary striatal cultures. We also observe many neurons that express the neuropeptide Y gene, and in so far as comparisons of the amounts of different mRNA species are possible, the neuropeptide Y gene appears to be expressed, at the mRNA level, at a lower level than the somatostatin gene. This parallels the situation in the normal striatum (Morris, 1989; 1992). Equally, within the limitations outlined above, it appears that considerably fewer neurons in culture contain somatostatin mRNA or neuropeptide Y mRNA, as compared to proenkephalin mRNA.

These results provide the first evidence that neurons in striatal culture also contain NADPH-diaphorase. While it has yet to be shown that somatostatin mRNA, neuropeptide Y mRNA and NADPH-diaphorase are all

contained in the same cell in vitro, our data demonstrate that the cultures contain neurons expressing each of these phenotypes. It now seems clear that the NADPH-diaphorase reaction detects the enzyme responsible for nitric oxide synthase (Hope et al., 1991). Release of nitric oxide from primary striatal cultures following glutamate receptor stimulation has recently been described, (Marin et al., 1992). Our results confirm that the origin of this release is likely to be neuronal. Nitric oxide is synthesised in the cytoplasm by the nitric oxide synthase/NADPH-diaphorase enzyme, and is then thought to diffuse randomly across membranes to effect any cells in the surrounding area. The evidence that nitric oxide is involved in a number of biochemical and physiological processes in the central nervous system, is increasing rapidly (Garthwaite, 1991).

The morphology of the NADPH-diaphorase-positive neurons appears to correspond surprisingly closely to the morphologies identifiable in intact tissue. The intense Golgi-like staining of the processes of the neurons containing NADPH-diaphorase enabled us to ascertain the extent of their connections with other cells in the culture wells. In every case, the diaphorase-positive neurons gave rise to a complex branching network of fibres covered in varicosity-like swellings. This reproduces closely the appearance of intact striatal tissue, which is a dense interwoven mesh of diaphorase-positive fibres with varicosities. We were able to observe these variscosities in contact with unstained neurons. For traditional neurotransmitters, evidence of synaptic specialisations, both pre- and post-synaptic, in electron micrographs, would be required for evidence of cell-cell communication in culture. However, the non-synaptic release of nitric oxide, and its ability to cross cellular membranes, means that a rough proximity of NADPH-diaphorase-containing fibres with another cell would be sufficient to allow intercellular communication of signals. These results therefore provide strong evidence that nitric oxide release from these cells may affect other neurons in culture.

The adult intact striatum also contains relatively rare cholinergic cells, larger than the other striatal neurons, and thought to be interneurons (Bolam et al., 1984; Phelps et al., 1985; Graybiel, 1990). In primary striatal culture we found that a very small percentage of striatal neurons stained heavily for acetylcholinesterase, which is an indication, although not an absolute proof that the cells are cholinergic. In other brain regions there are neurons which contain acetylcholinesterase, but no choline acetyl-transferase, and therefore do not synthesise the transmitter acetylcholine. However, in the striatum in vivo, it appears that acetylcholinesterase is expressed only in cholinergic neurons (Walker et al., 1987). The use of acetylcholinesterase as a marker for cholinergic neurons has the advantage that, in contrast to choline acetyl-transferase-immunostaining, the entire neuritic structure of the neurons is revealed.

The acetylcholinesterase-positive neurons that we observed in culture were larger than most of the other neurons, consistent with the situation in the intact striatum. The entire neuropil in normal striatal tissue stains very heavily for acetylcholinesterase, suggesting that neurites containing acetylcholineterase in vivo are virtually ubiquitous. It was noticeable that a small number of acetylcholinesterase-positive cells in culture displayed an intricate and widespread array of neurites, extending potentially to affect large areas of the culture well. It is therefore possible that this feature of the acetylcholinesterase-positive neurons in vitro mirrors an aspect of the functional cytoarchitecture of similar neurons in vivo.

The results shown here suggest that both NADPH-diaphorase-positive and the acetylcholinesterase-positive neurons make extensive connections with other neurons in culture. There is electrophysiological (Misgeld and Dietzel, 1989; Dubinsky, 1989) and biochemical (Weiss et al., 1986) evidence to support the presence of synaptic interactions between cultured striatal neurons. It should therefore be remembered that any neurotransmitter effects being

studied in such cultures may occur trans-synaptically, as well as directly on the cells under investigation.

Overall, these results suggest that primary cultures of striatal neurons may provide a surprisingly good model of the intact striatum. Neurons expressing proenkephalin mRNA, protachykinin mRNA, somatostatin mRNA and neuropeptide Y mRNA are all present, along with neurons staining for NADPH-diaphorase and acetylcholinesterase. The only neurotransmitter gene that apparently fails to be expressed from the striatal repertoire is the prodynorphin gene. Therefore primary cultures are a relatively good model of the intact striatum and will provide a suitable model for the identification of factors regulating the expression of these genes in culture.

Chapter 6

Dopaminergic Regulation of Immediate Early Genes in vitro

INTRODUCTION

6.0 Dopaminergic Regulation of Immediate Early Genes In Vitro

Many cell types in the mature central nervous system respond to changes in their local neurochemical environment by altering their pattern of gene expression (Goodman, 1990; Morris, 1993). These alterations in gene expression then persist for some time, allowing the cells to sustain a long-term response to the original stimulus. Perturbation of the normal level of dopaminergic transmission in the rat striatum, either by the administration of neuroleptics, which antagonise the actions of dopamine, or by lesioning the nigrostriatal pathway, results in sustained changes in the levels of peptide neurotransmitter mRNAs (Bannon et al., 1986; Merchant and Dorsa, 1993; Morris et al., 1988; Morris and Hunt, 1991; Young et al., 1986), associated with parallel changes in the rate of neuropeptide release (Lindefors et al., 1989).

Immediate early genes encode proteins whose structures fall into a number of distinct classes. They are rapidly induced in response to a variety of different stimuli such as growth hormones, increased synaptic activity and drug administration, and their proteins are thought to bind to specific sites in the genome to modulate the rate of transcription of downstream genes (Morgan and Curran, 1987; Sheng and Greenberg, 1990). Induction of immediate early genes is likely to provide a link between events at the cell membrane and long term alterations in neuronal gene expression. The immediate early genes c-fos (Morgan and Curran, 1987) and zif/268 (Millbrandt, 1987), also known as erg1, Krox 24, and NGFIA, are thought to be involved in functional plasticity in rat striatal neurons, in that they are induced in vivo by treatment with neuroleptic drugs (Dragunow et al., 1990; Nguyen et al., 1992; Robertson and

Fibiger, 1992; see also chapter 3). It is therefore of considerable interest to determine the mechanisms through which dopamine receptors are linked to the regulation of gene expression. A number of different dopamine receptors are known to be present in the striatum. The D1 and D5 (D1b) dopamine receptors correspond to the traditional D1-like receptors (Monsma et al., 1990; Sunahara et al., 1991; Tiberi et al., 1991) in that they show high affinity for the agonist SKF38393 and the antagonist SCH23390, and low affinity for D2 "selective" drugs such as quinpirole and eticlopride. Both D1 and D5 receptors are positively coupled to adenylate cyclase. Therefore the stimulation of D1-like receptors results in an increase in intracellular cAMP levels, with the consequent activation of cAMP-dependent protein kinase (PKA). However, there is also evidence that D1-like receptors can be linked to a stimulation of phospholipase C and consequently protein kinase C (PKC) activation (Felder et al., 1989; Mahan et al., 1990; Frail et al., 1993). The D2, D3, and D4 dopamine receptors correspond to the traditional D2-like receptor, in that they show relatively high affinity for the agonist quinpirole and the antagonist eticlopride, along with low affinity for SKF38393 and SCH23390. The D2 and D4 receptors are coupled to an inhibition of adenylate cyclase activity (Seeman and Van Tol, 1993).

At present there is very little evidence on the dopaminergic regulation of gene expression in striatal neurons maintained in cell culture, although there is some evidence for neurotransmitter regulated induction of immediate early genes in cell culture conditions. Stimulation of the N-methyl-D-aspartate receptor in primary cultures of cerebellar neurons has been shown to trigger the induction of c-fos and other immediate early genes (Szekely et al., 1987; Szekely et al., 1989; Szekely et al., 1990). More recently it has been shown that glutamate can increase c-fos, c-jun, jun-B, and zif/268 mRNAs by binding to selective glutamatergic receptor subtypes in cultured cortical and striatal neurons, and this induction is thought to be mediated by the enzyme PKC

(Vaccarino et al., 1992). Several neurotransmitters activate phosphoinositide hydrolysis in central nervous system neurons as the first step of a transmembrane signalling cascade that may lead to neuronal circuit modulation (Berridge et al., 1982; Gonzales and Crews, 1984; Batty et al., 1985; Schoepp and Johnson, 1988). Ambrosini and Meldolesi (1989) have shown that quisqualate and muscarinic receptor-induced phosphoinositide hydrolysis in primary cultures of striatal and hippocampal neurons involves differential mechanisms of activation. Activators of the cAMP second messenger pathway in primary cultures of neurons from rat cerebral cortex have also been shown to induce c-fos and other immediate early gene transcription factors directly (Vaccarino et al., 1993). Indeed, the D1-dopaminergic agonist, SKF38393, administered for short time periods, has been demonstrated to increase mRNA levels for the immediate early genes c-fos, jun-B, and zif/268 in cortical neurons (Vaccarino et al., 1993). Stimulation of glutamatergic NMDA receptors and metabotropic quisqualate receptors in cultured striatal neurons has previously been shown to increase the release of arachidonic acid, and therefore activate the arachidonic acid cascade (Dumuis et al., 1988; 1990).

There is little information on the regulation of late response genes in cell culture. However, Giraud and colleagues (1991) have demonstrated that preproenkephalin gene transcription can be activated by the cAMP-dependent protein kinase A and protein kinase C pathways in striatal cell cultures, with the use of selective drugs to target the respective enzymes. Similarly, treatment of striatal astrocytes with isoprenaline or with forskolin or 8-bromo-cAMP, agents which directly increase intracellular cAMP, have been shown to elevate the level of preproenkephalin mRNA (Batter and Kessler, 1991).

Therefore in this study, we have attempted to reproduce the dopaminergic stimulation of c-fos and zif/268 gene expression in primary cultures of embryonic rat striatal neurons, in order to identify the intracellular pathway(s) and second messenger system(s) involved. As much controversy

surrounds the location of dopaminergic D1 and D2 receptors in the caudate-putamen. Two proposals are currently well supported. Firstly, that the two subtypes of dopamine receptor are located on separate populations of cells, on the basis that certain neuropeptides have been colocalised with a particular subtype but not the other and secondly that both D1 and D2 subtypes are expressed on the same cell. In the following studies we have addressed this question using the cultured striatal neuron model.

RESULTS

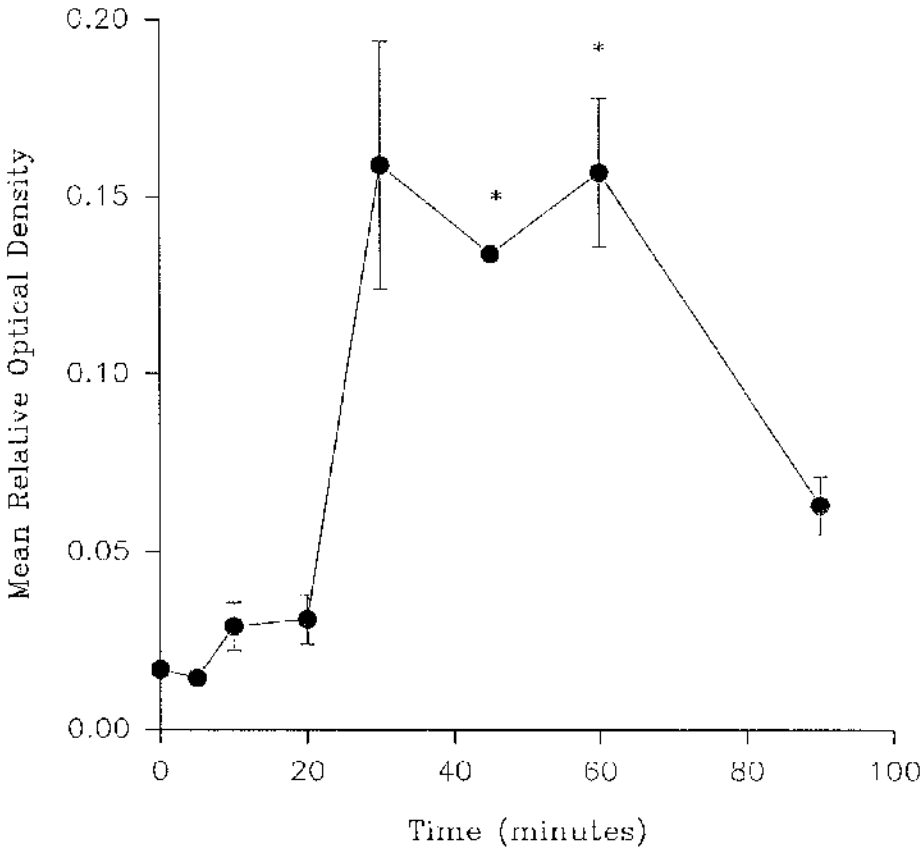
6.1 The time course effect of haloperidol on zif/268, jun-B and c-fos mRNA levels in cultured rat striatal neurons

Many IEGs including zif/268, jun-B and c-fos are known to be induced in vivo by the dopaminergic antagonist drug haloperidol (Nguyen et al, 1992). Therefore we have investigated the effect of haloperidol on these IEGs in a cultured striatal neuron system.

Neuronal cultures were exposed to dopamine (5μM) for two days. Haloperidol (2μM), when supplemented into the medium, was shown to induce the expression of zif/268 mRNA in vitro in a time-dependent manner. A dramatic increase in the levels of zif/268 mRNA was observed after 30 minutes, although a significant induction of zif/268 mRNA compared to the levels of expression at time zero, occurred only after 45 minutes [$F(2, 16) = 153.22$, $p< 0.05$] [Figure 6.1.1]. This maximal induction of zif/268 mRNA remained significantly high up to 60 minutes after haloperidol administration, $p< 0.05$ [Figure 6.1.1], and thereafter declined back to basal levels.

A similar, but more gradual induction of jun-B mRNA levels was observed with haloperidol (2μM). The induction of jun-B mRNA levels were significantly higher 20 minutes after treatment with haloperidol [$F(2,16) = 27.46$, $p= 0.006$ $p< 0.01$], than the levels expressed at time zero. A similar level of induction was observed 30 minutes after drug administration, $p< 0.01$. After 45 minutes the expression of jun-B mRNA remained significantly higher than at time zero, such that $p< 0.05$, after which the levels were not

Figure 6.1.1 : Effect of haloperidol on zif/268 mRNA levels in striatal cultures. Haloperidol (2μM) was added to cultures for 90, 60, 45, 30, 20, 10, and 5 minute time intervals before fixation in buffered formalin. Results, obtained from film autoradiographs, are expressed as mean relative optical density ± s.e.m.; n=3. Significance was determined by one-way ANOVA, followed by post-hoc Dunnett's test. * $p < 0.05$


0.20
0.15
0.10
0.05
0.00
Mean Relative Optical Density
*
*
0
20
40
60
80
100
Time (minutes)

significantly induced compared to the levels of jun-B mRNA at time zero (not shown).

Haloperidol (2μM), also had a tendency to increase the levels of c-fos mRNA in striatal neuronal cultures, although the increase was generally variable, and proved not to be significantly different from the levels expressed at time zero (not shown).

6.2 Effect of dopamine receptor activation on the levels of c-fos and zif/268 mRNA in cultured rat striatal neurons.

The previous experiment established that maximal induction of zif/268 mRNA occurred 45 minutes after drug application. Since the induction of jun-B mRNA and c-fos mRNA followed a similar pattern, this time point was consequently used for all subsequent experiments. The probes used in the following studies have all been extensively characterised under the conditions used (Wisden et al, 1990). .Nevertheless, the specificity of each probe used was confirmed in a number of preliminary experiments. Hybridisation of sense probes labelled to the same specific activity, gave no signal, and addition of a 25-fold excess of unlabelled antisense oligonucleotide to the hybridisation buffer completely displaced the hybridisation signal (refer to chapter 5).

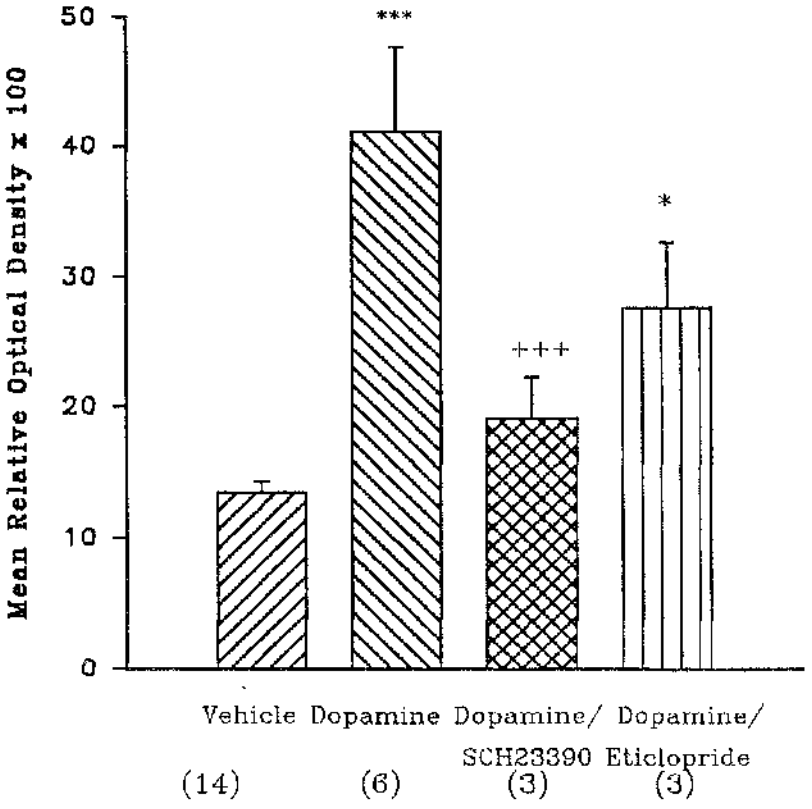
The hybridisation signal representing c-fos mRNA was generally low in unstimulated cells. In unstimulated cultures a small percentage of neurons were labelled for c-fos following treatment with vehicle alone (Hank's balanced salts solution).[Figure 6.2.1]. This was assumed similar to the low levels expressed in vivo when the cells remain unstimulated. Following treatment with dopamine

(5μM) a dramatic increase in the hybridisation signal for c-fos mRNA was observed. [Figure 6.2.1]. This induction was highly significant compared to the low levels of c-fos mRNA expressed in vehicle treated cultures [$F(3, 22) = 18.57$, $p= 0.0007$, $p< 0.001$]. The magnitude of induction was related to the concentration of dopamine applied. The levels of c-fos mRNA were induced by 100nM dopamine (not shown) , but a maximal induction was obtained at 5μM dopamine. Coadministration of the selective D1/D5 receptor antagonist, SCH23390 (1μM) with dopamine (5μM), clearly caused a reduction in the hybridisation signal due to c-fos mRNA.. This decrease was demonstrated to be significantly different from the signal due to dopamine alone [$F (3,22) =18.57$, $p< 0.05$] [Figure 6.2.1]. However ,coadministration of eticlopride (1μM), the selective dopamine D2 receptor antagonist, with dopamine proved not to be significantly different from dopamine alone, but was significantly different from vehicle alone [$F (3,22) =18.57$, $p< 0.05$] [Figure 6.2.1].

A very similar pattern of induction was observed for zif/268 mRNA in cultured striatal neurons. Low levels of zif/268 mRNA were expressed in vehicle treated cultures, which was assumed similar to the low levels expressed in vivo when the cells are unstimulated. Addition of a 25-fold excess of unlabelled probe to the hybridisation buffer, abolished the hybridisation signal completely (not shown), confirming specificity of the probe. Dopamine (5μM), clearly increased the hybridisation signal due to zif/268 mRNA , and this increase was significantly different from vehicle alone ($n= 8$) [$F(3,16) = 12.11$, $p= 0.0007$, $p< 0.001$] [Figure 6.2.2]. By coadministering SCH23390 (1μM) along with dopamine (5μM) a dramatic reduction in the hybridisation signal was observed, such that the reduction was different from dopamine alone [$F(3,16) = 12.11$, $p< 0.001$] [Figure 6.2.2]. , Coadministration of eticlopride (1μM) with dopamine (5μM) clearly did not reduce the signal representing the level of zif/268 mRNA compared to dopamine treatment alone, but was significantly different from vehicle treatment alone [$F (3,16) = 12.11$, $p< 0.001$] [Figure 6.2.2].

Figure 6.2.1 : Effect of dopamine receptor stimulation on c-fos mRNA levels in striatal cultures. Cultures were treated with vehicle or dopamine (5μM), or treated by coadministration of dopamine / SCH23390 (1μM), or coadministration of dopamine / Eticlopride (1μM) for 45 minutes. Results ,obtained from film autoradiographs, are expressed as mean relative optical density units x 100 +s.e.m. from the number of different cultures shown in parentheses. * $p< 0.05$, *** $p< 0.001$, relative to vehicle alone; +++ $p< 0.001$ relative to dopamine alone.

Figure 6.3.1 : Effect of dopamine D1 receptor stimulation on c-fos mRNA levels in striatal cultures. Cultures were treated with vehicle, or SKF38393 (1μM), or treated by coadministration of SKF38393 / SCH23390 (1μM), or coadministration of SCH23390 / Eticlopride (1μM) for 45 minutes. Results, obtained from film autoradiographs, are expressed as mean relative optical density units x 100 +s.e.m. from the number of different cultures shown in parentheses. * $p< 0.05$, *** $p< 0.001$, relative to vehicle alone; +++ $p< 0.001$ relative to SKF38393 alone.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40
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10
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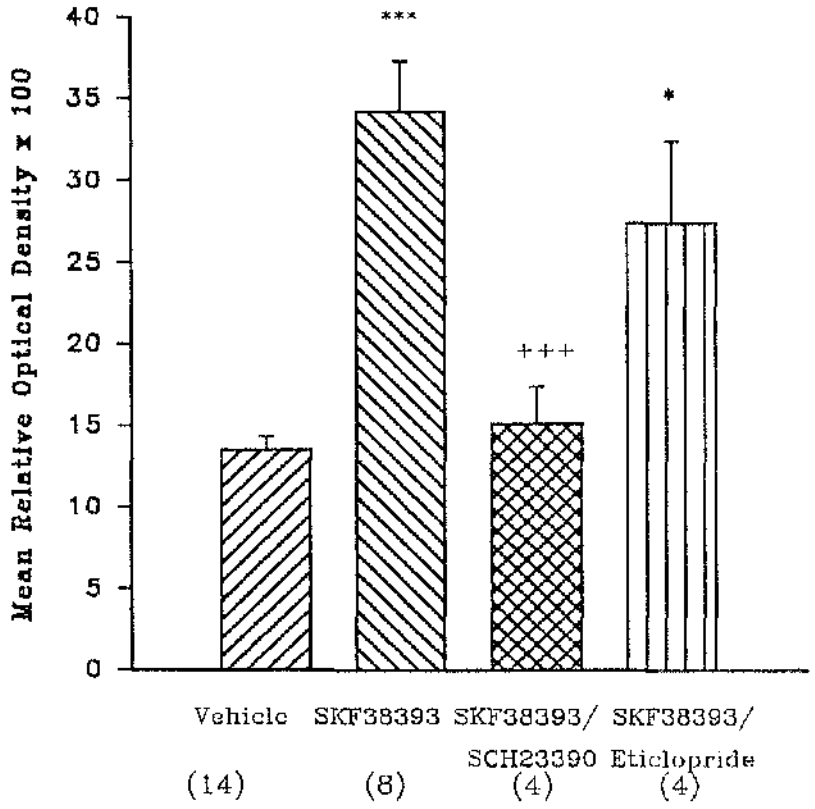
Dopamine
Dopamine/ SCH23390
Dopamine/ Eticlopride
(14)
(6)
(3)
(3)


Mean Relative Optical Density x 100
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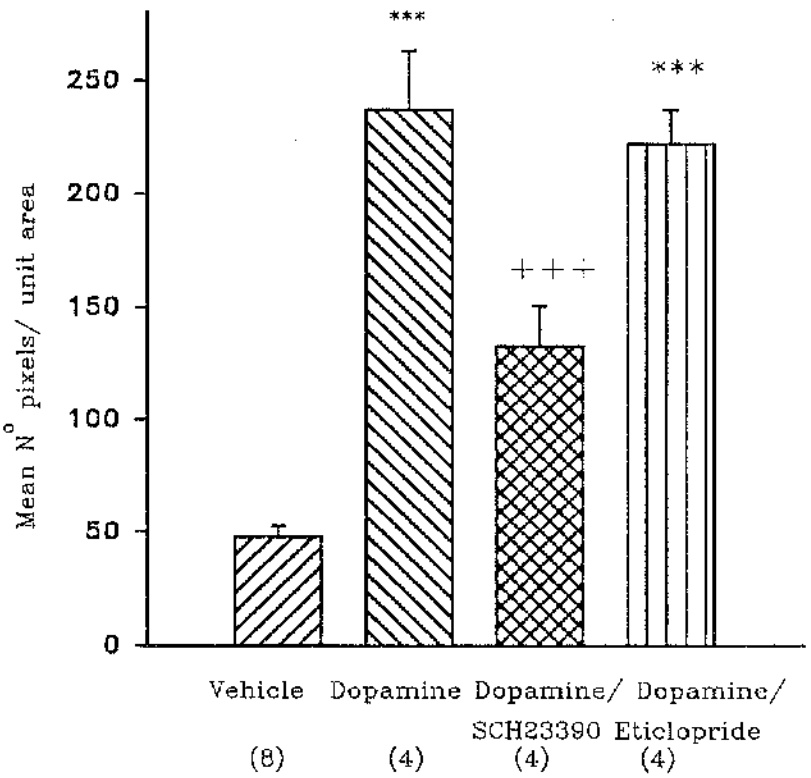
Vehicle
SKF38393
SKF38393/ SCH23390
SKF38393/ Eticlopride
(14)
(8)
(4)
(4)

Figure 6.2.2 : Effect of dopamine receptor stimulation on zif/268 mRNA levels in striatal cultures. Cultures were treated with vehicle, or dopamine (5μM), or treated by coadministration of dopamine / SCH23390 (1μM), or coadministration of dopamine / Eticlopride (1μM) for 45 minutes. Results are expressed as mean number of pixels / unit area +s.e.m. from the number of different cultures shown in parentheses. *** $p< 0.001$, relative to vehicle alone; +++ $p< 0.001$, relative to dopamine alone.

Figure 6.3.2 : Effect of dopamine D1 receptor stimulation on zif/268 mRNA levels in striatal cultures. Cultures were treated with vehicle, or SKF38393 (1μM), or treated by coadministration of SKF38393 / SCH23390 (1μM), or by coadministration of SKF38393 / Eticlopride (1μM) for 45 minutes. Results are expressed as mean number of pixels / unit area +s.e.m. from the number of different cultures shown in parentheses. *** $p< 0.001$, relative to vehicle alone; +++ $p< 0.001$, relative to SKF38393 alone.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Dopamine
Dopamine/ SCH23390
Dopamine/ Eticlopride
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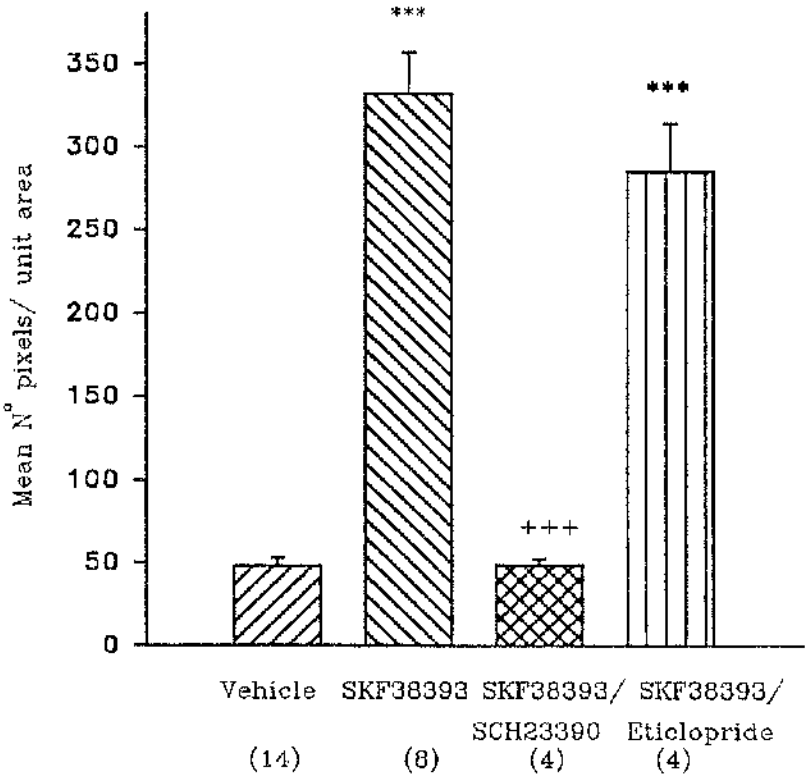
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Mean N° pixels/ unit area
Vehicle
SKF38393
SKF38393/ SCH23390
SKF38393/ Eticlopride
(14)
(8)
(4)
(4)

6.3 Effect of D1/D5 dopamine receptor stimulation on the levels of c-fos and zif/268 mRNA in cultured striatal neurons

Low levels of both c-fos and zif/268 mRNA were expressed in vehicle treated cultures. Following the addition of the D1/D5 receptor agonist, SKF38393 (1μM) a dramatic induction of c-fos mRNA was observed, and this increase was significantly different from vehicle alone [F (3,26) = 33.83, p= 0.0006, p< 0.001] [Figure 6.3.1]. Similarly, treatment with SKF38393 (1μM) caused a massive induction of zif/268 mRNA in these cultures [F (3,26) = 33.83, p< 0.001] [Figure 6.3.2, 6.4.3]. SCH23390 (1μM) when coadministered with SKF38393, was demonstrated to reduce the hybridisation signal for both c-fos and zif/268 [Figure 6.4.3] respectively. This decrease in the hybridisation signal was significantly different from SKF38393 alone for both c-fos [F (3,26) = 33.83, p< 0.001] [Figure 6.3.1] and zif/268 [F (3,26) = 33.83, p< 0.001] [Figure 6.3.2, 6.4.3]. However, coadministration of eticlopride (1μM) with SKF38393 was shown to cause an induction of c-fos mRNA [F (3,26) = 33.83, p< 0.05] [Figure 6.3.1] and zif/268 mRNA [F (3,26) = 33.83, p< 0.001] [Figure 6.3.2, 6.4.3], compared to vehicle alone (n= 14), but there was no significant decrease in the signal when compared to SKF38393 alone (n= 8).

In every case the increase in silver grain density was found to occur over the smaller, more intensely counterstained cells in the cultures. These were identified in preliminary immunocytochemical experiments as neurons. Despite a large percentage of neurons being labelled following application of dopaminergic agonists, there were still many neurons that did not show a

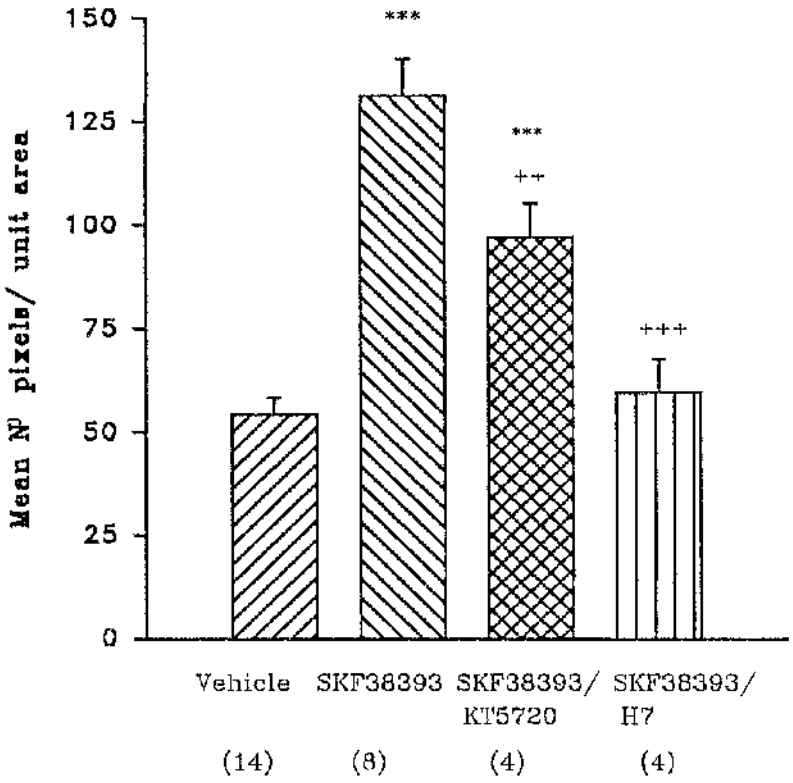
significant accumulation of silver grains. No increases in silver grain density were detected over the larger, more weakly counterstained non-neuronal cells.

6.4 Effect of inhibition of protein kinase A on c-fos and zif/268 mRNA levels in striatal cultures

The second messenger systems involved in the induction of the IEGs c-fos and zif/268 mRNA were investigated using selective inhibitors of serine/threonine protein kinases. The selective protein kinase A inhibitor, KT5720 (2μM) (Kase et al., 1987), when applied alone to striatal cultures had no effect on the expression of either IEG when compared to vehicle treated cultures (not shown). However pretreatment of the cultures for 30 minutes with KT5720, prior to the addition of SKF38393 (1μM) caused a small but significant reduction in the ability of SKF38393 to induce c-fos mRNA levels [$F(3,26) = 14.23$, $p = 0.0033$, $p < 0.01$] [Figure 6.4.1, 6.3.4]. In the presence of KT5720, the levels of c-fos mRNA due to indirect stimulation via SKF38393, were still significantly higher [$F(3,26) = 14.23$, $p<0.001$], than the levels expressed in vehicle alone. Pretreatment with a relatively non-selective inhibitor of all protein kinases, H7 (50μM), caused a more dramatic reduction in the hybridisation signal due to the induction of c-fos mRNA by SKF38393 [$F(3,26) = 14.23$, $p < 0.001$] when compared to SKF38393 alone [Figure 6.4.1]. This information suggests the involvement of a protein kinase other than protein kinase A.

Figure 6.4.1 : Effect of inhibition of protein kinase A on c-fos mRNA levels in striatal cultures. KT5720 (2μM), or H7 (50μM) or vehicle, were added 30 minutes prior to treatment with SKF38393 (1μM), for 45 minutes. Results are expressed as mean number of pixels / unit area + s.e.m. from the number of different cultures shown in parentheses. *** $p< 0.001$, relative to vehicle alone; ++ $p< 0.01$, +++ $p< 0.001$, relative to SKF38393 alone.

Figure 6.4.2 : Effect of inhibition of protein kinase A on zif/268 mRNA levels in striatal cultures. KT5720 (2μM), or H7 (50μM), or vehicle were added 30 minutes prior to treatment with SKF38393 (1μM), for 45 minutes. Results are expressed as mean number of pixels / unit area + s.e.m. from the number of different cultures shown in parentheses. * $p< 0.05$, *** $p< 0.001$, relative to vehicle alone; +++ $p< 0.001$, relative to SKF38393 alone.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SKF38393/ KT5720
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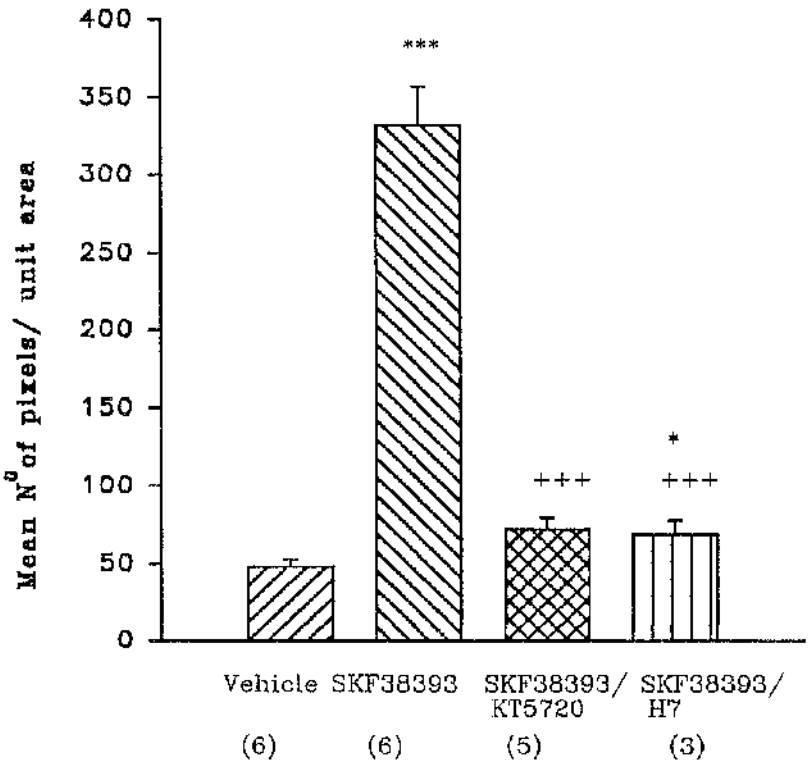
Mean N°of pixels/ unit area
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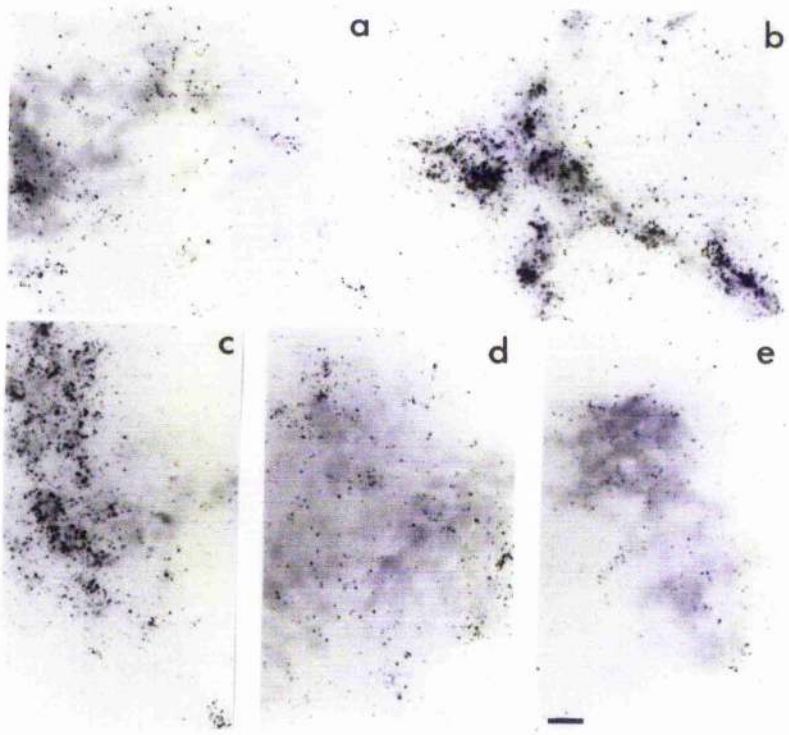
Vehicle
SKF38393
SKF38393/ KT5720
SKF38393/ H7
(6)
(6)
(5)
(3)

Figure 6.4.3 : Induction of zif/268 mRNA by D1-like dopaminergic agonists. Cultured rat striatal neurones are shown following hybridisation with the probe to detect zif/268 mRNA. Black silver grains overlying counterstained cell bodies represent the hybridisation signal. Cells were treated with (a) vehicle (HBSS) (b), SKF38393 (1μM) (c) SKF38393 (1μM) + eticlopride (1μM), (d) SKF38393 (1μM) + SCH23390 (1μM), (e) SKF38393 (1μM) after pretreatment with KT5720 (2μM). Scale bar represents 20μm.


a
b
c
d
e

Pretreatment of the cultures with KT5720 (2μM), dramatically inhibited the ability of SKF38393 to induce zif/268 mRNA by approximately 90%. This decrease in signal was shown to be significantly different from SKF38393 treatment alone, such that F (3,16) =66.40, p= 0.00076, p< 0.001, [Figure 6.4.2, 6.4.3]. Similarly, pretreatment with H7 (50μM) caused a significant reduction in the levels of zif/268 mRNA due to stimulation by SKF38393 [F (3,16) =66.40, p< 0.001] [Figure 6.4.2].

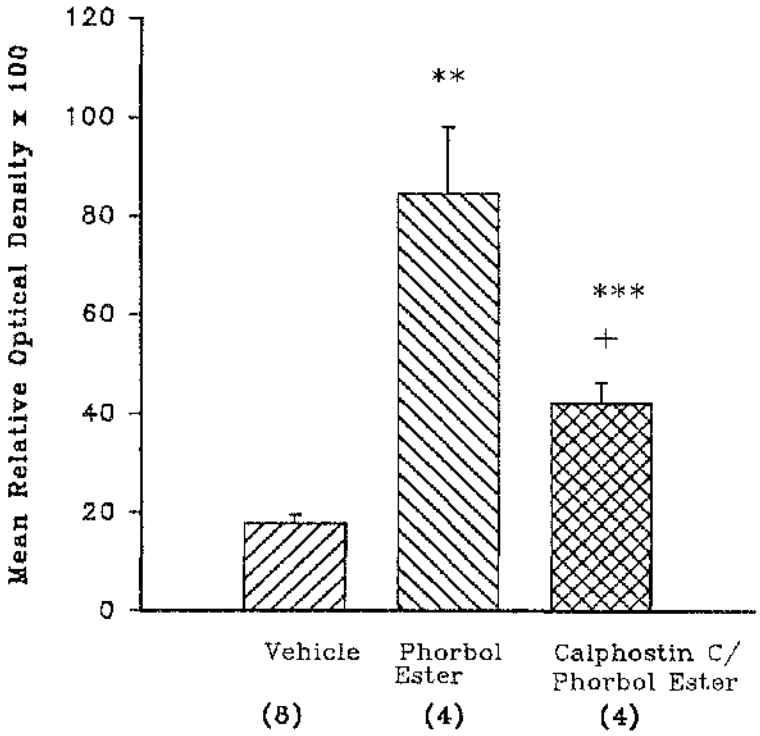
6.5 Effect of inhibition of protein kinase C on the levels of c-fos and zif/268 mRNA in rat striatal cultures

From the previous experiment, it is clear that the role of protein kinase enzymes in the induction of c-fos and of zif/268 mRNA is relatively complex. Therefore we went on to investigate the role of protein kinase C (PKC) in these responses using the selective PKC inhibitor, calphostin C (Bruns et al., 1991). This PKC inhibitor has been reported to be activated only by exposure to light (Bruns et al, 1991).

Initially the efficiency of PKC inhibition in striatal cultures was monitored by observing the ability of calphostin C to reduce the activation of c-fos and zif/268 gene expression by phorbol-12-myristate-13-acetate (PMA). A significant induction in the levels of c-fos mRNA [F (2,13) = 23.42, p= 0.007, p< 0.01] [Figure 6.5.1] and zif/268 mRNA [F (2,9) = 36.19, p= 0.0006, p< 0.001] [Figure 6.5.3], compared to treatment with vehicle alone,were observed following treatment with 100nM PMA. Pretreatment for 30 minutes with the selective PKC inhibitor, calphostin C, caused a marked reduction in the levels of c-fos and zif/268 mRNA (approximately 70%). The decrease in c-

Figure 6.5.1: Effect of calphostin C on the phorbol ester-induced expression of c-fos mRNA in striatal cultures. Calphostin C (1mM), or vehicle were added 30 minutes prior to treatment with phorbol-12-myristate-13-acetate (10^{-7}M) for 45 minutes. Results obtained from film autoradiographs, are expressed as mean relative optical density units x 100 +s.e.m..from the number of different cultures shown in parentheses. ** $p< 0.01$, *** $p< 0.001$, relative to vehicle alone; + $p< 0.05$ relative to phorbol-12-myristate-13-acetate alone.

Figure 6.5.2 : Effect of calphostin C on the SKF38393-induced expression of c-fos mRNA in striatal cultures. Calphostin C (1μM), or vehicle were added 30 minutes prior to treatment with of SKF38393 (1μM) for 45 minutes. Results, obtained from film autoradiographs, are expressed as mean relative optical density units x 100 +s.e.m. from the number of different cultures shown in parentheses. *** $p< 0.001$, relative to vehicle alone; +++ $p< 0.001$ relative to SKF38393 alone.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100
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Phorbol Ester
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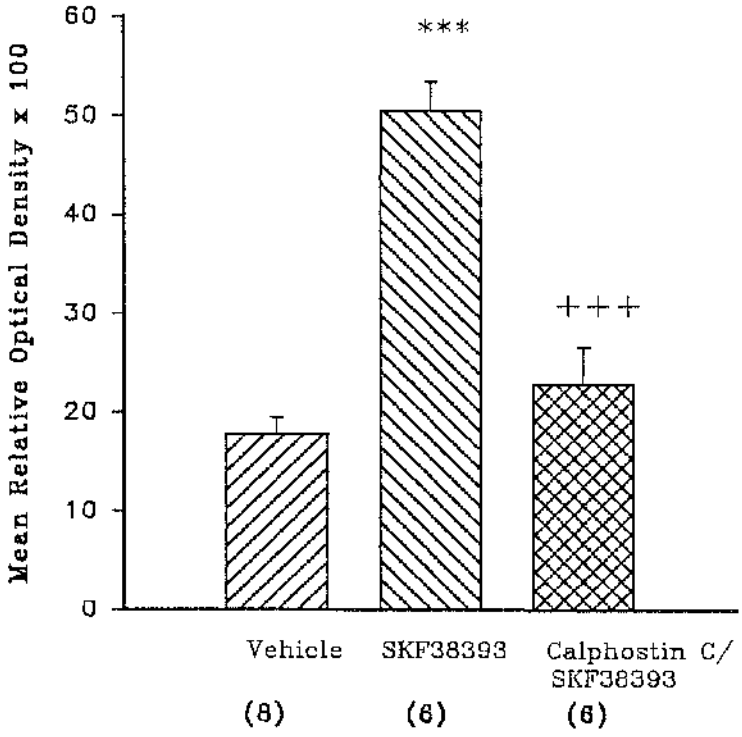
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SKF38393
Calphostin C/ SKF38393
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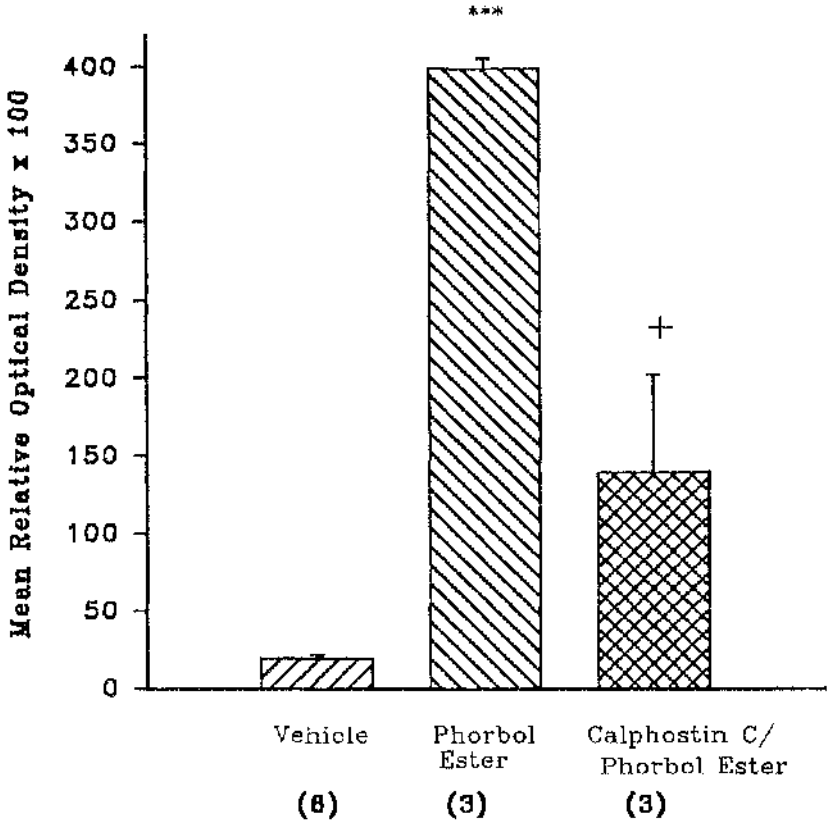
Figure 6.5.3 : Effect of calphostin C on phorbol ester-induced zif/268 mRNA levels in striatal cultures. Calphostin C (1μM), or vehicle were added to the cultures 30 minutes prior to treatment with phorbol-12-myristate-13-acetate (10^{-7}M) for 45 minutes. Results, obtained from film autoradiographs, are expressed as mean relative optical density units x 100 +s.e.m. from the number of different cultures shown in parentheses. *** $p< 0.001$, relative to vehicle alone; + $p< 0.05$, relative to phorbol-12-myristate-13-acetate alone.

Figure 6.5.4 : Effect of calphostin C on the SKF38393-induced expression of zif/268 mRNA levels in striatal cultures. Calphostin C (1μM), or vehicle were added 30 minutes prior to treatment with SKF38383 (1μM) for 45 minutes. Results, obtained from film autoradiographs, are expressed as mean relative optical density units x 100 +s.e.m. from the number of different cultures shown in parentheses. *** $p< 0.001$, relative to vehicle alone.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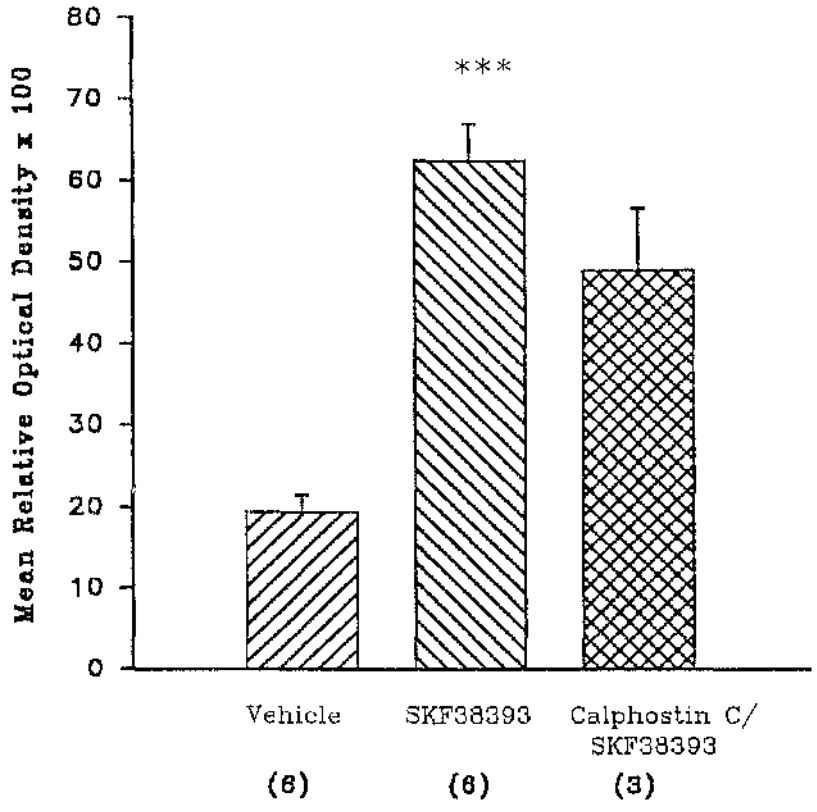
Mean Relative Optical Density x 100
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SKF38393
Calphostin C/ SKF38393
(6)
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fos mRNA levels was shown to be significantly different from cultures treated with phorbol ester alone [F (2,13) = 23.42, p< 0.05] [Figure 6.5.1], and similarly the reduction in zif/268 mRNA levels was also significantly different from phorbol ester stimulated cultures alone [F (2,9) = 36.19, p< 0.05] [Figure 6.5.3].

As in previous experiments, SKF38393(1μM) was shown significantly to induce the expression of c-fos mRNA [F (2,17) = 36.06, p= 0.00033, p< 0.001] [Figure 6.5.2]. Following pretreatment with calphostin C (1μM) for 30 minutes,the ability of SKF38393 (1μM) to induce the c-fos gene was dramatically inhibited. This reduction was demonstrated to be significantly different from SKF38393 treatment alone [F (2,17) = 36.06, p< 0.001] [Figure 6.5.2], such that the levels of c-fos mRNA were not significantly different from basal levels. The expression of zif/268 mRNA was also shown to be increased to significant levels after SKF38393 (1μM) treatment [F (2,12) = 37.14, p= 0.00086, p< 0.001] [Figure 6.5.4], as in previous experiments. However, pretreatment with calphostin C (1μM) was unable to cause a significant reduction in the increase in zif/268 mRNA after treatment with SKF38393 [Figure 6.5.4].

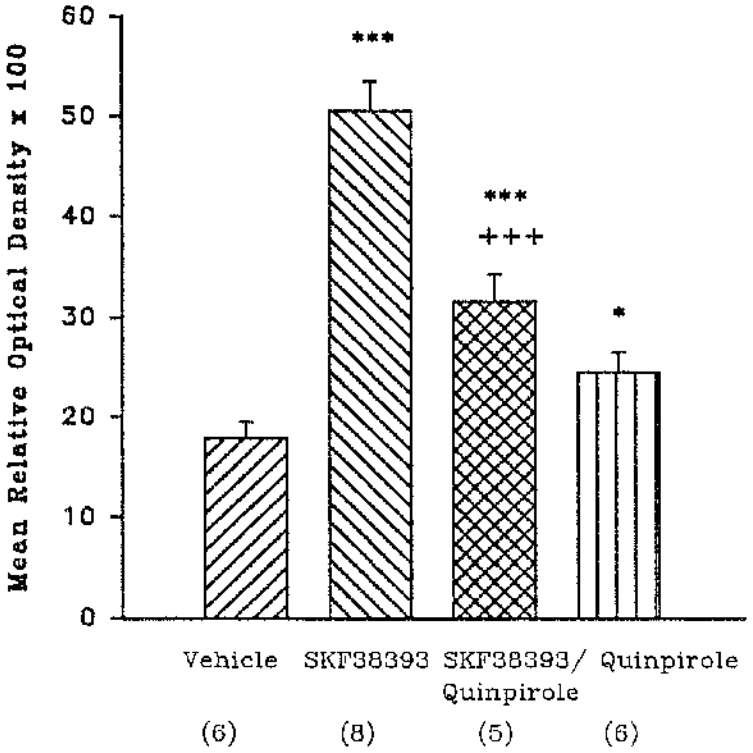
6.6 Effect of quinpirole on c-fos and zif/268 mRNA expression in cultured rat striatal neurons

Low levels of c-fos and zif/268 mRNA were expressed in vehicle treated cultures compared to the highly significant induction of both c-fos mRNA [F (3,19) = 33.14, p= 0.0006, p< 0.001] [Figure 6.6.1] and zif/268 mRNA [F(3,16) = 28.23, p= 0.0009, p< 0.001) [Figure 6.6.2] after SKF38393

treatment. Coadministration of SKF38393 (1μM) together with quinpirole (1μM), the selective D2 dopamine receptor agonist, caused a marked fall in the hybridisation signal representing c-fos mRNA, due to SKF38393 treatment. This decrease was significantly different from SKF38393 alone [$F(3,19) = 33.14$, $p < 0.01$] [Figure 6.6.1]. A similar suppression of the hybridisation signal for zif/268 mRNA was observed when quinpirole was coapplied with SKF38393, and this was significantly different from cultures treated with SKF38393 alone [$F(3,16) = 28.23$, $p < 0.001$]. [Figure 6.6.2]. Quinpirole (1μM) when applied alone had no effect on the levels of zif/268 mRNA expressed in these cultures [Figure 6.6.2]. However, the levels of c-fos were slightly increased in the presence of quinpirole alone. This increase was shown to be significantly different from vehicle treated cultures [$F(3,19) = 33.14$, $p < 0.05$] [Figure 6.6.1].

Figure 6.6.1: Effect of quinpirole on the SKF38393-induced expression of c-fos mRNA levels in striatal cultures. Quinpirole (1μM), or vehicle was added 30 minutes prior to treatment with SKF38393 (1μM), for 45 minutes. Results, obtained from film autoradiographs, are expressed as mean relative optical density units x 100 + s.e.m. from the number of different cultures shown in parentheses. * $p< 0.05$, *** $p< 0.001$, relative to vehicle alone; +++ $p< 0.001$, relative to SKF38393 alone.

Figure 6.6.2 : Effect of quinpirole on the SKF38393-induced expression of zif/268 mRNA levels in striatal cultures. Quinpirole (1mM), or vehicle were added 30 minutes prior to treatment with SKF38393 (1mM), for 45 minutes. Results, obtained from film autoradiographs, are expressed as mean relative optical density units x 100 + s.e.m. from the number of different cultures shown in parentheses. *** $p< 0.001$, relative to vehicle alone; +++ $p< 0.001$, relative to SKF38393 alone.


Mean Relative Optical Density x 100
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Quinpirole
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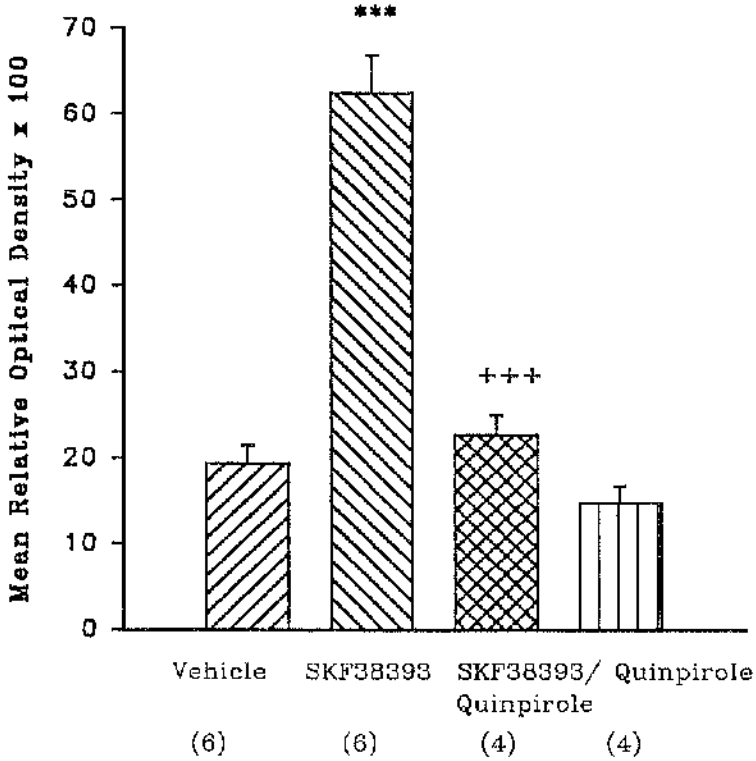
Mean Relative Optical Density x 100
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Vehicle
SKF38393
SKF38393/ Quinpirole
Quinpirole
(6)
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(4)
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DISCUSSION

Initially it was necessary to determine the time course of induction of the immediate early genes under investigation. Previous in vivo studies have suggested that peak induction of the immediate early genes c-fos and zif/268 mRNAs occurs thirty to sixty minutes after the initial injection of both psychostimulants or neuroleptic drugs in vivo (Robertson et al., 1989; Cole et al., 1992; Nguyen et al., 1992; Bhat et al., 1992). In agreement with this our results show that the immediate early gene zif/268 is maximally induced in primary culture approximately forty-five minutes after the addition of haloperidol to the culture medium. The rise in zif/268 mRNA levels in vivo, has been shown to be apparent as early as fifteen minutes after the injection (Cole et al., 1992). The lack of an induction of zif/268 mRNA at early time points in vitro, is likely to reflect differences in the neuronal environment such as temperature, and thus may be responsible for delayed signal transduction processes. Similar to the in vivo situation (Cole et al., 1992; Nguyen et al., 1992), zif/268 mRNA levels in vitro, decline back to basal levels by approximately three hours.

6.7 Dopaminergic Regulation of Immediate Early Genes In Vitro

In this study we have demonstrated that exposure of rat striatal primary cultures to dopamine resulted in dramatic increases in the levels of c-fos and zif/268 mRNAs. The use of in situ hybridisation to monitor these changes has the advantage that the cell types where the changes in gene expression occur can be identified with some confidence. It is worth noting that despite the small

proportion of non-neuronal cells in such cultures, some pharmacological agents can stimulate dramatic increases in immediate early gene expression in the glial cells. The induction of c-fos and zif/268 mRNA levels that we observed in these studies, following dopamine receptor stimulants appears to occur in the same population of cells that exhibit immunoreactivity for neuron-specific enolase. Therefore this induction of immediate early gene expression is occurring in the striatal neurons.

We have previously shown that a high proportion of striatal neurons in primary culture express either the proenkephalin gene or the preprotachykinin gene, and therefore are likely to represent the medium spiny projection neurons characterised in the mature striatum (Graybiel, 1986). Interneurons are thought to constitute only a small percentage of the number of neurons in the normal striatum (Graybiel, 1986), and we have shown earlier that the corresponding neurochemical phenotypes represent a similarly small proportion of striatal neurons in primary striatal culture. The high proportion of neurons containing c-fos mRNA or zif/268 mRNA in these experiments after dopaminergic stimulation suggests that immediate early gene induction is taking place in the culture counterpart of the medium spiny neuron. This is consistent with evidence suggesting that the induction of immediate early gene expression in striatal neurons in vivo following manipulation of the level of dopaminergic activity occurs in medium spiny neurons (Dragunow et al., 1990; Graybiel et al., 1990; Robertson et al., 1990; Robertson et al., 1992). The induction of c-fos or zif/268 following dopamine stimulation was observed with a doseage ranging from 100nM to 5μM, concentrations which would be expected to stimulate all dopamine receptor subtypes.

In rats with a unilateral 6-hydroxydopamine lesion of the nigrostriatal pathway, there is evidence that the induction of c-fos by SKF38393 occurs primarily in the medium spiny neurons expressing the preprotachykinin gene and projecting to the substantia nigra (Robertson et al., 1989; Robertson et al.,

1992). The number of neurons showing increased c-fos expression in culture after treatment with the dopaminergic D1 agonist, SKF38393, means that the preprotachykinin-containing neurons must be involved, but we would not rule out the possibility that enkephalinergic neurons in culture also respond to SKF38393 with an increased level of c-fos expression.

Since the addition of SKF38393 to the culture medium was able to reproduce the dopaminergic stimulation of c-fos and zif/268 mRNA, but the addition of quinpirole had no effect on zif/268 and a minor inductive effect on c-fos, it is most likely that this response is mediated by stimulation of a D1/ D5-like receptor. This is further supported by the observation that SCH23390, the D1 selective antagonist, was able to attenuate the inductive effect of SKF38393, but the selective D2 antagonist, eticlopride was unable to affect these responses. This is in accordance with previous work in vivo, which also suggests that the induction of c-fos and zif/268 after administration of dopaminergic agonists is likely to occur via stimulation of D1-like dopamine receptors (Robertson et al., 1989; Robertson et al., 1990; Graybiel et al., 1990; Arnauld et al., 1993; Cole et al., 1992; Mailleux et al., 1992; Moratalla et al., 1992). The effects of dopaminergic agonists on immediate early gene expression in primary cultures reported here therefore appear to reflect the corresponding in vivo actions very closely.

Significant increases in c-fos and zif/268 mRNA levels in striatal neurons were detected following treatment with SKF38393 at concentrations between 30nM and 5μM. The EC50 value obtained is very close to the K_D values reported for the binding of SKF38393 to striatal membranes (Seeman and Van Tol, 1993). This suggests that there is a low receptor reserve for SKF38393 in stimulating these changes, consistent with the biochemical evidence that, in striatal tissue, SKF38393 acts as an agonist with only moderate efficacy (Setler et al., 1978).

It is clear that, in a number of different cell types, increased transcription of both the c-fos and zif/268 genes can be observed following activation of either protein kinase A (PKA) or protein kinase C (PKC) (review by Sheng and Greenberg, 1990). This is in line with the presence of cyclic AMP- responsive enhancers and phorbol ester- responsive enhancers in the promoter regions of these genes. The biochemical evidence suggests that D1-like dopamine receptors can be coupled either to stimulation of adenylate cyclase or phospholipase C, with consequent activation of PKA or PKC respectively (Mahan et al., 1990; Monsma et al., 1990; Sunahara et al., 1991; Tiberi et al., 1991; Frail et al., 1993). The dopaminergic stimulation of c-fos and zif/268 gene expression in striatal neurons could therefore potentially be mediated via either PKA or PKC.

Highly selective inhibitors are now available which allow the intracellular actions of different serine / threonine kinases to be distinguished. The highly selective inhibitor of PKA, namely KT5720 (Kase et al., 1987), blocked the ability of SKF38393 to stimulate zif/268 expression by 90%. The Ki value for KT5720 against PKA is approximately 60nM, whereas its Ki value against other kinases such as PKC is greater than 2μM (Kase et al., 1987). The concentration of KT5720 used in this study would therefore be predicted to cause a 95% inhibition of PKA and less than 50% inhibition of PKC. The degree of attenuation of the effect of SKF38393 on zif/268 induction observed with KT5720 therefore strongly implicates protein kinase A in this effect.

Calphostin C is a highly selective inhibitor of PKC, which shows negligible activity against other serine/ threonine kinases (Kobayashi et al., 1989). The concentration of 1μM calphostin C used in these experiments, should consequently inhibit PKC by more than 90%, with only minimal effects on other serine/ threonine kinases. However, an important aspect concerning the use of calphostin C, is that its activity is dependent on exposure to light (Bruns et al., 1991). We observed that treatment of the cultures with the

phorbol ester, phorbol-12-myristate-13 acetate (PMA), resulted in a clear induction of both c-fos and zif/268 mRNA levels compared to controls. Pretreatment with calphostin C caused a 70% decrease in the ability of PMA to induce expression of both c-fos and zif/268 mRNA levels. While this may imply that the activation of calphostin C was not quite maximal in our protocol, it provides a definite indication of the degree of PKC inhibition achieved.

Consistent with the above evidence that the actions of dopaminergic agonists on zif/268 expression were mediated entirely by PKA, calphostin C pretreatment had no significant effect on the stimulation of zif/268 mRNA levels by SKF38393. In contrast, calphostin C pretreatment caused a dramatic reduction in the ability of SKF38393 to enhance c-fos expression. The relatively greater inhibition by calphostin C of the SKF38393 response as compared to the PMA response may simply reflect a sub-maximal activation of PKC by the endogenous intracellular pathway, subsequent to stimulation of D1-like dopamine receptors. It is of some interest that the SKF38393-induced increase in c-fos gene expression was also reduced by inhibition of PKA. This implies that PKA activity also participates in the D1-dopaminergic induction of c-fos expression.

These results therefore suggest that stimulation of c-fos expression by SKF38393 involves mainly PKC, but with an additional contribution from PKA. Conversely, the stimulation of zif/268 gene expression by SKF38393 appears to proceed almost entirely via PKA. However, it is important to remember that there are may be a further level of complexity involved in these effects, in that phosphorylation of cyclic AMP-response element binding proteins by PKA or PKC may affect their relative affinity for the cyclic AMP-reponsive and phorbol ester-responsive enhancers (Merino et al., 1989). In other words, even when only a single kinase is activated, its ability to modulate gene expression may be affected by altering the basal level of activity of another kinase.

Nevertheless, some important conclusions can be drawn from these results. The inratcellular pathways coupled to induction of IEGs vary in different cell types. It is clear that in non-neuronal cells and cell lines, both PKA and PKC are powerful stimulants for the induction of both c-fos and zif/268 (reviewed by Sheng and Greenberg, 1989). The information available on neuronal cell responses is rather scarce, but it appears that in primary cultures of cerebellar granule cells, c-fos expression can be stimulated by PKC, but not by PKA (Szekely et al., 1989). In contrast, in primary cultures of cerebral cortical neurons, activation of PKA can produce a mild enhancement of expression of the c-fos and zif/268 genes (Vaccarino et al., 1993). Our results represent, to the best of our knowledge, the first indication that both PKA and PKC can be coupled to stimulation of c-fos and zif/268 expression in striatal neurons. Furthermore, our data suggests that the population of D1-like dopamine receptors on striatal neurons includes receptors coupled to both adenylate cyclase and to phospholipase C. Until now, the evidence for coupling of D1-like receptors in the central nervous system to phospholipase C has been derived from in vitro experiments using translated endogenous and exogenous nucleic acids (Mahan et al., 1990; Monsma et al., 1990; Frail et al., 1993), or from extrapolation of results from kidney cells (Felder et al., 1989). The evidence reported here therefore provides a functional confirmation of the molecular evidence that D1-like receptors on striatal neurons can couple to both second messenger systems.

The different relative contribution of PKC- and PKA-dependent pathways to the dopaminergic induction of c-fos and zif/268 expression may have implications for the regulation of striatal gene expression by other neurotransmitters. For example a receptor negatively linked to adenylate cyclase might act to suppress zif/268 expression more than c-fos expression. It appears that, when activation of both PKC and PKA occurs in striatal neurons, the rate of zif/268 transcription may be more sensitive to changes in PKA

activity, while the rate of c-fos transcription may be more sensitive to changes in PKC activity. In the light of our results, investigations of the second messenger systems involved in the regulation of immediate early gene expression by other neurotransmitter receptors are clearly of some importance.

While we have shown that dopamine and SKF38393 are able to exert a powerful stimulatory influence on striatal immediate early gene expression by activating D1-like dopamine receptors, we also detected a relatively small enhancement of c-fos expression following treatment with the selective D2-like agonist quinpirole. This effect was specific for the c-fos gene, in that quinpirole did not affect zif/268 expression. At present the in vivo information suggests that D2-like agonists should act to supress immediate early gene expression. However, there are thought to be a relatively small number of D3 receptors in the rat striatum, and quinpirole is known to be an effective agonist at D3 receptors (Sokoloff et al., 1990). It has recently been reported that stimulation of D3 dopamine receptors can activate c-fos in a transfected cell line (Pilon et al., 1994). Our results raise the interesting possibility that stimulation of D3 receptors may activate c-fos gene expression in a population of striatal neurons. However, further work would be necessary to expolre this possibility.

We also report that activation of D2-like dopamine receptors by quinpirole reduces the stimulation of c-fos and zif/268 expression by SKF38393. Since the in vivo evidence suggests that tonic activity at D2-like receptors suppresses immediate early gene expression, (Dragunow et al., 1990; Nguyen et al., 1992; Robertson and Fibiger, 1992) this action of quinpirole conforms more closely to expectations. There has been considerable debate as to whether individual striatal neurons express both D1-like and D2-like dopamine receptors. Based on an inability to detect D1 receptor mRNA and D2 receptor mRNA in the same population of neurons by in situ hybridisation (Gerfen et al., 1990; Le Moine et al., 1991), it has been argued that these two receptor types are not colocalised in striatal neurons. In contrast functional and immunochemical evidence suggests

that medium spiny neurons contain both D1 and D2 receptors (Lester et al., 1993; Surmeier et al., 1992; Larson and Ariano, 1994).

Our results have demonstrated that quinpirole can act functionally to antagonise the effects of SKF38393 on striatal neurons. If it is assumed that SKF38393 and quinpirole are not acting trans-synaptically in our studies, then the data appears to confirm the coexpression of D1-like and D2- like receptors in the same neuronal population. There is evidence that striatal neurons in primary culture form functional synapses (Pin et al., 1986; Weiss et al., 1986: Dubinsky, 1989; Misgeld and Dietzel, 1989). However, the synapses appear to be predominantly GABAergic (Pin et al., 1986; Misgeld and Dietzel, 1989), so that an indirect action of quinpirole to inhibit synaptic activity would be predicted to enhance immediate early gene induction rather than to suppress it. Furthermore we have found that bicuculline does not influence c-fos or zif/268 mRNA levels (data not shown), suggesting that intrinsic GABAergic activity is not a major factor regulating immediate early gene expression in striatal neurons. Our results therefore lend further weight to the theory that medium spiny neurons express both D1-like and D2-like dopamine receptors, and that these two receptor classes are in functional opposition.

Chapter 7

Antisense Oligonucleotide Strategies

INTRODUCTION

7.0 Antisense Oligonucleotides

Single stranded oligonucleotides, complementary to a specific mRNA are called antisense oligonucleotides. Several investigators have used "antisense strategy" to block the synthesis of specific proteins (review by Dolnick, 1990; Vand der Krol et al., 1988). Although the exact mechanism of action of antisense oligonucleotides is unknown, they can achieve a translational arrest of a target mRNA when hybridisation occurs in vivo or in a cell-free translation system. Effective antisense oligonucleotides are generally of short length (15-20 bases). It is believed that this length is short enough to be taken up by cells, but long enough to provide specificity of binding to a unique base sequence of mRNA (Zamecnik and Stephenson, 1978; Zamecnik et al., 1986; Holt et al., 1988; Van der Krol et al., 1988; Dolnick, 1990). There is current evidence suggesting that the mechanism of oligonucleotide uptake is predominantly fluid-phase endocytosis (pinocytosis) (Stein and Cheng., 1993) which may be initiated by receptor-mediated recognition (Yakubov et al., 1989). The 'mechanisms of potential oligonucleotide interactions with target nucleic acids are very complex. According to the most popular theories, the antisense oligonucleotides exert their effects by inducing translational arrest of protein synthesis either by stimulating ribonuclease H-mediated degradation (Walder and Walder, 1988) or preventing the translocation of the ribosome along the targeted mRNA sequence (Boiziau et al., 1991).

Two types of antisense oligonucleotides are presently in use as experimental tools, either the unmodified phosphodiester oligonucleotides or the nuclease resistant phosphorothioate analogues. Different cell types have been shown to differ greatly with respect to oligonucleotide uptake as well as oligonucleotide

degradation. It has been suggested that many neuronal systems have a very low capacity to degrade oligonucleotides and that they are therefore amenable to treatment with either phosphodiester oligonucleotides or nuclease-stable analogues (Wahlestedt et al., 1993; Standifer et al., 1993; Akabayashi et al., 1994). It has previously been shown that phosphodiester oligonucleotides can enter cultured neurons at a concentration of up to 5% in the media (Caceres and Kosik, 1990). Effective treatment with antisense oligonucleotides is entirely dependent on the gene type under study and the turnover rate of the protein. In general, for receptor synthesis inhibition a minimum treatment time of two to five days has been shown necessary (Wahlestedt et al., 1993; Erlinge et al., 1993; Standifer et al., 1993).

The potential uses of antisense oligonucleotides has gradually become clear and a considerable focus on experimental use is currently in progress. In the past decade antisense oligonucleotides have been effective in inhibiting herpes simplex virus replication (Zamecnik and Stephenson, 1978) and in blocking the synthesis of human immunodeficiency viral proteins (Zamecnik et al., 1986). The antisense strategy has also been used in studying the function of cellular oncogenes and other proteins on cell proliferation and differentiation in vitro (Holt et al., 1988; Wickstrom et al., 1988; Becker et al., 1989; Tortora et al., 1990; Maier et al., 1990; Venturelli et al., 1990; Wang et al., 1992). In clonal cell lines antisense oligonucleotides have been used to target specific proteins involved in second messenger systems (Wagner et al., 1992). An antisense oligonucleotide has also been used against the NMDA receptor complex preventing NMDA receptor channel formation and functionally reducing neurotoxicity elicited by NMDA receptors in vitro (Wahlestedt et al., 1993). Both the muscarinic M2 and $GABA_B$ receptor proteins have been successfully decreased by more than 50% using antisense oligonucleotides and thus has eliminated the ability of these receptors when stimulated to inhibit cyclic AMP formation (Holpainen and Wojcik, 1992). Only a few reports have

shown the effectiveness of antisense oligonucleotides in primary cultured neurons (Caceres and Kosk, 1990; Listerud et al., 1991).

Direct administration of antisense oligonucleotides in vivo has recently proved effective against certain IEGs and receptor proteins. Direct infusion into the striatum of an antisense oligonucleotide against the IEG c-fos, has been shown to reduce amphetamine-induced production of fos-like immunoreactivity, without affecting zif/268 expression (Chiasson et al., 1992). Similarly, the ability of cocaine to induce c-fos protein in striatum and also locomotor stimulation was blocked when an antisense oligonucleotide against c-fos was injected into the nucleus accumbens (Heilig et al., 1993). Zhang and Creese (1993) have shown that an intracerebroventricular infusion of D2 antisense selectively decreases striatal D2 receptors by 50% in the rat, while not effecting D1, muscarinic and 5-HT2 receptor proteins. Direct infusion of D2 antisense has also been shown to inhibit rotational behaviour induced by quinpirole, a D2 agonist, but not that induced by the D1 agonist SKF38393, or the muscarinic agonist oxotremorine in the striatum of rats previously treated with 6-hydroxy dopamine (Weiss et al., 1993). The use of antisense strategy has also revealed a possible new subtype of D2 receptor on the basis of functional properties (Valerio et al., 1994).

The aim of these experiments was therefore to attempt to identify the functional dopamine receptor subtype, whose stimulation is involved in the induction of both c-fos and zif/268 mRNA, in striatal neurons, after treatment with SKF38393, a non-selective agonist for both D1 and D5 dopamine receptor subtypes.

RESULTS

7.1 Effect of blockade of dopamine D1 and D5 receptor synthesis on the levels of the IEGs c-fos and zif/268 mRNA using antisense oligonucleotide strategies in rat striatal neuron cultures

The use of antisense oligonucleotides to arrest the translation of receptor mRNAs and thus reduce receptor synthesis has recently been employed to investigate receptor mediated responses. In this study we were interested in the nature of the receptor(s) involved in the induction of c-fos and zif/268mRNA after treatment of cultures with SKF38393, the selective D1/D5 receptor agonist.

Initially the dose range of both the D1 and D5 antisense oligonucleotides was determined. We found that doses of 2μM and above were toxic to the neurons in culture, whilst doses of 1μM appeared to be effective. Addition of SKF38393 (1nM-5μM) to cultured striatal neurons was demonstrated to increase the expression of both c-fos mRNA and zif/268 mRNA in a dose-dependent manner. The EC_{50} for the SKF38393-induced stimulation of both c-fos and zif/268 mRNA was in the region of 95nM. There was no difference in the basal level of expression of c-fos mRNA in striatal cultures pretreated for 2 days with 15-mer antisense phosphorothioate oligonucleotides (1μM) complementary to a region of the dopamine D1 and D5 receptors alone compared to vehicle treated cultures. However, in cultures pretreated for 2 days with D1 antisense, followed by SKF38393 (1nM-5μM) treatment for 45 minutes, the slope of the dose response curve representing induction of c-fos mRNA had clearly shifted in a downward direction, implying that the D1 receptor is involved in this response. This shift in the dose response

curve to SKF38393 was demonstrated to be significantly different from the the dose response curve to SKF38393 alone (F= 118.64, p< 0.001, ANOVA minitab, n= 3). [Figure 7.1.1]. Less of a shift was observed in the presence of the dopamine D5 receptor antisense oligonucleotide, however, this overall shift was significantly different from the dose response curve to SKF38393 alone (F= 18.58, p< 0.001) [Figure 7.1.2]. As an appropriate control for the effects of the D1 and D5 receptor antisense oligonucleotides, a 15-mer D1 'sense' phosphorothioate oligonucleotide was synthesised, having the same sequence as a region of the D1 receptor mRNA. Pretreatment of the cultures with a 15-mer D1 sense oligonucleotide (1μM) for two days , followed by acute treatment with SKF38393 (1nM-5μM) for 45 minutes had no effects on the induction of c-fos mRNA by SKF38393 [Figure 7.1.3]. However, the dose response curve in the presence of the D1 sense oligonucleotide was significantly different from the dose response curve to SKF38393 in the presence of the D1 antisense oligonucleotide (F=22.98, p< 0.05) and similarly was significantly different from the curve to SKF38393 in the presence of the D5 antisense (F= 18.17, p< 0.05) [Figure 7.1.3].

The induction of zif/268 mRNA following SKF38393 treatment showed a similar dose dependent profile, with an EC_{50} of 95nM. However, when cultures were pretreated for two days with the D1 'sense' oligonucleotide, the dose response curve to SKF38393 (1nM-5μM) was clearly different from SKF38393 (1nM-5μM) alone, such that F= 37.55, p< 0.001, n= 3 [Figure 7.1.4]. This would suggest that the sense oligonucleotide can effect the ability of SKF38393 to induce zif/268 under these conditions. Pretreatment with the D1 antisense oligonucleotide (1μM) also caused a significant shift in the SKF38393 dose response curve (F= 81.57, p< 0.001, n= 3) [Figure 7.1.5] , and

similarly pretreatment with the D5 antisense oligonucleotide (1μM) resulted in a significant shift in the dose response curve to SKF38393 (1nM-5μM) ($F= 159.51$, $p< 0.001$, $n= 3$) [Figure 7.1.6]. The effects observed with the sense oligonucleotide suggests that the reduction in response seen with the two antisense oligonucleotides is likely to be non-specific.

Figure 7.1.1 : Effect of a dopamine D1 antisense oligonucleotide on c-fos mRNA levels in striatal cultures. Cells were pretreated for two days with a 15-mer phosphorothioate oligonucleotide corresponding to a D1 dopamine receptor antisense (1μM, n= 3). Cells were then exposed to SKF38393 (1nM-5μM, n= 5) alone or in the presence of D1 antisense (n= 3), or vehicle (HBSS) alone or in the presence of D1 antisense. After 45 minutes the cells were fixed in buffered formalin and then processed for in situ hybridisation with a specific radiolabelled probe for c-fos. Results are expressed as mean number of pixels per unit area ± s.e.m. The dose response curve in the presence of the D1 antisense was significantly different from SKF38393 treatment alone $p< 0.001$*** (two-way ANOVA, Minitab).

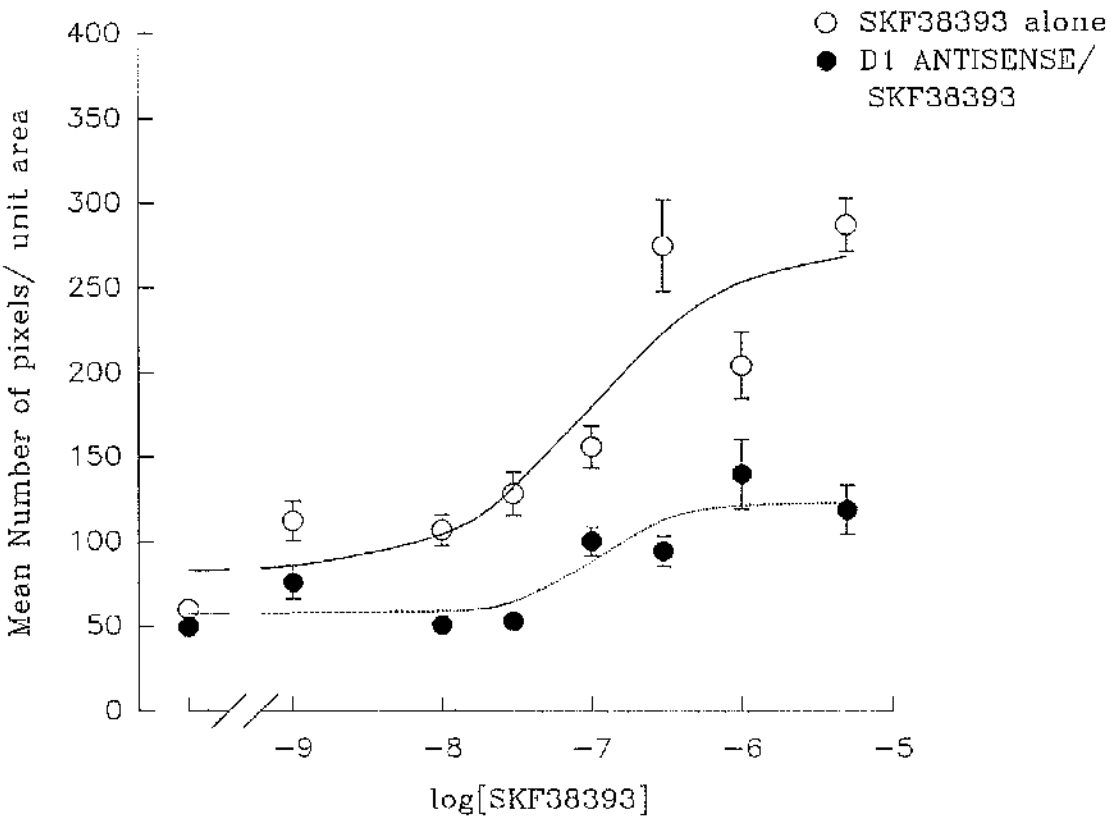
SKF38393 alone
D1 ANTISENSE/
SKF38393
Mean Number of pixels/ unit area
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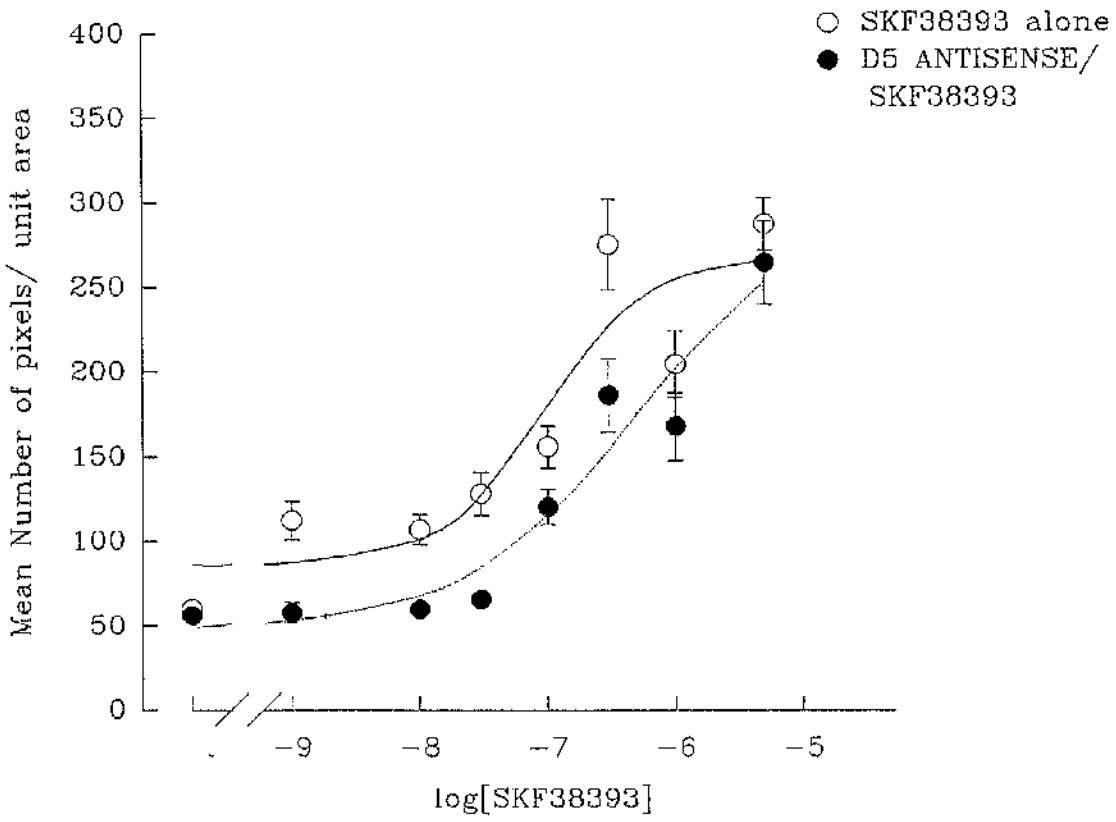
Figure 7.1.2 : Effect of a dopamine D5 antisense oligonucleotide on c-fos mRNA levels in striatal cultures. Cells were pretreated for two days with a 15-mer phosphorothioate oligonucleotide corresponding to a D5 dopamine receptor antisense (1μM, n= 3). Cells were then exposed to SKF38393 (1nM-5μM, n= 5) alone or in the presence of D5 antisense (n= 3), or vehicle (HBSS) alone or in the presence of D5 antisense. After 45 minutes the cells were fixed in buffered formalin and then processed for in situ hybridisation with a specific radiolabelled probe for c-fos. Results are expressed as mean number of pixels per unit area ± s.e.m. The dose response curve in the presence of the D5 antisense was significantly different from SKF38393 treatment alone $p< 0.001$*** (two-way ANOVA, Minitab).


SKF38393 alone
D5 ANTISENSE/
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Mean Number of pixels/ unit area
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Figure 7.1.3 : Effect of a dopamine D1 sense oligonucleotide on c-fos mRNA levels in striatal cultures. Cells were pretreated for two days with a 15-mer phosphorothioate oligonucleotide corresponding to a D1 dopamine receptor sense probe (1μM, n= 3). Cells were then exposed to SKF38393 (1nM-5μM, n= 5) alone or in the presence of D1 sense (n= 3), or vehicle (HBSS) alone or in the presence of D1 sense. After 45 minutes the cells were fixed in buffered formalin and then processed for in situ hybridisation with a specific radiolabelled probe for c-fos. Results are expressed as mean number of pixels per unit area ± s.e.m. The dose response curve in the presence of the D1 sense was not significantly different from SKF38393 treatment alone (two-way ANOVA, Minitab).

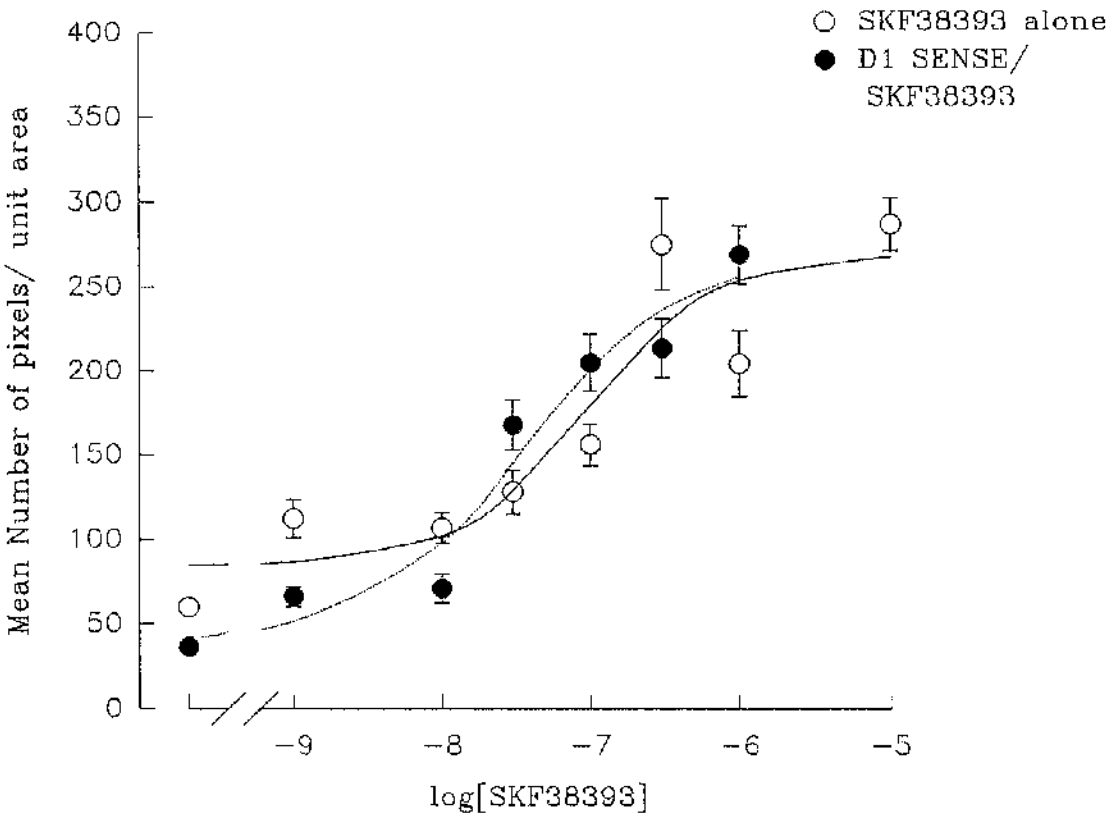
SKF38393 alone
D1 SENSE/
SKF38393
Mean Number of pixels/ unit area
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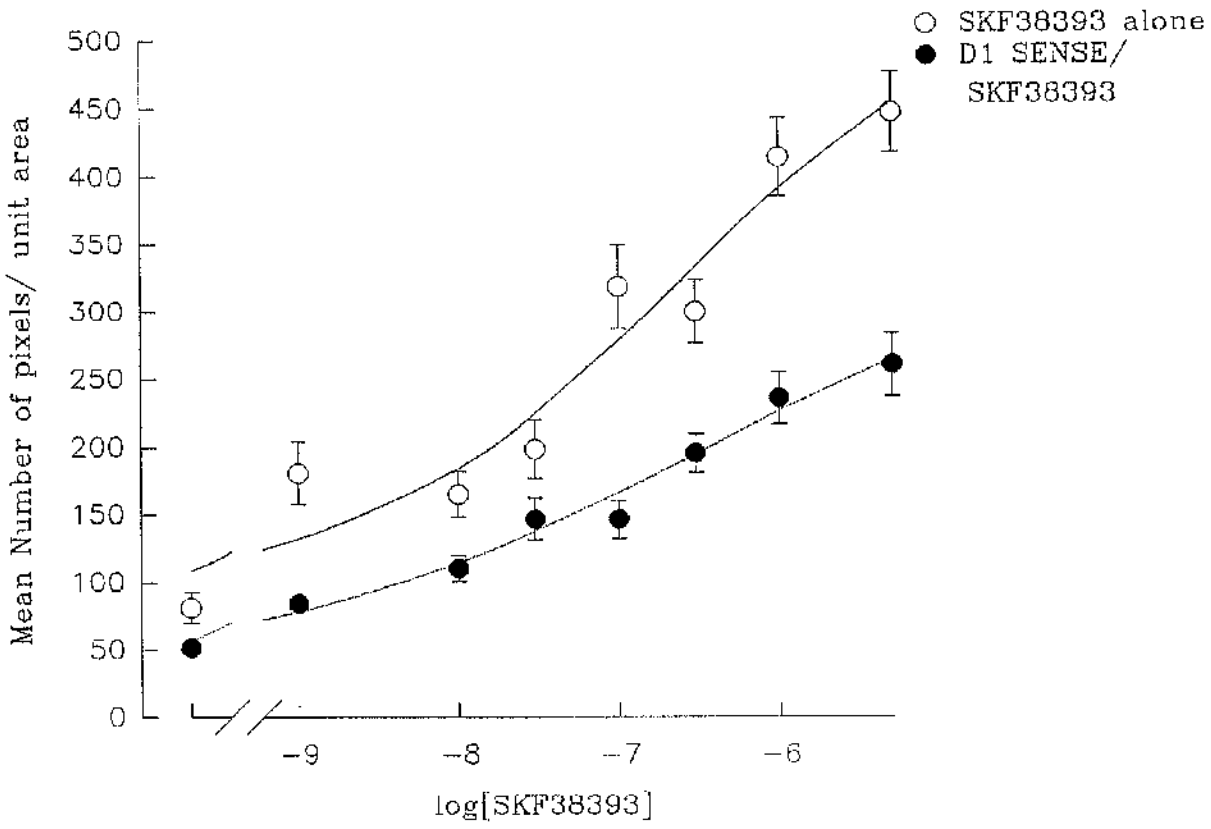
Figure 7.1.4 : Effect of a dopamine D1 sense oligonucleotide on zif/268 mRNA levels in striatal cultures. Cells were pretreated for two days with a 15-mer phosphorothioate oligonucleotide corresponding to a D1 dopamine receptor sense probe (1μM, n= 3). Cells were then exposed to SKF38393 (1nM-5μM, n= 5) alone or in the presence of D1 sense (n= 3), or vehicle (HBSS) alone or in the presence of D1 sense. After 45 minutes the cells were fixed in buffered formalin and then processed for in situ hybridisation with a specific radiolabelled probe for zif/268. Results are expressed as mean number of pixels per unit area ± s.e.m. The dose response curve in the presence of the D1 sense was significantly different from SKF38393 treatment alone $p< 0.001$ (two-way ANOVA, Minitab).


SKF38393 alone
D1 SENSE/
SKF38393
Mean Number of pixels/ unit area
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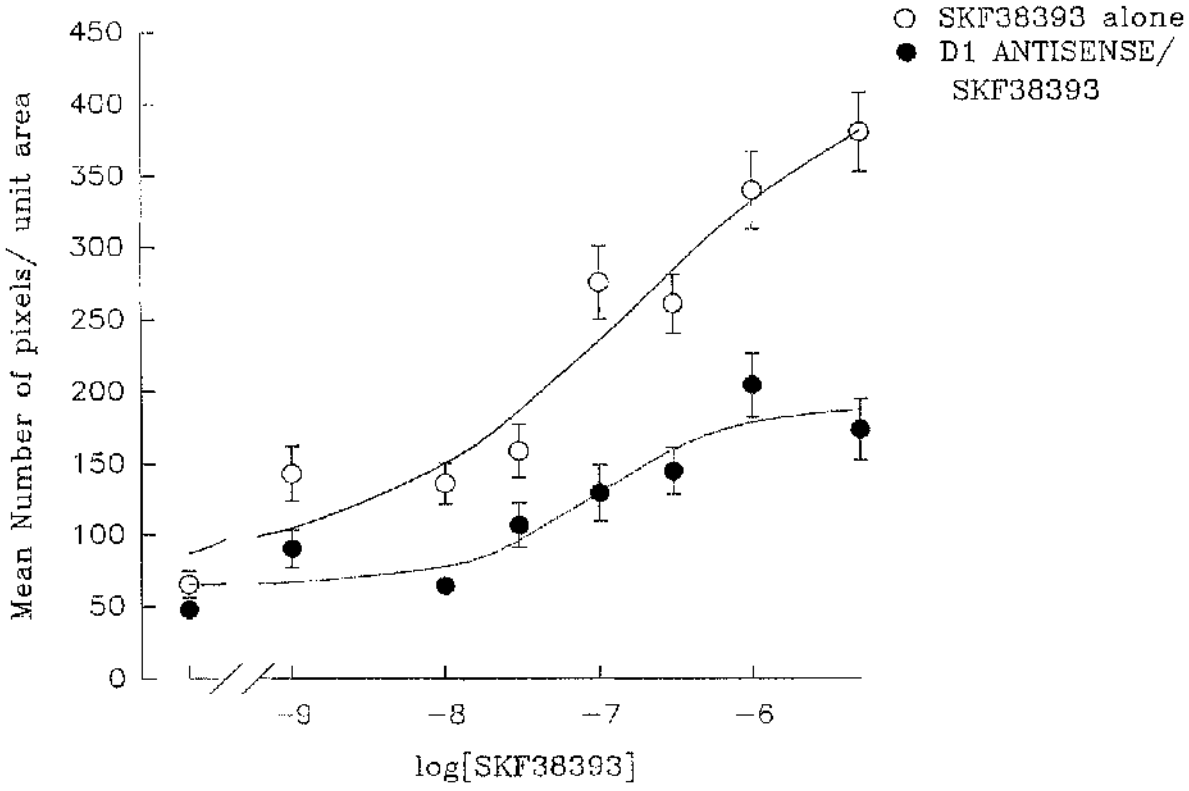
log[SKF38393]

Figure 7.1.5 : Effect of a dopamine D1 antisense oligonucleotide on zif/268 mRNA levels in striatal cultures. Cells were pretreated for two days with a 15-mer phosphorothioate oligonucleotide corresponding to a D1 dopamine receptor antisense (1μM, n= 3). Cells were then exposed to SKF38393 (1nM-5μM, n= 5) alone or in the presence of D1 antisense (n= 3), or vehicle (HBSS) alone or in the presence of D1 antisense. After 45 minutes the cells were fixed in buffered formalin and then processed for in situ hybridisation with a specific radiolabelled probe for zif/268. Results are expressed as mean number of pixels per unit area ± s.e.m. The dose response curve in the presence of the D1 antisense was significantly different from SKF38393 treatment alone $p< 0.001^{***}$ (two-way ANOVA, Minitab).


SKF38393 alone
D1 ANTISENSE/
SKF38393
Mean Number of pixels/ unit area
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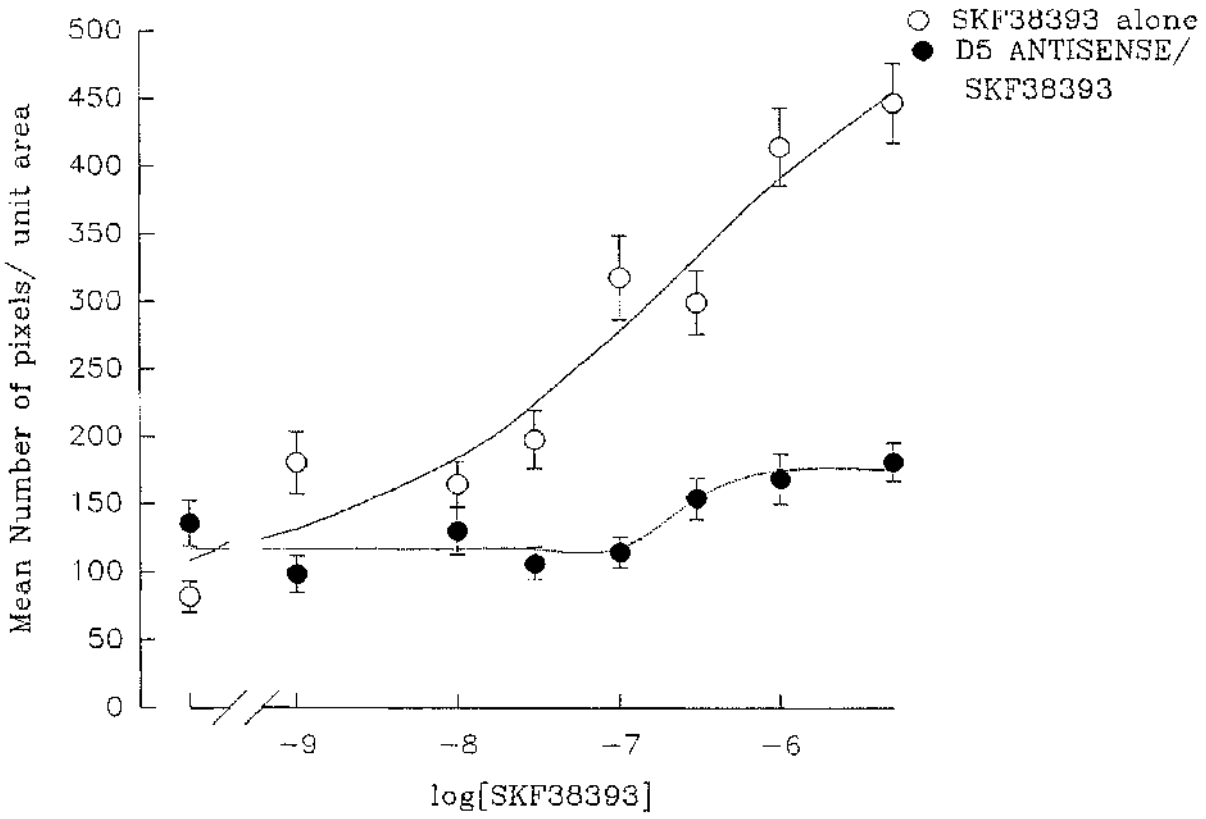
-8
-7
-6
log[SKF38393]

Figure 7.1.6 : Effect of a dopamine D5 antisense oligonucleotide on zif/268 mRNA levels in striatal cultures. Cells were pretreated for two days with a 15-mer phosphorothioate oligonucleotide corresponding to a D5 dopamine receptor antisense (1μM, n= 3). Cells were then exposed to SKF38393 (1nM-5μM, n= 5) alone or in the presence of D5 antisense (n= 3), or vehicle (HBSS) alone or in the presence of D5 antisense. After 45 minutes the cells were fixed in buffered formalin and then processed for in situ hybridisation with a specific radiolabelled probe for zif/268. Results are expressed as mean number of pixels per unit area ± s.e.m. The dose response curve in the presence of the D5 antisense was significantly different from SKF38393 treatment alone $p< 0.001^{***}$ (two-way ANOVA, Minitab).


SKF38393 alone
D5 ANTISENSE/
SKF38393
Mean Number of pixels/ unit area
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log[SKF38393]

DISCUSSION

7.2 Effect of dopamine receptor antisense oligonucleotides on immediate early gene expression in striatal cultures

In previous results we have demonstrated that acute treatment with SKF38393 can induce the expression of the immediate early genes c-fos and zif/268 in primary embryonic rat striatal cultures. This is in agreement with in vivo work where the induction of c-fos by SKF38393 has been shown, following a unilateral 6-hydroxydopamine lesion of the nigrostriatal pathway (Robertson et al., 1989; Robertson et al., 1992) and with in vitro research demonstrating the induction of c-fos with SKF38393 treatment in cerebral cortical cultures (Vaccarino et al., 1993).

SKF38393, a potent D1/ D5 dopamine receptor agonist, is thought to be producing this stimulatory effect on both c-fos and zif/268 gene expression by activating D1-like receptors in vivo and in vitro. Until now it has been impossible to distinguish D1-mediated effects from D5-mediated effects, simply because there are no selective ligands to differentiate between these two highly homologous dopamine receptor subtypes. However, with the introduction of antisense oligonucleotides targeted towards particular receptor mRNAs, it is now possible to achieve a substantial degree of protein synthesis inhibition. Two 15-nucleotide-long antisense oligonucleotides were designed to recognize a sequence of the D1 and D5 dopamine receptor genes respectively, in an attempt to answer this question more fully.

In this study we have demonstrated that c-fos mRNA can be dose-dependently increased by SKF38393 in vitro, with an EC_{50} value of approximately 110nM. This EC_{50} value obtained is very close to the K_D values previously reported for the binding of SKF38393 to striatal membranes

(Seeman and Van Tol, 1993). The levels of c-fos mRNA reached a plateau at around 5μM SKF38393. Pretreatment of primary striatal cultures with a D1 sense phosphorothioate oligonucleotide, at a concentration not causing toxic effects,demonstrated by no change in D2 receptor binding and no obvious change in cell numbers, clearly had no effect on the dose-dependent induction of c-fos gene expression by SKF38393, in rat striatal cultures. This would be expected, because the sense oligonucleotide is acting as a control here, in that it has the same sequence as part of the D1 receptor mRNA and therefore no binding interaction to cellular mRNA will occur if conditions are optimal, with non-specific binding reduced to a minimum. In contrast, pretreatment with the D1 phosphorothioate antisense oligonucleotide, at a concentration not causing toxicity, was clearly able to reduce the dose-dependent induction of c-fos mRNA by SKF38393. The D1 antisense caused a greater than 50% reduction of the maximum effect of SKF38393.

Similarly pretreatment with the phosphorothioate antisense oligonucleotide directed towards the D5 dopamine receptor protein reduced the overall effect of SKF38393, particularly at low concentrations of SKF38393. However, the maximum effect of SKF38393 was unaltered by pretreatment with the D5 antisense. In general, these results are suggesting that the D1 dopamine receptor primarily mediates the inductive effect of SKF38393 on c-fos gene expression in the rat striatum. However, there may be a smaller D5 component to this effect. It is known that the density of D5 dopamine receptors in the striatum is much lower than the density of D1 dopamine receptors, and that the affinity for the endogenous ligand dopamine, is ten-fold higher for the D5 receptor (Van Tol et al., 1991). These properties of the D5 receptor may be thought to functionally represent a 'fine control' over the dopaminergic system. Therefore stimulation of D1 and D5 dopamine receptors may contribute to the induction of c-fos by SKF38393. The results also indicate

that in rat primary striatal cultures, in the case of D1 receptors, there appears to be little receptor reserve.

The results obtained for the SKF38393- induced expression of zif/268 mRNA in the presence of the antisense oligonucleotides are much more difficult to interpret. Similar to the results obtained for c-fos, a dose-dependent increase in zif/268 mRNA was observed following acute treatment with SKF39383 alone,with a very similar EC_{50} value to that obtained for c-fos induction, of around 100nM. However, pretreatment with the D1 sense oligonucleotide clearly inhibited the dose-dependent effect of SKF38393 on zif/268 gene expression. This non-specific inhibitory effect of the D1 sense makes any effect of the D1 or D5 antisense very difficult to interpret as a specific effect. Therefore the effects of the dopamine D1 and D5 antisense on zif/268 expression after SKF38393 are likely to be non-specific in these experiments.

One of the major disadvantages in the use of 'knockdown' approaches such as antisense oligonucleotide inhibition, is the narrow experimental and therapeutic windows that accompany antisense use. With the D1 and D5 antisense a concentration of 1μM proved effective in reducing the SKF38393 effect on c-fos gene expression by 50%. However, we found that concentrations of 2μM and above proved toxic to the cells in primary striatal culture. Therefore the existence of a narrow window for effective concentration may not always parallel the therapeutic window. In particular, potential problems are associated with an incomplete effect of oligonucleotide treatment. If a biological phenomenon has a great deal of spare capacity or redundancy, for example, then only a fraction of receptors have to be occupied to elicit a full response, and the oligonucleotide treatment may become functionally 'silent'. In this study it would also be necessary to demonstrate reductions in D1-like receptor binding, and to rule out oligonucleotide effects on proteins other than the targeted D1 and D5 receptor proteins, by performing

appropriate ligand-binding experiments, which are at present still in progress in our laboratory. Further appropriate controls for these experiments would involve the use of random sequence oligonucleotides and mismatch oligonucleotides.

However, the results from this study are the first to provide an insight into the specific receptor mechanism involved in the inductive effect of SKF38393 on c-fos expression in the rat striatum. Antisense oligonucleotides targeted towards the D1 and D5 dopamine receptor proteins have been used to reduce the respective receptor protein and thus establish which dopamine receptor subtype(s) contribute to the stimulatory effect of SKF38393 on c-fos gene expression in rat striatal culture. We have produced evidence to suggest that D1 receptor stimulation is primarily involved in this effect, with a minor D5 component. However, the evidence that non-specific effects occur on another cellular process- the induction of zif/268 expression, suggests that extreme caution should always be observed when using antisense oligonucleotides.

Chapter 8

Serotoninergic Regulation of Immediate Early Gene Expression in vitro

INTRODUCTION

8.0 Serotoninergic Modulation of Immediate Early Gene Expression in the Central Nervous System

The serotoninergic system is involved in numerous physiological functions. In the central nervous system, serotonin (5-HT) is known to be involved in the homeostatic regulation of fundamental physiological functions such as sleep, appetite, locomotion, nociception, sexual activity and vascular contraction (Wilkinson and Dourish, 1991). Disruption of the serotoninergic system has also been implicated as a critical factor in mental disorders such as schizophrenia, anxiety, depression, parkinsonism and obsessive-compulsive disorders (Lopez-Ibor, 1988). This biogenic amine neurotransmitter is synthesised by neurons in the raphe nuclei of the brain stem that project throughout the central nervous system, with the highest density in basal ganglia and limbic structures (Steinbusch, 1984).

At present, at least seven classes of serotonin receptor have been cloned, each class being supported by a number of receptor subtypes. The functional significance, if any, in terms of the physiological role of many of these newly cloned receptors is as yet to be identified and therefore is of great importance in understanding the role of 5-HT as a regulator of many behavioural responses.

The relatively new field of research into immediate early genes is currently expanding. However, there is very litttle research, as yet, into the serotoninergic modulation of immediate early genes in the central nervous system. Recent work has demonstrated , using cultured spinal cord neurons, that the induction of the immediate early gene c-fos and subsequent transactivation of the prodynorphin gene, are coupled events triggered by 5-

HT1A receptor agonists (Lucas et al., 1993). Classical 5-HT1A receptor agonists are known to be inhibitory, although this induction of c-fos following a 5-HT1A receptor stimulation also resulted in a simultaneous increase in cAMP levels. This would appear to be an anomoly from the classical cloned 5-HT1A receptor subtype, suggesting the possibility of another subtype of the 5-HT1A receptor.

The serotoninergic system has also been implicated in the regulation of immediate early genes in the striatum. Recent in vivo research has suggested that the ability of cocaine to induce the expression of c-fos and zif/268 in brain neurons is partly due to its characteristic property of 5-HT uptake inhibition. Using the selective inhibitors of 5-HT uptake, fluoxetine and citalopram, the ability of mazindol, a dopamine and noradrenaline uptake blocker, to induce zif/268 and c-fos was shown to be potentiated (Bhat and Baraban, 1993). More recently, activation of the serotonin 2A/2C receptor has been demonstrated to cause a localised expression of the immediate early gene c-fos in the frontal cortex, amygdala, globus pallidus, nucleus accumbens and dorsomedial striatum, and this induction could be selectively blocked with the 2A/2C antagonist ritanserin in vivo (Leslie et al., 1993). There is also some evidence for the serotoninergic regulation of tachykinin gene expression in the rat neostriatum. A recent report has shown that by raising extracellular levels of serotonin with zimelidine, a 5-HT uptake inhibitor, the levels of preprotachykinin mRNA were increased (Walker et al., 1991). This suggests that neostriatal tachykinin biosynthesis is sensitive to alterations in 5-HT neurotransmission.

At present there are several suggestions with regard to the involvement of central serotoninergic mechanisms in the actions of antipsychotic drugs. The recently cloned 5-HT6 and 5-HT7 receptor subtypes have been demonstrated to exhibit a particularly high affinity for some atypical neuroleptic drugs, including clozapine. The mRNA encoding the 5-HT6 receptor has been shown

to be mainly present in the striatum, olfactory tubercles, nucleus accumbens and in the hippocampus (Monsma et al., 1992). More recently it has been demonstrated that the 5-HT6 receptor is present in primary striatal neuron cultures and it is positively coupled to adenylyl cyclase activity (Sebben et al., 1994). Similarly the 5-HT7 receptor subtype has also been shown to be positively coupled to adenylyl cyclase (Tsou et al., 1994). Therfore the 5-HT6 and 5-HT7 receptor subtypes may have a functional role in the manifestation and clinical treatment of certain disease states such as schizophrenia and parkinsonism.

Therefore, it was decided to investigate the role of serotonin in the induction of the immediate early genes c-fos and zif/268, and to elucidate the receptor mechanism(s) involved.

RESULTS

Much research has been done on the dopaminergic regulation of IEGs in the striatum. However, at present there is very little information on the serotoninergic modulation of IEGs in the striatum. Although there are implications for a serotoninergic stimulation of zif/268 in the striatum (Bhat et al, 1993). Therefore in this study we were interested in the serotoninergic modulation of the IEGs zif/268 and c-fos in rat striatal neuronal cultures and the receptor site(s) involved.

8.1 Effect of serotonin (5-HT) on the expression of the IEGs c-fos and zif/268 mRNA

Serotonin (5-HT) (1nM-5μM) was demonstrated to clearly increase the expression of both zif/268 and c-fos mRNA in striatal cultures in a dose dependent manner. The induction of zif/268 mRNA forty-five minutes after 5-HT (300nM) can be seen in figure 8.1.19, as an increase in the number of silver grains over cell bodies in striatal culture, compared to vehicle treated cultures. The EC_{50} of 5-HT to stimulate both zif/268 and c-fos mRNA in striatal culture was approximately 100nM. The induction occured in a large proportion of the cultured cells, suggesting that the increase in mRNA was observed mainly in medium spiny neurons [Figure 8.1.19]. Pretreatment of striatal cultures with ondansetron (200nM), the selective 5-HT3 receptor antagonist, clearly had no effect on the levels of zif/268 mRNA being expressed in culture after addition of 5-HT (1nM-5μM). [Figure 8.1.1]. Although c-fos mRNA levels due to stimulation by 5-HT were effected by the presence of ondansetron (200nM)

and this was shown to be significantly different from 5-HT (1nM-5μM) treatment alone ($F= 9.73$, $p< 0.01$; $n= 3$). [Figure 8.1.2].

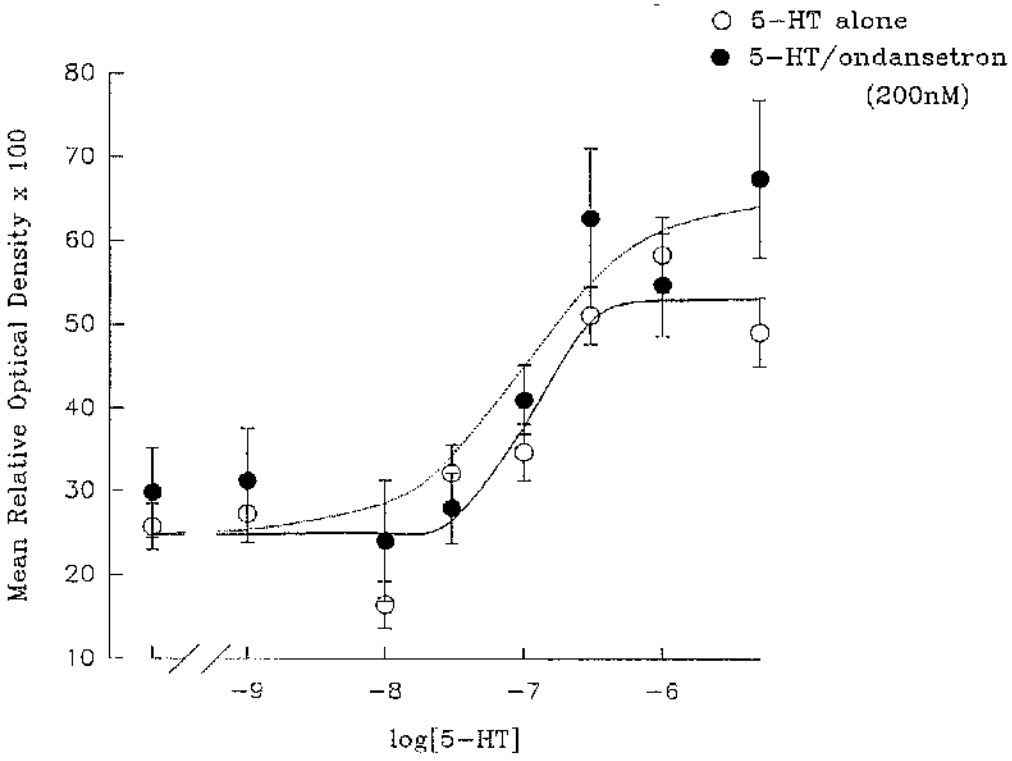
The cultures were also pretreated with a number of 5-HT1/2 receptor antagonists including cyproheptadine, methiothepin, mesulergine and spiperone. It was found that cyproheptadine (500nM) caused a dramatic reduction in the hybridisation signal representing the induction of zif/268 mRNA by 5-HT, and this was demonstrated to be significantly different from the expression of zif/268 mRNA due to 5-HT (1nM-5μM) alone ($F= 29.95$, $p< 0.001$; $n= 3$) [Figure 8.1.3, 8.1.19]. A similar reduction in the signal was observed for c-fos mRNA ($F= 19.57$, $p< 0.001$; $n= 3$) [Figure 8.1.4]. Pretreatment with methiothepin (200nM) clearly decreased the hybridisation signal for zif/268 mRNA in culture and was significantly different from 5-HT alone ($F= 39.95$, $p< 0.001$; $n= 3$) [Figure 8.1.5]. A reduction in the hybridisation signal for c-fos mRNA was also observed ($F= 33.89$, $p< 0.001$; $n= 3$) [Figure 8.1.6]. In cultures treated with mesulergine (100nM) prior to the addition of 5-HT (1nm-5μM) a marked decrease in zif/268 mRNA was observed and this was significantly different from 5-HT (1nM-5μM) alone ($F= 28.71$, $p< 0.001$; $n= 3$) [Figure 8.1.7]. Pretreatment with the lower concentration of 30nM mesulergine also resulted in a highly significant decrease in the levels of zif/268 mRNA in culture ($F= 17.36$, $p< 0.001$; $n= 3$) following a 5-HT-induced stimulation [Figure 8.1.8]. Mesulergine (100nM) caused a reduction in the levels of c-fos mRNA as a result of a 5-HT receptor stimulation. This decrease proved to be significantly different ($F= 7.10$, $p< 0.01$; $n= 3$) from 5-HT alone (1nM-5μM) [Figure 8.1.9]. Spiperone (100nM) pretreatment was also demonstrated to cause a massive inhibition of the 5-HT-induced stimulation of zif/268 mRNA in striatal cultures, and this decrease was shown to be significant ($F= 59.36$, $p< 0.001$; $n= 3$) compared to 5-HT (1nM-5μM) alone [Figure 8.1.10], as did the lower dose of 30nM spiperone ($F= 31.96$, $p< 0.001$; $n= 3$) [Figure 8.1.11, 8.1.19]. As spiperone has very low

affinity for the 5-HT1C receptor, the low dose used in this study would suggest that the increase in zif/268 mRNA observed, is not due to stimulation of the 5-HT2C receptor. Pretreatment with spiperone (30nM) also resulted in a dramatic and highly significant inhibition of the hybridisation signal for c-fos mRNA ($F= 40.86$, $p<0.001$; $n= 3$) compared to 5-HT treatment alone [Figure 8.1.12].

The effects of a number of 5-HT receptor agonists were investigated in striatal culture. Addition of the 5-HT2C receptor agonist mCPP (1nM-5μM) had no overall effects on the levels of both zif/268 mRNA [Figure 8.1.13] and c-fos mRNA [Figure 8.1.14]. Similarly the selective 5-HT1A receptor agonist 8OH-DPAT (1nM-5μM) had no significant effect on the expression of the zif/268 [Figure 8.1.15] and c-fos genes [Figure 8.1.16]. Finally the selective 5-HT2A/2C receptor agonist, ±- DOI (1nM-5μM) was shown to have a slight effect on the levels of zif/268 and c-fos mRNA in striatal culture, but this induction did not prove to be significant [Figures 8.1.17 and 8.1.18 respectively].

Figure 8.1.1 : Effect of selective inhibition of the serotononergic 5-HT3 receptor on the levels of expression of zif/268 mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with ondansetron (200nM) prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 8). Results, obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. The two dose response curves were not significantly different.

Figure 8.1.2 : Effect of selective inhibition of the serotononergic 5-HT3 receptor on the levels of expression of c-fos mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with ondansetron (200nM) prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 7). Results, obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. The two dose response curves were significantly different, $p< 0.01$ **.


○ 5-HT alone
● 5-HT/ondansetron
(200nM)
Mean Relative Optical Density x 100
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-6
log[5-HT]

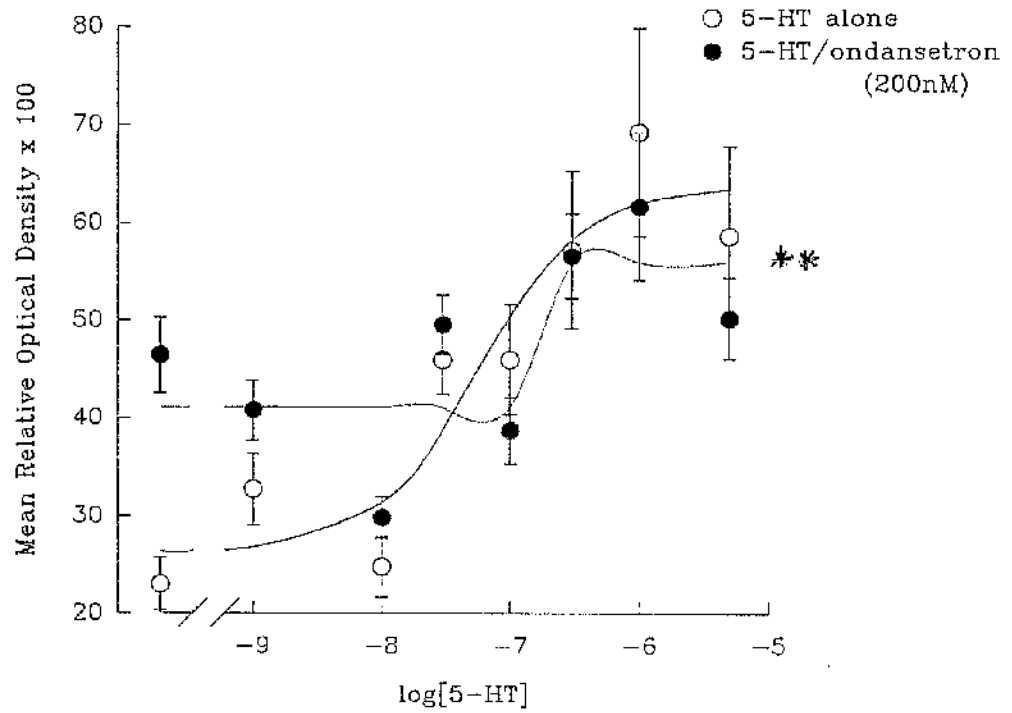
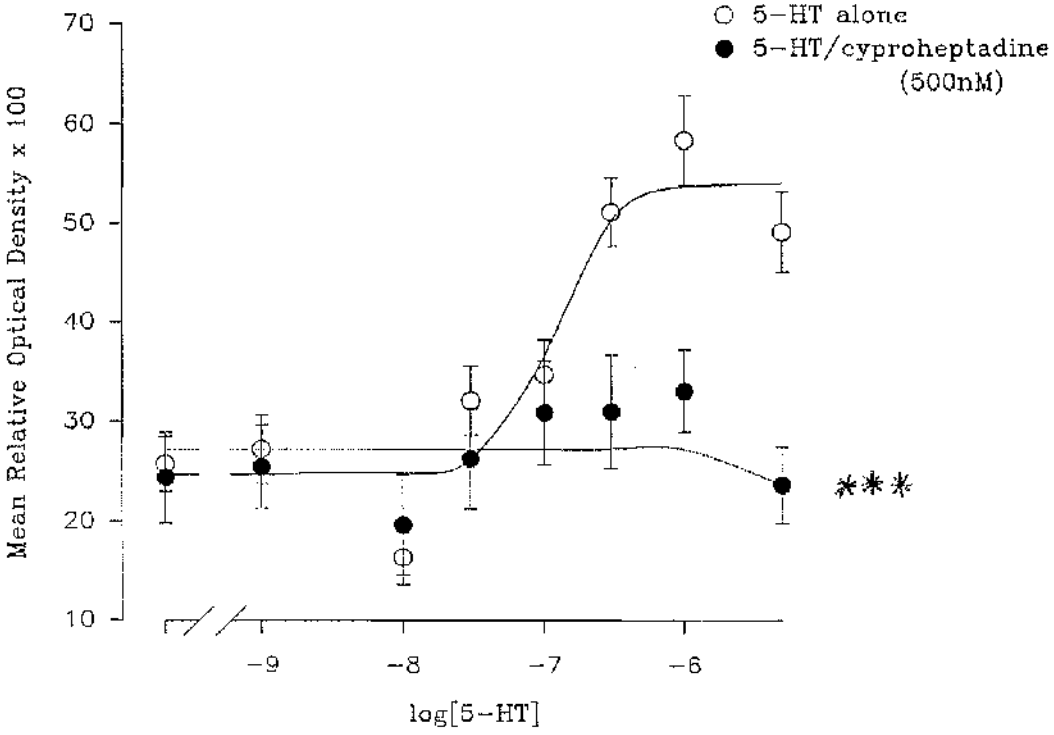

○ 5-HT alone
● 5-HT/ondansetron
(200nM)
Mean Relative Optical Density x 100
80
70
60
50
40
30
20
**
-9
-8
-7
-6
-5
log[5-HT]

Figure 8.1.3 : Effect of inhibition of serotoninergic 5-HT1/2 receptors on the levels of expression of zif/268 mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with cyproheptadine (500nM), prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 8). Results obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. The two dose response curves were significantly different, $p< 0.001$ ***.

Figure 8.1.4 : Effect of inhibition of serotoninergic 5-HT1/2 receptors on the levels of expression of c-fos mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with cyproheptadine (500nM), prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 7). Results obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. The two dose response curves were significantly different, $p< 0.001$ ***.


○ 5-HT alone
● 5-HT/cyproheptadine (500nM)
Mean Relative Optical Density x 100
70
60
50
40
30
20
10
-9
-8
-7
-6
log[5-HT]
***

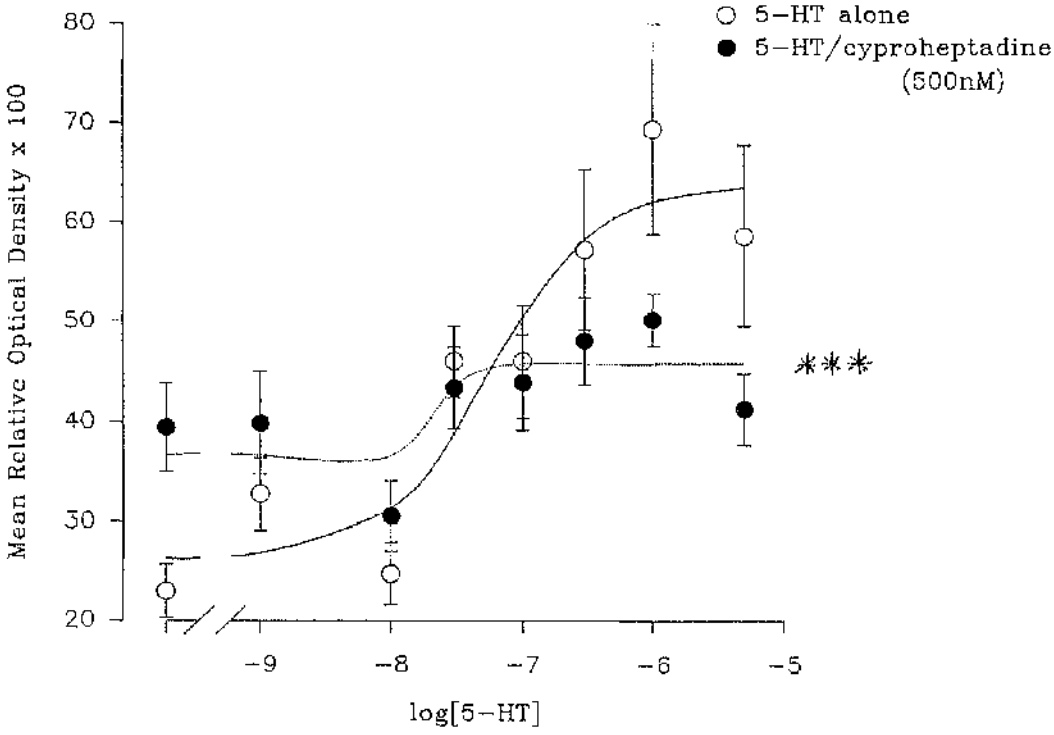
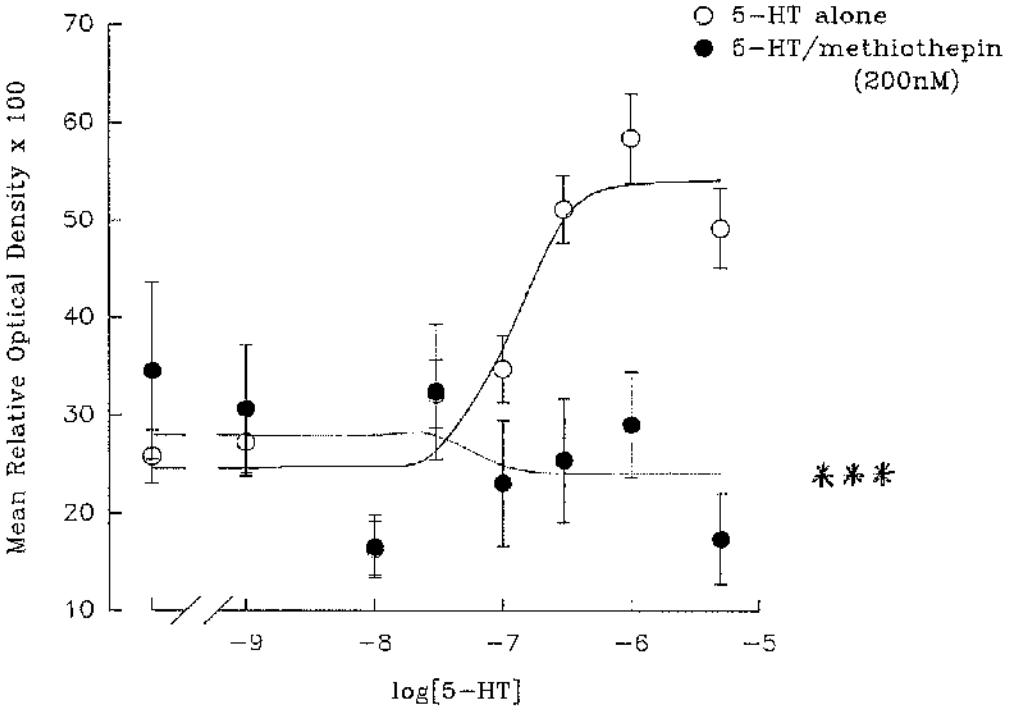

○ 5-HT alone
● 5-HT/cyproheptadine (500nM)
Mean Relative Optical Density x 100
80
70
60
50
40
30
20
-9
-8
-7
-6
-5
log[5-HT]
***

Figure 8.1.5 : Effect of inhibition of serotoninergic 5-HT1/2/6 receptors on the levels of expression of zif/268 mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with methiothepin (200nM), prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 8). Results obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab.The two dose response curves were significantly different, $p< 0.001$ ***.

Figure 8.1.6 : Effect of inhibition of serotoninergic 5-HT1/2/6 receptors on the levels of expression of c-fos mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with methiothepin (200nM), prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 7). Results obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. The two dose response curves were significantly different, $p< 0.001$ ***.


○ 5-HT alone
● 5-HT/methiothepin
(200nM)
Mean Relative Optical Density x 100
70
60
50
40
30
20
10
-9
-8
-7
-6
-5
log[5-HT]
***

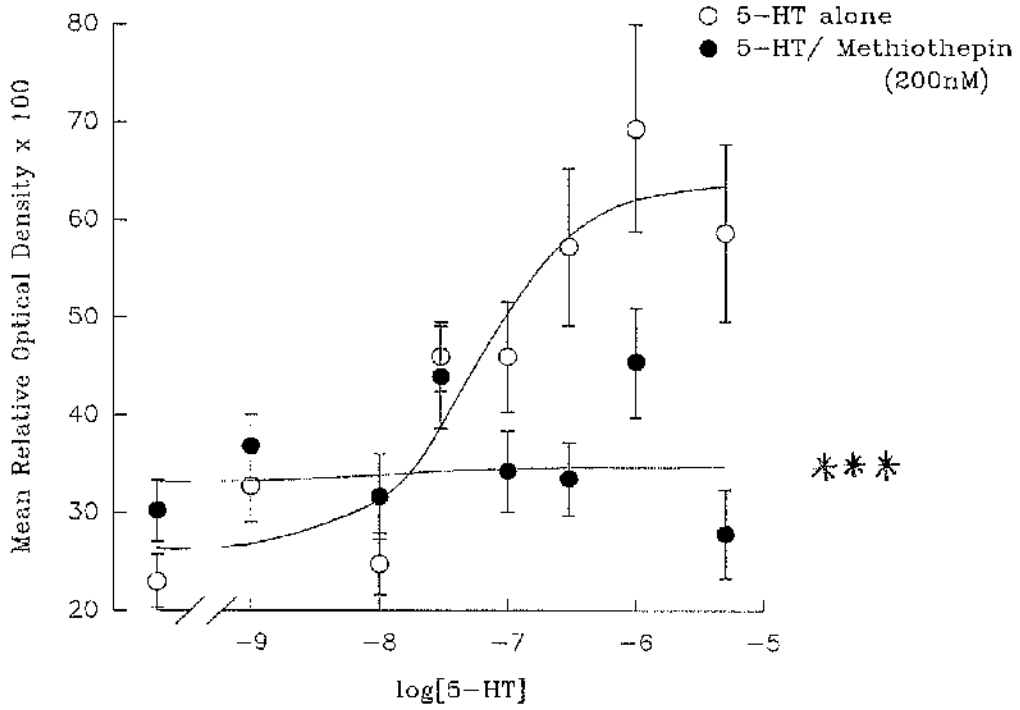
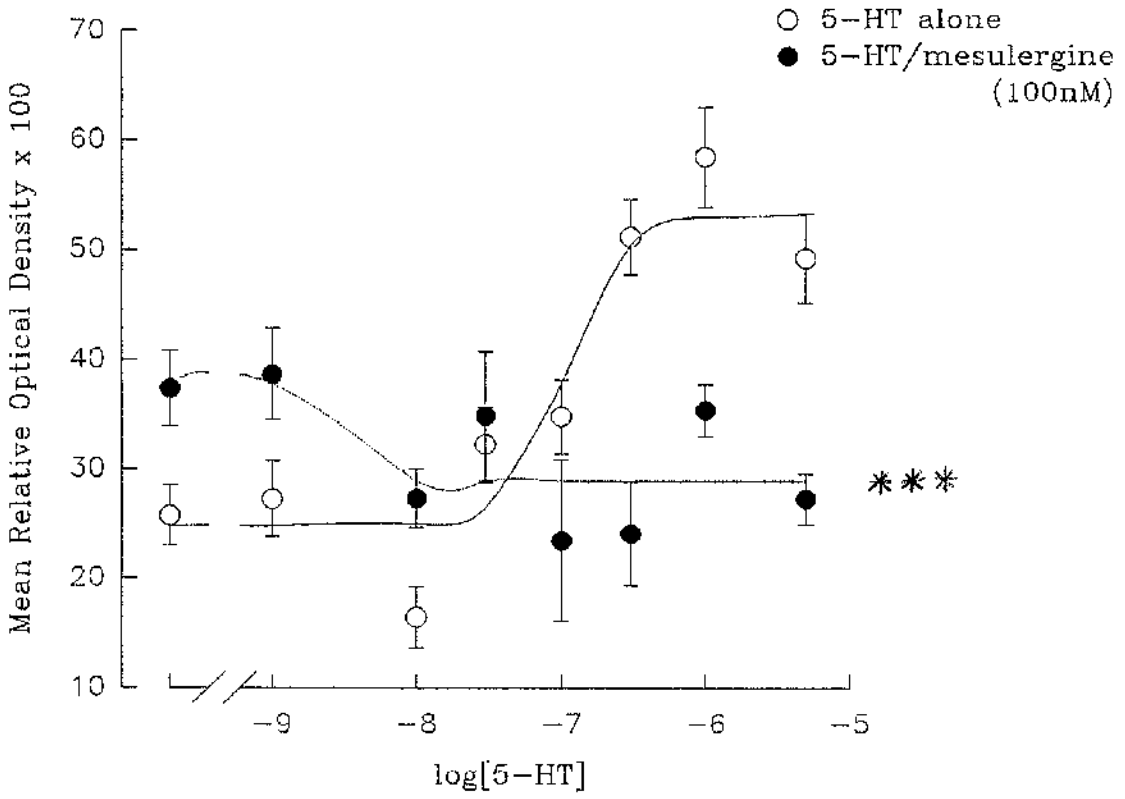

○ 5-HT alone
● 5-HT/ Methiothepin
(200nM)
Mean Relative Optical Density x 100
80
70
60
50
40
30
20
-9
-8
-7
-6
-5
log[5-HT]
***

Figure 8.1.7 : Effect of inhibition of serotoninergic 5-HT1A /2A /2C receptors on the levels of expression of zif/268 mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with mesulergine (100nM), prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 8). Results obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. The two dose response curves were significantly different, $p< 0.001$ ***.

Figure 8.1.8 : Effect of inhibition of serotoninergic 5-HT1A /2A /2C receptors on the levels of expression of zif/268 mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with mesulergine (30nM), prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 8). Results obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. The two dose response curves were significantly different, $p< 0.001$ ***.


5–HT alone
5–HT/mesulergine
(100nM)
Mean Relative Optical Density x 100
70
60
50
40
30
20
10
***
−9
−8
−7
−6
−5
log[5–HT]

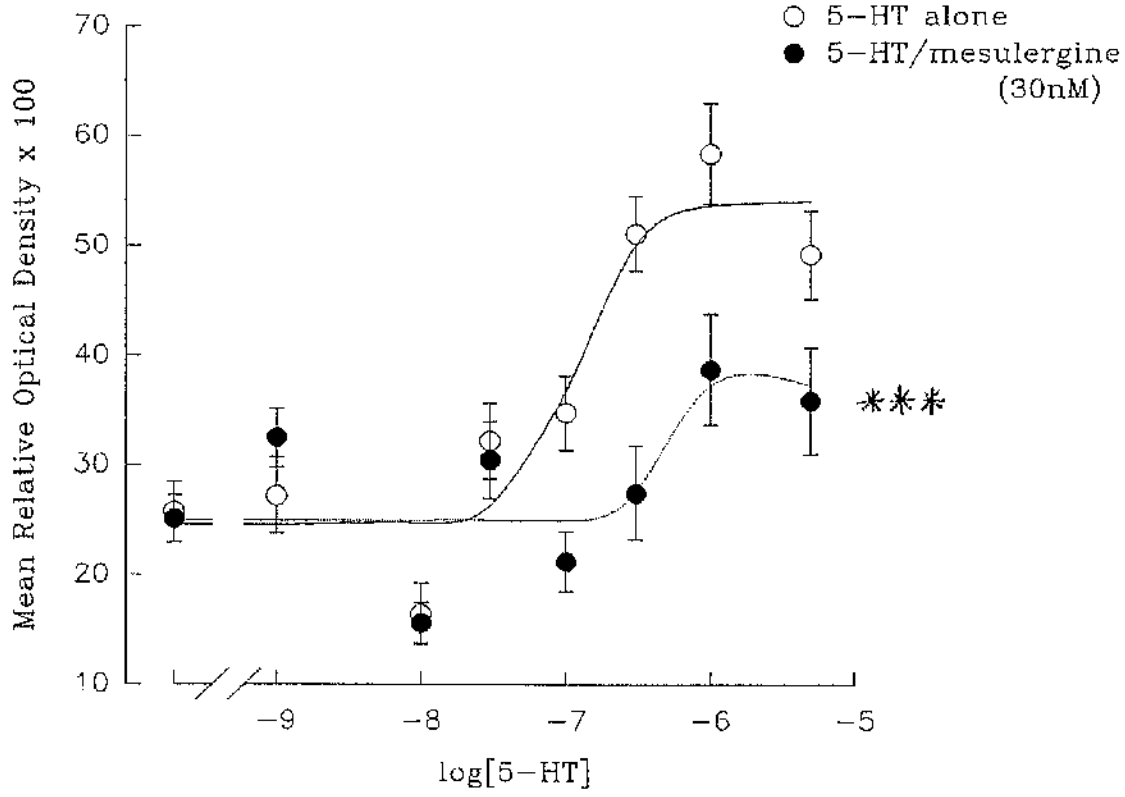

5–HT alone
5–HT/mesulergine
(30nM)
Mean Relative Optical Density x 100
70
60
50
40
30
20
10
***
−9
−8
−7
−6
−5
log[5–HT]

Figure 8.1.9 : Effect of inhibition of serotoninergic 5-HT1A /2A /2C receptors on the levels of expression of c-fos mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with mesulergine (100nM), prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 4) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 7). Results obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 $\pm$ s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. $p < 0.01$ ***.

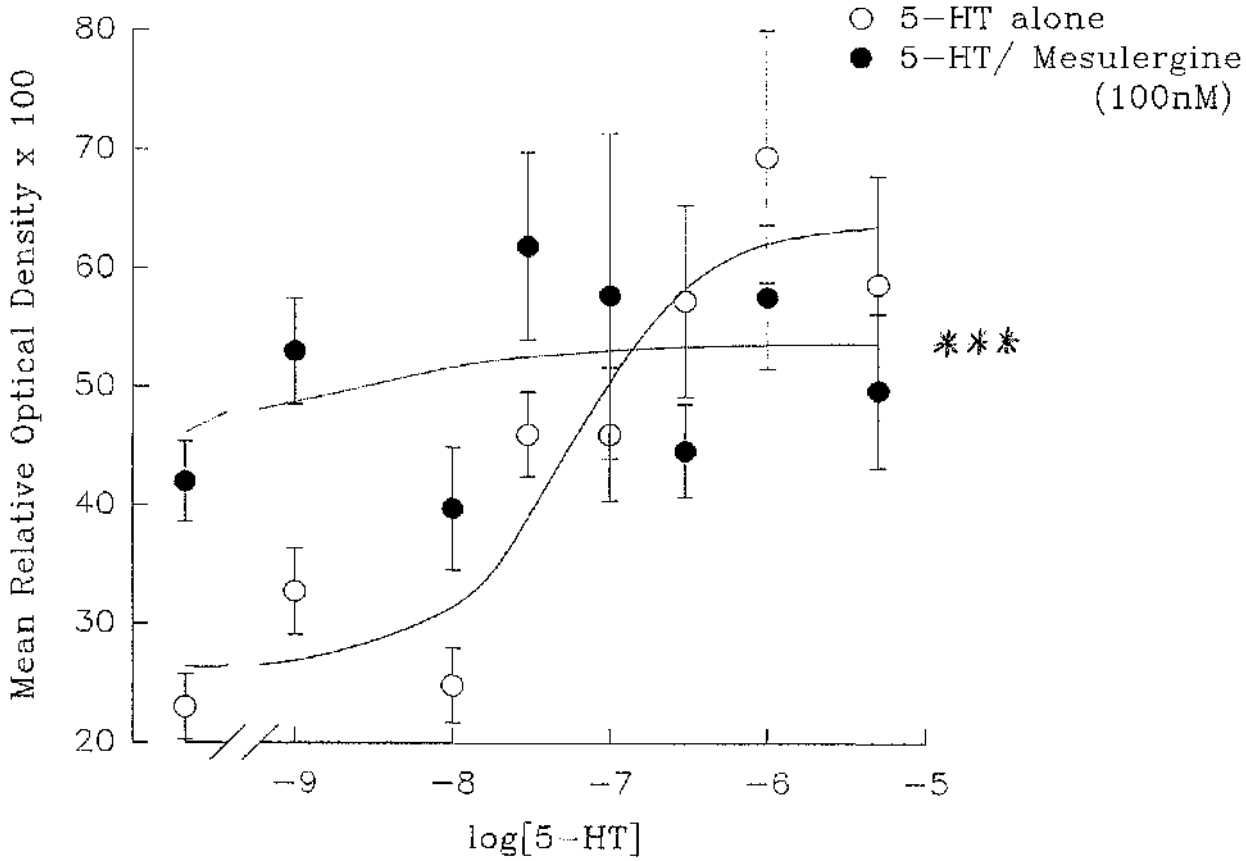
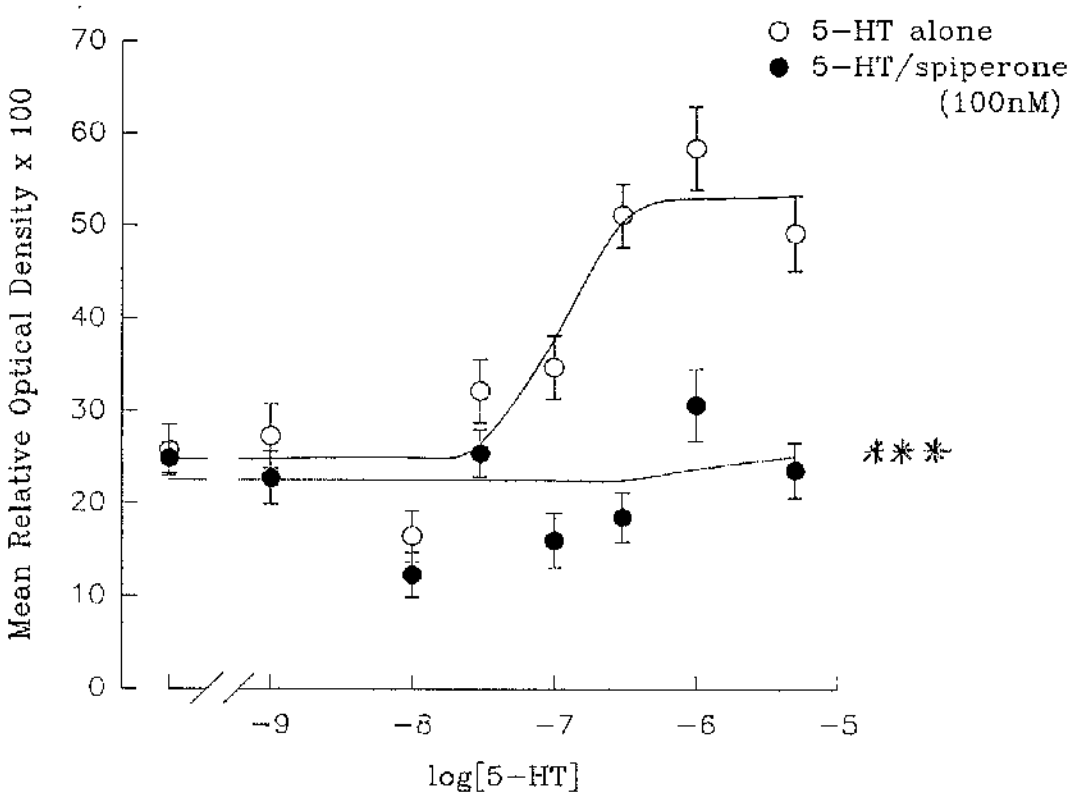

○ 5−HT alone
● 5−HT/ Mesulergine
(100nM)
Mean Relative Optical Density x 100
80
70
60
50
40
30
20
***
−9
−8
−7
−6
−5
log[5−HT]

Figure 8.1.10: Effect of inhibition of serotoninergic 5-HT1A /2A /2C / 7 receptors on the levels of expression of zif/268 mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with spiperone (100nM), prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 8). Results obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. The two dose response curves were significantly different, $p< 0.001$ ***.

Figure 8.1.11 : Effect of inhibition of serotoninergic 5-HT1A /2A /2C / 7 receptors on the levels of expression of zif/268 mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with spiperone (30nM), prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 8). Results obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. The two dose response curves were significantly different, $p< 0.001$ ***.


○ 5-HT alone
● 5-HT/spiperone
(100nM)
Mean Relative Optical Density x 100
70
60
50
40
30
20
10
0
-9
-8
-7
-6
-5
log[5-HT]
***

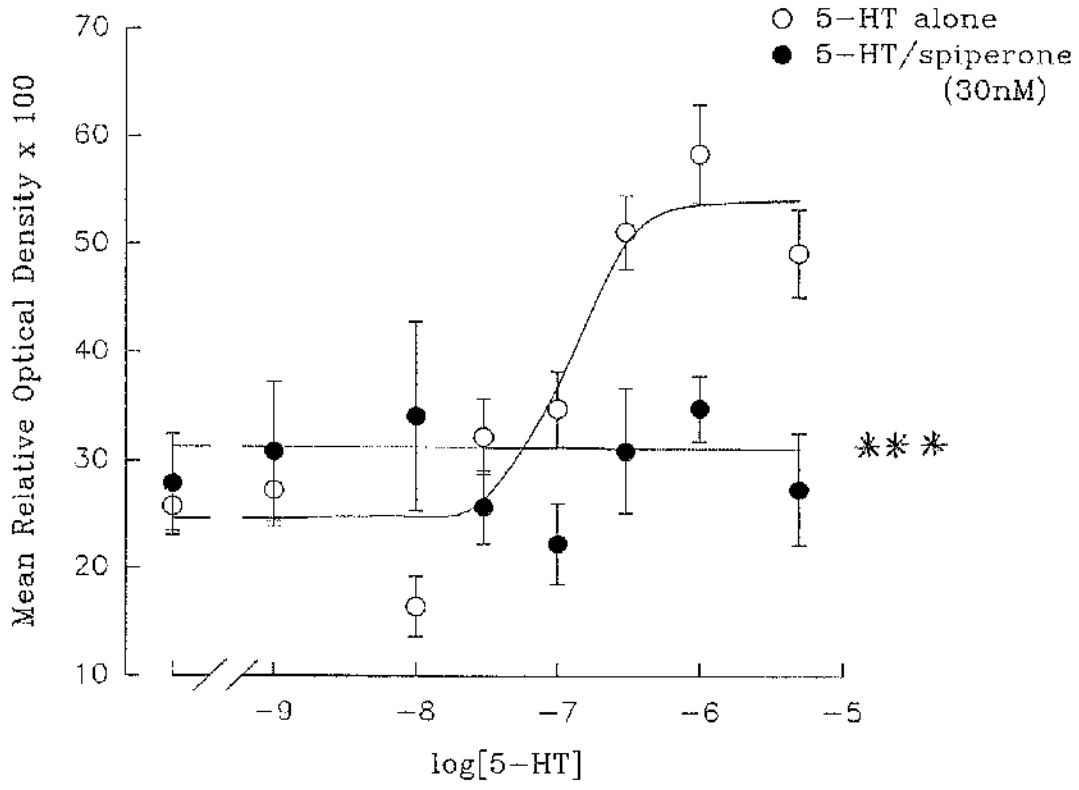

○ 5-HT alone
● 5-HT/spiperone
(30nM)
Mean Relative Optical Density x 100
70
60
50
40
30
20
10
-9
-8
-7
-6
-5
log[5-HT]
***

Figure 8.1.12 : Effect of inhibition of serotoninergic 5-HT1A /2A /2C / 7 receptors on the levels of expression of c-fos mRNA in rat striatal neuron cultures. Cultures were pretreated for 30 minutes with spiperone (30nM), prior to the addition of vehicle or 5-HT (1nM-5μM) (n= 3) or treated with vehicle or 5-HT (1nM-5μM) alone (n= 7). Results obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. Significance was determined by ANOVA, General Linear Model, Minitab. The two dose response curves were significantly different, $p < 0.001$ ***.

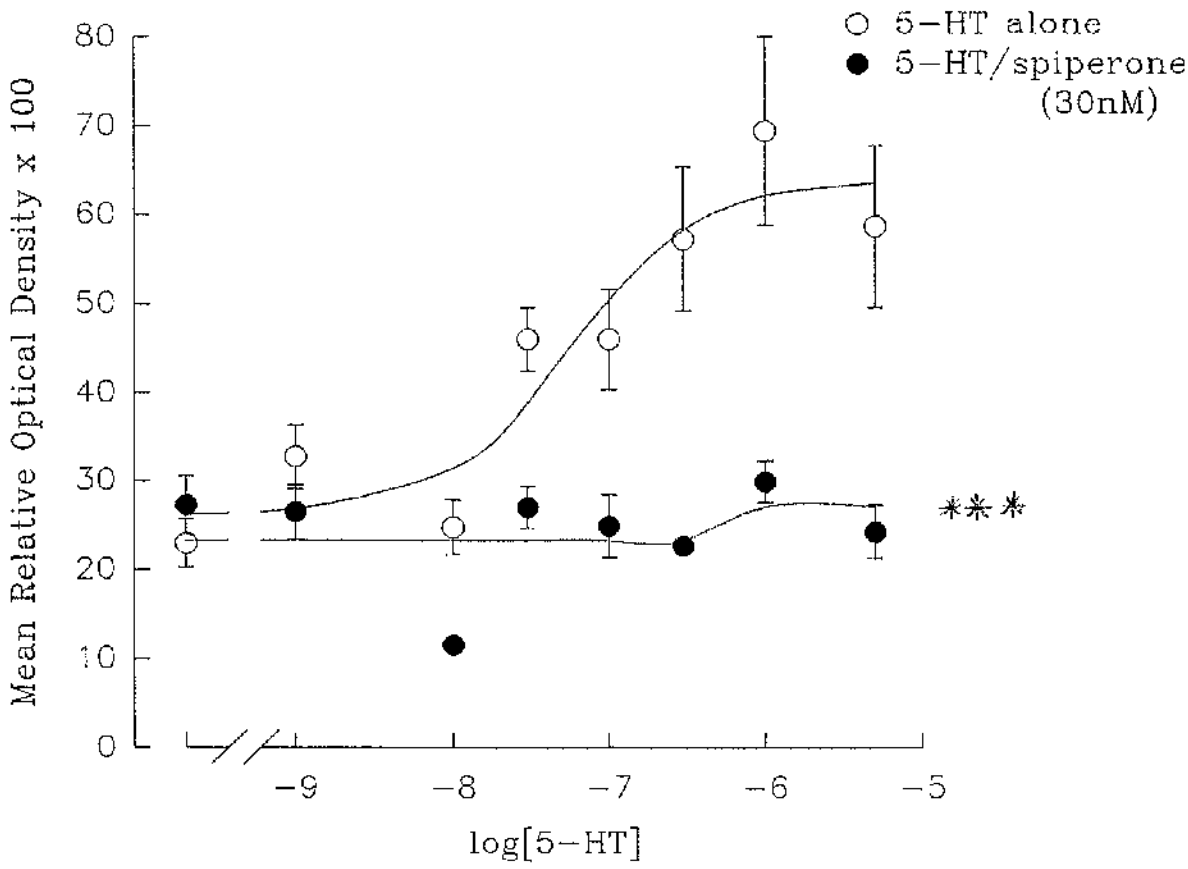
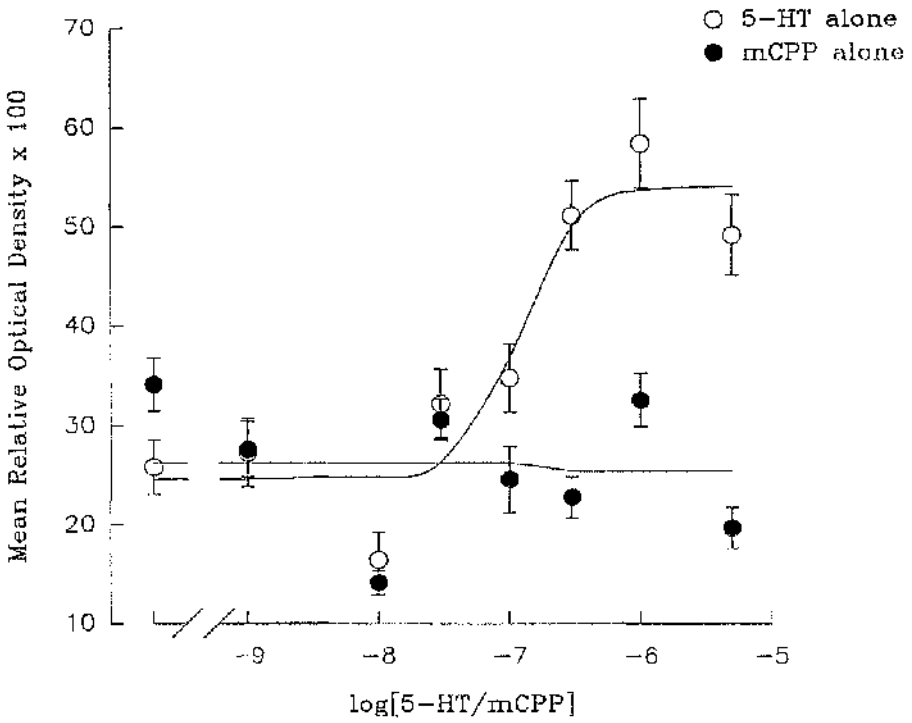

5−HT alone
5−HT/spiperone
(30nM)
Mean Relative Optical Density x 100
80
70
60
50
40
30
20
10
0
−9
−8
−7
−6
−5
log[5−HT]
***

Figure 8.1.13 : Effect of a 5-HT2C receptor stimulation on the level of expression of zif/268 mRNA in rat striatal neuron cultures. Cultures were treated for 45 minutes with vehicle (HBSS), mCPP (1nM-5μM, n= 5) alone or with 5-HT (1nM-5μM, n= 8) alone for comparison. Result, obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m.

Figure 8.1.14 : Effect of a 5-HT2C receptor stimulation on the level of expression of c-fos mRNA in rat striatal neuron cultures. Cultures were treated for 45 minutes with vehicle (HBSS), mCPP (1nM-5μM, n= 3) alone or with 5-HT (1nM-5μM, n= 7) alone for comparison. Results, obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m.


5-HT alone
mCPP alone
Mean Relative Optical Density x 100
70
60
50
40
30
20
10
-9
-8
-7
-6
-5
log[5-HT/mCPP]

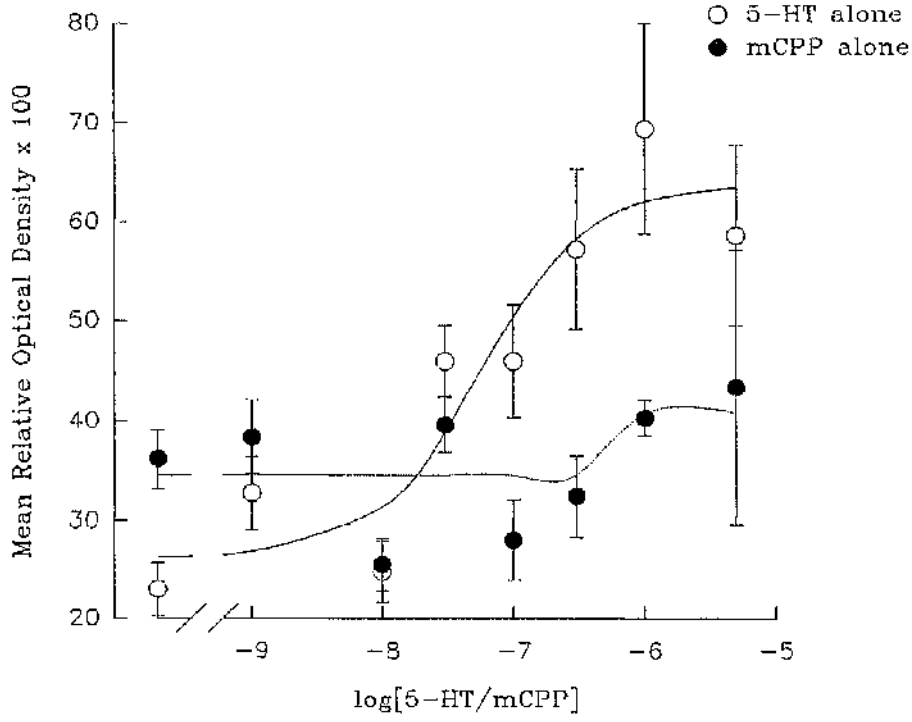
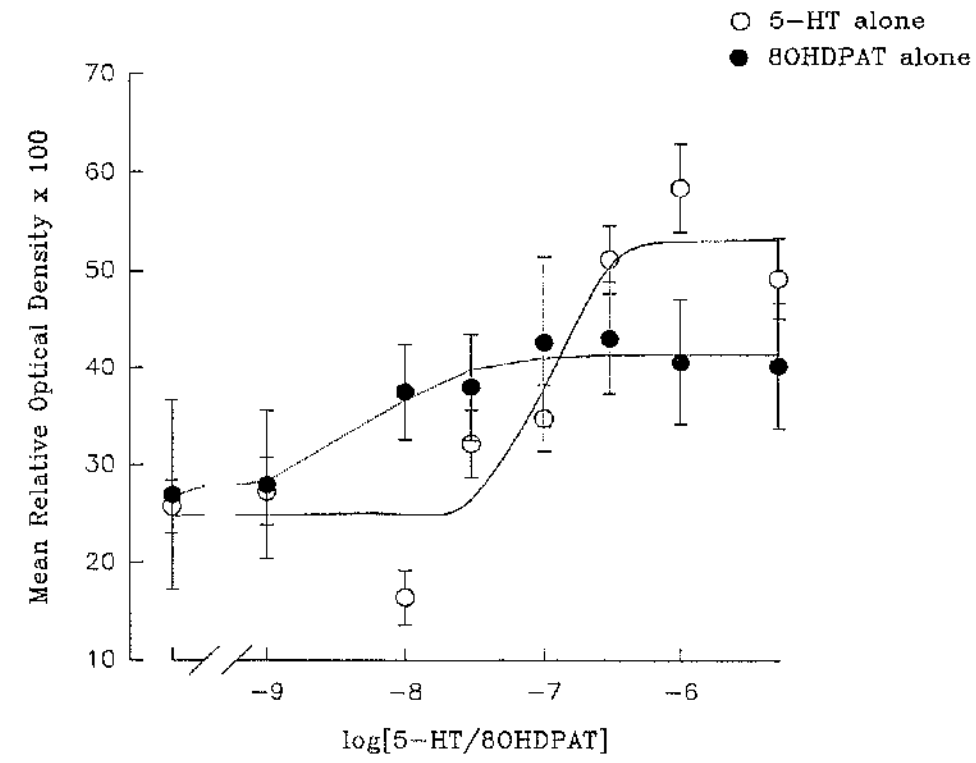

5-HT alone
mCPP alone
Mean Relative Optical Density x 100
80
70
60
50
40
30
20
-9
-8
-7
-6
-5
log[5-HT/mCPP]

Figure 8.1.15 : Effect of a 5-HT1A receptor stimulation on the level of expression of zif/268 mRNA in rat striatal neuron cultures. Cultures were treated for 45 minutes with vehicle (HBSS), 8OHDPAT (1nM-5μM, n= 4) alone or with 5-HT (1nM-5μM, n= 8) alone for comparison. Results, obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. No significant induction of zif/268 mRNA was observed.

Figure 8.1.16 : Effect of a 5-HT1A receptor stimulation on the level of expression of c-fos mRNA in rat striatal neuron cultures. Cultures were treated for 45 minutes with vehicle (HBSS), 8OHDPAT (1nM-5μM, n= 4) alone or with 5-HT (1nM-5μM, n= 7) alone for comparison. Results, obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. No significant induction of c-fos mRNA was observed.


5-HT alone
8OHDPAT alone
Mean Relative Optical Density x 100
70
60
50
40
30
20
10
-9
-8
-7
-6
log[5-HT/8OHDPAT]

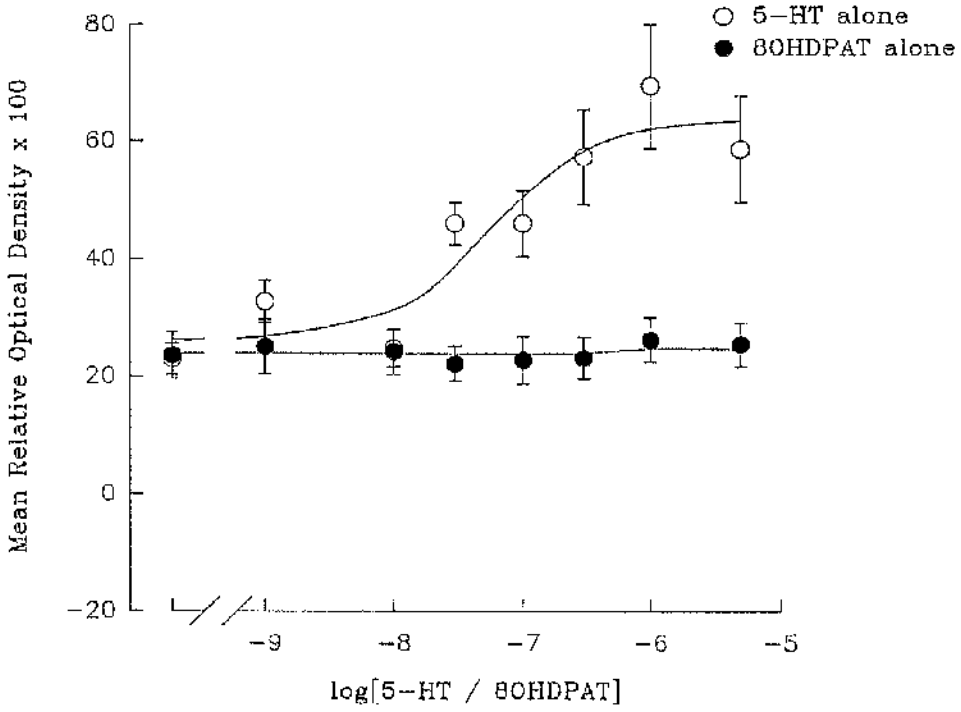
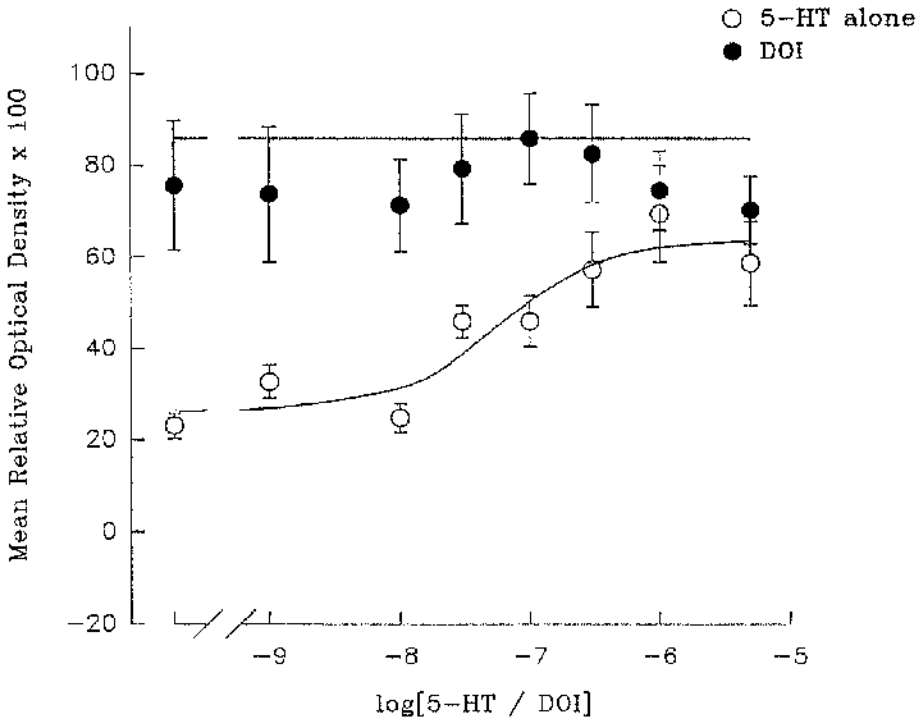

5-HT alone
8OHDPAT alone
Mean Relative Optical Density x 100
80
60
40
20
0
-20
-9
-8
-7
-6
-5
log[5-HT / 8OHDPAT]

Figure 8.1.17 : Effect of a 5-HT2A/ 2C receptor stimulation on the level of expression of zif/268 mRNA in rat striatal neuron cultures. Cultures were treated for 45 minutes with vehicle (HBSS), ± DOI (1nM-5μM, n= 3) alone or with 5-HT (1nM-5μM, n= 8) alone for comparison. Results, obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. No significant induction of zif/268 mRNA was observed.

Figure 8.1.18 : Effect of a 5-HT2A/ 2C receptor stimulation on the level of expression of c-fos mRNA in rat striatal neuron cultures. Cultures were treated for 45 minutes with vehicle (HBSS), ± DOI (1nM-5μM, n= 3) alone or with 5-HT (1nM-5μM, n= 7) alone for comparison. Results, obtained from film autoradiographs, are expressed as mean relative optical density x 10^2 ± s.e.m. No significant induction of c-fos mRNA was observed.


5-HT alone
DOI
Mean Relative Optical Density x 100
100
80
60
40
20
0
-20
-9
-8
-7
-6
-5
log[5-HT / DOI]

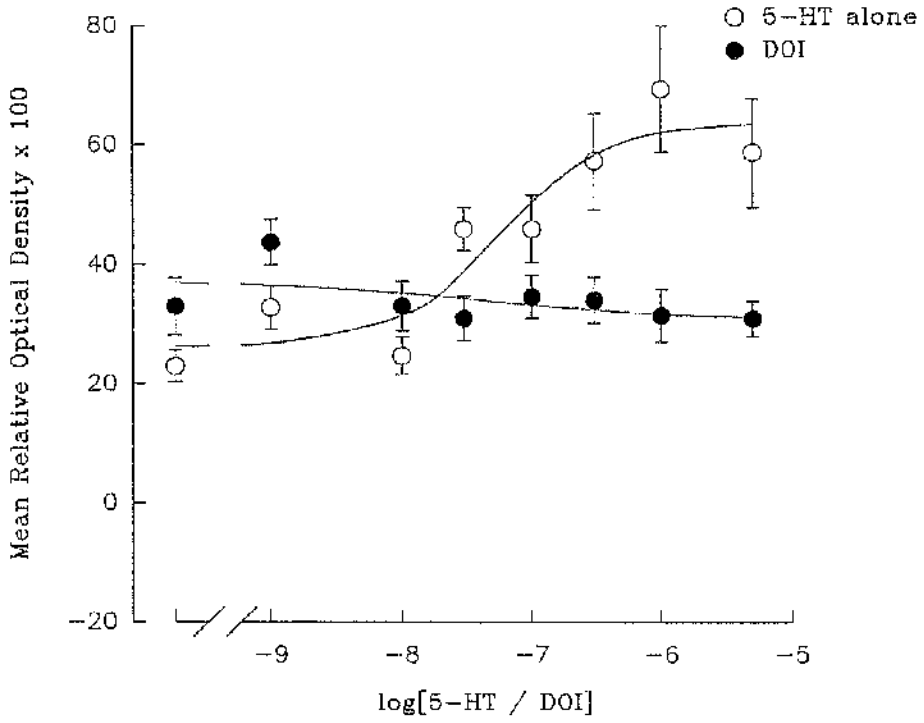
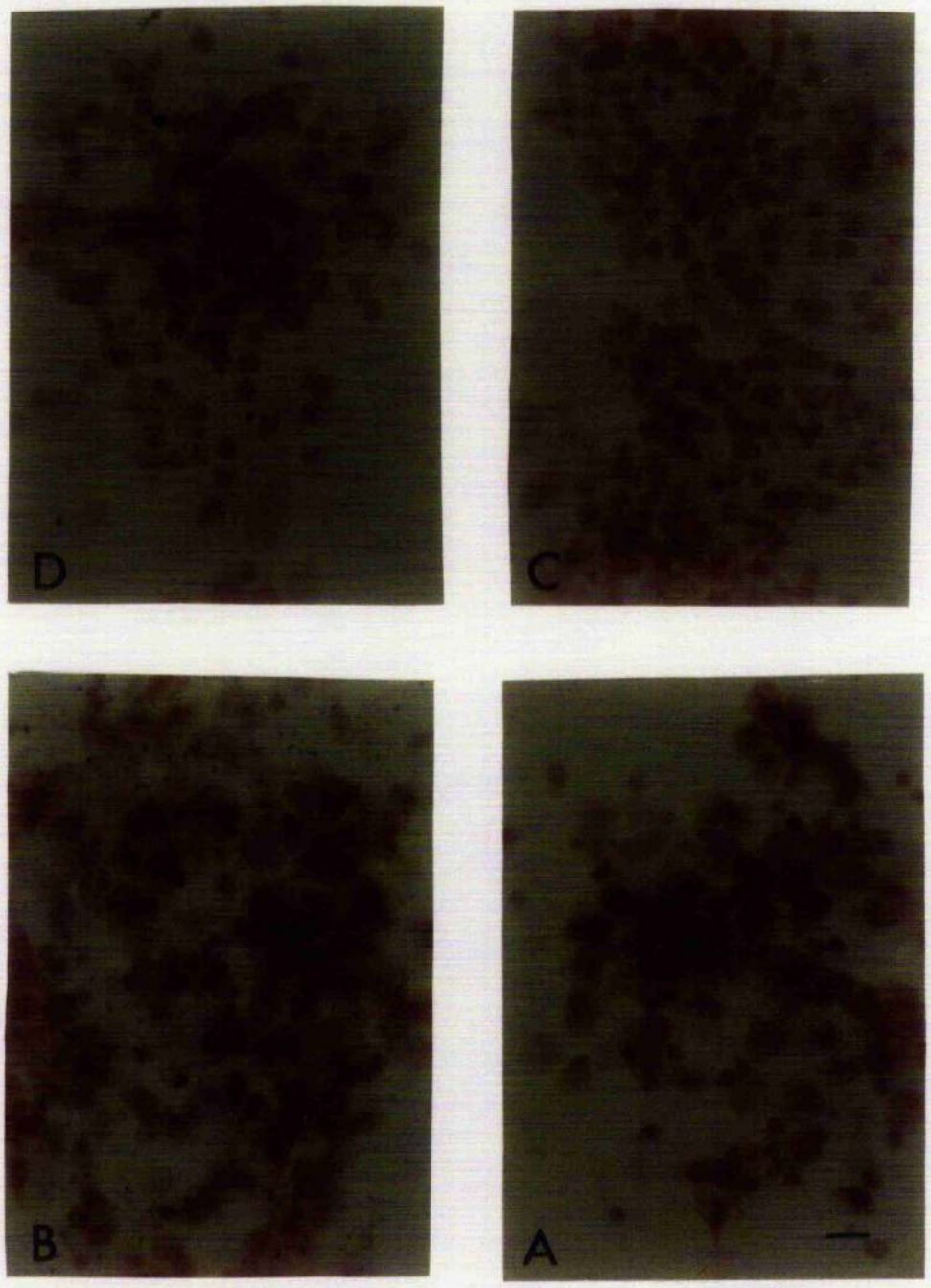

5-HT alone
DOI
Mean Relative Optical Density x 100
80
60
40
20
0
-20
-9
-8
-7
-6
-5
log[5-HT / DOI]

Figure 8.1.19 : Effect of serotoninergic antagonists on the level of expression of zif/268 mRNA in rat striatal neuron cultures. Photomicrographs show neuronal cultures treated with (A) vehicle, (B) 5-HT (1μM), (C) cyproheptadine (500nM)/ 5-HT (1μM) and (D) spiperone (30nM)/ 5-HT (1μM). An induction of zif/268 can be seen as an increase in the number of silver grains over the cell bodies. Scale bar represents 25μm.


D
C
B
A

DISCUSSION

8.2 Regulation of immediate early genes by serotonin in vitro

It has been known for some time that serotonin may play an important role in the manifestation of certain pathological disorders, including pain, anxiety, schizophrenia and parkinsonism. With recent evidence suggesting that serotonin may be involved in the modulation of immediate early gene expression both in vivo and in vitro, this study was undertaken in order to examine the pattern of immediate early gene expression in striatal neurons grown in culture, and to elucidate a possible receptor mechanism(s) involved.

The results show for the first time that serotonin can induce the expression of both c-fos and zif/268 mRNA in rat primary striatal culture when administered alone. This inductive effect of serotonin on c-fos and zif/268 gene expression proved to be dose-dependent. The EC_{50} value for both c-fos and zif/268 was very similar, and in the range of 100nM. This value is similar to an EC_{50} value of 125 nM, recently reported for the 5-HT stimulation of cyclic AMP production, in striatal neurons in culture (Sebben et al., 1994). Previous research indicates a balance between the dopaminergic and serotoninergic systems, and that the central serotoninergic system exerts an inhibitory influence on the central dopaminergic system. In particular this has been evident when looking at behavioural effects in rats. Serotonin agonists have been shown to potentiate the cataleptic effect of haloperidol in a dose dependent manner in rats, and pretreatment with serotonin antagonists was shown to reduce this effect (Balsara et al., 1979). It appears that the effect of neuroleptics apparently depends on the balance between the two systems (Kostowski et al., 1972; Balsara et al., 1979). Recent research has suggested that serotonin may act synergistically in the striatum with the neurotransmitter, dopamine, in order to activate transcription factor genes, including c-fos and

zif/268 in vivo (Bhat and Baraban, 1993). Our results demonstrate that serotonin may also have a solitary role in activating these transcription factor genes.

A variety of pharmacological agents known to be active at various subtypes of 5-HT receptors were used to characterize the subtype of receptor responsible for this stimulation of c-fos and zif/268 mRNA by serotonin. It was thought to be unlikely that this response was mediated by a 5-HT3 subtype of receptor, because pretreatment with the selective 5-HT3 receptor antagonist, ondansetron, had no effect on the expression of zif/268 mRNA and little effect on c-fos mRNA levels after 5-HT stimulation. Receptors of the 5-HT1 subclass generally couple to inhibition of adenylyl cyclase, although there are reports of the 5-HT1A receptor coupling to stimulation of this second messenger (Shenker et al., 1987). The inactivity of 8-hydroxy-DPAT, a potent 5-HT1A agonist (Gozlan et al., 1983; Dourish et al., 1987), indicates that the 5-HT1A receptor is not responsible for the stimulation of zif/268 or c-fos mRNA. The involvement of a 5-HT1B or 5-HT1D receptor was eliminated by the finding that the drugs spiperone, mesulergine and cyproheptadine, all having very low affinity for these receptor subtypes (Peroutka, 1986; Van Wijngaarden et al., 1990), were clearly effective antagonists of the inductive response of c-fos and zif/268 mRNA, to serotonin stimulation. The lack of agonist activity for mCPP at high doses, also suggests that the response is not mediated by the 5-HT1B receptor subtype (Hoyer and Schoeffter, 1991). A 5-HT1E-like receptor is known to be highly expressed in the rat striatum, although the primary structure of this receptor suggests it may be coupled to an inhibition of adenylate cyclase through an inhibitory G_i-like protein (Lovenberg et al., 1992). It is unlikely that the induction of zif/268 and c-fos is due to stimulation of a receptor negatively coupled to adenylate cyclase. This, together with the reported low affinity of mesulergine at this receptor subtype makes it an unlikely candidate. The 5-HT4 subtype was also eliminated as a possible

candidate, because the drugs spiperone and mesulergine have been reported to be inactive at this receptor (Schiavi et al., 1993). The 5-HT5A and 5-HT5B receptors can also be eliminated, because of their moderate affinity for methiothepin and the knowledge that these receptors are unlikely to be coupled to either adenylate cyclase or phospholipase C (Matthes et al., 1992). Similarly stimulation of the newly cloned 5-HT6 receptor is unlikely to be reponsible for the inductive effect on c-fos and zif/268 gene expression, because spiperone is known to have a very low affinity at this receptor site (Roth et al., 1993), which does not correspond to its action as a highly potent antagonist seen in our experiments. However, the affinity of spiperone at the 5-HT7 receptor is much higher than at the 5-HT6 receptor subtype. The 5-HT7 or 5-HTx receptor as it is also known, is expressed in the forebrain region in rat brain (Plassat et al., 1993) and has a high affinity for atypical antipsychotic drugs such as clozapine (Roth et al., 1993). This receptor subtype can not be ruled out totally, in view of the current evidence.

The presence of a 5-HT-stimulated increase in zif/268 and c-fos mRNA initially suggested the involvement of a 5-HT2-like receptor. It is known that this receptor subtype is coupled to phospholipase C and the consequent cascade leading to diacylglycerol and inositol triphosphate production (Conn and Sanders-Bush, 1986; Conn et al., 1986). In previous results we have suggested that both c-fos and zif/268 stimulation may be linked to protein kinase C, an enzyme responsible for the phosphorylation of cellular proteins. This further suggests that the response to 5-HT may involve a 5-HT2-like receptor subtype. However, the 5-HT2C (formerly known as 5-HT1C) subtype is unlikely to be responsible for mediating the induction of c-fos and zif/268 mRNA in vitro, due to the low affinity of spiperone at this subtype of 5-HT2 receptor (Hoyer et al., 1985).The lack of an agonist effect of mCPP and ±-DOI also suggests that the 5-HT2C receptor is not involved in mediating this effect. Similarly the 5-HT2B receptor (formerly known as 5-HT2F) is most unlikely to

be mediating the induction of c-fos and zif/268 mRNA in culture. This is due to the low affinity of spiperone for this receptor subtype (Wainscott et al., 1992), together with the low abundance of this receptor subtype in the brain (Pompeiano et al., 1993). The 5-HT2A subtype is possibly the most feasible candidate, however there are some discrepancies which do not coincide with the pharmacologically described 5-HT2A receptor. The 5-HT2A has high affinity for spiperone, mesulergine and cyproheptadine (Zifa and Fillion, 1992), which is in agreement with our results describing potent antagonism of the 5-HT-stimulated increase in c-fos and zif/268 mRNA with these three drugs. The 5-HT2A subtype has also been shown to be expressed at moderate levels in the caudate-putamen in the adult rat (Pompeiano et al., 1993). In humans, 5-HT2 binding sites have been predominantly located in striosomes in the basal ganglia (Waeber and Palacios, 1994). However, we found that the selective agonist at 5-HT2A and 5-HT2C receptor subtypes, ±-DOI, was unable to stimulate the induction of zif/268 or c-fos mRNA in striatal culture. In previous reports ±-DOI has been suggested to be a mixed agonist-antagonist (Dabire et al., 1989) and also be able to inhibit 5-HT-induced neuronal activity in the dorsal raphe, an effect not blocked by classical 5-HT2 receptor antagonists (Garratt et al., 1991). This suggests that there are properties of ±-DOI aside from the known agonist activity at 5-HT2 receptors, that as yet, have not been fully investigated.

These results indicate that the serotonin receptor responsible for the stimulation of both c-fos and zif/268 mRNA in rat striatal culture may be a 5-HT2A-like receptor. However, there are still open avenues of investigation concerning the 5-HT7 receptor, which has recently been reported to have a high affinity for antipsychotic drugs such as clozapine. As yet, we have been unable to obtain a result with clozapine in this experimental model.

Ultimately the physiological significance of our results is suggestive that serotonin can modulate cellular gene expression which in turn may affect

behavioural responses. Accumulated experimental evidence implies that the immediate early genes are 'third messengers' in signal transduction pathways (Goelet et al., 1986; Curran and Morgan, 1987). In the central nervous system, certain late response genes have been identified as targets for transactivation based on the presence of the appropriate regulatory sequences in their promoters (Comb et al., 1988; Sonnenberg et al., 1989; Naranjo et al., 1991).

It is well characterised that neuroleptics, the major treatment for diseases such as schizophrenia, can cause the induction of a number of immediate early genes in the brain, in addition to affecting the levels of many late response genes. Therefore a functional link has been implied between the induction of immediate early genes and the consequent increase or decrease in late response genes such as enkephalin (Sonnenberg et al., 1989). In view of the affinity of many neuroleptic drugs for 5-HT receptors, the current awareness is suggesting that neuroleptics may be affecting the serotoninergic sytem as easily as the dopaminergic system, and in fact the atypical drug clozapine has a particularly high affinity for some serotonin receptor subtypes. The properties of clozapine and similar atypical compounds result in fewer extrapyramidal side effects when given clinically, and it has been postulated that clozapine's actions on the serotoninergic system may be an important contributing factor to its atypical drug profile.

General Discussion

As a whole, this work has not only uncovered some novel findings, but demonstrated the complexity of the functioning of the basal ganglia. In addition to demonstrating an induction of c-fos and zif/268 gene expression in the rat striatal model in vitro following a dopaminergic D1/D5 receptor stimulation, the expression of these immediate early genes and others, was also increased by dopaminergic D2-like antagonists in vivo. Dopamine D1-like-induced

expression of c-fos in the caudate in vivo has been shown to occur only after a 6-hydroxy-dopamine-induced lesion (Robertson et al., 1989; 1990), although the D1/D5 receptor antagonist SCH23390 has been demonstrated to reduce the mRNA levels of the transcription factor zif/268 in the adult rat intact striatum (Mailleux et al., 1992). A possible expla nation for the D2-like mediated mRNA induction of these immediate early genes, may be that blockade of D2-like receptors results in an unopposed D1 stimulation.

We have also demonstrated that a further complexity may exist concerning the second messenger pathway prior to immediate early gene induction after a D1-like receptor stimulation. The results shown provide the first evidence that both PKA and PKC participate in the regulation of c-fos and zif/268 gene expression by dopamine in striatal neurones. Further work is clearly required to discover the functional significance of these control mechanisms.

The serotoninergic regulation of c-fos and zif/268 gene expression in the striatal model in vitro is the first evidence to suggest that serotonin alone can induce these immediate early genes. In addition, we have attempted to reveal a possible receptor mechanism for this induction. Our results are suggestive that the induction may be mediated by a 5-HT2A-like receptor, although conclusive evidence is hampered by the few selective drugs available for serotonin receptor subtypes. Perhaps an improved receptor antisense technology may provide the answer in the future.

We have also shown that the recently cloned transcription factor gene BF-1, present in the striatum, can be regulated by the atypical neuroleptic, clozapine, in vivo. This is a novel finding, which may be functionally important considering clozapine has a unique drug profile that provides clinical advantages in schizophrenia.

An important consideration when attempting to explain many of the findings in this research work, is the possiblity of interaction occurring between different neuronal systems. The therapeutic efficacy of atypical antipsychotic agents such as clozapine may be attributed to the ability of the drug to act on different transmitter/receptor systems in vivo. From this, it may be suggested that drug efficacy may rely on a complex compensatory mechanism between neuronal systems. Indeed, one explanation for the selective behavioural and physiological effects of atypical agents on the function of the A10 dopamine system is an interaction with other neuronal systems which may compensate for dopamine receptor antagonism in the nigrostriatal system. Since clozapine is known to have a high affinity for a number of 5-HT receptor subtypes in vitro, of which several are known to be expressed in the caudate-putamen, tonic 5-HT activity may be crucial for the resultant physiological effects of this drug. Wilmot and Szczepanik (1989), have previously demonstrated that both acute and chronic intraperitoneal administration of clozapine, at relatively high doses, can decrease the maximal number of cortical 5-HT2 receptors by greater than 50% of control, whereas typical drugs such as haloperidol, had no effect. The functional significance of 5-HT2 receptor down-regulation following chronic treatment with an atypical antipsychotic remains to be determined.

The existence of a relationship between the differential effects of clozapine and haloperidol on subcortical D2 dopamine receptors and cortical or subcortical 5-HT2 serotonin receptors is not presently known. However, the cortical control of striatal function is regionally specific. The lateral striatum, which shows the greatest changes in D2 receptors following chronic treatment with haloperidol, receives afferents from the somatosensory frontoparietal cortex. It has been suggested that clozapine may indirectly affect the development of striatal D2 receptor up-regulation via its effects on cortical somatosensory neurons. Another explanation is that clozapine, as a 5-HT2 antagonist, directly affects raphe neurons which then influence dopamine neuronal function (Wilmot and Szczepanik, 1989). It has previously been

shown that acute treatment with 5-HT2 antagonists can antagonize the haloperidol-induced increase in levels of striatal dihydroxyphenylacetic acid (DOPAC) and haloperidol-induced catalepsy (Balsara et al., 1979). Lesions of the dorsal raphe are also known to prevent haloperidol-induced catalepsy (Kostowski et al., 1972). Therefore it would seem that there is a definite interaction between the dopamine and serotonin transmitter systems in the central nervous system.

Regulation of tachykinin biosynthesis by serotonin has recently been shown in the neostriatum (Walker et al., 1991). Depletion of 5-HT with p-chlorophenylalanine treatment decreased preprotachykinin (the prohormone precursor to substance P) mRNA levels in the neostriatum. By contrast, raising extracellular levels of 5-HT with zimelidine, a 5-HT uptake inhibitor, or clorgyline, a monoamine oxidase inhibitor resulted in increased levels pf preprotachykinin mRNA. Therefore it may be suggested that certain peptides in the striatum may be regulated by serotonin neurotransmission. Typical neuroleptics such as haloperidol given acutely or chronically, have been shown to decrease the levels of preprotachykinin (PPT) mRNA in the striatum (Bannon et al., 1986). The proposed mechanism for this biochemical change is a decrease in dopamine neurotransmission. In addition to the effect on the dopamine system, haloperidol may also be affecting the serotonin system. Haloperidol has been shown to have an affinity at a number of serotonin receptor subtypes, including the 5-HT2 receptor (Canton et al., 1993), therefore the decrease in PPT mRNA seen after haloperidol may be due to a decrease in serotonin neurotransmission in addition to a decrease in dopamine neurotransmission. Tonic serotonin levels may therefore have a regulatory role in controlling the expression of neuropeptides in the basal ganglia. The increase in the immediate early genes c-fos and zif/268 which we observed after treatment with 5-HT could also be blocked by 5-HT2-like receptor antagonist compounds. As yet there is no clear evidence for a functional role of immediate early genes in the brain, although it has been suggested that they may play a part in the regulation of gene expression. Similarly glutamate must also be considered to be a possible interacting component in the

basal ganglia. A glutamatergic projection from the cortex to the striatum suggests that tonic glutamate may also have a homeostatic function in controlling the level of neurotransmitters /peptides in the striatum.

This research has also demonstrated an induction of proenkephalin mRNA after acute typical neuroleptic drug treatment. This is in agreement with previous studies that have shown an induction of proenkephalin mRNA and enkephalin protein in the rat striatum (Hong et al., 1978; Sabol et al., 1983; Tang et al., 1983; Angulo et al., 1986; Sivam et al., 1986; Romano et al., 1987; Morris et al., 1988). We suggest that the increase of proenkephalin mRNA after 24 hours may infact be related to the induction of a number of immediate early genes prior to this time point. This would be in agreement with the idea that immediate early genes act as "third messengers" in the sequence of events leading to changes in gene expression. However, this is only indirect evidence, and therefore a relationship between immediate early genes and "late response genes", such as enkephalin, is only circumstantial.

Tonic dopamine is thought to have an inhibitory control on enkephalin gene expression. Enkephalin peptide is thought to be located predominantly in striatopallidal neurons (Gerfen and Young, 1988), and striatopallidal neurons have been shown to be a distinct population of neurons expressing a relatively high proportion of D2-like receptors (Le Moine et al., 1990). Blockade of D2-like dopamine receptors with typical neuroleptic drugs such as haloperidol cause an increase in proenkephalin mRNA, as the tonic inhibiton is removed. Lesioning the nigrostriatal system, which effectively removes the tonic dopamine acting predominantly through D2-like receptors to maintain an inhibitory control on enkephalin gene expression, also causes an induction of enkephalin mRNA. Therefore it would seem that the D2-mediated inhibitory effect of tonic dopamine on the level of enkephalin gene expression predominates in the 'normal' brain.

Despite the evidence previously described suggesting that D1-like and D2-like dopamine receptors are expressed on different populations of cells in vivo

(Gerfen et al., 1990; Le Moine et al., 1990), our results indicate that both D1-like and D2-like receptors are expressed on the same cell in vitro. Administration of the D2 agonist quinpirole has been shown to antagonize the SKF38393-induced expression of both c-fos and zif/268 mRNA. If it is assumed that our model of striatal neurons in culture closely resembles the phenotype of striatal neurons in vivo, then this suggests that a population of neurons in vivo may express both D1-like and D2-like dopamine receptors. There is already a large body of evidence in agreement with this idea (Seeman et al., 1989; Bertorello et al., 1990; Piomelli et al., 1991; Surmeier et al., 1993). Dopaminergic D1-like receptors, when stimulated, appear to be in opposition to dopaminergic D2-like receptors in the striatum. However, it must be remembered that the population size which possess both D1-like and D2-like dopamine receptor subtypes is unknown. It is possible that a relatively small population of neurons possess both phenotypes. Perhaps the proportion of D1:D2 phenotype on neuronal populations within the basal ganglia differ such that one phenotype predominates over the other in vivo. The tonic activity of dopamine on different neuronal populations within the caudate may therefore have localized effects in terms of gene regulation, dependent on the predominant phenotype of dopamine receptor. Several serotonin receptor subtypes are known to be present in the striatum. The physiological significance of these receptors has yet to be determined. Similar to the dopaminergic system, it is not unlikely that these receptor subtypes may be localised to particular neuronal cell populations and in turn regulate gene expression.

The relatively new concept of 'antisense strategy' to block the synthesis of specific proteins, has been employed in this research. The phosphorothioate antisense oligonucleotide directed towards the D1 dopamine receptor clearly reduced c-fos mRNA expression in striatal neurons after treatment with a range of doses of the D1-like receptor agonist SKF38393. The effect on c-fos mRNA expression after treatment with the D5 antisense oligonucleotide was less robust than that observed after treatment with the D1 antisense, although a significant

decline in mRNA levels were still observed. We have suggested that the physiological effect of c-fos induction following SKF38393, is predominantly mediated through stimulation of the D1 dopamine receptor subtype. However, as the D5 antisense was also effective, it may not be dismissed that the induction of c-fos mRNA in striatal neurons may also by mediated, but to a lesser extent, by the D5 dopamine receptor subtype. We have proposed that the D5 subtype may represent the 'fine control' over the dopaminergic system, due to only a small percentage of D5 receptors being expressed in the striatum, and dopamine, the endogenous ligand in vivo, having a higher affinity for the D5 dopamine receptor subtype than for D1 subtype.

At present there are a number of disadvantages with this method of protein 'knockdown'. Clearly, from the data on zif/268 mRNA expression after antisense use, non-specific effects can be a major problem. A number of separate controls, including the use of random sequence oligonucleotides, 'mismatch' oligonucleotides, radioligand binding studies and toxicity testing, should be performed in addition to the 'sense' control oligonucleotide that was used in our study in an attempt to clarify these results.

This research as a whole, has provided an important insight into the changes in gene expression in the basal ganglia. We have investigated the transient changes in immediate early gene expression, together with the more sustained changes in peptide gene expression in the striatum, in response to neuroleptic drug treatments, the clinical therapy for many neurodegenerative diseases. We have uncovered a complex cascade of events prior to the change in expression of the immediate early genes c-fos and zif/268 in the striatum after a dopaminergic stimulus. In addition we have also investigated another major transmitter system in the striatum, namely the serotoninergic system, and attempted to reveal the receptor(s) involved in the induction of the immediate early genes c-fos and zif/268 after serotonin treatment.

The complexity of the basal ganglia has undoubtedly left several questions unanswered until a future date.

REFERENCES

Aceves, J., Floran, B., Martinez-Fong, D., Sierra, A., Hernandez, S., & Mariscal, S.(1991) L-Dopa stimulates the release of [3H] gamma-aminobutyric acid in the basal ganglia of 6-hydroxydopamine lesioned rats. Neurosci. Letts. **121(1-2),** 223-226.

Adler, G.K., Smas, C.M. & Majzoub, J.A. (1988) Expression and dexamethasone regulation of the human corticotrophin-releasing hormone gene in a mouse anterior pituitary cell line. J. Biol. Chem. **263**, 5846-5852.

Agid, Y. & Javoy-Agid, F. (1985) Peptides and Parkinson's disease. Trends in Neurosci. 7, 30-35.

Ahlenius, S. (1989) Antipsychotic-like properties of the 5-HT1A agonist 8-OH-DPAT in the rat. Pharmacol. Toxicol. **64**, 3-5.

Akabayashi, A., Wahlestedt, C., Alexander, J.T. & Leibowitz, S.F. (1994) Specific inhibition of endogenous neuropeptide Y synthesis in arcuate nucleus by antisense oligonucleotides suppresses feeding behaviour and insulin secretion. Brain Res.- Mol. Brain Res. **21(1-2),** 55-61.

Ambrosini, A. & Meldolesi, J. (1989) Muscarinic and quisqualate receptor-induced phosphoinositide hydrolysis in primary cultures of striatal and hippocampal neurons. Evidence for differential mechanisms of activation. J. Neurochem. **53(3),** 825-832.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P., & Karrin, M. (1987) Phorbol ester inducible genes containing a common cis element recognised by a TPA-modulated trans-acting factor. Cell **49**, 729-739.

Angulo J.S., Davis L.G., Burkhart B.A., & Christoph G.R. (1986) Reduction of striatal dopaminergic neurotransmission elevates striatal proenkephalin mRNA. Eur. J. Pharmacol. **130(3),** 341-343.

Angulo, J.R. (1992) Involvment of dopamine D1 and D2 receptors in the regulation of proenkephalin mRNA abundance in the striatum and accumbens of rat brain. J. Neurochem. **58(3),** 1104-1109.

Ariano, M.A., Fisher, R.S., Smyk-Randall, E., Sibley, D.R., & Levine, M.S. (1993) D2 dopamine receptor distribution in the rodent CNS using antipeptide antisera. Brain Res. **609(1-2),** 71-80.

Ariano, M.A., Stromski, C.J., Smyk-Randall, E.M., & Sibley, D.R. (1992) D2 dopamine receptor localisation on striatonigral neurons. Neurosci. Letts. **44(1-2),** 215-220.

Arias-Montano, J.A., Martinez-Fong, D. & Aceves, J. (1992) Glutamate stimulation of tyrosine hydroxylase is mediated by NMDA receptors in the rat striatum. Brain Res. **569(2),** 317-322.

Arnauld, E., Arsaut, J. & Demotes-Mainard, J. (1993) Functional heterogeneity of the caudate-putamen as revealed by c-fos induction in response to D1 receptor activation. Mol. Brain Res. **18,** 339-342.

Ashby, C.R., Edwards, E., Harkins, K., & Wang, R.Y. (1989) Characterisation of 5-hydroxytryptamine3 receptors in the medial prefrontal cortex: a microiontophoretic study. Eur. J. Pharmacol. **173(2-3),** 193-196.

Audinot V., Canton H., Jaques V., Verriele L., and Millan M.J.(1993). Receptor profiles of atypical Antipsychotics at cloned, human dopamine D2 and D3 receptors and at rat D2 and 5-HT1C/2 receptors in vitro/ex vivo.(1993). Society of Neuroscience Abstracts **19,** 1169.

Augood, S.J., Kiyama, H., Faull, R.L.M. & Emson, P.C. (1991) Differential effects of acute dopaminergic D1 and D2 receptor antagonists on

proneurotensin mRNA expression in rat striatum. Mol. Brain Res. **9**, 341-346.

Ayd F.J.,(1961). A survey of drug induced extrapyramidal reactions. JAMA **175,** 1054-1060.

Baldessarini, R.J. (1979) The "neurologic" antipsychotic drugs. 2. Neurologic side effects. Postgrad. Medicine **65(4),** 123-128.

Baldessarini, R.J. (1985) Drugs and treatment of psychiatric disorders. In The Pharmacological Basis of Therapeutics, ed. by A.S. Gilman, L.S. Goodman, T.W. Rall & F. Mural. Macmillan, New York 387-445.

Balsara, J.J., Jadhav, J.H., & Chandorkar, A.G. (1979) Effects of drugs influencing central serotoninergic mechanisms on haloperidol-induced catalepsy. Psychopharmacol.- Berl. **62(1),** 67-69.

Bannon M.J., Lee J.M., Giraud P., Young A., Affolter H.U. & Bonner T.I.(1986). The dopamine antagionist haloperidol decreases substance P, substance K and preprotachykinin mRNA's in striatonigral neurones. J. Biol.Chem. **261,** 6640-6644.

Bannon, M.J., Elliot, P.J., & Bunney, E.B. (1987) Striatal tachykinin biosynthesis: Regulation of mRNA and peptide levels by dopamine agonists and antagonists. Mol. Brain Res. **3**, 31-37.

Bartel, D.P., Sheng, M., Lau, L.F., & Greenberg, M.E. (1989) Growth factors and membrane depolarisation activate distinct programs of early response gene expression: dissociation of fos and jun induction. Genes. Dev. **3(3),** 304-313.

Batter, D.K. & Kessler, J.A. (1991) Region-specific regulation of proenkephalin mRNA in cultured astrocytes. Mol. Brain Res. **11**, 65-69.

Batty, I.R., Nahorski, S.R. & Irvine, R.F. (1985) Rapid formation of inositol 1,3,4,5-tetrakisphosphate following muscarinic receptor stimulation of rat cerebral cortical slices. J. Biochem. **232(1),** 211-215.

Beal, M.F. & Martin, J.B. (1984) Effects of neuroleptic drugs on brain somatostatin-like immunoreactivity. Neurosci. Letts. **47(2),** 125-130.

Beavo, J.A., Bechtel, P.J., & Krebs, E.G. (1975) Mechanisms of control for cAMP-dependent protein kinase from skeletal muscle. Adv. Cyclic Nucleotide Res. **5,** 241-251.

Becker, D., Meier, C.B., & Herlyn, M. (1989) Proliferation of human malignant melanomas is inhibited by antisense oligodeoxynucleotides targeted against basic fibroblast growth factor. The EMBO J. **8(12),** 3685-3691.

Beretta, S., Robertson, H.A. & Graybiel, A.M. (1992) Dopamine and glutamate agonists stimulate neuron-specific expression of fos-like protein in striatum. J. Neurophysiol. **68**, 767-777.

Berg, K.A., Clarke, W.P., Chen, Y., Ebersole, B.J., McKay, R.D.G., & Maayani, S. (1994) 5-hydroxytryptamine 2A receptors regulate cyclic AMP accumulation in a neuronal cell line by protein kinase C dependent and calcium/ calmodulin-dependent mechanisms. Mol. Pharmacol. **45**, 826-836.

Berridge, M.J. (1987) Inositol triphosphate and diacylglycerol: two interacting second messengers. Annal. Rev. Biochem. **56**, 159-193.

Berridge, M.J., Downes, C.P. & Hanley, M.R. (1982) Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. J. Biochem. **206(3)**, 587-595.

Bersani, G., Grispini, A., Marini, S., Pasini, A., Valducci, M. & Ciani, N. (1990) 5-HT2 antagonist ritanserin in neuroleptic-induced Parkinsonism: a double blind with orphenadrine and placebo. Clin. Neuropsychopharmacol. **13**, 500-506.

Bertorello, A.M., Hopfield, J.F., Aperia, A., & Greengard, P. (1990) Inhibition by dopamine of $(Na^+ + K^+)$ ATPase activity in neostriatal neurons

through D1 and D2 dopamine receptor synergism. Nature **347**, 386-388.

Besson, M.J., Graybiel, A.M. & Quinn, B. (1990) Co-expression of neuropeptides in the cats striatum: an immunohistochemical study of substance P, dynorphin B and enkephalin. Neuroscience **39**, 33-58.

Bhat, R.V. & Baraban, J.M. (1993) Activation of transcription factor genes in the striatum by cocaine: role of both serotonin and dopamine systems. J. Pharmacol. and Exp. Ther. **267(1),** 496-501.

Bhat, R.V., Cole, A.J., Worley, P.F. & Baraban, J.M. (1992) Activation of the zinc finger encoding gene Krox-20 in adult rat brain: Comparison with zif/268. Mol. Brain Res. **13**, 263-266.

Bielenberg, G.W. & Burkhardt, M. (1990) 5-hydroxytryptamine1A agonists: A new therapeutic principle for stroke treatment. Stroke **21(IV),** 161-163.

Bischoff, S., Heinrich, M., Krauss, J., Sills, M.A., Williams, M. & Vassout, A. (1988) Interaction of the D1 receptor antagonist SCH23390 with the central 5-HT system: Radioligand binding studies, measurements of biochemical parameters and effects on L-5-HTP syndrome. J. Receptor Res. **8(1-4)**, 107-120.

Blackshear, M.A., Freidman, R.L., & Sanders-Bush, E. (1983) Acute and chronic effects of serotonin antagonists on serotonin binding sites. Naunyn-Schmiedebergs Arch. Pharmacol. **324**, 125-129.

Blandina, P., Goldfarb, J. & Green, J.P. (1988) Activation of a 5-HT3 receptor releases dopamine from rat striatal slice. Eur. J.Pharmacol. **155(3),** 349-350.

Bloom, F.E., Costa, E. & Salmoiraghi, G.C. (1965) Anaesthesia and the responsiveness of individual neurons of the caudate nucleus of the cat to acetylcholine, norepinephrine and dopamine administered by microelectrophoresis. J. Pharmacol. and Exp. Ther. **150**, 244-252.

Blumberg, P.M., Butler-Gralla, E., & Herschman, H.R. (1981) Analysis of phorbol ester receptors in phorbol ester unresponsive 3T3 cell variants. Biochem.Biophys. Res. Commun. **102(3)**, 818-823.

Blunt, S.B., Jenner, P. & Marsden, C.D. (1992) Autoradiographic study of striatal D1 and D2 dopamine receptors in 6-OHDA-lesioned rats receiving foetal ventral mesencephalic grafts and chronic treatment with L-DOPA and carbidopa. Brain Res. **582(2),** 299-311.

Bockaert, J., Sebben, M. & Dumuis, A. (1989) Pharmacological characterisation of 5-hydroxytryptamine (5-HT4) receptors positively coupled to adenylate cyclase in adult guinea pig hippocampal membranes: effect of substituted benzamide derivatives. Mol. Pharmacol. **37**, 408-411.

Bockaert, J., Sebben, M., and Dumuis, A. (1990) Pharmacological characterisation of 5-hydroxytryptamine4 (5-HT4) receptors positively coupled to adenylate cyclase in adult guinea pig hippocampal membranes: Effect of substitiuted benzamide derivatives. Mol. Pharmacol. **37**, 408-411.

Boddeke, H.W.G.M., Hoffman, B.J., Palacios, J.M., Knot, J.M., & Hoyer, D. (1993) Characterisation of functional responses in A9 cells transfected with cloned rat 5-HT1C receptors. Naunyn-Schmiedebergs Arch. Pharmacol. **347**, 119-124.

Bode-Greuel, K.M., Klisch, J., Horvath, E., Glaser, T., & Traber, J. (1990) Effects of 5-hydroxytryptamine receptor agonists on hippocampal damage after transient forebrain ischaemia in the mongolian gerbil. Stroke **21 (IV),** 164-166.

Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K., & Tjian, R. (1987) Human protooncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science **238**, 1386-1392.

Boiziau, C., Kurfurst, R., Cazenave, C., Roig, V., Thuong, N.T., & Toulme, J.J. (1991) Inhibition of translation initiation by antisense oligonucleotides via an RNase-H independent mechanism. Nucleic Acids Res. **19**, 1113-1115.

Bolam, J.P., Powell, J.F., Wu, J-Y., & Smith, A.D. (1985) Glutamate-decarboxylase-immunoreactive structures in the neostriatum: a correlated light and electron microscopic study including a combination of golgi impregnation and immunocytochemistry. J.Comp. Neurol. 237, 1-20.

Bolam, J.P., Wainer, B.H., & Smith, A.D. (1984) Characterisation of cholinergic neurons in the rat neostriatum: a combination of choline acetyltransferase immunohistochemistry, Golgi impregnation and electron microscopy. Neuroscience **12**, 711-718.

Borison, R.L. & Diamond, B.I. (1983) Regional selectivity of neuroleptic drugs: an argument for site specificity. Brain Res. Bull. **11(2)**, 215-218.

Borison, R.L., Hitri, A., Blowers, A.J. & Diamond, B.I. (1983) Antipsychotic drug action: clinical, biochemical, pharmacological evidence for site specificity of action. Clin. Neuropharmacol. **6(2)**, 137-150.

Bos, T.J., Rauscher, F.J., Curran, T., &Vogt, P.K. (1989) The carboxy terminus of the viral jun oncoprotein is required for complex formation with the cellular fos protein. Oncogene **4**, 123-126.

Boundy, V.A., Luedtke, R.R., & Molinoff, P.B. (1993) Development of polyclonal anti-D2 receptor antibodies to fusion proteins: inhibition of D2 receptor G-protein interaction. J. Neurochem. **60(6)**, 2181-2191.

Bowen, D.M., Francis, P.T., Pangalos, M.N., Stephens, P.H. & Proctor, A.W. (1992) Treatment strategies for Alzheimers disease. Lancet **339**, 132-133.

Boysen S.J., McGonigle P. & Molinoff P.B.(1986). Quantitative autoradiographic localisation of the D1 and D2 subtypes of dopamine receptors in rat brain. J. Neurosci. **6,** 3177-3188.

Brandt, S.J., Niedel, J.E., Bell, R.M., & Young, W.S. (1987) Distinct patterns of expression of different protein kinase C mRNAs in rat tissues. Cell **49(1),** 57-63.

Brocco, M.J., Koek, W., Degryse, A.D. & Colpaert, F.C. (1990) Comparative studies on the antipunishment effects of chlordiazepoxide, buspirone, and ritanserin in the pigeon, Geller-Seifter and Vogel conflict procedures. Behav. Pharmacol. **1,** 403-418.

Brostrom, C.O., Corbin, J.D., King, C.A., & Krebs, E.G. (1971) Interaction of the subunits of adenosine 3' 5'-cyclic monophosphate-dependent protein kinase of muscle. Proc. Natl. Acad. Sci. U.S.A. **68(10),** 2444-2447.

Bruns, R.F., Miller, F.D., Merriman, R.L., Howbert, J.J., Heath, W.F., Kobayashi, E., Takahashi, I., Tamaoki, T. & Nakano, H. (1991) Inhibition of protein kinase C by calphostin C is light dependent. Biochem. Biophy Res. Commun. **176,** 289-294.

Buckland, P.R., O' Donovan, M.C. & McGuffin, P. (1992) Atypical antipsychotics elevate dopamine D3 but not D1 or D2 receptor mRNA levels in rat brain. Eur. J. Neuropsychopharmacol. **2,** 348.

Buckland, P.R., O' Donovan, M.C. & McGuffin, P. (1992) Lack of effect of chronic antipsychotic treatment on dopamine D5 receptor mRNA level. Eur. J. Neuropsychopharmacol. **2,** 405-409.

Buckland, P.R., O' Donovan, M.C., & McGuffin, P. (1993) Clozapine and sulpiride up-regulate dopamine D3 receptor mRNA levels. Neuropharmacol. **32(9),** 901-907.

Bunzow, J.R., Van Tol, H.H.M., Grandy, D.K., Albert, P., Salon, J., Christie, M., Machida, C.A., Neve, K.A. & Civelli, O. (1988). Cloning and

expression of a rat D2 dopamine receptor cDNA. Nature **336,** 783-787.

Burgess, G.M., Dooley, R.K., Mckinney, J.S., Nanberg, E., & Putney, J.W. (1986) Further studies on the interactions between the calcium mobalisation and cyclic AMP pathways in guinea pig hepatocytes. Mol. Pharmacol. **30(4),** 315-320.

Caceres, A., & Kosik, K.S. (1990) Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. Nature **343**, 461-463.

Cadd, G & McKnight, G.S. (1989) Distinct patterns of cAMP-dependent protein kinase gene expression in mouse brain. Neuron **3(1),** 71-79.

Cadet, J.L., hu, S.M., & Angulo, J.A. (1992) Quantitative in situ hybridisation evidence for differential regulation of proenkephalin and dopamine D2 receptor mRNA levels in the rat striatum: effects of unilateral intrastriatal injections of 6-hydroxydopamine. Mol. Brain Res. **12,** 59-67.

Cadoret, M.-A., Jaber, M. & Bloch, B. (1993) Prenatal D1, D1b, and D3 dopamine receptor gene expression in the rat forebrain: detection by reverse polmerase chain reaction. Neurosci. Letts. 155, 92-95.

Canton, H., Audinot V., Verriele L., JaquesV., & Millan M.J.(1993). Actions of antipsychotic drugs at 5-HT1C and 5-HT2 receptors: Evidence for potent 5-HT1C receptor antagonism by clozapine but not haloperidol both invitro and invivo.J. Neurochem. Abstracts **61,** Supplement 91D.

Carlsson, A. (1978) Antipsychotic drugs, neurotransmitters and schizophrenia. Am. J. Psychiatry **135(2),** 165-173.

Ceballos, M.L., Fernandez, A., Jenner, P. & Marsden, C.D. (1993) Parallelalterations in met-enkephalin and substance P levels in medial globus pallidus in Parkinson's disease patients. Neurosci. Letts. **160,** 163-166.

Cenci, M.A., Kalen, P., Mandel, R.J., & Bjorklund, A. (1992) Regional differences in the regulation of dopamine and noradrenaline release in medial frontal cortex, nucleus accumbens and caudate-putamen: a microdialysis study in the rat. Brain Res. **581(2),** 217-228.

Cervo, L., Grignaschi, G., & Samanin, R. (1988) 8-Hydroxy-2-(di-n-propylamino)tetralin, a selective serotonin1A receptor agonist, reduces the immobility of rats in the forced swimming test by acting on the nucleus raphe dorsalis. Eur. J. Pharmacol. **158,** 53-59.

Chan, B.L., Chao, M.V., & Saltiel, A.R. (1989) Nerve growth factor stimulates the hydrolysis of glycosylphosphatidylinositol in PC-12 cells: a mechanism of protein kinase C regulation. Proc. Natl. Acad. Sci. U.S.A. **86(6),** 1756-1760.

Chan-Palay, V. (1987) Somatostatin immunoreactive neurones in the human hippocampus and cortex shown by immunogold/silver intensification on vibratome sections: Coexistence with neuropeptide Y neurones, and effects of Alzheimers type dementia. J. Comp. Neurol. **260**, 201-223.

Changelian, P.S., Feng, P., King, T.C. & Milbrandt, J. (1989) Structure of the NGFI-A gene and detection of upstream sequences responsible for its transcriptional induction by nerve growth factor. Proc. Natl. Acad. Sci. U.S.A. **86(1),** 377-381.

Cheramy, A., Leviel, V., & Glowinski, J. (1981) Dendritic release of dopamine in the substantia nigra. Nature **289**, 537-542.

Chiang, T.M., Cagen, L.M. & Kang, A.H. (1981) Effects of 12-0-tetradecanoyl phorbol 13-acetate on platelet aggregation. Thrombosis Res. **21(6),** 611-622.

Chiasson, B.J., Hooper, M.L., Murphy, P.R. & Robertson, H.A. (1992) Antisense oligonucleotide eliminates in vivo expression of c-fos in

mammalian brain. Eur. J. Pharmacol. - Mol. Pharmacol. section **227**, 451-453.

Chiodo, L.A. (1988) Dopamine-containing neurons in the mammalian central nervous system: Electrophysiology and pharmacology. Neuroscience Biobehavioral Review **12,** 49-91.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**, 156-159.

Christensen, A.V., Arnt, J., Hyttel, J., Larsen, J.-J., & Svendsen, O. (1984) Pharmacological effects of a specific dopamine D1 antagonist SCH23390 in comparison with neuroleptics. Life Sci. **34**, 1529-1540.

Christy, B.A., Lau, L.F. & Nathans, D. (1988) A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc. Natl. Acad. Sci. U.S.A. **85(21),** 7857-7861.

Chui, R., Angel, P. & Karin, M. (1989) Jun-B differs in its biological properties from, and is a negative regulator of, c-jun. Cell **59**, 979-986.

Civelli, O., Douglass, J., Goldstein, A. & Herbert, E. (1985). Sequence and expression of the rat prodynorphin gene. Proc. Natl. Acad. Sci. U.S.A. **82**, 4291-4295.

Claghorn J.L., Abuzzahab F.S., Wang R., Larson C., Gelenberg A.J., Klerman G.L., Tuason V., & Steinbook R.(1983) The current status of clozapine. Psychopharmacol. Bull., **19**, 138-140.

Claghorn, J., Honigfeld, G., Abuzzahab, F.S.Sr., Wang, R., Steinbook, R., Tuasson, V. & Klerman, G. (1987) The risks and benefits of clozapine versus chlorpromazine. J. Clin. Psychopharmacol. **7(6),** 377-384.

Clarke, D.E., Craig, D.R. & Fozard, J.R. (1989) The 5-HT4 receptor: naughty, but nice. Trends in Pharmacological Sci. **10**, 385-386.

Clevens, R.A. & Beal, M.F. (1989) Substance-P-like immunoreactivity in brains with pathological features of Parkinson's and Alzheimer's diseases. Brain Res. **486**, 387-390.

Clow, D. & Jhamandas, K. (1989) Characterisation of L-glutamate action on the release of endogenous dopamine from the rat caudate-putamen. J. Pharmacol.and Exp. Therap. **248**, 722-728.

Cohen, A.I., Todd, R.D., Harmon, S. & O'Malley, K.L. (1992) Photoreceptors of mouse retinas possess D4 receptors coupled to adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **89**, 12093-12097.

Cohen, B.M. & Lipinski, J.F. (1986) In vivo potencies of antipsychotic drugs in blocking alpha-1 noradrenergic and dopamine D2 receptors : Implications for drug mechanisms of actions. Life Sci. **39**, 2571-2580.

Cole A.J., Bhat R.V., Patt c., Worl y P.F., & Baraban J.M.(1992). D1 dopamine receptor activation of multiple transcription factor genes in the rat striatum. J. Neurochem. **58**, 1420-1426.

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E., & Goodman, H.M. (1986) A cyclic AMP and phorbol ester inducible element. Nature **323(6086),** 353-356.

Conn, P.J. & Sanders-Bush, E. (1986) Regulation of serotonin stimulated phosphoinositide hydrolysis: relation to the serotonin 5-HT2 binding sites. The J. Neurosci. **6**, 3669-3675.

Conn, P.J., Sanders-Bush, E., Hoffman, B. & Hartig, P.R. (1986) A unique serotonin receptor in choroid plexus is linked to phosphatidylinositol turnover. Proc. Natl. Acad. Sci. U.S.A. **83**, 4086-4088.

Cools, A.R. & Van Rossum, J.M. (1976) Excitation-mediating and inhibition-mediating dopamine receptors. A new concept towards a better understanding of electrophysiological, biochemical, pharmacological, clinical and functional data. Psychpharmacologia **45**, 243-254.

Corbin, J.D., Sugden, P.H., West, L., Flockhart, D.A., Lincoln, T.M., & McCarthy, D. (1978) Studies on the properties and mode of action of the purified regulatory subunit of bovine heart adenosine 3'5'-monophosphate-dependent protein kinase. J. Biol. Chem. **253(11),** 3997-4003.

Cori, G.T. & Cori, C.F. (1945) The enzymatic conversion of phosphorylase a to b. J. Biol. Chem. **158**, 321-332.

Craig, D.A. & Clarke, D.E. (1990) Pharmacological characterisation of a neuronal receptor for 5-hydroxytryptamine in guinea-pig ileum with properties similar to the 5-hydroxytryptamine4 receptor. J. Pharmacol. and Exp. Ther. **252(3),** 1378-386.

Cramer, H., Rissler, K., Rosler, N., Strubel, D., Schaudt, D., & Kuntzmann, F. (1989) mmunoreactive substance P and somatostatin in the cerebrospinal fluid of enile parkinsonian patients. Eur. J. Neurol. **29(1)**, 1-5.

Creese, I., Burt, D.R., & Snyder, S.H. (1976) Dopamine receptor binding predicts linical and pharmacological potencies of antischizophrenic drugs. Science **92(4238),** 481-483.

Cross, A.J., Crow, T.J., & Owen, F. (1981) 3H-Flupenthixol binding in post-mortem brains of schizophrenics : evidence for a selective increase in dopamine D2 receptors. Psychopharmacol.-Berl. **74(2),** 122-124.

Crow, T.J., Deaki, J.F.W. & Longden, A. (1977) The nucleus accumbens-possible site of antisychotic action of neuroleptic drugs? Psychol. Medicine 7, 213-221.

Curran T.& Morgan J.I.(1987). Memories of fos. Bioessays **7,** 255-258.

Curran, T. & Morgan, J.I. (1986) Barium modulates c-fos expression and post-translational modification. Proc. Natl. Acad. Sci. U.S.A. **83(22),** 8521-8524.

Curran, T. & Franza, B.R. (1988) Fos and jun: The AP-1 connection. Cell **55**, 395-397.

Dabire, H., Chaouche-Teyara, K., Cherqui, C., Fournier, B. & Schmitt, H. (1989) DOI is a mixed agonist-antagonist at postjunctional 5-HT2 receptors in the pithed rat. Eur. J. Pharmacol. **170(1-2)**, 109-111.

Dacko, S. & Schneider, J.S. (1993) Met-enkephalin immunoreactivity in the basal ganglia in symptomatic and asymptomatic MPTP-exposed monkeys: correlation with degree of parkinsonian symptoms. Neurosci. Letts. **127**, 49- 52.

De Camilli, P., Macconi, D. & Spada, A. (1979) Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenoma. Nature **278**, 252-254.

De Vivo, M. & Mayaani, S. (1990) Stimulation and inhibition of adenylyl cyclase by distinct 5-hydroxytryptamine receptors. Biochem. Pharmacol. **40(7)**, 1551-1558.

De Vries, T.L., Mulder, A.H. & Schoffelmeer, A.N.M. (1992) Differential ontogeny of functional dopamine and muscarinine receptors mediating presynaptic inhibition of neurotransmitter release and postsynaptic regulation of adenylate cyclase activity in rat striatum. Dev. Brain Res. **66**, 91-96.

Deakin, J.F.W., Slater, P., Simpson, M.D.C., Gilchrist, A.C., Skan, W.J., Royston, M.C., Reynolds, G.P. & Cross, A.J. (1989) Frontal cortical and left temporal glutamatergic dysfunction in schizophrenia. J. Neurochem. **52**, 1781-1786.

Dearry, A., Gingrich, J.A., Falardeau, P., Fremeau, R.T., Bates, M.D. & Caron, M.G. (1990) Molecular cloning and expression for the gene for a human dopamine D1 receptor Nature **347**, 72-76.

Delay, A., Deniker, P. & Harl, J.-M. (1952) Traitement des etats d'excitation et d'agitation par une methode medicamenteuse derivee de l'hibetnotherapie. Annals Med. Psychol. **110(2)**, 267-273.

Demchyshn, L., Sunahara, R., Miller, K., Teitler, M., Hoffman, B., Kennedy, J.L., Seeman, P., Van Tol, H.H.M. & Niznik, H.B. (1992) A human serotonin 1D receptor variant (5-HT1Dβ) encoded by an intronless gene on chromosome 6. Proc. Natl. Acad. Sci. U.S.A. **89**, 5522-5526.

Deskin, R., Sleidler, F.J., Whitmore, W.L. & Slotkin, T.A. (1981) Develpoment of α-noradrenergic and dopaminergic receptor systems depends on maturation of their presynaptic nerve terminals in the rat brain. J. Neurochem. **36**, 1683-1690.

Deutch A.Y., Lee M.C. & Iadarola M.J.(1992). Regionally specific effects of atypical antipsychotic drugs on striatal fos expression: The nucleus accumbens shell as a locus of antipsychotic action. Mol. Cell. Neurosci. **3**, 332-341.

De Vries, T.J., Mulder, A.H. & Schoeffelmeer, A.N.M. (1992) Differential ontogeny of functional dopamine and muscarinic receptors mediating presynaptic inhibition of neurotransmitter release and postsynaptic regulation of adenylate cyclase activity in rat striatum. Dev. Brain Res. **66**, 91-96.

Dillon-Carter, O. & Chuang, D.M. (1989) Homologous desensitisation of muscarinic cholinergic, histaminergic, adrenergic and serotoninergic receptors coupled to phospholipase C in cerebellar granule cells. J. Neurochem. **52,** 598-603.

Divac, I., Fonnum, F. & Storm-Mathisen, J. (1977) High affinity uptake of glutamate in terminals of corticostriatal axons. Nature **266**(5600), 377-378.

Dolnick, B.J. (1990) Antisense agents in pharmacology. J. Biochem. Pharmacol. **40,** 670-675.

Donoghue, J.P. & Herkenham, M. (1986) Neostriatal projections from individual cortical fields conform to histochemically distinct striatal compartments in the rat. Brain Res. **365(2),** 397-403.

Donovan D.M., Takemura M., O'Hara B.F., Brannock M.T. & Uhl G.R.(1992) Preproenkephalin promoter cassette confers brain expression and synaptic regulation in transgenic mice. Proc. Natl. Acad. Sci. U.S.A., **89**, 2345-2349.

Doran, A.R., Rubinow, D.R., Wolkowitz, O.M., Roy, A., Breier, A. & Pickar, D. (1989) Fluphenazine treatment reduces CSF somatostatin in patients with schizophrenia: correlation with CSF HVA. Biol. Psychiatry **25(4)**, 431-439.

Dourish, C.R., Hutson, P.H. & Ahlenius, S. (1987) Eds. Brain 5-HT1A receptors. Chichester press, Harwood.

Dragunow M., Leah J.D. & Faull R.L.M.(1991). Prolonged and selective induction of fos-related antigens in striatal neurons after 6-hydroxydopamine lesions of the rat substantia nigra pars compacta. Mol. Brain Res. **10**, 355-358.

Dragunow M., Robertson G.S., Faull R.L.M., Robertson H.A. & Jansen K.(1990). D2 dopamine receptor antagonists induce fos and related proteins in rat striatal neurons. Neuroscience **37**, 287-294.

Dubinsky, J.M. (1989) development of synapses among striatal neurons in vitro. J. Neurosci. **9**, 3955-3965.

Dubois, A. & Scatton, B. (1985) Heterogenous distribution of dopamine D2 receptors within the rat striatum as revealed by autoradiography of [3H] N-n-propylapomorphine binding sites. Neurosci. Letts. **57(1),** 7-12.

Dumuis, A., Bouhelal, R., Sebben, M. & Bockaert, J. (1988) A 5-HT receptor in the central nervous system positively coupled to adenylate cyclase is antaginised by ICS 205930. Eur. J. Pharmacol. **146**, 187-188.

Dumuis, A., Bouhelal, R., Sebben, M., Cory, R. & Bockaert, J. (1988) A non-classical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. Mol. Pharmacol. **34**, 880-887.

Dumuis, A., Pin, J-P., Omagari, K., Sebben, M. & Bockaert, J. (1990) Arachidonic acid released from striatal neurons by joint stimulation of ionotropic and metabotropic quisqualate receptors. Nature **347**, 182-184.

Dumuis, A., Sebben, M. & Bockaert, J. (1989) BRL 24924: a potent agonist at the non-classical 5-HT receptor positively coupled to adenylate cyclase in colliculi neurons. Eur. J. Pharmacol. **162**, 381-384.

Dumuis, A., Sebben, M., Haynes, L., Pin, J-P. & Bockaert, J. (1988) NMDA receptors activate the arachidonic acid cascade in striatal neurons. Nature **336**, 68-70.

Engber, T.M., Boldry, R.C., Kuo, S. & Chase, T.N. (1992) Dopaminergic modulation of striatal neuropeptides: differential effects of D1 and D2 receptor stimulation on somatostatin, neuropeptide Y, neurotensin, dynorphin and enkephalin. Brain Res. **581(2),** 261-268.

Engber, T.M., Susel, Z., Kuo, S., Gerfen, C.R. & Chase, T.N. (1991) Levodopa replacement therapy alters enzyme activities in striatum and neuropeptide content in striatal output regions of 6-hydroxydopamine lesioned rats. Brain Res. **552,** 113-118.

Enjalbert, A., Hamon, M., Bourgoin, S. & Bockaert, J. (1978) Post-synaptic serotonin sensitive adenylate cyclase in the central nervous system. Mol. Pharmacol. **14**, 11-23.

Erlander, M.G., Lovenberg, T.W., Baron, B.M., De Lecea, L., Danielson, P.E., Racke, M., Slone, A.L., Siegel, B.W., Foye, P.E. & Cannon, K. (1993) Two members of a distinct subfamily of 5-hydroxytrypyamine

receptors differentially expressed in rat brain. Pro. Natl. Acad. Sci. U.S.A. **90(8)**, 3452-3456.

Erlinge, D., Edvinsson, L., Brunkwall, J., Yee, F. & Wahlestedt, C. (1993) Human neuropeptide Y Y1 receptor antisense oligonucleotide specifically inhibits neuropeptide Y -evoked vasoconstriction. Eur. J. Pharmacol. **240(1)** , 77-80.

Everitt, B.J., Hokfelt, T., Terenius, L., Tatemoto, K., Mutt, V. & Goldstein, M. (1984) Differential coexistence of neuropeptide Y-like immunoreactivity with catecholamines in the central nervous system of the rat. Neuroscience **11**, 442-462.

Fargin, A., Raymond, J.R., Lohse, M.J., Kobilka, B.K., Caron, M.G. & Hen, R. (1988) The genomic clone G-21 which resembles the β- adrenergic receptor sequence encodes the 5-HT1A receptor. Nature **335**, 358-360.

Felder, C.C., Jose, P.A. & Axelrod, J. (1989) The dopamine agonist, SKF82526, stimulates phospholipase C activity independent of adenylate cyclase. J. Pharmacol. and Exp. Ther. **248**, 171-175.

Felder, C.C., Kanterman, R.Y., Ma, A.L. & Axelrod, J. (1990) Serotonin stimulates phospholipase A2 and the release of arachidonic acid in hippocampal neurons by a type 2 serotonin receptor that is independent of inositolphospholipid hydrolysis. Proc. Natl. Acad. Sci. U.S.A. **87**, 2187-2191.

Foguet, M., Hoyer, D., Pardo, L.A., Parekh, A., Kluxen, F.W., Kalkman, H.O., Stuhmer, W. & Lubbert, H. (1992) Cloning and functional characterisation of the rat stomach fundus serotonin receptor. The EMBO J. **11**, 3481-3487.

Forno, L.S. (1982) Pathology of Parkinson's disease. In Marsden CD, et al (eds): "Movement disorders". London: Butterworths Scientific 25-40.

Foster, G.A., Schultzberg, M., Hokfelt, M., Goldstein, M., Hemmings, H.C., Ouimet, C.C., Walaas, S.I. & Greengard, P. (1987) Development of a dopamine and cyclic adenosine 3'5'-monophosphate regulated phosphoprotein (DARPP-32) in the prenatal rat central nervous system and its relationship to the arrival of presumptive dopaminergic innervation. J. Neurosci. 7, 1994-2016.

Frail, D.E., Manelli, A.M., Witte, D.G., Lui, C.W., Steffey, M.E. & Mackenzie, R.G. (1993) Cloning and characterisation of a truncated D1 dopamine receptor from goldfish retina: stimulation of cAMP production and calcium mobalisation. Mol. Pharmacol. **44**, 1113-1118.

Fraser, A., Mayaani, S. & Wolfe, B.B. (1990) Subtypes of receptors for serotonin. Annal. Rev. Pharmacol. Toxicol. **30**, 307-348.

Friedman, E., Gianutsos, G. & Kuster, J. (1983) Chronic fluphenazine and clozapine elicit opposite changes in brain muscarinic receptor binding: Implications for understanding tardive dyskinesia. J. Pharmacol. and Exp. Ther. **226(1)**, 7-12.

Garratt, J.C., Kidd, E.J., Wright, I.K. & Marsden, C.A. (1991) Inhibition of 5-hydroxytryptamine neuronal activity by the 5-HT agonist, DOI. Eur. J. Pharmacol. **199(3)**, 349-355.

Garthwaite, G. (1991) Glutamate, nitric oxide and cell-cell signalling in the nervous system. Trends in Neurosci. **14**, 60-67.

Gatti, A., Watsuji, T. & Hanley, M. (1991) Apparent calcium-independent phorbol ester binding to protein kinase C. Biochem. Soc. Trans. **19(2)**, 150S.

Gentz, R., Rauscher, F.J., Abate, C. & Curran, T. (1989) Parallel association of fos and jun leucine zippers juxtaposes DNA binding domains. Science **243(4899)**, 1695-1699.

Gerfen, C.R. (1992) The neostriatal mosaic: multiple levels of compartmental organisation. Trends in Neurosci. **15(4)**, 133-139.

Gerfen, C.R. & Young, W.S. (1988) Distribution of striatonigral and striatopallidal peptidergic neurons in both patch and matrix compartments: an in situ hybridisation histochemistry and flourescent retrograde tracing study. Brain Res. **460(1),** 161-167.

Gerfen, C.R., Engber, T.M., Mahan, L.C., Susel, R.M.., Chase, T.N., Monsma, F.J. & Sibley, D.R. (1990) D1 and D2 dopamine receptor regulated gene expression of striatanigral and striatopallidal neurons. Science **250(4986),** 1429-1432.

Gerfen, C.R., McGinty, J.F. & Young, W.S. III (1991) Dopamine differentially regulates dynorphin, substance P and enkephalin expression in striatal neurones: In situ hybridisation histochemical analysis. J. Neurosci. **11**, 1016-1031.

Gianutsos, G. & Suzdak, P.D. (1984) Evidence for down regulation of GABA receptors following long-term gamma-butyrolactone. Naunyn. Schmiedebergs. Arch. Pharmacol. **328(1),** 62-68.

Gill, G.N. & Garren, L.D. (1971) Role of the receptor in the mechanism of action of adenosine 3' 5'-cyclic monophosphate. Proc. Natl. Acad. Sci. U.S.A. **68(4),** 786-790.

Gilman, M.Z. (1988) The c-fos serum response element responds to protein kinase C-dependent and independent signals but not to cyclic AMP. Genes. Dev. **2(4),** 394-402.

Gilman, M.Z., Wilson, R.N. & Weinberg, R.A. (1986) Multiple protein binding sites in the 5'-flanking region regulates c-fos expression. Mol. Cell. Biol. **6(12),** 4305-4316.

Giraud, P., Kowalski, C., Barthel, F., Becquet, D., Renard, M., Grino, M., Boudouresque, F. & Loeffler, J.P. (1991) Striatal proenkephalin

turnover and gene transcription are regulated by cyclic AMP and protein kinase C-related pathways. Neuroscience **43(1)**, 67-79.

Giros, B., Martres, M-P., Sokoloff, P.& Schwartz, J-C. (1990) Clonage du gene du receptor dopaminergique D3 humain et identification de son chromosome. Comptes Rendu Academic Science Paris **311**, 501-508.

Glaser, T. (1988) Isapirone, a potent and selective 5-HT1A receptor ligand with anxiolytic and antidepressant properties. Drug. Future **13**, 424-439.

Gleeson, S., Ahlers, S.T., Mansbach, R.S., Foust, J.M. & Barrett, J.E. (1989) Behavioural studies with anxiolytic drugs. VI. Effects on punished responding of drugs interacting with serotonin receptor subtypes. J. Pharmacol. and Exp. Ther. **250**, 809-817.

Glennon, R.A. (1990) Serotonin receptors: Clinical implications. Neurosci. Behav. Rev. **14**, 35-47.

Goelet, P., Castellucci, V.F., Schacher, S. & Kandel, E.R. (1986) The long and the short of long-term memory- A molecular framework. Nature **322**, 419-422.

Gonzales, R.A. & Crews, F.T. (1984) Characterisation of the cholinergic stimulation of phosphoinositide hydrolysis in rat brain slices. J. Neurosci. **4(12)**, 3120-3127.

Gonzalez, G.A. & Montminy,M.R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell **59(4)**, 675-680.

Gonzalez, G.A., Yamamoto, K.K., Fischer, W.H., Karr, D., Menzel, P., Biggs, W., Vale, W.W. & Montminy, M.R. (1989) A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature **337(6209)**, 749-752.

Goodman, R.H. (1990) Regulation of neuropeptide gene expression. Annal. Rev. Neurosci. **13**, 111-127.

Gozlan, H.S., Mestikawy, E., Pitchat, L., Glowinski, J. & Hamon, M. (1983) Identification of presynaptic serotonin autoreceptors using a new ligand: [^{3}H]-8OHPAT. Nature (London) **305**, 140-143.

Grandy, D.K., Zhang, Y.A., Bouvier, C., Zhou, Q.Y., Johnson, R.A., Allen, L., Buck, K., Bunzow, J.R., Salon, J. & Civelli, O. (1991) Multiple human D5 dopamine receptor genes: a functional receptor and two pseudogenes. Proc. Natl. Acad. Sci. U.S.A. **88(20),** 9175-9179.

Graybiel A.M.(1986). Neuropeptides in the basal ganglia. In neuropeptides in neurolgic and psychiatric disease.(J.B. Martin and J.D. Barchas Eds) Raven press, New York, 135-161.

Graybiel, A.M. (1990) Neurotransmitters and neuromodulators in the basal ganglia. Trends Neurosci. 13, 244 - 254.

Graybiel, A.M. & Ragsdale, C.W. (1978) Histochemically distinct compartments in the striatum of human, monkey, and cat demonstrated by acetylcholinesterase staining. Proc. Natl. Acad. Sci. U.S.A. **75(11),** 5723-5726.

Greenberg, M.E. & Ziff, E.B. (1984) Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature **311**, 433-438.

Greenberg, M.E., Greene, L.A. & Ziff, E.B. (1985) Nerve growth factor and epidermal growth factor induce rapid and transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem. **260(26),** 14101-14110.

Greenberg, M.E., Hermankowski, A.L. & Ziff, E.B. (1986) Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc, and actin gene transcription. Mol. Cell. Biol. **6(4),** 1050-1057.

Greenberg, M.E., Siegfried, Z. & Ziff, E.B. (1987) Mutation of the c-fos gene dyad symmetry element inhibits serum inducibility of transcription in vivo and the nuclear regulatory factor binding in vitro. Mol. Cell. Biol. **7(3),** 1217-1225.

Grohmann, R., Ruther, E., Sassim, N. & Schmidt, L.G. (1989) Adverse effects of clozapine. Psychopharmacol. **99**, S101-S104.

Guennoun, R. & Bloch, B. (1991) D2 dopamine receptor gene expression in the rat striatum during ontogeny: an in situ hybridisation study. Dev. Brain Res. **60**, 79-87.

Guennoun, R. & Bloch, B. (1992) Ontogeny of D1 and DARPP-32 gene expression in the rat striatum: an in situ hybridisation study. Mol. Brain Res. **12,** 131-139.

Halzonetis, T.D., Georgopoulo, K., Greenberg, M.E. & Leder, P. (1988) c-jun dimerizes with itself and with c-fos, forming complexes with different DNA binding affinities. Cell **55**, 917-914.

Hamblin, M. & Metcalf, M. (1991) Primary structure and functional expression of a human 5-hydroxytryptamine 1D-type serotonin receptor. Mol. Pharmacol. **40**, 143-148.

Hanson, G., Matsuda, L. & Gibb, J. (1987) Effects of cocaine on metamphetamine-induced biochemical changes: Characterisation of cocaine as a monoamine uptake blocker. J. Pharmacol. and Exp. Ther. **242**, 507-513.

Hanson, G.R., Alphs, L., Wolf, W., Levine, R. & Lovenberg, W. (1981) Haloperidol-induced reduction of nigral substance P-like immunoreactivity: a probe for the interactions between dopamine and substance P neuronal systems. J. Pharmacol. and Exp. Ther. **218(2),** 568-574.

Hartig, P.R., Adham, N., Zgombick, J., Weinshank, R. & Branchek, T. (1992) Molecular biology of the 5-HT1 receptor subfamily. Drug Dev. Res. **26,** 215-224.

Hartig, P.R., Branchek, T.A. & Weinshank, R.L. (1992) A subfamily of 5-HT1D receptor genes. Trends in Pharmacol. Sci. **13(4),** 152-159.

Hartog, J. & Wouters, W. (1988) Flesinoxan hydrochloride: Drugs Future. **13**, 31-33.

Haverstick, D.M., Rubenstein, A. & Bannon, M. J. (1989) Striatal tachykinin gene expression regulated by interaction of D1 and D2 dopamine receptors. J. Pharmacol. and Exp. Ther. **248(2),** 858-862.

He, X. & Rosenfeld, M.G. (1991) Mechanisms of complex transcriptional regulation: Implications for brain development. Neuron 7, 183-196.

Heffner, T.G., Zigmond, M.J. & Stricker, E.M. (1977) Effects of dopaminergic agonists and antagonists on feeding in intact and 6-hydroxydopamine-treated rats. J. Pharmacol. and Exp. Ther **201(2),** 386-399.

Heilig, M., Engel, J.A. & Soderpalm, B. (1993) C-fos antisense in the nucleus accumbens blocks the locomotor stimulant action of cocaine. Eur. J. Pharmacol. **236**, 339-340.

Heiss,W-D. & Hoyer, J. (1974) Dopamine receptor blockade by neuroleptic drugs in Aplysia neurons. Experientia **30,** 1318-1320.

Herve, D., Levi, S.M., Marey-Semper, T., Verney, C., Tassin, J.P., Glowinski, J. & Girault, J.A. (1993) G(olf) and Gs in rat basal ganglia: possible involvement of G(olf) in the coupling of dopamine D1 receptor with adenylyl cyclase. J. Neurosci. **13(5),** 2237-2248.

Heuring, R.E. & Peroutka, S.J. (1987) Characterisation of a novel [3H]5-hydroxytryptamine binding site subtype in bovine brain membranes. J. Neurosci. 7, 894-903.

Hicks, P.B. (1990) The effect of serotoninergic agents on haloperidol-induced catalepsy. Life Sci. 47, 1609-1615.

Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L. & Habener, J.F. (1988) Cyclic AMP responsive DNA binding protein: structure based on a cloned placental cDNA. Science **242(4884),** 1430-1433.

Hokfelt, T., Lundberg, J.M., Tatemoto, K., Mutt, V., Terenius, L., Polak, J., Bloom, S., Sasek, C., Elde, R. & Goldstein, M. (1983)

Neuropeptide Y and FMRFamide-like immunoreactivities in catecholamine neurones of the rat medulla oblongata. Acta. Physiol. Scandanavia **117**, 315-318.

Hollister, A.S., Breese, G.R. & Cooper, B.R. (1974) Comparison of tyrosine hydroxylase and dopamine beta hydroxylase inhibition with the effects of various 6-hydroxydopamine treatments on d-amphetamine-induced motor activity. Psychopharmacologia **36(1),** 1-16.

Holopainen, I. & Wojcik, W.J. (1992) A specific antisense oligodeoxynucleotide to mRNAs encoding receptores with seven transmembrane spanning regions decreases muscarinic M2 and γ-aminobutyric acid$_B$ receptors in cerebellar granule cells. J. Pharmacol. and Exp. Ther. **264(1),** 423-430.

Holt, J.T., Redner, R.L. & Nienhuis, A.W. (1988) An oligomer complementary to c-myc mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. Mol. Cell. Biol. **8**, 963-973.

Hong J.S., Yang H.Y., Gillin J.C. & Costa E.(1980). Effects of long term administration of antipsychotic drugs on enkephalinergic neurons. Adv. Biochem. Psychopharmacol. **24,** 223-243.

Hong, J.-S., Tilson, H.A. & Yoshikava, K. (1983) Effects of lithium and haloperidol administration on the rat brain levels of substance P. J. Pharmacol. and Exp. Ther. **224**, 590-593.

Hong, J.S., Yang, H.Y., Fratta, W. & Costa, E. (1978) Rat striatal methionine-enkephalin content after chronic treatment with catoleptogenic and noncatoleptogenic antischizophrenic drugs. J. Pharmacol. and Exp. Ther. **205(1),** 141-147.

Hope B., Kosofsky B., Hyman S.E. & Nestler E.J.(1992). Regulation of immediate early gene expression and AP-1 binding in the rat nucleus accumbens by chronic cocaine. Proc. Natl. Acad. Sci. U.S.A. **89,** 5764-5768.

Hope, B.T., Michael, G.J., Knigge, K.M. & Vincent, S.R. (1991) Neuronal NADPH-diaphorase is a nitric oxide synthase. Proc. Natl. Acad. Sci. U.S.A. **88**, 2811-2814.

Hornykiewicz, O. & Kish, S.J. (1987) Biochemical pathophysiology of parkinson's disease. Adv. Neurol. **45**, 19-34.

Hoyer, D. (1988) Functional correlates of 5-HT1 recognition sites. J. Receptor Res. **8**, 59-81.

Hoyer, D. & Schoeffter, P. (1991) 5-HT receptors: Subtypes and second messengers. J. Receptor Res. **11**, 197-241.

Hoyer, D., Schoeffter, P., Waeber, C. & Palacios, J.M. (1990) Serotonin 5-nigrostriatal dopamine projection increase the inhibitory effects of D1 and D2 dopamine agonists on caudate-putamen neurons and relieve D2 receptors from the necessity of D1 receptor stimulation. J. Neurosci. **10(7)**, 2318-2329.

Huang, F.L., Yoshida, Y., Nakabayashi, H., Knopf, J.L., Young, W.S. & Huang, K.P. (1987) Immunochemical identification of protein kinase C isozymes as products of discrete genes. Biochem. Biophys. Res.

Hunt, S.P., Emson, P.C., Gilbert, R., Goldstein, M. & Kimmel, J.R. (1981) Presence of avian pancreatic polypeptide-like immunoreactivity in catecholamine and methionine enkephalin-containing neurons within the central nervous system. Neurosci. Letts. **21**, 125-130.

Hunter, T. (1987) A thousand and one protein kinases. Cell **50(6)**, 823-829.

Irvine, R.F. (1989) Functions of inositol phosphates. Inositol lipids in cell signalling (R.H. Mitchell, A.H. Drummond, and C.P.Downes eds.) Academic press, San Diego, 135-161.

Ivins, K.J. & Molinoff, P.B. (1991) Desensitisation and downregulation of 5-HT2 receptors in P11 cells. J. Pharmacol. and Exp. Ther. **259**, 423-429.

Jaber, M., Fournier, M.C. & Bloch, B. (1992) Reserpine treatment stimulates enkephalin and D2 dopamine receptor gene expression in the rat striatum. Mol. Brain Res. **15,** 189-194.

Jaken, S. (1989) Diacylglycerol: the role of stimulated production in activation of protein kinase C. Inositol lipids in cell signalling (R.H. Mitchell, A.H. Drummond, and C.P. Downes eds.) Academic press, San Diego, 163-178.

Javitt, D.C. and Zukin, S.R. (1991) Recent advances in the phencyclidine model of schizophrenia. Am. J. Psychiatry **148(10),** 1301-1308.

Jin, H., Oksenberg, D., Ashkenasi, A., Peroutka, S.J., Duncan, A.M.V., Rozmahel, R., Yang, Y., Mengod, G., Palacios, J.M. & O'Dowd, B.F. (1992) Characterisation of the human 5-hydroxytryptamine1B receptor. J. Biol. Chem. **15,** 31-39.

Joyce, J.N. (1991) Differential response of striatal dopamine and muscarinic receptor subtypes to the loss of dopamine. II. Effects of 6-hydroxydopamine or colchicine microinjections into the VTA or reserpine treatment. Exp. Neurol. **113(3),** 277-290.

Joyce, J.N., Lexow, N., Bird, E. & Winokur, A. (1988) Organisation of dopamine D1 and D2 receptors in human striatum: receptor autoradiographic studies in Huntington's disease and schizophrenia. Synapse **2(5),** 546-557.

Kaibuchi, K., Takai, Y. & Nishizuka, Y. (1981) Cooperative roles of various membrabe phospholipids in the activation of calcium-activated, phospholipid-dependent protein kinase. J. Biol. Chem. **256(14),** 7146-7149.

Kanterman, R.Y., Felder, C.C., Brenneman, D.E., Ma, L., Fitzgerald, S. & Axelrod, J. (1990) Alpha-1 adrenergic receptor mediates arachidonic

acid release in spinal cord neurons independent of inositol phospholipid turnover. J. Neurochem. **54(4),** 1225-1232.

Kanterman, R.Y., Mahan, L.C., Briley, E.M., Monsma, F.J. & Sibley, D.R. (1990) Transfected D2 dopamine receptors mediate the potentiation of arachidonic acid release in chinese hamster ovary cells. Mol. Pharmacol. **39**, 364-369.

Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A. & Kaneka, M. (1987) K252 compounds: novel and potent inhibitors of protein kinase C and cyclic-nucleotide-dependent kinases. Biochem. Biophys. Res. Commun. **142,** 436-440.

Kebabian, J.W. & Calne, D.G. (1979) Multiple receptors for dopamine. Nature **277,** 93-96.

Kebabian, J.W. & Greengard, P. (1971) Dopamine-sensitive adenylyl cyclase. Possible role in synaptic transmission. Science **174**, 1346-1349.

Kelly, K., Cochran, C.H., Stiles, C.D. & Leder, P. (1983) Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**, 603-610.

Kelly, P.H. & Miller, R.J. (1975) The interaction of neuroleptic and muscarinic agents with central dopaminergic systems. Br. J. Pharmacol. **54(1),** 115-121.

Kemp, B.E., Benjamini, E., & Krebs, E.G. (1976) Synthetic hexapeptide substrates and inhibitors of 3' 5'-cyclic AMP-dependent protein kinase. Proc. Natl. Acad. Sci. U.S.A. 73(4), 1038-1042.

Kemp, B.E., Bylund, D.B., Huang, T.S. & Krebs, E.G. (1975) Substrate specificity of the cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. U.S.A. **72(9),** 3448-3452.

Kemp, B.E., Graves, D.J., Benjamini, E., & Krebs, E.G. (1977) Role of multiple basic residues in determining the substrate specificity of cyclic AMP- dependent protein kinase. J. Biol. Chem. **252(14),** 4888-4894.

Kerkerian, L., Salin, P. & Nieoullon, A. (1988) Pharmacological characterisation of dopaminergic influence on expression of neuropeptide Y immunoreactivity by rat striatal neurons. Neuroscience **26(3),** 809-817.

Kessler, J.A. (1986) Differential regulation of cholinergic and peptidergic development in the rat striatum in culture. Dev. Biol. **113**, 77-89.

Kilpatrick, G.J., Jones, B.J. & Tyers, M.B. (1987) Identification and distribution of 5-HT3 receptors in rat brain using radioligand binding. Nature **330**, 736-742.

Kishimoto, A. (1980) Studies on calcium-activated, phospholipid-dependent protein kinase: isolation and characterisation of a catalytically active fragment. Kobe. J. Med. Sci. **26(3),** 183-206.

Klawans, H.L., Goetz, C.G. & Perlik, S. (1980) Tardive dyskinesia: review and update. Am. J. Psychol. **137(8),** 900-908.

Kobayashi, E., Nakano, H., Morimoto, M. & Tamaoki, T. (1989) Calphostin C (UCN- 1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem. Biophys. Res. Commun. **159**, 548-553.

Koblika, B.K., Matsui, H.., Kobilka, T.S., Yang-Feng, T.L., Francke, U., Caron, M.G., Leftkowitz, R.J. & Regan, J.W. (1987) Cloning, sequencing and expression of the gene coding for the human platelet α_2- adrenergic receptor. Science **238**, 650-556.

Konradi C., Kobrerski L.A. & Nguyen T.V.(1993). The CAMP-response-element binding protein interacts, but fos protein does not interact with the proenkephalin enhancer in rat striatum. Proc. Natl. Acad. Sci. U.S.A. **90**, 7005-7009.

Koob, G.F. & Bloom, F.E. (1988) Cellular and molecular mechanisms of drug dependence. Science **242**, 715-723.

Kostowski, W., Gumulka, W. & Czlonkowski, A. (1972) Reduced cataleptogenic effects of some neuroleptics in rats with lesioned midbrain raphe and treated with p-chlorophenylalanine. Brain Res. **48**, 443-446.

Kouzarides, T., and Ziff, E. (1988) The role of the leucine zipper in the fos-jun interaction. Nature **336**, 646-651.

Krause, J.E., Chirgwin, J.M., Carter, M.S., Xu, Z.S. & Hershey, A.D. (1987). Three rat preprotachykinin mRNA's encode the neuropeptides substance P and neurokinin A. Proc. Natl. Acad. Sci. U.S.A. **84**, 881-885.

Krebs, E.G. (1985) The phosphorylation of proteins : a major machanism for biological regulation. Fourteenth Sir Frederick Gowland Hopkins memorial lecture. Biochemical Society Translation. **13(5),** 813-820.

Kuhar, M.J., Ritz, M.C. & Boja, J.W. (1991) The dopamine hypothesis of the reinforcing properties of cocaine. Trends in Neurol. Sci. **14**, 299-302.

Kumon, A., Nishiyama, K., Yamamura, H. & Nishizuka, Y. (1972) Multiplicity of adenosine 3' 5'-monophosphate-dependent protein kinases from rat liver and mode of action of nucleoside 3' 5'-monophosphate. J. Biol. Chem. **247(12),** 3726-3735.

Kursar, J.D., Nelson, D.L., Wainscott, D.B., Cohen, M.L. & Baez, M. (1992) Molecular cloning, functional expression and pharmacological characterisation of a novel serotonin receptor (5-hydroxytryptamine2F) from rat stomach fundus. Mol. Pharmacol. **42**, 549-557.

La Hoste, G.J. & Marshall, J.F. (1991) Chronic eticlopride and dopamine denervation induce equal nonadditive increases in striatal D2 receptor density: autoradiographic evidence against the dual mechanism hypothesis. Neuroscience **41(2-3),** 473-481.

Landschultz, W.H., Johnson, P.F. & Mc Knight, S.L. (1988) The leucine zipper, a hypothetical structure common to a new class of DNA binding proteins. Science **240**, 1759-1764.

Langston, J.W. (1985) MPTP and Parkinson's Disease. Trends in Neurosci. **2**, 79-82.

Lankford, K.L., DeMello, F.G. & Klein, W.L. (1988) D1-type dopamine receptors inhibit growth cone motility in cultured retina neurons: evidence that neurotransmitter acts as morphogenic growth regulators in the developing central nervous system. Proc. Natl. Acad. Sci. U.S.A. **85**, 4567-4571.

Larhammar, L., Ericsson, A. & Peersson, H. (1987). Structure and expression of the rat neuropeptide Y gene. Proc. Natl. Acad. Sci. U.S.A. **84**, 2068-2072.

Larson, E.R. & Ariano, M.A. (1994) Dopamine receptor binding on identified striatonigral neurones. Neurosci. Letts. **172**, 101-106.

Le Douarin, C., Oblin, A., Fage, D. & Scatton, B. (1983) Influence of lithium on biochemical manifestations of striatal dopamine target cell supersensitivity induced by prolonged haloperidol treatment. Eur. J. Pharmacol. **93(1-2)**, 55-62.

Le Moine, C. & Young, W.S. (1992). RHS2, a POU domain-containing gene, and its expression in the developing and adult rat. Proc. Natl. Acad. Sci. U.S.A. **89**, 3285-3289.

Le Moine, C., Normand, E. & Bloch, B. (1991) Phenotypical characterisation of the rat striatal neurons expressing the D1 dopamine receptor gene. Proc. Natl. Acad. Sci. U.S.A. **88(10)**, 4205-4209.

Le Moine, C., Normand, E., Guitteny, A.F., Fouque, B., Teoule, R. & Bloch, B. (1990) Dopamine receptor gene expression by enkephalin neurons in rat forebrain. Proc. Natl. Acad. Sci. U.S.A. **87(1)**, 230-234.

Leake, A. & Ferrier, I.N. (1993) Alterations in neuropeptides in aging and disease. Pathophysiology and potential for clinical intervention. Drugs-Aging **3(5),** 408-427.

Lee, C.Q., Yun, Y.D., Hoeffler, J.P. & Habener, J.F. (1990) Cyclic AMP responsive transcriptional activation of CREB-327 involves interdependent phosphorylated subdomains. The EMBO J. **9(13),** 4455-4465.

Lee, T. & Tang, S.W. (1984) Loxapine and clozapine decrease serotonin (S2) but not elevate dopamine (D2) receptor numbers in the rat brain. Psychiatry Res. **12(4),** 277-285.

Lee, T., Seeman, P., Hornykiewicz, O., Bilbao, J., Deck, J. & Tourtellotte, W.W. (1981) Parkinson's disease : low density and presynaptic location of D3 dopamine receptors. Brain Res. **212(2),** 494-498.

Lee, W., Mitchell, P. & Tjian, R. (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49,** 741-752.

Leslie, R.A., Moorman, J.M., Coulson, A. & Grahame-Smith, D.G. (1993) Serotonin 2/1C receptor activation causes a localised expression of the immediate early gene c-fos in the rat brain: evidence for invovlement of dorsal raphe nucleus projection fibres. Neuroscience **53(2),** 457-463.

Lester, J., Fink, J.S., Aronin, H. & DiFiglia, M. (1993) Colocaisation of D1 and D2 dopamine receptor mRNAs in striatal neurones. Brain Res. **621,** 106-110.

Levesque, D., Diaz, J., Pilon, C., Martres, M.P., Giros, B., Souil, E., Schott, D., Morgat, J.L., Schwartz, J.C. & Sokoloff, P. (1992) Identification, characterisation and localisation of the dopamine D3 receptor in rat brain using 7-[^{3}H]hydroxy-N,N-di-n-propyl-2-amino tetralin. Proc. Natl. Acad. Sci. U.S.A. **89,** 8155-8159.

Leysen, J.E. & Niemegeers, C.J.E. (1985) Neuroleptics. In Lajtha, A. (Ed.), Handbook of Neurochemistry, Plennum Press , 331-361.

Li, S.J., Huang, H.K., Stachowiak, M.S., Hudson, P.M., Owyang, V., Nanry, K., Tilson, H.A. & Hong, J.S. (1990) Influence of nigrostriatal dopaminergic tone on the biosynthesis of dynorphin and enkephalin in rat striatum. Brain Res. (Mol. Brain Res.) **8(3),** 219-225.

Lieberman, J.A., Kane, J.M., Sarantakos, S., Gadleta, D., Woerner, M., Alvir, J. & Ramos-Lorenzi, J. (1987) Prediction of relapse in schizophrenia. Arch. Gen. Psychiatry **44(7),** 597-603.

Lindefors N., Brodin E., Tossman U., Segovia J. & Ungerstedt U.(1989). Tissue levels and invivo release of tachykinins and GABA in striatum and substantia nigra of rat brain after unilateral striatal dopamine denervation. Exp. Brain Res. **74,** 527-534.

Listerud, M., Brussaard, A.B., Devay, P., Colman, D.R. & Role, L.W. (1991) Functional contribution of AChR subunits revealed by antisense oligonucleotides. Science **254,** 1518-1521.

Lloyd, K.G. & Bartholini, G. (1974) The effect of methiothepin on cerebral monoamine neurones. Adv. Biochem. Psychopharmacol. **10,** 305-309.

Lopez-Ibor, J.J. (1988) The involvment of serotonin in psychiatric disorders and behaviour. Br. J. Psychiatry **153(3),** 26-39.

Loric, S., Launay, J.M., Colas, J.F. & Maroteaux, L. (1992) New mouse 5-HT2 like receptor expression in the brain, heart and intestine. FEBS Letts. **312,** 203-207.

Lovenberg, T.W., Erlander, M.G., Baron, B., Racke, M., Slone, A.L., Siegel, B.W., Craft, C.M., Burns, J.E., Danielson, P.E. & Sutcliffe, J.G. (1992) Molecular cloning and functional expression of 5-HT1E-like rat and human 5-hydroxytryptamine receptor genes. Proc. Natl. Acad. Sci. U.S.A. **90,** 2184-2188.

Lucas, J.J., Mellstrom, B., Cooado, M.I. & Naranjo, J.R. (1993) Molecular mechanisms of pain: serotonin 1A receptor agonists trigger

transactivation by c-fos of the prodynorphin gene in spinal cord neurons. Neuron **10**, 599-611.

Lutz, E.M., Mitchell, R., Johnson, M.S. & MacEwan, D. (1993) functional expression of 5-HT1C receptor cDNA in COS-7 cells and its influence on protein kinase C. FEBS Letts. **316**, 228-232.

Mack, K.J., O'Malley, K.L. & Todd, R.D. (1991) Differential expression of dopaminergic D2 receptor mRNAs during development. Dev. Brain Res. **59**, 249-251.

Mahan, L.C., Burch, R.M., Monsma, F.J. & Sibley, D.R. (1990) Expression of striatal D1 dopamine receptors coupled to inositol phosphate production and calcium mobalisation in xenopus oocytes. Proc. Natl. Acad. Sci. U.S.A. **87**, 2196-2200.

Maier, J.A.M., Voulalas, P., Roeder, D. & Maciag, T. (1990) Extension of the life span of human endothelial cells by an interleukin-1α antisense oligomer. Science **249**, 1570-1573.

Mailleux P., Zhang F. & Vanderhaeghen J.(1992). The dopamine D1 receptor antagonist SCH-23390 decreases the mRNA levels of the transcription factor zif/268(krox-24) in adult rat intact striatum- an in situ hybridisation study. Neurosci. Letts. **147**, 182-184.

Maki, Y., Bos, T.J., Davis, C., Starbuck, M. & Vogt, P.K. (1987) Avian sarcoma virus 17 carries the jun oncogene. Proc. Natl. Acad. Sci. U.S.A. **84**, 2848-2852.

Malenka, R.C., Ayoub, G.S. & Nicoll, R.A. (1987) Phorbol esters enhance transmitter release in rat hippocampal slices. Brain Res. **403(1)**, 198-203.

Malenka, R.C., Madison, D.V., Andrade, R. & Nicoll, R.A. (1986) Phorbol esters mimic some cholinergic actions in hippocampal pyramidal neurons. J. Neurosci. **6(2)**, 475-480.

Mandal, A.K., Kellar, K.J., Friedman, E., Pineo, S.V., Hamosh, P. & Gillis, R.A. (1989) Importance of central nervous system serotonin-1A receptors for mediating the hypotensive effects of urapidil. J. Pharmacol. and Exp.Ther. **251**, 563-570.

Maricq, A.V., Peterson, A.S., Brake, A.J., Myers, R.M. & Julius, D.A. (1991) Primary structure and functional expression of the 5-HT3 receptor, a serotonin-gated ion channel. Science (Washington D.C.) **254**, 432-437.

Marin, P., Lafon-Cazal, M. & Bockaert, J. (1992) A nitric oxide synthase activity selectively stimulated by NMDA receptors depends on protein kinase C activation in mouse striatal neurons. Eur. J. Neurosci. **4**, 425-432.

Martin, P.R., Adinoff, B., Eckardt, M.J., Stapleton, J.M., Bone, G.A., Rubinow, D.R., Lane, E.A. & Linnila, M. (1989) Effective pharmacotherapy of alcoholic amnestic disorder with fluvoxamine. Arch. Gen. Psychiatry **46(7)**, 617-621.

Mason, S.L. & Reynolds, G.P. (1992) Clozapine has sub-micromolar affinity for 5-HT1A receptors in human brain tissue. Eur. J. Pharmacol. **221**, 397-398.

Matthes, H., Boschert, U., Amlaiky, N., Grailhe, R., Plassat, J-L., Muscatelli, F., Mattei, M. & Hen, R. (1992) Mouse 5-hydroxytryptamine5A and 5-hydroxytryptamine5B receptors define a new family of serotonin receptors: Cloning, functional expression, and chromosomal localisation. Mol. Pharmacol. **43**, 313-319.

Matthysse, S. (1973) Antipsychotic drug actions : a clue to the neuropathology of schizophrenia? Fed. Proc. **32(2)**, 200-205.

Mattson, M.P. (1988) Neurotransmitters in the regulation of neuronal cytoarchitecture. Brain Res. Rev. **13**, 179-212.

Matz, R., Rick, W., Thompson, H. & Gershon, S. (1974) Clozapine - a potential antipsychotic agent without extrapyramidal manifestations. Curr. Ther.Res. and Clin. Exp. **16(7),** 687-695.

Mauborgne, A., Javoy-Agid, F., Legrand, J.C., Agid, Y. & Cesselin, F. (1983) Decrease in substance P-like immunoreactivity in the substantia nigra and pallidum of parkinsonian brain. Brain Res. **268**, 167-170.

McAllister, G., Charlesworth, A., Snodin, C., Beer, M.S., Noble, A.J., Middlemass, D.N., Iverson, L.L. & Whiting, P. (1992) Molecular cloning of a serotonin receptor from human brain (5-HT1E): A fifth 5-HT1-like subtype. Proc. Natl. Acad. Sci. U.S.A. **89**, 5517-5521.

McLennan, H., York, D.H. (1967) The action of dopamine on neurons of the caudate nucleus. J. Physiol. (London) **189**, 393-482.

McVittie, L.D., Ariano, M.A. & Sibley, D.R. (1991) Characterisation of antipeptide antibodies for the localisation of D2 dopamine receptors in rat striatum. Proc. Natl. Acad. Sci. U.S.A. **88(4),** 1441-1445.

Meador-Woodruff, J.H., Mansour, A., Grandy, D.K., Damask, S.P., Civelli, O. and Watson, S.J. (1992) Distribution of D5 dopamine receptor mRNA in rat brain. Neurosci. Letts. **145(2),** 209-212.

Meador-Woodruff, J.H., Mansour, A., Healy, D.J., Kuehn, R., Zhou, Q,Y,m Bunzow, J.R., Akil, H., Civelli, O. & Watson, S.J. (1991) Comparison of the distribution of D1 and D2 dopamine receptor mRNAs in rat brain. Neuropsychopharmacol. 5(4), 231-242.

Meltzer, H.Y. & Nash, J.F. (1991) Effects of antipsychotic drugs on serotonin receptors. Pharmacol. Rev. **43(4)**, 587-604.

Meltzer, H.Y. (1988) New insights into schizophrenia through atypical antipsychotic drugs. Neuropsychopharmacol. **1**, 193-196.

Meltzer, H.Y., Burnett, S. & Bastani, B. (1990) Effect of six months of clozapine treatment on the quality of life of chronic schizophrenic patients. Hospital and Community Psychiatry **41**, 892-897.

Meltzer, H.Y., Matsubara, S. & Lee, J.-C. (1989) Classification of typical and atypical antipsychotic drugs on the basis of dopamine D1, D2 and serotonin2 pKi values. J. Pharmacol. and Exp. Ther. **251**, 238-246.

Merchant K.M. & Dorsa D.M.(1993). Differential induction of neurotensin and c-fos gene expression by typical versus atypical antipsychotics. Proc. Natl. Acad. Sci. U.S.A. **90**, 3447-3451.

Mercugliano, M. & Cheeselet, M-F. (1991) Clozapine decreases enkephalin mRNA in rat striatum. Neurosci. Letts. **136**, 10-14.

Mercugliano, M., Cheeselet, M.-F., Saller, C., Salama, A. & U'Prichard, D. (1989) Effects of chronic neuroleptic treatments on the level of glutamic acid decarboxylase (GAD) mRNA in the globus pallidus (GP) of the rat as revealed by in situ hybridisation histochemistry (ISHH). Neurosci.Abstract **15**, 991.

Merino, A., Buckbinder, L., Mermelstein, L. & Reinberg, D. (1989) Phosphorylation of cellular proteins regulates their binding to the cyclic AMP response element. J. Biol. Chem. **264**, 21266-21276.

Messer, A. (1981) Primary monolayer cultures of the rat corpus striatum. Neuroscience **6**, 2677-2687.

Millan, M.J., Rivet, J.M., Canton, H., Lejeune, F., Bervoets, K., Brocco, M., Gobert, A., Lefebvre de Ladonchamps, B., Le Marouille-Girardon, S., Verriele, L., Laubie, M. & Lavielle, G. (1992) S14671: A naphthylpiperazine 5-hydroxytryptamine1A agonist of exceptional potency and high efficacy possessing antagonist activity at 5-hydroxytryptamine1C/2 receptors. J. Pharmacol. and Exp. Ther. **262(2)**, 451-465.

Millbrandt J.(1987). A nerve growth factor -induced gene encodes a possible transcription regulatory factor. Science **238**, 797-799.

Miller J.C.(1990). Induction of c-fos mRNA expression in rat striatum by neuroleptic drugs. J. Neurochem. **54**, 1453-1455.

Miller, F.D., Naus, C.C.G., Durand, M., Bloom, F.E. & Milner, R.J. (1987) Subtypes of α- tubulin are differentially regulated during neuronal maturation. J. Cell Biol. **105(6)**, 3065-3073.

Miller, R.J. & Hilley, C.R. (1974) Antimuscarinic properties of neuroleptics and drug-induced Parkinsonism. Nature **248**, 596-597.

Misgeld, U. & Dietzel, I. (1989) Synaptic potentials in the rat neostriatum in dissociated embryonic culture. Brain Res. **492**, 149-157.

Monsma, F.J., Mahan, L.C., McVittie, L.D., Gerfen, C.R. & Sibley, D.R. (1990) Molecular cloning and expression of a D1 dopamine receptor linked to adenylyl cyclase activation. Proc. Natl. Acad. Sci. U.S.A. **87**, 6723-6727.

Monsma, F.J., Shen, Y., Ward, R.P., Hamblin, M.W. & Sibley, D.R. (1992) Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. Mol. Pharmacol. **43**, 320-327.

Montminy, M.R. & Bilezikjian, L.M. (1987) Binding of a nuclear protein to the cyclic AMP response element of the somatostatin gene. Nature **328(6126)**, 175-178.

Montminy, M.R., Goodman, R.H., Horovitch, S.J. & Habener, J.F. (1984). Primary structure of the gene encoding rat preprosomatostatin. Proc. Natl. Acad. Sci. U.S.A. **81**, 3337-3340.

Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. & Goodman, R.H. (1986) Identification of cyclic AMP responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. U.S.A. **83(18)**, 6682-6686.

Moratalla, R., Robertson, H.A. & Graybiel, A.M. (1992) Dynamic regulation of the NGF-IA (zif/268, ergl) gene expression in the striatum. J. Neurosci. **12**, 2609-2622.

Morgan, J.I. & Curran, T. (1989) Stimulus-transcription coupling in neurons: Role of cellular immediate early genes. Trends in Neurol. Sci. **12**, 459-462.

Morris B.J., Hollt V. & Herz A.(1988). Dopaminergic regulation of striatal proenkephalin mRNA and prodynorphin mRNA: contrasting effects of D1 and D2 antagonists. Neuroscience, **25**, 525-532.

Morris, B.J. (1989) Neuronal localisation of neuropeptide Y gene expression. J.Comp. Neurol. **290**, 358-368.

Morris, B.J. (1992) Neuropeptide Y gene expression. In Methods in Neurosciences (ed. P.M. Conn) **9**, 151-166. Academic press N.Y.

Morris, B.J. (1993) Controlof receptor sensitivity at the mRNA level. Mol. Neurobiol. 7, 1-17.

Morris, B.J. & Hunt, S. P. (1991) Proenkephalin mRNA levels in rat striatum are increased and decreased, respectively, by selective D2 and D1 dopamine receptor antagonists. Neurosci. Letts. **125**, 201-204.

Morris, B.J., Haarman, I., Kempter, B., Hollt, V. & Herz, A. (1986). Localisation of prodynorphin mRNA in rat brain by in situ hybridisation using a synthetic oligonucleotide probe. Neurosci. Letts. **69**, 104-108.

Morris, B.J., Herz, A. & Hollt, V. (1989) Localisation of striatal opioid gene expression, and its modulation by the mesostriatal dopamine pathway: an in situ hybridisation study. J. Mol. Neurosci. **1(1)**, 9-18.

Morris, B.J., Hicks, A.A., Wisden, W., Darlison, M.G., Hunt, S.P. & Barnard, E.A. (1990) Distinct regional expression of nicotinic acetylcholine receptor genes in chick brain. Mol. Brain Res. 7, 305-315.

Muller, P. & Seeman, P. (1977) Brain neurotransmitter receptors after long-term haloperidol: Dopamine, acetylcholine, serotonin, alpha-adrenergic, and naloxone receptors. Life Sci. **21**, 1751-1758.

Murphy, S.N., Thayer, S.A. & Miller, R.J. (1987) The effects of excitatory amino acids on intracellular calcium in single mouse striatal neurons in vitro. J.Neurosci. 7, 4145-4158.

Murrin, L.C., Gale, K. & Kuhar, M.J. (1979) Autoradiographic localisation of neuroleptic and dopamine receptors in the caudate-putamen and substantia nigra : effects of lesions. Eur. J. Pharmacol. **60**, 229-235.

Nakabeppu, Y., Ryder, K. & Nathans, D. (1988) DNA binding activities of three murine jun proteins: Stimulation by fos. Cell 55, 907-915.

Naranjo, J.R., Mellstrom, B., Achaval, M. & Sassone-Corsi, P. (1991) Molecular pathways of pain: fos/jun-mediated activation of the prodynorphin gene through a non-canonical AP-1 site. Neuron **6**, 607-617.

Nelson, D.L., Herbet, A., Pichat, L., Glowinski, J. & Hamon, M. (1979) In vitro and in vivo disposition of 3H-methiothepin in brain tissues. Naunyn-Schmeideberg's Arch. Pharmacol. **310**, 25-33.

Neuberg, M., Adamkiewicz, J., Hunter, J.B. & Muller, R. (1989) A fos protein containing the jun leucine zipper forms a homodimer which binds to the AP-1 binding site. Nature **341(6239)**, 243-245.

Neuberg, M., Schuermann, M., Hunter, J.B. & Muller, R. (1989) Two functionally different regions in fos are required for the sequence specific DNA interaction of the fos/jun protein complex. Nature **338**, 589-590.

Nguyen T.V., Kosofsky B. E., Birbaum R., Cohen B.M. & Hymans S.E.(1992). Differential expression of c-fos and zif/268 in rat striatum after haloperidol, clozapine, and amphetamine. Proc. Natl. Acad. Sci. U.S.A. **89**, 4270-4274.

Nishikawa, T., Takashima, M. & Toru, M. (1983) Increased [3H] kainic acid binding in the prefrontal cortex in schizophrenia. Neurosci. Letts. **40(3),** 245-250.

Norman, A.B., Ford, L.M., Kolmompunporn, M. & Sanberg, P.R. (1990) Chronic treatment with MK801 increases the quinolinic acid-induced loss of D1 dopamine receptors in rat striatum. E.ur. J. Pharmacol. **176,** 363-366.

O'Connell, M.L., Canipari, R. & Strickland, S. (1987) Hormonal regulation of tissue plasminogen activator secretion and mRNA levels in rat granulosa cells. J. Biol Chem. **262,** 2339-2344.

O'Dell, S.J., La Hoste, G.J., Widmark, C.B., Shapiro, R.M., Potkin, S.G. & Marshall, J.F. (1990) Chronic treatment with clozapine or haloperidol differentially regulates dopamine and serotonin receptors in rat brain. Synapse **6(2),** 146-153.

O'Malley, K.L., Harmon, S., Tang, L. & Todd, R. (1991) The rat dopamine D4 receptor : sequence, gene structure, and demonstration of expression in the cardiovascular system. The New Biologist **4(2),** 137-146.

O'Shea, E.K., Rutkowski, R., Stafford, W.F. & Kim, P.S. (1989) Preferential heterodimer formation by isolated leucine zippers from fos and jun. Science **245(4918),** 646-648.

Oblin, A., Zivkovic, B. & Bartholini, G. (1984) Involvement of the D2 dopamine receptor in the neuroleptic-induced decrease in nigral substance P. Eur. J.Pharmacol. **105(1-2),** 175-177.

Orr, W.B., Gardiner, T.W., Stricker, E.M., Zigmond, M.J. & Berger, T.W. (1986) Short-term effects of dopamine-depleting brain lesions on spontaneous activity of striatal neurons: relation to local dopamine concentration and behaviour. Brain Res. **376(1),** 20-28.

Palacios, J.M., Rigo, M., Chinaglia, G. & Probst, A. (1990) Reduced density of striatal somatostatin receptors in Huntington's chorea. Brain Res. **552(2),** 342-346.

Palacios, J.M., waeber, C., Hoyer, D. & Mengod, G. (1990) Distribution of serotonin receptors. The neuropharmacology of serotonin. Annual N. Y. Acad. Sci. **600,** 36-52.

Paul, M.L., Graybiel, A.M., David, J.-C. & Robertson, H.A. (1992) D1-like and D2-like dopamine receptors synergistically activate rotation and c-fos expression in the dopamine-depleted striatum in a rat model of Parkinson's disease. J. Neurosci. **12,** 3729-3742.

Pedigo, N.W., Yamamura, H.I. & Nelson, D.L. (1981) Discrimination of multiple [H]-5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. J. Neurochem. **36,** 220-226.

Peroutka, S.J. & Snyder, S.H. (1980) Relationship of neuroleptic drug effects at brain dopamine, serotonin, alpha-adrenergic, and histamine receptors to clinical potency. Am. J. Psychiatry **137,** 1518-1522.

Peroutka, S.J. (1986) Pharmacological differentiation and characterisation of 5-HT1A, 5-HT1B and 5-HT1C binding sites in rat frontal cortex. J. Neurochem. **47,** 529-540.

Phelps, P.E., Houser, C.R., & Vaugn, J.E. (1985) Immunocytochemical localisation of choline acetyl transferase within the rat neostriatum. J. Comp. Neurol. **238,** 286-307.

Pin, J.-P., Weiss, S., Sebben, M., Kemp, D.E. & Bockaert, J. (1986) Release of endogenous amino acids from striatal neurones in primary culture. J. Neurochem. **47,** 594-603.

Piomelli, D., Pilon, C., Giros, B., Sokoloff, P., Martres, M.P., & Schwartz, J.C. (1991) Dopamine activation of the arachidonic acid cascade as a basis for D1/D2 receptor synergism. Nature **353(6340),** 164-167.

Plassat, J.L., Amlaiky, N. & Hen, R. (1993) Molecular cloning of a mammalian serotonin receptor that activates adenylate cyclase. Mol. Pharmacol. **44(2)**, 229-236.

Plassat, J.L., Boschert, U., Amlaiky, N. & Hen, R. (1992) The mouse 5-HT5 receptor reveals a remarkable heterogeneity within the 5-HT1D receptor family. The EMBO J. **11**, 4779-4786.

Pollack, A.E. & Wooten, G.F. (1992) D2 dopaminergic regulation of striatal preproenkephalin mRNA levels is mediated at least in part through cholinergic interneurons. Mol. Brain Res. **13**, 35-41.

Pompeiano, M., Palacios, J.M. & Mengod, G. (1994) Distribution of the serotonin 5-HT2 receptor family mRNAs: comparison between 5-HT_{2A} and 5-HT_{2C} receptors. Mol. Brain Res. **23**, 163-178.

Pritchett, D.B., Bach, A.W., Wozny, M., Taleb, O., Dal Toso, R., Shih, J.C. & Seeberg, P.H. (1988) Structure and functional expression of cloned rat 5-HT2 receptor. The EMBO J. **7**, 4135-4140.

Prywes, R. and Roeder, R.G. (1986) Inducible binding of a factor to the c-fos enhancer. Cell **47(5)**, 777-784.

Quik, M., Emson, P.C. & Joyce, E. (1979) Dissociation between the presynaptic dopamine-sensitive adenylate cyclase and 3H-spiperone binding sites in rat substantia nigra. Brain Res. **167**, 355-365.

Radke, J.M., MacLennan, A.J., Vincent, S.R. & Fibiger, H.C. (1988) Comparison between short and long-term haloperidol administration on somatostatin and substance P concentrations in the rat brain. Brain Res. **445(1)**, 55-60.

Rauscher, F.J., Sambucetti, L.C., Curran, T., Distel, R.J. & Spiegel mann B.M. (1988) A common DNA binding site for fos protein complexes and transcription factor AP-1. Cell **52**, 471-480.

Rauscher, F.J., Voulalas, P.J., Franza, B.R. & Curran, T. (1988) Fos and jun bind cooperatively to the AP-1 site: reconstruction in vitro. Genes. Dev. **2**, 1687-1699.

Reinikainen, K.J., Koponen, H., Jolkkonen, J. & Riekkinen, P.J. (1990) Decreased somatostatin-like immunoreactivity in the cerebrospinal fluid of chronic schizophrenic patients with cognitive impairment. Psychiatry Res. **33(3)**, 307-312.

Reynolds, G.P. (1988) Post-mortem neurochemistry of schizophrenia. Psychol. and Med. **18(4)**, 793-797.

Reynolds, G.P. & Czudek, C. (1988) Status of the dopaminergic system in post-mortem brain in schizophrenia. Psychopharmacol. Bull. **24(3)**, 345-347.

Reyntjens, A., Gelders, Y.G., Hoppenbrouwers, M.-L.J.A. & Vanden Bussche, G. (1986) Thymostenic effects of ritanserin (R 55667), a centrally acting serotonin S2 receptor blocker. Drugs and Dev.l Res. **8**, 205-211.

Richelson, E. (1984) Neuroleptic affinities for human brain receptors and their use in predicting adverse effects. J. Clin. Psychiatry **45(8)**, 331-336.

Robertson G.S. & Fibiger H.C. (1992). Neuroleptics increase c-fos expression in the forebrain: contrasting effects of haloperidol and clozapine. Neuroscience. **46**, 315-328.

Robertson G.S., Vincent S.R. & Fibiger H.C. (1990). Striatonigral projection neurons contain D1 dopamine receptor- activated c-fos. Brain Res. 523(2), 288-290.

Robertson G.S., Vincent S.R. & Fibiger H.C. (1992). D1 and D2 dopamine receptors differentially regulate c-fos expression in striatonigral and striatopallidal neurons. Neuroscience **49**, 285-296.

Robertson, G.S., Herrera, D.G., Dragunow, M. & Robertson, H.A. (1989) L-DOPA activates c-fos in the striatum ipsilateral to a 6-

hydroxydopamine lesion of the substantia nigra. Eur. J. Pharmacol. **159**, 99-100.

Robertson, H.A. (1992) Dopamine receptor interactions: some implications for the treatment of Parkinson's disease. Trends in Neurosci. **15(6)**, 201-206.

Robinson, D.S., Alms, D.R., Shrotriya, R.C., Messina, M. & Wickramaratne, P. (1989) Serotoninergic anxiolytics and treatment of depression. Psychopathology **22**, 27-36.

Robinson, T.E. & Whishaw, I.Q. (1988) Normalisation of extracellular dopamine in striatum following recovery from a partial unilateral 6-OHDA lesion of the substantia nigra : a microdialysis study in freely moving rats. Brain Res. **450(1-2)**, 209-224.

Robison, G.A. & Sutherland, E.W. (1971) Cyclic AMP and the function of eukaryotic cells: an introduction. Annal. New York Acad. Sci. **185**, 5-9.

Roesler, W.J., Vandenbark, G.R. & Hanson, R.W. (1989) Identification of multiple protein binding domains in the promoter-regulatory region of the phosphoenolpyruvate carboxykinase (GTP) gene. J. Biol. Chem. **264(16)**, 9657-9664.

Rogaeva, E.A., Chumakova, M.M., Derunova, N.N., Pozharitskaia, D.A., Kopeiko, G.I., Tsutsul'kovskaia, M.I. & Tsibezov, V.V. (1990) Anti somatostatin autoantibodies in the blood serum of patients with schizophrenia. Zh. Nevropatol. Psikhiatr. Im. S.S. Korsakova. **90(7)**, 82-84.

Romano G.J., Shivers B.D., Harlan R.E. & Pfaff D.W.(1987) Haloperidol increases proenkephalin mRNA levels in the caudate-putamen of the rat: A quantitative study at the cellular level using insitu hybridisation. Mol. Brain Res. **2**, 33-41.

Rosen, O.M., Erlichman, J. & Rubin, C.S. (1975) Molecular structure and characterisation of bovine heart protein kinase. Adv. Cyclic Nucleotide Res. **5**, 253-263.

Rossum, J.M.-van. (1967) The effect of psychostimulants on the central and autonomic nervous system. Schweiz. Z. Sportmed. **15(1)**, 26-40.

Roth, B.L., Ciaranello, R.D. & Meltzer, H.Y. (1991) Binding of typical and atypical antipsychotic agents to transiently expressed 5-HT1c receptors. J. Pharmacol. and Exp. Ther. **260(3)**, 1361-1365.

Roth, B.L., Craigo, S.C., Choudhary, M.S., Uluer, A., Monsma, F.J., Shen, Y., Meltzer, H. & Sibley, D.R. (1993) Binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors. J. Pharmacol. and Exp. Ther. **268(3)**, 1403-1410.

Rupniak, N.M., Kilpatrick, G., Hall, M.D., Jenner, P. & Marsden, C.D. (1984) Differential alterations in striatal dopamine receptors ensitivity induced by repeated administration of clinically equivalent doses of haloperidol. Psychopharmacol.- Berl. **84(4)**, 512-519.

Ryder K., Lau L.F. & Nathens D.(1988). A gene activated by growth factors is related to the oncogene v-jun. Proc. Natl. Acad. Sci. U.S.A. **85**, 1487-1491.

Sabol S.L., Yoshikawa K. & Hong J.S.(1983). Regulation of met-enkephalin precursor mRNA in rat striatum by haloperidol and lithium. Biochem. Biophys. Res. Commun. **113**, 391-399.

Sales, N., Martres, M.P., Bouthenet, M.L. & Schwartz, J.C. (1989) Ontogeny of dopaminergic D2 receptors in the rat nervous system: characterisation and detailed autoradiographic mapping with [^{125}I]iodosulpiride. Neuroscience **28**, 673-700.

Salin P., Mercugliano M. & Chesselet M.F.(1990). Differential effects of chronic treatment with haloperidol and clozapine on the level of

preprosomatostatin mRNA in the striatum, nucleus accumbens, and frontal cortex of the rat. Cell. Mol. Neurobiol. **10,** 127-145.

Saller C.F., Kreamer L.D., Adamovage L.A. & Salama A.I. (1989). Dopamine receptor occupancy invivo: measurement using N-ethoxycarbonyl-2-ethoxy-,2-dihydroquinoline(EEDQ). Life Sci. **45**, 917-929.

Saller, C.F. & Salama, A.I. (1986) D1 and D2 dopamine receptor blockade: interactive effects in vitro and in vivo. J. Pharmacol. and Exp. Ther. **236(3),** 714-720.

Saller, C.F., Cyzupryana, M.J. & Salama, A.I. (1990) 5-HT2 receptor blockade by ICI 169,369 and other 5-HT2 antagonists modulates the effects of D2 dopamine receptor blockade. J. Pharmacol. and Exp. Ther. **253**, 1162-1170.

Samuel, D., Errami, M. & Nieoullon, A.J. (1990) Localization of N-methyl-D-aspartate receptors in the rat striatum : effects of specific lesions on the 3H-(2- carboxypiperazin- 4-yl) propyl- 1- phosphonic acid binding. J. Neurochem. **54**, 1926-1933.

Sandell, J.H., Graybiel, A.M. & Cheeselet, M.F. (1986) A new enzyme marker for striatal compartmentalization: NADPH diaphorase activity in the caudate nucleus and putamen of the cat. J. Comp. Neurol. **243(3),** 326-334.

Sanders-Bush, E. & Breeding, M. (1988) Putative and selective 5-HT2 receptor antagonists block serotonin 5-HT1C receptors in choroid plexus. J. Pharmacol. and Exp. Ther. **247**, 169-173.

Sanders-Bush, E. and Breeding, M. (1990) Serotonin 1C receptor reserve in choroid plexus masks receptor subsensitivity. J. Pharmacol. and Expl Ther. **252**, 984-988.

Savasta, M., Dubois, A., Benavides, J. & Scatton, B. (1986) Different neuronal location of [3H] SCH23390 binding sites in pars reticulata and pars

compacta of the substantia nigra in the rat. Neurosci. Letts. **72(3)**, 265-271.

Schiavi, G.B., Brunet, S., Rizzi, C.A. & Ladinsky, H. (1993) Identification of serotonin 5-HT4 recognition sites in the porcine caudate nucleus by radioligand binding. Neuropharmacol. **33(3,4)**, 543-549.

Schoepp, D.D., Johnson, B.J., Smith, E.C.R. & Mcquaid, L.A. (1990) Stereoselectivity and mode of inhibition of phosphoinositide-coupled excitatory amino acid receptors by 2-amino-3-phosphonopropionic acid. Mol. Pharmacol. **38**, 222-228.

Schotte, A., Janssen, P.M.F., Megens, A.A.H.P. & Leysen, J.E. (1993) Occupancy of central neurotransmitter receptors by risperidone, clozapine and haloperidol, measured ex vivo by quantitative autoradiography. Brain Res.**631**, 191-202.

Schutte, J., Viallet, J., Nau, M., Segal, S., Fedorka, J. & Minna, J. (1989) Jun-B inhibits and c-fos stimulates the transforming and trans-activating activities of c-jun. Cell **59**, 987-997.

Schwartz, J.P. & Simantov, R. (1988) Developmental expression of preproenkephalin mRNA in rat striatum and in striatal cultures. Dev. Brain Res. **40**, 311-314.

Sculptoreanu, A., Scheuer, T. & Catterall, W.A. (1993) Voltage dependent potentiation of L-type calcium channels due to phosphorylation by cAMP-dependent protein kinase. Nature **364(6434)**, 240-243.

Sebben, M., Ansanay, H., Bockaert, J. & Dumuis, A. (1994) 5-HT6 receptors positively coupled to adenylyl cyclase in striatal neurones in culture. Neuroreport **5**, 2553-2557.

Seeger, T.F., Thal, L. & Gardner, E.L. (1982) Behavioural and biochemical aspects of neuroleptic-induced dopaminergic supersensitivity: studies with chronic clozapine and haloperidol. Psychopharmacol.-Berl. **76(2)**, 182-187.

Seeman, P. (1980) Brain dopamine receptors. Pharmacol. Rev. **32(3),** 229-312.

Seeman, P. (1987) Dopamine receptors and the dopamine hypothesis of schizophrenia. Synapse **1(2),** 133-152.

Seeman, P. & Van Tol, H.H.M. (1993) Dopamine receptor pharmacology. Curr. Opinions on Neurol. and Neurosurgery **6(4),** 602-608.

Seeman, P., Chau-Wong, M., Tedesco, J. & Wong, K. (1975) Brain receptors for antipsychotic drugs and dopamine: Binding assays. Proc. Natl. Acad. Sci. U.S.A. **72(11),** 4376-4380.

Seeman, P., Guan, H.C. & Van Tol, H.H.M. (1993) Dopamine D4 receptors elevated in schizophrenia. Nature **365(6445),** 441-445.

Seeman, P., Guan, H.C., Van Tol, H.H. & Niznik, H.B. (1993) Low density of dopamine D4 receptors in Parkinson's, schizophrenia, and control brain striata. Synapse **14(4),** 247-253.

Seeman, P., Lee, T., Chau-Wong, M. & Wong, K. (1976) Antipsychotic drug doses and neuroleptic/ dopamine receptors. Nature **61,** 717-719.

Seeman, P., Niznik, H.B., Guan, H-C., Booth, G. & Ulpian, C. (1989) Link between D1 and D2 dopamine receptors is reduced in schizophrenia and Huntington diseased brains. Proc. Natl. Acad. Sci. U.S.A. **86,** 10156-10160.

Setler, P.E., Sarau, H.M., Zirkle, C.L. & Saunders, H.L. (1978) The central effects of a novel dopamine agonist. Eur. J. Pharmacol. **50,** 419-430.

Severson, J.A., Robinson, H.E. & Simpson, G.M. (1984) Neuroleptic-induced striatal dopamine receptor supersensitivity in mice: relationship to dose and drug. Psychopharmacol.-Berl. **84(1),** 155-158.

Shen, Y., Monsma, F.J., Metcalf, M.A., Jose, P.A., Hamblin, M.W. & Sibley, D.W. (1993) Molecular cloning and expression of a 5-hydroxytryptamine7 serotonin receptor subtype. J. Biol. Chem. **268,** 18200-18204.

Sheng, M. & Greenberg, M.E. (1990) The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron **4**, 477-485.

Sheng, M., Dougan, S.T., McFadden, G. and Greenberg, M.E. (1988) Calcium and growth factor pathways of c-fos transcriptional activation require distinct upstream regulatory sequences. Mol. Cell. Biol. **6(7)**, 2787-2796.

Sheng, M., McFadden, G. & Greenberg, M.E. (1990) Membrane depolarisation and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron 4(4), 571-582.

Shenker, A., Maayani, S., Weinstein, H. & Green, J.P. (1987) Pharmacological characterisation of two 5-hydroxytryptamine receptors coupled to adenylate cyclase in guinea pig hippocampal membranes. Mol. Pharmacol. **31**, 357-367.

Shibata, K., Haverstick, D.M. & Bannon, M.J. (1990) Tachykinin gene expression in rat limbic nuclei: Modulation by dopamine antagonists. J. Pharmacol. Exp. Ther. 255(1), 388-392.

Shiomi, H., Watson, S.J., Kelsey, J.E. & Akil, H. (1986) Pretranslational and posttranslational mechanisms for regulating beta-endorphin-adrenocorticotropin of the anterior pituitary. Endocrinology **199**, 1793-1799.

Sirinathsinghji, D.J.S., Morris, B.J., Wisden, W., Northrop, A., Hunt, S.P. & Dunnett, S.B. (1990) Gene expression in striatal grafts I: cellular localisation of neurotransmitter mRNA's. Neuroscience **34**, 675-686.

Sirinathsinghji, D.J.S., Schulidoi, R., Heavens, R.P., Dixon, A. Iversen, S.D. & Hill, R.G. (1994) Temporal changes in the messenger RNA levels of cellular immediate early genes and neurotransmitter/ receptor genes in the rat neostriatum and substantia nigra after acute treatment with

eticlopride, a dopamine D2 receptor antagonist. Neuroscience **62(2)**, 407-423.

Sivam S.P., Strunk C., Smith D.R. & Hong J.S.(1986). Proenkephalin A gene regulation in rat striatum.Influence of lithium and haloperidol. Mol. Pharmacol. **30**, 186-191.

Sivam, S.P., Breese, G.R., Krause, J.E., Napier, T.C., Muller, R.A. & Hong, J.S. (1987) Neonatal and adult 6-hydroxydopamine-induced lesions differentially alter tachykinin and enkephalin gene expression. J. Neurochem. **49**, 1623-1633.

Smith, Y. & Parent, A. (1986) Neuropeptide Y immunoreactive neurons in the striatum of cat and monkey: morphological characteristics, intrinsic organisation and co-localisation with somatostatin. Brain Res. **372(2)**, 241-245.

Snell, L. & Johnson, K. (1986) Characterisation of the inhibition of excitatory amino-acid-induced neurotransmitter release in the rat striatum by phencyclidine-like drugs. J. Pharmacol. and Exp. Ther. **238**, 938-946.

Sokoloff, P., Giros, B., Martres, M.P., Bouthenet, M.L. & Schwartz, J.C. (1990) Molecular cloning and characterisation of a novel dopamine receptor (D3) as a target for neuroleptics. Nature **347**, 146-151.

Sokoloff, P., Levesque, D., Martres, M.P., Lannfelt, L., Diaz, G. & Pilon, C. (1992) The dopamine D3 receptor as a key target for antipsychotics. Clin. Neuropharmacol. **15(1)**, 456A-457A.

Sonnnenberg J.L., Rauscher III F.J., Morgan J.I. & Curran T.(1989). Regulation of proenkephalin by fos and jun. Science, **246**, 1622-1625.

Spencer, H.J. (1976) Antagonism of cortical excitation of striatal neurons by glutamic acid diethyl ester: evidence for glutamic acid as an excitatory transmitter in the rat striatum. Brain Res. **102(1)**, 91-101.

Spinelli, W. & Ishii, D.N. (1983) Tumour promoter receptors regulating neurite formation in cultured human neuroblastoma cells. Cancer Res. **43(9),** 4119-4125.

Sproson, T.S. & Woodruff, G.N. (1990) Polyamines potentiate NMDA-induced whole cell currents in cultured striatal neurons. Eur. J. Pharmacol. **179,** 477-478.

Standifer, K.M., Chien, C.C., Wahlestedt, C., Brown, G.P. & Pasternak, G.K. (1993) Selective loss of delta opioid analgesia and binding by antisense oligonucleotides to a delta opioid receptor. Neuron, **12(4),** 805-810.

Stein, C.A. & Cheng, Y.C. (1993) Antisense oligonucleotides as therapeutic agents- is the bullet really magical. Science **261(5124),** 1004-1012.

Steinbusch, H.W.M. (1984) in handbook of chemical neuroanatomy: classical transmitters and transmitter receptors in the CNS, eds. Bjorklund, A., Hokfelt, T. and Kuhar, M.J. (Elsevier, Amsterdam), Part II (3), 68-125.

Steiner, H. & Gerfen, C.R. (1993) Cocaine-induced c-fos messenger RNA is inversely related to dynorphin expression in striatum. J. Neurosci. **13(12),** 5066-5081.

Stevens, J.R. (1973) An anatomy of schizophrenia? Arch. Gen. Psychiatry. **29(2),** 177-189.

Stoof, J.C. & Kebabian, J.W. (1981) Opposing roles for D1 and D2 dopamine receptors in efflux of cyclic AMP from rat neostriatum. Nature **294(5839),** 366-368.

Struyker Boudier, H.A.J., Gielen, W., Cools, A.R. & van Rossum, J.M. (1974) Pharmacological analysis of dopamine-induced inhibition and excitation of neurons of the snail Helix Aspersa. Arch. International Pharmacodynopia and Ther. **209,** 324-331.

Stubbs, C.M., Connor, H.E. & Feniuk, W. (1991) BMY 7378 is an agonist at 5-HT1A receptors mediating hypotension and renal-sympatho inhibition in anaesthetised cats. Eur. J. Pharmacol. **197,** 113-116.

Sunahara, R.K., Niznik, H.B., Wiener, D.M., Stormann, T.M., Brann, M.R., Kennedy, J.L., Gelernter, J.E., Rozmahel, R., Yang, Y., Israel, Y., Seeman, P. & O'Dowd, B.F. (1990) Human dopamine D1 receptor encoded on an intronless gene on chromosome 5. Nature **347**, 80-83.

Surmeier, D.J., Eberwine, J., Wilson, C.J., Stefani, A. & Kitai, S.T. (1992) Dopamine receptor subtypes colocalise in rat striatonigral neurons. Proc. Natl. Acad. Sci. U.S.A., 89(21), 10178-10182.

Surmeier, D.J., Kita, H. & Kitai, S.T. (1988). The expression of g-aminobutyric acid and leu-enkephalin immunoreactivity in primary monolayer cultures of rat striatum. Dev. Brain Res. **42,** 265-282.

Sutherland, E.W. & Wosilait, W.D. (1955) Inactivation and activation of liver phosphorylase. Nature, **175**, 169.

Szekely, A.M., Barbaccia, M.L., Alho, H. & Costa, E. (1989) In primary cultures of cerebellar granule cells the activation of N-methyl-D-aspartate-sensitive glutamate receptors induces c-fos mRNA expression. Mol. Pharmacol. **35,** 401-408.

Szekely, A.M., Barbaccia, M.L. & Costa, E. (1987) Activation of specific glutamate receptor subtypes increases c-fos proto-oncogene expression in primary cultures of cerebellar granule cells. Neuropharmacol. **26,** 1770-1782.

Szekely, A.M., Costa, E. & Grayson, D.R. (1990) Transcriptional program coordination by N-methyl-D-aspartate-sensitive glutamate receptor stimulation in primary cultures of cerebellar neurons. Mol. Pharmacol. **38**, 624-633.

Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. & Nishizuka, Y. (1979) Unsaturated diacylglycerol as a possible messemger for the activation of calcium-activated, phospholipid-dependent protein kinase system. Biochem. Biophys. Res. Commun. **91(4)**, 1218-1224.

Tang, F., Costa, E. & Schwartz, J.P. (1983) Increase of proenkephalin mRNA and enkephalin content of rat striatum after daily injection of haloperidol for 2-3 weeks. Proc. Natl. Acad. Sci. U.S.A. **80(12),** 3841-3844.

Tao, M., Salas, M.L. & Lipmann, F. (1970) Mechanism of activation of adenosine 3', 5'-monophosphate of a protein phosphokinase from rabbit reticulocytes. Proc. Natl. Acad. Sci. U.S.A. **67(1),** 408-414.

Tao, W. & Lai, E. (1992). Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. Neuron **8**, 957-966.

Taquet, H., Javoy-Avid, F., Giraud, P., Legrand, J.C., Agid, Y. & Cesselin, F. (1985) Dynorphin levels in Parkinsonian patients: Leu-enkephalin production from either proenkephalin A or prodynorphin in human brain. Brain Res. **341**, 390-392.

Taquet, H., Javoy-Avid, F., Hamon, M., Legrand, J.C., Agid, Y. & Cesselin, F. (1983) Parkinson's disease affects differently Met and Leu-enkephalin in the human brain. Brain Res. **280**, 379-382.

Tenovno, O., Runne, V.K. & Viljanen, M.K. (1984) Substance P immunoreactivity in the post-mortem parkinsonian brain. Brain Res. **303**, 113-116.

Thayer, S.A., Murphy, S.N. & Miller, R.J. (1986). Widespread distribution of dihydropyridine-sensitive calcium channels in the central nervous system. Mol. Pharmacol. **30**, 505-509.

Tiberi, M., Jarvie, K.R., Silvia, C., Falardeau, P., Gingrich, J.A., Godinot, N., Bertrand, L., Yang-Feng, T.L., Fremeau, R.J. & Caron, M.G. (1991) Cloning, molecular characterisation and chromosomal assignment of a gene encoding a second D1 dopamine receptor subtype: differential expression pattern in rat brain compared with the D1a receptor. Proc. Natl. Acad. Sci. U.S.A. **88**, 7491-7495.

Tortora, G., Clair, T. & Cho-Chung, Y.S., (1990) An antisense oligonucleotide targeted against the type II_β regulatory subunit mRNA of protein kinase inhibits cAMP-induced differentiation in HL-60 leukaemia cells without effecting phorbol ester effects Proc. Natl. Acad. Sci. U.S.A. **87**, 705-708.

Traber, J., and Glaser, T. (1987) 5-HT1A receptor related anxiolytics. Trends in Pharmacol. Sci. **8**, 432-437.

Treisman, R. (1986) Identification of a protein-binding site that mediates transcriptional response of the c-fos gene to serum factors. Cell. **46(4)**, 567-574.

Tsou, A.P., Kosaka, A., Bach, C., Zuppan, P., Yee, C., Tom, L., Alvarez, R., Ramsey, S., Bonhaus, D.W. & Stefanich, E. (1994) Cloning and expression of a 5-hydroxytryptamine-7 receptor positively coulped to adenylate cyclase. J. Neurochem. **63(2)**, 456-464.

Turner, R. & Tjian, R. (1989) Leucine repeats and an adjacent DNA binding domain mediate the formation of functional c-fos-c-jun heterodimers. Science **243(4899)**, 1689-1694.

Ugedo, L., Grenhoff, J. & Svensson, T.H. (1989) Ritanserin, a 5-HT2 receptor antagonist, activates midbrain dopamine neurons by blocking serotoninergic inhibition. Psychopharmacol. **28**, 45-50.

Ungerstedt, U. (1971) Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigrostriatal dopamine system. Acta. Physiol. Scandanavia. Supplement 367, 69-93.

Unsworth, C.D. and Molinoff, P.B. (1993) Characterisation of a 5-hydroxytryptamine receptor in mouse neuroblastoma N18TG2 cells. J. Pharmacol. and Exp. Ther. **269(1)**, 246-270.

Vaccarino, F.M., Hayward, M.D., Le, H.N., Hartigan, D.J., Duman, R.S. & Nestler, E.J. (1993) Induction of immediate early genes by cyclic

AMP in primary cultures of neurons from rat cerebral cortex. Mol. Brain Res. **19**, 76-82.

Vaccarino, F.M., Hayward, M.D., Nestler, E.J., Duman, R.S. & Tallman, J.F. (1992) Differential induction of immediate early genes by excitatory amino acid receptor types in primary cultures of cortical and striatal neurons. Mol. Brain Res. **12**, 233-241.

Valerio, A., Alberici, A., Tinti, C., Spano, P. & Memo, M. (1994) Antisense strategy unravels a dopamine receptor distinct from the D2 subtype, uncoupled with adenylyl cyclase, inhibiting prolactin release from rat pituitary cells. J. Neurochem. **62(4)**, 1260-1266.

Vallar, L., Muca, C., Magni, M., Albert, P., Bunzow, J., Meldolesi, J. & Civelli, O. (1990) Differential coupling of dopaminergic D2 receptors expressed in different cell types. Stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis in Ltk- fibroblasts, hyperpolarisation, and cytosolic-free calcium concentration decrease in GH4Cl cells. J. Biol. Chem. **265(18)**, 10320-10326.

Valtschanoff, J.G., Weinberg, R.J. and Rustioni, A. (1992). NADPH diaphorase in the spinal cord of rats. J. Comp. Neurol. **321**, 209-222.

Van Beveren, C., van Straaten, F., Curran, T., Muller, R. & Verma, I.M. (1983) Analysis of FBJ-MuSV provirus and c-fos (mouse) gene reveals that viral and cellular fos gene products have differentcarboxy termini. Cell 32, 1241-1255.

Van Der Krol, A.R., Mol, J.N.M. & Stuitje, A.R. (1988) Modulation of eukaryotic gene expression by complementary RNA or DNA sequences. Biotechniques **6**, 958-976.

Van Tol, H.H.M., Bunzow J.R., Guan H.C., Sunahara R.K., Seemen P., Niznik H.B., & Civelli O.(1991) Cloning of the gene for a human dopamine D4

receptor with high affinity for the antipsychotic clozapine. Nature **350**, 610-614.

Van Wijngaarden, I., Tulp, M. & Soudijn, W. (1990) The concept of selectivity in 5-HT receptor research. Eur. J. Pharmacol. **188**, 301-312.

Venturelli, D., Travali, S. & Calabretta, B. (1990) Inhibition of T-cell proliferation by a MYB antisense oligomer is accompanied by selective down-regulation of DNA polymerase α expression. Proc. Natl. Acad. Sci. U.S.A. **87**, 5963-5967.

Vilijn, M-H., Vaysse, P. J-J., Zukin, S. & Kessler J.A. (1988). Expression of proenkephalin mRNA by cultured astrocytes and neurons. Proc. Natl. Acad. Sci. U.S.A. **85**, 6551-6555.

Vincent, S.R. & Johansson, O. (1983) Striatal neurones containing both somatostatin and avian pancreatic poly-peptide-like immunoreactivities and NADPH-diaphorase activity. J. Comp. Neurol. **217**, 264-270.

Voigt, M.M., Laurie, D.J., Seeberg, P.H. & Bach, A. (1991) Molecular cloning and characterisation of a rat cDNA encoding a 5-hydroxytryptamine1B receptor. The EMBO J. **10(13)**, 4017-4023.

Von-Voigtlander, P.F. & Moore, K.E. (1973) Involvement of nigrostriatal neurons in the in vivo release of dopamine by amphetamine, amantadine and tyramine. J. Pharmacol. and Exp. Ther. **184(3)**, 542-552.

Vuillet, J., Kerkerian-Le Goff, L., Kachidian, P., Dusticier, G., Bosler, O. & Nieoullon, A. (1990) Striatal neuropeptide Y-containing neurons receive GABA-ergic afferents and may also contain GABA. Eur. J. Neurosci. **2**, 672-681.

Waeber, C. & Palacios, J.M. (1994) Binding sites for 5-hydroxytryptamine-2 receptor agonists are predominantly located in striosomes in the human basal ganglia. Brain Res.- Mol. Brain Res. section **24(1-4)**, 199-209.

Waeber, C., Schoeffter, P., Palacios, J.M. & Hoyer, D. (1989) 5-HT1D receptors in guinea-pig and pigeon brain. Radioligand binding and biochemical studies. Naunyn. Schmiedebergs Arch. Pharmacol. **340(5),** 479-485.

Wagner, J.A., McDonald, T.V., Ngheim, P.T., Lowe, A.W., Schulman, H., Gruenert, D.C., Struyer, L. & Gardner, P. (1992) Antisense oligodeoxynucleotides to the cystic fibrosis transmembrane conductance regulator inhibit cAMP-activated but not calcium-activated currents. Proc. Natl. Acad. Sci. U.S.A. **89**, 6785-6789.

Wahlestedt, C. (1994) Antisense oligonucleotide strategies in neuropharmacology. Trends in Pharmacol. Sci. **15**, 42-45.

Wahlestedt, C., Golanov, E., Yamamoto, S., Yee, F., Ericson, H., Yoo, H., Inturissi, C.E. & Reiss, D.J. (1993) Antisense oligodeoxynucleotides to NMDA-R1 receptor channel protect cortical neurons from excitotoxicity and reduces focal ischaemic infarctions. Nature **363**, 260-263.

Wahlestedt, C., Pich, E.M., Koob, G.F., Yee, F. & Heilig, M. (1993) Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides. Science **259**, 528-531.

Wainscott, D.B., Cohen, M.L., Schenk, K.W., Audia, J.E., Nissen, J.S., Baez, M., Kursar, J.D., Lucaites, V.L. & Nelson, D.L. (1993) Pharmacological characteristics of the newly cloned rat 5-hydroxytryptamine 2F receptor. Mol. Pharmacol. **43**, 419-426.

Walder, R.Y. & Walder, J.A. (1988) Role of RNase H in hybrid arrested translation by antisense oligodeoxynucleotides. Proc. Natl. Acad. Sci. U.S.A. **85**, 5011-5015.

Walker, P.D., Chovanes, G.I. & McAllister, J.P. (1987) Identification of acetylcholinesterase reactive neurones and neuropil in neostriatal transplants. J. Comp. Neurol. **259**, 1-12.

Walker, P.D., Riley, L.A., Hart, R.P. & Jonakait, G.M. (1991) Serotonin regulation of tachykinin biosynthesis in the rat neostriatum. Brain Res. **546**, 33-39.

Wander, T.J., Nelson, A., Okazaki, H. & Richelson, E. (1987) Antagonism by neuroleptics of serotonin 5-HT1A and 5-HT2 receptors of normal human brain in vitro. Eur. J. Pharmacol. **143**, 279-282.

Wang, H., Watkins, D.C. & Malbon, C.C. (1992) Antisense oligodeoxynucleotides to G_s protein α-subunit sequence accelerate differentiation of fibroblasts to adipocytes. Nature **358**, 334-337.

Watling, K.J., Beer, M.S., Stanton, J.A. & Newberry, N.R. (1990) Interaction of the atypical neuroleptic clozapine with 5-HT3 receptors in the cerebral cortex and superior ganglion of the rat. Eur. J. Pharmacol. **182**, 465-472.

Weick, B.G. & Walters, J.R. (1987) Effects of D1 and D2 dopamine receptor stimulation on the activity of substantia nigra pars reticulata neurons in 6-OHDA lesioned rats : D1/D2 coactivation induces potentiated responses. Brain Res. **405**, 234-246.

Weihmuller, F.B., Ulas, J., Nguyen, L., Cotman, C.W. & Marshall, J.F. (1992) Elevated NMDA receptors in parkinsonian striatum. Neuroreport **3**, 977-980.

Weinshank, R.L., Adham, N., Macchi, M., Olsen, M.A., Branchek, T.A. & Hartig, P.R. (1991) Molecular cloning and characterisation of a high affinity dopamine receptor (D1 beta) and its pseudogene. J. Biol. Chem. **266(33)**, 22427-22435.

Weiss, B., Zhou, L.-W., Zhang, S.-P. & Quin, Z.-H. (1993) Antisense oligodeoxynucleotide inhibits D2 dopamine receptor-mediated behaviour and D2 messenger RNA. Neuroscience **55(3)**, 607-612.

Weiss, S., Pin, J-P., Sebben, M., Kemp, D.E., Sladeczek, F., Gabrion, J. & Bockaert, J. (1986) Synaptogenesis of cultured striatal neurons in

serum- free medium: a morphological and biochemical study. Proc. Nal. Acad. Sci. U.S.A. **83**, 2238-2242.

Wickstrom, E.L., Bacon, T.A., Gonzalez, A., Freeman, D.L., Lyman, G.H. & Wickstrom, E. (1988) c-myc protein expression are inhibited by an antisense pentadecaoxynucleotide targeted against c-myc mRNA. Proc. Natl. Acad. Sci. U.S.A. **85**, 1028-1032.

Wilk, S., Watson, E. & Stanley, M.E. (1975) Differential sensitivity of two dopaminergic structures in rat brain to haloperidol and to clozapine. J. Pharmacol. and Exp. Ther. 195(2), 265-270.

Wilkinson, L.O. & Dourish, C.T. (1991) Serotonin and animal behaviour. in serotonin receptor subtypes: basic and clinical aspects (S.J. Peroutka, eds.), Wiley-Liss, New York, 147-210.

Williams, J.S., Berbekar, I. & Weiss, S. (1991) NMDA evokes the release of somatostatin from striatal interneurones in primary culture. Neuroscience **43**, 437-444.

Wilmot, C.A. & Szczepanik, A.M. (1989) Effects of acute and chronic treatment with clozapine and haloperidol on serotonin (5-HT2) and dopamine (D2) receptors in the rat brain. Brain Res. **487(2)**, 288-298.

Wisden, W. Morris, B.J., Darlinson, M.G., Hunt, S.P. & Barnard E.A. (1988). Distinct $GABA_A$ receptor a-subunit mRNA's show differential patterns of expression in bovine brain. Neuron **1**, 937-947.

Wisden, W., Morris, B.J. & Hunt, S.P. (1991). In situ hybridisation with synthetic DNA probes. Molecular Neurobiology- A practical approach, **volume II**, 205-225.

Yakubov, L.A., Deeva, E.A., Zarytova, V.F., Ivanova, E.M., Ryte, S., Yurchenk, L.A. & Vlassov, V.V. (1989) Mechanism of oligonucleotide uptake by cells: involvment of specific receptors? Proc. Natl. Acad. Sci. U.S.A. **86**, 6454-6458.

Yamamoto, K.K., Gonzalez, G.A., Biggs, W.H. & Montminy, M.R. (1988) Phosphorylation induced binding and transcriptional efficacy of nuclear factor CREB. Nature 334(6182), 494-498.

Yamamoto, K.K., Gonzalez, G.A., Menzel, P., Rivier, J. & Montminy, M.R. (1990) Characterisation of a bipartite activator domain in transcription factor CREB. Cell. 60(4), 611-617.

Yoshikawa, K., Williams, C. & Sabol, S.L. (1984). Rat brain proenkephalin mRNA. J. Biol. Chem. **25**, 14,301-14,308.

Young, S.T., Porrino, L.J. & Iadarola, M.J. (1991) Cocaine induces striatal c-fos immunoreactive proyeins via dopaminergic D1 receptors. Proc. Natl. Acad. Sci. U.S.A. **88**, 1291-1295.

Young, W.S., Bonner, T.I. & Brann, M.R. (1986) Mesencephalic dopaminergic neurons regulate the expression of neuropeptide mRNAs in the rat forebrain. Proc. Natl. Acad. Sci. U.S.A. **83,** 9827-9831.

Zamecnik, P.C. & Stephenson, M.L. (1978) Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc. Natl. Acad. Sci. U.S.A. 75, 280-284.

Zamecnik, P.C., Goodchild, J., Taguchi, Y. & Sarin, P.S. (1986) Inhibition of replication and expression of human T-cell lymphotropic virus type III in culture cells by exogenous synthetic oligonucleotides complementary to viral RNA. Proc. Natl. Acad. Sci. U.S.A. **83**, 4143-4146.

Zang, M. & Creese, I (1993) Antisense oligonucleotide reduces brain dopamine D2 receptors: Behavioural correlates. Neurosci. Letts. **161**, 223-226.

Zerial, M., Toschi, L., Ryseck, R.P., Schuermann, M., Muller, R. & Bravo, R. (1989) The product of a novel growth factor activated gene, fos-B, interacts with jun proteins enhancing their DNA binding activity. The EMBO J. **8**, 805-813.

Zgombick, J.M., Weinshank, R.L., Macchi, M., Schecter, L.E., Branchek, T.A. & Hartig, P.R. (1991) Expression and pharmacological characterisation of a canine 5-hydroxytrptamine 1D receptor subtype. Mol. Pharmacol. **40(6),** 1036-1042.

Zhang, W.C., Ding, M.C. & Jiao, S.S. (1988) Intracaudate grafting of the adrenal medulla in the treatment of parkinson's disease. Chung Hua, Wai. Ko Tsa Chih. **26(1),** 1-5, 59.

Zhou, Q-Y, Grandy, D.K., Thambi, L., Kushner, J.A., Van Tol, H.H.M., Cone, R., Pribnow, D., Salon, J., Bunzow, J.R. & Civelli, O. (1990) Cloning and expression of human and rat D1 receptors. Nature **347**, 76-79.

Zifa E. & Fillion G.(1992). 5-hydroxytryptamine receptors. Pharmacol. Rev. **44,** 401-448.

Zigmond, M.J., Acheson, A.L., Stachowiak, M.K. & Stricker, E.M. (1984) Neurochemical compensation after nigrostriatal bundle injury in an animal model of preclinical parkinsonism. Arch. Neurol. **41(8),** 856-861.

Zigmond, R.E., Schwarzchild, M.A. & Rittenhouse, A.R. (1989) Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. Annal. Rev. Neurosci. **12,** 415-461.

Dubinsky, J.M. (1989) Development of inhibitory synapses among striatal neurons in vitro. J. Neurosci. **9(11)**, 3955-3965.

Mathis, J.M. (1992) Brain 4: a novel POU domain transcription factor exhibiting restricted brain-specific expression. EMBO J. **11(7)**, 2551-2561.

Wisden, W., Errington, M.L., Williams, S., Dunnett, S.B., Waters, C., Hitchcock, D., Evan, G., Bliss, T.V. & Hunt, S.P. (1990) Differential expression of immediate early genes in the hippocampus and spinal cord. Neuron **4(4)**, 603-614.

Wisden, W., & Morris, B.J. (1994) In situ hybridisation protocols for the brain. In Biological Techniques: A series of practical guides to new methods in modern biology. *Academic Press (Harecourt Brace & Company, Publishers)*

Civelli, O., Bunzow, J.R., & Grandy D.K. (1993). Molecular diversity of the dopamine receptors. Ann. Rev. Pharmacol and Toxicol 32, 281-307.