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ANTIDOPAMINERGIC EFFECT OF BENZODIAZEPINES AND MELATONIN IN RAT STRIATUM

By

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ABSTRACT

Benzodiazepines (BZ) are a class of drugs that are extensively used for their anxiolytic, anticonvulsant and sedative properties. The therapeutic actions of these drugs may be mediated through the central-type BZ receptors that are linked to the $GABA_A$ receptor complex ($BZ/GABA_A$). When administered in large doses, the pineal hormone, melatonin, can interact with BZ receptors. The pharmacological actions of melatonin, similar to those listed above for BZs, appear to be mediated primarily by $BZ/GABA_A$ receptors, although other BZ receptors may be involved. Recently, pharmacological doses of melatonin were found to decrease apomorphine-induced rotations in 6-hydroxydopamine lesioned rats.

The main objective of this thesis was to determine the mechanism(s) underlying the antidopaminergic effect of melatonin as well as BZs using the 6-hydroxydopamine lesion model of dopaminergic supersensitivity. It was hypothesized that the antidopaminergic action of BZs and melatonin was due to the ability of these drugs to either enhance GABAergic activity (since GABA can suppress striatal dopaminergic activity) or block cyclic AMP (cAMP) production since dopamine enhances cAMP formation in the striatum.

The major findings may be summarized as follows: 1) Clonazepam, a central-type BZ agonist, and melatonin blocked apomorphine-induced turning in lesioned animals; 2) intrastriatal injection of the $GABA_A$ antagonist, bicuculline, caused a significant reduction in the antidopaminergic effect of clonazepam and melatonin; 3) the peripheral-type BZ antagonist, PK 11195, attenuated the antidopaminergic effect of these drugs but with much

less potency than bicuculline; 4) the combination of bicuculline and PK 11195, injected directly into the striatum, completely blocked the antidopaminergic action of clonazepam or melatonin; 5) PK 11195 also blocked BZ-induced inhibition of cAMP production which is involved in striatal dopaminergic function. Therefore, in addition to a GABAergic mechanism, inhibition of a cAMP pathway may be a secondary mechanism in the antidopaminergic action of clonazepam and melatonin. In studying the effects of BZs on the cAMP pathway, a significant increase in the inhibitory effect of diazepam on adenylate cyclase (AC) activity was observed in striatal membranes from lesioned animals. Further studies indicated that this sensitization to the inhibitory effect of diazepam on AC activity may involve upregulation of BZ receptors as well as enhanced functional coupling of these receptors to inhibitory G proteins.

Taken together these findings indicate that the antidopaminergic effect of clonazepam and melatonin in lesioned animals involved at least two distinct mechanisms: 1) a predominant GABAergic action and 2) possibly the suppression of cAMP production in the striatum.

This thesis is dedicated to my grandmother, Alberta Louise Kow, who passed away two months after I started my doctorate.

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2-IMEL	2-iodomelatonin
5-HT	serotonin; 5-hydroxytryptamine
6-CLMEL	6-chloromelatonin
6-OHDA	6-hydroxydopamine
AC	adenylyl cyclase
ANOVA	analysis of variance
APO	apomorphine
ATP	adenosine 5'-triphosphate
BIC	bicuculline ($GABA_A$ receptor antagonist)
B_{MAX}	maximal binding site density
BSA	bovine serum albumin
BZ	benzodiazepine
$BZ/GABA_A$	$GABA_A$ receptor (central-type benzodiazepine receptor)
cAMP	cyclic adenosine 3',5' monophosphate
CHO	chinese hamster ovary cells
Cl^-	chloride ion
CLO	clonazepam; central-type benzodiazepine receptor agonist
CNS	central nervous system
CONTRA	contralateral (opposite side)
DIAZ	diazepam
DMSO	dimethylsulfoxide
DTT	dithiothreitol

EC_{50}	concentration of compound producing 50% of the maximal effect
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra acetic acid
ENK	enkephalin
FLU	flunitrazepam
FMZ	flumazenil; central-type benzodiazepine receptor antagonist
FSK	forskolin
G protein	guanine nucleotide binding protein
G_i (α)	G protein coupled to inhibition of adenylate cyclase (the α subunit of this G protein)
G_o	G (other) protein
G_s	G protein coupled to stimulation of adenylate cyclase
GABA	gamma-aminobutyric acid
GABA-T	α-oxoglutarate transaminase
GAD	glutamic acid decarboxylase
GDP	guanosine 5'-diphosphate
GLU	glutamine
GTP	guanosine 5'-triphosphate
HEPES	(N-[2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid])
IC_{50}	inhibitory concentration reducing radioligand binding by 50%
ip	intraperitoneal
IPSI	ipsilateral (same side)
kD	kilodalton
K_d	receptor ligand dissociation constant

MEL	melatonin; N-acetyl5-methoxytryptamine
mM	millimolar
NaCl	sodium chloride
NAS	N-acetylserotonin
nM	nanomolar
mRNA	messenger ribonuclei acid
PBR	peripheral-type benzodiazepine receptor
PK	PK 11195; peripheral-type benzodiazepine receptor antagonist
PKA	protein kinase A
pmol	picomolar
PMSF	phenylmethlysulfonylfluoride
QUIN	quinpirole (D_2 dopamine receptor agonist)
Ro5-4864	4'-chlorodiazepam
SCN	suprachiasmatic nuclei
SDS/PAGE	sodium dodecyl sulfate/polyacrylamide gel electrophoresis
SKF	SKF 38393 (D_1 dopamine receptor agonist)
SN_C	substantia nigra pars compacta
SN_R	substantia nigra pars reticulata
SP	substance P
STN	subthalamic nuclei
TBPS	t,butylbicyclophosphorothionate
TRIS	tris(hydroxymethyl)aminomethane
μM	micromolar

CHAPTER 1.0

General Introduction

Chapter 1.0 *GENERAL INTRODUCTION*

In this introduction, the biochemistry of gamma-aminobutyric acid (GABA) synthesis and metabolism is briefly described. Benzodiazepines and their receptors as well as the interaction of melatonin with these binding sites are reviewed. A brief description of dopamine, its receptors, its role in the basal ganglia and its interaction with GABA follows. Finally, the use of the 6-hydroxydopamine model which has been used to study dopaminergic mechanisms, will be described. The present project was designed to determine the mechanism(s) underlying the observed inhibitory effect of benzodiazepines and melatonin in the 6-hydroxydopamine model

1.1 GAMMA- AMINOBUTYRIC ACID

The amino acid, gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain (Macdonald and Olsen, 1994). Although widely distributed in the central nervous system, the concentration of GABA varies across brain regions with the highest concentration present in the basal ganglia, hippocampus, cerebellum and hypothalamus and in the dorsal horn of the spinal cord (Goodchild, 1993).

1.1.1 GABA Metabolism and Transport

GABA is formed *in vivo* by a metabolic pathway referred to as the GABA shunt. The shunt is a closed loop that acts to conserve the supply of GABA. GABA is synthesized from glutamate a precursor mainly derived from glutamine. The first step in the shunt process is the transamination of α-ketoglutarate, an intermediate in the Krebs cycle, by GABA α-oxoglutarate transaminase (GABA-T) into glutamic acid (glutamate). Glutamic acid decarboxylate (GAD) catalyzes the decarboxylation of glutamic acid to form GABA. GAD is expressed only in the cytoplasm of neurons that use GABA as a neurotransmitter. GABA is metabolized by GABA-T to form succinic semialdehyde. This transamination step occurs when the initial parent compound, α-ketoglutarate is present to accept the amino group from GABA, reforming glutamic acid. Succinic semialdehyde is oxidized by succinic semialdehyde dehydrogenase (SSADH) into succinic acid which can re-enter the Krebs cycle, completing the loop (Delorey and Olsen 1994). (see figure 1).

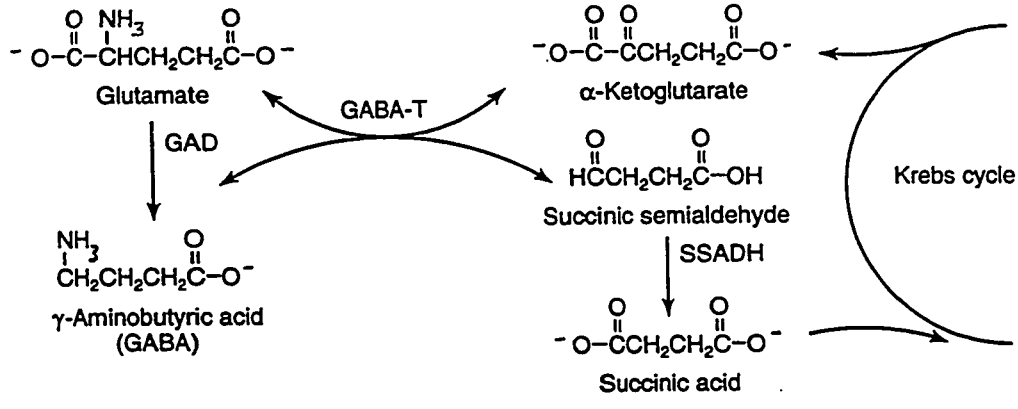


Figure 1. Reactions involved in the GABA shunt.
(Ref: Delorey and Olsen, 1994).

After being released GABA interacts with its own receptors. GABA agonists such as muscimol, mimic the action of the native neurotransmitter, while antagonists such as bicuculline or picrotoxin block the effects of GABA. The action of GABA is terminated by reuptake of GABA into presynaptic nerve terminals and surrounding glial cells. A membrane transport system mediates the reuptake of GABA. GABA taken back up into nerve terminals is 'recycled', however the GABA in glia is metabolized to succinic semialdehyde by GABA-T. The glutamate formed cannot be converted to GABA in glia due to the lack of GAD. Therefore, GABA is converted to glutamine and transferred back to the neuron where glutaminase can convert it to glutamate which is then able to re-enter the GABA shunt (DeLorey and Olsen, 1994).

1.1.2 GABA Receptors

GABA released from GABAergic neurons binds to two major receptors- the $GABA_A$ and $GABA_B$ receptors to produce fast and slow inhibitory responses, respectively (Mody et al. 1994). The $GABA_B$ receptor indirectly enhances potassium and suppresses calcium channels as a G protein-coupled receptor. The $GABA_B$ receptors are insensitive to bicuculline but are stimulated by selective $GABA_B$ agonists such as baclofen (Bowery, 1989). Not much is known about the molecular biology and pharmacology of the $GABA_B$ receptor when compared to its counterpart, the $GABA_A$ receptor. These receptors seem to be coupled to calcium or potassium channels by second messenger systems (Bowery, 1993). Recently, Chapman et al. (1993) reported that GABA and $GABA_B$ receptor agonists inhibited the cough reflex in cats and guinea pig. $GABA_B$ agonists were found to inhibit "a variety of responses

in the airways such as neuronal induced cholinergic- and tachykinin-mediated airway smooth muscle contraction, airway microvascular leakage and anaphylactic reactions in the airways". These findings suggest that a selective $GABA_B$ agonist maybe beneficial in treating respiratory disorders (Chapman et al. 1993).

The $GABA_A$ receptors are important because they not only play a role in regulation of brain excitability but their function is allosterically regulated by several classes of therapeutic compounds including the benzodiazepines. The $GABA_A$ receptors which are associated with central-type benzodiazepine receptors are described below.

1.2 BENZODIAZEPINES

Benzodiazepines (BZ) are one of the most widely prescribed class of psychoactive drugs in current therapeutic use. In addition to their anxiolytic, hypnotic and anticonvulsant properties, BZs produce sedation, muscle relaxation, as well as strongly potentiate the central nervous system (CNS) depressant effects of barbiturates and alcohol (Haefely, 1986). Since the introduction of the two prototype BZs, chlorodiazepoxide (Librium) and diazepam (Valium), many attempts have been made in order to gain insight into the molecular mechanisms of actions of the BZs. A major breakthrough in understanding the mechanism of action of these drugs came with the demonstration of high affinity binding sites for [^{3}H]BZs in mammalian brain (Braestrup and Squires 1977; Mohler and Okada, 1977).

To date, there are at least four types of BZ receptors: 1) a high affinity central-type with an affinity (K_d) for diazepam in the nanomolar range: the central-type BZ receptor is part of a complex that includes gamma-aminobutyric $acid_A$ ($GABA_A$) receptors and chloride ion (Cl^-)

channels (Mohler et al. 1977); 2) a peripheral-type BZ receptor with a K_d in the nanomolar range for Ro5-4864 (Tallman et al, 1980); 3) a low affinity type with a K_d for diazepam in the micromolar range (Bowling et al. 1982), though the existence of such this BZ receptor is controversial; 4) and finally a type that is coupled to a G protein (Dan'ura et al. 1988).

1.2.1 Central-type BZ Receptor (BZ/GABA$_A$ receptor)

The BZ/GABA$_A$ receptor complex is a pentameric protein reported to be composed of 2α, 2β and either γ, δ or ρ subunits to make up the native receptor. The complexity of this receptor is increased by the multiple subtypes of the subunits which have been identified. So far, a total of six α, three β, four γ, one δ and two ρ subunits have been cloned and sequenced (Macdonald and Olsen, 1994). The α subunits regulate the sensitivity of the receptor for GABA and appear essential for the full expression of receptor activity. Together with the α subunit, the β subunit is necessary for the channel to operate efficiently. The presence of the γ subunit confers BZ sensitivity to the receptor (Knoflach et al. 1991). Little is known about the functional properties of the δ and ρ subunits. The ρ subunit was first identified in human retina (Cutting et al. 1991). When expressed as a homooligomer, a ρ1 receptor is responsive to GABA and is picrotoxin-sensitive and bicuculline-insensitive (Shimada et al. 1992).

Each BZ/GABA$_A$ receptor subunit cDNA encodes for a polypeptide of approximately 50 kD with putative N-glycosylation sites and four helical hydrophobic membrane-spanning regions (Olsen and Tobin 1990). Between the third and fourth membrane-spanning region is a hydrophilic cytoplasmic region that may be phosphorylated by intracellular cAMP-

dependent protein kinase (Olsen and Tobin, 1990). The subunits form an enclosed structure around the ion channel such that each subunit contributes to the wall of the channel (Olsen and Tobin 1990). The model of the BZ/$GABA_A$ receptor is based on an analogy with the nicotinic acetylcholine receptor which belongs to a superfamily of ligand-gated ion channel receptors.

The $GABA_A$ receptor is activated by GABA and structural analogues of GABA such as muscimol, isoguavacine and 4,5,6,7 tetra-hydroiso-xazolo[5,4-c]pyridin 3-ol (THIP- a bicyclic muscimol analogue). Activation of a $GABA_A$ receptor by GABA agonist opens the Cl^- channels, leading to an influx of Cl^- ions. This results in hyperpolarization of the neuronal membrane leading to inhibition of neuronal firing. In addition to the GABA agonist binding site, a number of allosteric sites mediate the modulatory action of various drugs which can enhance or in some instances, inhibit the ability of GABA to open the channel. These sites are specific for the following drugs: benzodiazepines, barbiturates, neuroactive steroids, ethanol, zinc and picrotoxin. In the presence of GABA, BZs potentiate the inhibitory responses by an increase in the frequency of chloride ion channel opening (Olsen and Tobin, 1990). Compounds acting at the BZ binding site of the $GABA_A$ receptor include agonists that enhance the effects of GABA (which are sedative, anticonvulsant and anxiolytic), inverse agonists that produce exactly the opposite effects to those of the BZ agonists (which are proconvulsant) or antagonists which block the effects of agonists and inverse agonists (Goodchild, 1993).

1.2.2 Peripheral-type BZ Receptor

In addition to the central-type BZ receptors, the peripheral BZ receptor (PBR) has been identified in peripheral tissues (Braestrup and Squires, 1977; Syapin and Skolnick, 1979; Benavides et al. 1983), as well as in the brain (Braestrup and Squires, 1977; Schoemaker et al. 1981; Marangos et al. 1982). These receptors differ from the central-type BZ receptor in their lack of coupling to $GABA_A$ receptors and their ligand specificity. The central-type BZ receptor exhibits high affinity for clonazepam, but not for Ro5-4864 (4'-chlorodiazepam) or PK 11195. The reverse is true for peripheral-type BZ receptors, which have a high affinity for Ro5-4864 and PK 11195 but low affinity for clonazepam (Gavish et al. 1992). However, the PBR has a nanomolar affinity for diazepam (Braestrup and Squires 1977) which is a non-selective BZ agonist. The PBRs were originally thought to reside only on the outer mitochondrial membrane of peripheral tissues and to be involved in mitochondrial respiration, however, they were later shown to be also present in the central nervous system (Shoemaker et al. 1981). The functional roles of PBR are not clear, however, these receptors are thought to be involved in steroidogenesis, cell proliferation and immunoreactivity of non-neuronal tissues (Parola, 1993; Mukhin et al. 1989; Papadolpoulos, 1990; Bruce et al. 1991).

1.2.3 Micromolar BZ Receptor

The third type of receptors for BZs bind with micromolar affinity and have been identified in the rat central nervous system (Bowling and DeLorenzo, 1982). This binding site might be associated with an inhibition of the calcium-calmodulin stimulated membrane protein phosphorylation (DeLorenzo et al. 1981). These micromolar BZ binding sites differ from the

central- and peripheral-type BZ receptors not only in their much lower affinity for BZs but also in their binding properties, kinetic and pharmacological characteristics (Bowling and DeLorenzo, 1982). There is controversy surrounding the existence of the micromolar site. It has been suggested that the 'non-neuronal' type BZs cross react with dihydropyridine binding sites which are also linked to calcium channels. Cantor et al. (1984) reported that dihydropyridine calcium channel blockers, nifedipine and nitrendipine, displaced the binding of [^{3}H]Ro5-4864 in a competitive manner (ie. the affinity for [^{3}H]Ro5-4864 had decreased in the presence of the channel blockers when compared to controls). Nifedipine and nitrendipine were ineffective at displacing the central type agonist, [^{3}H]flurazepam (Cantor et al. 1984). These investigators suggested that at micromolar concentrations, the dihydropyridines and 'non-neuronal' type BZs are capable of crossreacting at each group's binding sites and perhaps the 'micromolar' site is in fact the same site as that for dihydropyridines. However, Doble et al. (1985) disagrees with this proposal as they have found that in rat cardiac membranes, dihydropyridines and BZs do not bind to the same site but the subcellular distribution for both binding sites in the heart is localized in the same membrane compartments-the sarcolemma and sarcoplasmic reticulum (Doble et al. 1985).

1.2.4 G_i - coupled BZ Receptor

The fourth type of BZ receptor is one that was first identified in the rat brain membranes and found to be coupled to an inhibitory guanine nucleotide binding protein (G_i) (Dan'ura et al. 1988). Various BZs (diazepam, flunitrazepam and clonazepam) were shown to inhibit adenylyl cyclase activity in rat brain. The inhibitory effects of these BZs were found not to be

antagonized by the central-type BZ antagonist, flumazenil, (Dan'ura et al. 1988; Niles and Hashemi, 1990). Pertussis toxin acts by uncoupling the inhibitory G-protein from its receptor, thus blocking signal transduction. Addition of this toxin to rat brain membranes reduced the inhibitory effect of BZs on cAMP production. The suppression by diazepam of cAMP production was enhanced in the presence of either guanyl-5'-imidodiphosphate (GppNHp), a stable analog, or NaF-$AlCl_3$ (Dan'ura et al. 1988). Addition of GABA or pentetrazole had no effect on the inhibitory action of diazepam indicating that the high affinity central site was not involved (Dan'ura et al. 1988). Ro5-4864 has also been shown to inhibit forskolin-stimulation of cAMP production in rat brain membranes (Niles and Hashemi, 1990). Thus, Ro5-4864, which was once thought to be a specific peripheral BZ receptor agonist is also able to act at the G_i-coupled BZ receptor.

1.3 MELATONIN

The synthesis and secretion of the pineal hormone, melatonin (N-acetyl-5-methoxy tryptamine), is principally controlled by the light/dark environment, acting via the hypothalamic suprachiasmatic nuclei (SCN) (Cassone, 1990). Melatonin synthesis is suppressed by light and stimulated by darkness. In rats, the photic signals from the retina pass via the retinohypothalmic tract to the SCN. SCN efferents project to cells in the paraventricular nucleus of the hypothalamus that in turn synapses on neurons in the intermediolateral cell column of the spinal cord. These neurons provide preganglionic fibers to the superior cervical sympathetic ganglia whose postganglionic sympathetic fibers then project to the pineal gland (Krause and Dubocovich, 1990; Ebadi et al. 1993).

The endocrine cells of the pineal gland, the pinealocytes, receive sympathetic nerve endings which release the neurotransmitter, noradrenaline, during darkness. After noradrenaline stimulation, the synthesis of the intracellular second messenger cyclic AMP is amplified, leading to melatonin synthesis (Reiter, 1991). Noradrenaline acting on β-adrenergic receptors stimulates tryptophan uptake and the synthesis of melatonin from the precursor serotonin after several enzymes have been activated (Krause and Dubocovich, 1990).

Since melatonin is highly lipophilic, once it is produced, it rapidly passes into the blood stream and circulates throughout the body. Melatonin is primarily metabolized in the liver to 6-hydroxymelatonin which is conjugated to either glucuronide or sulphate. The main metabolite excreted is urinary 6-sulphatoxy melatonin (Arendt et al. 1985). There is a high correlation between pineal and circulating melatonin levels and urinary 6-sulphatoxymelatonin levels (Arendt et al. 1985; Matthews et al. 1991).

Melatonin, at physiological levels (picomolar-nanomolar), is reported to influence circadian and seasonal timing of a variety of physiological and behaviourial processes (Tamarkin et al. 1985; Cassone, 1990). The physiological binding sites for melatonin are present on high-affinity G protein-coupled receptors in the hypothalamus and other tissues (Krause and Dubocovich 1990; Niles et al. 1991; Tenn and Niles 1993). Recently, the cDNA for a high affinity melatonin receptor from *Xenopus laevis* dermal melanophore was isolated using a expression cloning strategy (Ebisawa 1994). In addition, Reppert et al. (1994) cloned and characterized a melatonin receptor from sheep and human. Expression of these receptors in COS-7 cells results in high affinity [^{125}I]iodomelatonin binding as well as pharmacological characteristics that are similar to the endogenous high affinity receptors. When sheep

melatonin receptor cDNA was transfected in NIH 3T3 cells, it was shown that the expressed receptor was functionally coupled to inhibition of adenylyl cyclase (Reppert et al. 1994). This agrees with previous studies that show melatonin receptors mediate inhibition of adenylyl cyclase via activation of a pertussis toxin-sensitive G protein (Morgan et al. 1994). Studies using in situ hybridization, have shown the presence of mRNA for the receptor in areas such as pars tuberalis of the pituitary gland of sheep and the SCN of rat and hamster (Reppert et al. 1994).

1.3.1 Melatonin Interaction with Benzodiazepine Sites

Previous studies have shown that the pharmacological effects of melatonin may be mediated through the BZ/$GABA_A$ receptor complex (Niles 1991; Hales and Lambert, 1992). Niles (1991) found that melatonin (in micromolar concentrations) competitively inhibited [^{3}H]diazepam binding in human and bovine cortex. Melatonin in pharmacological (micromolar) doses enhances *in vitro* and *in vivo* binding of GABA in rat brain (Coloma and Niles, 1988; Niles et al. 1987; Niles, 1991). In addition, Hales and Lambert (1992) found that BZs produced increases similar to melatonin in the binding of GABA to BZ/$GABA_A$ receptors on brain membranes. Furthermore, melatonin has a micromolar affinity for central-type BZ receptors (Marangos et al. 1981) suggesting that its pharmacological modulation of $GABA_A$ binding may be due to its action on these BZ receptor sites. Melatonin is capable of inhibiting TBPS (t-butylbicyclophosphorothionate, a convulsant compound) binding to GABA gated chloride channels (Niles and Peace 1990), which provides further evidence that melatonin is involved in modulating GABAergic function. Moreover, melatonin is currently

considered to have important behaviourial effects due to its sedative, anxiolytic and anticonvulsant actions (Sugden, 1983, Naranjo-Rodriguez et al. 1992; Golombek et al. 1993). These properties are like those exhibited by BZs, indicating that the pharmacological actions of melatonin are mediated by BZ receptors linked to the enhancement of GABAergic activity (Niles, 1991).

1.4 DOPAMINE

Since the discovery of dopamine in the central nervous system (Carlsson et al. 1958; Montagu, 1957) and its localization to specific neuronal systems in the brain (Carlsson et al. 1962), this neurotransmitter has been extensively investigated. Histoflouresence methods provided detailed anatomical localization of dopamine pathways (Anden et al. 1964; Dalström et al. 1964) and characterized the major dopamine containing fibre tracts such as the nigrostriatal, mesolimbic, and mesocortical pathways (Moore and Bloom 1978; Ungerstedt 1971; Lindvall and Björklund 1987). In humans, dopaminergic systems have been suggested to be involved in several neurodegenerative and neuropsychiatric disorders (Reynolds 1992; Yurek and Sladek, 1990). Drugs used in the treatment of Parkinson's disease or in the treatment of schizophrenia are known to interact with specific dopamine receptors in the brain (Seeman and Van Tol, 1994). The existence of dopamine receptors was first proposed to explain the action of neuroleptics on dopamine turnover (Carlsson and Lindquest, 1963). Since then, experimental evidence has accumulated which indicates the existence of more than one type of dopamine receptor (Seeman and Van Tol, 1994).

1.4.1 Dopamine Receptors

Dopamine receptors were originally classified into two main groups, D_1 and D_2 (Kebabian and Calne, 1979). The five dopamine receptors currently known and cloned fall into these two groups. The dopamine D_1-like receptors include D_1 and D_5 ; the dopamine D_2-like receptors include D_2 , D_3 and D_4 (Seeman and Van Tol , 1994). The D_1 and D_2 receptor subtypes are distinguished by their selective affinity for specific radioligands and their different coupling to signal transduction pathways (Kebabian et al. 1984).

The main difference in the D_1 -like receptors is that D_5 shows a higher affinity for dopamine and a lower affinity for butaclamol when compared to D_1. The D_1 receptors operate via adenylyl cyclase activation and formation of the second messenger, cyclic AMP (cAMP). The mRNA for D_1 receptors is present in high concentration in the striatum (caudate/putamen), nucleus accumbens and olfactory tubercle (Dearry et al. 1990; Miester et al. 1991). The mRNA for D_5 receptors is present at low levels in brain regions such as caudate/putamen, nucleus accumbens, hippocampus, hypothalamus and frontal cortex (Sunahara et al. 1991).

The dopamine D_2 isoforms were initially cloned in 1988 (Bunzow et al. 1988). It became apparent that there were 2 forms of the D_2 receptor which were termed D_2 (short) and D_2 (long) (see review by Strange, 1990). These were derived by alternative splicing of a common genomic sequence. The two splice variants code for proteins that were identical except for an insertion of 29 amino acids into the third intracellular loop of the receptor, therefore the names D_2 (short) and D_2 (long).

The D_3 and D_4 isoforms were identified by gene cloning (Sokoloff et al. 1990; Van Tol et al. 1991). The D_3 also has long and short forms (Van Tol et al. 1992). The D_4 receptor has many variants in humans. Each variant has a different number of repeat units which consist of 16 amino acids. The repeat units are located in the third cytoplasmic loop of the receptor protein. Most humans have four repeats known as the dopamine $D_{4.4}$ receptor (Van Tol et al. 1992; Lichter et al. 1993).

Each isoform of the D_2 - like dopamine receptors when expressed in animal cells, was found to bind to dopamine antagonists (e.g. haloperidol and spiperone) with high affinity (Seeman and Van Tol, 1994). In comparing the 3 isoforms, some trends are discernable. The D_3 isoform is distinguished from D_2 by its lower affinity for binding antagonists such as spiperone. Agonist affinities are higher at the D_3 than at D_2 receptors. The D_4 receptor binds some typical dopamine antagonists with similar affinity to D_2 but has a lower affinity for others (e.g. substituted benzamide antagonists).Most antipsychotic drugs work by blocking D_2 receptors except for clozapine which is more potent at the D_4 than D_2 receptors (Seeman and Van Tol 1994).

The D_2 isoforms both inhibit adenylyl cyclase when expressed in CHO cells (Castro and Strange, 1993). Initially, the D_3 receptor when expressed in a mammalian cell line showed no evidence of coupling to G protein (Sokoloff et al. 1990), however, recently Castro and Strange (1993) reported that D_3 receptor expressed in the same cell line (CHO cells) do show coupling to the endogenous G proteins of the cells. One explanation that was offered, was that in isolating the recombinant cell line, a clone was obtained which allowed receptor/G protein interaction (Castro and Strange, 1993). The results from these two studies indicate

the importance of identifying if or what G protein the D_3 receptor couples to *in situ* for understanding the receptor function. The D_4 receptor when expressed in COS-7 cells does couple to G proteins (Van Tol et al. 1991).

For the D_2 receptor isoforms, the mRNA concentrations are highest in the ventral tegmental area, the substantia nigra pars compacta, the caudate/putamen and areas of the limbic regions. The D_3 receptor appears to be concentrated in limbic regions of the brain with very low levels in the striatum. The D_4 receptors are present in much lower levels in the brain than D_2 receptors (Van Tol et al. 1991). However, high levels of the D_4 receptors have been reported in the cardiovascular system, making it predominantly a peripheral dopamine receptor (O'Malley et al. 1992).

1.5 G PROTEIN-COUPLED RECEPTORS

These receptors are coupled to a GTP binding protein that in turn activates an enzyme to produce a second messenger such as cyclic AMP or inositol trisphosphates or directly regulates ion channel activity (Neer, 1994). These G protein coupled receptors share a common structural motif in which a single polypeptide with an extracellular amino terminus and intracellular carboxy terminus spans the plasma membrane seven times (Dohlman et al. 1991). Generally, the ligand -binding domain is comprised of the amino terminal segment and/or portions of the membrane-spanning domains, while the G protein interaction domains involve intracellular loops and the carboxyl terminus. The G proteins are heterotrimers consisting of an α subunit that binds guanine nucleotides with high affinity and specificity and β and γ subunits that form a tightly linked dimer. The heterotrimeric form of the G protein

is required for high-affinity interaction with the receptor (Clapham and Neer 1993). The α subunits are proteins with a molecular weight from 39-52 KDa. The molecular weights for β and γ subunits are 35 and 8 KDa units, respectively.

1.5.1 G-protein Regulatory Cycle

The G protein-coupled pathway is regulated by a GTPase cycle. In the basal (inactive) state, the G protein heterotrimer has GDP tightly bound to its α subunit. Upon binding of an agonists, the receptor becomes activated and undergoes a conformational change, causing the affinity of the α subunit for GDP to decrease. GDP is released from the active site allowing GTP to bind. Once GTP is bound, the α subunit assumes an activated conformation and dissociates from the receptor and from the βγ complex. Both the α and βγ subunits are then free to interact with effectors and modulate their activity (Clapham and Neer 1993). An intrinsic GTPase activity hydrolyzes the γ phosphate of the α subunit-bound GTP, converting it to GDP (Birnbaumer, 1990). In some cases, effectors may act like GTPase activating proteins, which are associated with *ras* and other low molecular weight GTP-binding proteins, to stimulate the GTPase activity of the α subunit (Bourne et al. 1992). Once GTP is cleaved to GDP, the α subunit reassociates with the βγ subunits to re-establish the basal state of the heterotrimer (Birnbaumer 1990)(see figure 2).

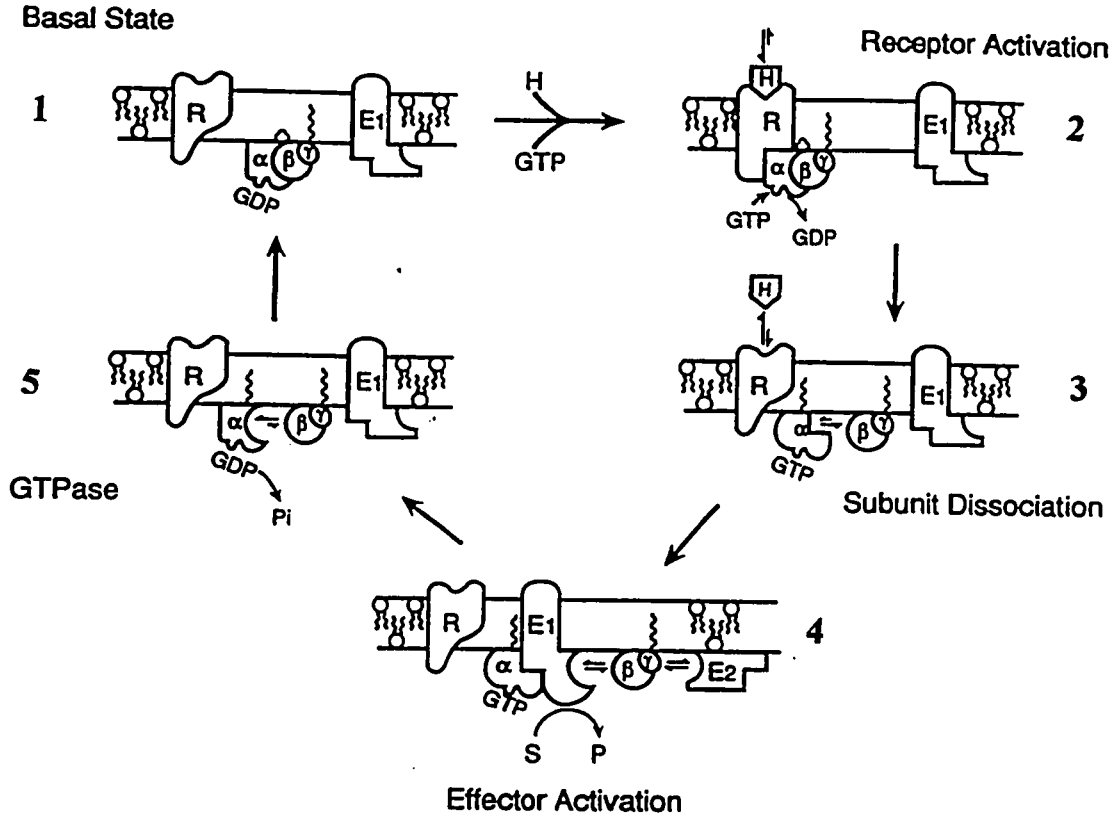


Figure 2. *G-protein-mediated transmembrane signalling.* (1) G proteins exist as heterotrimers with GDP bound tightly to the α subunit in the basal state. The receptor (R) is unoccupied and the effector (E) is unregulated. (2) Upon hormone (H) binding , the receptor is activated and undergoes a conformational change, causing a decrease in the affinity of α for GDP. GTP is exchanged for GDP, the α subunit assumes an activated form which dissociates from the receptor and the βγ complex (3). Both the α and βγ subunits are then free to interact with effectors (E1 or E2) (4). The α subunit possess an intrinsic GTPase activity which hydrolyzes the phosphate of the α subunit-bound GTP to GDP causing dissociation and deactivation of the active complex (5). The α subunit then reassociates with the βγ complex returning the system to the basal state. (Ref: Helper and Gilman, 1992).

G proteins have generally been classified by their α subunit. With the diversity in β and γ subunits and the possibility of their interactions with each other and with α subunits, there is potential for a large number of heterotrimers (Helper and Gilman 1992; Birnbaumer 1992). G proteins range in expression from highly localized (for example, transducin is found only in the retina) to widespread (for example G_s that couples many different receptors to stimulation of adenylyl cyclase) (Helper and Gilman 1992). G proteins can be grouped into subfamilies based on the amino acid sequence similarities of their α subunits (Helper and Gilman 1992; Birnbaumer, 1992). The α_s class was first recognized by its ability to stimulate adenylyl cyclase activity and includes α_s and α_{olf} (from the olfactory neuroepithelium). The α_I class named for the ability of some members to inhibit adenylyl cyclase includes α_{i-1}, α_{I-2}, α_{I-3} and α_o which is found predominantly in the brain (Simon et al. 1991). The retinal α subunits are α_t and α_{t2}. The α_q class includes α_q, α_{11}, α_{16}. Members of this class activate phospholipase C. The fourth class includes α_{12} and α_{13} whose function is still undetermined (Simon et al. 1991)

1.5.2 Signalling through cyclic AMP

Adenylyl cyclase is under the control of both the stimulatory and inhibitory G proteins, G_s and G_i. Binding of $G_s\alpha$ to adenylyl cyclase activates the catalytic subunit resulting in increased production of cAMP. Conversely, $G_i\alpha$ binding results in inhibition of the catalytic subunit. Often the effects mediated by cAMP have been identified by examining the actions of cholera toxin and forskolin, both of which increase cAMP levels by activating adenylyl cyclase. Cholera toxin acts at the level of the G protein (G_s) while forskolin activates the

enzyme directly (Birnbaumer, 1990; Laurenza et al. 1989). Pertussis toxin which is produced by the bacterium, *Bordetella pertussis*, interferes with the ability of agonists to inhibit adenylyl cyclase activity (Reisine, 1990). The toxin was found to cause ADP-ribosylation of the cysteine residue close to the c-terminus of $G_i\alpha$, thus preventing the interaction between the G protein and its receptor (Katada and Ui 1982; Racine, 1990).

1.6 Basic circuitry of the basal ganglia

Specific cortical areas send glutamatergic projections to select regions of the striatum. The basal ganglia output nuclei (globus pallidus and substantia nigra pars reticulata) exert a tonic GABA-mediated inhibitory effect on the thalamus (Graybiel, 1990). The inhibitory outflow appears to be differentially modulated by two opposing but parallel pathways that pass from the striatum to the basal ganglia output nuclei.

The 'direct' pathway to the output nuclei arises from inhibitory striatal efferents that contain GABA and substance P. Activation of this pathway tends to disinhibit the thalamic part of the circuit. The 'indirect' pathway, goes to the globus pallidus via striatal projection neurons that contain GABA as well as enkephalin. From the globus pallidus, there is a GABAergic projection to the subthalamic nucleus which sends an excitatory (probably glutamatergic) projection to the output nuclei. The pallidal neurons exert a tonic inhibitory influence on the subthalamic nucleus. Activation of the inhibitory GABA/enkephalin projection from the striatum tends to suppress the activity of the pallidal neurons and thus disinhibits the subthalamic nucleus. Disinhibition of this nucleus, leads to an increase in the excitatory 'drive' on the output nuclei and increases the inhibition of target neurons within

the thalamus (see figure 3). The two striatal efferent systems of each circuit appear to have opposing effects upon the basal ganglia output nuclei and therefore upon the thalamic targets of basal ganglia output (Gerfen et al. 1990; Graybiel, 1990).

During the execution of specific motor acts, movement-related neurons within the basal ganglia output nuclei may show either increases or decreases in their spontaneous discharge (Anderson et al. 1985; Mitchell et al. 1987). It has been reported that decreases in nigral discharge play an important role in motor control by disinhibiting the ventrolateral thalamus and thereby facilitating cortical initiated movement (by excitatory thalamocortical connection). Increases in nigral discharge may have the opposite effect (Albin et al. 1989; Alexander et al. 1990).

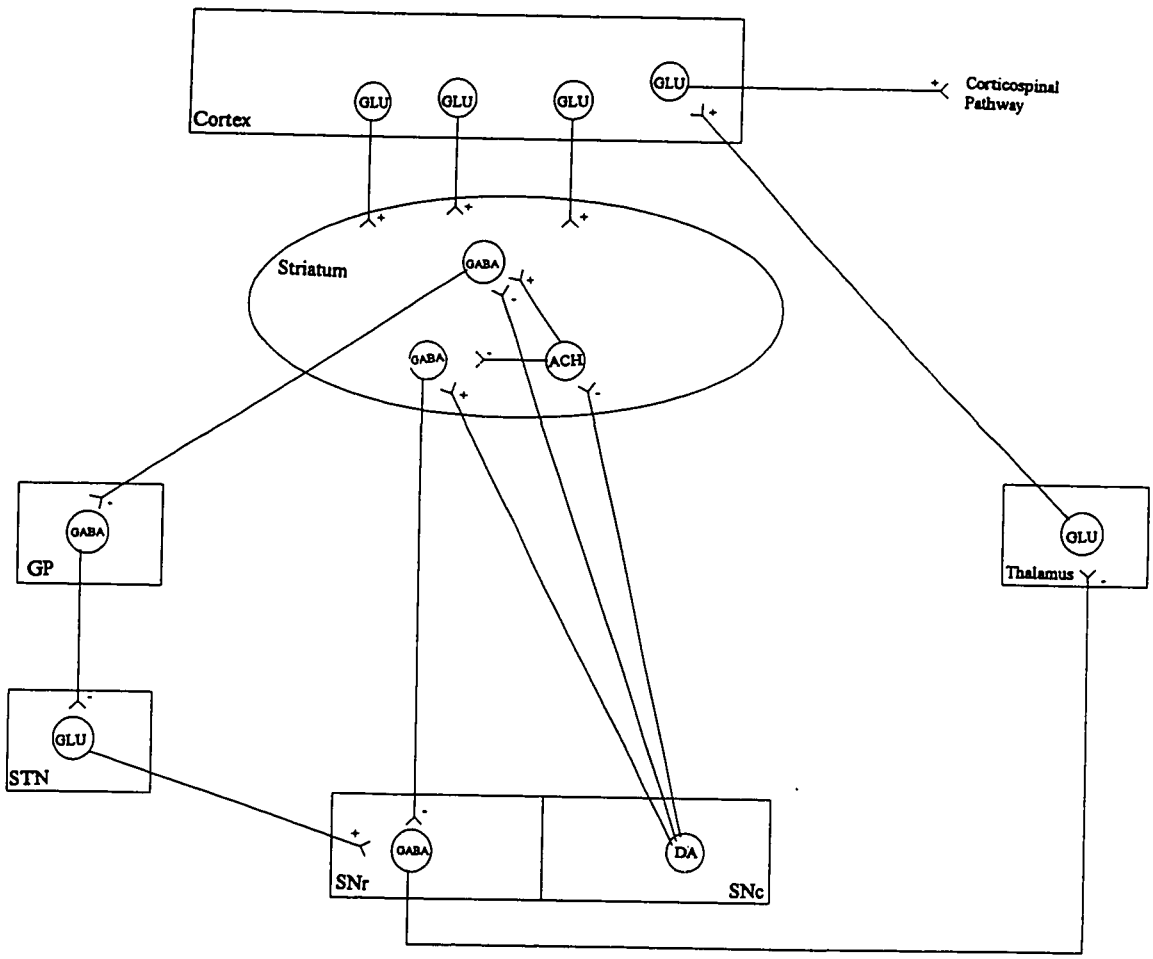


Figure 3. *Schematic diagram of the basal ganglia.* The striatum is the principal output structure of the basal ganglia and receives excitatory glutamatergic input from many areas of the cortex. Outflow from the striatum proceeds along two routes. The 'direct' pathway from the striatum to the substantia nigra par reticulata (SNr) uses the inhibitory transmitter GABA. The 'indirect' pathway from the striatum through the globus pallidus (GP) and the subthalamic nucleus (STN) to the SNr consists of two inhibitory GABAergic links on excitatory glutamatergic projection. The substantia nigra pars compacta (SNc) sends dopaminergic projections to the striatal neurons giving rise to both the direct and indirect pathways. The SNr provides feedback to the cortex through the thalamic nuclei. (Ref: Carlsson and Carlsson, 1990; Gerfen et al. 1990; Graybiel, 1990).

1.7 Role of Dopamine within the Basal Ganglia

The role of dopamine in the basal ganglia is very complex and many issues remain undetermined. There are two major output pathways from the striatum on which dopamine exerts contrasting effects. One of the striatal output pathways projects directly to the substantia nigra and the other indirectly from the striatum to the basal ganglia output nuclei via the globus pallidus and subthalamic nucleus. The majority of the striatal neuronal population are medium spiny neurons which give rise to the two major striatal output pathways (Gerfen et al. 1990). These neurons use the inhibitory neurotransmitter GABA (Kita and Kitai 1988). Dopaminergic input has an excitatory effect on striatal neurons that contains GABA and the neuropeptide, substance P which projects to the basal output nuclei (via the direct pathway). These striatal neurons appear to express D_1 dopamine receptors. Activation of this pathway leads to inhibition of the nigral GABAergic neurons and disinhibition of the glutamatergic neurons in the thalamus resulting in an excitatory effect on the cortex. Whereas dopaminergic input have an inhibitory effect on striatal neurons that send GABA/enkephalin projections to the globus pallidus. These striatal neurons express D_2 receptors. Pallidal neurons send inhibitory GABAergic projections to the subthalamic nucleus which provide an excitatory input to the substantia nigra. Activation of this indirect pathway suppresses the activity of the pallidal neurons which results in an increase excitatory drive to the output nuclei resulting in an increase in the inhibitory GABAergic output to the thalamic neurons (see figure 3). The overall influence of dopamine within the striatum is to modulate the balance of cortical and thalamic excitation of the 'direct' and 'indirect' pathways (Gerfen et al. 1990).

1.8 6-Hydroxydopamine Lesion

The primary defect in Parkinson's disease is the destruction of the dopaminergic neurons of the substantia nigra par compacta (SNc) resulting in loss of dopaminergic input to the striatum. This leads to a differential effect on the two outflow pathways; the 'direct' GABAergic pathway to the substantia nigra pars reticulata (SNr) is less active, while the 'indirect' pathway activity is increased. The net effect is that GABAergic neurons in the SNr become more active leading to an increased inhibition of the glutamateric neurons of the thalamus and reduced excitation of the motor cortex (see figure 4).

In the animal model of Parkinson's disease, rats with unilateral destruction of the ascending nigrostriatal dopaminergic neurons with the neurotoxin, 6-hydroxydopamine (6-OHDA), develop a supersensitivity to dopamine agonists (Ungerstedt, 1971a; Schoenfeld Uretsky, 1972). A consequence of this lesion is that the animals exhibit a characteristic turning response to dopamine agonists such as apomorphine (Ungerstedt, 1971a). Apomorphine directly stimulates the dopamine receptors resulting in contraversive turning (away from the side of the lesion) (Ungerstedt, 1971b). In contrast, when injected with amphetamine, which releases as well as blocks the reuptake of dopamine from the intact terminals, turning is ipsiversive (towards the side of the lesion)(Ungerstedt, 1971a). It was suggested that the animal would turn away from the side with greater dopaminergic activity in the striatum (Ungerstedt, 1971b).

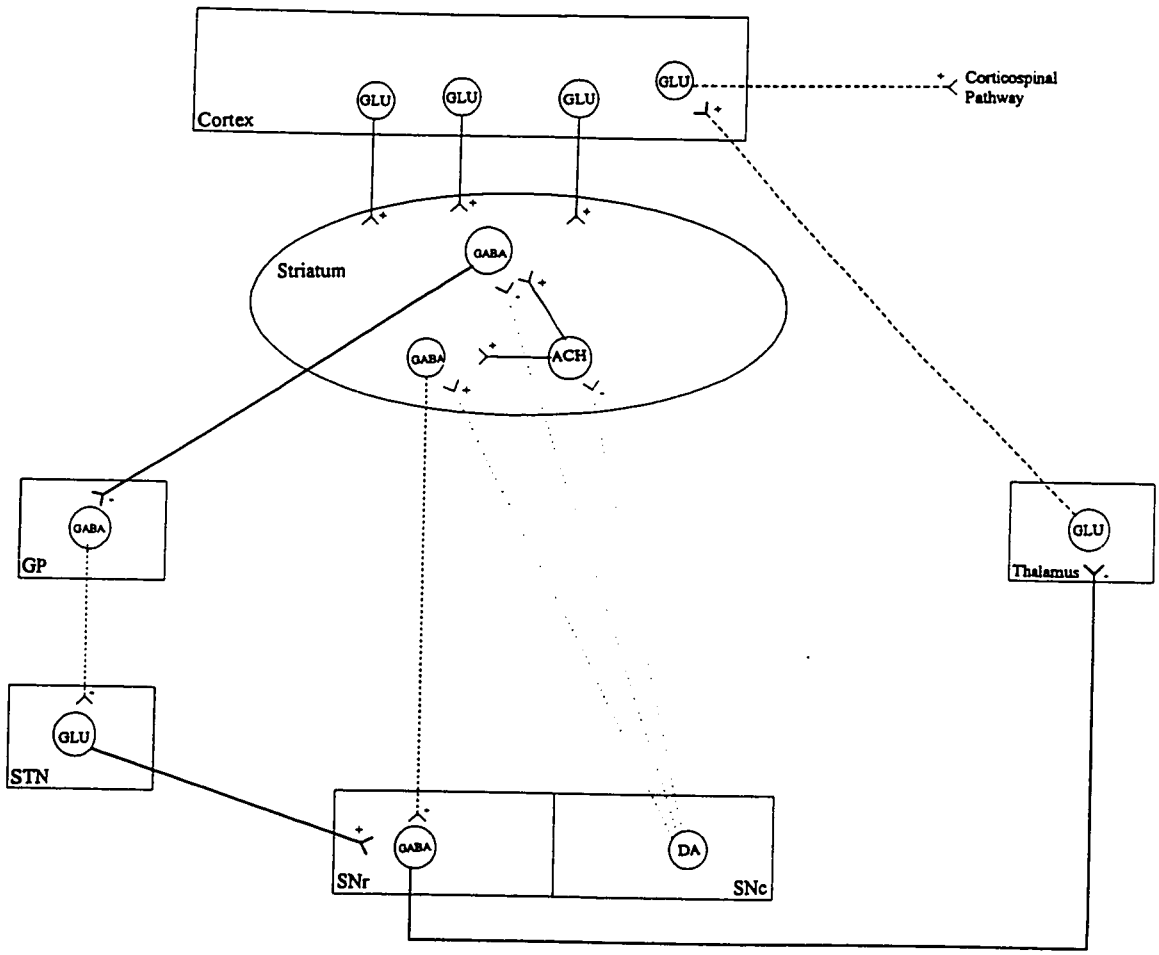


Figure 4. *Destruction of nigral dopaminergic neurons.* Loss of dopaminergic neurons of the SNc has a differential effect on the two outflow pathways; the direct pathway to the SNr is less active, whereas the activity of the indirect pathway is increased. The net effect is that the GABAergic neurons in the SNr become more active, resulting in increased inhibition of the thalamus and reduced excitatory input to the cortex. Broken line, reduced pathway; Bold lines, increased pathway. (Ref: Albin et al. 1989; Gerfen et al. 1990; Graybiel, 1990).

This unilateral 6-OHDA lesion model has been used extensively to study dopaminergic mechanisms. While several radioligand binding studies have shown upregulation of striatal D_2 receptors (Creese et al. 1977; Mishra et al. 1980; Savasta et al. 1987), the results for D_1 receptor upregulation after dopaminergic denervation of the striatum are ambiguous (Buonamici et al. 1986; Marshall et al. 1989; Savasta et al. 1987). In addition, there have been reports of sensitization of dopamine-stimulated AC activity following denervation in the striatum (Mishra et al. 1974; 1980; Robertson et al. 1986; Pifl et al. 1992), as well as studies which showed that the second messenger system was not affected following striatal denervation (Von Voigtlander, 1973).

Recently, micromolar concentrations of melatonin were found to reduce apomorphine induced turning behaviour in 6-hydroxydopamine and quinolinic acid lesioned rats (Burton et al. 1991). This report supports the idea that melatonin may have a role in modulating central dopaminergic function. In contrast to animals lesioned with 6-OHDA, which produces a sensitivity in dopamine receptors, in quinolinic acid treated rats, the receptors are 'normosensitive' (Burton et al. 1991). Therefore, since melatonin reduced the rotational behaviour induced by apomorphine in quinolinic acid lesioned rats, Burton and coworkers (1991) concluded it was unlikely that melatonin was acting by 'normalizing receptor sensitivity'. Although these investigators suggested that melatonin may be acting via other pathways such as noradrenergic, serotonergic, cholinergic or GABAergic to modify the rotational response (Burton et al. 1991), the mechanism underlying the action of melatonin in lesioned animals was left undetermined.

Burton et al. (1991) had administered 1 mg/kg and 10 mg/kg of melatonin, which translates into micromolar concentrations of the indoleamine, and observed the inhibition of apomorphine-induced turning (an antidopaminergic effect) in lesioned animals. Since melatonin at these levels will bind to benzodiazepine receptors, it was hypothesized that the antidopaminergic effect of melatonin in lesioned animals was due to either 1) enhancement of GABAergic activity since GABA can suppress dopaminergic activity in the striatum or 2) the ability of these drugs to block cAMP production since dopamine enhances cAMP production in the striatum since dopamine agonists have been shown to enhance cAMP formation.

1.9 Aims of the Present Study

The primary aim of the present project was to determine the mechanism(s) underlying the antidopaminergic effect of benzodiazepines and melatonin, using the 6-hydroxydopamine lesion model of dopaminergic supersensitivity, and in doing so, the following question were addressed.

The first study looked at the following:

1) Could clonazepam, a central-type BZ agonist, like melatonin, suppress apomorphine-induced turning in 6-OHDA lesioned animals? If the mechanism underlying the suppressive effect of melatonin on apomorphine-induced turning in lesioned animals involved a GABAergic action, then the central-type BZ agonist, clonazepam should also inhibit rotation in lesioned animals.
2) Could the central-type BZ antagonist, flumazenil, block the effect of clonazepam and

melatonin on apomorphine-induced rotation? Does the $GABA_A$ antagonist, bicuculline, have any effect on the inhibitory actions of clonazepam or melatonin? Does the peripheral-type BZ antagonist, PK 11195, have any effect on the inhibitory actions of these drugs? Since clonazepam and melatonin inhibited apomorphine-induced turning, supression of the antidopaminergic effect of these drugs by pretreatment with either the central-type antagonist, flumazenil or the $GABA_A$ antagonist, bicuculline, was examined. Since neither the central-type antagonist nor the $GABA_A$ antagonist completely blocked the effect of clonazepam or melatonin, pretreatment with the peripheral-type antagonist, PK 11195, was also examined.

The second study examined the G_i -coupled BZ receptor and asked:

3) Does the peripheral-type BZ antagonist, PK 11195, have an effect on the inhibitory action of Ro5-4864 and diazepam on forskolin-induced AC activity? Both diazepam and the peripheral-type BZ receptor agonist, Ro5-4864 had previously been shown to inhibit forskolin-stimulated AC activity. Flumazenil, a central-type BZ receptor antagonist, had no effect on AC activity or on its inhibition by diazepam and Ro5-4864. Since Ro5-4864 and diazepam will bind to the peripheral-type BZ receptors, an investigation of the effects of a peripheral-type antagonist, PK 11195, on the inhibitory effect of these drugs on AC activity was carried out.

The third study was designed to answer the following questions:

4) Was the incomplete blockade of the antidopaminergic effect by bicuculline injected intraperitoneally (in the first study), due to the low dose utilized or was there a secondary mechanism that could account for the remaining effect not blocked by the $GABA_A$

antagonist? Due to the potent effect of bicuculline only a subconvulsant dose was administered in the first study. In order to clarify the extent of GABAergic involvement, the effect of intrastriatal injection of the $GABA_A$ antagonist on the antidopaminergic effect of clonazepam and melatonin in lesioned animals was examined.

5) Does lesioning the nigrostriatal dopaminergic pathway have any effect on the inhibition of AC activity by diazepam? The results from the third study indicated the involvement of at least two distinct mechanisms, one being enhancement of GABAergic activity. Since BZs can act on pertussis toxin-sensitive G protein coupled BZ receptors to suppress AC activity in the striatum, the possibility that inhibition of cAMP production maybe a secondary mechanism involved in the antidopaminergic effect of BZs and melatonin was investigated.

6) Does lesioning the nigrostriatal dopaminergic pathway have any effect on [^{3}H]diazepam binding to striatal membranes or on GTP binding to striatal G proteins? It was proposed that the increase in the inhibitory effects of diazepam on forskolin-stimulated AC activity in the striatum was associated with changes in the BZ receptors and in the cAMP pathway of the denervated striatum.

The final study examined the following:

7) Since the naphthalenic compound, S-20098 is similar in structure and has a high (picomolar) affinity for melatonin binding sites, could high doses of this compound also interact at BZ sites to suppress apomorphine-induced rotation?

CHAPTER 2.0

CHAPTER 2. CENTRAL-TYPE BENZODIAZEPINE RECEPTORS MEDIATE THE ANTIDOPAMINERGIC EFFECT OF CLONAZEPAM AND MELATONIN IN 6-HYDROXYDOPAMINE LESIONED RATS: INVOLVEMENT OF A GABAERGIC MECHANISM. *CC. Tenn and LP. Niles. J. Pharm. Exp. Ther. 274:84-89 (1995).*

2.1 *Abstract*

In this study, we examined the effect of the central-type benzodiazepine agonist, clonazepam, and the indoleamine hormone, melatonin, on central dopaminergic function using the 6-hydroxydopamine model of dopamine receptor supersensitivity. Unilateral lesioning of the nigrostriatal pathway with 6-hydroxydopamine was carried out in Sprague Dawley rats. Two weeks following surgery, the animals were examined for the presence of dopaminergic supersensitivity by their response to the dopamine receptor agonist, apomorphine. Clonazepam, melatonin and its analogs, 6-chloromelatonin and 2-iodomelatonin, significantly inhibited apomorphine-induced turning behaviour ($p<0.01$). Pretreatment with a central-type benzodiazepine antagonist, flumazenil, significantly reduced the effect of melatonin and clonazepam ($p<0.01$). The peripheral-type benzodiazepine antagonist, PK 11195, caused some attenuation of melatonin's effect ($p<0.05$), but it was significantly less potent than flumazenil. Bicuculline, a $GABA_A$ receptor antagonist, was also found to reduce the inhibitory effect of melatonin on the induced rotational response ($p<0.01$). These results indicate that the anti-dopaminergic effect of clonazepam and melatonin is mediated predominantly by central-type benzodiazepine receptors in the central nervous system, via a GABAergic mechanism.

2.2 *Introduction*

Unilateral 6-hydroxydopamine (6-OHDA) lesions of the dopaminergic neurons in the substantia nigra of the rat produce an often used model of dopamine receptor supersensitivity. The administration of dopamine agonists induce contralateral turning behaviour by stimulating the supersensitive receptors in the denervated striatum of these animals (Ungerstedt 1971).

A functional interaction exists between the GABAergic and dopaminergic systems (Sandoval and Palmero-Neto, 1989). There is evidence that GABAergic descending neurons regulate the activity of nigral dopaminergic neurons. GABAergic nerve terminals are localized in both the pars compacta and pars reticulata of the substantia nigra. Most of these GABAergic projections originate in the striatum and the globus pallidus (Graybiel,1990). An increase in the levels of GABA in the denervated striatum as compared to the unoperated side has been reported in nigrostriatal lesioned rats (Tanaka et al.1986). This increase in GABA release in the dopaminergic-denervated striatum may be due to a decrease in dopamine mediated inhibition of local GABA neurons (Lindefors et al.1989). Furthermore, a reduction in striatal dopamine release was observed when GABA receptor agonists were injected into the substantia nigra (Reid et al. 1988).

The benzodiazepine (BZ) drugs possess anxiolytic, anticonvulsant, sedative and hypnotic properties (Sieghart, 1994). These therapeutic actions are believed to involve central-type BZ receptors which are linked to $GABA_A$ receptors ($BZ/GABA_A$) in the central nervous system. Benzodiazepines and related ligands act as allosteric modulators of GABAergic activity (Haefely et al. 1985) by enhancing the binding of GABA to the $GABA_A$ receptor complex,

thereby increasing the frequency at which the associated chloride ion channel is open (Haefely and Polc 1986). In keeping with their enhancement of GABAergic activity, benzodiazepines decrease the release of dopamine in the striatum and nucleus accumbens (Ivernizzi et al. 1991; Zetterstrom et al. 1990). Conversely, the central-type BZ antagonist, flumazenil, increases dopamine levels, and this effect is associated with a decrease in GABA transmission initiated by the blockade of BZ receptors (Gruen et al. 1992).

The pineal hormone melatonin has micromolar affinity for the BZ receptors labelled by [^{3}H]diazepam (Marangos et al. 1981). Moreover, melatonin like BZs, enhances $GABA_A$ binding (Coloma and Niles 1988) and inhibits TBPS binding in the brain (Niles and Peace, 1990). Melatonin's pharmacological (micromolar) modulation of $GABA_A$ binding appears to be due to its action on central-type BZ receptor sites present on the $GABA_A$ receptor complex (Niles, 1991). Thus, the ability of pharmacological doses of melatonin to inhibit dopaminergic activity in striatal and limbic regions (Bradbury et al., 1985), and to block apomorphine-induced contralateral turning in 6-OHDA-lesioned animals (Burton et al., 1991), could involve a GABAergic mechanism. The objective of the present study was to investigate the mechanism(s) underlying the anti-dopaminergic effect of BZs and melatonin on central dopaminergic function using the 6-OHDA model of striatal dopaminergic receptor supersensitivity.

2.3 *Materials and Methods*

2.3.1 *Animals.* Male Sprague Dawley rats weighing 300-400g were housed 2 per cage prior to surgery and individually thereafter. The housing room was temperature controlled at 22 ± 1°C and the lights were on from 0800h-2000h. Food and water were freely available.

2.3.2 *Radioligand and chemicals.* [^{3}H]flunitrazepam (79 Ci/mmol) was obtained from New England Nuclear and Amersham. Clonazepam, flumazenil and diazepam were gifts from Hoffman La Roche. Other drugs were obtained from the sources indicated in parentheses: PK 11195 (Rhone Poulenc Sante), melatonin, N-acetylserotonin, serotonin, apomorphine hydrochloride and 6-hydroxydopamine (Sigma), 2-iodomelatonin, bicuculline, SKF 38393 and quinpirole (RBI). 6-Chloromelatonin was a gift from Bristol Myers Squibb Co.

2.3.3 *Surgery.* Twenty minutes prior to surgery, rats were injected with desipramine (Sigma, 15 mg/kg bwt;ip) to protect noradrenergic neurons from the toxic effects of 6-hydroxydopamine hydrochloride (6-OHDA). Rats were anaesthetized with sodium pentobarbital (52 mg/kg bwt) and placed in a stereotaxic instrument. The skull was oriented according to the Paxinos and Watson (1982) stereotaxic atlas. A Hamilton syringe with a cannula of 0.045 cm diameter was used to inject 8µg of 6-OHDA dissolved in 4µl of a vehicle solution (cold saline with 0.1% ascorbic acid) at a rate of 1µl/min. The cannula was left in situ for a further 4 minutes following drug injection to allow for passive diffusion away from the tip of the cannula. Sham-operated animals underwent the same surgical procedure but were injected with 4µl of the vehicle solution. A second group of control animals was left

untouched.

2.3.4 *Turning behaviour*. Rotational behaviour was observed by placing each animal in a transparent plexiglass bowl, 60 cm in diameter. The total number of complete 360 degree turns made by the animal in a 15 minute period was recorded manually, with contralateral (i.e. away from lesioned side) turns being scored as positive and ipsilateral (i.e. towards lesioned side) turns as negative. The net total turns were obtained as the sum of the contralateral and ipsilateral turns. Two weeks after surgery, animals were tested for circling responses 5 minutes after injection of apomorphine (0.25 mg/kg bwt; sc). The effect of clonazepam (1 mg/kg bwt; ip) or melatonin (10 mg/kg bwt; ip) on apomorphine-induced turning, was tested by injecting these drugs 5 minutes prior to apomorphine. Flumazenil or PK 11195 at a dose of 10 mg/kg bwt (ip) was injected 15 minutes prior to melatonin or clonazepam treatment. Pretreatment with bicuculline at a subconvulsive dose of 2.5 mg/kg (ip) was carried out 5 minutes before melatonin. The melatonin analogs, 6-chloromelatonin and 2-iodomelatonin, and its precursors, serotonin and N-acetylserotonin were injected at a dose of 1 mg/kg (ip) in testing for their effects on apomorphine-induced turning. The effect of melatonin on the rotational response induced by SKF 38393(2.0 mg/kg, sc) and quinpirole (0.5 mg/kg, sc) was examined. All drugs were prepared on the day of the experiment. Subsequent tests were carried out using the same animals but after a wash-out period of at least one week. For each test, animals were randomly assigned to one of the treatment groups.

2.3.5 *Preparation of striatal membranes.* Male Sprague-Dawley rats (250-375g) were decapitated and brain tissues were rapidly dissected on ice. Striatal membranes were homogenized in 10 vol of 0.32M sucrose using a hand held glass homogenizer. The homogenate was centrifuged at 1000 g for 10 minutes. The pellet was discarded and the supernatant was centrifuged at 10,000 g for 30 minutes. The P_2 pellet was washed twice in 10 volumes of 50mM Tris-HCl buffer (pH 7.4 at 4^0C) by centrifugation (27,500 g for 10 minutes) and resuspension before use in assays.

2.3.6 *Receptor Binding Assays.* For competition assays, striatal membranes were incubated with 1nM [^{3}H]flunitrazepam for 1 hour on ice, in the presence or absence of various concentrations (0.1-1mM) of melatonin. Bound radioligand was separated by rapid filtration. Specific binding was defined as total bound radioactivity minus binding in the presence of 10μM clonazepam. Protein content was measured by the method of Lowry et al (1951).

2.3.7 *Statistics.* Experimental data were analyzed by one-way analysis of variance and group differences were assessed by Scheffe's test.

2.4 *Results*

2.4.1 *Control groups.* Lesioned animals treated with vehicle solutions and sham-operated animals treated with both vehicle and drug did not differ in their behaviour from unoperated animals treated with vehicle. Therefore, the data from these groups were pooled and are shown as the control group in figures.

2.4.2 *Effect of melatonin on apomorphine-induced rotation.* As shown in Fig. 1, the turning response to apomorphine in 6-OHDA lesioned rats was several-fold greater than the response seen in controls. When melatonin was administered alone, it caused a slight decrease in net total turns as compared to controls, but this was not significant (figure 1). In fact, when the total number of turns (regardless of direction), was taken into account, the melatonin treated group showed a slight increase in turning behaviour (control: 15.4 ± 1.08 turns per 15 min; melatonin treated group: 21.2 ± 2.7 turns per 15 min). Furthermore, the melatonin treated group exhibited exploratory and grooming behaviour which was similar to that of control rats.

However, when various doses of melatonin were administered prior to apomorphine, a dose-dependent decrease in turning behaviour was observed, with the maximal effect occurring at a dose of 5-10 mg/kg (Fig. 2). At an EC_{50} dose of approximately 0.40 mg/kg, half the net total turns were inhibited.

2.4.3 *Effects of clonazepam, flumazenil and PK 11195.* Like melatonin, clonazepam, a central-type BZ receptor agonist also blocked the effect of apomorphine in lesioned animals (Fig. 3). When flumazenil was administered 15 minutes prior to either melatonin or

clonazepam, this central-type BZ antagonist significantly ($p<0.01$) attenuated (by 67 ± 2.6% and 65 ± 2.5% respectively, n=3) the ability of either drug to block apomorphine-induced turning behaviour. In contrast, pretreatment with the peripheral-type BZ receptor antagonist, PK 11195, caused only a slight decrease in melatonin's effect (Fig.4). These results indicate that the anti-dopaminergic effect, of both melatonin and clonazepam in lesioned animals, is mediated primarily by central-type BZ receptors in the striatum.

2.4.4 *Effects of melatonin analogs on apomorphine-induced rotation.* In testing the effects of melatonin and related analogs, it was observed that at a dose of 1 mg/kg, 6-chloromelatonin and 2-iodomelatonin were significantly more potent than melatonin in blocking turning behaviour in lesioned animals (Fig. 5). In contrast, there was no significant effect on apomorphine-induced rotational behaviour when either serotonin or N-acetylserotonin were injected prior to the dopamine agonist.

2.4.5 ***Effect of $GABA_A$ receptor blockade by bicuculline.*** The next set of experiments, as shown in Fig. 6, involved the use of a $GABA_A$ receptor antagonist, bicuculline. At a dose of 2.5 mg/kg, bicuculline significantly ($p<0.01$) suppressed the anti-dopaminergic effect of melatonin by 67 ± 4% (n=5), as observed earlier with flumazenil. Although there seemed to be a slight decrease in the net total turns in the bicuculline/apomorphine treated animals as compared to the apomorphine treated group, this was not significant (apomorphine group:65.7 ± 1.5 turns/15 min; bicuculline/apomorphine group:59.3 ± 1.3 turns/15 min).

2.4.6 ***Effect of melatonin on rotation induced by selective dopaminergic agonists.*** In addition to its blockade of the effect of apomorphine, which is a mixed D_1/D_2 dopamine agonist, melatonin also significantly inhibited the rotation caused by the selective D_1 agonist, SKF-38393, and the D_2-agonist, quinpirole (Fig. 7).

2.4.7 ***Effect of melatonin on benzodiazepine binding in the striatum.*** In competition experiments, utilizing [^{3}H]flunitrazepam to label central-type BZ sites in the striatum, melatonin inhibited binding in a concentration-dependent manner with an IC_{50} of about 300 μM (Fig. 8).

2.5 *Discussion*

The results from this study show that while flumazenil, a central-type BZ antagonist, suppresses the inhibitory effect of clonazepam and melatonin on apomorphine-induced turning behaviour, the peripheral-type BZ receptor antagonist, PK 11195, has very little effect. This indicates that central-type BZ receptors mediate the anti-dopaminergic effect of clonazepam and melatonin. In accordance with the above findings, it has been shown that BZs such as midazolam and flunitrazepam could decrease striatal dopamine release in a dose-dependent fashion. The inhibitory effects of these BZs were reportedly mediated by the BZ/$GABA_A$ receptor complex as they were abolished by flumazenil (Takada et al. 1993). Similarly, the behavioral effects of pharmacological doses of melatonin are suppressed by pretreatment with flumazenil (Golombek et al. 1993).

The ability of melatonin in doses of 10 mg/kg (ip) or higher to induce sedation has previously been reported (Holmes and Sudgen, 1982). However, in the present study, animals injected with melatonin (10 mg/kg, ip) exhibited similar turning, grooming and exploratory activity as compared with controls. Furthermore, dose-response studies indicated that a range of melatonin doses, significantly below its sedative threshold, was effective in blocking the effect of apomorphine, with an EC_{50} of 0.40 mg/kg (ip). Therefore, the anti-dopaminergic effect of melatonin was not due to sedation.

It is well known that BZ agonists, such as clonazepam and diazepam act on the BZ/$GABA_A$ receptor complex to enhance GABAergic activity (Haefely and Polc, 1986). In addition, both in vivo and in vitro studies have shown that melatonin enhances $GABA_A$ receptor binding (Niles et al., 1987; Coloma and Niles, 1988). Therefore, it is very likely that

the anti-dopaminergic action of these drugs involves facilitation of GABA's inhibitory effects on the dopaminergic system. The idea that melatonin's action is due to its ability to enhance GABAergic activity is further supported by the fact that bicuculline, a $GABA_A$ antagonist, inhibited melatonin's effect. Although bicuculline significantly suppressed the effect of melatonin, it did not completely abolish it. Perhaps, at a higher dose than was used here, bicuculline would have more of an impact on melatonin's action. However, because of the risk of convulsions at higher doses, only a subconvulsive dose was used.

As noted earlier, the slight reduction in apomorphine-induced turning observed in the presence of bicuculline was not significant (Figure 6). Nonetheless, this tendency towards decreased rotational behaviour is somewhat paradoxical and may be related to the ability of bicuculline to alter dopaminergic function. A decrease in striatal dopamine release, after intranigral injection of bicuculline (70 nmol) was observed in anesthetized animals(Reid et al. 1990). Therefore, the even higher dose (3.8μmol) of bicuculline utilized in this study could have decreased striatal dopamine release thereby attenuating apomorphine's effect.

Interestingly, while melatonin inhibited the circling behaviour induced by either the D_1 or D_2 dopamine agonists, SKF 38393 and quinpirole respectively, it was significantly more potent[1] in blocking the effect of the D_1 agonist. The mechanism underlying the differential interaction of melatonin with D_1 and D_2 pathways is presently unclear. However, the difference in the densities of dopamine receptor subtypes in the striatum may be involved.

[1] Melatonin blocked SKF 38393-induced rotations more effectively than quinpirole-induced turning.

It has been reported that the density of D_1 receptors in the striatum is approximately 2-3 times that of D_2 receptors (Boyson et al. 1986; Camp et al. 1990). Therefore, it is possible that there is a higher density of D_1 receptors on striatal neurons that also express BZ/$GABA_A$ receptor complexes-the site of melatonin action.

Activation of the D_1 receptor has been linked to stimulation of adenylyl cyclase in a variety of tissues while the D_2 has either an inhibitory or no effect on the enzyme (Kebabian & Calne, 1979; Weiner & Molinoff, 1994). Therefore, the greater inhibitory action of melatonin on SKF 38393-induced circling behaviour could also involve its ability to inhibit cAMP production in the striatum as observed previously (Niles and Hashemi, 1990). This view is supported by evidence that G_i-coupled BZ receptors, which mediate inhibition of adenylyl cyclase activity, are present in the striatum and other brain areas (Dan'ura et al. 1988; Niles and Hashemi, 1990). Moreover, Satoh et al. (1976) reported that the dopamine antagonist, haloperidol, not only blocks apomorphine-stimulated cAMP but also the induced-rotational behaviour. These investigators suggested that cAMP may be implicated in the rotational behaviour induced by dopaminergic agonists. Thus, in addition to enhancement of GABAergic activity, which predominantly mediates the anti-dopaminergic action of BZs and melatonin, suppression of cAMP production may provide a supplementary mechanism for this action.

The melatonin analogs, 6-chloromelatonin and 2-iodomelatonin, were found to be significantly more potent than melatonin at blocking turning behaviour in lesioned animals. This is probably due to the fact that these halogenated analogs are more lipophilic than melatonin, and may have a higher affinity for central BZ sites than melatonin. Indoles, such

as 6-chloromelatonin, also have a longer half-life than melatonin, as the halogenation on carbon-6 protects against degradation via 6-hydroxylation. In contrast to the above agents, the melatonin precursors, serotonin and N-acetylserotonin, did not block turning behaviour in lesioned animals, indicating the structural specificity of the action of melatonin and other indolealkylamines with a 5-methoxy substituent. It is unlikely that the ineffectiveness of the more hydrophilic indoles, serotonin and N-acetylserotonin, was due to their limited entry into the central nervous system, as these compounds do not compete for BZ binding sites (Marangos et al., 1981), and do not modulate BZ/$GABA_A$ activity *in vitro* (Niles and Peace, 1990).

Understanding the mechanism(s) underlying the anti-dopaminergic effect of BZs, melatonin and related drugs could have important clinical implications. The benzodiazepine, clonazepam, has an anti-dyskinetic effect in patients with tardive dyskinesia (TD), which is associated with dopamine receptor supersensitivity, resulting from chronic use of neuroleptics (Thaker et al., 1990). It is believed that the effect of clonazepam is due to its ability to enhance the actions of GABA at the BZ/GABA-chloride channel (Turner & Whittle, 1983; Haefely, 1989). However, although, benzodiazepines have been proposed for clinical use in treating dyskinetic patients, there are problems with the use of these drugs which cause undesirable side effects and to which tolerance is quickly developed (Thaker et al., 1990). Therefore, the natural non-toxic neuromodulator, melatonin, and related analogs that can allosterically interact with the BZ/$GABA_A$ receptor complex to enhance GABAergic transmission, may be useful in alleviating the dyskinetic symptoms associated with the chronic use of neuroleptics.

Fig. 1. Effect of melatonin on the rotational response induced by apomorphine in 6-OHDA lesioned rats. Melatonin (10 mg/kg, ip) was injected 5 minutes before apomorphine (0.25 mg/kg, sc) treatment. Values represent mean ± S.E.M of the net total turns, for the number of animals shown in parentheses. [a]$p < 0.01$ versus control, melatonin and melatonin/apomorphine treated groups.

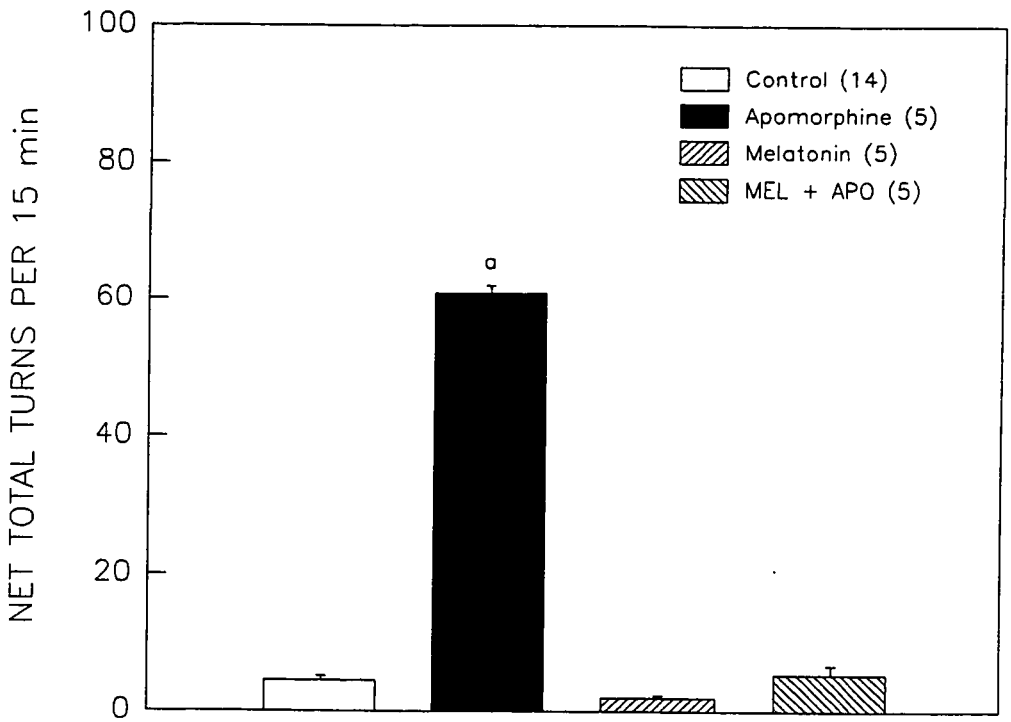

NET TOTAL TURNS PER 15 min
100
80
60
40
20
0
Control (14)
Apomorphine (5)
Melatonin (5)
MEL + APO (5)
a

Fig. 2. Dose-dependent effect of melatonin on the rotational response to apomorphine. Lesioned animals were injected with melatonin (ip; n=3/dose) 5 minutes before apomorphine administration (0.25 mg/kg, sc). Values represent mean ± S.E.M of the net total turns.

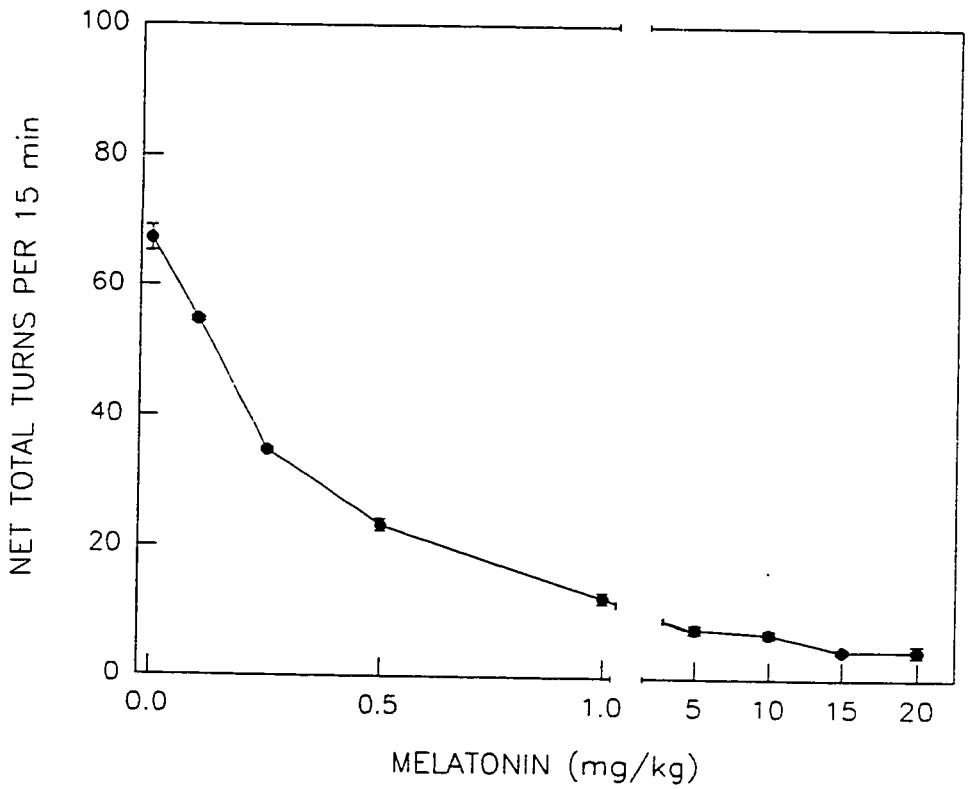

100
80
60
40
20
0
NET TOTAL TURNS PER 15 min
0.0
0.5
1.0
5
10
15
20
MELATONIN (mg/kg)

Fig. 3. Flumazenil blockade of the anti-dopaminergic effect of clonazepam and melatonin in 6-OHDA lesioned animals. Flumazenil (FMZ, 10 mg/kg, ip) was injected 15 minutes before clonazepam (CLO, 1mg/kg, ip) or melatonin (MEL, 10 mg/kg, ip), and 20 minutes before apomorphine (0.25 mg/kg, sc) was administered. Values represent the mean ± S.E.M of the net total turns, for the number of animals shown in parentheses. $^{a}p<0.01$ versus control, melatonin, clonazepam and flumazenil treated groups. $^{bc}p<0.01$ versus flumazenil treated groups.

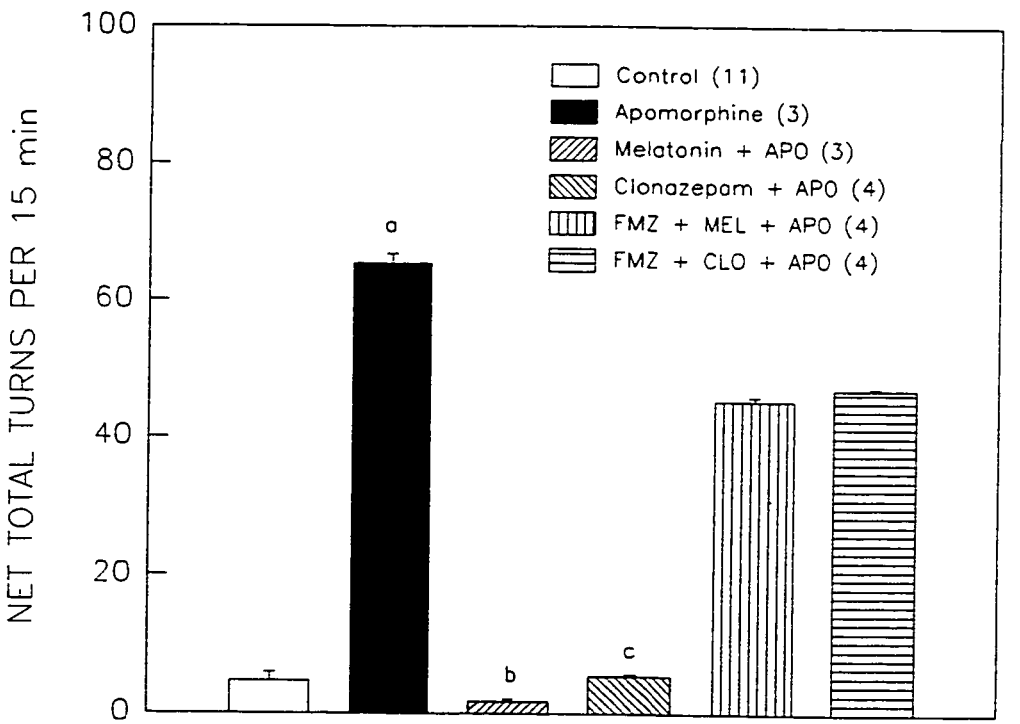

NET TOTAL TURNS PER 15 min
100
80
60
40
20
0
Control (11)
Apomorphine (3)
Melatonin + APO (3)
Clonazepam + APO (4)
FMZ + MEL + APO (4)
FMZ + CLO + APO (4)
a
b
c

Fig. 4. Effect of PK 11195 on the rotational response induced by apomorphine. PK 11195 (PK,10 mg/kg,ip) was injected 15 minutes prior to the administration of melatonin (10 mg/kg,ip). Values represent the mean ± S.E.M of the net total turns, for the number of animals shown in parentheses. $^{ab}p<0.01$ versus control and melatonin treated groups. $^{c}p<0.05$ versus control and melatonin/apormorphine treated groups.

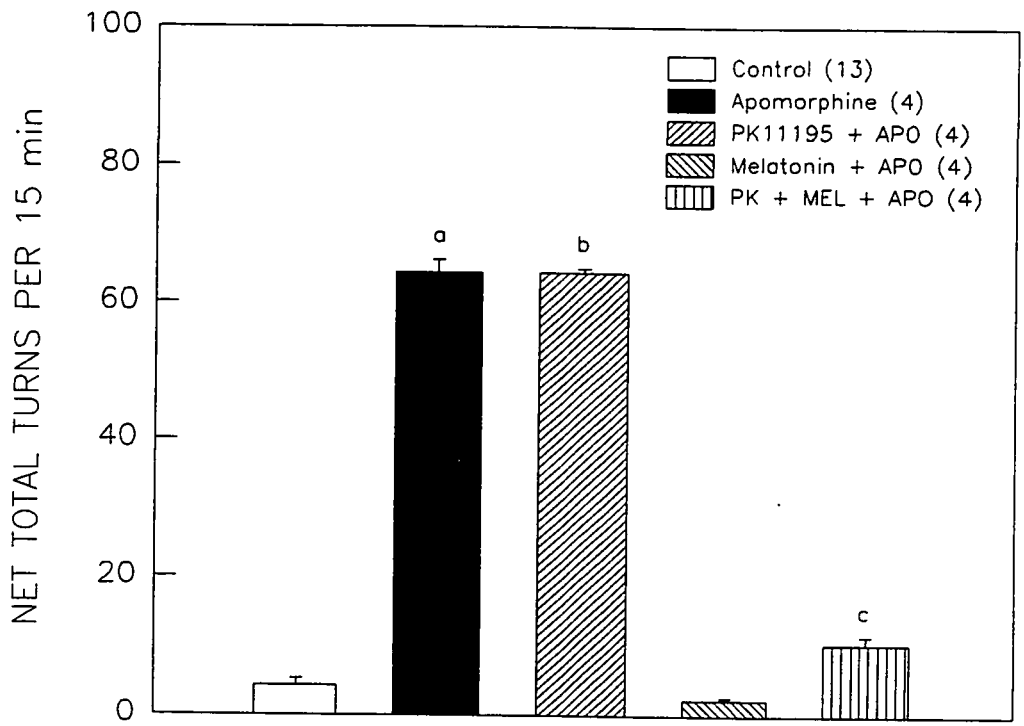

NET TOTAL TURNS PER 15 min
100
80
60
40
20
0
Control (13)
Apomorphine (4)
PK11195 + APO (4)
Melatonin + APO (4)
PK + MEL + APO (4)
a
b
c

Fig. 5. Effect of melatonin and related analogs on the rotational response induced by apomorphine. All drugs (1 mg/kg, ip) were injected 5 minutes before apomorphine (0.25 mg/kg, sc). Values represent the mean ± S.E.M of the net total turns, for the number of animals shown in parentheses. $^{abc}p<0.01$ versus control, melatonin/apomorphine, 6-chloromelatonin/apomorphine and 2-iodomelatonin/apomorphine treated groups. $^{d}p<0.01$ versus control, 6-chloromelatonin/apomorphine, and 2-iodomelatonin/apomorphine treated groups.

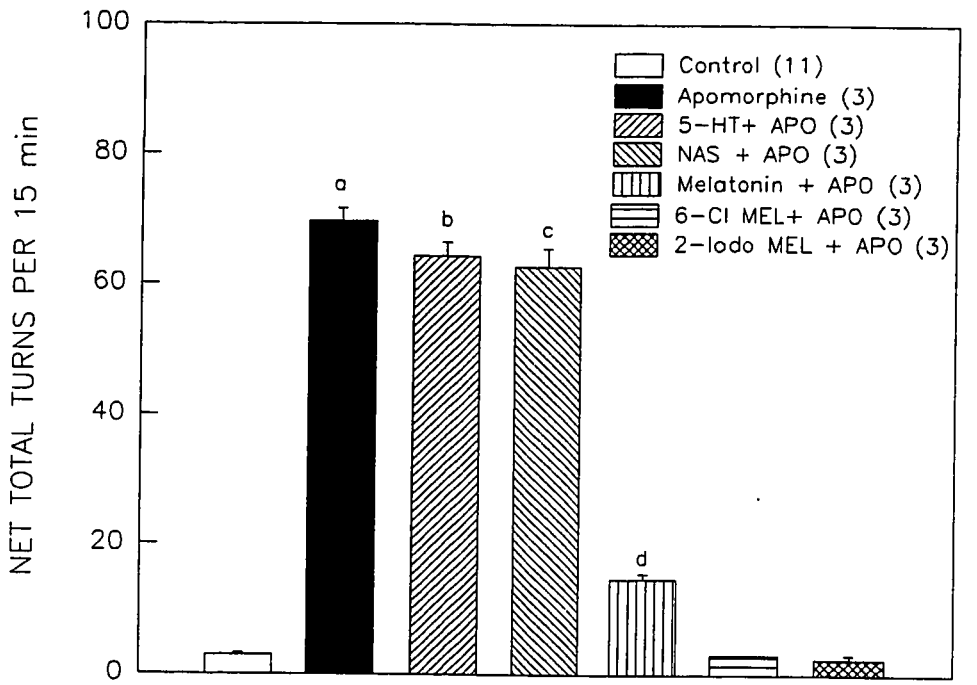

NET TOTAL TURNS PER 15 min
100
80
60
40
20
0
a
b
c
d
Control (11)
Apomorphine (3)
5-HT+ APO (3)
NAS + APO (3)
Melatonin + APO (3)
6-Cl MEL+ APO (3)
2-Iodo MEL + APO (3)

Fig. 6. Bicuculline blockade of the anti-dopaminergic effect of melatonin in 6-OHDA lesioned animals. Bicuculline (BIC, 2.5 mg/kg,ip) was injected 5 minutes before melatonin (10 mg/kg, ip), and 10 minutes before apomorphine (0.25 mg/kg sc). Values represent the mean ± S.E.M of the net total turns, for the number of animals shown in parentheses. $^{ab}P<0.01$ versus control, melatonin/apomorphine and bicuculline/melatonin/apomorphine treated groups. $^{c}P<0.01$. versus control and melatonin/apomorphine treated groups.

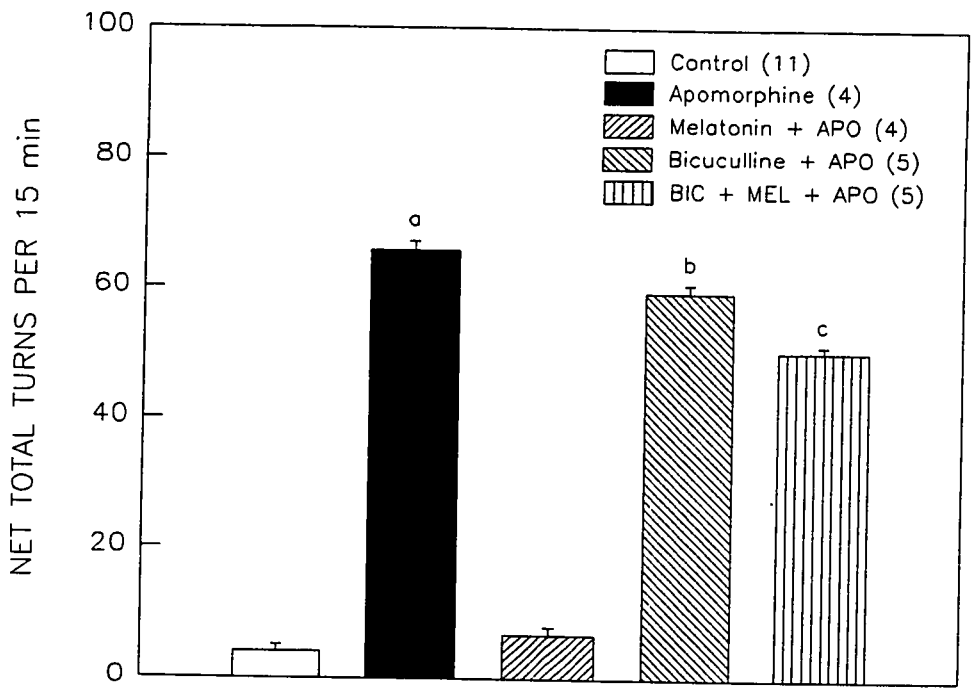

NET TOTAL TURNS PER 15 min
100
80
60
40
20
0
Control (11)
Apomorphine (4)
Melatonin + APO (4)
Bicuculline + APO (5)
BIC + MEL + APO (5)
a
b
c

Fig. 7. The effect of melatonin on the rotational response induced by SKF 38393 or quinpirole. The D_1 agonist, SKF 38393 (SKF, 2 mg/kg, sc), or the D_2 agonist, quinpirole (QUIN, 0.5 mg/kg, sc), was administered 5 minutes after melatonin (10 mg/kg,ip) injection. Values represent the mean ± S.E.M of the net total turns, for the number of animals shown in parentheses. [a]$p<0.01$ versus control, quinpirole, melatonin/SKF 38393 and melatonin/quinpirole treated groups. [b]$p<0.01$ versus control, melatonin/SKF 38393 and melatonin/quinpirole treated groups. [c]$p<0.01$ versus control and melatonin/SKF 38393 treated groups.

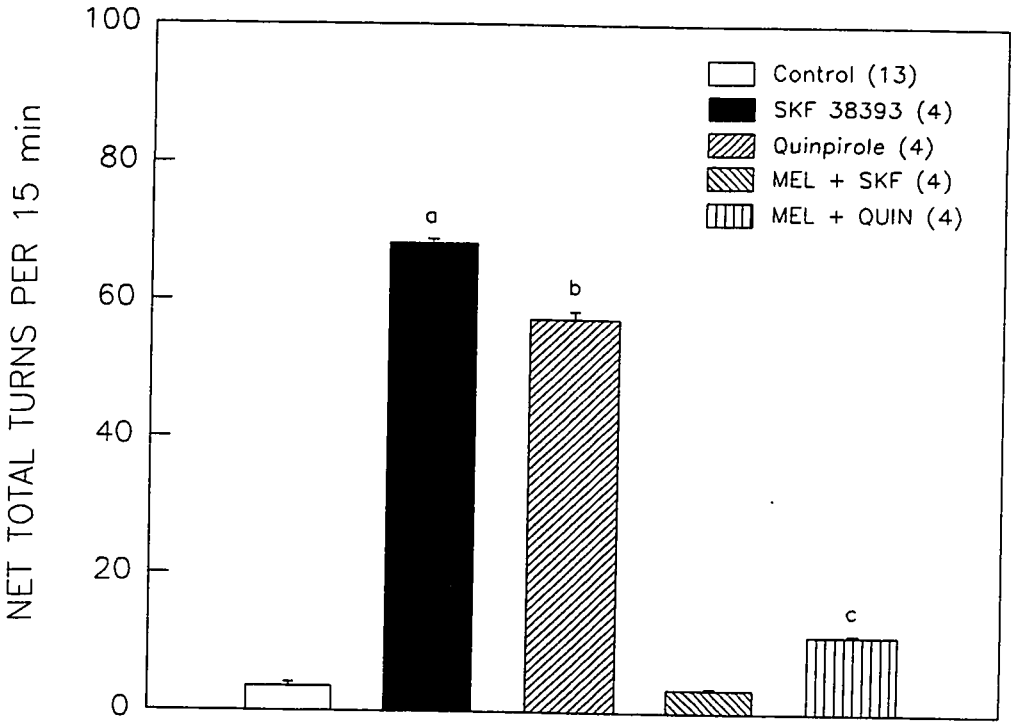
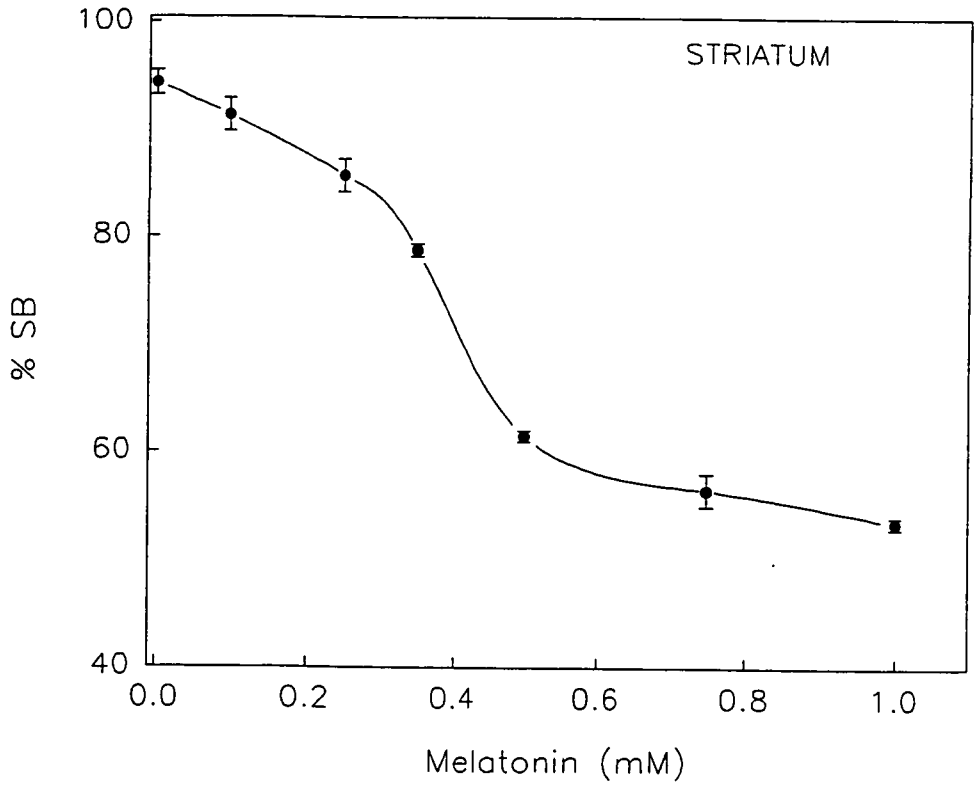

NET TOTAL TURNS PER 15 min
100
80
60
40
20
0
Control (13)
SKF 38393 (4)
Quinpirole (4)
MEL + SKF (4)
MEL + QUIN (4)
a
b
c

Fig. 8. Competition for [^{3}H]flunitrazepam binding in the rat striatum by melatonin. A synaptosomal membrane preparation (P2) was used in the binding assay as described under Methods section. Each point represents the mean ± S.E.M of 2-3 separate experiments performed in triplicate.


STRIATUM
100
80
60
40
% SB
0.0
0.2
0.4
0.6
0.8
1.0
Melatonin (mM)

2.6 *References*

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CHAPTER 3.0

CHAPTER 3.0 PK 11195 BLOCKADE OF BENZODIAZEPINE-INDUCED INHIBITION OF FORSKOLIN-STIMULATED ADENYLATE CYCLASE ACTIVITY IN THE STRIATUM. *CC. Tenn, JM. Neu & LP. Niles.(1996) Br. J. Pharm. 119:223-228.*

3.1 *Summary*

1 The effects of benzodiazepine receptor antagonists on the inhibition of forskolin-stimulated adenylate cyclase (AC) activity by various benzodiazepine(BZ) and indoleamine agonists in the rat striatum were investigated.

2 A biphasic inhibition of forskolin-stimulated AC activity by the peripheral-type agonist, Ro5-4864, and a multiphasic inhibition by the non-selective BZ, diazepam, was observed. One phase of AC inhibition is consistent with a G_i -coupled receptor-mediated action, whereas the other phases appear to involve a direct effect on the enzyme itself.

3 While the central-type antagonist, flumazenil, had no effect on the ability of Ro5-4864 to inhibit AC activity, the peripheral-type receptor ligand, PK 11195, abolished the first phase of inhibition.

4 PK 11195 and pertussis toxin were found to block the inhibitory effect of various BZs and the indoleamines, melatonin and 2-Iodomelatonin, on induced AC activity.

5 Saturation binding studies, conducted at 30 ^{0}C with [^{3}H]diazepam revealed a single binding site in the rat striatum (K_D = 19.3± 0.80 nM) which significantly decreased in affinity in the presence of GTP (K_D = 30.5± 2.6 nM; $p<0.05$). No significant change in B_{MAX} was observed.

6 These findings indicate the presence of G_i - coupled BZ receptors in the rat striatum. Thus, suppression of cAMP production may contribute to the diverse neuropharmacological effects of BZs, melatonin and related drugs.

3.2 *Introduction*

Benzodiazepines are effective anticonvulsant, anxiolytic and hypnotic drugs. It has been suggested that their therapeutic effects are mediated by specific central benzodiazepine (BZ) receptors which are coupled to γ-aminobutyric acid$_A$ ($GABA_A$) receptors (Tallman et al. ,1980; Olsen and Tobin, 1990). In addition to these central-type BZ receptors, high densities of BZ binding sites have been found in a variety of peripheral tissues (Braestrup and Nielsen, 1983; Parola et al., 1993). These peripheral binding sites differ from the central-type BZ receptor in that they are not coupled to $GABA_A$ receptors and have different pharmacological and molecular characteristics. For example, clonazepam has a high affinity for central-type receptors but is very weak at peripheral sites. By contrast, Ro5-4864 has a high affinity for peripheral-type binding sites but very low affinity for central-type receptors. The peripheral-type BZ receptor (PBR) is present in not only peripheral tissues, such as the kidney, but also the central nervous system (Braestrup and Squires, 1977; Marangos et al. 1982). Recent cloning of a PBR component responsible for the receptor binding properties, showed no apparent sequence homology with any of the cloned $GABA_A$ receptor subunits (Parola et al. 1993). Thus, the peripheral binding sites are distinctly different from the central-type BZ receptors.

Recent reports have suggested the presence of another BZ receptor which is coupled to an inhibitory guanine nucleotide binding protein (G_i) in the CNS (Dan'ura et al., 1988; Niles and Hashemi, 1990). Dan'ura et al., (1988) found that micromolar concentrations of diazepam could inhibit adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] (AC) activity in rat whole brain and this effect could be inhibited by pertussis toxin.

Furthermore, Ro15-1788 (flumazenil), a central-type BZ receptor antagonist, had no effect on AC activity or on its inhibition by diazepam (Dan'ura et al., 1988). This finding was later confirmed by Niles and Hashemi (1990) who also found that flumazenil failed to block the inhibition of forskolin-stimulated AC activity by Ro5-4864 and pharmacological concentrations of the indoleamine hormone, melatonin, in the rat striatum. Moreover, it was suggested that micromolar concentrations of melatonin and related indoleamines could inhibit AC activity via G_i - coupled BZ receptors, which are quite distinct from the physiological high-affinity receptors for melatonin (Niles and Hashemi, 1990). Since Ro5-4864, an agonist at peripheral-type BZ receptors, was found to inhibit forskolin-stimulated AC activity in rat brain (Niles and Hashemi, 1990), we investigated the effects of PK 11195, a potential antagonist at these receptors, on this inhibitory effect of various BZs and indoleamines.

3.3 *Materials and methods*

3.3.1 Membrane preparation

Male Sprague Dawley rats weighing 200-275 g were housed 2 per cage with free access to food and water. Animals were maintained under a 12L:12D lighting regimen for at least one week before use. For adenylate cyclase assays, animals were sacrificed by decapitation approximately 3 hours after lights on. Striatal tissue was dissected from rat brains on ice and hand homogenized in cold 50 mM HEPES/NaOH buffer containing 5 mM $MgCl_2$, 1 mM dithiothreitol and 0.2 mM EGTA (pH 7.4 at 30 ^{0}C). The homogenate was centrifuged at 39,000 g for 10 min and membranes were subsequently washed twice by resuspension and centrifugation in the above buffer. Finally, membranes were resuspended in 50 mM Hepes/NaOH (pH 7.4 at 30 ^{0}C) buffer for assays.

For binding assays, the striatum was rapidly dissected out on ice. Fresh striatal tissue was homogenized in 50 volumes of 50 mM Tris/HCl (pH 7.4 at 4 ^{0}C) for 5 sec by a polytron homogenizer. The homogenate was centrifuged at 39,000 g for 15 min at 4 ^{0}C and the pellet was washed twice in the same buffer. Membranes were then resuspended in 50 mM Tris/HCl (pH 7.4 at 30 ^{0}C) at a concentration of approximately 5 mg/ml (wet weight/volume) for binding assays.

3.3.2 Adenylate cyclase assays

Assays were performed as we have previously reported (Niles and Hashemi, 1990) with slight modifications. Striatal membrane aliquots containing about 80-100 μg protein were preincubated with various drugs at 30 ^{0}C for 30 min. Following pre-incubation, a reaction

mixture containing 4 mM $MgCl_2$, 100 µM GTP, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM cAMP, 1 mg/ml BSA, 1 mM rolipram, 20 mM creatine phosphate and 20 units/ml of creatine phosphokinase was added to a final volume of 150 µl. Forskolin (50 µM) or dopamine (100 µM) was added to all tubes except basal and background samples and 0.5 mM ATP containing ~ 1 µCi [α^{32}P]ATP was then added to all tubes and incubation carried out at 30 ^{0}C for 10 min. The reaction was stopped by adding 4 mM cAMP and boiling for 5 min. Samples were centrifuged at 10,000 g for 10 min in a refrigerated Mikro Rapid centrifuge. [^{3}H]cAMP was added to monitor recovery and the [^{32}P]cAMP formed was separated from the supernatant by sequential chromatography on Dowex and alumina columns as described by Salomon et al. (1974).

3.3.3 Pertussis Toxin Treatment

Pertussis toxin experiments were carried out as we have previously described (Niles et al.,1991) with some modifications. Fresh rat striatal tissue was placed into culture dishes containing 2 ml of α-minimum essential medium in 10 mM HEPES (pH 7.4 at 37 ^{0}C) supplemented with 1% fetal bovine serum. Each striatum was manually cut into 6 sections and immersed in the medium which was saturated with 95%O_2 / 5% CO_2. Pertussis toxin was reconstituted in 1 ml of 1 mg/ml of BSA in deionized water and then added at a concentration of 8.5 µg/ml to one culture dish while an equivalent volume of the reconstitution buffer was added to another dish. The dishes were then incubated at 37 ^{0}C for 15 1/2 h under 95% O_2 / 5% CO_2. After incubation, the culture dishes containing tissue slices and medium were frozen at -20 ^{0}C until used for AC assays. For assays, culture dishes were thawed at room

temperature and tissue and medium transferred to centrifuge tubes, hand homogenized in 50 mM HEPES/NaOH buffer containing 5 mM $MgCl_2$, 1 mM dithiothreitol and 0.2 mM EGTA (pH 7.4 at 30 °C). The homogenate was centrifuged at 39,000 g for 10 min. Tissue pellets were washed twice by resuspension and centrifugation and then resuspended in 50 mM HEPES/NaOH (pH 7.4 at 30 °C) buffer for AC assays as described above.

3.3.4 Receptor binding assays

For saturation binding assays, tissue preparations were incubated at 30 °C for 60 min with varying concentrations of [^{3}H]diazepam (0.25-83 nM) with or without 1 mM GTP. Non-specific binding was determined in the presence of 10 μM diazepam. After incubation, samples were filtered and washed with 4 x 3 ml ice cold 50 mM Tris/HCl (pH 7.4 at 4 °C). Samples were then counted in a Beckman LS 6500 liquid scintillation counter (efficiency about 70 %). Protein concentrations were determined using the method of Lowry et al. (1951).

3.3.5 Chemicals

The following drugs were used: adenosine 5'[α-^{32}P]triphosphate triethylammmonium salt (3,000 Ci/mmol), and [^{3}H]diazepam (85 Ci/mmol) (Amersham, Oakville, Canada); [2,8-^{3}H]adenosine 3',5'-cyclic monophosphate (ICN Radiochemicals, Montreal, Canada); 2-Iodomelatonin and Ro5-4864 [7-chloro-5-(4-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one] (Research Biochemicals Inc, Natick, USA); PK 11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide] (a gift from Rhône

Poulenc Santé, Vitry sur Seine, France); Flumazenil (a gift from Hoffmann-La Roche Ltd Basle, Switzerland); Rolipram (a gift from Schering AG, Berlin); Melatonin and all other chemicals(Sigma, St. Louis, MO, USA).

3.3.6 Data Analysis

Drug EC_{50} values in AC assays were calculated by non-linear regression analysis using the computer program CDATA (EMF software).[1] The EC_{50} values from at least three separate experiments are expressed as means with 95% confidence limits. Statistical analyses of enzyme data were performed by one-way analysis (ANOVA) and Scheffe's test. Saturation binding data were analyzed by non-linear regression using the BDATA program (EMF software) to determine the equilibrium dissociation constant (K_D) and the maximum number of binding sites (B_{MAX}).

[1] The data was fit to either one-, two- or three-sites using a non-linear least squares parametric analysis program. To determine whether the data was best fit to more than one site, a F-test was calculated as shown below:

$$F = \frac{(SSR_1 - SSR_2)/ df_1 - df_2}{SSR_2/df_2}$$

SSR_1 = sum of squared residuals for the first model
SSR_2 = sum of squared residuals for the model with an additional set of binding sites
df = degrees of freedom

The calculated ratio is compared to the tabulated value for the F statistic with (df_1 - df_2) and df_2 degree of freedom (Munson, PJ. and Robard, D (1980) Anal. Biochem 107:220-239)

3.4 *Results*

3.4.1 Effect of diazepam and Ro5-4864 on Forskolin-stimulated AC activity

Functional studies with Ro5-4864 and diazepam (10^{-11}-10^{-3} M) revealed a concentration-dependent inhibition of forskolin-stimulated AC activity as shown in Figure 1. A F-test indicated a significantly better fit to two-site and three-site models for Ro5-4864 and diazepam, respectively ($p<0.05$). For the first phase of inhibition by Ro5-4864, this drug had an EC_{50} of 1.36 nM (0.64-2.08). The second phase of inhibition by Ro5-4864 was steep with an EC_{50} of 131 μM (87.9-174.0). Diazepam, which was more potent than Ro5-4864, inhibited forskolin-stimulated AC activity with an EC_{50} of 0.43 nM (0.33-0.53) for the first phase and 1.0 μM (0.68-1.32) and 194.0 μM (157.0-231.1) for the other phases.

In other studies, diazepam was also found to inhibit dopamine-stimulated AC activity in the rat striatum. However, it was more potent against forskolin-stimulated AC, presumably due to the greater stimulation produced by forskolin (Table 1).

3.4.2 Effect of flumazenil on Ro5-4864 inhibition of AC activity

Previously, single point assays had shown that the central-type BZ receptor antagonist, flumazenil, failed to block the inhibitory effect of diazepam and Ro5-4864 on forskolin-stimulated AC activity (Niles and Hashemi, 1990). In the present study, a detailed examination of the effects of flumazenil, in dose response studies, indicated that this drug, enhanced the effect of Ro5-4864 at lower doses (10^{-11} - 10^{-10} M, $p<0.001$ vs Ro5-4864 in the absence of flumazenil)(Figure 2). No difference was observed for basal AC activity in the absence or presence of flumazenil (376.3 ±14.2 pmol cAMP/mg protein/min and 369.3 ±11.3

pmol cAMP/mg protein/min, respectively). There was also no difference in forskolin-stimulation of AC activity in the absence or presence of flumazenil (2213±67 pmol cAMP/mg protein/min and 2193 ±94 pmol cAMP/mg protein/min respectively). Thus, the enhanced effect of Ro5-4864 in the presence of flumazenil is not due to an additive effect between these compounds.

3.4.3 Effect of PK 11195 on BZ and indoleamine inhibition of AC activity

There was no difference in basal AC activity in the absence or presence of PK 11195 (265.7 ±1.1 pmol cAMP/mg protein/min and 270.3 ±7.2 pmol cAMP/mg protein/min, respectively). In addition, PK 11195 did not affect forskolin-stimulated AC activity (1965 ±253 pmol cAMP/mg protein/min and 1966±227 pmol cAMP/mg protein/min, in the absence or presence of PK 11195, respectively). Preliminary experiments indicated that in the presence of 1 μM, 5 μM or 10 μM of PK 11195, the inhibitory effect of Ro5-4864 on AC activity was blocked. For example, in control incubates, Ro5-4864 (10 μM) suppressed stimulated AC activity by 26±2.2% as compared with 11 ± 2.8%, 4 ±1.0% and 6 ±1.0%, in the presence of 1 μM, 5 μM and 10 μM of PK 11195, respectively. On the basis of these findings, a 5 μM concentration of PK 11195 was used in subsequent dose response experiments, which indicated that the first phase of Ro5-4864-induced inhibition of AC activity was blocked with no significant effect on the second phase of inhibition (Figure 2).

PK 11195 also blocked the inhibitory effect of diazepam and flunitrazepam as well as that of the indoleamines, melatonin and its iodinated analog, 2-iodomelatonin, on forskolin-stimulated AC activity in the striatum (Figure 3). Diazepam was found to be the most potent

of the BZs examined, in inhibiting forskolin-stimulated AC activity, while 2-iodomelatonin was significantly more potent than melatonin (Figure 3).

3.4.4 *Effect of pertussis toxin on BZ and indoleamine inhibition of AC activity*

Pretreatment of striatal tissues with pertussis toxin did not affect basal or forskolin-stimulated AC activity. Control values were: basal:117.6±5.7 pmol cAMP /mg protein/min; forskolin:458 ±121 pmol cAMP/mg protein/min. Following pertussis toxin treatment, AC activities were: basal:114±9.2 pmol cAMP/mg protein/min; forskolin : 446 ±131 pmol cAMP/mg protein/min. However, the toxin blocked the inhibitory effect of diazepam and Ro5-4864, as well as that of melatonin and 2-iodomelatonin, on forskolin-stimulated AC activity (Figure 4). While pertussis toxin did not affect basal or forskolin-stimulated AC activity, with prolonged incubation (15 1/2 hours), enzyme activity was decreased as compared with that in fresh tissues (see Table 1).

3.4.5 Effect of GTP on [^{3}H]diazepam binding

Saturation binding with [^{3}H]diazepam in the rat striatum revealed a sensitivity to GTP as shown in Figure 5. In the absence of GTP, binding parameters were: K_D = 19.3±0.8 nM; B_{MAX} =631.9 ±21.1 fmol/mg protein, n=3. In the presence of GTP, a modest but significant ($p<0.05$) decrease in affinity was seen, but binding site density was not affected:K_D =30.5±2.6 nM;B_{MAX} = 688.4 ±76.6 fmol/mg protein, n=3.

3.5 *Discussion*

In the present study, the multiphasic dose-response curves observed for benzodiazepine inhibition of AC activity suggest that more than one site or mechanism is involved. The first phase of inhibition is very similar to that mediated by other inhibitory receptor types including striatal and hippocampal G_i - coupled receptors such as $5HT_{1A}$ (De Vivo and Maayani, 1986) and D_2 (Olianas and Onali, 1987). In contrast, the extremely steep and nearly linear secondary phase of inhibition observed with micromolar amounts of BZs supports earlier evidence that high concentrations of these drugs can act directly on the AC enzyme (Dan'ura et al., 1988).

Interestingly, while Ro5-4864 exhibited a biphasic inhibitory action against AC activity, inhibition by diazepam was triphasic. The high-affinity component (EC_{50} = 0.43±0.05 nM) of inhibition by diazepam, like that of Ro5-4864 (EC_{50} = 1.36±0.37 nM), appears to be receptor mediated as discussed below. As seen in Figure 2, the Ro5-4864 curve plateaus between concentrations of 1 μM and 10 μM in a manner typical of a receptor-mediated effect. However, at similar concentrations, the diazepam curve does not plateau, but continues a dose-dependent inhibition of AC activity. Thus, the secondary phase of the diazepam inhibitory curve (EC_{50} = 1± 0.16 μM) may involve both the G-coupled BZ receptor and a direct effect on the AC enzyme. This is supported by the fact that pertussis toxin, which blocks G_i-protein mediated activity, was not as effective in reversing the inhibitory action of diazepam on AC activity as it was with Ro5-4864, as shown in Figure 4.

The fact that the central-type BZ antagonist, flumazenil, failed to block the inhibitory effects of Ro5-4864 on AC activity in this study, as well as that of diazepam in previous

studies (Dan'ura et al. 1988; Niles and Hashemi, 1990), indicates that it is inactive at the G_i-coupled BZ receptor. In contrast to flumazenil, the peripheral-type BZ receptor ligand, PK 11195, blocked the first phase of inhibition by Ro5-4864, suggesting involvement of a receptor with PBR-like pharmacological characteristics. Although PK 11195 may act as a partial agonist in some systems, it did not affect basal or stimulated AC activity, but blocked the inhibitory effect of various other BZs and pharmacological doses of indoleamines, which are thought to act at the same BZ receptor (Niles and Hashemi, 1990). This suggests that PK 11195 acts as a functional antagonist at the G_i-coupled BZ receptor. Furthermore, the inhibition of forskolin-stimulated AC activity in the rat striatum by BZs and indoleamines, was blocked following pretreatment with pertussis toxin. These results provide further evidence for the presence of a G_i - coupled BZ receptor, in the striatal plasma membrane, which mediates the first phase of AC inhibition by various BZs, melatonin and indoleamine analogs.

Melatonin has been shown to inhibit AC activity via a high-affinity receptor in vertebrate brain which is coupled to a pertussis toxin-sensitive G-protein (Niles et al. 1991; Ying et al. 1992). However, it should be emphasized that the inhibition of striatal AC activity, by melatonin and 2-iodomelatonin, does not involve the high-affinity G_i-coupled receptor for melatonin. This is indicated by evidence that: (1) micromolar concentrations of these indoleamines are required to inhibit AC activity in the striatum, as compared to the picomolar to nanomolar concentrations which are effective at the high-affinity receptor for melatonin; (2) autoradiographic and other studies in the rat brain have shown that the high-affinity G_i - coupled receptor for melatonin is primarily localized in the suprachiasmatic nucleus of the hypothalamus, but not present in the striatum; (3) the ability of the PBR antagonist, PK

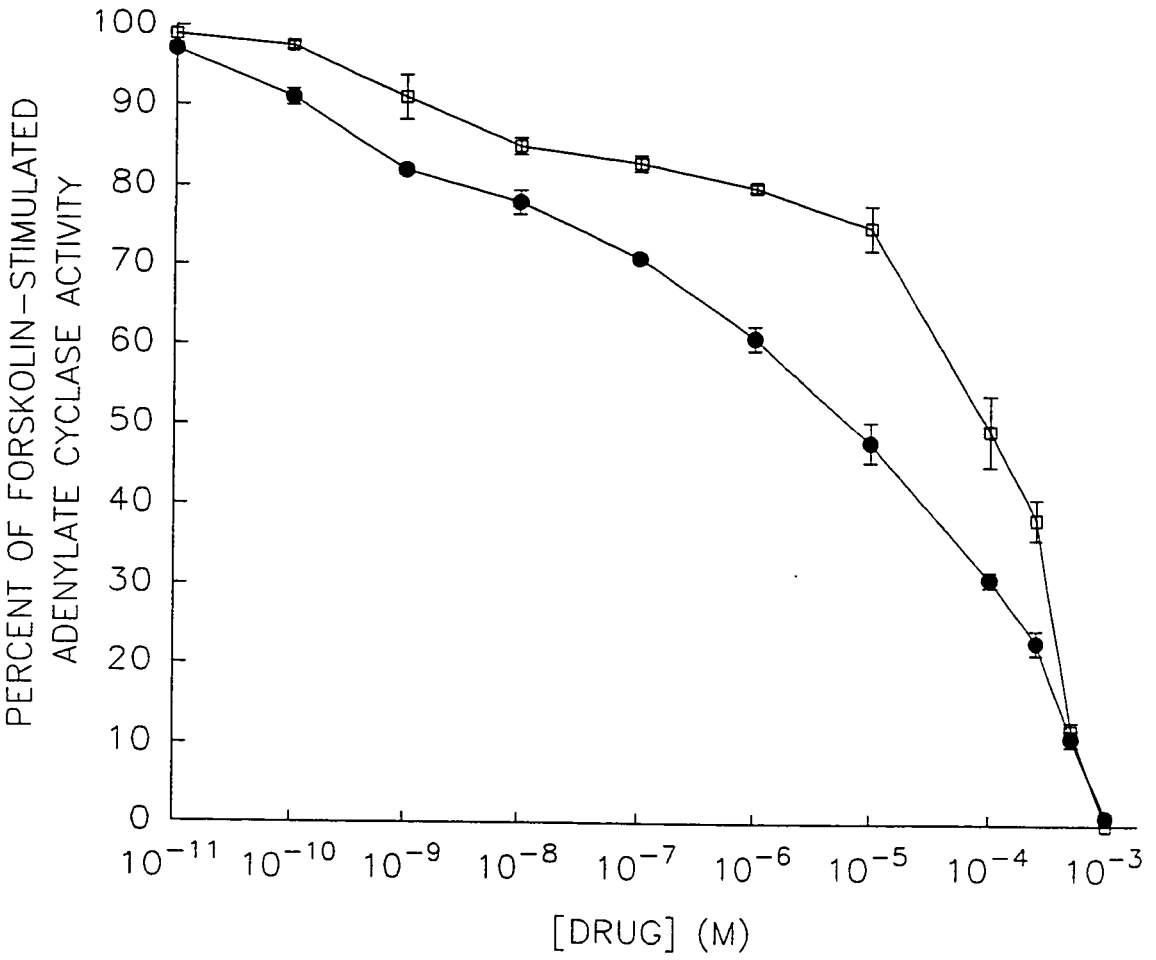
11195, to block the inhibitory effect of melatonin and 2-iodomelatonin in the striatum provides further evidence that a BZ receptor, and not a melatonin receptor, is involved.

Although diazepam is a non-selective BZ agonist, it was more potent than Ro5-4864 in inhibiting stimulated AC activity, therefore, [^{3}H]diazepam was used in binding experiments which were conducted at 30 ^{0}C, in order to minimize binding to the temperature-sensitive central site. Both the binding and functional data suggest that a high (nanomolar)-affinity G_i - coupled BZ receptor mediates inhibitory signalling via the AC pathway by BZ agonists. This is in contrast to the micromolar affinity site reported by Dan'ura et al. (1988). However, this discrepancy could involve tissue differences as their studies were carried out using rat whole brain preparations while striatal tissues were utilized in this study.

In conclusion, the multiphasic nature of AC inhibition observed suggests involvement of multiple sites or mechanisms of BZ action on this signalling pathway, with the high-affinity phase of inhibition being receptor mediated. Therefore, G_i - coupled BZ receptors in the brain may mediate suppression of the ubiquitous second messenger, cAMP, contributing to the diverse psychotropic effects of the BZs, melatonin and related indoleamines. There is evidence that cAMP/protein kinase A (PKA)-dependent mechanisms are involved in the regulation of excitatory synaptic transmission in the CNS (Colwell and Levine, 1995). In keeping with the above, the α_2 -adrenoceptor agonist, clonidine, which inhibits adenylate cyclase activity and cAMP synthesis in the rat brain (Kitamura et al., 1985), is a potent anticonvulsant (Papanicolaou et al., 1982) and anxiolytic drug (Scheinin et al. 1989). It is therefore possible that the anticonvulsant (Albertson et al., 1981; Champney and Champney, 1992; Golombek et al., 1992) anxiolytic (Guardiola-Lemaitre et al., 1992; Pierrefiche et al., 1993) and other

central effects of benzodiazepines, melatonin and related drugs, may involve modulation of the cAMP/PKA pathway. An important question is whether behaviourally effective doses of BZs, such as diazepam, are present in the brain in concentrations sufficient to significantly alter cAMP production, and thus involve this pathway in the neuropharmacological actions of these drugs. Diazepam has been shown to produce its anxiolytic and anticonvulsant effects in rats with ED_{50}s of 1.25 mg/kg, i.p. and 1.31 mg/kg, i.p., respectively (Caccia et al. 1980). Fifteen minutes, post-injection of diazepam (1.25 mg/kg ;ip), 0.04±0.01 μg/ml or about 140 nM of the drug was detectable in plasma. More importantly, over 5 times as much diazepam was present in the brain (Caccia et al.1980), which is enough to cause a significant suppression of central production of cAMP, assuming that *in vivo* efficacy is reasonably comparable to that observed *in vitro* (see figure 1). Thus, in addition to the ability of these drugs to enhance GABAergic activity (Haefely 1989; Tenn and Niles, 1995), inhibition of cAMP production in the CNS appears to be a second mechanism underlying the neuropharmacological effects of BZs, melatonin and analogs.

Figure 1. The effects of diazepam and Ro5-4864 on forskolin-stimulated adenylate cyclase (AC) activity in rat striatum. Membranes were preincubated with the indicated drug concentrations at 30 °C for 30 min. ●, diazepam; □, Ro5-4864. AC assays were performed as described in materials and methods. Results are presented as a percentage of forskolin-stimulated AC activity. Data are the means±S.E.M for 3-4 experiments carried out in duplicate.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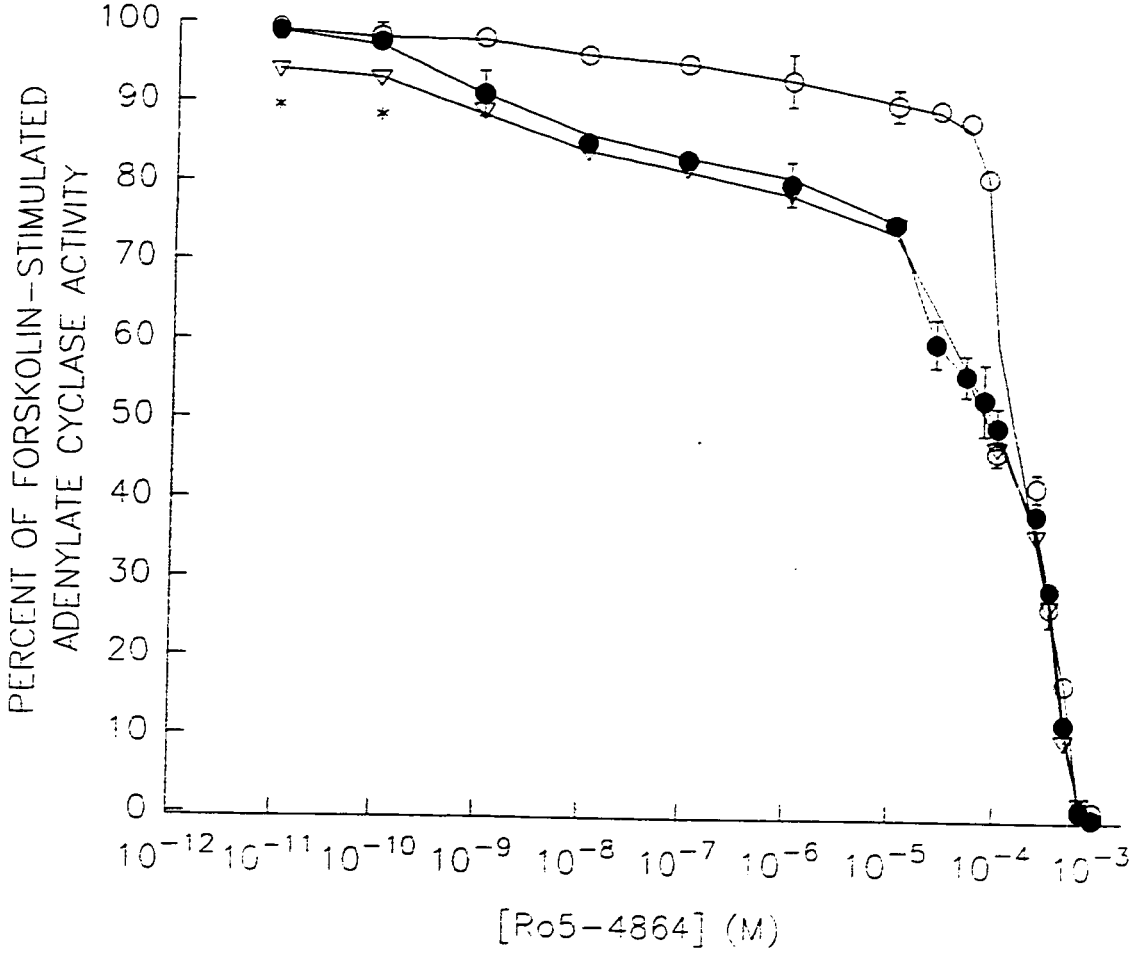
[DRUG] (M)

Figure 2. The effects of BZ antagonists on Ro5-4864-induced inhibition of AC activity. Striatal membranes were preincubated with the indicated concentrations of Ro5-4864, in the absence(●) or presence (○) of 5 μM of PK 11195 or 1 μM of Ro15-1788 (▽). AC assays were performed as described in materials and methods and results are presented as described in figure 1. Data are means ±S.E.M for 3-4 experiments carried out in duplicate. * $p<0.001$ vs control.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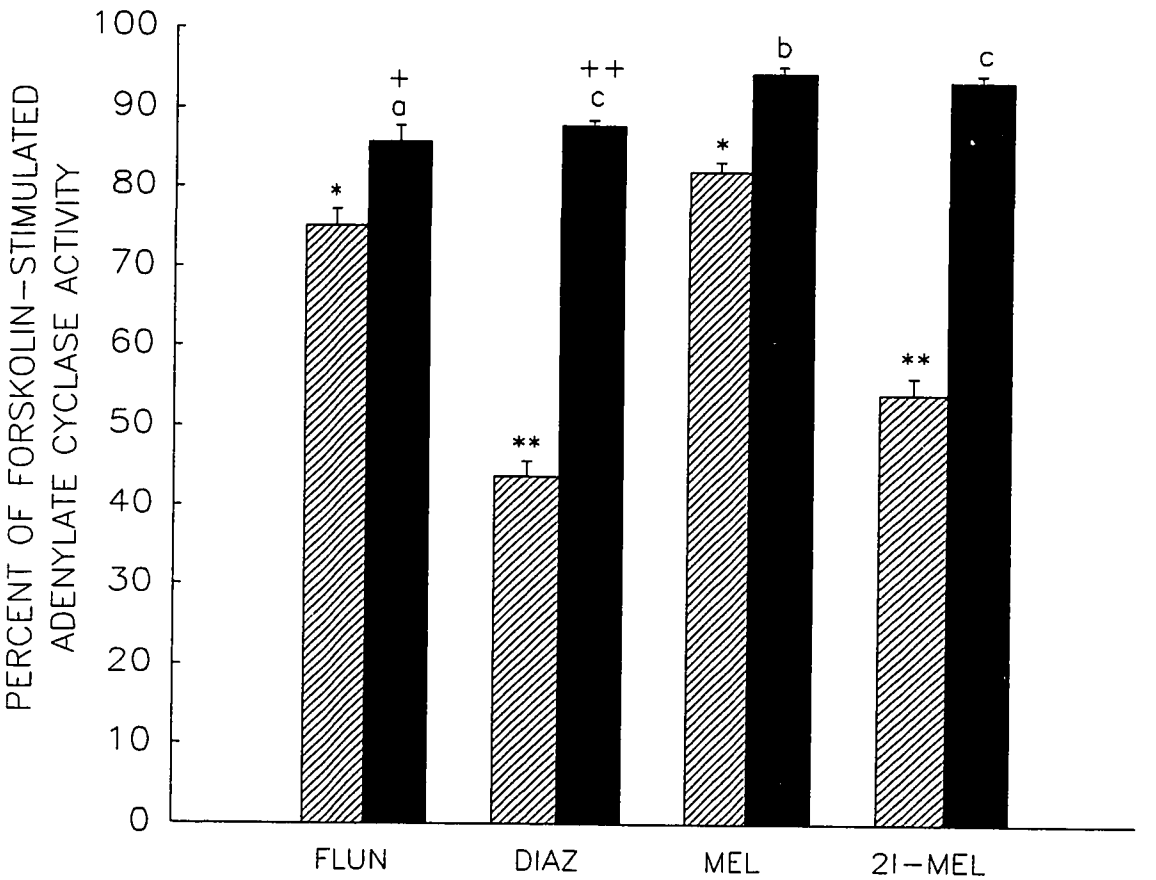
[Ro5-4864] (M)

Figure 3. Effect of PK 11195 on the inhibitory action of BZs and indoleamines on stimulated AC activity. Striatal membranes were preincubated with the indicated drug concentrations, in the absence or presence of PK 11195 (5 μM). FLUN, flunitrazepam (500 μM); DIAZ, diazepam (10 μM); MEL, melatonin (500 μM); 2I-MEL, 2-Iodomelatonin (500 μM). ▨, drug; ■, + PK 11195. AC assays were performed as described in materials and methods. Data are means ± S.E.M of 3-4 experiments carried out in triplicate. $^{*}p<0.01$ and $^{**}p<0.001$ vs control. $^{a}p<0.05$, $^{b}p< 0.001$ and $^{c}p<0.001$ vs drug in the absence of PK 11195. $^{+}p<0.05$ and $^{++}p<0.01$ vs PK 11195 control.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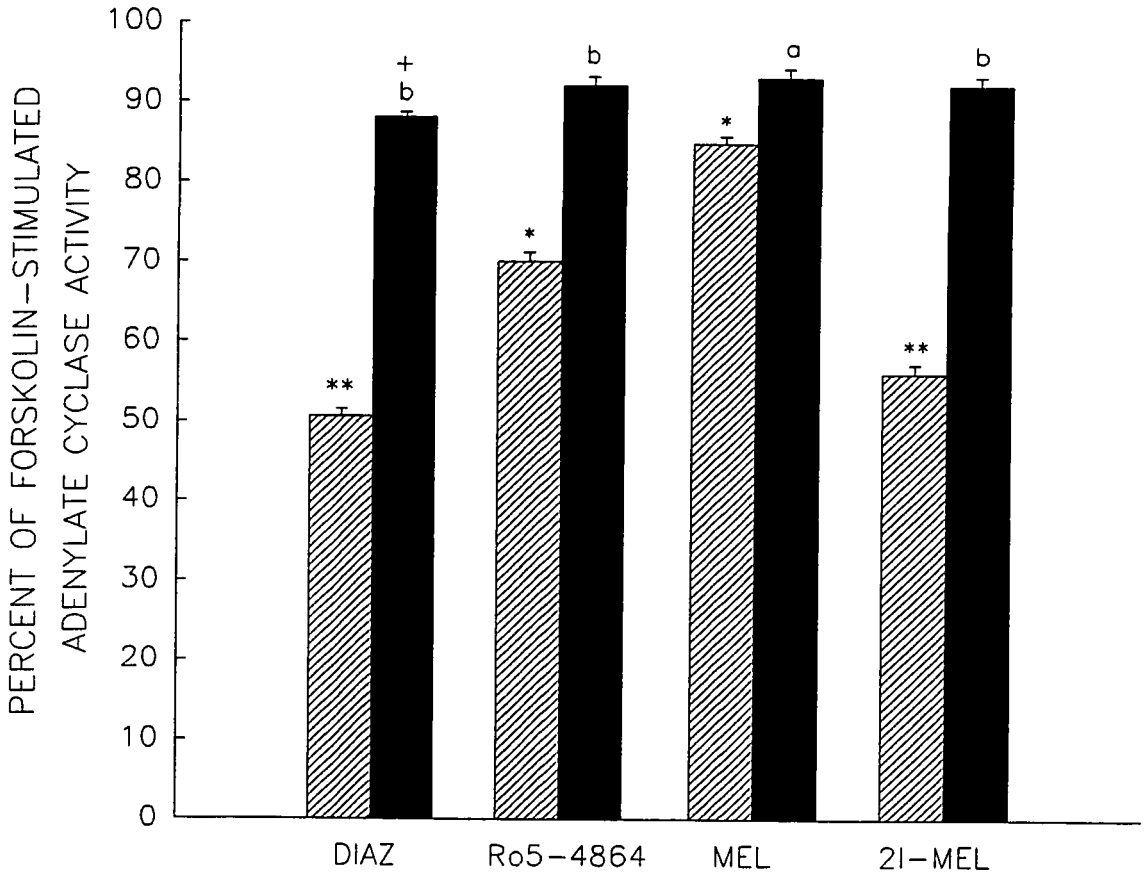
c
FLUN
DIAZ
MEL
21-MEL

Figure 4. Effect of pertussis toxin on the inhibitory action of BZs and indoleamines on stimulated AC activity. Striatal slices were pretreated with pertussis toxin (8.5 μg/ml) for 15 1/2 h as described in materials and methods. Subsequently, membranes were prepared and preincubated with DIAZ; diazepam (10 μM); Ro5-4864 (50 μM); MEL, melatonin (500 μM) and 2I-MEL, 2-Iodomelatonin (500 μM). ▨ drug ; ■ + pertussis toxin. AC assays were performed as described in materials and methods. Data are means ± S.E.M of 3 experiments carried out in either duplicate or triplicate. $^{*}p< 0.01$ and $^{*}p<0.001$ vs control. $^{a}p<0.005$ and $^{b}p<0.001$ vs drug in the absence of pertussis toxin treatment. $^{+}$ $p<0.05$ vs pertussis toxin control.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Ro5-4864
MEL
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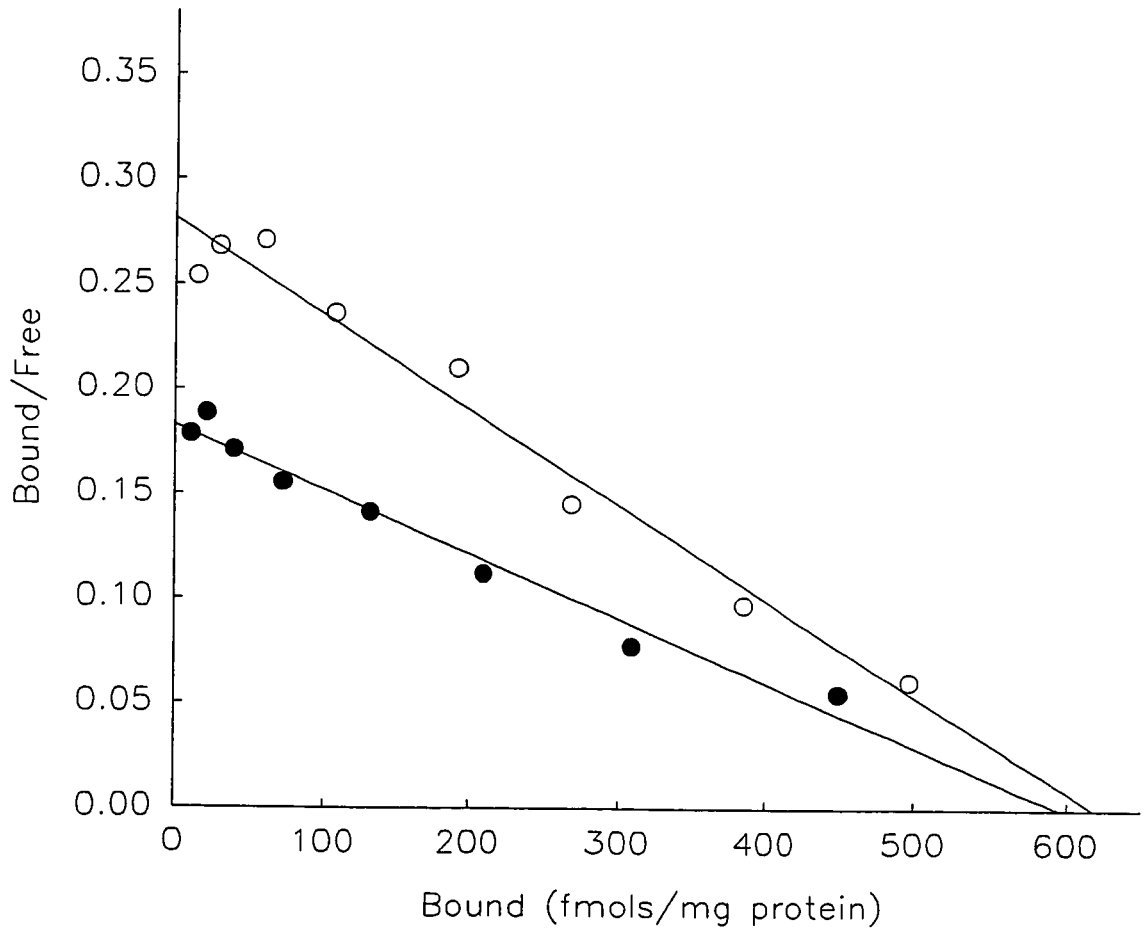
Figure 5. Scatchard plot of [^{3}H]diazepam binding in the rat striatum in the absence (○) and presence (●) of GTP (1 mM). Striatal membranes were incubated at 30 ^{0}C for 60 min with [^{3}H]diazepam (0.25-83 nM) in the presence or absence of diazepam (10 μM). Means of triplicate determination are presented for one of three separate experiments.


0.35
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0.15
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Bound/Free
0
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200
300
400
500
600
Bound (fmols/mg protein)

Table 1. Effect of diazepam on dopamine- or forskolin-stimulated AC activity in the rat striatum.

Drug	Adenylate cyclase activity (pmol cAMP/mg protein/min) Dopamine	% inhibition	Forskolin	% inhibition
none	562 ± 46.9		1474 ± 160	
Diazepam (10 μM)	392 ± 36.1*	30.2	746 ± 79.2*	49
Diazepam (100 μM)	264± 21.1**	53.0	467 ± 66.4**	68

Fresh striatal membranes were preincubated with diazepam at 30 °C for 30 min. Dopamine (100 μM) or forskolin (50 μM) was added and following incubation at 30 °C for 10 min, enzyme activity was measured. Basal enzyme activity was 282± 18.9 pmol cAMP/mg protein/min. Mean ± SEM are presented for N=3. *$p < 0.05$; **$p < 0.005$.

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CHAPTER 4.0

Chapter 4.0 GABAERGIC MEDIATION OF THE ANTIDOPAMINERGIC EFFECT OFCLONAZEPAM AND MELATONIN IN STRIATUM: SENSITIZATION TO THE INHIBITORY EFFECT OF DIAZEPAM ON ADENYLYL CYCLASE ACTIVITY IN 6-HYDROXYDOPAMINE LESIONED ANIMALS. *CC. Tenn and LP. Niles. (1996) J.Neurosci (submitted).*

4.1 *Abstract*

Intrastriatal injection of the $GABA_A$ antagonist, bicuculline, blocked the inhibitory effect of the central-type benzodiazepine (BZ) agonist, clonazepam and the indoleamine hormone, melatonin, on apomorphine-induced rotation in a 6-hydroxydopamine model of dopaminergic supersensitivity. The peripheral-type BZ antagonist, PK 11195, (intrastriatally or intraperitoneally) also attenuated the antidopaminergic effect of these drugs but with much less potency than bicuculline. However, the combination of both bicuculline and PK 11195, injected directly into the striatum, completely blocked the antidopaminergic action of clonazepam or melatonin. Earlier, PK 11195 was found to also block BZ-induced inhibition of cAMP production which is involved in striatal dopaminergic function. Therefore, in addition to a predominant GABAergic mechanism, inhibition of a cAMP pathway may be a secondary mechanism in the antidopaminergic action of clonazepam and melatonin. In other studies, an increase in [^{3}H]diazepam binding was observed in denervated striata with no change in affinity. A marked sensitization to the inhibitory effect of diazepam on AC activity was observed in striatal membranes of lesioned animals. Two distinct striatal proteins with apparent molecular masses of 40- and 45- kD, which are suggestive of the alpha subunits of G_i and G_o respectively, were labeled by [^{32}P]GTP. There was a significant increase in both

basal and diazepam stimulated [^{32}P]GTP binding in lesioned striata. These data indicate that the sensitization of the receptor-mediated inhibitory effect of diazepam on AC activity in denervated striata, may involve upregulation of BZ receptors as well as enhanced functional coupling of these receptors to inhibitory G proteins.

4.2 *Introduction*

Benzodiazepine (BZ) drugs are extensively used because of their anxiolytic, anticonvulsant and sedative properties. The therapeutic actions of these drugs are believed to be mediated through the high affinity central-type BZ receptor which is a component of the macromolecular complex of the benzodiazepine-$GABA_A$ ($BZ/GABA_A$) receptor/chloride channel (Haefely et al. 1985; Olsen and Tobin, 1990; Sieghart, 1994).The $BZ/GABA_A$ receptor is a pentameric protein complex consisting of several membrane spanning glycoprotein subunits. The complexity of this receptor system is increased given the diversity of the subunits identified (Schofield, 1989). So far, a total of 6α- 3β-, 4γ- , 1δ- and 2ρ-subunits of the $BZ/GABA_A$ receptor have been cloned and sequenced from mammalian brain (Macdonald and Olsen, 1994). The complex consists of separate and non-overlapping binding sites for GABA, BZs, barbiturates, picrotoxin, anesthetic steroids and other agents. The potency of GABA, BZs and other modulators of GABA action at these receptors is influenced by the subunits that form the $GABA_A$ receptor (Wisden et al. 1992). The interaction of BZ agonists with central-type BZ sites on the $BZ/GABA_A$ receptor, allosterically enhances GABAergic transmission (Olsen and Tobin, 1990).

Pharmacological concentrations of the pineal hormone, melatonin, can interact with the $BZ/GABA_A$ receptor complex (Niles and Peace, 1990; Niles, 1991). The behavioural effects, such as the anticonvulsant and anxiolytic actions of pharmacological doses of melatonin (Sugden 1983; Golombek et al. 1993), are similar to those of BZs appear to be mediated primarily by $BZ/GABA_A$ receptors (Niles, 1991; Golombek et al. 1993; Tenn and Niles, 1995), although other BZ receptors may also be involved (Tenn et al. 1996).

Following unilateral 6-hydroxydopamine (6-OHDA) lesioning, denervation supersensitivity develops in the rat striatum as shown by contralateral turning when a dopaminergic agonist, such as apomorphine, is administered (Ungerstedt, 1971; Herrera-Marshitz et al. 1985). Using this model of dopaminergic supersensitivity, we have shown that blockade of apomorphine-induced rotation by clonazepam or melatonin is significantly suppressed by pretreatment with either the central-type BZ receptor antagonist, flumazenil, or with the $GABA_A$ receptor antagonist, bicuculline (Tenn and Niles, 1995). The intraperitoneal administration of either flumazenil or bicuculline , caused a significant decrease (65-70%) in the antidopaminergic action of clonazepam or melatonin (Tenn and Niles, 1995). However, due to the potent effect of bicuculline, only a subconvulsant dose was injected. Therefore, it was unclear whether the incomplete blockade by this $GABA_A$ antagonist was due to the low dose utilized or if a secondary mechanism accounts for the remaining 30-35% of the effect not blocked by bicuculline. In order to clarify the extent of GABAergic involvement in the antidopaminergic action described above, we have now utilized direct intrastriatal injection of bicuculline in 6-hydroxydopamine lesioned animals. In view of recent evidence that BZs can act on pertussis toxin-sensitive G protein - coupled BZ receptors to suppress adenylyl cyclase activity in the striatum (Tenn et al. 1996), we have also investigated the possibility that inhibition of cAMP production may be a secondary mechanism involved in the antidopaminergic effect of BZs and melatonin . In addition, we have examined the possible mechanisms underlying the apparent sensitization of the adenylyl cyclase- cAMP pathway to inhibition by BZs in lesioned animals.

4.3 *Methods and Materials*

4.3.1 *Animals*

Male Sprague Dawley rats weighing 250-300 g were singly housed in a room with lights on from 0800-2000h. The temperature was controlled at 22 $\pm$ 1^{0}C. Food and water were freely available and animals were maintained for at least one week before use.

4.3.2 *Chemicals*

The following drugs were obtained from Sigma: melatonin, clonazepam, diazepam, apomorphine, 6-hydroxydopamine and bicuculline. PK 11195 [1-2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide] was a gift from Rhône Poulenc Santé (Vitry sur Seine France). [^{3}H]diazepam (83 ci/mmol) and [α-^{32} P]GTP (3,000 ci/mmol) were purchased from Mandel.[α-^{32} P]ATP (3,000 ci/mmol) was bought from Amersham Life Sciences and [^{3}H]cAMP was obtained from ICN Radiochemicals.

4.3.3 *Cannulation and Intrastriatal Injection*

Unilateral lesioning of the nigrostriatal system was carried out with 6-hydroxydopamine, as previously described (Tenn and Niles, 1995). Approximately 20-30 min prior to anesthesia with Somnotol (55 mg/kg, ip), rats were injected with 15 mg/kg (ip) desipramine to protect noradrenergic neurons. A 30 gauge stainless steel cannula was stereotaxically place in the right or left substantia nigra pars compacta (coordinates- Bregma A-4.8 mm L $\pm$ 1.6 mm V-7.5 mm) (Paxinos and Watson, 1982). Injections of the neurotoxin (8 µg/ 4 µl dissolved in 0.1 % ascorbate saline) were made over a 4 minute period, after which the cannula was left

in place for an additional 4 minutes. Sham operated rats received an equivalent volume of the vehicle solution. A 26 gauge stainless steel guide cannula (Plastics One, USA) was implanted in the striatum ipsilaterally to the lesion (coordinates-Bregma, A-1 mm, L- $\pm$ 3 mm, V- 5 mm) (Paxinos and Watson, 1982), and fixed to the skull by dental cement and screws. In order to prevent clogging, "dummy" cannulas (Plastics One, USA) were inserted into the guide cannulas so that they protruded approximately 0.5 mm from the tip. Two weeks post-surgery, the lesion was evaluated by measuring the circling response to apomorphine (0.25 mg/kg; sc). For intrastriatal injection, BZ antagonists were made fresh on the day of the experiment and a 1 µl volume was injected at a constant rate of 0.2 µl/min, using a 33 gauge stainless steel internal cannula (Plastics One, USA) connected to a syringe infusion pump (Harvard Apparatus).

Lesioned and sham animals were randomly assigned to either vehicle or drug treated groups. All treatment conditions were controlled for with the appropriate vehicle injections. Intrastriatal injection of bicuculline (1-10 nmoles) or PK 11195 (1-50 nmoles) (singly or combined) was followed by intraperitoneal (ip) administration of clonazepam (1 mg/kg) or melatonin (10 mg/kg). Apomorphine was injected 5 min after either clonazepam or melatonin treatment and rotational behaviour was recorded as previously described (Tenn and Niles, 1995). Behavioural experiments were conducted over several months and after a two to three week wash-out period, some of the animals were sacrificed and fresh striata used immediately in adenylyl cyclase or receptor binding assays.

4.3.4 *Adenylyl Cyclase Assay*

Striatal tissue was hand homogenized in cold 50 mM HEPES/NaOH buffer containing 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 0.2 mM EGTA (pH 7.4 at 30 ^{0}C). The homogenate was centrifuged at 39,000 x g for 10 min and membranes were subsequently washed twice by resuspension and centrifugation in the above buffer. Finally, membranes were resuspended in 50 mM HEPES/NaOH buffer (pH 7.4 at 30 ^{0}C). Adenylyl cyclase assays were performed as we have reported previously (Niles and Hashemi, 1990), with slight modifications. Striatal membrane aliquots containing approximately 80-100 µg protein were pre-incubated with diazepam at 30 ^{0}C for 30 min. Following pre-incubation, a reaction mixture, containing 4 mM $MgCl_2$, 100 µM GTP, 1 mM EGTA, 1mM DTT, 0.5 mM cAMP, 1 mg/ml BSA, 1 mM rolipram, 20 mM creatine phosphate and 20 units/ml creatine phosphokinase, was added to a final volume of 150 µl. Forskolin (50 µM) was added to all tubes except basal and background samples. ATP (0.5 mM) containing ~ 1 µCi[α - ^{32}P]ATP was then added to all tubes and incubation carried out at 30 ^{0}C for 10 min. The reaction was stopped by adding 4 mM cAMP and boiling for 5 min. Samples were centrifuged at 10,000 x g for 10 min in a refrigerated Mikro Rapid centrifuge. [^{3}H]cAMP was added to monitor recovery, and the [^{32}P]cAMP formed was separated from the supernatant by sequential chromatography on Dowex and alumina columns, as described by Salomon et al. (1974). Protein concentrations were measured as described by Lowry et al. (1951).

4.3.5 *Receptor Binding Studies*

4.3.5.1 *Tissue preparation:* Fresh striatal tissue from sham or lesioned animals was homogenized in 50 volumes of 50 mM Tris/HCl (pH 7.4 at 4 ^{0}C) by a hand held glass homogenizer. The homogenate was then centrifuged at 39,000 g for 15 min at 4 ^{0}C and the pellet washed twice in the same buffer. Tissue preparations were then resuspended in 50 mM Tris/HCl (pH 7.4 at 4 ^{0}C) at a concentration of approximately 5 mg/ml (wet weight/vol).

4.3.5.2 *Assay:* Tissue preparations were incubated on ice for 60 min with varying concentrations of [^{3}H]diazepam. Non-specific binding was determined in the presence of 10 µM of diazepam. After incubation, samples were filtered and washed with 4 X 3 ml of ice cold 50 mM Tris/HCl buffer (pH 7.4 at 4 ^{0}C). Filters were transferred to minivials with 4.5 ml scintillation fluid (EcoLite). After 5-7 hours, the samples were counted for 2 min in a Beckman LS 7000 liquid scintillation counter (efficiency approximately 40%).

4.3.6 *Guanine Nucleotide Binding Studies*

4.3.6.1 *Basal [α-^{32}P] GTP binding*: The assay was carried out as previously described by Friedman et al. (1993). Briefly, fresh striatal tissue was homogenized in 2 ml of ice cold HED buffer (20 mM HEPES, pH 7.4, 1 mM EGTA, 0.5 mM DTT) using a ground glass homogenizer and volume made up to 5 ml with HED buffer. The homogenate was centrifuged at 700 x g for 5 min at 4 ^{0}C and the supernatant was centrifuged at ~ 46,000 x g for 15 min at 4 ^{0}C. The supernatant was discarded and the pellet washed twice in 5 ml of HEND (HED buffer containing 100 mM NaCl) buffer and centrifuged at ~ 46,000 x g for 10 min at 4 ^{0}C. Membranes (~ 200 µg protein) were incubated in HEND buffer containing 2 mM $MgCl_2$ and

approximately 50-70 nM [α - ^{32}P]GTP for 5, 10 or 15 min. Samples were diluted with 3 volumes of ice cold HEND buffer and centrifuged at 10,000 x g for 15 min at 4 ^{0}C. The supernatant was carefully decanted and the pellets resuspended in 50 µl of sample buffer (62.5 mM Tris HCl pH 6.8, 10% glycerol, 2% SDS, 5 % 2-mercaptoethanol and 0.1% bromophenol blue). The samples were incubated at room temperature for 1 hour with gentle shaking. Various aliquots containing 20 - 40 µg of protein were then separated by the discontinuous method (Laemmli, 1970) with a 12% acrylamide separation gel and a 4% stacking gel. Proteins were transferred to nitrocellulose membranes and then exposed to X-ray film with intensifying screens at -70 ^{0}C overnight. Quantitation of ^{32}P labeled proteins was performed by densitometry of the autoradiograms, using a computerized image-analysis system (MCID Imaging Research, St. Catherines, Ontario).

4.3.6.2 *Receptor-Stimulated [α^{32} P]GTP binding:* The assay was carried out as previously described by Friedman et al. (1993), with modifications. Fresh striatal tissue was homogenized with a ground glass homogenizer in 10 volumes of ice-cold 25 mM HEPES (pH 7.4) buffer which contained 1mM EGTA, 0.1 M sucrose, 50 µg/ml of leupeptin, 0.04 mM phenylmethlysulfonyl fluoride (PMSF), 2 µg/ml of soybean trypsin inhibitor and 0.2% 2-mercaptoethanol. The homogenates were centrifuged at 700 x g for 5 min at 4 ^{0}C and the resulting supernatant was centrifuged at ~ 46,000 x g for 15 min at 4 ^{0}C. The supernatant was discarded and the pellet washed twice in 10 volumes of 25 mM HEPES (pH 7.4) containing 100 mM NaCl, 50 µg/ml leupeptin, 0.04 mM PMSF and 0.2% 2-mercaptoethanol (buffer A). Membranes (~ 200 µg protein/assay tube) were preincubated for 2 min at 30 ^{0}C in buffer A that contained an additional 1 mM $MgCl_2$ and 100 µM GDP. After addition of 50 nM [α

^{32}P]GTP plus buffer (control) or agonist, the incubation continued for 5 min. The reactions were terminated with 3 volumes of ice-cold buffer A containing 1 mM EGTA and immediately centrifuged at approximately 10,000 x g for 15 min at 4 ^{0}C. The supernatants were discarded and the pellets were resuspended in 50 µl of sample buffer. The samples were incubated at room temperature for 1 hour with gentle shaking. Various aliquots containing 20- 40 µg of protein were analyzed by SDS/PAGE as described above. Proteins were transferred to nitrocellulose membranes and exposed to X-ray film with intensifying screens at -70 ^{0}C overnight. Quantitation of ^{32}P labeled proteins was performed by densitometry of the autoradiograms as described above.

4.3.7 *Data Analysis:* Saturation binding data were analyzed by non-linear regression using the BDATA program (EMF software), to determine the equilibrium dissociation constant (K_D) and the maximum number of binding sites(B_{MAX}). For statistical evaluation, experimental data were analyzed by either one-way or two-way analysis of variance and group differences were assessed by Scheffe's test or Student's t-test, where appropriate.

4.4 *Results*

Lesioned animals treated with vehicle solutions and sham-operated animals treated with both vehicle and drug did not differ in their behaviour. The data from these groups were pooled and are shown as the control group in figures.

4.4.1 *Effect of intrastriatal infusion of bicuculline on clonazepam suppression of apomorphine-induced rotation.*

Intrastriatal infusion of bicuculline in doses of 1,5 or 10 nanomoles significantly reduced the effect of clonazepam on apomorphine-induced turning in lesioned animals. Five nanomoles was found to be a maximally effective dose and was used in subsequent experiments. Bicuculline administered intrastriatally was found to be more effective than intraperitoneal injections; however, the antagonist still did not completely abolish the antidopaminergic action of clonazepam (figure 1).

4.4.2 *Effect of intrastriatal infusion of bicuculline in combination with PK 11195.*

Previously, we had shown PK 11195 (10 mg/kg; ip) gave a slight but significant decrease in the effect of melatonin on apomorphine-induced turning behaviour (Tenn and Niles, 1995). In preliminary experiments, intrastriatal infusion of PK 11195 in doses of 1-50 nmoles was tested and optimal dose of 20 nmoles selected for subsequent experiments. PK 11195 at a dose of 20 nmoles caused a modest but significant decrease in the inhibitory action of clonazepam on apomorphine-induced turning. Subsequent experiments examined the effect of intrastriatal pretreatment with bicuculline (5 nmoles) in combination with PK 11195 (20

nmoles) on the antidopaminergic action of BZs and melatonin. Intrastriatal administration of the combined antagonists, completely blocked suppression of apomorphine-induced rotation by either clonazepam or melatonin (figures 1 and 2).

4.4.3 *Effect of Diazepam on AC Activity in Denervated Striatum*

In single point studies, the inhibitory effect of diazepam (10 μM or 100 μM) on adenylyl cyclase activity in the denervated striatum, was significantly greater in the presence of 120 mM NaCl (Figure 3). In contrast, there were no differences in the inhibitory effect of diazepam on enzyme activity in striata from sham-operated animals or intact striata from unilaterally lesioned animals, regardless of whether NaCl was present or absent. The basal activity of adenylyl cyclase was significantly higher in denervated striata as compared to intact striata (309.7 ± 12.8 pmol cAMP/mg prot/min vs 220 ± 12.5 pmol cAMP/mg/prot/min; $p< 0.01$).

Although in preliminary dose-response studies diazepam tended to be more potent in inhibiting forskolin-stimulated AC activity in the denervated striatum, this was not significantly different from the contralateral intact striata. However, with the addition of 120 mM NaCl, the difference between the two sides became significant. The inhibition of AC activity by diazepam produced a biphasic curve in the denervated and intact striata (Figure 4). While there was no difference in the inhibitory effect of diazepam on forskolin- stimulated enzyme activity between striata from sham-operated animals, there was a significant difference between the intact and denervated striata of lesioned rats. For the first phase of diazepam-induced inhibition, the EC_{50} s were 10.4 ± 1.1 nM and 4.8 ± 0.9 nM, ($p<0.05$; n=3),

for intact and lesioned striata, respectively. The second phase of inhibition showed no significant differences, with EC_{50} s of 79.4 ± 9.2 μM and 72.9 ± 6.0 μM for intact and denervated striata, respectively (Figure 4).

4.4.4 *Receptor binding studies*

Saturation binding with [^{3}H]diazepam in the striatum ipsilateral to the lesion revealed no difference in affinity when compared to the intact side. However, the binding site density in the denervated striatum was approximately 23% greater than that of the contralateral (B_{MAX} = 730 ± 49 fmol/mg protein vs 595 ± 12 fmol/mg protein) (Figure 5). There were no differences in either binding affinity or site density between striata from sham-operated animals. Furthermore, the binding characteristics of the intact side of lesioned animals did not differ from those of the sham-operated striata.

4.4.5 *Effects of 6-hydroxydopamine lesion on basal and stimulated [α-^{32}P] GTP binding to striatal proteins*

Basal [^{32} P]GTP binding[1] to striatal membranes was assessed in denervated and intact striata of lesioned animals at various incubation times. Separation on SDS/PAGE indicated binding to both 40- and 45-kD proteins which increased with longer incubation times.

[1] Due to the intrinsic GTPase activity associated with membrane G proteins, [^{32}P]GTP may be converted to [^{32}P]GDP during experimentation. Therefore, the [α- ^{32} P]GTP binding discussed here may involve a combination of [^{32}P]GTP and [^{32}P]GDP bound to membrane G proteins.

Two-way analysis of variance revealed a significant treatment by time interaction for only the 45 kD protein [$F_{(2,8)}$= 25.2, $p<0.001$]. The time-dependent increase in GTP binding to 45 kD proteins was significantly enhanced in the striata of 6-OHDA lesioned animals. There was a significant time effect [$F_{(2,8)}$ = 455.9, $p<0.001$] and treatment effect [$F_{(1,4)}$ = 139.4, $p<0.001$], but no interaction for the 40 kD protein. There was no difference between the 10 min and 15 min incubation times, however, [^{32}P]GTP binding to the 40 kD protein was significantly greater at these times than at 5 min. In addition, there was a greater enhancement of [α-^{32}P]GTP binding in membranes taken from lesioned striata (figure 6). A five minute incubation of striatal membranes in the presence of 10 μM or 50 μM of diazepam, produced an increase in GTP binding to the 40-kD proteins from striata, when compared to basal levels (figure 7). A significant treatment by stimulation interaction [$F_{(2,8)}$ = 54.0, $p<0.001$] was found. While, agonist-induced stimulation of [^{32}P]GTP binding was enhanced in all striata, this effect was significantly greater in denervated tissue (figure 7).

4.5 *Discussion*

Intrastriatal injection of the $GABA_A$ antagonist, bicuculline, was more effective at blocking the antidopaminergic action of clonazepam and melatonin (~ 75 % blockade) than intraperitoneal injection (~65% blockade; Tenn and Niles, 1995). Although significant, the incomplete blockade by bicuculline suggested that, in addition to GABAergic activation, other mechanisms may also be involved in the acute antidopaminergic effect of BZs and melatonin, as previously discussed (Tenn and Niles, 1995).

The possibility that a secondary mechanism involves a cAMP pathway was investigated since: 1) apomorphine, which induces contralateral rotations in lesioned animals, stimulates cAMP production in the striatum (Mishra et al. 1974; Satoh et al. 1976); 2) benzodiazepines, melatonin and melatonin analogs can suppress AC activity via a pertussis toxin-sensitive G protein-coupled BZ receptor (Dan'ura et al. 1988; Niles and Hashemi, 1990; Tenn et al. 1996); 3) Pretreatment with the peripheral-type BZ antagonist, PK 11195, which blocks BZ-induced suppression of AC activity in the striatum (Tenn et al. 1996), caused a slight but significant attenuation of the antidopaminergic effect of clonazepam and melatonin, in lesioned animals (Tenn and Niles, 1995).

PK 11195 was found to be slightly more effective when administered intrastriatally than via intraperitoneal injection. More importantly, pretreatment with the combination of bicuculline and PK 11195 completely blocked the antidopaminergic action of clonazepam and melatonin. These findings indicate the involvement of at least two distinct cellular mechanisms: the primary one being enhancement of GABAergic activity and a secondary mechanism possibly involving suppression of cAMP production in the striatum.

Evidence that the antidopaminergic action of clonazepam and melatonin primarily involves a GABAergic mechanism is consistent with previous studies indicating an extensive interaction between dopaminergic and GABAergic systems in the basal ganglia (Scheel-Krüger, 1983). The majority of the striatal neuronal population are medium spiny GABAergic neurons which give rise to two major output pathways (Kita and Kitai 1988; Gerfen et al. 1990). Dopamine exerts contrasting effects on the two striatal output pathways by utilizing D_1 receptors which mediate stimulation of the striatonigral GABAergic pathway and D_2 receptors which are linked to inhibition the striatopallidal GABAergic pathway (Gerfen et al. 1990; Reid et al. 1990). Conversely, GABA is thought to exert an inhibitory control on the nigrostriatal dopamine system (Gale and Casu, 1981) as intranigral infusion of the GABA antagonists, bicuculline and picrotoxin, was shown to stimulate dopamine release in the striatum (Santiago and Westerink, 1992). Thus, GABA may also modulate striatal dopaminergic activity via its action on the nigrostriatal and other pathways.

With regard to other mechanisms, Satoh et al. (1976) have reported the possibility that cAMP acts as a mediator in the rotational behaviour induced by dopamine receptor stimulation in rats with nigral lesions. In addition to dopamine, dibutyryl cAMP was found to induce rotational behaviour towards the intact side of lesioned animals. Moreover, apomorphine which causes turning behaviour, increased cAMP content in the caudate nucleus when injected intraperitoneally, while the phosphodiesterase inhibitor, theophylline, was found to potentiate this effect (Satoh et al. 1976). These results indicate that cAMP acts as a second messenger in striatal dopaminergic pathways involved in locomotor activity. This is consistent with the importance of cAMP in regulating the protein kinase A (PKA)-dependent

phosphorylation linked to excitatory synaptic transmission by dopamine and other neurotransmitters (Cole et al. 1994; Colwell and Levine, 1995). Thus, suppression of cAMP production in the striatum may be an additional mechanism underlying the antidopaminergic action of clonazepam and melatonin in lesioned animals.

Previously, Pifl et al. (1992) reported a sensitization of dopamine-stimulated AC activity in the striatum of rats with lesions of the nigrostriatal dopamine pathway. This sensitization of AC in the denervated striatum was observed only in the presence of a physiological concentration of NaCl. This finding is in keeping with the ability of sodium ions to modulate the function of receptors coupled to an inhibitory G protein (G_i) (Limbird et al. 1982; Ying et al. 1992). We have also detected a functional sensitization in the striatal membranes of lesioned animals. This significant sensitization to the inhibitory effect of diazepam on AC activity was, like that to dopamine in the above study by Pifl et al. (1992), only detectable in the presence of NaCl.

Studies have shown that denervation supersensitivity occurs following neurotoxin or surgically induced lesions of dopamine neurons, in the mammalian central nervous system (Ungerstedt, 1971; Mishra et al. 1974). This denervation supersensitivity is associated with enhanced dopamine D_2 receptor binding due to an increase in the number of receptors, without any change in the dissociation constant (Mishra et al. 1980). In examining the effect of removing dopaminergic input to the striatum on [^{3}H]diazepam binding, we have also observed an increase in the number of binding sites in the denervated striata as compared to the intact side, with no change in the affinity. Although previous studies with 6-OHDA lesioned animals either failed to observe any changes (Reisine et al. 1979) or found a decrease

(Pan et al. 1985) in striatal [^{3}H]flunitrazepam binding, the difference in ligands employed to measure the benzodiazepine receptor levels may be a factor. While [^{3}H]flunitrazepam is more selective for the central-type BZ receptors, [^{3}H]diazepam is less selective as it labels central-type as well as peripheral-type BZ receptors. A significant increase in the binding of [^{3}H]Ro5-4864, a ligand for peripheral-type BZ sites, was observed following intracisternal injection of 6-OHDA, which could be associated with gliosis as a result of dopaminergic denervation in the striatum (Basile and Skolnick, 1988). Since a portion of the peripheral-type BZ receptors in the brain are localized on glia (Shoemaker et al. 1982), the increase in [^{3}H]diazepam binding observed may be due in part to gliosis in the denervated striatum.

The present results also demonstrate that basal [^{32}P]GTP binding to 40- and 45- kD proteins was higher in lesioned striata. The molecular weights of these detected bands are consistent with the α subunits of G_i or G_o (~40 kD) and G_s (~45 kD) (Hepler and Gilman, 1992). Similarly, Butkerait et al. (1994) have shown in control and reserpine treated animals, that [α- ^{32}P]GTP labelled striatal proteins with molecular masses of 40- and 45- kD, which they had previously identified as the alpha subunits of $G_{i/o}$ and G_s , respectively (Friedman et al. 1993). In the diazepam-stimulated GTP binding experiments, only the 40 kD band, which presumably represents $G_i\alpha$, was detected and the intensity of this band increased in lesioned tissue. Since G_i is in greater abundance than G_s in the brain (Goldsmith et al. 1987), this could account for only the $G_i\alpha$ subunit beinglabelled in the diazepam-stimulated GTP binding experiments, which were conducted for shorter incubation times than in the basal studies.

The increase in basal and agonist-stimulated [^{32}P]GTP binding may be associated with the increase in BZ binding sites observed in lesioned striata. It has been suggested that after

dopamine depletion by reserpine, the increase in D_2 receptor density, which occurs without changes in the levels of G_i, may account for the enhanced coupling of this receptor to its G protein resulting in enhanced GTP binding (Butkerait et al. 1994). This is supported by evidence that there were no changes in overall G_i levels in the striatum at 16 days following 6-OHDA treatment (Marcotte et al. 1994). In view of the foregoing, our data suggest that a similar mechanism, involving upregulation of BZ receptors and increased coupling of these receptors to their inhibitory G proteins in denervated striatum, underlies the sensitization to the inhibitory effect of diazepam on the AC pathway.

A schematic representation of the possible interaction of benzodiazepines or melatonin with both BZ/$GABA_A$ receptors and G_i - coupled BZ receptors in the striatum, is shown in figure 8. While both the BZ/$GABA_A$ receptor complex and the G_i - coupled BZ receptor are depicted on the same cell, it is also possible that these receptors are localized on separate neurons in the striatum. In summary, these findings indicate that the antidopaminergic effect of clonazepam and melatonin involve at least two distinct cellular mechanisms: 1) a predominant GABAergic action and 2) the suppression of cAMP production or other mechanisms in the striatum.

Figure 1. Effect of intrastriatal infusion of bicuculline in combination with PK 11195 on clonazepam suppression of apomorphine-induced rotation. Bicuculline (Bic, 5 nmoles) and PK 11195 (PK, 20 nmoles) were combined and infused 5 min before clonazepam (Clon, 1 mg/kg; ip) and 10 min before apomorphine (Apo, 0.25 mg/kg; ip). Values represent the mean ± SEM of the net total turns for the number of animals shown in parentheses. *P<0.01 vs control; ª P <0.01 vs apomorphine group.

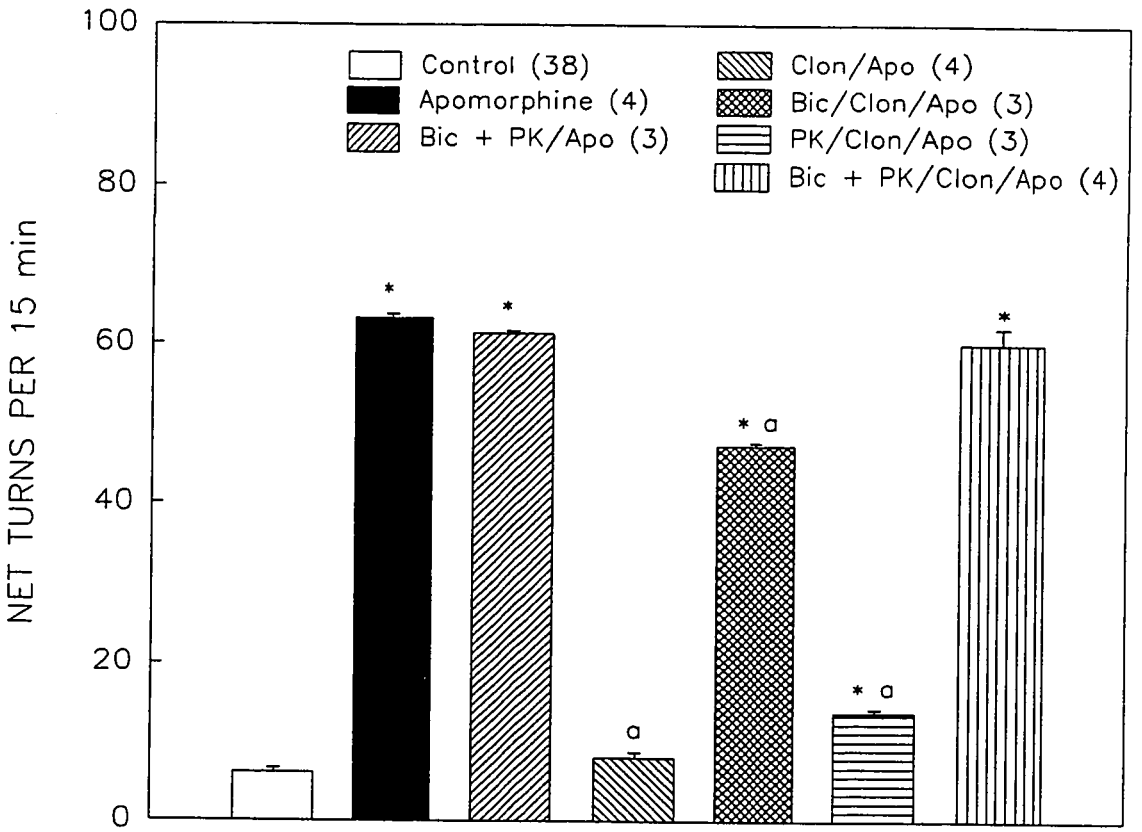

100
80
60
40
20
0
NET TURNS PER 15 min
Control (38)
Apomorphine (4)
Bic + PK/Apo (3)
Clon/Apo (4)
Bic/Clon/Apo (3)
PK/Clon/Apo (3)
Bic + PK/Clon/Apo (4)
*
*
a
* a
* a
*

Figure 2. Effect of intrastriatal infusion of bicuculline in combination with PK 11195 on melatonin suppression of apomorphine-induced rotation. Bicuculline (Bic, 5 nomoles) and PK 11195 (PK, 20 nmoles) were combined and infused 5 min before melatonin (Mel, 10 mg/kg; ip) and 10 min before apomorphine (Apo, 0.25 mg/kg; ip). Values represent the mean ± SEM of the net total turns for the number of animals shown in parenthesis. *P<0.01 vs control; [a] P <0.01 vs apomorphine group.

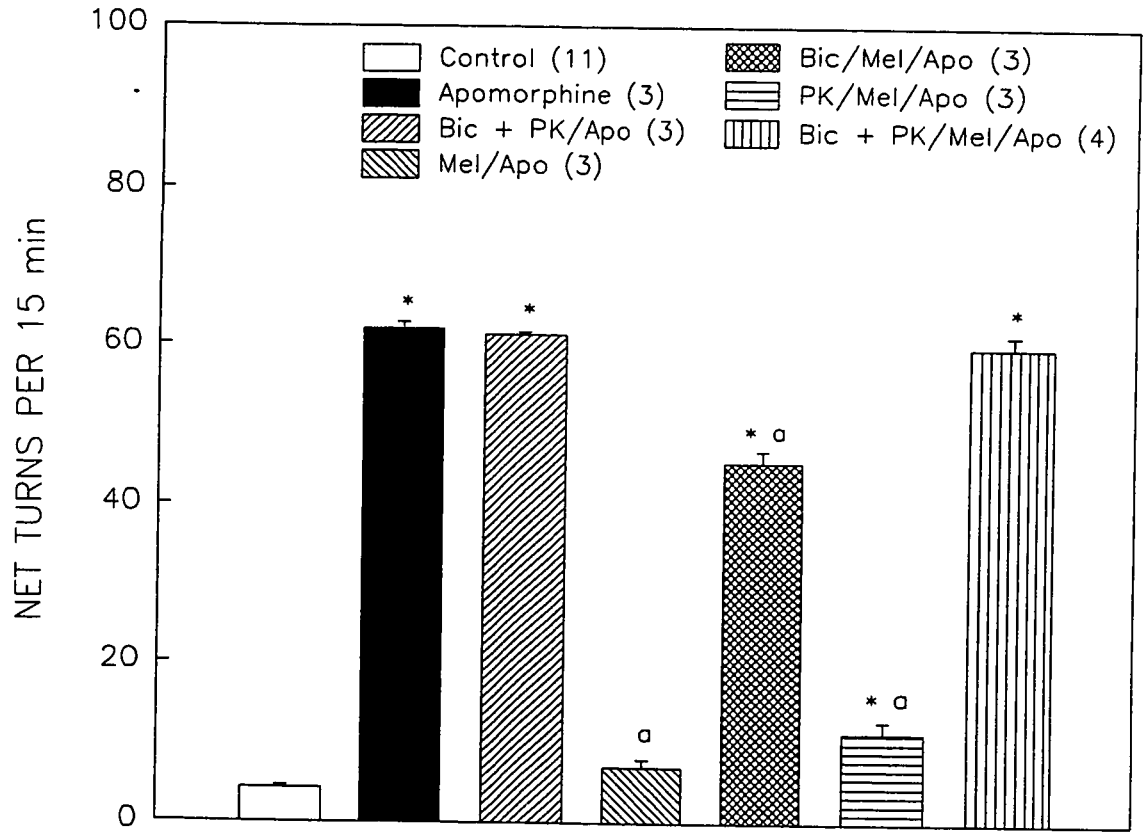
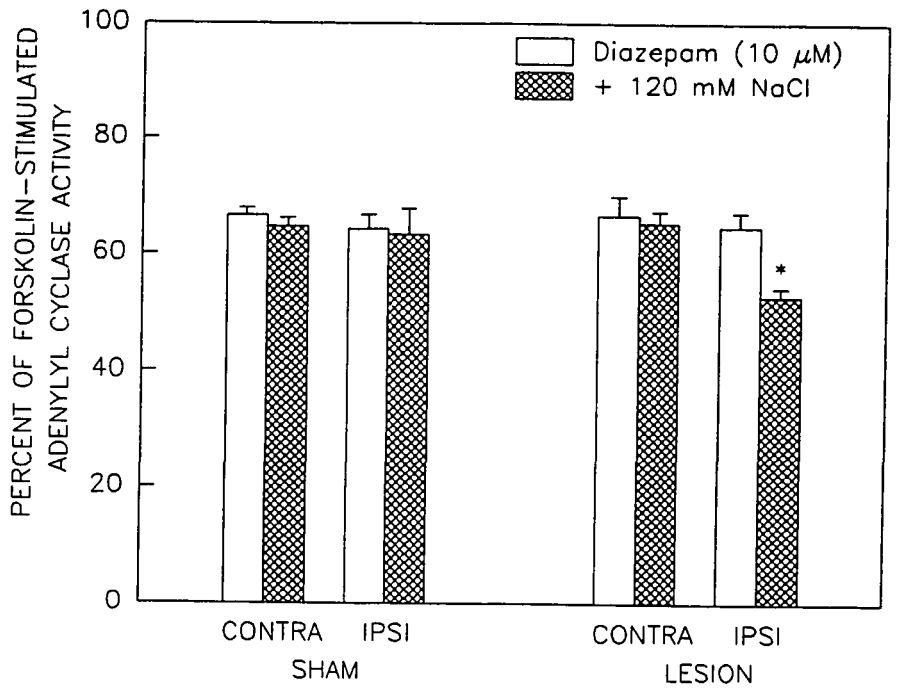

100
80
60
40
20
0
NET TURNS PER 15 min
Control (11)
Apomorphine (3)
Bic + PK/Apo (3)
Mel/Apo (3)
Bic/Mel/Apo (3)
PK/Mel/Apo (3)
Bic + PK/Mel/Apo (4)
*
*
a
* a
* a
*

Figure 3. Inhibition of forskolin-stimulated adenylyl cyclase activity in striatum by diazepam in 6-OHDA lesioned animals. The effects of diazepam (10 μM, upper panel and 100 μM, lower panel) in the presence or absence of 120 mM NaCl are shown. Bars represent mean ± SEM for 3 experiments carried out in duplicate. CONTRA; contralateral; IPSI, ipsilateral. * $p<0.05$ vs denervated tissue in the absence of NaCl.

A


PERCENT OF FORSKOLIN-STIMULATED ADENYLYL CYCLASE ACTIVITY
100
80
60
40
20
0
Diazepam (10 μM)
+ 120 mM NaCl
*
CONTRA
IPSI
SHAM
CONTRA
IPSI
LESION

B

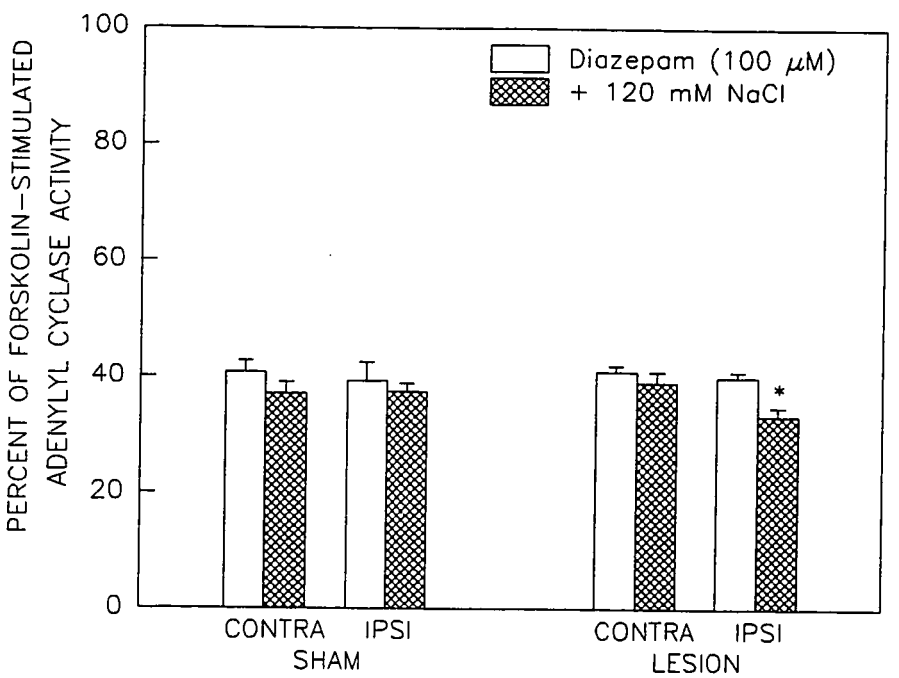

PERCENT OF FORSKOLIN-STIMULATED ADENYLYL CYCLASE ACTIVITY
100
80
60
40
20
0
Diazepam (100 μM)
+ 120 mM NaCl
*
CONTRA
IPSI
SHAM
CONTRA
IPSI
LESION

Figure 4. Inhibition of forskolin-stimulated adenylyl cyclase activity by diazepam in striatum of 6-hydroxydopamine lesioned animals. The values are the mean ± SEM of 3 experiments carried out in duplicate. Basal activity: Denervated: 309.7 ± 12.8 pmol cAMP/mg protein/ min; Intact: 220 ± pmol cAMP/mg protein/min.

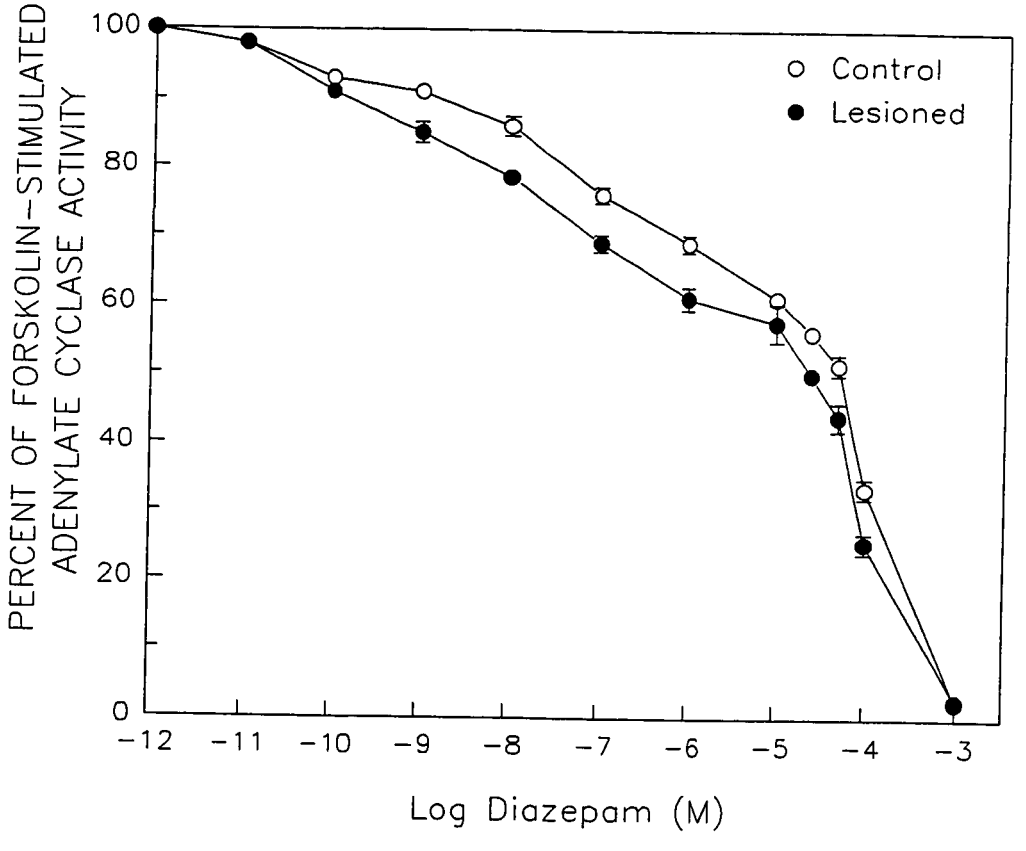

PERCENT OF FORSKOLIN-STIMULATED ADENYLATE CYCLASE ACTIVITY
100
80
60
40
20
0
−12
−11
−10
−9
−8
−7
−6
−5
−4
−3
Log Diazepam (M)
Control
Lesioned

Figure 5. Scatchard plot of [^{3}H]diazepam binding to intact (○) and denervated (●) rat striatum.Striatal membranes were incubated at 0 ^{0}C for 60 min with [^{3}H]diazepam (0.3-41 nM) in the presence or absence of diazepam (10 μM). Data are means of 3 separate experiments performed in triplicate.

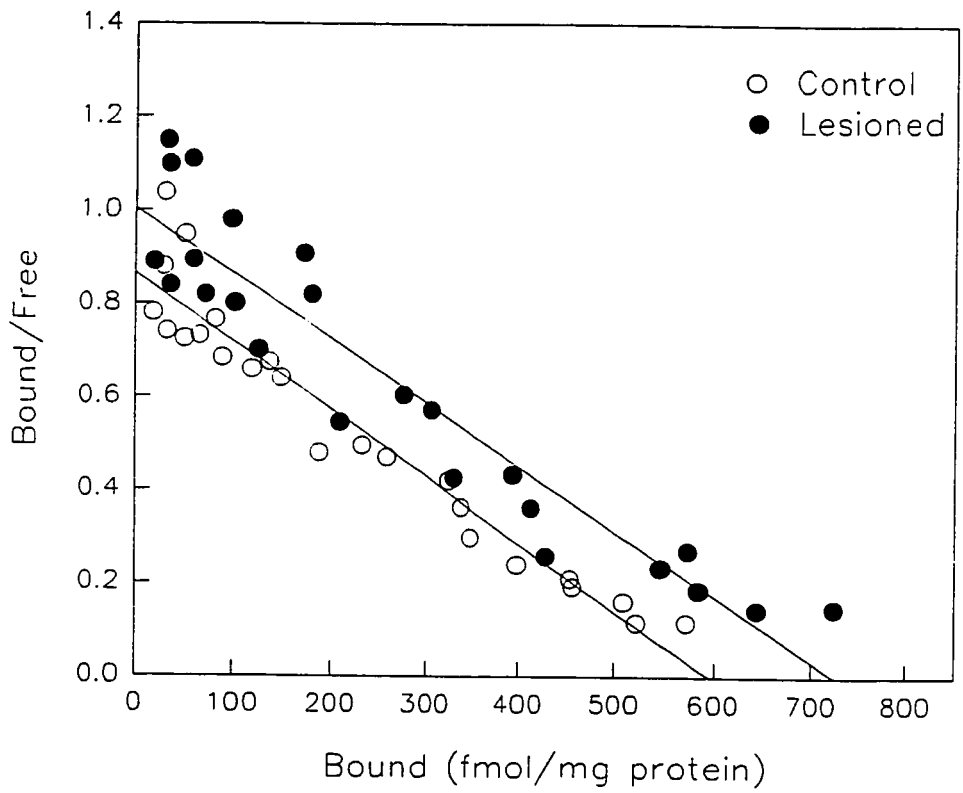
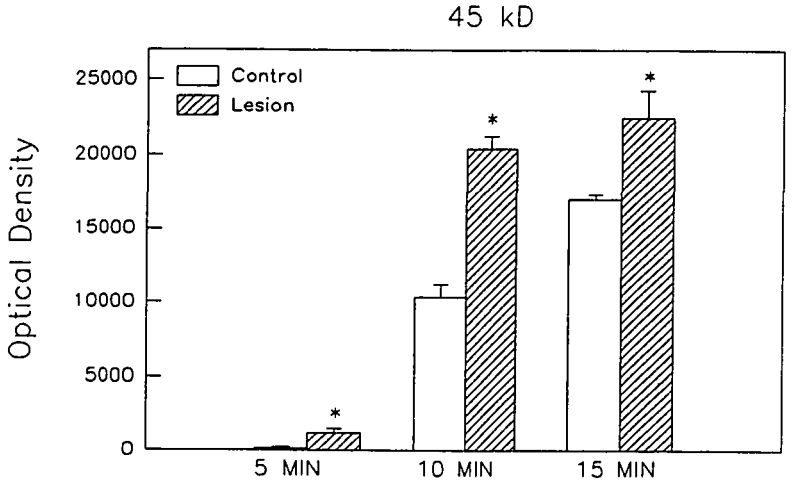

Control
Lesioned
Bound/Free
Bound (fmol/mg protein)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
100
200
300
400
500
600
700
800

Figure 6. The effect of 6-hydroxydopamine treatment on basal GTP binding to striatal proteins. Membranes from control (C) and lesioned (L) striata were incubated with approximately 50 nM [α ^{32}P]GTP for 5, 10 or 15 min as described in Methods and Materials. A) Representative autoradiogram of [^{32}P]GTP labeled proteins separated on SDS/PAGE. B) and C) Quantitation of basal [^{32}P] GTP binding to 45- and 40- kD proteins, respectively, as analyzed by densitometry. Data are presented as the mean ± SD optical density (arbitrary units) representing the amount of bound guanine nucleotide determined from 2-3 separate experiments. There was a treatment x time interaction for the 45 kD proteins ($p<0.001$). *$p<0.001$, Scheffe's test.

A


45 kD-
40 kD-
C L C L C L

B


45 kD
Optical Density
25000
20000
15000
10000
5000
0
Control
Lesion
*
5 MIN
10 MIN
15 MIN

C

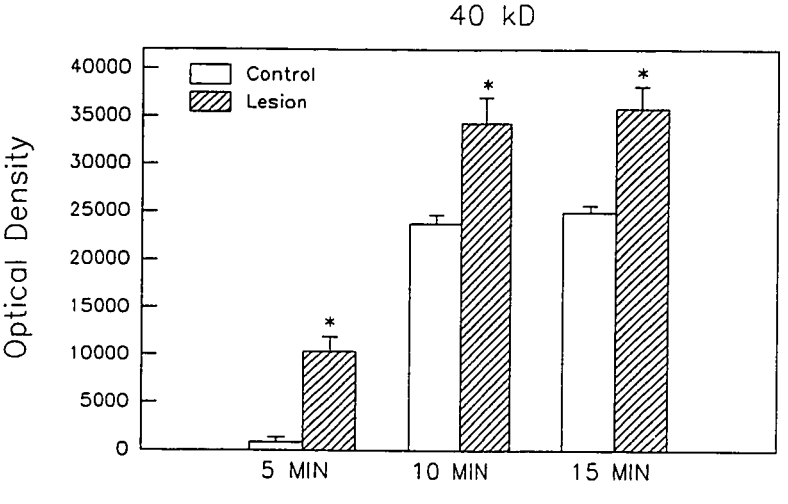

40 kD
Optical Density
40000
35000
30000
25000
20000
15000
10000
5000
0
Control
Lesion
*
5 MIN
10 MIN
15 MIN

Figure 7. Effects of 6-hydroxydopamine treatment on striatal diazepam-stimulated [α-^{32}P]GTP binding to a 40 kD protein in the striatum. Membranes from control (C) and lesioned (L) striata were pre-incubated for 2 mins without agonist. Following pre-incubation, striatal membranes were incubated in the presence of either vehicle (basal), 10 μM diazepam or 50 μM diazepam. A) Representative autoradiogram of [^{32}P]GTP labeled striatal proteins separated on SDS/PAGE. B) Quantitation of diazepam-stimulated [α-^{32}P]GTP binding to 40 kD membrane proteins, as analyzed by densitometry. Data are presented as the mean ± SD optical density (arbitary units) which represent the amount of bound guanine nucleotide determined from 2-3 separate experiments. There was a treatment x stimulation interaction ($p<0.001$). *$p<0.001$, Scheffe's test.

A


40 kD-
C
L
C
L
C
L

B

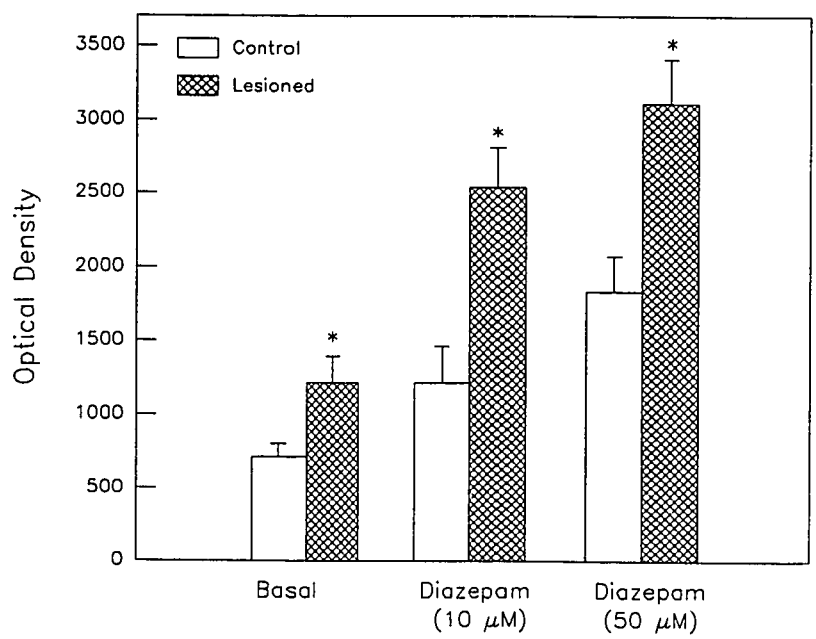
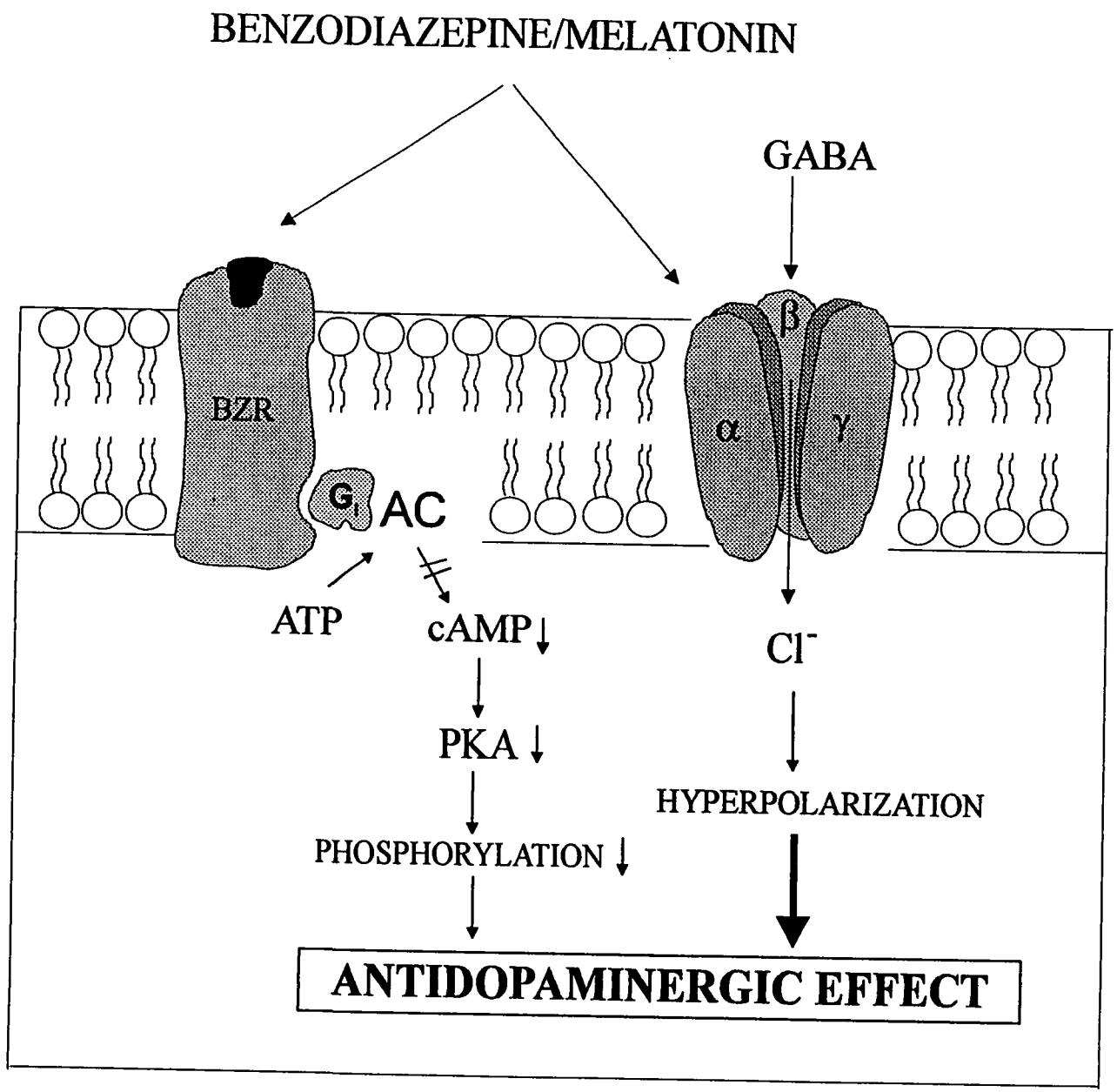

Optical Density
3500
3000
2500
2000
1500
1000
500
0
Control
Lesioned
*
*
*
Basal
Diazepam
(10 μM)
Diazepam
(50 μM)

Figure 8. Diagrammatic representation of the BZ/$GABA_A$ receptor complex and G_i - coupled BZ receptors in the striatum. The BZ/$GABA_A$ receptor ion channel is shown as a pentameric structure composed of α, β, and γ subunits. It is proposed that the interaction of BZ or melatonin with the BZ/$GABA_A$ receptor complex facilitates GABA-mediated chloride influx resulting in hyperpolarization of target cells with suppression of dopaminergic function in the striatum. Activation by BZ or melatonin of another BZ receptor (BZR), which is coupled to an inhibitory guanine nucleotide binding protein (G_i) causes inhibition adenylyl cyclase (AC) activity and cAMP production. Consequently, the activity of the cAMP-dependent protein kinase (PKA) is reduced leading to a decrease in phosphorylation of cellular function. Thus, in addition to a predominant GABAergic mechanism, suppression of cAMP production may also play a role in the antidopaminergic action of BZs and melatonin in the striatum.


BENZODIAZEPINE/MELATONIN
GABA
BZR
β
α
γ
G_i
AC
ATP
cAMP↓
Cl^-
PKA ↓
HYPERPOLARIZATION
PHOSPHORYLATION ↓
ANTIDOPAMINERGIC EFFECT

4.6 *References*

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CHAPTER 5.0

Chapter 5.0 The Antidopaminergic Action of S-20098 is Mediated by Benzodiazepine/$GABA_A$ Receptors in the Striatum *CC. Tenn and LP. Niles. Brain Res. (Submitted)*

5.1 *Abstract*

The naphthalenic compound, S-20098, which is a melatonergic agonist, inhibits [^{3}H]diazepam binding in striatal membranes. S-20098 also inhibits apomorphine-induced turning in 6-hydroxydopamine lesioned rats. This antidopaminergic effect is blocked by either intraperitoneal injection of the central-type benzodiazepine antagonist, flumazenil, or intrastriatal injection of the $GABA_A$ antagonist, bicuculline. These findings indicate that S-20098 can activate central-type BZ receptors and its antidopaminergic action, like that of melatonin, involves a GABAergic mechanism in the striatum.

5.2 *Introduction*

Recently, S-20098 (N[2-(7-methoxy napht-1-yl)ethyl]acetamide) which has a naphthalene nucleus substituted for the indole ring of melatonin, was shown to have a high affinity for melatonin binding sites in ovine pars tuberalis cells [6,22]. These physiological binding sites for melatonin are present on high-affinity G protein-coupled receptors in the hypothalamus, pars tuberalis and other regions [8,19]. In behavioural studies, the naphthalenic compound had similar effects as melatonin such as re-entrainment of circadian activity rhythms [12,18] and restoration of normal sleep pattern in rats infected with a parasite which severely alters slow wave synchronized sleep [5].

Previously, it was shown that melatonin has a micromolar affinity for the central-type benzodiazepine (BZ) receptors [10] and this indoleamine could enhance binding of GABA in rat brain [3,13]. Thus, apart from its own receptors, pharmacological concentrations of melatonin also interact with central-type BZ binding sites on the $GABA_A$ chloride ionophore ($BZ/GABA_A$) [14,15]. The $BZ/GABA_A$ receptors are distributed throughout the central nervous system and mediate the anxiolytic, sedative and anticonvulsant actions of benzodiazepines. Similar behaviourial effects of pharmacological doses of melatonin as an anxiolytic are suppressed by the central-type BZ antagonist, flumazenil, indicating involvement of $BZ/GABA_A$ receptors [4,20].

Recently, pharmacological concentrations of melatonin have been shown to inhibit apomorphine-induced turning behaviour in 6-hydroxydopamine lesioned animals [2,20]. This antidopaminergic effect of melatonin is mediated by BZ receptors and predominantly involves the enhancement of GABAergic activity [20]. In view of the structural and functional

similarities between melatonin and S-20098, we hypothesized that the latter compound would also interact at BZ sites to modulate dopaminergic activity, as has been shown for melatonin [20].

5.3 *Methods and Materials*

Male Sprague Dawley rats (250-300 g) were singly housed with lights on from 0800-2000h. Food and water were freely available and the temperature was controlled at 22 ± 1 °C. Binding experiments were carried out on P_2 membrane fractions prepared from striatum as previously described [20]. Striatal membranes were incubated with 1.5- 2 nM [^{3}H]diazepam (Mandel, 83 Ci/mMol) for 1 hr on ice, in the presence or absence of various concentrations (10^{-15} - 10^{3} M) of S-20098, melatonin, 2-iodomelatonin, diazepam or clonazepam. After incubation, samples were filtered and washed with 4 x 3 ml of ice-cold 50 mM Tris/HCl (pH 7.4 at 4 °C). Samples were then counted in a Beckman LS 6500 liquid scintillation counter (efficiency about 70%). Specific binding was defined as total bound radioactivity minus binding in the presence of 10 μM diazepam. Protein content was measured by the method of Lowry et al. [9].

Unilateral lesioning of the nigrostriatal system was carried out as previously described [20]. Animals were cannulated ipsilaterally to the lesion. A 26 gauge stainless steel guide cannula (Plastics One, USA) was implanted in the striatum (coordinates-Bregma, A-1mm, L- ± 3 mm, V - 5mm) [16]. Guide cannulae were fixed to the skull by screws and dental cement. Two weeks post-surgery, animals were examined for the presence of dopaminergic supersensitivity by their response to apomorphine. Drugs were freshly prepared on the day of the experiment and bicuculline (5 nmoles in 1 μl of 50% DMSO/saline) or vehicle solution was injected intrastriatally at a constant rate of 0.2 μl/min, using a 33 gauge stainless steel internal cannula (Plastics One, USA) connected to a syringe infusion pump (Harvard Apparatus).

Lesioned and sham animals were assigned to either vehicle or drug treated groups. All treatment conditions were controlled for with the appropriate vehicle injections. Injection of flumazenil (10 mg/kg; ip) or bicuculline (5 nmoles; intrastriatally) was followed by administration of S-20098 (5mg/kg; ip). Apomorphine (0.25 mg/kg; sc) was injected 5 min post S-20098 treatment. The rotational behaviour was recorded as previously described [20]. The total number of complete 360 degree turns made by the animal in a 15 min period was recorded manually with contralateral (ie. away from lesioned side) turns being scored as positive and ipsilateral (ie. toward lesioned side) turns as negative. The net total turns were obtained as the sum of the contralateral and ipsilateral turns. Experimental data were analyzed by one-way analysis of variance and group differences were assessed by Scheffe's test. The IC_{50} values for various drugs used in competition binding assays were calculated by the computer program CDATA (EMF software).

5.4 *Results*

The structural similarities between melatonin and S-20098 are illustrated in figure 1. Except for replacing the indole nucleus with a naphthalene structure, these compounds have similar substituents including the methoxy as well as the N-acetyl amino side groups. Interestingly, both of these compounds exhibit micromolar affinity for BZ receptors, yet structurally they are very different from the prototype BZ, diazepam, as shown.

The effects of S-20098 and other drugs on [^{3}H]diazepam binding in the striatum are shown in figure 2. Melatonin and S-20098 were equipotent as inhibitors of diazepam binding, however, they were significantly less potent than the melatonin analog, 2-iodomelatonin. Of the two BZs studied, the central-type BZ agonist, clonazepam, was more potent than diazepam. Inhibition of [^{3}H]diazepam binding to rat striatal membranes by S-20098, indoleamines and BZs had a rank order of potency, with the IC_{50} values shown in parentheses, as follows: clonazepam (0.7 nM) > diazepam (6.2 nM) >> 2-iodomelatonin (1.6 μM) > melatonin (107 μM) ≥ S-20098 (120 μM).

In the behavioural studies, lesioned animals treated with vehicle solutions and sham-operated animals treated with either vehicle or drug did not differ in their responses. Therefore, data from these groups were pooled and are shown as the control group in figure 3. As seen in this figure, the net total turns in response to apomorphine was more than 10 fold greater than that seen in controls. When S-20098 was administered alone, there was no difference in the net total turns as compared to controls. However, when S-20098 was administered prior to apomorphine it completely blocked the turning behaviour induced by this dopaminergic agonist ($p<0.01$).

Pretreatment with the central-type BZ antagonist, flumazenil, 15 min before injection of S-20098, attenuated significantly the ability of the naphthalenic compound to block apomorphine-induced rotation. Similarly, bicuculline, the $GABA_A$ receptor antagonist, caused a significant suppression of the antidopaminergic effect of S-20098 ($p<0.01$).

5.5 *Discussion*

The results from the present study show that S-20098, like melatonin, competes for BZ binding sites in rat striatal membranes, but with much lower affinity than that of the BZs. Substituting a naphthalene structure for the indole nucleus of melatonin, did not affect the potency of S-20098 as an inhibitor of diazepam binding when compared to melatonin. However, a 2-iodo substitution on melatonin produced an analog with about 65-75 fold greater potency than either melatonin or S-20098. The central-type BZ agonist, clonazepam, was found to be the most potent of the compounds tested in inhibiting diazepam binding in the striatum, indicating the involvement of central-type BZ receptors.

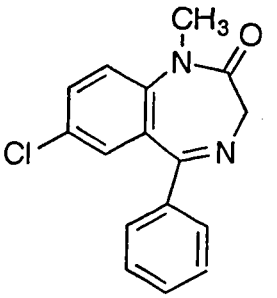
Earlier studies have shown that melatonin inhibits diazepam binding with an IC_{50} of approximately 500 μM in the rat forebrain [10]. In contrast, the present study shows that melatonin inhibits diazepam binding at a significantly lower concentration (IC_{50} = 107 μM) in the rat striatum. A similar higher affinity for melatonin at BZ sites was also found in human and bovine cortex (IC_{50} = 50-100 μM) [15]. While some of these differences may be species related, the heterogeneity of central-type BZ receptors across brain regions, may account for the differential effects of melatonin in rat cortex versus striatum. Two receptor subtypes, type I and type II, were originally suggested for central-type BZ receptors, based on their differential affinity for triazolopyridazines and β-carbolines [1,7]. Examination of the pharmacological characteristics of BZ/$GABA_A$ receptors produced by recombination of the α subunits indicated that those containing the α_1 subunit behaved like type I BZ receptors while those with α_2 and α_3 subunits behaved like type II receptors [17]. Areas such as the striatum which are enriched with the α_2 subunit [21] would be associated with type II

receptors. Therefore, the present data suggests that melatonin has a higher affinity for the type II BZ receptors found in the striatum. In addition, [^{3}H]diazepam labels both central-type and peripheral-type BZ receptors, while melatonin has a 6-fold greater affinity for peripheral-type sites [11]. Therefore, regional differences in the relative densities of these BZ receptors could also influence the affinities observed in binding studies.

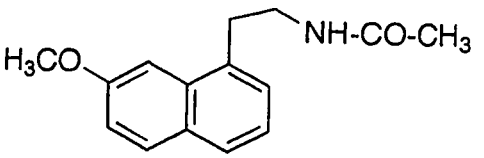
The behavioural study shows that S-20098 exerts a potent antidopaminergic effect in 6-hydroxydopamine lesioned animals. The ability of flumazenil, a central-type benzodiazepine antagonist, to attenuate this inhibitory effect, implicates activation of central-type BZ receptors in the antidopaminergic effect of S-20098. Moreover, the similar blocking effect observed after intrastriatal injection of the $GABA_A$ antagonist, bicuculline, indicates that the antidopaminergic action of S-20098 involves a BZ receptor-mediated enhancement of GABAergic activity in the striatum. These results are similar to those we have reported recently for melatonin and clonazepam, where the antidopaminergic action of these drugs was found to primarily involve a GABAergic mechanism [20].

However, it has been reported that 20 minutes after administration of 5 or 10 mg/kg of melatonin, micromolar levels of this lipophilic indoleamine are present throughout the brain [4]. Presumably, the similarly lipophilic compound S-20098, at the dose of 5 mg/kg utilized in this study, would also be initially present in micromolar concentrations, allowing it to interact with central-type BZ receptors in the striatum and elsewhere in the brain. Thus, like melatonin, micromolar concentrations of S-20098 induce antidopaminergic and other behavioural effects that are mediated by predominantly central-type BZ receptors in the central nervous system.

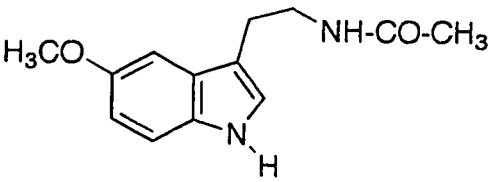
Figure 1. Structural formulae of diazepam, melatonin and S-20098.



Diazepam



S-20098



Melatonin

Figure 2. Competition curves showing inhibition of [^{3}H]diazepam (1.5-2.0 nM) binding by clonazepam, diazepam, 2-iodomelatonin, melatonin and S-20098 in striatal membranes. The assays were carried out at 0 ^{0}C for 1 hour. The values shown are means of 2-3 experiments carried out in duplicate.

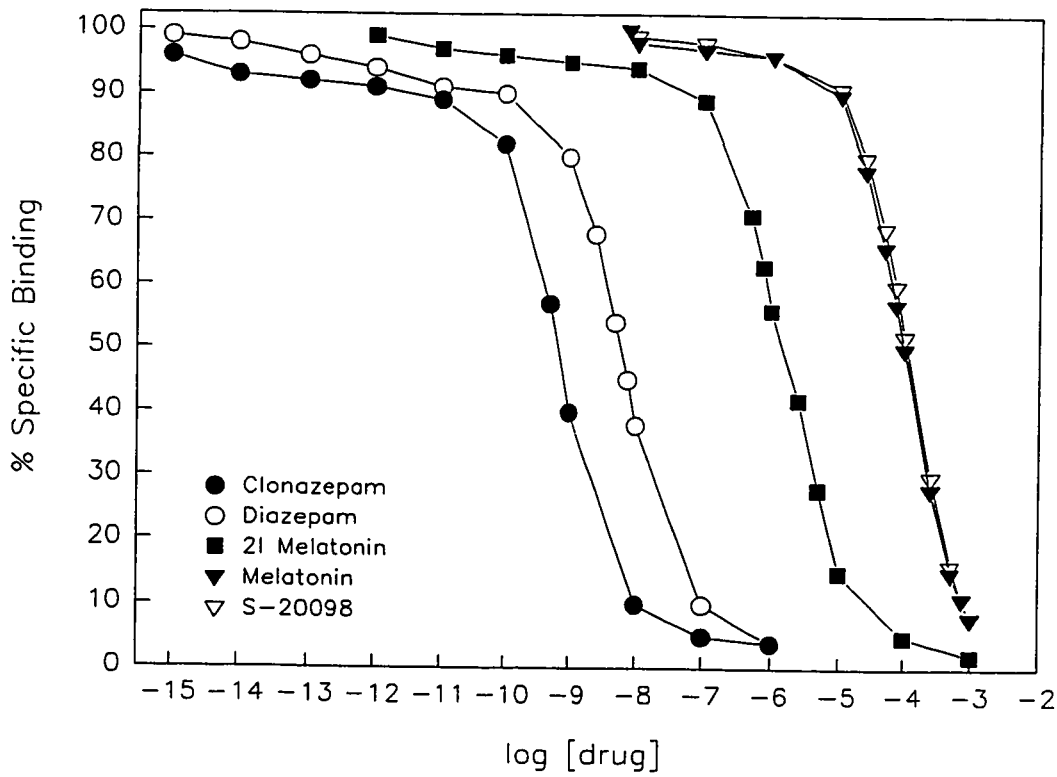
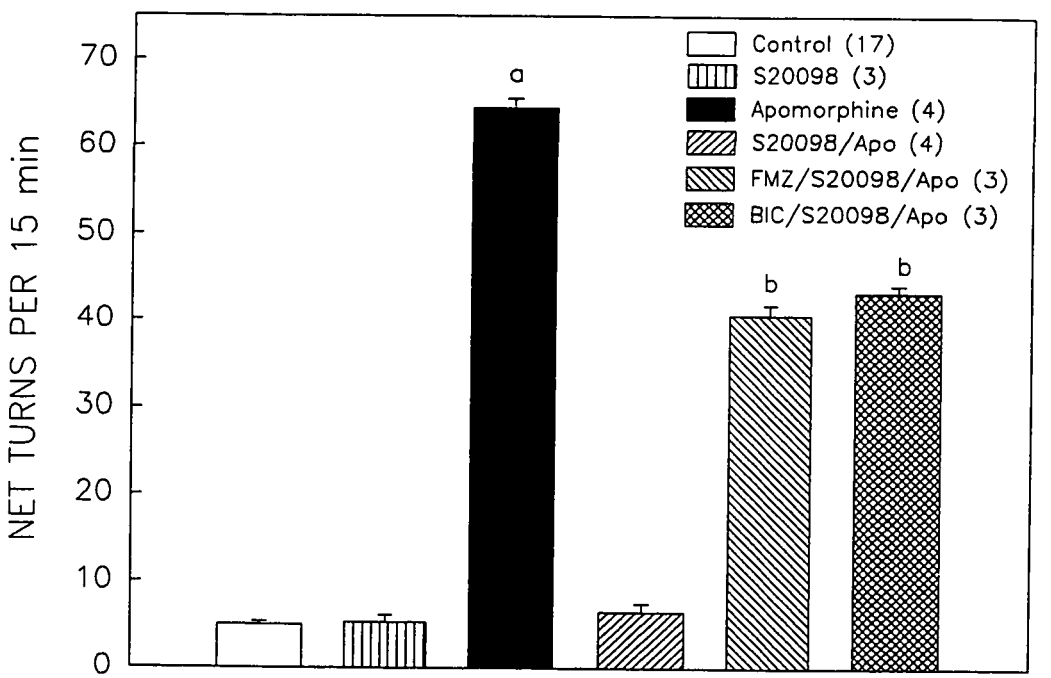

% Specific Binding
100
90
80
70
60
50
40
30
20
10
0
−15 −14 −13 −12 −11 −10 −9 −8 −7 −6 −5 −4 −3 −2
log [drug]
Clonazepam
Diazepam
2I Melatonin
Melatonin
S−20098

Figure 3. Effect of flumazenil or bicuculline on S-20098 inhibition of apomorphine-induced rotation. Flumazenil (FMZ 10 mg/kg; ip) was injected 15 min before S-20098 (5 mg/kg; ip) and 20 min before apomorphine (APO, 0.25 mg/kg; ip). Bicuculline (BIC, 5 nanomoles; intrastriatal) was administered 5 min before S-20098 and 10 min before apomorphine. Values represent the mean ± SEM of the net total turns for the number of animals shown in parentheses. [a] $P< 0.01$ vs all other groups.[b] $P<0.01$ vs control, S-20098 and S-20098+ apomorphine-treated groups.


NET TURNS PER 15 min
70
60
50
40
30
20
10
0
a
b
b
Control (17)
S20098 (3)
Apomorphine (4)
S20098/Apo (4)
FMZ/S20098/Apo (3)
BIC/S20098/Apo (3)

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CHAPTER 6.0

Summary and General Discussion

Chapter 6.0 *SUMMARY AND GENERAL DISCUSSION*

6.1 *Summary of findings*

In the following section, the major findings from the studies presented in the thesis are summarized.

Using the 6-hydroxydopamine model of striatal dopaminergic receptor supersensitivity it was shown that clonazepam and melatonin inhibited apomorphine-induced turning behaviour. The antidopaminergic effect of these drugs was significantly suppressed by pre-treatment with either the central-type BZ receptor antagonist, flumazenil or with the BZ/$GABA_A$ receptor antagonist, bicuculline. These findings indicated that the antidopaminergic effect of clonazepam and melatonin is mediated predominantly by central-type BZ receptors in the CNS via a GABAergic mechanism. However, administration of either of these antagonists intraperitoneally, did not completely block the antidopaminergic action of clonazepam or melatonin. Limitations in the use of the convulsant, bicuculline, made it difficult to determine the extent of GABAergic involvement in the above inhibitory action of melatonin and clonazepam. In order to circumvent this problem, direct intrastriatal injection of the $GABA_A$ antagonists in 6-OHDA lesioned animals was examined.

In a follow up study, intrastriatal injection of bicuculline was more effective than intraperitoneal treatment in suppressing the effect of clonazepam and melatonin, but did not completely abolish it. The peripheral-type BZ antagonist, PK 11195 (intraperitoneally or intrastriatally), only slightly attenuated the effects of clonazepam and melatonin. However, when both antagonists were combined and injected directly into the striatum, the suppression

of apomorphine-induced rotation by clonazepam or melatonin was completely blocked. These results indicate the involvement of at least two distinct cellular mechanisms, the primary one being enhancement of GABAergic activity. Since BZs can act on pertussis toxin -sensitive G protein coupled BZ receptors to suppress AC activity in the striatum, the possibility that inhibition of cAMP production may be a secondary mechanism involved in the antidopaminergic effect of BZs and melatonin was investigated.

The third study characterized further the G_i - coupled BZ receptor by examining the effect of the peripheral-type BZ receptor antagonist, PK 11195, on the inhibition of forskolin-stimulated AC activity by BZs. A biphasic inhibition of AC activity by the peripheral-type BZ receptor agonist, Ro5-4864 and diazepam was observed. The biphasic nature of the inhibition suggests two distinct mechanisms of action for the benzodiazepines, one being receptor mediated and the other possibly a direct effect on the enzyme itself. The receptor-mediated phase of inhibition was abolished by PK 11195 and as well as by pertussis toxin. Saturation binding studies revealed a GTP- sensitive binding site in the striatum. These results strongly suggest the presence of a pertussis toxin-sensitive G protein coupled BZ binding site in the rat striatum. Since PK 11195 was found to also suppress the antidopaminergic effects of BZs and melatonin in 6-OHDA lesioned animals, suppression of cAMP production may contribute to the antidopaminergic effect of clonazepam and melatonin.

Subsequently, the effect of diazepam on AC activity in lesioned animals was examined. A sensitization to the receptor-mediated inhibitory effect of diazepam on AC activity was observed in the denervated striatum. Saturation binding with [^{3}H]diazepam revealed an increase in the number of BZ binding sites in the denervated striatum with no change in

affinity. In examining the functional activity of G proteins in striatal membranes, both basal and diazepam-stimulated [^{32}P]GTP binding were found to be significantly increased in the denervated striata. These findings suggest that the increased sensitivity to the inhibitory effect of diazepam may be associated with the upregulation of BZ binding sites and enhanced coupling of these receptors with inhibitory G proteins in denervated striata.

The final study examined the antidopaminergic action of the naphthalenic compound, S-20098, which binds to the melatonin receptor with high affinity in the brain. It was hypothesized that in high doses, like melatonin, S-20098 would interact with BZ binding sites to modulate dopaminergic activity. This suggestion was supported by the finding that S-20098 inhibited [^{3}H]diazepam binding in striatal membranes with an efficacy similar to that of melatonin. In addition, the antidopaminergic effect of S-20098 was suppressed by intrastriatal injection of the $GABA_A$ antagonist, bicuculline. These findings indicate that the antidopaminergic action of S-20098 in the striatum of lesioned animals also involves a GABAergic mechanism.

In summary, the findings in this thesis suggest that there are at least two distinct cellular mechanisms underlying the inhibitory effect of clonazepam and melatonin on central dopaminergic function. The antidopaminergic action of these drugs involves mainly a GABAergic action (via BZ/$GABA_A$ receptor complex) with suppression of cAMP production (via G_i- coupled BZ receptor) as a possible secondary mechanism (see figure 8, Chapter 4).

6.2 *General Discussion*

Since the antidopaminergic effect of clonazepam and melatonin was predominantly mediated by a GABAergic mechanism, the following section will discuss the relevance of this finding, and specifically how the basal ganglia and various neurotransmitters may be involved. For clarity and simplicity the two distinct pathways that pass from the striatum to the basal ganglia output nuclei will be dealt with separately.

The striatal neurons giving rise to the direct (striatonigral) pathway express primarily the excitatory D_1 dopamine receptors (Gerfen et al. 1990). Under normal basal conditions, the presence of dopamine in the striatum has a stimulatory effect via D_1 receptors on striatal substance P/GABA (SP/GABA) neurons (Dichara et al. 1994). In addition, dopamine acts on presynaptic D_2 receptors to reduce acetylcholine (ACh) and cortical glutamatergic release, thereby reducing the inhibitory effect mediated by cholinergic M4 binding sites on SP/GABA neurons (Dichiara et al. 1994). Nigral GABAergic neurons tonically inhibit thalamic projection neurons. Lesioning of the nigrostriatal pathway results in less activity along the direct (striatonigral) pathway to the nigral output nuclei. Reduction of striatal dopamine intensifies the effects of the inhibitory cholinergic system which is driven by excitatory glutamatergic input. Consequently, striatonigral SP/GABA neuronal activity decreases, while GABAergic nigral efferent neurons inhibit thalamic projection neurons. The end result is a decrease in locomotor activity (see figure 1a).

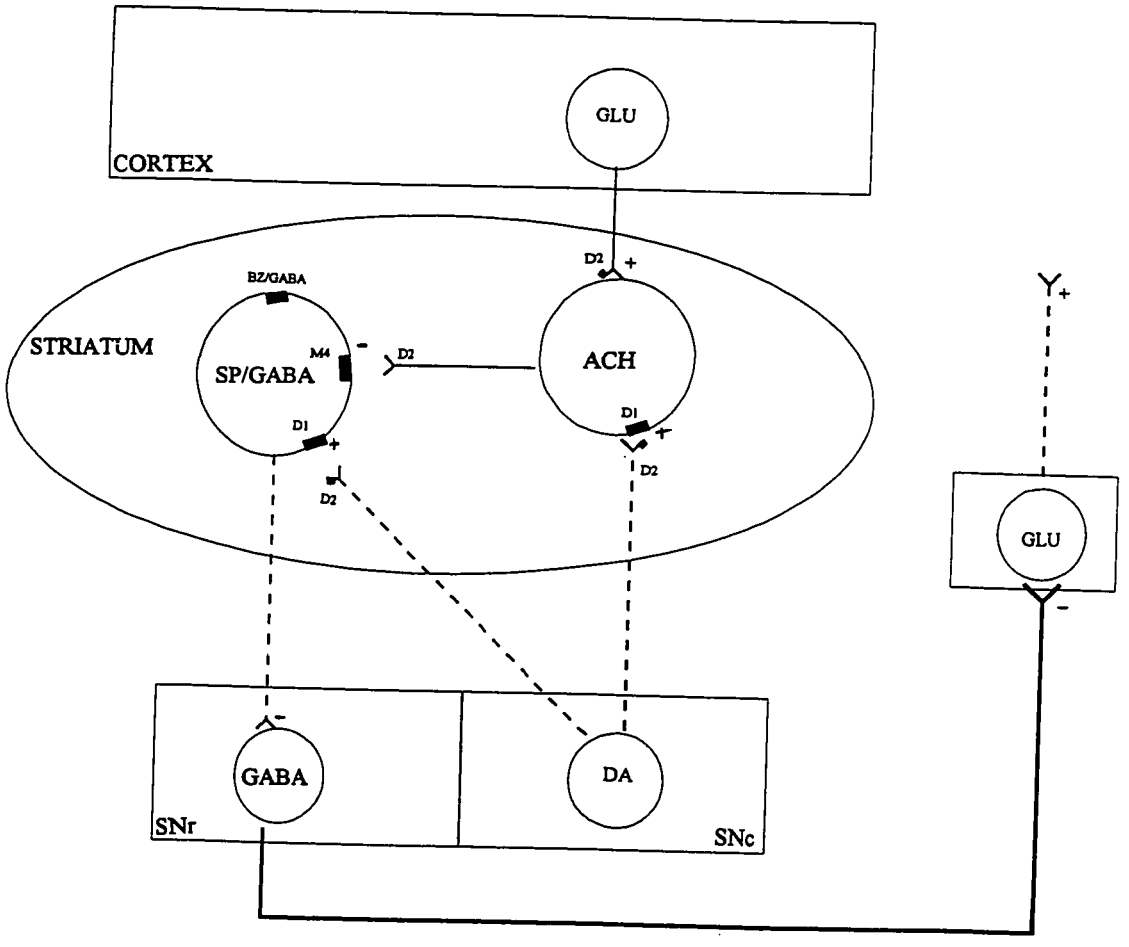

Figure 1a. Interactions of dopamine/acetylcholine/GABA in the striatonigral pathway of the basal ganglia of a lesioned animal. Broken lines, decrease in pathway activity; Bold lines, increase in pathway activity. ACH- acetylcholine; DA- dopamine; GLU- glutamate; SP- substance P. D1/ D2 - dopamine receptors; M4-muscarinic binding site (inhibitory); SNr- substantia nigra pars reticulata; SNc-substantia nigra pars compacta. +, excitatory; - , inhibitory. (Ref: Dichiara et al. 1994; Gerfen et al. 1990; Carlsson and Carlsson, 1990).

Administration of apomorphine, a D_1/D_2 agonist stimulates D_1 receptors located on the striatal SP/GABA neurons. Through the action of the presynaptic D_2 receptors located on glutamatergic and cholinergic terminals, apomorphine also reduces inhibitory cholinergic activity mediated by the M4 pathway (coupled to adenylate cyclase). The overall effect is **stimulation of the striatonigral pathway resulting in inhibition** of the nigral GABAergic influence on the thalamic projection neurons and an increase in locomotor activity (see figure 1b)

Administration of melatonin or clonazepam results in blockade of the apomorphine effect by interacting with the BZ/GABAA receptor complex on striatal SP/GABA neurons (figure 1 b). As a result of enhanced GABA-induced hyperpolarization following treatment with clonazepam or melatonin, striatonigral transmission is **decreased** causing disinhibition of nigral output neurons and a decrease in locomotor activity. Blockade of the effect of melatonin or clonazepam by pretreatment with the $GABA_A$ antagonist, bicuculline, allows apomorphine-induced **stimulation** of the striatonigral pathway and locomotor activity as described above.

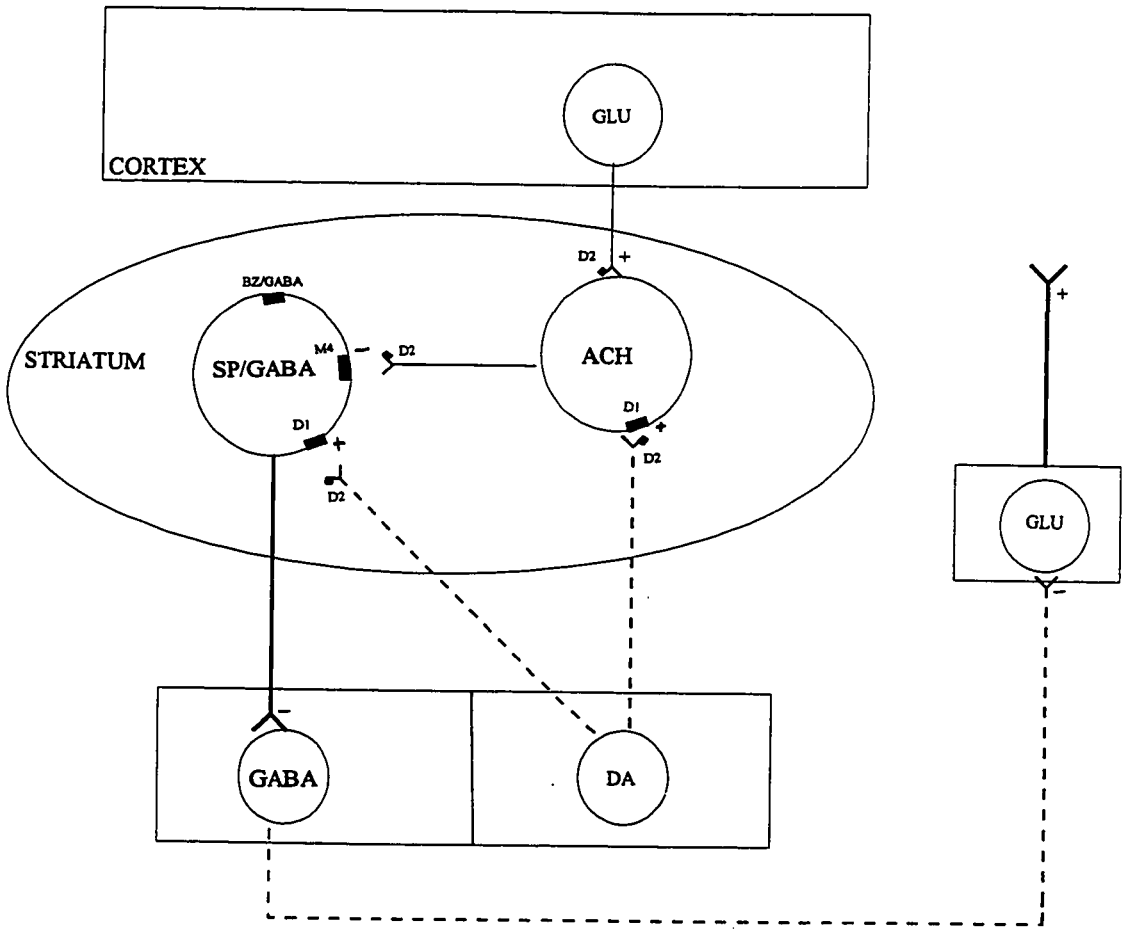

Figure 1b. Schematic diagram of the interactions of dopamine/acetylcholine/GABA in the striatonigral pathway of a lesioned animal following administration of apomorphine.+, excitatory; -, inhibitory. (Ref: Dichiara et al. 1994; Gerfen et al. 1990; Carlsson and Carlsson, 1990).

The striatopallidal pathway expresses primarily inhibitory D_2 receptors. Under normal basal conditions, the presence of dopamine in the striatum has an inhibitory effect via D_2 receptors on striatal enkephalin/ GABA (ENK/GABA) neurons. Activation of presynaptic D_2 receptors results in reducing the **stimulatory** effect mediated by cholinergic M1 receptors (linked to phosphoinositol turnover)on ENK/GABA neurons (Dichiara et al. 1994). The medium-size spiny GABAergic neurons tonically inhibits cholinergic interneurons as well as ENK/GABA neurons. Thus, the striatopallidal transmission decreases. Disinhibition of the pallidal GABAergic neurons results in suppression of glutamatergic neurons of the subthalamic nuclei thus reducing the excitatory input on nigral GABAergic neurons. Inhibition of nigral output neurons results in glutamatergic activation in the thalamus and locomotor activity.

Lesioning of the nigrastriatal pathway results in increased activity along the striatopallidal pathway to the nigral outflow. This increased activity in the striatal ENK/GABA neurons is due to the stimulatory input via cholinergic interneurons. Inhibition of pallidal GABAergic neurons results in stimulation of nigral GABAergic inhibitory neurons and inhibition of thalamic neurons. Locomotor activity decreases (see figure 2a).

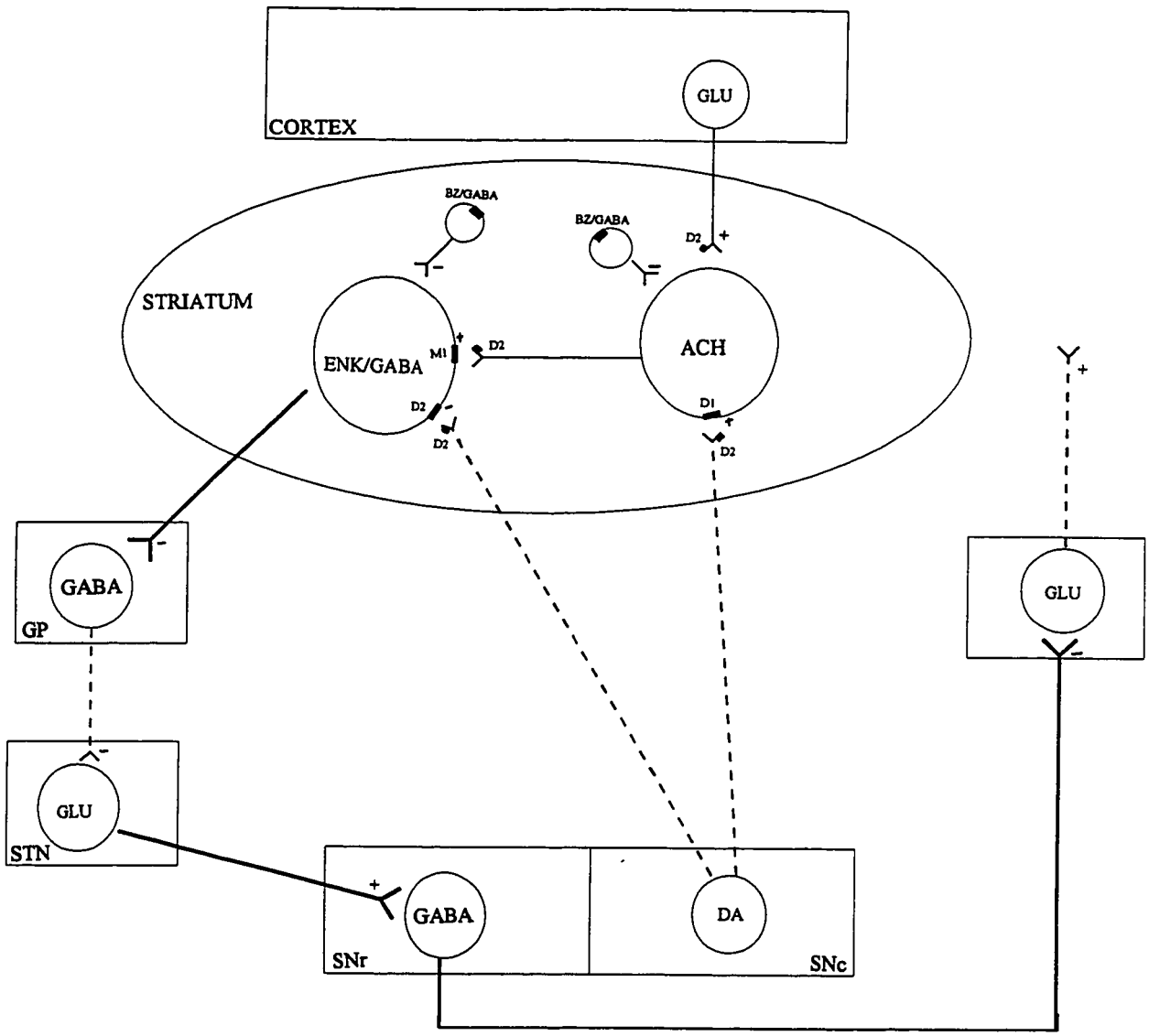

Figure 2a. Interaction of dopamine /acetylcholine/GABA in striatopallidal pathway of a lesioned animal. Broken line, decrease in pathway activity; Bold lines, increase in pathway activity. Ach- acetylcholine; DA- dopamine; ENK- enkephalin; GLU- glutamate; BZ/GABA-BA/$GABA_A$ binding site; M1-muscarinic binding site (excitatory); GP- globus pallidus; STN-subthalamic nuceli; SNr-subtantia nigra pars recticula; SNc- substantia nigra pars compact; +, excitatory; -, inhibitory.
(Ref:Dichiara et al. 1994; Gerfen et al. 1990; Carlsson and Carlsson, 1990).

Administration of apomorphine, activates D_2 inhibitory receptors located on the ENK/GABA neurons. In addition, the release of acetylcholine and glutamate are reduced due to the activation of presynaptic D_2 receptors. The inhibitory influence of ENK/GABA is reduced which removes the inhibition on pallidal GABAergic neurons. As a result of this, the activity of nigral output neurons is reduced and locomotor activity increases (see figure 2b).

Administration of melatonin or clonazepam in lesioned animals results in blockade of the apomorphine effect by interacting with the BZ/GABA binding sites which maybe located on medium-size spiny neurons. The BZ/GABA binding sites may also be located on the cholinergic and ENK/GABA neurons. However, due to the predominance of the medium spiny GABAergic neurons in the striatum, binding of melatonin or clonazepam to these sites would result in an overall effect of stimulation of the ENK/GABA neurons. Removal of the inhibitory effect on ENK/GABA neurons and cholinergic interneurons, presumably counteracts the action of apomorphine at D_2 receptors located on ENK/GABA neurons. The overall effect is stimulation of the ENK/GABA neurons and reduction in pallidal GABAergic neuronal activity. The increase in inhibitory nigral neuronal output results in decrease in locomotor activity.

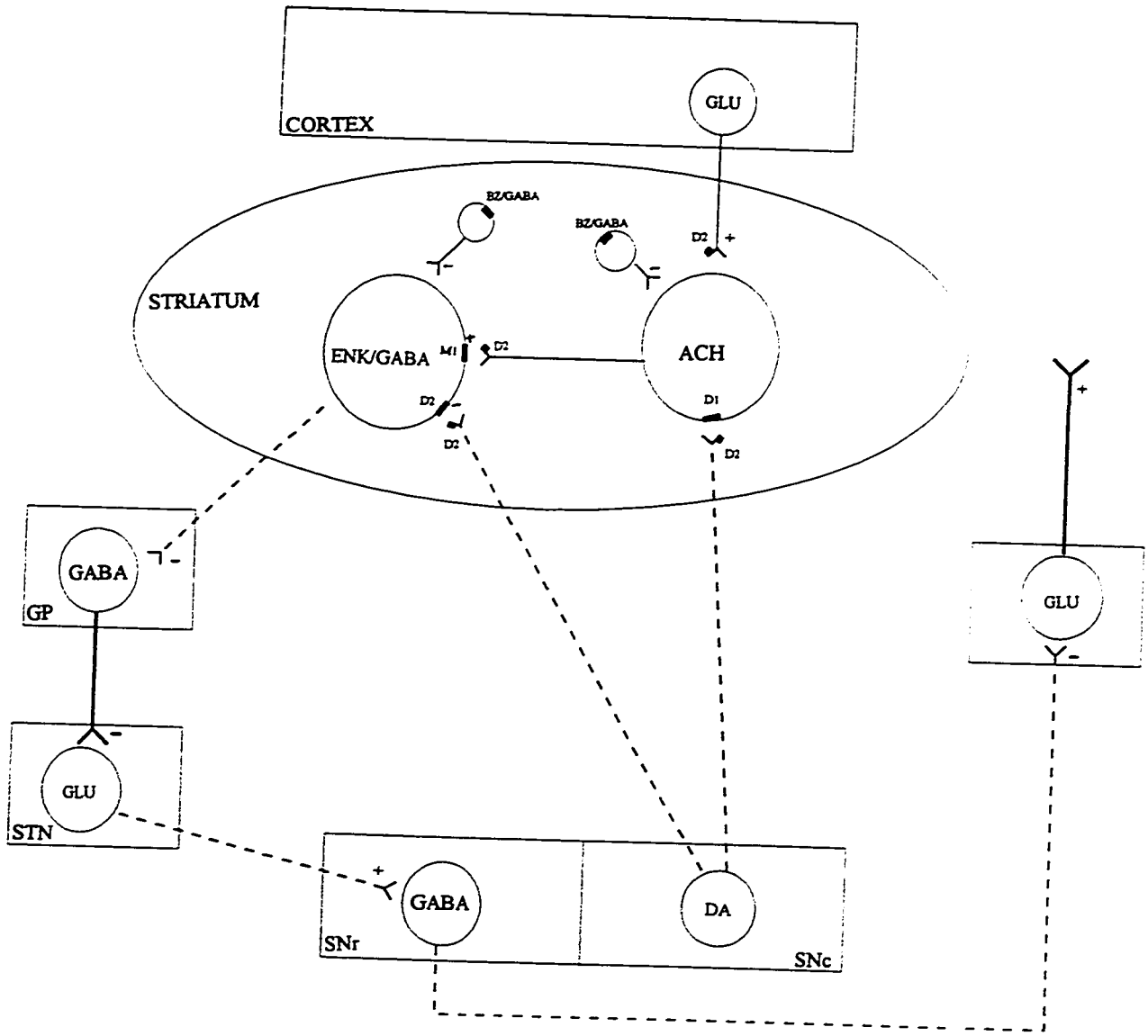

Figure 2b. Schematic diagram of the interactions of dopamine/acetylcholine/GABA in striatopallidal pathway of a lesioned animals following administration of apomorphine. +, excitatory; -, inhibitory. (Ref: DiChiara et al. 1994; Gerfen et al. 1990; Carlsson and Carlsson, 1990).

Bicuculline acts at the BZ/GABA sites on the medium spiny neurons to block the effect of melatonin or clonazepam. As a result of this, ENK/GABA neurons are inhibited, leading to the reduction of glutamate release and a decrease in nigral output. The inhibitory effect of GABAergic nigral efferents on thalamic projection neurons decreases resulting in locomotor activity. Thus, intrastriatal administration of bicuculline results in the $GABA_A$ antagonist blocking melatonin and clonazepam effect by acting on the BZ/GABA receptor complex on medium spiny neurons in the striatopallidal pathway and on striatal SP/GABA neurons in the striatonigral pathway. The overall effect is inhibition of the nigral GABAergic influence on the thalamic projection neurons and an increase in locomotor activity.

In chapter 2, quinpirole, the D_2 agonist was found to be not as potent as the D_1 agonist, SKF 38393, in inducing turning. Moreover, melatonin was not as effective in blocking quinpirole -induced rotation as compared with SKF 38393-induced locomotion. If $BZ/GABA_A$ binding sites are located on the cell bodies of the SP/GABA neurons as depicted in the above figures, then melatonin acting at the BZ site on the $BZ/GABA_A$ complex would directly block SKF 38393 action postsynaptically (see figures 1a and b). In the case of quinpirole, blockade by melatonin maybe indirect and possibly less effective since the $BZ/GABA_A$ complex is on GABAergic neurons that synapse on the ENK/GABA neurons and cholinergic interneurons. In addition, the greater effect of melatonin in blocking SKF 38393-induced activity, could involve its ability to inhibit D_1-mediated stimulation of cAMP production. This is not applicable to the D_2 pathway which suppresses cAMP formation.

The results from the present study do not support the idea that melatonin is acting via its physiological high-affinity G protein-coupled binding sites to modulate striatal dopamine activity for the following reasons: 1) both flumazenil, the central-type BZ receptor antagonist and bicuculline, the $GABA_A$ antagonist, were found to block the suppressive effect of melatonin on apomorphine-induced turning behaviour; 2) receptor autoradiography has demonstrated the localization of the high-affinity melatonin receptors in various areas of the brain such as the hypothalamus but not the striatum; 3) the concentrations required for the antidopaminergic action are in the micromolar range which greatly exceeds the picomolar-nanomolar concentrations which activate the physiological binding sites for melatonin.

In addition to the high affinity G protein-coupled melatonin binding sites, a low affinity (nanomolar) site has been reported in hamster brain (Krause and Dubocovich, 1991; Pickering and Niles, 1992). This site has an equipotent or higher affinity for N-acetylserotonin (NAS) than for melatonin. The physiological significance of this site has been questioned since endogenous melatonin levels are too low to interact with it. It is unlikely this low affinity site is responsible for the antidopaminergic action reported here, since unlike melatonin, NAS did not block apomorphine-induced rotation in lesioned animals. In addition, *in vitro* binding studies have shown that in micromolar concentrations, melatonin but not NAS, interacts at BZ/GABA binding sites (Niles and Peace, 1990).

6.3 Implications

These studies which were aimed at clarifying the mechanisms underlying the antidopaminergic effects of clonazepam and melatonin could have important clinical implications. Melatonin administered in high doses has been shown to modulate dopaminergic activity in the central nervous system probably via the BZ/$GABA_A$ receptor complex. Therefore, melatonin may be useful in the treatment of human disorders associated with increase in dopaminergic activity or sensitivity such as schizophrenia and tardive dyskinesia (TD). In contrast, melatonin, may not be useful in the treatment of Parkinson's disease since there is already a decrease in striatal dopaminergic activity as a result of loss of nigral dopaminergic neurons. Presently, BZs such as clonazepam are used in the treatment of patients with TD, a disorder that occurs due to chronic use of neuroleptic drugs, however, various side effects and tolerance for the drug is quickly developed (Thaker et al.1990). Since melatonin is a non-toxic endogenous substance, it may be administered in high doses to interact with BZ receptors to produce similar neuropharmacological actions without the side effects.

With the increase in use of melatonin by the public, one of the questions that should be addressed is how much of the indoleamine actually reaches the brain, and is this sufficient to act at the BZ/$GABA_A$ binding sites? In this study, we have shown that the antidopaminergic effect of melatonin at a dose of 10 mg/kg was blocked by flumazenil. It was recently reported in mice that the anxiolytic action of melatonin at a dose as low as 2.5 mg/kg could be blocked by the central-type BZ receptor antagonist (Pierrefiche et al. 1993). Clearly, as shown in the dose response curve from Chapter 2, as low as 0.4 mg/kg of melatonin could significantly reduce apomorphine-induced rotation. It seems unlikely that at this low dose, melatonin is

acting at the central-type BZ binding site, since high pharmacological (micromolar) doses of melatonin are required to act at these sites. Therefore, it is possible that some other site(s) are also involved in the antidopaminergic action of melatonin. The elucidation of the mechanism that underlies the effect of melatonin at doses which may be too low to act directly on central BZ sites will require further studies. However, one possibility is that melatonin is interacting with other sites on the BZ/$GABA_A$ complex. Previously, it was shown that a Ro5-4864 site existed on the BZ/$GABA_A$ receptor complex (Gee et al. 1988). Since melatonin has a higher affinity for the peripheral-type BZ binding site than the central sites (Marangos et al. 1982), at the lower end of the dose-dependent curve shown in chapter 2, melatonin may be acting at the Ro5-4864 site on the BZ/$GABA_A$ complex. The peripheral-type BZ receptor antagonist, PK 11195, has been shown to block the effects of Ro5-4864 on [^{35}S]TBPS binding to a site on or near the BZ/$GABA_A$ receptor linked chloride ionophore, indicating that PK 11195 may modulate $GABA_A$ receptors by binding to the Ro5-4864 site (Gee, 1987). If this is the case, the 25-30% of the suppression of the antidopaminergic effect by melatonin not blocked by bicuculline could be because melatonin binds to Ro5-4864 site. This idea is in keeping with the findings that it was the combination of bicuculline and PK 11195 that completely blocked the effect of melatonin on apomorphine-induced rotation. At higher doses, melatonin may produce the antidopaminergic effect through interaction with central-type BZ receptors as the effect was blocked by flumazenil.

Understanding the mechanisms underlying the central effects of melatonin will become important with the increase in use of this drug by the public as a nutritional supplement or for treatment of insomnia and other problems. Clinicians should be aware that melatonin may potentiate the effects of BZs such as clonazepam and advise patients accordingly.

CHAPTER 7.0

References

Chapter 7.0 *REFERENCES*

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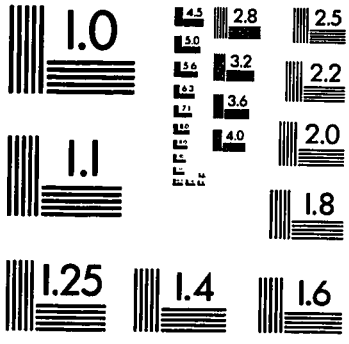

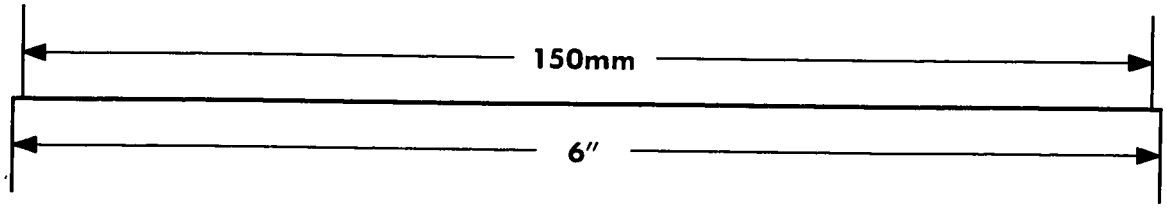

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