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CHOLECYSTOKININ MODULATES COCAINE-EVOKED DOPAMINE RELEASE VIA CCK-A AND CCK-B RECEPTORS IN RAT STRIATUM

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

CHOLECYSTOKININ MODULATES COCAINE-EVOKED DOPAMINE RELEASE VIA CCK-A AND CCK-B RECEPTORS IN RAT STRIATUM

By

Thomas M. Loonam

Adviser: Professor Jesus A. Angulo, PhD

The dopamine system has been the focus of much research during the past 30 years, mainly because alterations in dopamine transmission are involved, directly or indirectly in several brain dysfunctions. Cocaine has been well established as an indirect dopamine agonist by means of binding to the dopamine transporter (DAT) and blocking synaptic dopamine from being transported into the terminal. Using *in vivo* microdialysis, we assessed the effects of three treatment paradigms (acute, chronic, and early withdrawal) on cocaine-evoked extracellular dopamine in the rat caudate-putamen. Repeated administration of cocaine (10 mg/kg i.p., once daily) produces a progressive augmentation in extracellular dopamine concentration. However, when daily cocaine administration was discontinued for 3 days, animals in early withdrawal displayed a decrease in extracellular dopamine in response to a cocaine challenge compared to a cocaine challenge administered in naïve and chronically treated animals. The development of tolerance to cocaine-induced elevations in extracellular dopamine during early withdrawal was shown not to be a result of an increase in DAT sites as shown with receptor autoradiography. As a result of the anatomical distribution

of the neuropeptide cholecystokinin (CCK) within the striatal dopaminergic system, we investigated a possible role for CCK in the development of tolerance in cocaine-evoked extracellular dopamine. By perfusing CCK-8S, CCK-A receptor antagonist L-364, 769, and CCK-B receptor antagonist L-369,293 directly into the caudate-putamen via the microdialysis probe, we assessed these effects on cocaine-evoked dopamine release. Both L-364,769 and L-369,293 elevated and CCK-8S further decreased cocaine-evoked extracellular dopamine levels, respectively. Interestingly in naïve animals, L-364,769 blocked cocaine-evoked elevations and L-369,293 increased cocaine-evoked elevations in extracellular dopamine levels. In chronically treated animals, both L-364,769 and L-369,293 elevated, while CCK-8S slightly decreased dopamine levels. Receptor autoradiography demonstrated a significant increase in binding for CCK receptors in early withdrawal animals compared to naïve and chronic groups, supporting the neurochemical data obtained above. It is concluded that CCK appears to play a significant modulatory role, via both CCK-A and CCK-B receptors, in the development of tolerance in extracellular dopamine to the effects of daily cocaine administration, and also in cocaine-evoked extracellular dopamine concentrations in naïve and chronically treated animals.

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However, this work was not completed alone. The constant loving support and encouragement from my confidant and fiancé Daniella Rondanelli was of immeasurable importance in allowing me to persevere and complete my doctoral work. Daniella has been my foundation during the numerous difficult times I encountered during this dissertation. The positive atmosphere and support provided by my family and friends were especially helpful, especially the few times I needed to be reminded how remarkable it is to earn a PhD. I would also like to acknowledge the unconditional love provided by my dog Buster, who has the ability to make me smile during trying times.

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INTRODUCTION

1.1 Drug Abuse and Addiction

Drug abuse and drug addiction are among the largest and most challenging problems facing society today. According to a 1997 survey conducted by the National Institute on Drug Abuse, more than 70 million adult Americans used an illicit drug at some time in their lifetime and an estimated 1.5 million Americans age 12 and older were chronic cocaine users (National Institute on Drug Abuse, NIDA). Although this is an improvement over the 1985 estimate of 5.7 million users (NIDA), we still have a substantial distance to go in reducing the use of this addictive stimulant and all drugs of abuse. It is important to understand that drug abuse and addiction are complex social and public health issues that require complex approaches.

Scientific advances have contributed significantly to understanding drug abuse and addiction, but we are not near a cure capable of making these problems disappear. We now know that drug addiction is a treatable brain disease in which the addicted brain is different in its neurobiology from the non-addicted brain (Leshner, NIDA). However, there still exists a tremendous gap between what science tells us and the clinical application of these findings.

Chronic drug abuse changes the brain in fundamental and long-lasting ways. It is believed that these long-lasting brain changes or neuroadaptations are a major component of the addiction itself (Leshner, NIDA). These adaptations can be thought of as though

there is a "switch" in the brain that "flips" at some point during an individual's drug use (Leshner,NIDA). The switch flips at different points for different individuals and in a sense changes the person from a drug user/abuser to a drug addict. Neuroadaptations could occur in many ways or routes: at the neurotransmitter level as a change in the amount of neurotransmitter being released, a change in the number of receptors that activate or are activated by the neurotransmitter, a change in the transporters that deactivate the neurotransmitter and a change in the intracellular signal transduction machinery that responds to the neurotransmitter. And although we do not know the precise molecular nature of this switch, we are beginning to understand some of the mechanisms that are involved when drug-induced brain neuroadaptations changes occur.

1.2 Psychostimulants

Psychostimulants are a class of highly addictive drugs known to create feelings of pleasure or euphoria by mediating their effects on brain catecholamines, primarily dopamine released in forebrain structures of the basal ganglia. Cocaine and methamphetamine (METH) are psychostimulants that behave as indirect dopamine (DA) agonists which cause synaptic dopamine overflow in terminal field regions (Sulzer et al.,1995). To stay within the scope of this thesis, the rest of the discussion of psychostimulants will focus on cocaine and its neurophysiological effects on the brain.

1.2.1 Cocaine

Cocaine is one of the oldest known drugs and is a powerful addictive psychostimulant that directly affects the brain. The pure chemical cocaine hydrochloride, which is extracted from the leaf of the *Erythroxylon* coca bush, has been an abused substance for

more than 100 years, and coco leaves themselves have been ingested for thousands of years (NIDA). In the early 1900's, cocaine became the main stimulant drug used in most of the tonics/elixirs that were developed to treat a wide variety of illnesses. In modern times, the principle routes of cocaine administration are oral, intranasal, intravenous and inhalation (NIDA). The most common method of snorting is the process of inhaling cocaine powder through the nostrils, where it is absorbed into the bloodstream through nasal tissue. Cocaine in its freebase/smokable form, known on the street as "crack", is enormously addictive and highly popular because of its rapid absorption into the bloodstream via the lungs and is inexpensive both to produce and buy. The duration of cocaine's effects are also dependent on the route of administration. The faster the absorption, the more intense the high is but the shorter the duration (NIDA).

1.2.2 Acute Effects of Cocaine

As mentioned earlier, cocaine mediates its euphoric effects by behaving as an indirect DA agonist or releasing agent. The molecular mechanism of action of cocaine involves the dopamine transporter (DAT) located on presynaptic dopaminergic terminals. The DAT is critical in determining the concentration of extracellular dopamine. Under normal conditions, the DAT removes excess dopamine from the synapse and transports it back into the terminal for recycling, thus rapidly terminating dopamine-mediated potentials. Cocaine has a similar molecular structure to dopamine and when introduced into the brain binds to the DAT. By blocking the DAT, cocaine allows released DA to persist in the extracellular space, extending DA receptor stimulation and prolonging DA-

mediated responses. Therefore, cocaine offers an important research tool to manipulate dopaminergic pathways and study important cause and effect type activities of the brain.

Accumulating evidence indicates that midbrain dopaminergic neurons, particularly their terminals, are the principle targets of cocaine in the brain. These neurons project to forebrain structures caudate-putamen(CPu) and nucleus accumbens(Nac), together referred to as the neostriatum, and are involved in mediating movement (motor activity and stereotyped behaviors) and pleasure (reward and reinforcement), respectively (Angulo and McEwen, 1994). Studies have shown that cocaine-induced motor excitation in rats is prevented by microinjection of dopamine antagonists into the neostriatum (Kalivas and Duffy, 1993a), and motor activity can be produced by direct cocaine administration into the neostriatum (Delfs et al., 1990). In humans, after intravenous administration, cocaine accumulates in dopamine-rich regions of the forebrain (CPu and Nac) (Madras et al., 1998). In these regions, a single dose of cocaine raises the extracellular concentration of DA, and the rise and decline of dopamine correspond closely to cocaine levels in the blood and brain (Madras et al., 1998). Also, behaviors such as motor excitation and hyperactivity are seen in individuals who administer psychostimulants (Woolverton and Balster, 1982). These clinical observations have generated considerable interest for the development of animal models of psychostimulant psychosis. Rodents are the species that are studied most thoroughly and systematically with respect to the behavioral and neurochemical effects of psychostimulants (Segal et al., 1981; Kalivas et al., 1993). In rats, we and others have found that acute injections of cocaine lead to elevated hyperlocomotor activity and an increase in extracellular dopamine in rat neostriatum (Zhang et al., 2001). Also, an increase in stereotyped

behaviors such as head bobbing and rearing are seen in rodents exposed to a single dose of cocaine (Robinson and Becker, 1986).

1.2.3 Chronic Effects of Cocaine

Studies in laboratory animals suggest that chronic cocaine administration changes the function of the dopamine system. Repeated administration of cocaine to rats leads to a number of changes in the acute effects of the drug, depending on several factors, including: dose, route, and frequency of administration (White et al., 1995). For example, in the case of tolerance, drug effects are decreased which is represented by a shift to the right of the dose-response curve. Behavioral and neurochemical tolerance to cocaine can be produced when it is administered in high doses and/or multiple times daily for several days (Kalivas and Duffy, 1993a). Another well characterized effect of repeated administration of cocaine in rats is the development of reverse tolerance or "behavioral sensitization", which is a leftward shift of the dose-response curve. As stated earler, increased locomotor activity and stereotyped behaviors are typical rodent responses to cocaine. With repeated, intermittent administration of a constant moderate dose of cocaine, the magnitude of these elicited behaviors becomes progressively augmented and the animal is referred to as being sensitized. In rats, behavioral sensitization can persist for as long as 87 days after a short period of cocaine administration (Cass et al., 1993). We and others have demonstrated that chronically treated rats display behavioral sensitization and have an elevated concentration of extracellular dopamine in the striatum, compared to acute animals (Kalivas and Duffy, 1993a; Zhang et al., 2001). Cocaine is rapidly cleared from the system (less than 1 hour)

implying that the behavioral and neurochemical changes are not due to the cumulative effects of the drug.

Chronic exposure of moderate to high doses of psychostimulants in rodents seems to mimic the stimulant abuse seen in human addicts. Some authors contend the phenomenon of behavioral sensitization in animals might represent an analogue of "drug-craving" in humans, therefore may have clinical relevance (Robinson and Becker, 1986; Robinson and Berridge, 1993). It is proposed that sensitization to cocaine, in humans, represents a central nervous system mechanism that induces drug-seeking behavior and increases the probability of relapse in ex-addicts (Robinson and Berridge, 1993). A particularly menacing aspect of behavioral sensitization, as in rodents, is that it is relatively enduring, even in the absence of continued drug use. Thus, while cocaine-induced behaviors generally dissipate when drug use is discontinued, the sensitized behavioral pathologies can be reinstated by an acute drug challenge up to a decade later (Sato, 1983; Sato et al., 1992). Moreover, the sensitizing properties of cocaine and all psychostimulants are believed to account for the emergence of psychopathologies such as paranoid psychosis, anxiety disorders and panic attacks (Ellinwood et al., 1973; Segal et al., 1981; Post et al., 1988). When these changes develop, they supersede the euphoric effects of acute cocaine use and become the primary behavioral characteristics of drug abuse (Post et al., 1988; Gawin, 1991). As a result of the above data, it is quite apparent that there is a critical need to identify the neural alterations mediating sensitization, and to determine appropriate treatments for reversing the changes and blunting their behavioral consequences.

1.3 Basal Ganglia

1.3.1 Anatomy

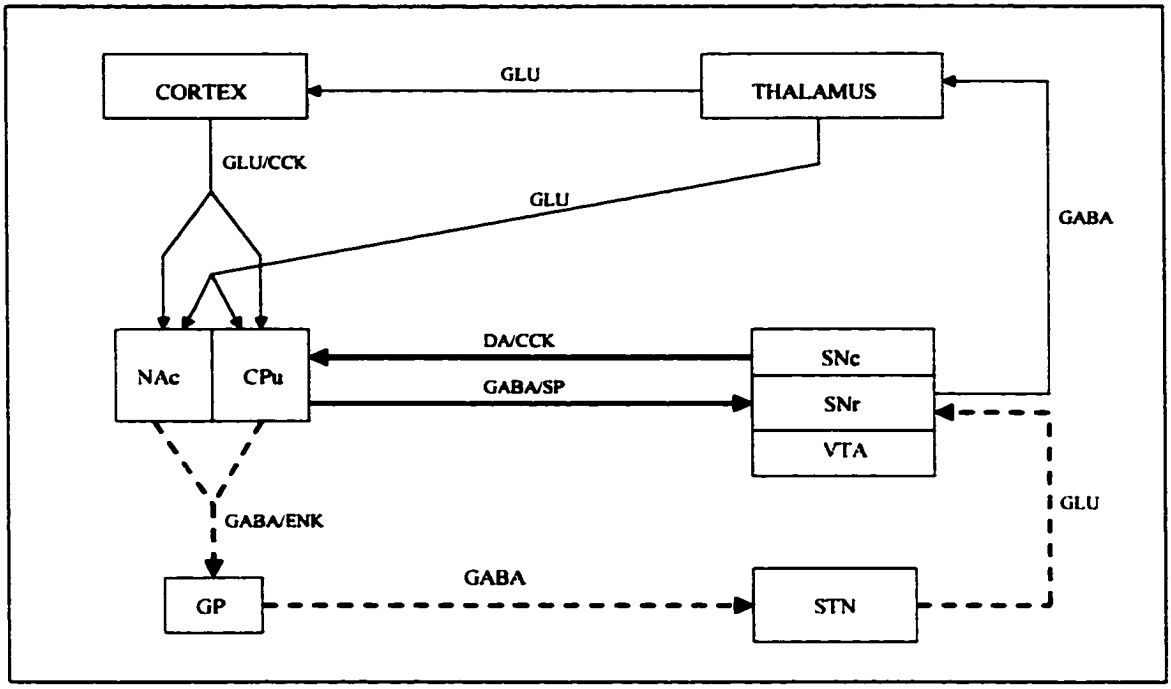
The basal ganglia are a group of nuclei located in subcortical regions. These nuclei are highly interconnected and project to numerous brain regions (Fig. 1). The components of the basal ganglia include the caudate-putamen (CPu), nucleus accumbens (NAc), globus pallidus (GP), subthalamic nucleus (STN), substantia nigra compacta (SNc), substantia nigra reticulata (SNr), and ventral tegmental area. The caudate-putamen and nucleus accumbens are similar in their structures, are located in the forebrain and together are referred to as the neostriatum. The neostriatum represents the input structure of the basal ganglia. The globus pallidus receives input via the indirect pathway from and lies posterior to the neostriatum. The subthalamic nucleus is a small structure that resides between the GP and the SN. The substantia nigra is located in the midbrain and is composed of two areas: the SNr which contains GABAergic neurons and is the primary output structure and the SNc which contains DA and DA/CCK neurons. The VTA resides in the midbrain and also contains DA neurons which project to the NAc.

1.3.2 Dopamine and Dopaminergic Pathways

Dopamine is the major catecholamine in the central nervous system. It is involved in the regulation of a variety of functions, including locomotor activity, emotion and affect, and neuroendocrine secretion (Jaber et al., 1996). The dopamine system has been the focus of much research during the past 30 years, mainly because alterations in dopamine

neurotranmission has been directly or indirectly linked to several brain dysfunctions, including Parkinson's and Huntington's disease and schizophrenia.

Fig. 1 Basal Ganglia Circuitry



Diagramatic representation of the rat basal ganglia. The thick black arrows represent the direct loop and the dashed arrows represent the indirect loop. SNc – Substantia Nigra compacta; SNr – Substantia Nigra reticulata; VTA – Ventral Tegmental Area; CPu – Caudate-Putamen; NAc – Nucleus Accumbens; GP - Globus Pallidus; STN – Subthalamic Nucleus.

Dopamine synthesis, like that of all catecholamines in the central nervous system, originates from the amino acid precursor tyrosine, which is transported across the blood-brain barrier into the dopamine neuron. The rate-limiting step in dopamine synthesis, once tyrosine gains entry into the neuron, is the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase. L-DOPA is subsequently converted to dopamine by L-aromatic amino acid decarboxylase.

Release of dopamine from the nerve terminal is calcium-dependent and is thought to occur in response to invasion of the terminal by an action potential. Microdialysis studies have shown that rat striatal extracellular dopamine levels decrease by 50% when perfused with a calcium-free medium, under both basal and K+-depolarizing conditions (You et al., 1994a). The extent of dopamine release appears to be a function of the rate and pattern of firing. Dopamine release is also modulated by presynaptic release-modulating autoreceptors, namely D2 dopamine receptors. In general, dopamine agonists inhibit, while dopamine antagonists facilitate the release of dopamine via inhibitory autoreceptors.

Following its release, dopamine is quickly removed from the synapse and transported back into the nerve terminal for recycling by the dopamine uptake transporter. Once dopamine is transported into the terminal, it is converted to dihydroxyphenylacetic acid (DOPAC) by intraneuronal monoamine oxidase (MAO). Released dopamine is also converted to homovanillic acid (HVA) at an extraneural site through the sequential action of catechol-O-methyltransferase (COMT) and MAO.

In the central nervous system, the majority of dopamine neurons are located in the basal ganglia, specifically the ventral mesencephalon (midbrain) from which the two major dopaminergic projections originate: the nigrostriatal and mesolimbic dopamine system. The nigrostriatal pathway originates from dopamine neurons located in the SNc and project axons that innervate the CPu (Fig. 1). The nigrostriatal pathway is involved in mediating motor activity and stereotypical behaviors (Angulo and McEwen, 1994).

The mesolimbic pathway emanates from dopamine cell bodies of the VTA and projects to the NAc. The mesolimbic pathway has been implicated in drug reinforcement

and reward (Angulo and McEwen, 1994). Pharmacological and behavioral studies have demonstrated that cocaine administration affects both nigrostriatal and mesocorticolimbic systems (Kalivas and Duffy, 1993a, b).

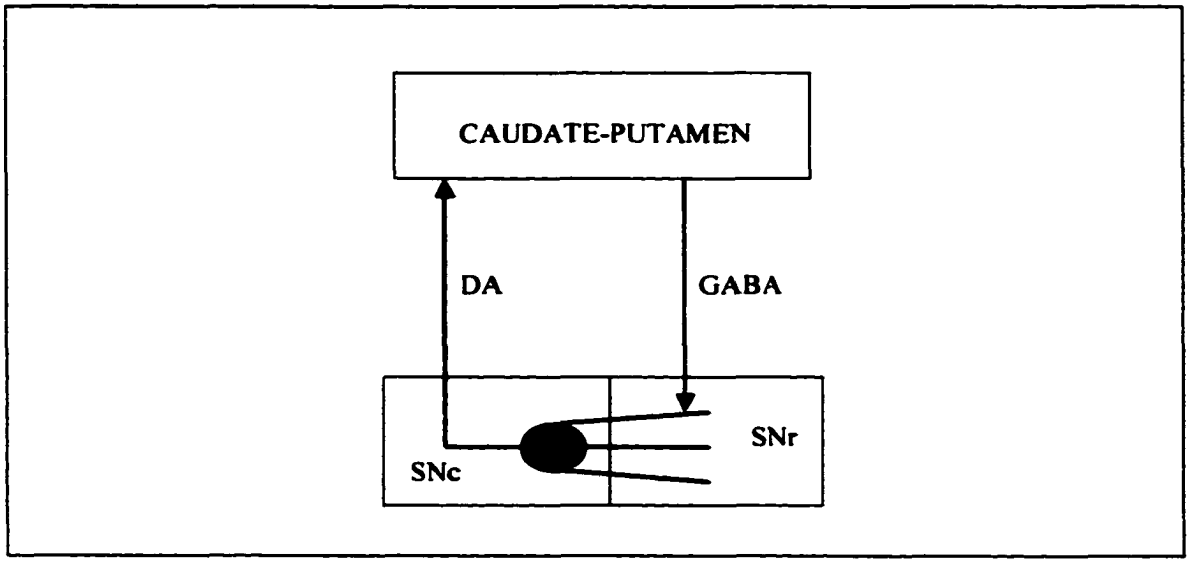
It has been proposed that striatonigral and mesolimbic systems are discrete and segregated parallel circuits (Alexander and Crutcher, 1990), however, there is reason to believe that extensive crosstalk between circuits occurs within the basal ganglia (Heimer et al., 1991). For example, the substantia nigra reticulata, the primary output region of the basal ganglia, receives innervation from pathways originating from the Cpu and pathways originating from the NAc.

1.3.3 Striatal Organization and Projection Neurons

The principal input component of the basal ganglia is the striatum or CPu, and along with the NAc compromise the neostriatum. As mentioned earlier, the striatum receives massive intrinsic dopaminergic innervation via the nigrostriatal pathway. The major striatal projection cells are medium spiny neurons that account for approximately 90 – 95% of striatal neurons (Gerfen, 1992a, b). All of the striatal medium spiny neurons utilize γ-aminobutyric acid (GABA) as a neurotransmitter (Kita, 1993). The medium spiny neurons are separated into two major types based on their axonal projections, neuropeptide content and dopamine receptor expression (Gerfen et al., 1990). The striatal projection neurons that directly innervate the SNr (direct striatonigral pathway) utilize GABA and employ neuropeptides substance P and dynorphin as co-transmitters, express dopamine-1 receptor mRNA, and supply inhibitory input to nigrothalamic GABAergic neurons located in the SNr (Gerfen, 1992a, b). These striatal neurons also synapse onto dendrites of dopaminergic perikarya of the SNc which extend into the SNr

(Fig 2). Striatal projection neurons that innervate the substantia nigra indirectly via the globus palidus (indirect striatonigral pathway) utilize GABA and the neuropeptide

Fig. 2 Direct Reciprocal Pathway



Reciprocal pathway from the substantia nigra to the caudate-putamen and then back to the subatantia nigra. The dendrites of the dopaminergic neurons of the SNc extend into the terminal field area of the striatonigral neurons in the SNr where release of GABA could influence the output of dopamine.

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enkephalin (ENK) as a co-transmitter, express dopamine-2 receptor mRNA, and also supply inhibitory input to the GP (Gerfen, 1992a, b). The three peptides mentioned above, substance P, dynorphin, and enkephalin, are regulated by the dopaminergic input from the substantia nigra, as evidenced by various manipulations. Thus, attenuation of dopamine transmission in the striatum, for example by lesioning the nigrostriatal pathway with 6-hydroxydopamine or treatment with dopamine receptor antagonists, results in increased expression of enkephalin and a decrease in dynorphin and substance P, whereas

dopamine agonists increase levels of dynorphin and substance P but not enkephelin (Steiner and Gerfen, 1996).

In addition, striatal projection neurons also receive bilateral and topographically widespread input from cortical regions (Graybiel and Ragsdale, 1979; Fonnum et al., 1981). The caudate putamen of the rat is inervated by several cortical areas both ipsilaterally and contralaterally (Wilson, 1993). It is generally accepted that this input to the striatum is excitatory and that glutamate in the major transmitter in this pathway (Fagg and Foster, 1983). In addition, the neuropeptide cholecystokinin (CCK) is present in cortical neurons and is believed to be the major source of striatal extracellular CCK (Morino et al., 1994; You et al., 1994a; You et al., 1998). Functionally it is believed the corticostriatal pathway with glutamate and CCK as a neurotransmitter/neuromodulator respectively, plays a modulatory role in the activity, metabolism and responsiveness of dopamine neurons and will be discussed more in depth later in this paper, particularly regarding CCK and its neuromodulatory role (Cheramy et al., 1986; Leviel et al., 1990; Gerfen, 1992a).

Within the striatum two interdigitating tissue compartments have been identified that can be distinguished on connectional and cytochemical grounds. The larger of these compartments, called the matrix, represents about 85% of the total volume of the striatum, and is perforated throughout by trabeculae formed from the other compartment, called patches or striosomes (Graybiel, 1990; Gerfen, 1992a, b). These two compartments have a similar composition but receive different inputs and project to different regions. The striosomes receive cortical afferents from prefrontal and limbic cortices while the matrix receives cortical afferents from primary motor and

somatosensory cortex (Gerfen, 1992a, b). Projections from the striosomes go mainly back to the SNc while matrix projections go mainly to the globus pallidus and and SNr (Graybiel and Ragsdale, 1979; Gerfen et al., 1987).

1.3.4 Striatal Interneurons

Four major classes of interneurons have now been identified using cytochemical, physiological and morphological methods, and account for the majority of aspiny neurons present in the neostriatum. They are (1) the large cholinergic neurons, which are identifiable by the presence of choline acetyltransferase (ChAT); (2) GABAergic interneurons that contain parvalbumin, one of the calcium-binding proteins; (3) GABAergic interneurons that contain calretinin; and (4) a class of interneurons that contain somatostatin, and nitric oxide synthase (NOS), and perhaps employ GABA as well (Kawaguchi et al., 1997).

The best known of the interneurons, now known as giant cholinergic interneurons, have long been recognized as a separate cell type, owing to their large somatic size (20-50 μm) and constitute about 2% of all striatal neurons. The key to their identification as interneurons was the discovery that they are the source of Ach and ChAT in the striatum, and that striatal projections are devoid of any cholinergic component (Malthe-Sorenssen et al., 1974). The main role they seem to play are as afferent relay neurons from midbrain dopaminergic projections to the projection neurons. While a large concentration of dopaminergic afferents synapse directly onto projection neurons, cholinergic cells do receive dopamine inputs (Chang, 1988), do make most of their synapses on the spiny projection neurons (Izzo and Bolam, 1988), and the cholinergic

interneurons have the ability to respond to D2 receptor agonists (D2 inhibits Ach release)(Gerfen et al., 1991;Stoof et al., 1992). In addition, these cells have widespread dendritic trees, making them also capable of integrating synaptic inputs from other projections, such as corticostriatal and/or thalamostriatal afferents. Spiny projection neurons of both major types (GABA-SP or GABA-ENK) are relatively enriched in the m1 and m4 muscarinic Ach receptors (Kawaguchi et al., 1997), but the synaptic influence of cholinergic interneurons on spiny projection neurons still retains some mystery.

Neurochemical studies have established that a group of interneurons in the striatum stain positive for both GABA and the peptide parvalbumin, and constitute 3-5% of all striatal neurons (Gerfen et al., 1985). Parvalbumin-positive GABAergic interneurons receive powerful excitation from the cerebral cortex (Kita, 1993). They also make synaptic contacts amongst themselves, which are presumably inhibitory (Kita et al., 1990). Parvalbumin-positive interneurons are hypothesized to play an inhibitory function in the striatum. Experimental studies of projection neurons using *in vitro* striatal slices have indicated an inhibitory role for these interneurons (Kita, 1993), but a definitive role has not yet been determined.

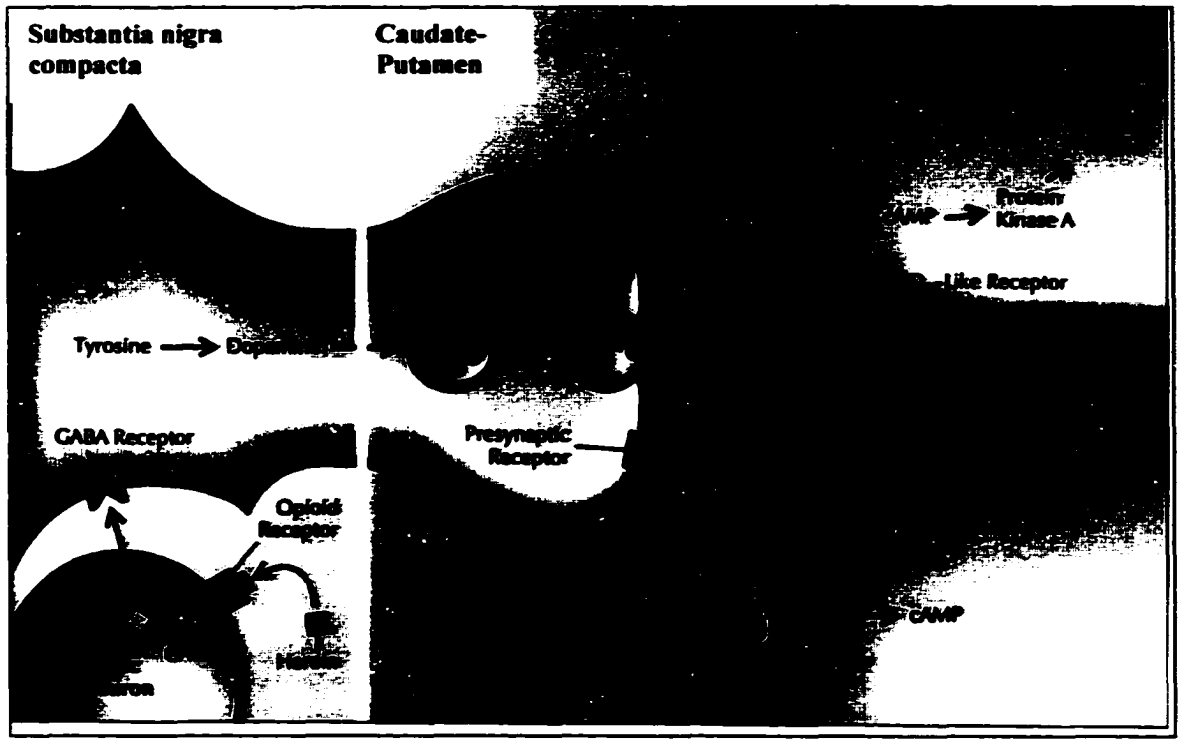
Another population of aspiny GABAergic interneurons also stain positive for calretinin, another cacium-binding protein. Light- and electron-microscopic studies showed that these cells are medium-sized aspiny neurons with indented nuclei, represent 1-2% of striatal neurons, and are distinct from neurons that contain ChAT, parvalbumin or NOS (Bennett and Bolam, 1993). The receptor phenotype and physiology of these neurons remain to be elucidated.

Somatostatin-containing interneurons are aspiny cells which represent 2% of striatal neurons. In comparison with cholinergic and parvalbumin-positive cells, neurons of this class have fewer dendritic branches and somatostatin-positive cells receive direct cortical inputs (Vuillet et al., 1989). These cells are not enriched in transcripts for Glu1-GLU4 receptors, thus if cortical input to these cells utilize glutamate, it is mediated through different types of glutamate receptors from those used by other striatal cells. Somatostatin-positive cells also receive cholinergic (Vuillet et al., 1992), and dopaminergic innervation (Vuillet et al., 1989), and express the NK-1 (SP) receptor (Li et al., 2001). The terminals of these cells, like those of parvalbumim-immunoreactive neurons, make synapses on perikarya and dendrites of striatal cells, which include spiny projection neurons (Vuillet et al, 1989). Both somatostatin- and parvalbumin-containing neurons receive direct cortical input and innervate spiny projection neurons, thus both types can mediate feed-forward processing of inputs from the cortex to projection cells.

1.3.5 Dopamine Receptors

The actions of dopamine are mediated via a family of receptors designated D1 – D5 that belong to the larger superfamily of G-protein coupled receptors that have seven membrane-spanning domains. D1 type receptors (D1 and D5) constitute a subfamily that stimulates adenylyl cyclase activity and increases cAMP (cyclic adenosine-mono-phosphate) formation and the D2 type receptor (D2, D3 and D4) subfamily inhibits adenylyl cyclase activity and cAMP formation (Kebabian and Calne, 1979) (Fig 3). For example, cAMP efflux in the striatum elicited by D1 agonists can be effectively blocked by D2 agonists (Stoof and Kebabian, 1984). Also, the inhibitory effect of D2 agonists on striatal cAMP efflux is blocked by D2 anatagonists (Weiss et al., 1985).

Fig. 3 Dopamine-mediated Actions on D1 and D2-like Receptors



Cocaine mediates its effects by binding to the presynaptic dopamine uptake transporter. The resulting increase of dopamine in synaptic clefts heightens the transmitter's effects on dopamine receptors, classified as D1-like and D2-like. Through their association with stimulatory G (Gs) proteins, the D1 receptors trigger intracellular responses dependent on the second messenger cAMP. D2 receptors mediate the opposite effect, through their association with inhibitory G (Gi) proteins. Figure from A Special Report, NIDA.

Receptor autoradiographic methods demonstrate the presence of high levels of dopamine D1 and D2 receptor binding throughout the caudate-putamen (Altar and Marien, 1987; Closse et al., 1988). In the striatum, D1 receptors are primarily on striatal medium spiny neurons that utilize the neurotransmitter GABA and neuropeptides substance P and dynorphin. Evidence for the localization of D1 receptors include

experiments where lesioning the SNc dopamine neurons did not significantly alter dopamine D1 receptors in the Cpu (Joyce, 1991). Also, *in situ* hybridization studies show GABAergic medium spiny neurons that co-express preprotachykinin-A (precursor for substance P), Preprodynorphin, and dopamine D1 receptor mRNA project directly to the SNr (direct striatonigral projection, Fig. 1) (Gerfen, 1992b; Surmeier et al., 1996). Lastly, *in situ* hybridization studies show that D1 mRNA is not expressed by dopamine neurons in the SNc (Mansour et al., 1992).

D2 receptors are found postsynaptically on medium spiny neurons co-expressing GABA and enkephalin (Gerfen, 1992a, b). Evidence for D2 localization also include in situ hybridization studies which reveal a subset of GABAergic medium spiny neurons co-expressing preproenkephalin and dopamine D2 receptor mRNA which project to the globus pallidus (Gerfen, 1992a; Surmeier et al., 1996). These neurons initiate the indirect striatonigral pathway. D2 receptors are also present presynaptically as autoreceptors on dopaminergic terminals (Silvia et al., 1994). In addition, D2 receptors are found on large aspiny cholinergic interneurons and function to inhibit Ach release (Gerfen, 1991; Stoof et al., 1992).

In the midbrain, D1 dopamine receptors are found in both the SNc and VTA. Lesion studies of striatal GABA/substance P neurons show that D1 receptors are located primarily on the striatonigral terminals (Altar and Marien, 1987). Also, *in situ* hybridization studies reveal that D1 mRNA is not expressed by midbrain dopamine neurons (Mansour et al., 1992). Stimulation of D1 receptors in the SNr enhances neurotransmitter release from the striatonigral terminals.

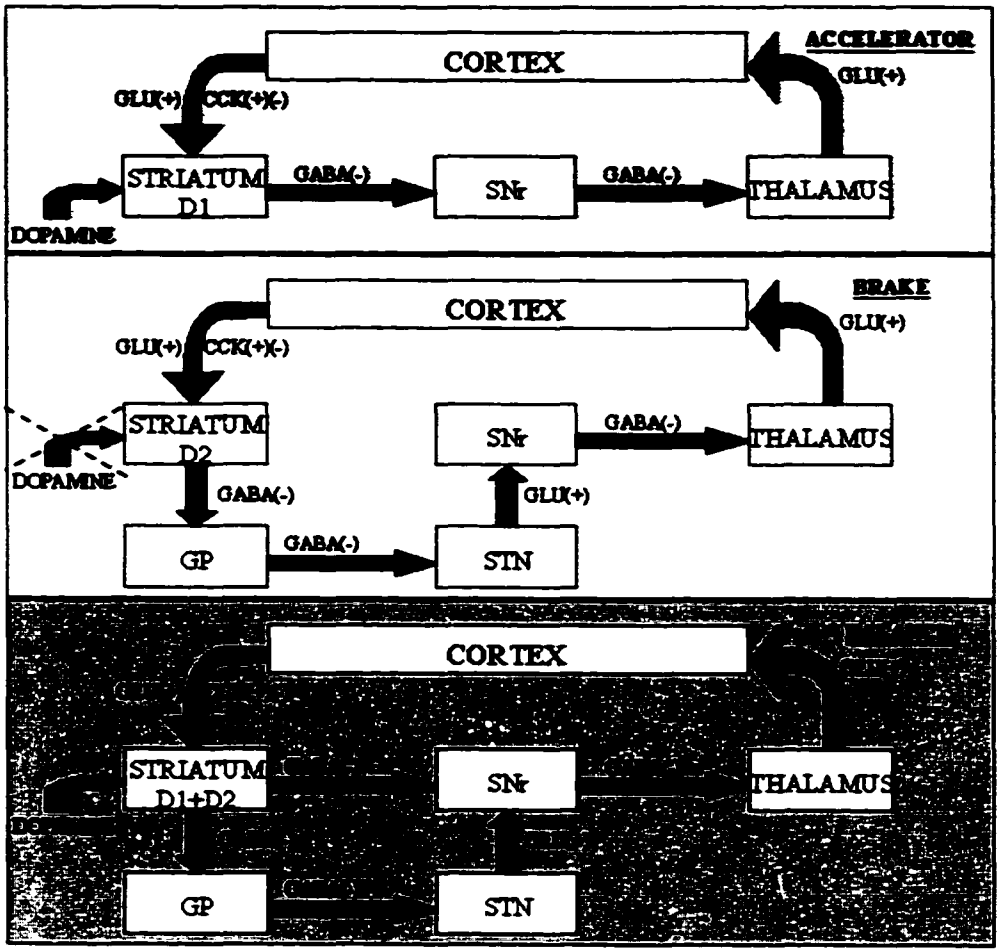
D2 dopamine receptors are found on the soma and dendrites of dopamine neurons, and this is supported by the presence of D2 mRNA in midbrain dopamine neurons (Meador-Woodruff et al., 1991). Stimulation of D2 receptors by somatodendritically released dopamine hyperpolarizes the dopamine neuron, causing an inhibitory post synaptic potential (IPSP) due to a G-protein-dependent increase in K+ conduction (Lacey et al., 1987).

1.3.6 Dopamine Neurophysiology and Motor Activity

Dopamine projections from the midbrain exert opposing effects on the direct and indirect striatonigral pathways. Dopamine has an excitatory influence on GABAergic medium spiny neurons containing substance P and dynorphin, via D1 receptors (Twery et al., 1993). These direct striatonigral neurons, when activated by dopamine, inhibit GABAergic neurons in the SNr, which then disinhibits its target nuclei in the thalamus (Fig. 4). This allows excitatory thalamic input to the motor cortex to initiate movement.

When active, the indirect striatonigral pathway is responsible for the tonic activity of GABAergic neurons in the SNr, thus inhibiting its target nuclei in the thalamus and inhibition of motor activity or movement (Angulo and McEwen, 1994). Dopamine released in the striatum inhibits GABAergic medium spiny neurons containing enkephalin via D2 receptors (Fig. 4). This then disinhibits the GABAergic neurons of the globus pallidus and allows inhibitory input to the subthalamic nucleus. The STN then cannot stimulate the inhibitory GABAergic neurons in the SNr, disinhibiting the thalamus and allowing it to send excitatory input to the cortex and cause motor excitation. Thus, the overall effect of dopamine on the striatum is to reinforce movement by facilitating the direct striatonigral pathway and suppress the indirect striatonigral pathway.

Fig. 4 Control of Motor Activity



The brake-accelerator model for the control of movement by the basal ganglia. The direct pathway or 'accelerator' (leading to the release of movement) consists of two successive inhibitory GABAergic connections, from the striatum to the SNr and from the SNr to the thalamus. Dopamine activates striatal D1 receptors in this pathway. In the indirect pathway or 'brake' (leading to inhibition of movement), inhibitory D2 receptors on striatal GABAergic neurons are not activated thus allowing GABAergic inhibition of the GP and excites the inhibitory outputs of the SNr to the thalamus, resulting in tonic inhibition of the motor cortex. Balance is achieved when these antagonistic systems are activated together under normal circumstances. Model is a modified version from Grabiel, 2000.

1.4 Basal Ganglia Disorders

The dopamine system has been the focus of much research during the past 30 years, mainly because alterations in dopamine transmission are involved, directly or indirectly, in several brain dysfunctions. For instance, degeneration of dopamine neurons in the sustantia nigra contributes to the pathogenisis of Parkinson's disease. Moreover, imbalances in the limbic dopamine pathways are thought to contribute to psychotic disorders such as schizophrenia. However, disorders of the basal ganglia involving hyperkinetic dysfunction, such as Huntington's disease, are not well understood.

1.4.1 Parkinson's Disease

Parkinson's disease (PD) is a progressive, neurodegenerative and hypokinetic disorder of the basal ganglia which is characterized by tremor, muscular rigidity, difficulty in initiating motor activity, and loss of postural reflexes (Albin et al., 1988). It has been known for decades that PD is characterized by loss of dopamine in the striatum, and now it is clear that PD can be defined in biochemical terms as a loss of dopamine neurons in the nigrostriatal system due to degeneration or injury (Langston, 1987). Even in patients with mild symptoms, a striatal dopamine loss of 70-80% is observed, while severely impaired subjects have striatal dopamine depletions in excess of 90% (Hornykiewicz and Kish, 1987). Since the dopamine transporter is heavily expressed in the terminals of dopamine neurons that are lost in PD, it is not surprising that striatal

binding of agents which label this site, such as cocaine, are lost in the Parkinsonian striatum.

There are a number of theoretical strategies for drug therapy in PD, including substrate supplementation, direct and indirect dopamine agonists, metabolic inhibitors (MAO-inhibitors), and dopamine uptake inhibitors. The most common and successful treatment to today has been the use of the dopamine precursor L-DOPA, which helps to compensate for the lack of endogenously produced dopamine (Langston, 1987). However, drug therapies have multiple side effects linked essentially to their action on the dopamine system. For example, involuntary movements seen in Huntington's disease and psychotic reactions seen in schizophrenia are sometimes observed during chronic administration of L-DOPA to patients suffering from PD (Langston, 1987). Direct dopamine agonists also have some benefit in patients whose responsiveness to L-DOPA is greatly reduced or erratic. The dopamine agonist that has found extensive use in PD is bromocriptine, primarily a D2 agonist (Wolf and Roth, 1990). It is important to continue to investigate dopamine-releasing agents or mechanisms because they should prove useful in the future as supplements or alternatives to drug therapies used today for PD.

1.4.2 Huntington's Disease

Huntington's disease (HD) is an autosomal dominant hereditary neurodegenerative condition in which the most pronounced pathologic changes occur in the striatum (Martin and Gusella, 1986), including striatal neuronal death (Albin et al., 1990). HD is a hyperkinetic movement disorder characterized by uncontrollable and rapid motor acts intruding into normal flow of motor activity (Albin and Young, 1988). Characteristics of HD are also observed in patients with PD who have been overdosed with dopamine

replacement therapy (L-DOPA). The abnormal movements seen in HD are suppressed by the administration of D2 receptor antagonists and exacerbated by dopamine agonists (Bruyn and Padberg, 1984). While pharmacology has given us some clues regarding HD, hyperkinetic disorders are still poorly understood.

1.4.3 Schizophrenia

Schizophrenia is a psychopathological disease characterized by symptoms of euphoria, auditory hallucinations, akathisia or the inability to remain inactive, in which a hyperactive dopaminergic system is thought to be at the center of this disorder. The hypothesis that the dopaminergic system is overactive in schizophrenia is based on the finding that neuroleptics, which are used in the successful management of some symptoms of this disorder, selectively block dopamine receptors (Kane and Freeman, 1994; Sigmundson, 1994). The dopamine hypothesis was further strengthened by the fact that psychostimulants, such as cocaine, which increase dopaminergic transmission, induce psychotic states resembling those observed in the symptoms of schizophrenia.

From a pharmacological point of view, D2 receptor antagonists have been shown to treat these symptoms effectively, but are less effective in managing the negative symptoms of schizophrenia such as loss of motivation and slowed body movement (Seeman et al., 1975; Creese et al., 1976). Prolonged treatment with antipsychotic drugs has a major drawback, since 90% of patients under medication suffer from extreme movement disorders known as extrapyramidal side effects (EPS). From this perspective, it is of great interest to find other methods to alleviate the psychotic symptoms of schizophrenia.

1.5 Cholecystokinin

1.5.1 Structure and Localization

Cholecystokinin (CCK) is a gut-brain peptide first discovered in the gastrointestinal system, where it stimulates gallbladder contraction and enhances pancreatic enzyme secretion (Liddle, 1994). It is more abundant in brain than intestine, and is one of the most abundant peptides in the cerebral cortex, striatum and hippocampus. The discovery of CCK in the nervous system is credited to Vanderhaeghen, Signeau, and Gepts (1975), who reported the presence of a brain peptide that cross-reacted with antibodies directed against gastrin (a closely-related gut peptide possessing a C-terminal pentapeptide sequence that is identical to that of CCK). CCK is a member of a family of peptides derived from a common precursor, prepro-CCK. Although present in various fragments with different peptide lengths, the sulfated octapeptide CCK-8S, Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2, is the most prominate form in the central nervous system (Larsson and Rehfeld, 1979), and unless otherwise stated, in this paper CCK refers to sulphated CCK (CCK-8S).

Recently, CCK has received much attention because its distribution throughout the brain, giving perspective to being a modulator of a broad spectrum of brain functions such as memory consolidation, satiety, anxiety and pain sensation (Cooper et al., 1992). A second reason for the interest in CCK in addition to its neuromodulatory potential, is that CCK fulfills the criteria of being a neurotransmitter. The peptide has been localized in nerve endings (Hokfelt et al., 1980a; Hokfelt et al., 1980b), secreted from them in a regulatory way, and acts on G-protein coupled receptors (Carlberg et al., 1990). Thirdly,

CCK regularly co-localizes with other, more classic, neurotransmitters in central neurons. This latter property gives the neuron the possibility to vary its communication with other neurons depending on stimulation pattern and localization.

Biochemical and immunohistochemical studies have shown localization of CCK-like immunoreactivity in cell bodies and fibers in a widespread distribution in the brain (Straus et al., 1977), mainly representing CCK-8S (Dockray, 1978; Dockray et al., 1978). The cortex is the region with the highest concentration of CCK, particularly layers 2 and 3 (Rehfeld and Hansen, 1986), where the peptide is rapidly and extensively synthesized as a precurser that is then metabolized to the active sulfated form of CCK (Goltermann et al., 1980). In separate studies, the antibody to pro-CCK labeled a small number of cell bodies in the cortex, located mainly in layers II, III, V and VI, with no immunoreactivity found in layers I and IV (You et al., 1994a; You et al., 1998) and some of these neurons were pyramidal in shape and have been shown to have long projections (Seroogy et al., 1985). While CCK-like immunoreactivity in the cortex was only moderately seen, a large number of cortical cells were shown to express CCK mRNA (Burgunder and Young, 1990). This suggests a possibility that CCK produced in the cortex is rapidly transported to terminal field areas and retrograde tracing studies have revealed the cortical neurons containing CCK mRNA project to the striatum (Burgunder and Young, 1990; Morino et al., 1994b), and most of the retrograde labeled cells could be double-stained with the antiserum to pro-CCK in both cortical hemispheres (Morino et al., 1994b)(Morino et al., 1994). In addition, studies have provided some morphological evidence for a partially crossed cortico-striatal CCK pathway in the rat (Morino et al., 1992). For example, it was found that a unilateral decortication plus a callosotomy lesion

produced a decrease in CCK-like immunoreactivity and extracellular CCK in the ipsilateral striatum by approximately 70%, under both basal and K+-depolarizing conditions (Morino et al., 1992; Morino et al., 1994; (You et al., 1994a). Lastly, it has been suggested that CCK may also coexist with glutamate in corticostriatal pathways (Morino et al., 1992). The same unilateral decortication plus callosotomy lesion that produced a 70% ipsilateral decrease in extracellular striatal CCK also produced an ipsilateral decrease in extracellular striatal glutamate by 60% under both basal and K^+-depolarizing conditions (You et al., 1994(You et al., 1994b). While it is generally accepted that glutamate is the major neurotransmitter of the corticostriatal projection it remains to be established whether or not CCK and glutamate actually coexist in this pathway. Containment of both CCK and classical neurotransmitters such as glutamate would provide the synapse with the opportunity to secrete multiple chemical signals to neighboring neurons.

In the striatum of the rat under normal conditions, extracellular levels of CCK are relatively low (Herrera-Marschitz et al., 1992; You et al., 1994a), but can be dramatically increased by perfusion with K^+, an effect that is Ca^{++}-dependent, indicating a neuronal source for releasable CCK (You et al., 1994). Intrinsic and extrinsic CCK systems have been described (Hokfelt et al., 1988), with the extrinsic systems originating in both the cortex and midbrain (Morino et al., 1992). Electron microscopy studies revealed that corticostriatal CCK terminals make direct contact with spiny and aspiny interneurons, providing a morphological basis for direct interaction of CCK with striatal projection neurons and modulatory interneurons (Grofova et al., 1982). The caudate-putamen has been shown to contain a vast majority of CCK-like immunoreactivity coeluted with the

sulfated form (Rose et al., 1988). No, or only minute amounts of, immunoreactivity could be detected for CCK-4, and the unsulfated CCK-8 (CCK-8US) (Rose et al., 1988) which are considered less potent CCKb receptor agonists. The majority of CCK-like immunoreactivity has mainly been found in axons and terminals, suggesting a highly extrinsic origin of striatal CCK (Hokfelt et al., 1988). In rats, light microscopy has revealed dot-like CCK-positive patches of fibers, mainly in the medial aspects of the striatum (Morino et al., 1994). In addition, no positive patches of immunoreactive fibers or terminals for the antibody to pro-CCK were detectible in the striatum (Morino et al., 1994). However, small numbers of cell bodies containing CCK-like immunoreactivity have been described in the striatum of the rat (Hokfelt et al., 1988). Also, intrinsic to the neostriatum, medium-sized aspiny interneurons have been shown to be positive for CCK-like immunoreactivity (Hokfelt et al., 1988). In contrast, *in situ* hybridization studies did not reveal striatal neurons expressing CCK mRNA in the normal adult rat (Burgunder and Young, 1990). Thus, the CCK-like immunoreactivity seen in the striatum could be a result of the internalization of CCK-bound receptors (CCK peptide/receptor complex) on intrinsic striatal cell bodies.

In the midbrain, there is evidence that subpopulations of nigrostriatal dopamine neurons contain CCK as a co-transmitter (Hokfelt et al., 1980a; Hokfelt et al., 1980b). Studies demonstrate that CCK coexists in at least 40% of the neurons of the midbrain . In both rats and primates, CCK-like immunoreactivity and CCK mRNA are visible in VTA and substantia nigra neurons which also contain tyrosine hydroxylase-like immunoreactivity and tyrosine hydroxylase mRNA (Hokfelt et al., 1985; Schalling et al., 1989). In addition, studies using rats have revealed immunoreactive pro-CCK-positive

cell bodies in the substantia nigra compacta and VTA, paralleling the distribution of CCK-8 positive cell bodies (Morino et al., 1994). However, lesions of nigrostriatal pathway did not significantly alter striatal CCK, conversely lesions of the mesocorticolimbic pathway which contains DA/CCK projections to the posterior nucleus accumbens altered accumbens CCK (You et al., 1998). Also, it was shown that CCK/DA co-localization in mesocorticolimbic terminals in the posterior nucleus accumbens are found primarily in separate nerve terminals in the anterior nucleus accumbens and caudate putamen (Seroogy et al., 1989). It appears that the majority of striatal CCK is released from corticostriatal projections and the functional significance of CCK co-localized with DA in nigral midbrain neurons in not known, but could involve an autoregulatory mechanism since CCK receptors were found to be present in the substantia nigra (see CCK receptors/physiology).

1.5.2 Regulation and Modulation of CCK Release

As mentioned above, CCK regularly co-localizes with classic neurotransmitters in nerve terminals, and is most likely stored in morphologically distinct vesicles at remote sites from the membrane site where the classic neurotransmitters are secreted (Verhage et al., 1991). These dense-core vescicles have a large diameter of around 100nm in contrast to classic neurotransmitter vescicles which are clear-cored and smaller in diameter, around 35nm (Verhage et al., 1991). Often, the multiple and complex chemical signals produced by the co-localized neurotransmitters exert different functions postsynaptically (Deutch and Zahm, 1992) and presynaptically in terms of reciprocal modulation of the release process (Marshall et al., 1991). The synapse is adapted to this task by differentiating in release dynamics and calcium regulation of the secretion of both

transmitter classes. This phenomenon has been observed in some systems in which low-frequency stimulation evokes the release of only the classic neurotransmitter, whereas higher frequencies or bursts of impulses lead to release of the co-existing peptide as well (Hokfelt et al., 1991; Hokfelt et al., 2000).

Being a neurotransmitter, presynaptic CCK release is regulated by depolarization of the terminal plasmamembrane upon arrival of a train of action potentials, which leads to Ca^{++}-entry through high-voltage activated calcium channels. The subsequent intrasynaptic rise in Ca^{++} concentration triggers CCK release from dense-cored vescicles. In microdialysis studies, extracellular CCK was decreased by >80% from perfusion with a Ca^{++}-free medium under both basal and K^{+}-depolarizing conditions (You et al., 1994). In a separate study, a slight enhancement of CCK release was observed when the extracellular Ca^{++} concentration was elevated, even in the absence of depolarization (Verhage et al., 1991). After its release, CCK is rapidly degraded by extracellular peptidases. The peptidase responsible for inactivation of endogenous CCK has been identified and purified from brain (Rose et al., 1996).

As a highly abundant peptide heterogeneously distributed in various areas of the brain, CCK release is subject to modulation by a variety of transmitters. In striatal slices, different effects of dopaminergic receptors of D1 and D2 type on CCK-release were found. Initial studies indicated inhibition of CCK-release by D1 receptors, and stimulation by D2 receptors (Bartfai et al., 1988). However later studies suggested that CCK-release in slices of different rat brain regions was increased by D1-receptor stimulation, whereas no D2-receptor mediated effects could be found (Brog and Beinfeld, 1992). Other neurotransmitters have been found to modulate CCK-release in rat brain.

In rat cortex synaptosomes a GABA-B-autoreceptor mediated inhibition of CCK-release was reported (Gemignani et al., 1994). Similarly, broad metabotropic glutamate receptor (mGLuR) agonist t-ACPD inhibited CCK release in hippocampal synaptosomes (Breukel et al., 1997). Additionally, as mentioned earlier, CCK can be modulated by frequency-dependent release. In general, facilitation of release of classic transmitters was observed at stimulation frequencies from 1 to 10Hz, whereas for CCK release a stimulation between 5 and 50Hz was required. This difference would imply that in most cases nerve terminals will release mainly classic neurotransmitters, whereas only under synchronous high-frequency stimulation neuropeptides will be secreted as well.

1.5.3 CCK Receptors and Physiology

CCK is reported to have various functions as a neuropeptide in the brain and acts via two receptor types, which have been classified as CCK-A and CCK-B subtypes (Moran and McHugh, 1982; Moran and Schwartz, 1994), and have also been classified recently as CCK_1 and CCK_2 receptors, respectively (Mercer et al., 2000). Both CCK-A and CCK-B receptors belong to the superfamily of seven transmembrane domain G protein-coupled receptors and were both cloned in the rat (Wank et al., 1992). CCK receptor subtypes have not been well established although reports indicate that these receptors mediate anxiety, panic attacks, satiety, and the perception of pain (Moran and Schwartz, 1994). CCK-A receptors (referred to as peripheral receptors, A = alimentary) are predominantly found in the periphery and CCK-B receptors are predominantly found in the central nervous system (B = brain), however this classification is an oversimplification since studies have demonstrated each receptor subtype can be found both centrally and peripherally (Woodruff et al., 1991). Both CCK-A and CCK-B receptors have a similar

affinity for the agonist CCK-8S, however the unsulfated octapeptide (CCK-8US) and the tetrapeptide (CCK-4) residues show higher affinities for the CCK-B receptors (Innis and Snyder, 1980). There have been conflicting reports concerning the effects and cellular mechanisms mediated by the two receptor subtypes (Saint and Buckett, 1991; Branchereau et al., 1993; Boden and Woodruff, 1994). CCK has been shown to mediate excitation via CCK-B receptors (Branchereau et al., 1993), but CCK-A receptors are reported to close (Boden and Woodruff, 1994) or to open (Branchereau et al., 1993) potassium channels.

Receptor autoradiographic studies using selective antagonists to displace [^{125}I]-Bolton Hunter CCK-8S from either CCK-A or CCK-B receptors confirm that CCK-B receptors are the predominate subtype in the brain. In rats, moderate to high densities of these receptors have been found in the cortex, olfactory bulbs, caudate putamen, nucleus accumbens, and various regions of the limbic system, diencephalon and brain stem (Carlberg et al., 1990; Knapp et al., 1990). CCK-A receptor binding was shown to reside in discrete areas of the rat brain, including the nucleus tractus solitarius, interpenduncular nucleus, area postrema and the posterior hypothalamus (Woodruff et al., 1991), whereas in primates CCK-A receptors were shown to be also present in the midbrain and neostriatum (Hill et al., 1990). It is also likely that CCK-A receptors exist in other areas of the rat brain but a large CCK-B receptor population might mask their presence. There is physiological evidence to support CCK-A receptor localization in many other regions of the brain, including the midbrain, striatum and nucleus accumbens of the rat. For example, CCK-A antagonists could block excitatory or seldom-occurring inhibitory effects of CCK-8S on striatal neurons (Davidowa et al., 1995). In addition, selective

CCK-A receptor agonists were shown to influence neuronal activity in the rat neostriatum (Davidowa et al., 1997). Also, in the posterior nucleus accumbens, CCK-A receptor agonists caused an increase in potassium-stimulated dopamine release and this increase was blocked by CCK-A receptor antagonists (Marshall et al., 1991). Lastly, increased substantia nigral dopamine levels following intranigral perfusion with CCK was antagonized by CCK-A receptor antagonists and not CCK-B receptor antagonists, indicating an involvement of CCK-A receptors (You et al., 1996).

The role and physiological mechanism of action of CCK in the caudate putamen is still not clear. Striatal neurons are mainly excited by CCK and appear to be mediated by CCK-A or CCK-B receptors (Moran et al., 1986; Davidowa et al., 1995). The majority of CCK innervation of the striatum has been shown to originate from the cortex (Morino et al., 1992; (Davidowa et al., 1995). The CCK receptors associated with this afferent pathway have been shown to be located mainly on somata and dendrites of intrinsic neurons (Beresford et al., 1987; Hill et al., 1987). This receptor distiribution would indicate a modulatory action of striatal projection neurons, local interneurons, and/or possibly other corticostriatal afferents such as glutamate. CCK-B receptors have been reported to mediate amino acid release from the striatum. An interesting interaction between CCK and N-methyl-D-Apartate (NMDA)-receptors has been found in the striatum, where the striatal medium spiny projection neurons synthesize a dopamine- and cAMP-regulated phosphoprotein (DARPP-32). DARPP-32 is phosphorylated following activation of the D1 receptor (Hemmings et al., 1984) and dephosphorylated following activation of the NMDA receptor (Halpain et al., 1990). It has been demonstrated that, in striatal slice preparation, CCK produces a decrease in the phosphorylation of DARPP-32

(Snyder et al., 1993). This effect was counteracted by simultaneous application of not only the CCK-B antagonist CI-988, but also by the non-competitive NMDA-antagonist MK-801, indicating that CCK may stimulate the release of glutamate, or activate aspartate interneurons shown to be present in the striatum (Snyder et al., 1993; You et al., 1996). This proposal was supported when it was shown that local administration of CCK via microdialysis probe caused a robust elevation of extracellular levels of glutamate and aspartate in the caudate-putamen, and these increases were prevented by co-adminitration of CCK-B antagonist L-365,260, but not with CCK-A antagonist L-364,718 (Ge et al., 1998). Another microdialysis study showed that CCK-induced GABA release in the nucleus accumbens was completely blocked by the selective CCK-B antagonist PD134308 (Ferraro et al., 1996). Moreover, PD134308 by its own provoked a rapid inhibition of GABA release, and this inhibition was blocked by the CCK-A antagonist L-364,718 (Ferraro et al., 1996). It was also found that in one case the effect of CCK-evoked release of striatal amino acids was not dependent on the presence of external calcium (Barnes et al., 1991). CCK receptors have also been implicated in the modulation of dopamine release and will be discussed further in the next section.

1.5.4 CCK/Dopamine Interactions

As discussed above, high concentrations of CCK are found in the nucleus accumbens and the caudate putamen, important projection areas of the mesolimbic and nigrostriatal dopamine pathways, respectively. Furthermore, CCK and dopamine co-exist in many of the midbrain dopamine neurons that project to forebrain structures. It is therefore not surprising that the possible interactions of these co-transmitters have been of great interest to many investigators. To date, a number of CCK-dopamine interactions have

been found using various electrophysiological, neurochemical, pharmacological, and behavioral approaches. However, the majority of studies have concentrated on CCK/dopamine interactions in the nucleus accumbens where a substantial amount of midbrain CCK is co-localized in and released from mesolimbic dopamine neuron terminals.

Several lines of evidence show that exogenous CCK produces opposite effects on dopaminergic function in the anterior versus the posterior nucleus accumbens, and that different receptor subtypes of CCK may mediate these effects. In the anterior nucleus accumbens, CCK decreased DA-stimulated adenylate cyclase activity in tissue homogenates (Studler et al., 1986) and decreased potassium-stimulated DA release from tissue slice preparations (Marshall et al., 1991). The effects of CCK on DA release in the anterior NAc were blocked by L-365,260, a selective CCK-B antagonist, but were not blocked by L-364,718, a selective CCK-A receptor antagonist (Marshall et al., 1991). Additionally in a separate study, CCK injected directly into the anterior NAc inhibited dopamine-induced hyperactivity in rats (Crawley, 1992), possibly by an action on CCK-B receptors. Conversely, in the posterior NAc, CCK increased DA-stimulated adenylate cyclase activity in tissue homogenates (Studler et al., 1986), and increased potassium-stimulated DA release from tissue slice preparations (Marshall et al.. 1991). The effects of CCK on DA in this region of the accumbens were blocked by L-364,718, but not by L-365.260 (Marshall et al., 1991). Direct administration of CCK into the posterior NAc potentiated dopamine-induced hyperactivity (Crawley et al., 1992), supporting the above data. The different effects of CCK on the posterior and anterior NAc may be related not only to the receptor subtypes involved, but also to the different sources of CCK in each

case. CCK in the posterior region is co-localized with dopamine in the mesolimbic nerve terminals, whereas CCK in the anterior accumbens appears to arise from other regions, such as the cortex.

In *in vivo* microdialysis experiments, perfusion of high concentrations of the CCK-B antagonist CI-988 through a microdialysis probe significantly increased dopamine overflow in the caudate-putamen and both anterior and posterior nucleus accumbens, and was shown not to be calcium-dependent (Corwin et al., 1995). However, at lower doses of around 100μM, CI-988 had no effect on DA overflow and at the higher concentrations used, the compound is not selective for the CCK-A or CCK-B receptor subtype.

Recent findings have provided evidence for CCK/dopamine receptor interactions in the striatum and nucleus accumbens. Autoradiographic and biochemical binding studies indicate that CCK reduces the affinity of D2 receptors in the striatum and nucleus accumbens (Li et al., 1994). It was demonstrated using *in vitro* filter wipe-off technique, that the reduction in affinity of the D2 agonist binding sites in the striatum by CCK is mediated via the activation of the CCK-B receptor subtype, since this effect was counteracted by the CCK-B receptor antagonist PD134308 (Ferraro et al., 1996). Such an inhibitory regulation of D2 receptors by CCK via CCK-B receptors is also observed in fibroblast cells cotransfected with human CCK-B and D2 receptor cDNAs (Dasgupta et al., 1996). Lastly, in support of the CCK-B/D2 receptor interactions described above, it was demonstrated that a distinct population of D2 mRNA-positive striatal nerve cells contain CCK-B receptor mRNA signals (Hansson et al., 1998).

In other receptor binding studies, chronic treatment with the dopamine receptor antagonist haloperidol significantly increases the number of CCK receptors in the

neostriatum and cortex (Fukamauchi et al., 1987). The increase in number of CCK binding sites appears to be functionally significant, in that chronic haloperidol also increases the responsiveness of neostriatal neurons to the excitatory effects of CCK (Debonnel et al., 1990). Conversely, acute and chronic CCK administration significantly increases the number of D2 receptor binding sites in the neostriatum and cortex (Agnati et al., 1985).

1.5.5 CCK and Neurobiological Disorders

The biological actions of CCK at different physiological areas have not been clearly nor completely defined. Nevertheless, the development of specific and sensitive immuno-bioassays for identification of CCK and the recent discovery of potent and selective CCK receptor blockers has led to a better comprehension of the multiple roles of CCK. At the central level, many studies have focused on the possible role of CCK peptides and receptors in the treatments of mental disease. Several studies have shown that CCK is largely involved in the etiology of anxiety and panic disorders (Crawley, 1994; Crawley and Corwin, 1994). In different *in vivo* studies, exogenous CCK, and in particular CCK-4, a CCK-B receptor agonist, induced panic attacks in humans and produced a distinct sensation similar to anxiety and hallucination (Ravard and Dourish, 1990; Bourin et al., 1998). It was also demonstrated that pretreatment of patients with the CCK-B receptor antagonist dose dependently blocked CCK-4-induced panic attacks (Bourin et al., 1996; Bourin et al., 1998).

The anatomical distribution of CCK within the larger dopaminergic system raises the possibility of that CCK may play a critical role in dopaminergic pathologies such as Parkinson's disease, Huntington's disease, schizophrenia, and drug addiction. Despite

few reports that have directly assessed a link between CCK and neuropathological and psychopathological diseases, several lines of evidence point to CCK playing a role in either the pathology of the disease or having therapeutic implications. For example, reports indicate that CCK receptors are reduced in the neostriatum of patients who suffer from degenerative diseases such as Huntington's disease (Hays and Paul, 1982). Another study showed Huntington's disease patients have a decrease in CCK-like immunoreactivity in the globus pallidus and substantia nigra (Emson et al., 1980). CCK-like immunoreactivity was also found to be decreased in several brain regions among schizophrenic patients (Bourin et al., 1996). Additionally, CCK-B antagonists are being investigated as a potential antipsychotic treatment in schizophrenia patients, and one report showed that the CCK-B antagonist LY262291 decreased midbrain dopamine unit activity in schizophrenic patients (Emson et al., 1980; Rasmussen et al., 1991). A potential therapeutic role was found for CCK when reports by Boyce and colleagues indicated that the CCK-B antagonist L-365,260 potentiates the therapeutic efficacy of L-DOPA in a monkey model of Parkinsonism, whereas CCK treatment inhibits the occurrence of dyskinesias, the undesirable effects of chronic L-DOPA administration (Boyce et al., 1990). Presynaptic modification of CCK release by CCK receptor antagonists is an important way to fine-tune local CCK responses in the brain, and may provide additional future targets for therapeutic intervention.

1.6 Central Questions

It has been proposed that drug addiction is the result of neuroadaptive processes within the central nervous system that oppose the acute reinforcing actions of abuse

(Koob and Bloom, 1988; Koob et al., 1998). The acute reinforcing actions are believed to be the increase in DA in terminal field areas. Psychostimulant-induced dopamine overflow is detrimental to dopaminergic neurons and their axonal terminal field areas in the neostriatum (Ricaurte et al., 1984b; Ricaurte et al., 1984a), and while there has not been any evidence linking cocaine use to dopamine neuron degeneration, it is conceivable that the cocaine-induced elevated dopamine levels that are significantly above physiological levels may be toxic and damaging over time. Due to the questions that still surround neuropathological diseases, such as Parkinson's disease, it is of great interest to discover neural mechanisms that protect neurons from the damaging effects of dopamine overflow induced by psychostimulants. Neuropeptides, such as cholecystokinin, are usually found co-expressed or in close proximity with classical transmitters such as dopamine and conceivably modulate the activity of these neurotransmitters, thus protecting neurons from excessive excitation resulting in neural damage and restoring physiological conditions. **This introduces the central question(s) of this thesis:**

Does cholecystokinin modulate cocaine-evoked dopamine release in the striatum?

First, the effects and pattern of cocaine-evoked synaptic dopamine release in the caudate-putamen during three treatment paradigms will be determined by collecting and analyzing perfusate using microdialysis and high performance liquid chromatography (HPLC). We will then micro-infuse several different doses of the agonist, sulfated cholecystokinin octapeptide (CCK8-S), and selective non-peptide CCK receptor antagonists through the microdialysis probe into the caudate putamen in the early

withdrawal treatment group and measure its effects on cocaine-evoked synaptic dopamine. If it is found that CCK-8S peptide agonist or selective CCK-receptor antagonists modulate cocaine-evoked dopamine release during early withdrawal, then:

What effects are mediated through each CCK receptor subtype and do they modulate dopamine differently in naïve (acute) compared to chronically treated and early withdrawal animals?

The optimal dose of CCK-8S agonist and selective antagonists will be administered in acute and chronic cocaine treatment groups and their effects will be compared among the three treatment groups. If there are differences in the effects mediated through CCK receptors during the three different treatment paradigms, then:

Are there changes in receptor-binding during the three cocaine treatment paradigms?

To assess the possibility of the dopaminergic effects being mediated by a change in CCK receptor-binding or dopamine transporter sites, receptor autoradiography will be used for quantitative receptor comparison of the three treatment groups. And lastly, to begin to determine the mechanism by which dopamine is modulated, then:

Are the effects of CCK and/or CCK-receptor antagonists on cocaine-evoked dopamine release calcium dependent?

A calcium-channel blocker will be infused through the microdialysis probe along with the CCK antagonists to observe if the antagonist-induced dopamine augmentations are mediated via a calcium-dependent mechanism.

Thousands of articles have been published, but the main question still remains: What regulates dopamine release in the brain and how? In addition, the role and physiological mechanism of action of CCK in the striatum is not clear. There are conflicting reports concerning how CCK modulates dopamine and the effects and cellular mechanisms mediated by the two CCK receptor subtypes. The above central questions outline specific aims, **however the relevance of this study is 3-fold:**

1) Study neuroadaptive changes that occur as a result of chronic psychostimulant abuse, which presents a compelling model of neuroplasticity

2) Use cocaine as a research tool to perturb or activate the basal ganglia and investigate *in vivo* the mechanisms of neurotransmitters and their interactions with each other.

3) The possible therapeutic implications of cholecystokinin and CCK receptor antagonists in dopaminergic pathologies, such as Parkinson's disease, Huntington's disease, schizophrenia and drug addiction.

Considering the critical role of the basal ganglia and dopamine in Parkinson's disease, Huntington's disease, and schizophrenia, and possibly other neuropathological and psychopathological conditions, the ability of CCK to modulate dopaminergic functioning has important therapeutic implications.

Chapter 2

MATERIALS AND METHODS

2.1 Animals

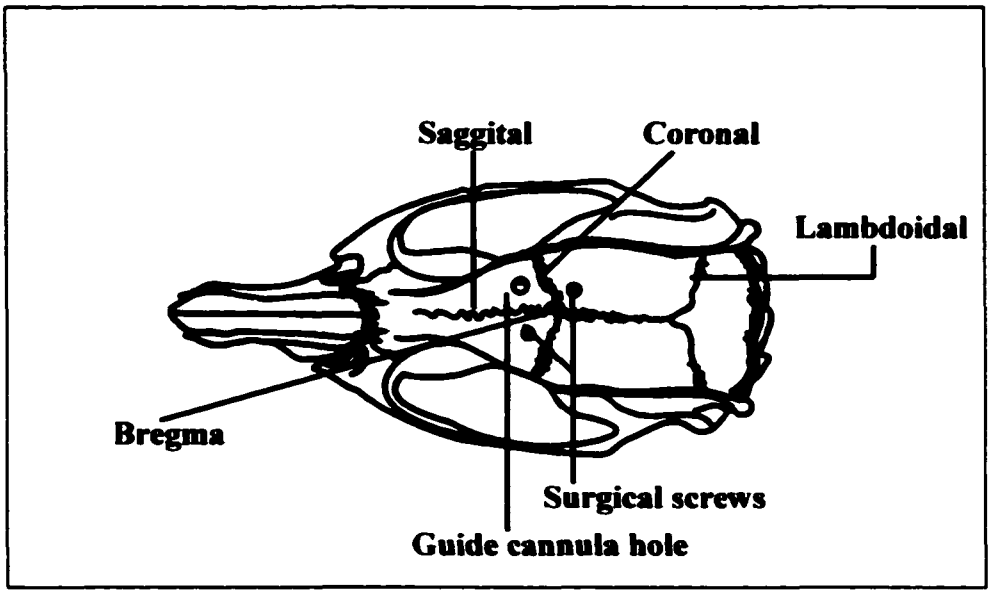
All experiments were performed on male Sprague-Dawley rats (Harlan-Sprague Dawley) weighing approximately 300g at the time of surgery. All animals were allowed at least 7 days to acclimate to their new environment and were handled daily before any experimental manipulation. They were housed in their home cages in groups of two in a temperature and humidity controlled environment on a 12 hour light/dark cycle (light on at 9:00 a.m.), with access to food and water *ad libitum*. The experimental protocols performed in the present study were approved by the Institutional Animal Care and Use Committee.

2.2 Surgery

Rats were anesthetized by an i.p. injection of a mixture of Ketamine/Promace, the scalp was shaved with electric clippers, and were placed in a stereotaxic apparatus (Stoelting). Ophthalmic ointment was added to the eyes for lubrication and protection and the scalp was swabbed three times each with iodine scrub, iodine solution, and sterile saline solution. A midline incision in the scalp was made, the area was cleaned and the saggital, coronal, and lambdoidal sutures and bregma were exposed (Fig 5). The bregma, which is the location where the coronal and saggital sutures intersect, was used as a zero

point of reference for the coordinates of the guide cannula. A hole is drilled into the skull and the guide cannula(CMA/Microdialysis) was slowly lowered into the brain. The coordinates used for guide cannula implantation into the left

Fig. 5 Dorsal View of Rat Skull



Close-up view of the rat skull with a Trephine-drilled hole for the guide cannula and two anchor screws placed in two different bone plates. The coordinates used for guide cannula insertion into the caudate-putamen were in reference to the bregma or zero point. Two stainless steel screws were tapped into the skull to anchor the cannula with dental cement.

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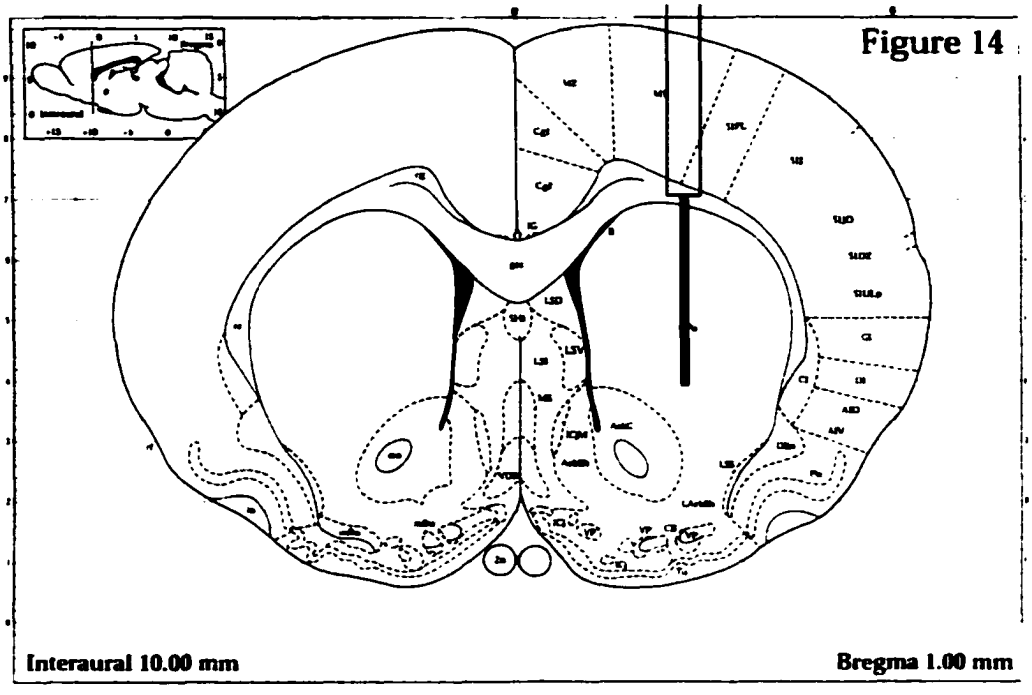
or right caudate-putamen were: A = +1.6mm. L = +/-2.2mm. D = -2.8mm; according to the atlas of Paxinos and Watson (Fig. 6). Two stainless steel screws are also tapped into the skull in the surrounding area and dental cement was used to secure the cannula to the skull and screws. A dummy probe is placed in the opening of the guide cannula to prevent pathogens from entering the brain. The wound was sutured, cleaned, and topical

antibiotic was applied to the surgical area. The animals were then transported back to their home cages and allowed 3 days to recover before testing and/or drug treatment.

Fig. 6 Microdialysis Guide Cannula Coordinates



A coronal view of the rat brain from the stereotaxic atlas of Paxinos and Watson, sectioned at the level of forebrain structures caudate-putamen (Cpu) and nucleus accumbens (Acb), which has morphologically distinct regions, the core (AcbC) and the shell (AcbSh). The coordinates used for guide cannula implantation (thin black rectangle, L = 2.2mm, D = 2.8mm; each large box equals 1mm) allows the active membrane of the microdialysis probe (thick black line, 3mm in length) to be centrally located in the Cpu.

2.3 Systemic Drug Treatment

Cocaine was dissolved in phosphate-buffered saline (pH 7.4) and injected intraperitoneally with plastic 1.0ml syringes. Animals received drug with vehicle or saline vehicle only (control animals) in a total volume of 0.5ml. Cocaine was obtained

from Sigma Chemical Co. and was administered at 10mg/kg of body weight once daily. Animals were weighed daily to ensure proper growth, to monitor health and to determine appropriate dose of drug. Three treatment paradigms for each cocaine and control group were used and are as follows: acute groups received one injection and then tested; chronic groups received one injection daily for seven days and were tested on the 7th day; and early withdrawal groups received one injection daily for seven days, had three days without drug, and received one challenge dose and tested on day 11 (Fig. 7).

Fig. 7 Cocaine and Saline Treatment Paradigms

Days	1	2	3	4	5	6	7	8	9	10	11
Acute	I+C										
Chronic	I	I	I	I	I	I	I + C				
E.W.	I	I	I	I	I	I	I	N	N	N	I + C

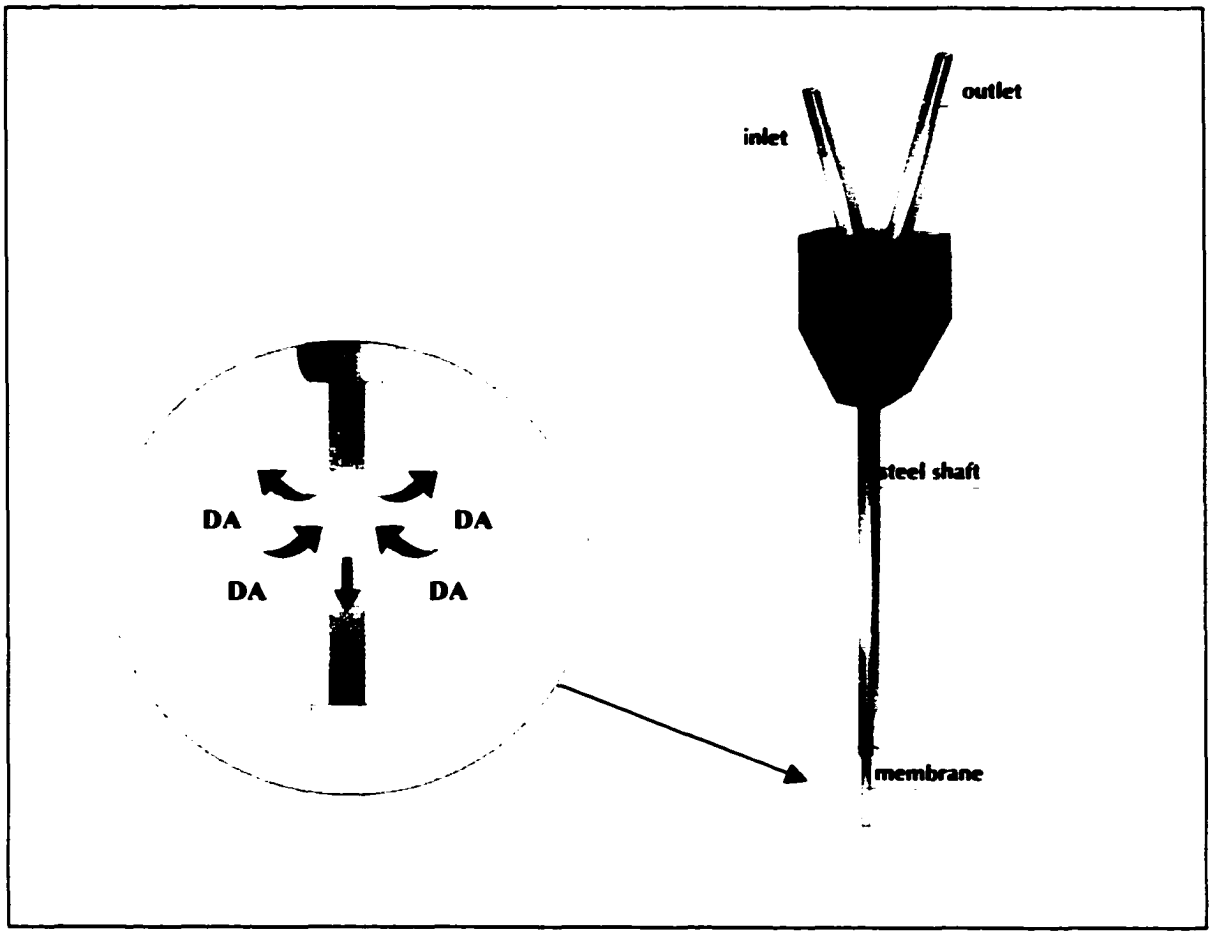
An outline of the three cocaine and saline treatment paradigms (I = Inject; C = Collect; N = No drug treatment). Animals receive cocaine at 10mg/kg i.p. once daily.

2.4 In vivo Microdialysis

All animals were transported from their home cages and placed in testing cages with food and water *ad libitum* the night before testing. The dummy probes were removed and a microdialysis probe with a 3mm active dialysing membrane, 0.5mm diameter and a 20,000 Da molecular cutoff (CMA12/03) was inserted through the guide cannula

(Fig. 8). This period allowed the animals to become accustomed to the system and minimized disturbances due to the insertion of the probe. The animals were fitted with a

Fig. 8 In Vivo Microdialysis Probe



The CMA/12 3mm microdialysis probe. The semi-permeable membrane is constructed of a polycarbonate and its molecular cut-off is 20,000 Daltons.

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plastic collar, which was connected to a tether attached to a dual channel swivel (Instech) and were permitted with relatively unrestricted movement. The probe was connected via tubing (CMA) attached to the tether to an infusion pump(CMA 102) via the dual channel swivel and artificial cerebrospinal fliud (145 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl, 1.0

mM $MgCl_2$, pH 7.4) was perfused at 0.6µl/min overnight. The following morning, the infusion pump flow rate was increased to 1.0µl/min and sample fractions of brain dialysate were collected every 30 min in plastic 250µl vials containing 3.0µl of preservative (5N $HClO_4$) to inhibit the degredation of dopamine. For experiments without intracranial drug administration, three baseline samples were collected and the animals were treated with an i.p. injection of cocaine. Four more 30 min samples were then collected and the microdialysis probe was removed. The animals were then transported back to the animal facility where they were placed in a CO_2 chamber and sacrificed and their brains were removed for histological verification of probe placement.

2.5 Intracranial Drug Treatment

After three baseline samples were collected, the perfusion medium was changed from aCSF to aCSF containing one of the compounds; CCK-8S, L-369,293, L-364,769, and/or Verapamil, an L-type calcium channel blocker (Sigma Chemical Co). The aCSF perfusion syringe was carefully replaced with aCSF/drug by hand, taking care not to introduce bubbles in the tubing. The non-selective peptide receptor agonist CCK-8S (Bachem) was dissolved in ddH_20 as a stock of 10mM and then was diluted to the proper molarity in aCSF. The final solution was adjusted to a final pH of 7.4. The selective CCK-A receptor antagonist L-364,769 (Dr. Roger Freidinger, Merck Research Laboratories, West Point, PA) is a non-peptide compound that was dissolved into a mixture of ddH_2O and 20% DMSO at a stock concentration of 10mM and then was diluted to the proper molarity in aCSF and 10% DMSO. The selective CCK-B receptor antagonist L-369,293 (Dr. Roger Freidinger, Merck) is also a non-peptide compound that

was dissolved in ddH_2O at a stock concentration of 10mM and then was diluted to the proper molarity in aCSF. Verapamil was dissolved in ddH_2O and diluted in either aCSF with L-364,769 or aCSF with L-369,293 to the proper molarity. All aCSF/drug mixtures were adjusted to a final pH of 7.4 before perfusion.

2.6 In Vitro Recovery

The following procedure was used to estimate the in vitro recovery of dopamine using a 3mm dialysis probe. The probe was placed in an eppendorf tube containing dopamine of a known concentration (10^{-7} M) and the probe was perfused at a flow rate of 1µl/min with aCSF free of dopamine. The perfusate was collected at 30-min intervals in tubes with the same preservative as mentioned above and dopamine was measured using HPLC with electrochemical detection. The percentage of recovery was estimated for dopamine by the following ratio: concentration of dopamine in the collected dialysate sample / concentration of the known amount in the outside medium. An average was taken of six (n=6) samples and the recovery was approximately 18.7±1.63% for dopamine.

In order to estimate the in vitro perfusion of CCK-8S through the microdialysis probe, the probe was placed in an eppendorf tube containing 1ml of aCSF free of CCK-8S and perfused with 1ml of aCSF containing 10^{-6} M CCK-8S with 500cpm/µl of [^{3}H]CCK-8S added as a tracer, at a flow rate of 1µl/min. The eppendorf tube was replaced every 30-min and the radioactivity was counted in a liquid scintillation β-counter. The recovered amount of perfused CCK-8S out of the microdialysis probe was estimated using the following ratio: radioactivity counted in the tube / radioactive drug perfused through the

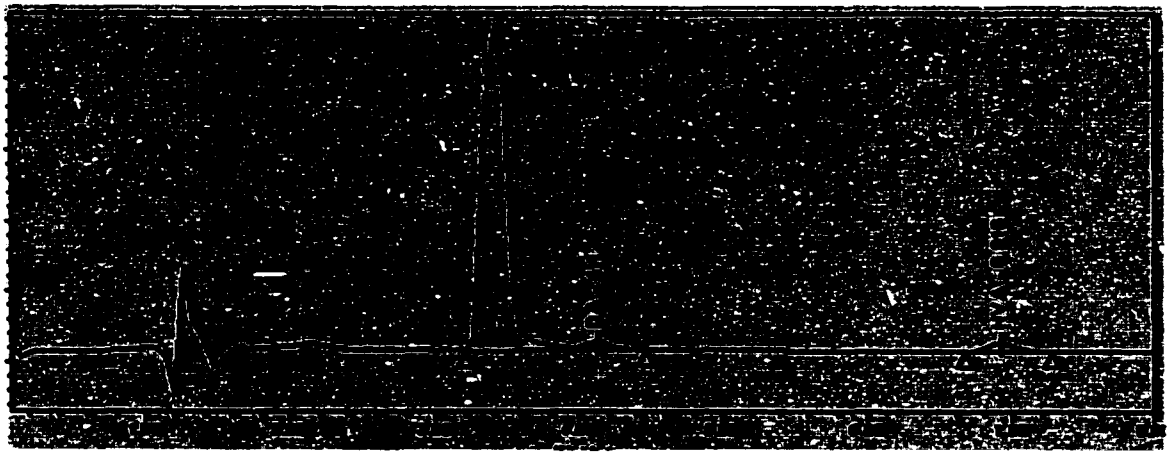
probe during 30 min. An average was taken of seven (n=7) samples and the recovery was estimated at 9.36 ±1.13% for CCK-8S.

2.7 High-Performance Liquid Chromatography

Brain dialysate samples were analyzed for dopamine and its metabolites DOPAC and HVA using HPLC coupled with electrochemical detection. The HPLC system consisted of a Waters 515 pump linked to a 717plus autosampler. An MD-150 column (ESA, 3 x 150mm) was used to separate the amines and their metabolites from a 10µl dialysate sample. They were detected using a Choulochem II detector (ESA) using three electrodes, an ESA 5020 guard cell and a graphite coulometric analytical cell (ESA 5014b) containing two working electrodes in series. The guard cell was set at 300mV and placed between the pump and injector to decrease mobile phase contaminants and background noise resulting from oxidizable compounds. The working electrodes were set at: electrode 1, -150mV; electrode 2, 200mV. By selectively choosing the applied potentials, interferences can be removed by the first (upstream) electrode leaving the second (downstream) electrode free to measure low levels of analytes. The mobile phase was an acetonitrile-based mobile phase (MD-TM, ESA) for catecholamine detection. Dopamine and its metabolites were eluted at a mobile phase flow rate of 0.55ml/min within 15 minutes. The peaks were monitored and analyzed using Milleneum integrating software (Waters Corp.). Standard samples containing DA, DOPAC, and HVA were injected prior to each individual animals sample set to determine peak identification and quantification (Fig. 9). Changes in dopamine concentration in unknown experimental

samples were expressed as a percent change relative to the average baseline of two samples.

Fig. 9 Chromatographic Separation of Dopamine



A sample chromatogram showing separation of dopamine and its metabolites. DOPAC and HVA as a function of millivolts per unit time.

2.8 Histology

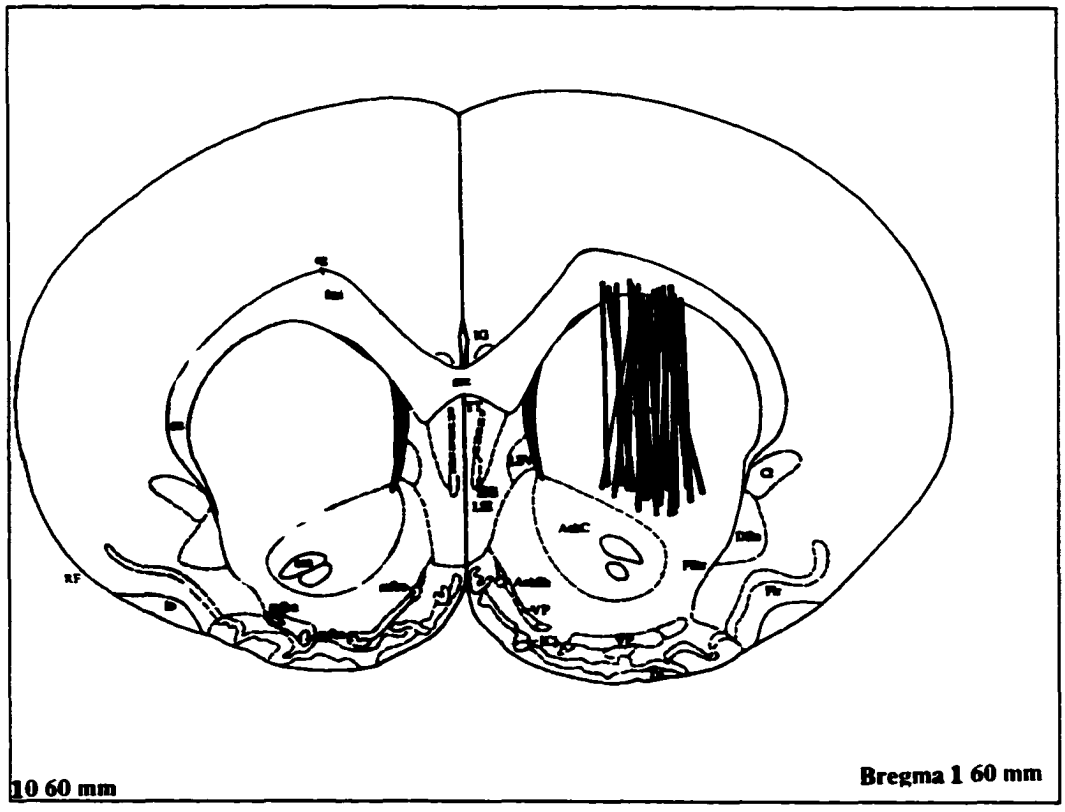
Following microdialysis testing, the animals were sacrificed in a CO_2 chamber and their brains were immediately removed and frozen on dry ice. The brains were sectioned (20μM) in a cryostat and the location of the microdialysis probe implantation was examined using low magnification and verified according to the atlas of Paxinos and Watson (Fig. 10). Only animals with correctly implanted probes were included in the statistics.

2.9 Receptor Autoradiography

2.9.1 Autoradiographic Tissue Preparation

Animals were administered cocaine or saline according to three treatment paradigms (Fig. 7). Following their last treatment, they were sacrificed with CO_2 and their brains

Fig. 10 Illustration of Probe Placement



A sample of histological localizations of 3mm microdialysis probe membranes in the caudate-putamen from a cocaine-only groups. The number of lines is less than the number of rats used in the groups (n = 20) because overlapping placements among several animals. The coordinates used were A = 1.6mm, L = 2.2, D = 2.8, all in reference to the bregma. In this study and in all studies, rats that had dialysis membranes outside of the caudate-putamen were not included in data analysis. The illustration was derived from the atlas of Paxinos and Watson, 1986.

were immediately removed and frozen on dry ice. They were sectioned (20μM) on a cryostat and thaw-mounted onto Fisher Superfrost/Plus slides. All slides were stored dessicated at -80°C until ready for autoradiographic studies. Twenty slides with three sections on each slide were used from each rat brain.

2.9.2 CCK Receptor Autoradiography

Tissue sectioned slides were air dried overnight in a vacuumed dessicator at 4°C before running the assay. The next day, the sections were pre-incubated at room temperature for 30 minutes in 50mM Tris buffer (pH 7.4) containing 5mM $MgCl_2$ and 0.2% bovine serum albumin. The slides were then transferred to the Tris buffer containing the above with 0.02% bacitracin and 100pM Bolton-Hunter labeled [125I]CCK-8S (Amersham Pharmacia Biotech, specific activity = 2000Ci/mmol) and incubated for 60 minutes at room temperature. Incubations were terminated by two consecutive washes in the pre-incubation buffer (0-4°C, 10 min. each) followed by rinsing in ddH_2O (0-4°C, 5 sec. each). Non-specific binding was determined by co-incubation with 1uM CCK-8S and was calculated as the difference in [125I]CCK-8S binding in the presence or absence of CCK-8S, which was approximately 15%. For CCK-A/CCK-B receptor competition experiments, sections were incubated under the conditions outlined above except unlabeled CCK-A receptor antagonist (L-364,769; 100nM) or unlabeled CCK-B receptor antagonist (L-369,293; 10nM) was added to the incubation buffer to selectively block one of the receptors. Immediately following the assay, the slides were dried by fan overnight and exposed to Hyperfilm MP (Amersham)

for 21 days. The gray levels were quantified in the Cpu using an image analysis system utilizing the NIH Image 1.49 software VDM. Six sections per animal were quantified and the values averaged to generate an optical density value that corresponds to CCK receptor levels per animal.

2.9.2 Dopamine Transporter Autoradiography

Tissue sectioned slides were air dried overnight in a vacuumed dessicator at 4°C before running the assay. Sections were incubated in assay buffer containing; 137mM NaCl, 2.7mM KCl, 10.14mM Na_2PO_4, 1.76mM KH_2PO_4, 10mM NaI containing 0.073nM [^{125}I]RTI-121 (PerkinElmer Life Sciences, specific activity = 2200Ci/mmol), for 60 minutes at room temperature. The incubations were terminated by two consecutive washes in the assay buffer described above (without [^{125}I]RTI-121) at 0-4°C for 20 minutes each. The slides were then washed three times in ddH_20 (0-4°C 5sec each). Non-specific binding was determined by co-incubation with 10μM non-labeled GBR-12909 and was calculated as the difference in [125I]RTI-121 binding in the presence or absence of GBR-12909. Specific binding was found to be greater than 90% of total binding. Immediately following the assay, the slides were dried by fan overnight and exposed to Hyperfilm MP (Amersham) for 51-53 hours. The gray levels were quantified in the caudate-putamen using an image analysis system utilizing the NIH Image 1.49 software VDM. Six sections per animal were quantified and the values averaged to generate an optical density value that corresponds to DAT levels per animal.

2.10 Statistical Analysis

The neurochemical time course data for dopamine in each microdialysis sample was converted to a percentage of change from the average of three baseline measurements and the average baseline for each animal was normalized to 100%. This is necessitated by the observation that baseline values are highly variable between animals so percent differences in neurotransmitter release relative to baseline eliminates this variable and are highly comparable. The data was then analyzed using a two-way ANOVA with repeated measures over time. Post hoc analysis was performed using Fisher's test and significance was obtained with P values of 0.05 or smaller. Under the curve analysis for each cocaine treatment group was determined by adding all consecutive post-cocaine administration dopamine peak values for each animal (four 30 min. samples summed together) in corresponding agonist/antagonist groups. Differences among agonist/ antagonist groups were examined by a one-way ANOVA, followed by Fishers post-hoc analysis.

Autoradiographic receptor levels in different treatment groups were analyzed using ANOVA. Untreated (control) animals were normalized to 100% and the results of cocaine and saline treatment groups were represented as percent change relative to control animal receptor levels. Post hoc analyses were performed using Scheffes test for comparison of differences between individual groups.

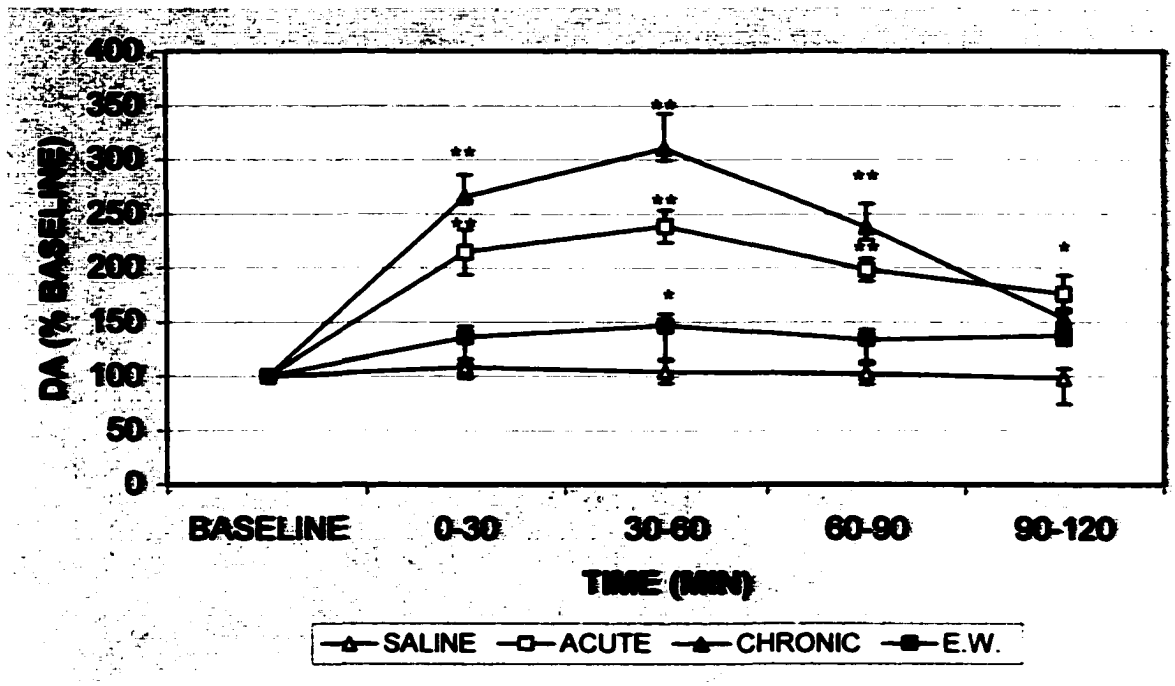
Chapter 3

RESULTS

3.1 Cocaine-Evoked Dopamine Release

Using *in vivo* microdialysis and high performance liquid chromatography (HPLC), we assessed the effects of three treatment paradigms on cocaine-induced dopamine neurotransmission in the caudate-putamen Fig. 10). Rats were treated with cocaine (10mg/kg) i.p. once daily for either 1 day (acute or naïve), 7 consecutive days (chronic), or 7 consecutive days followed by the same dose challenge after 3 days of withdrawal (early withdrawal) (Fig. 7). Following cocaine administration, samples were collected every 30 minutes for 120 minutes. Acute administration of cocaine increased extracellular dopamine content in the dCPU by an average of 207% and had a maximum increase of 248%; chronic administration increased dopamine content by an average of 241% and had a maximum increase of 310%; and animals in the early withdrawal group yielded an average extracellular dopamine increase of 138% with a maximum increase of 147% (percent values are expressed relative to baseline dopamine release which was averaged and normalized to 100%). In all saline groups, the extracellular dopamine levels were similar to their respective baseline levels, and never were significantly above baseline. Also, there was no significant difference between the three saline groups, therefore, all groups were averaged and the results represented as one saline group on the graph.

Fig. 11 Cocaine-Induced Dopamine Release in Acute, Chronic and E.W. Animals



The effect of acute, chronic and early withdrawal cocaine and saline in rats on extracellular dopamine in the caudate-putamen. The data were normalized by dividing all values by the average of the three baseline measurements made before the injection of cocaine (i.p. 10mg/kg) or saline. Each treatment group is represented by the number of animals: acute, n = 6; chronic, n = 8; e.w., n = 6. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$; ** = $p < 0.0001$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for acute dopamine group, $F = 13.225$, $p < 0.0001$; chronic group, $F = 18.185$, $p < 0.0001$; early withdrawal group, $F = 1.8$, $p > 0.05$.

3.2 Dose Response Curves

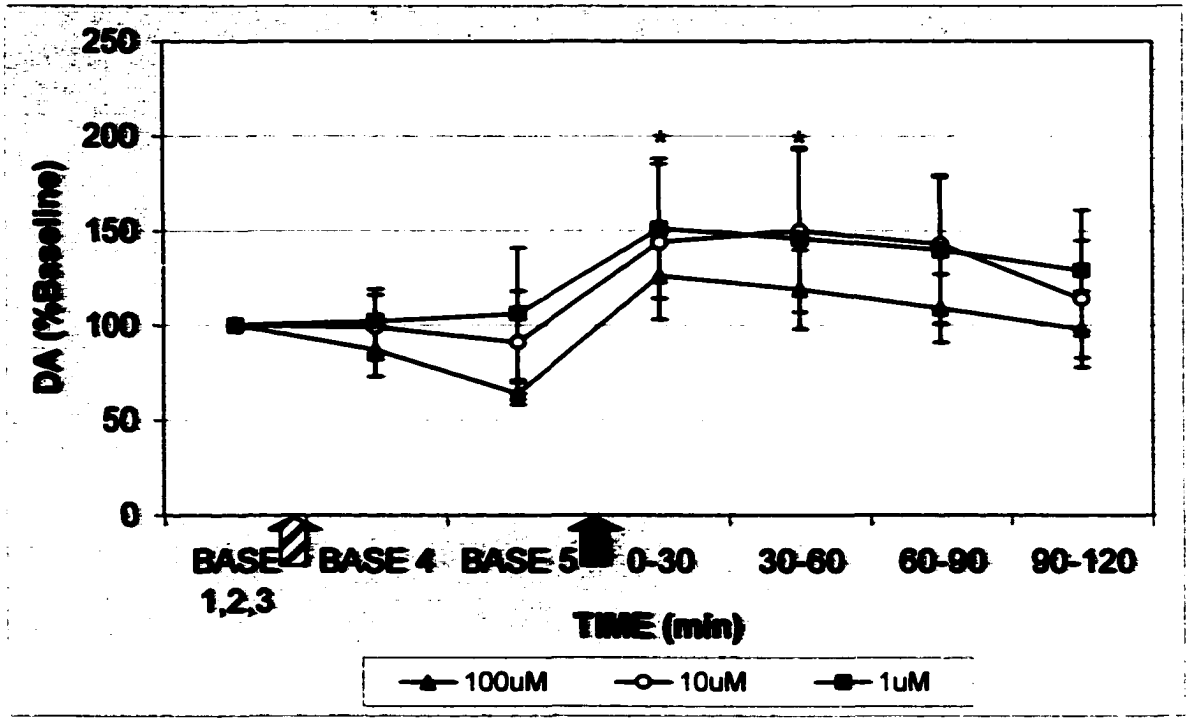
3.2.1 CCK-8S Dose Response

Using the techniques outlined in the previous study, dopamine levels were measured in the three groups of rats treated with cocaine, except in this study the CCK receptor

agonist CCK-8S was continuously infused through the microdialysis probe into the caudate-putamen. After three baseline samples were taken, three different doses of CCK-8S (100μM, 10μM, 1μM) were added to the perfusion medium and were continuously administered during the rest of the experiment. Before cocaine administration, two samples were collected to measure the effect of the infused drug on basal dopamine levels (internal standard). After cocaine administration, four 30 min. samples were collected. Animals in early withdrawal were chosen for this study because extracellular dopamine levels were found to be depleted in this group (day 11), and the effects of CCK-8S on dopamine in the caudate-putamen were not conclusive in the few published studies, so our hypothesis was that CCK-8S infused into the caudate-putamen would increase dopamine levels back to acute or chronic levels. When perfused with 100μM CCK-8S (Fig. 12), animals in early withdrawal had a decrease in basal dopamine levels before cocaine administration, which fell below baseline levels to 64%. Cocaine administration failed to significantly elevate dopamine levels in the Cpu, compared to baseline, when perfused with 100μM CCK-8S. The average dopamine level after cocaine administration was 113%, with a maximum increase of 126%. There was a significant difference between the CCK-8S-evoked decrease in basal dopamine of 64% (baseline 5) and post-cocaine-evoked dopamine levels of 119% and 123%. When perfused with 10μM CCK-8S, the inhibitory effect on dopamine observed with the 100μM concentration was negligible, with a maximum decrease in pre-cocaine dopamine levels to 91%. The average dopamine level after cocaine administration was 138%, which was the same average levels as early withdrawal animals that received cocaine without CCK-8S. There was a significant increase in cocaine-evoked extracellular

dopamine (150% peak) when compared to baseline and CCK-8S basal levels (baseline 5). At 1μM, CCK-8S did not have any effect on pre- and post-cocaine dopamine levels in the Cpu, with pre-cocaine levels averaging 104% and post-cocaine levels averaging 142%.

Fig. 12 CCK-8S Dose Response

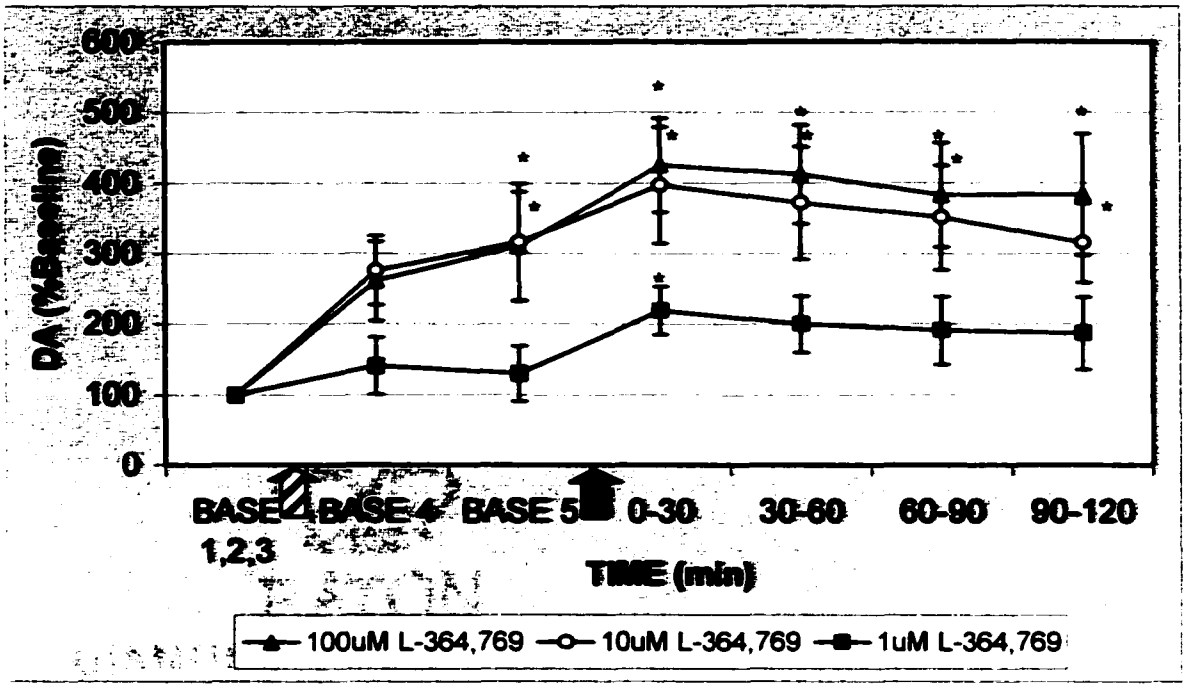


CCK-8S dose response on caudate-putamen extracellular dopamine levels in early withdrawal animals. CCK-8S was administered continuously after baseline measurements into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM, n= 7; 10μM, n = 9; and 1μM, n = 6. Cocaine was administered i.p. (10mg/kg) 1 hour after the start of the CCK-8S infusion, and is represented as the solid arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the administration of CCK-8S. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: 100μM, F = 1.908; 10μM, F = 2.134; 1μM, F = 2.192. P values for all groups were > 0.05.

3.2.2 CCK-A antagonist L-364,769 Dose Response

Microdialysis samples were collected and dopamine was measured using the methods outlined in the CCK-8S dose response except after three baseline samples, three different doses (100μM, 10μM, 1μM) of the highly selective CCK-A receptor antagonist L-364,769 was administered into the caudate-putamen through the microdialysis probe, continuously throughout the duration of the experiment (Fig. 13). After observing the inhibitory effects of CCK-8S on pre-cocaine basal and cocaine-evoked extracellular dopamine in early withdrawal animals, the L-364,769 dose response was also administered in early withdrawal animals (day 11), hypothesizing that it would have the opposite effect and facilitate dopamine release. Perfusion of 100μM L-364,769 significantly increased pre-cocaine basal extracellular dopamine levels by an average of 286% and as much as 310%. Following cocaine administration, L-364,769 elevated the depleted cocaine-evoked dopamine levels observed in early withdrawal animals by an average of 401%, with a maximum increase of 425%. All post-cocaine dopamine levels were significantly above baseline levels in response to 100μM L-364,769. The 10μM concentration also significantly increased pre-cocaine basal dopamine levels to 316%, and significantly elevated cocaine-evoked dopamine levels to an average of 358%, with a maximum increase of 396%. Lastly, the 1μM concentration did not significantly elevate pre-cocaine basal dopamine levels and only increased cocaine-evoked extracellular dopamine to a significant level during the first 30 min. post-cocaine treatment.

Fig. 13 CCK-A Receptor Antagonist L-364,769 Dose Response



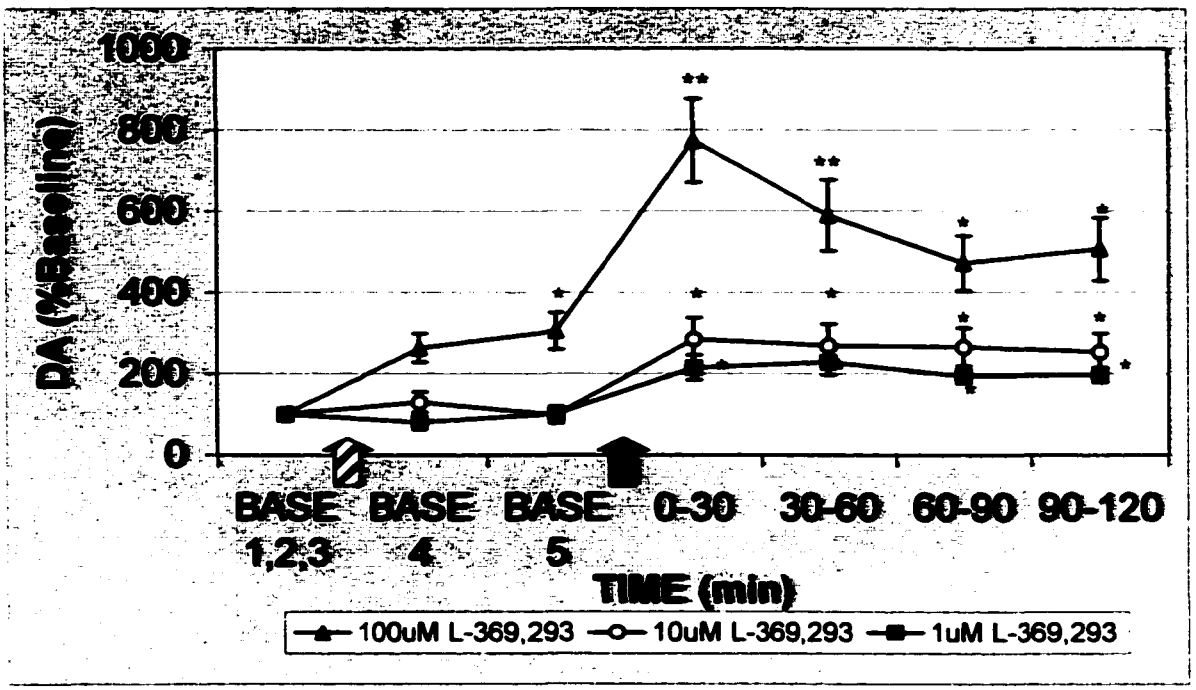
CCK-A receptor antagonist L-364,769 dose response on caudate-putamen extracellular dopamine levels in early withdrawal animals. L-364,769 was administered continuously after baseline measurements into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM, n= 7; 10μM, n = 6; and 1μM, n = 7. Cocaine was administered i.p. (10mg/kg) 1 hour after the start of the L-364,769 infusion, and is represented as the solid arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the administration of L-364,769. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: 100μM, $F = 2.956$, $P < 0.05$; 10μM, $F = 2.174$, $P > 0.05$; 1μM, $F = 1.219$ $P > 0.05$.

3.2.3 CCK-B Receptor Antagonist L-369,293 Dose Response

Again, three different doses of the selective CCK-B receptor antagonist L-369,293 were infused into the caudate-putamen of animals in early withdrawal and their effects on basal and cocaine-evoked dopamine release was measured. We used the early

withdrawal group again hypothesizing that by blocking CCK-B receptors, the depleted extracellular dopamine levels observed on day 11 would be elevated, as was the results with the CCK-A antagonist L-364,769. The highest concentration (100μM) elevated pre-cocaine basal dopamine levels by an average of 284% (Fig. 14), with a significant maximum increase of 305%. However, the most robust effect was observed in post-cocaine dopamine measurements. Here, L-369,293 elevated cocaine-evoked dopamine levels by as much as 774%, with an average increase of 586%. All cocaine-evoked dopamine levels were significant when administered with L-369,293. The 10μM concentration also resulted in considerable dopamine increases. Pre-cocaine basal dopamine levels were not increased by 10μM L-369,293 alone, with a dopamine average of 115% compared to baseline. The 10μM L-369,293 dose significantly increased cocaine-evoked dopamine levels to an average of 268%, however to a much less effect when compared to 100μM. The lowest dose of 1μM did not have an effect on basal dopamine levels, but did significantly increase cocaine-evoked dopamine levels to maximum of 229%, with an average of 208%.

Fig. 14 CCK-B Receptor Antagonist L-369,293 Dose Response



CCK-B receptor antagonist L-369,293 dose response on caudate-putamen extracellular dopamine levels in early withdrawal animals. L-369,293 was administered continuously after baseline measurements into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM, n= 8; 10μM, n = 7; and 1μM, n = 7. Cocaine was administered i.p. (10mg/kg) 1 hour after the start of the L-369,293 infusion, and is represented as the solid arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the administration of L-369,293. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$; ** = $p < 0.0001$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: 100μM, $F = 7.240$, $P < 0.0001$; 10μM, $F = 4.705$, $P < 0.05$; 1μM, $F = 9.406$ $P < 0.0001$.

3.3 Acute Cocaine Groups

3.3.1 Effects of CCK-8S in Acute Cocaine-Treated Animals

This study consisted of observing the effects of CCK-8S perfused into the caudate-putamen of naïve animals, or animals that have not had any previous cocaine exposure. The concentration of 100μM CCK-8S was administered because of the results obtained in the dose response study, in which the highest dose of CCK-8S prevented cocaine-

evoked extracellular dopamine of significantly reaching elevated levels and also decreased basal dopamine levels. In naïve animals, 100μM CCK-8S had the opposite effect on pre-cocaine basal dopamine levels than that found in the early withdrawal group. CCK-8S-evoked basal dopamine levels were significantly increased to 156%. Cocaine-evoked dopamine levels were significantly elevated above baseline and the CCK-8S-evoked basal dopamine levels (baseline 5) at an average of 210%.

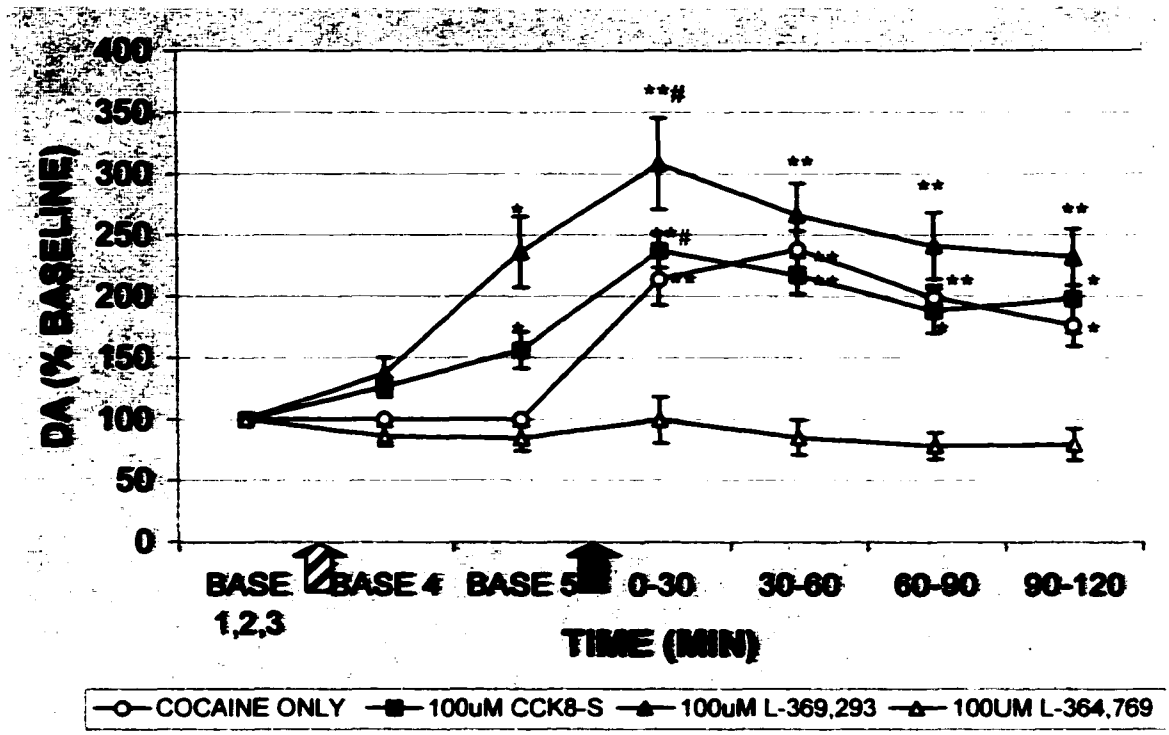
3.3.2 Effects of CCK-A Antagonist L-364,769 in Acute Cocaine-Treated Animals

The concentration of 100μM L-364,769 was used because it had the most robust effect on cocaine-evoked extracellular dopamine in early withdrawal animals. In naïve animals, 100μM L-364,769 had an inhibitory effect on basal dopamine levels, which were decreased to 84% of baseline (Fig. 15). The increased cocaine-evoked dopamine levels previously observed in naïve animals (207%) were blocked completely with L-364,769, with the average extracellular dopamine measurements after cocaine administration falling below baseline (84%).

3.3.3 Effects of CCK-B Antagonist L-369,293 in Acute Cocaine-Treated Animals

The concentration of 100μM L-369,293 was used because it had the most robust effect on cocaine-evoked extracellular dopamine in early withdrawal animals. In naïve animals, 100μM L-369,293 significantly increased dopamine levels above baseline to 236% (Fig. 15). Extracellular dopamine levels after cocaine administration were significantly increased with a maximum increase of 308%, which was also significantly higher than L-369,293-evoked basal dopamine levels (baseline 5).

Fig. 15 Acute Cocaine Groups

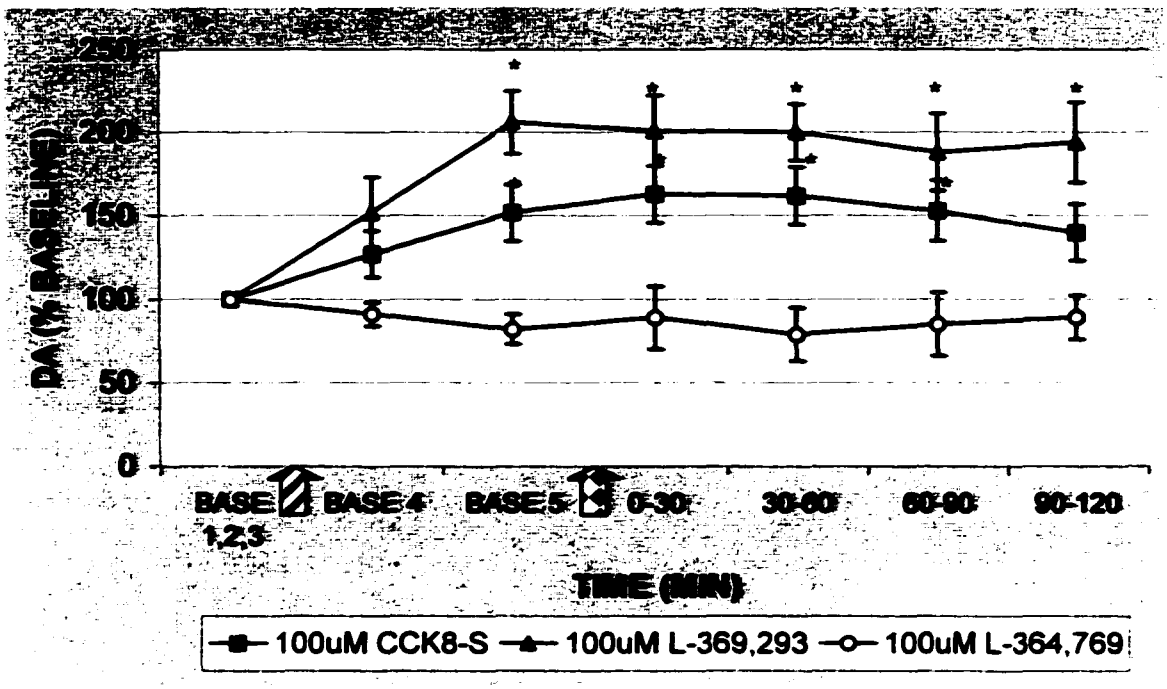


The effects of acute cocaine only, CCK-8S, CCK-A receptor antagonist L-364,769, and CCK-B receptor antagonist L-369,293 on cocaine-evoked caudate-putamen extracellular dopamine levels in naïve animals. All agonist/antagonist were administered continuously after baseline measurements (except cocaine only group) into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM. The number of animals used were as follows: COCAINE ONLY, N = 6; CCK-8S, N = 6; L-364,769, N = 6, L-369,293, N = 10. Cocaine was administered i.p. (10mg/kg) 1 hour after the start of the intracranial drug infusion, and is represented as the solid arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the perfusion of drug. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$; ** = $p < 0.0001$; # = significant difference from baseline 5 of at least $p < 0.05$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: cocaine only, $F = 13.225$, $P < 0.0001$; CCK-8S, $F = 9.788$, $P < 0.0001$; L-364,769, $F = 0.533$ $P > 0.05$; L-369,293, $F = 8.603$ $P < 0.0001$.

3.4 Acute Control Groups

In agonist and antagonist control group studies, animals receive their normal cocaine treatment paradigms, but are administered saline on the last day or microdialysis sampling day. In this situation, naïve animals without prior cocaine administration were infused with an agonist or antagonist throughout the experiment, after three baseline measurements, and received saline instead of cocaine. The control group studies are necessitated by the fact that the perfused agonist/antagonist usually display a trend in extracellular dopamine measurements during the first hour before cocaine administration. This study allowed us to observe if the dopaminergic trend caused by the infused drug continued beyond one hour. It also allowed us to observe if the extracellular dopamine change (if any) after cocaine administration were due to the cocaine and the infused drug, and not the infused drug alone. In the acute control group for CCK-8S (Fig. 16), the effects of 100μM CCK-8S on extracellular dopamine peaked at 162% during the 2nd hour and was not significantly different than9 the effect produced during the first hour of perfusion (152%). In the acute control group for L-364,769, the inhibitory effects on dopamine did not significantly change after one hour of administration. In the 1st hour, the largest decrease in dopamine was 82% and only had a maximum decrease of 79% during the rest of the experiment. The effects of L-369,293 displayed an increase in extracellular dopamine of 206% and averaged 195% throughout the rest of the experiment. In all drug groups, there was not any further change in extracellular dopamine after the first hour of administration.

Fig. 16 Acute Control Groups



The effects of CCK-8S, CCK-A receptor antagonist L-364,769, and CCK-B receptor antagonist L-369,293 on caudate-putamen extracellular dopamine levels in naïve animals. All agonist/antagonist were administered continuously after baseline measurements into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM. The number of animals used were as follows: CCK-8S, N = 6; L-364,769, N = 6, L-369,293, N = 6. Saline was administered i.p. 1 hour after the start of the intracranial drug infusion, and is represented as the checkered arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the perfusion of drug. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: CCK-8S, $F = 2.090$, $P > 0.05$; L-364,769, $F = 0.651$ $P > 0.05$; L-369,293, $F = 3.987$ $P < 0.05$.

3.5 Chronic Cocaine Groups

3.5.1 Effects of CCK-8S in Chronic Cocaine-Treated Animals

When CCK-8S (100μM) was perfused into the caudate-putamen of chronically treated animals it caused pre-cocaine basal dopamine levels to significantly increase up to 150% and had an average increase of 132% during the first hour of perfusion (Fig. 17).

When the animal was given cocaine, the dopamine levels increased by as much as 187%, with an average of 177%, however there was not a significant difference between post-cocaine dopamine levels and pre-cocaine dopamine levels (baseline 5).

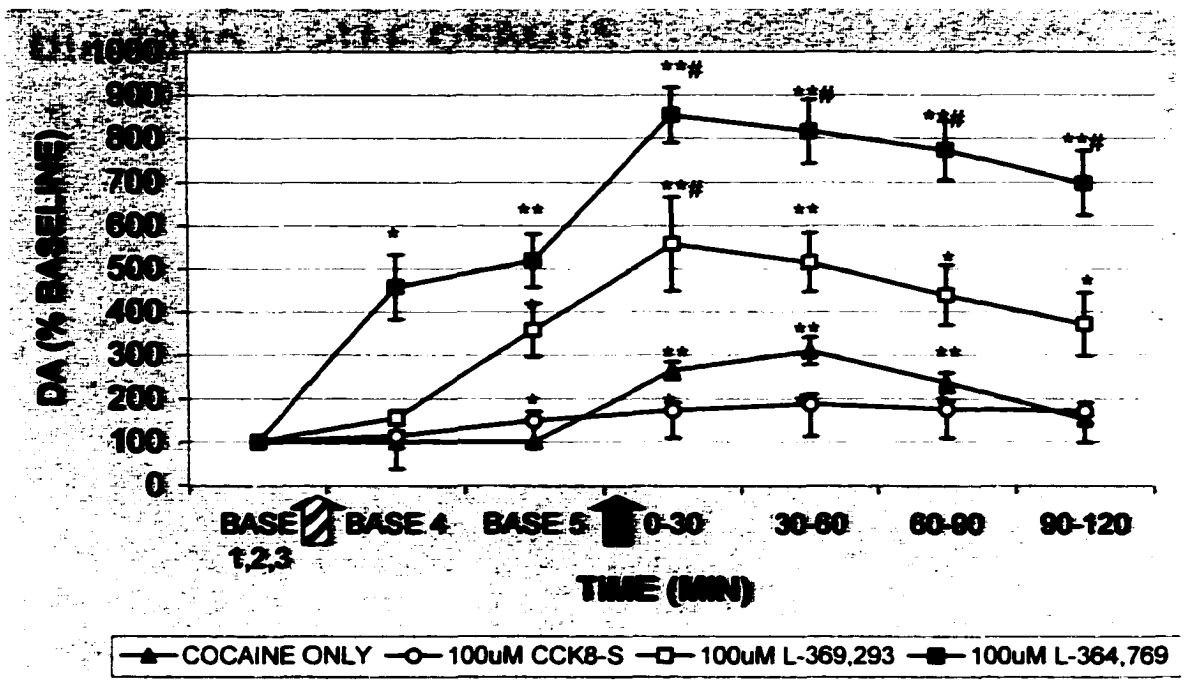
3.5.2 Effects of CCK-A Antagonist L-364,769 in Chronic Cocaine-Treated Animals

In chronic animals, 100μM L-364,769 had a robust effect on basal dopamine levels, significantly increasing them to as much as 519% of baseline, with an average of 488% (Fig. 17). When cocaine was administered, the elevated basal values increased further to a peak measurement of 854%, with an average post-cocaine increase in extracellular dopamine of 785%. All post-cocaine dopamine levels were significantly different from baseline 5. Interestingly, the last measurement taken still had elevated dopamine levels of 697% of baseline. These results are in total contrast to the effects observed in acute (naïve) animals when administered with L-364,769.

3.5.3 Effects of CCK-B Antagonist L-369,293 in Chronic Cocaine-Treated Animals

As in the acute cocaine group, L-369,393 significantly increased dopamine levels in chronic cocaine animals. The pre-cocaine basal dopamine levels reached 358%, with an average increase of 257% (Fig. 17). Following a cocaine injection, extracellular dopamine levels increased to 557%, with an average increase of 470% of baseline. There was a significant increase in dopamine in the first post-cocaine sample when compared to baseline 5.

Fig. 17 Chronic Cocaine Groups



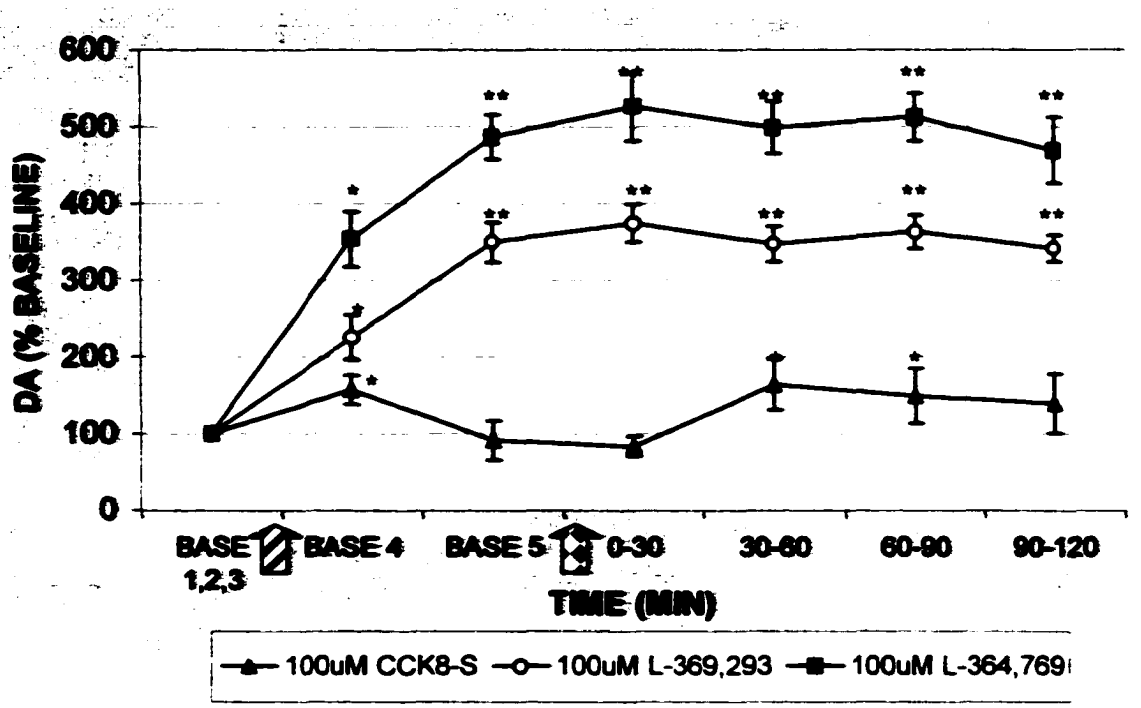
The effects of chronic cocaine only, CCK-8S, CCK-A receptor antagonist L-364,769, and CCK-B receptor antagonist L-369,293 on cocaine-evoked caudate-putamen extracellular dopamine levels in chronic animals. All agonist/antagonist were administered continuously after baseline measurements (except cocaine only group) into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM. The number of animals used were as follows: COCAINE ONLY, N = 8; CCK-8S, N = 6; L-364,769, N = 8, L-369,293, N = 7. Cocaine was administered i.p. (10mg/kg) 1 hour after the start of the intracranial drug infusion, and is represented as the solid arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the perfusion of drug. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$; ** = $p < 0.0001$; # = significant difference from basline 5 of at least $p < 0.05$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: cocaine only, $F = 18.185$, $P < 0.0001$; CCK-8S, $F = 4.075$, $P < 0.05$; L-364,769, $F = 17.217$ $P < 0.0001$; L-369,293, $F = 6.823$ $P < 0.0001$.

3.6 Chronic Control Groups

Chronic control animals received cocaine once a day for six days and on the 7th day when brain dialysate is collected, the animals receive saline while being perfused with 100μM agonist or antagonist drug. In the chronic control group for CCK-8S (Fig. 18), the effects of 100μM CCK-8S on extracellular dopamine peaked at 164%, which was not

statistically significant from the first 30 min. measurement of 157%. The chronic control group for L-364,769 displayed a maximum increase in dopamine levels of 526% during the third 30 min. sample, which was not significant from the first hour peak measurement of 487%. The effects of L-369,293 displayed a maximum increase in extracellular dopamine of 374% during the third 30 min. sample, which was not significantly different from the first hour sample peak of 349%.

Fig. 18 Chronic Control Groups



The effects of CCK-8S, CCK-A receptor antagonist L-364,769, and CCK-B receptor antagonist L-369,293 on caudate-putamen extracellular dopamine levels in chronic animals. All agonist/antagonist were administered continuously after baseline measurements into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM. The number of animals used were as follows: CCK-8S, N = 6; L-364,769, N = 6. L-369,293, N = 6. Saline was administered i.p. 1 hour after the start of the intracranial drug infusion, and is represented as the checkered arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the perfusion of drug. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$; ** = $p < 0.0001$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: CCK-8S, $F = 2.861$, $P < 0.05$; L-364,769, $F = 21.583$ $P < 0.001$; L-369,293, $F = 24.372$ $P < 0.0001$.

3.7 Early Withdrawal Cocaine Groups

The data from this study was obtained from the previous dose response experiments. Each agonist/antagonist group represented in the graph (Fig. 18) used the optimal dose of perfused drug found in each study, which happened to be 100μM for all groups. In the graph (Fig. 19), we also compared perfusion drug animal groups with animals that received cocaine only on day 11.

3.7.1 Effects of CCK-8S in Early Withdrawal Cocaine-Treated Animals

CCK-8S (100μM) decreased pre-cocaine basal levels to 64%, which were then elevated to a maximum increase of 126% following cocaine administration. The post-cocaine elevation peak of 126% was not significant when compared to the average baseline, however when compared to the CCK-8S-evoked basal dopamine decrease of 64%, it was statistically significant (Fig. 19).

3.7.2 Effects of CCK-A Antagonist L-364,769 in Early Withdrawal Cocaine-Treated Animals

CCK-A selective antagonist L-364,769 (100μM) significantly increased pre-cocaine basal extracellular dopamine levels by as much as 310% (Fig. 19). Following cocaine administration, L-364,769 had a maximum increase in dopamine of 425% during the first time point after the cocaine injection, and dopamine was significantly elevated above baseline in each of the four 30 min. measurements after cocaine treatment. The maximum post-cocaine dopamine peak of 425% was not statistically significant from baseline 5.

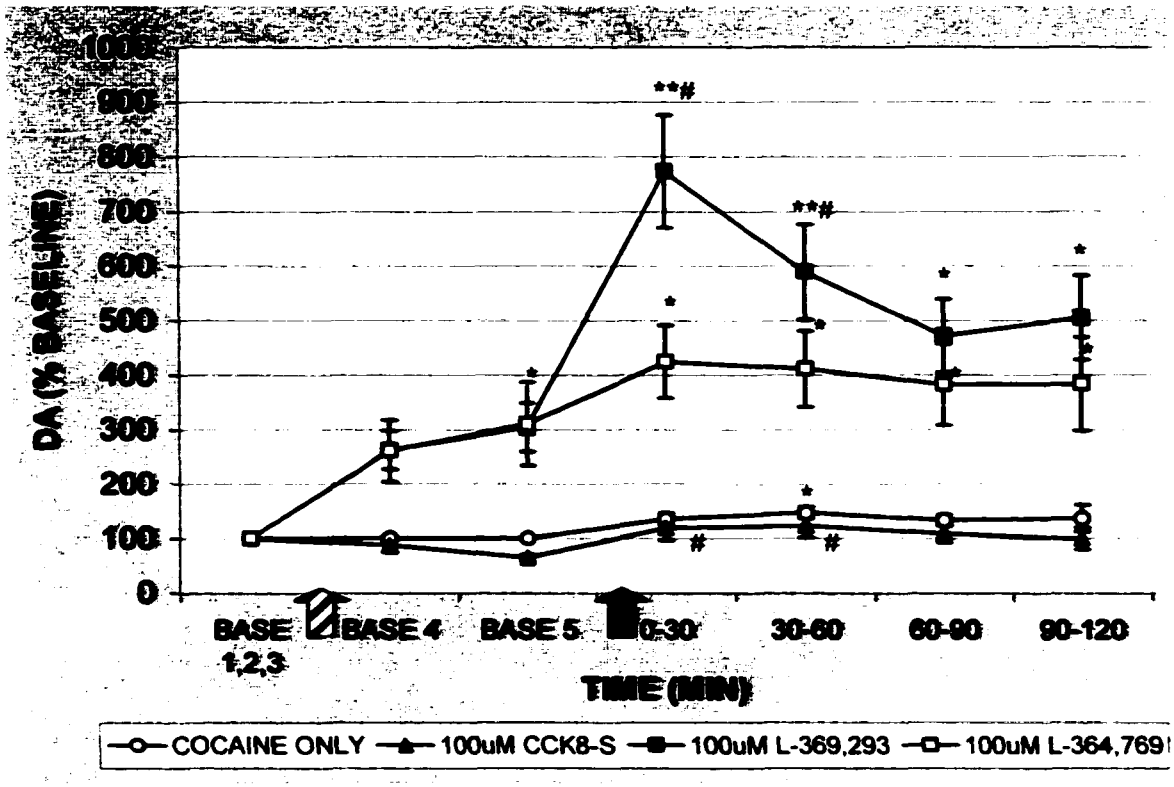
3.7.3 Effects of CCK-B Antagonist L-369,293 in Early Withdrawal Cocaine-Treated Animals

CCK-B selective antagonist L-369,293 (100μM) significantly elevated pre-cocaine dopamine levels by 305% (Fig. 19). Following cocaine administration, there was a robust increase in dopamine levels to 774%, with an average post-cocaine dopamine increase of 586%. All post-cocaine dopamine elevations were statistically significant when compared to the average baseline. The first two post-cocaine time points were statistically significant when compared to baseline 5.

3.8 Early Withdrawal Control Groups

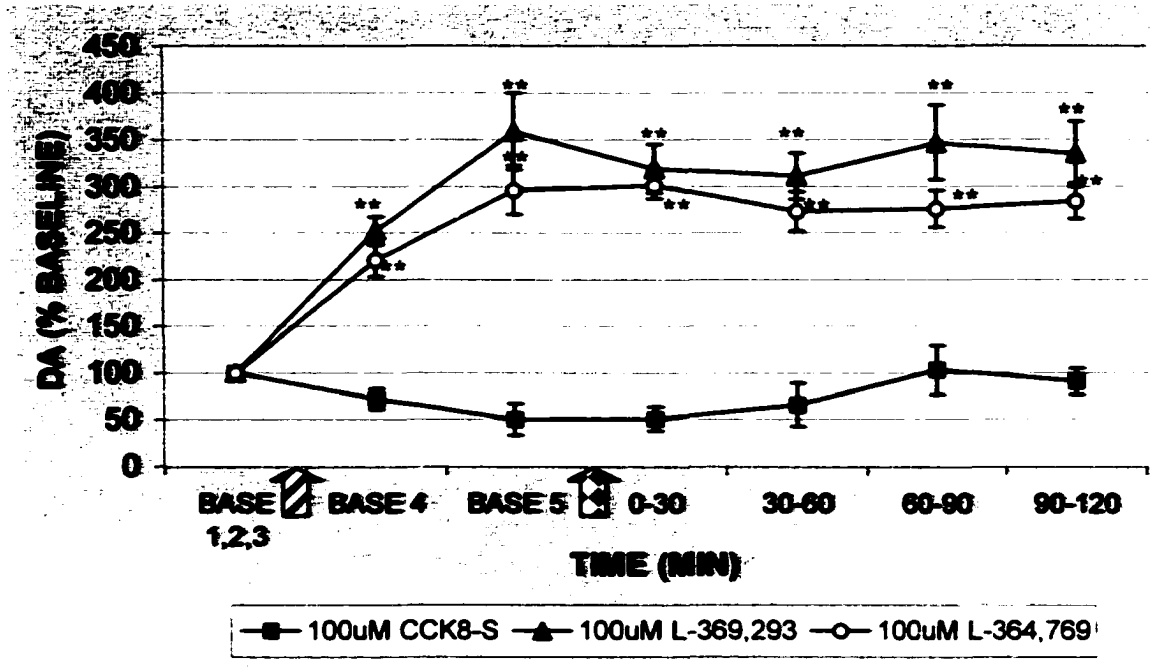
Early withdrawal control animals received cocaine once a day for seven days, had three days without cocaine and on the 11^{th} day, when brain dialysate is collected, the animals received saline while being perfused with 100μM agonist or antagonist drug. In the early withdrawal control group for CCK-8S (Fig. 20), the maximum inhibitory effects of 100μM CCK-8S on extracellular dopamine were observed during the first hour of perfusion, with dopamine levels shifting back to baseline. The early withdrawal control group for L-364,769 displayed a maximum increase in dopamine levels of 301% during the third 30 min. sample, which was not significant from the first hour peak measurement of 296%. The effects of L-369,293 displayed a maximum increase in extracellular dopamine of 359% during the second 30 min. sample, which was not significantly different from dopamine levels measured at later times.

Fig. 19 Early Withdrawal Cocaine Groups



The effects of the early withdrawal cocaine paradigm only, CCK-8S, CCK-A receptor antagonist L-364,769, and CCK-B receptor antagonist L-369,293 on cocaine-evoked caudate-putamen extracellular dopamine levels in early withdrawal animals. All agonist/antagonist were administered continuously after baseline measurements (except cocaine only group) into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM. The number of animals used were as follows: COCAINE ONLY, N = 6; CCK-8S, N = 7; L-364,769, N = 7, L-369,293, N = 8. Cocaine was administered i.p. (10mg/kg) 1 hour after the start of the intracranial drug infusion, and is represented as the solid arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the perfusion of drug. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$; ** = $p < 0.0001$; # = significant difference from basline 5 of at least $p < 0.05$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: cocaine only, $F = 1.800$, $P > 0.05$; CCK-8S, $F = 1.908$, $P > 0.05$; L-364,769, $F = 2.956$, $P < 0.05$; L-369,293, $F = 7.240$ $P < 0.0001$.

Fig. 20 Early Withdrawal Control Groups



The effects of CCK-8S, CCK-A receptor antagonist L-364,769, and CCK-B receptor antagonist L-369,293 on caudate-putamen extracellular dopamine levels in early withdrawal animals. All agonist/antagonist were administered continuously after baseline measurements into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM. The number of animals used were as follows: CCK-8S, N = 6; L-364,769, N = 6, L-369,293, N = 6. Saline was administered i.p. 1 hour after the start of the intracranial drug infusion, and is represented as the checkered arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the perfusion of drug. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$; ** = $p < 0.0001$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: CCK-8S, $F = 0.696$, $P > 0.05$; L-364,769, $F = 14.712$ $P < 0.0001$; L-369,293, $F = 9.529$ $P < 0.0001$.

3.9 Calcium Channel Blocker Studies

During chronic and early withdrawal cocaine and CCK agonist/antagonist administration studies, both the CCK-A receptor antagonist L-364,769 and the CCK-B receptor antagonist L-369,293, increased dopamine levels before and after cocaine treatment. To begin to understand the physiological mechanism of action, it is necessary to investigate whether the CCK receptor antagonist-evoked dopamine release is mediated by calcium-dependent mechanism. In past studies, dopamine release has been shown to be calcium-dependent, however, at the present time, there are no published studies using L-364,769 or L-369,293 involving its modulation of dopamine. The experimental design of these studies were the same as the agonist/antagonist perfusion studies previously discussed, except that the L-type calcium channel blocker Verapamil was infused (100μM) with either L-364,769 or L-369,293 during microdialysis. The L-type calcium blocker was chosen as a result of its use in previous studies investigating dopamine release.

3.9.1 Effects of Verapamil on L-364,769-Evoked Dopamine Release

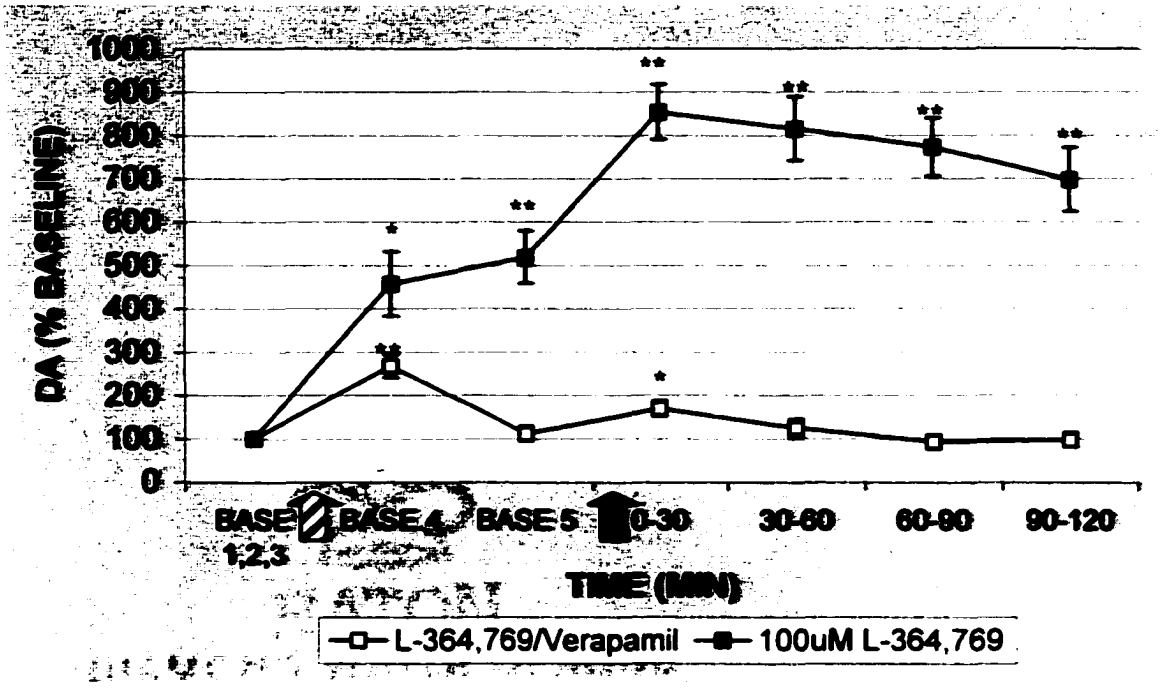
This study was carried out in the chronic cocaine group because of the robust increases in dopamine observed when L-364,769 was perfused into the caudate-putamen. If the L-364,769-evoked increases in extracellular dopamine levels are mediated by a calcium-dependent mechanism, verapamil should significantly block the elevations of dopamine previously observed in chronic animals by L-364,769 perfusion. During the first 30 min. (baseline 4) interval, there was a significant elevation of dopamine to 268% in response to the co-perfusion of L-364,769/Verapamil, however this level decreased

back towards baseline levels (112%) during the next 30 min. interval (baseline 5). Dopamine levels were then significantly elevated to 170% in response to cocaine administration, but were quickly decreased back to baseline levels (124%, 92%, 97%; respectfully) during the rest of the study.

3.9.2 Effects of Verapamil on L-369,293-Evoked Dopamine Release

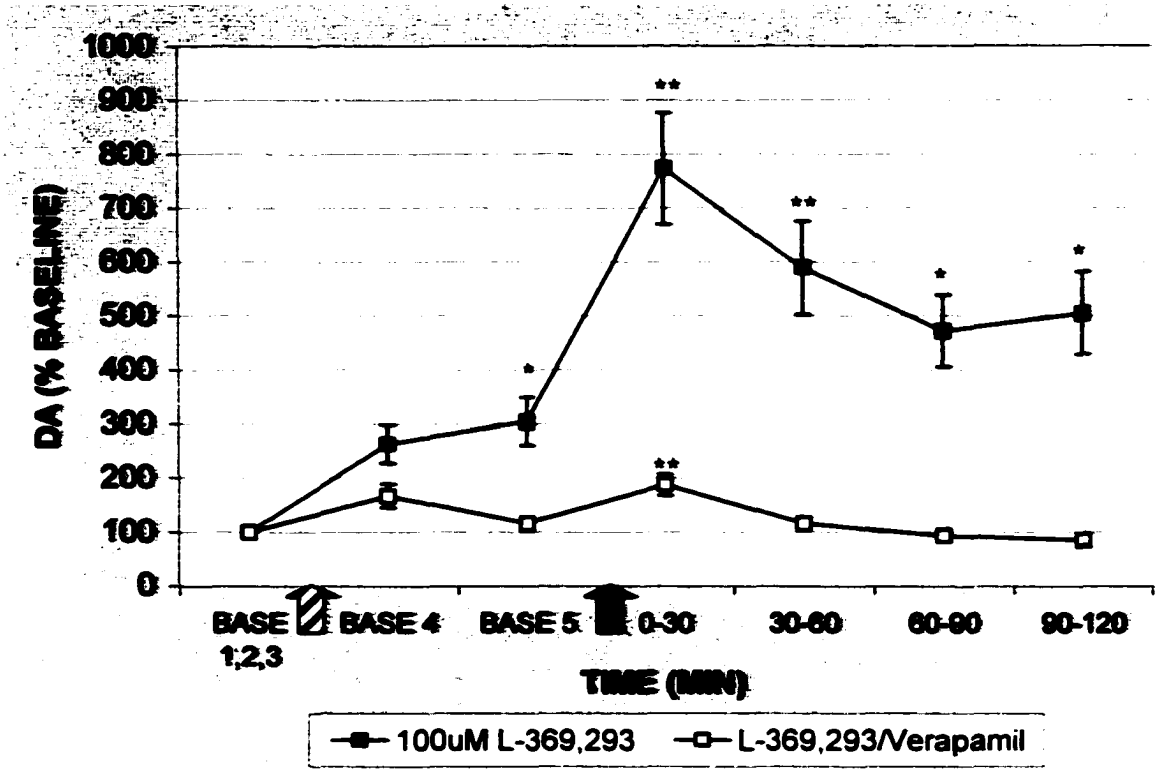
This study was carried out in the early withdrawal cocaine group because L-364,769 had the largest facilitory effects on extracellular dopamine in this group. Again, if the L-369,293-evoked increases in extracellular dopamine levels are mediated by a calcium-dependent mechanism, verapamil should significantly block the elevations of dopamine previously observed in early withdrawal animals by L-369,293 perfusion. When verapamil was co-perfused with L-369,293, dopamine levels were significantly elevated to 167% during the first 30 min. (baseline 4), however, dopamine levels decreased to 115% during the second 30 min. interval (baseline 5). Cocaine significantly elevated dopamine levels to 186%, but again they were quickly decreased to 116% during the next 30 min. interval and continued to drop below baseline levels to 84%.

Fig. 21 L-364,769 and Calcium Blocker Administration in Chronic Animals



The effects of CCK-A receptor antagonist L-364,769, and L-364,769 with the L-type calcium channel blocker verapamil on cocaine-evoked caudate-putamen extracellular dopamine levels in chronic animals. L-364,769 with Verapamil was administered continuously after baseline measurements into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM. The number of animals used were as follows: L-364,769, N = 8, L-364,769/Verapamil, N = 8. Cocaine was administered i.p. (10mg/kg) 1 hour after the start of the intracranial drug infusion, and is represented as the solid arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the perfusion of drug. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$; ** = $p < 0.0001$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: L-364,769, $F = 17.217$ $P < 0.0001$; L-364,769/Verapamil, $F = 12.004$ $P < 0.0001$.

Fig. 22 L-369,293 and Calcium Blocker Administration in Early Withdrawal Animals



The effects of CCK-B receptor antagonist L-369,293, and L-369,293 with the L-type calcium channel blocker verapamil on cocaine-evoked caudate-putamen extracellular dopamine levels in early withdrawal animals. L-369,293 with Verapamil was administered continuously after baseline measurements into the caudate-putamen through the microdialysis probe, represented as the striped arrow, at concentrations of 100μM. The number of animals used were as follows: L-369,293, N = 8, L-364,769/Verapamil, N = 6. Cocaine was administered i.p. (10mg/kg) 1 hour after the start of the intracranial drug infusion, and is represented as the solid arrow. The data were normalized by dividing all values by the average of the three baseline measurements made before the perfusion of drug. The data are shown as mean ± SEM percentage change of dopamine where * = $p < 0.05$; ** = $p < 0.0001$. All data were analyzed using a two-way ANOVA with repeated measures over time followed by Fisher's PLSD. F scores for each group were as follows: L-369,293, $F = 7.240$ $P < 0.0001$; L-364,769/Verapamil, $F = 8.768$ $P < 0.0001$.

3.10 Area Under the Curve Analysis (AUCA)

These studies consist of the data obtained in all groups during acute, chronic and early withdrawal cocaine administration paradigms. The area under the curve data was determined by adding all dopamine peak values in each animal using the four consecutive post-cocaine 30-min. samples for a total of 2 hours. The statistical analysis performed previously in each group included all dopamine levels being compared to baseline and all post-cocaine dopamine levels being compared to pre-cocaine agonist/antagonist-evoked dopamine levels. However, what is equally important and necessary is to study and compare with each other the overall effect of each perfused drug on cocaine-evoked dopamine release and also compare these effects to groups that received cocaine only. The area under the curve analysis allows a comprehensible and logical way for the study of these group comparisons.

3.10.1 AUCA of Acute Cocaine Groups

In the acute cocaine group paradigm, CCK-8S perfused into the caudate-putamen produced no overall effect on cocaine-evoked extracellular dopamine levels (Fig. 23). When perfused with CCK-A receptor antagonist L-364,769, extracellular dopamine levels were significantly decreased when compared to cocaine only and CCK-8S groups. The CCK-B receptor antagonist L-369,293 significantly elevated cocaine-evoked extracellular dopamine levels compared to cocaine only and L-364,769 group over the 2 hour period.

3.10.2 AUCA of Chronic Cocaine Groups

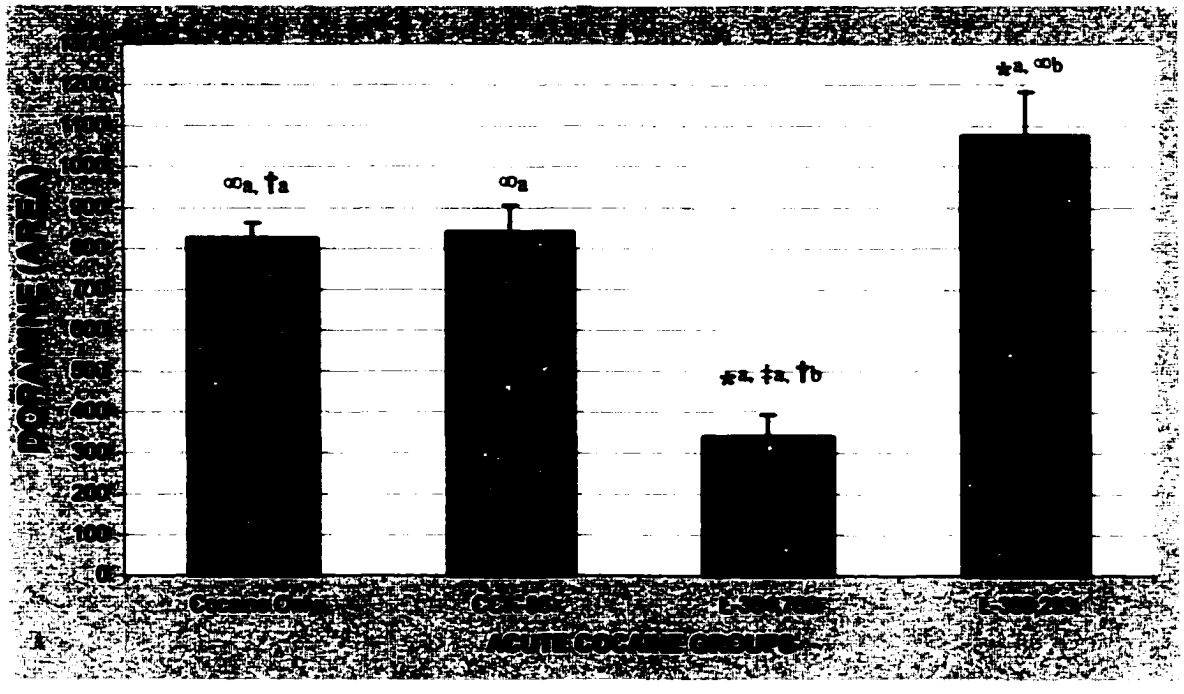
In animals treated chronically with cocaine, perfused CCK-8S did not produce a significant change in extracellular dopamine levels when compared to the cocaine only group, however there was significant difference when compared to L-364,769 and L-369,293 groups (Fig. 23). As discovered earlier, L-364,769 administration into the caudate-putamen caused a robust increase in extracellular dopamine when compared to baseline, and in group comparisons, L-364,769-elevated cocaine-evoked dopamine levels were significantly larger than the cocaine-only group as well as all other chronic groups. L-369,293 also produced an overall significant increase in cocaine-evoked extracellular dopamine, however the increase was roughly half the effect of L-364,769 over two hours post-cocaine administration. When the L-type calcium channel blocker Verapamil was co-perfused with L-364,769, the robust dopamine increases were fully blocked and decreased to below cocaine only-evoked dopamine levels (Fig. 23), suggesting the increased dopamine levels induced by L-364,769 were mediated by a calcium-dependent mechanism.

3.10.3 AUCA of Early Withdrawal Cocaine Groups

As discussed earlier, extracellular dopamine levels were depleted in animals that were in early cocaine withdrawal. When perfused with CCK-8S, these levels were decreased even further when compared to baseline, but the overall effect was not significantly different from cocaine only groups (Fig. 24). L-364,769 significantly elevated dopamine levels during 2 hours post-cocaine treatment compared to the group that received cocaine only. When perfused with L-369,293, cocaine-evoked extracellular levels were nearly 4

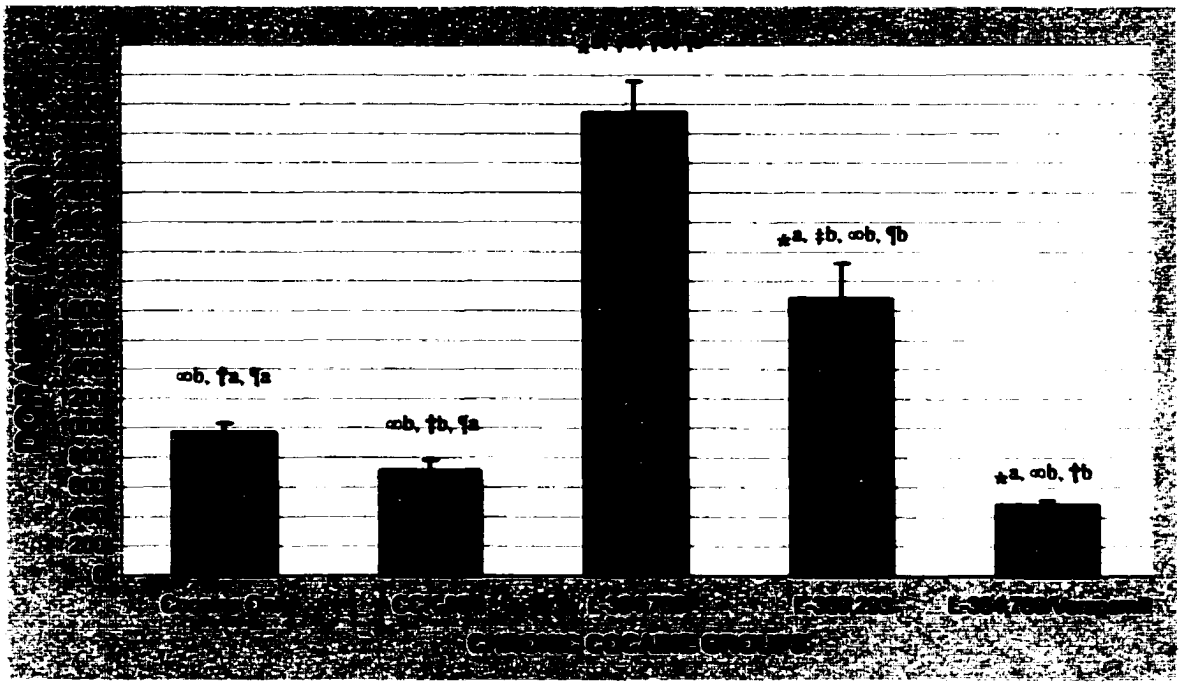
½ times larger during 120-min. after injection of cocaine. The robust effect of L-369,293 was blocked when Verapamil was co-perfused into the caudate-putamen.

Fig. 23 Area Under the Curve Analysis for Acute Cocaine Groups



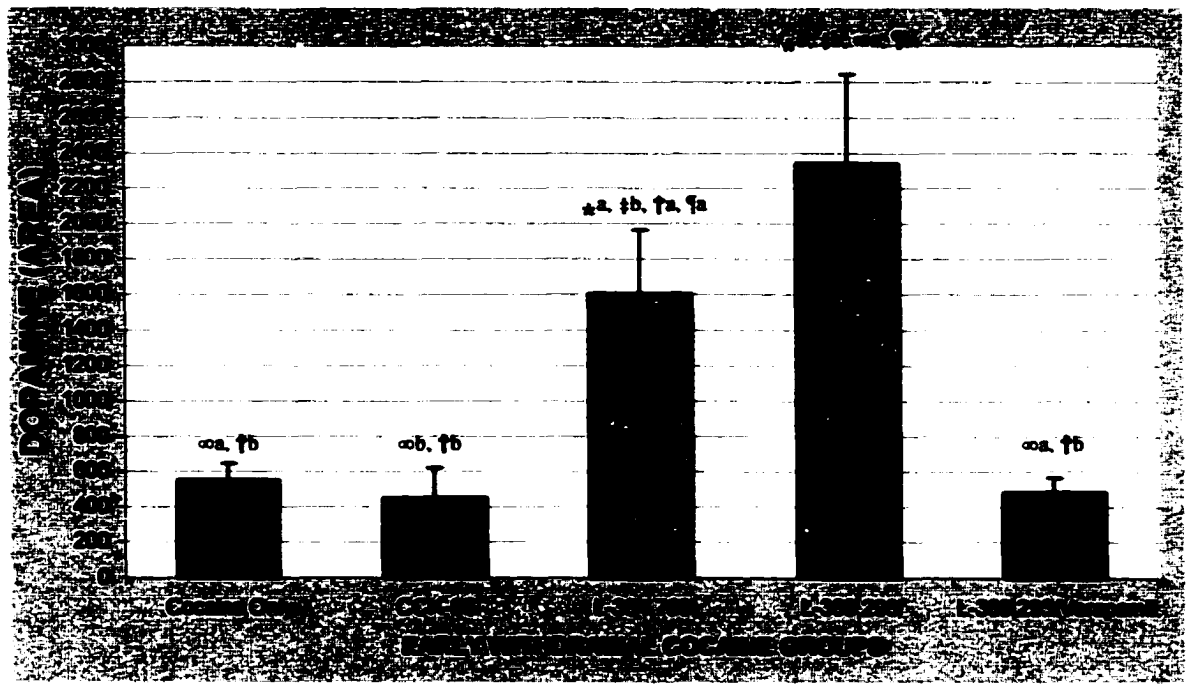
Area under the curve for extracellular dopamine during the first 120 min after injection of cocaine. Data are from acute groups that were administered cocaine only and from groups that were administered cocaine and perfused with CCK-8S, CCK-A receptor antagonist L-364,769, and CCK-B receptor antagonist L-369,293 into the caudate-putamen. Comparisons were made between each and all groups. Each group is represented by the following number of animals: cocaine only, n = 6; CCK-8S, n = 6; L-364,769, n = 6, L-369,293, n = 10. The data was calculated by adding all consecutive post-cocaine dopamine peak values for each animal and their mean was represented as a group dopamine area, where * = significantly different from cocaine only; ‡ = significantly different from CCK-8S (100μM); ∞ = significantly different from L-364,769 (100μM); † = significantly different from L-369,293 (100μM), where ^{a} = $p < 0.05$, ^{b} = $p < 0.0001$ using a one-way ANOVA followed by Fisher's PLSD for multiple comparisons. F scores for group analysis were, $F = 12.766$, $P < 0.0001$.

Fig. 24 Area Under the Curve Analysis for Chronic Cocaine Groups



Area under the curve for extracellular dopamine during the first 120 min after injection of cocaine. Data are from chronic groups that were administered cocaine only and from groups that were administered cocaine and perfused with CCK-8S, CCK-A receptor antagonist L-364,769, CCK-B receptor antagonist L-369,293, and L-364,769 co-perfused with Verapamil, an L-type calcium channel blocker, into the caudate-putamen. Comparisons were made between each and all groups. Each group is represented by the following number of animals: cocaine only, n = 8; CCK-8S, n = 6; L-364,769, n = 8, L-369,293, n = 7; L-364,769/Verapamil, n = 8. The data was calculated by adding all consecutive post-cocaine dopamine peak values for each animal and their mean was represented as a group dopamine area, where * = significantly different from cocaine only; ‡ = significantly different from CCK-8S (100μM); ∞ = significantly different from L-364,769 (100μM); † = significantly different from L-369,293 (100μM); ¶ = significantly different from L-364,769/Verapamil (100μM), where ^{a} = $p < 0.05$, ^{b} = $p < 0.0001$ using a one-way ANOVA followed by Fisher's PLSD for multiple comparisons. F scores for the group analysis were, $F = 53.985$, $P < 0.0001$.

Fig. 25 Area Under the Curve Analysis for Early Withdrawal Cocaine Groups



Area under the curve for extracellular dopamine during the first 120 min after injection of cocaine. Data are from early withdrawal groups that were administered cocaine only and from groups that were administered cocaine and perfused with CCK-8S, CCK-A receptor antagonist L-364,769, CCK-B receptor antagonist L-369,293, and L-360,293 co-perfused with Verapamil, an L-type calcium channel blocker, into the caudate-putamen. Comparisons were made between each and all groups. Each group is represented by the following number of animals: cocaine only, n = 6; CCK-8S, n = 7; L-364,769, n = 7, L-369,293, n = 8; L-364,769/Verapamil, n = 6. The data was calculated by adding all consecutive post-cocaine dopamine peak values for each animal and their mean was represented as a group dopamine area, where * = significantly different from cocaine only; ‡ = significantly different from CCK-8S (100μM); ∞ = significantly different from L-364,769 (100μM); † = significantly different from L-369,293 (100μM); ¶ = significantly different from L-364,769/Verapamil (100μM), where ^{a} = $p < 0.05$, ^{b} = $p < 0.0001$ using a one-way ANOVA followed by Fisher's PLSD for multiple comparisons. F scores for the group analysis was, $F = 21.563$, $P < 0.0001$.

3.11 Receptor Autoradiography Studies

3.11.1 Dopamine Transporter Autoradiography

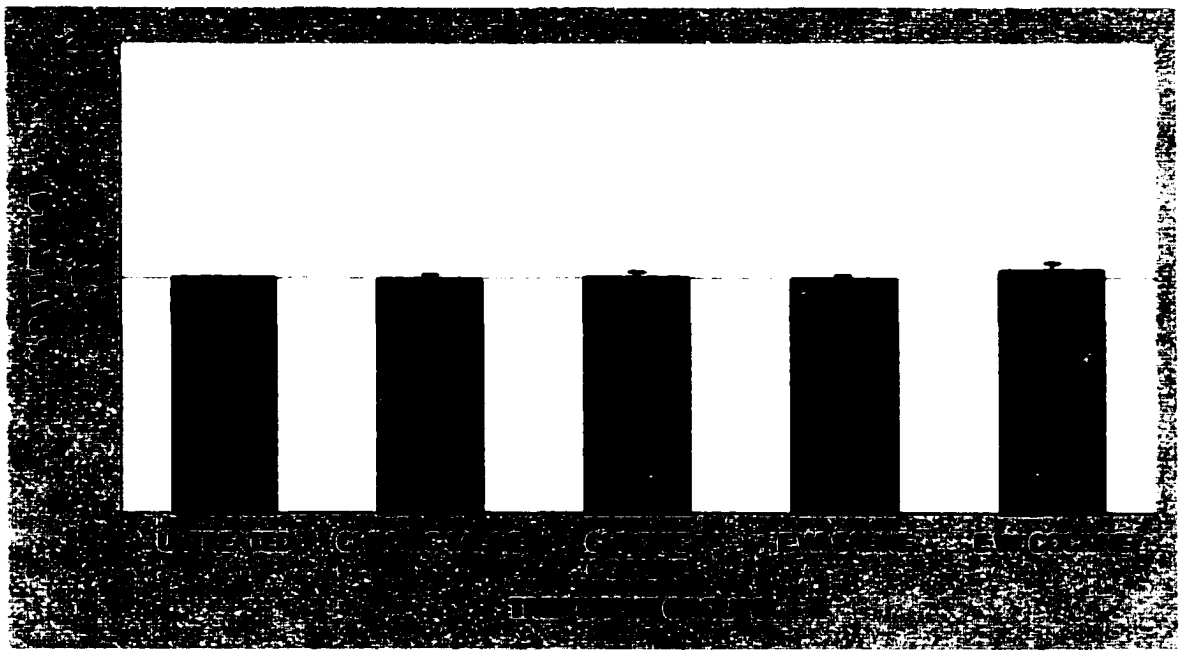
As reported earlier, cocaine-evoked extracellular dopamine levels in the caudate-putamen are decreased on day 11 (early withdrawal) when compared to day 7 (chronic) or day 1 (acute). A possible mechanism could involve an increase in dopamine transporter (DAT) sites during day 11, which would reduce extracellular dopamine levels. To assess this possibility, DAT sites in the caudate-putamen were labeled and quantified in untreated animals (acute), saline and cocaine groups, and saline and cocaine early withdrawal groups. The labeled DAT sites in acute and chronic groups were quantified as amount of specific activity per mg of tissue (nCi/mg) and were expressed as a percent change compared to the untreated group. DAT levels labeled with [^{125}I]RTI-121 did not change in any of the treatment groups (Fig.26). Specific activity levels in all groups were approximately 85nCi/mg.

3.11.2 CCK Receptor Autoradiography

Autoradiography was used to investigate if there were changes in CCK receptor binding during acute, chronic, and early withdrawal saline and cocaine treatment paradigms. There was no change in total CCK receptor binding in the two chronic groups and in the early withdrawal saline group (Fig.28) when compared to the untreated group. There was a significant elevation of 27% in total CCK receptor binding sites in the early withdrawal cocaine group when compared to the untreated group. When the CCK-A receptors were selectively blocked to allow selective labeling of the CCK-B receptor type, there was no significant difference between the two chronic groups and the early withdrawal saline group (Fig. 29). The early withdrawal group did display a small

increase in CCK-B receptors of 9% when compared to the untreated group, however it was not statistically significant and there was no observable binding difference between the two groups on film. When the CCK-B receptors were blocked, the gray regions of the caudate-putamen were around the levels of unspecific binding, so they could not be quantified.

Fig. 26 Dopamine Transporter Autoradiography
A.



B. Autoradiograph of DAT Sites

UNTREATED **CHRONIC COCAINE** **E.W. COCAINE**

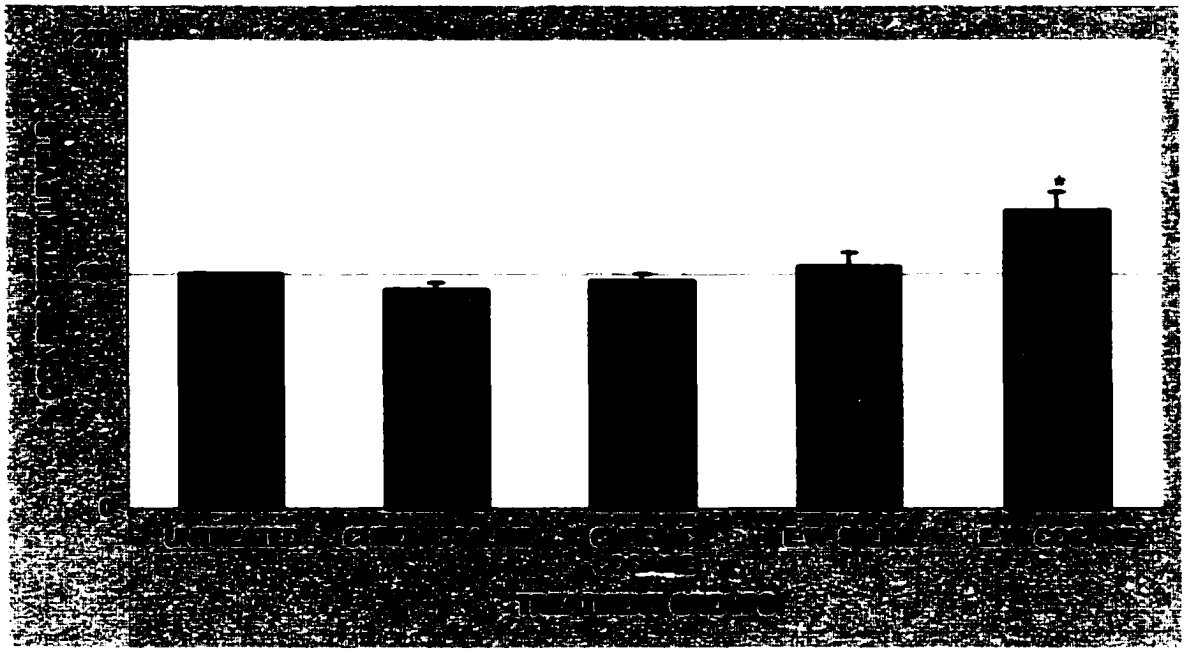
C.

UNTREATED	CHRONIC SALINE	CHRONIC COCAINE	E.W. SALINE	E.W.COCAINE
87.4 nCi/mg	84.2 nCi/mg	87.4 nCi/mg	84.2 nCi/mg	97 nCi/mg

Figure 26A illustrates the labeled dopamine transporter sites in the caudate-putamen. The untreated group was normalized to 100% and the other groups were represented as a percent change compared to the untreated group. Each group was represented by n = 6, and there was no difference among groups. B. The total DAT binding in the caudate-putamen in each cocaine treatment group. C. Concentration of labeled DAT sites among groups in the caudate-putamen.

Fig. 27 CCK Receptor Autoradiography

A.



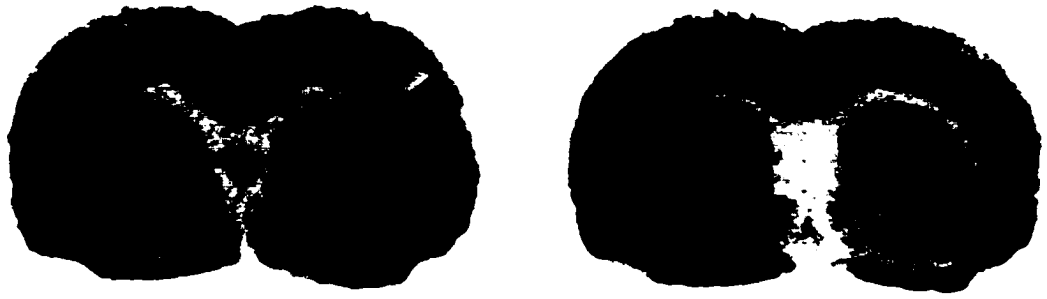
B.

UNTREATED	CHRONIC SALINE	CHRONIC COCAINE	E.W. SALINE	E.W.COCAINE
2.91 nCi/mg	2.53 nCi/mg	2.62 nCi/mg	3.07 nCi/mg	4.80 nCi/mg*

Figure 27A illustrates the labeled CCK receptor sites in the caudate-putamen. The untreated group was normalized to 100% and the other groups were represented as a percent change compared to the untreated group. Each group was represented by n = 6. where statistical significance is illustrated as * = $p < 0.05$. Fig. 27B shows the concentration of labeled CCK receptor sites among groups in the caudate-putamen.

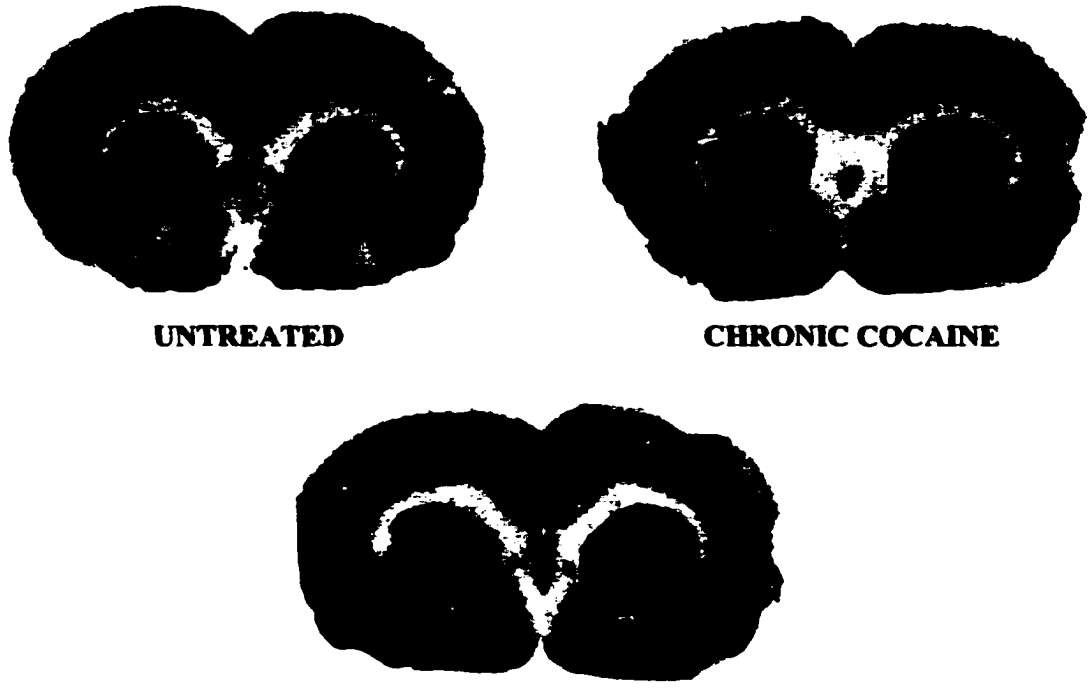
Fig. 28 Autoradiograph of CCK Receptors

A. CCK Receptor Total Binding



UNTREATED EARLY WITHDRAWAL COCAINE

B. CCK-B Receptor Binding

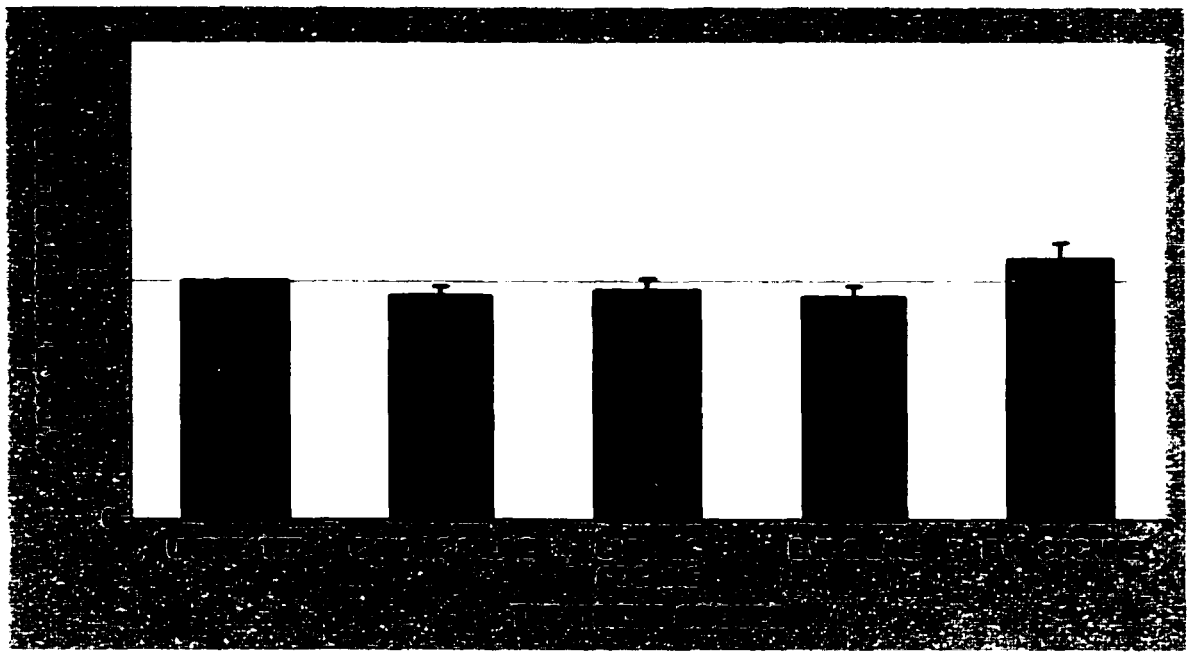


UNTREATED CHRONIC COCAINE

EARLY WITHDRAWAL COCAINE

A. & B. Autoradiographic images of rat brain slices viewing the caudate-putamen. A. The dark regions indicate CCK receptor binding sites. On the left, an untreated brain representing CCK-receptor binding in the acute (naïve) animal, and on the right, early withdrawal cocaine animals display a 27% increase in CCK receptor binding in the caudate-putamen. B. Dark regions indicate CCK-B receptor binding. There were no significant changes in binding found in chronic or early withdrawal groups when compared to the untreated group, however a minor increase in CCK-B receptor binding of 9% was found in early withdrawal cocaine animals (bottom).

Fig. 29 CCK-B Receptor Autoradiography
A.



B.

UNTREATED	CHRONIC SALINE	CHRONIC COCAINE	E.W. SALINE	E.W.COCAINE
2.57 nCi/mg	2.22 nCi/mg	2.33 nCi/mg	2.16 nCi/mg	2.96 nCi/mg

Figure 30 illustrates labeled CCK-B receptors in the caudate-putamen. The untreated group was normalized to 100% and the other groups were represented as a percent change compared to the untreated group. Each group was represented by n = 6, and there was no significant difference among groups. Fig. 30B shows the concentration of labeled CCK-B receptor sites among groups in the caudate-putamen.

Chapter 4

DISCUSSION

The present study has demonstrated that repeated daily cocaine injections augment the extracellular dopamine response observed in an acute cocaine treatment in naïve animals (Zhang et al., 2001). This is in agreement with studies examining dopamine overflow in response to repeated administration of cocaine and other psychostimulants (Kalivas et al., 1993; Kalivas et al., 1998). However, when a cocaine challenge is administered after 3 days of discontinuation of daily treatments, the augmented extracellular dopaminergic response observed in naïve and chronic animals is decreased (Zhang et al., 2001). This "tolerance"-type dopamine decrease was observed by others when using high (15mg/kg, 30mg/kg i.p.) doses of cocaine (Segal and Kuczenski, 1992; Kalivas and Duffy, 1993a). There are a number of possible mechanisms for the development of tolerance to the effects of cocaine on extracellular dopamine. One possibility is that repeated cocaine administration may reduce or deplete the concentration of intracellular releaseable dopamine in terminal field regions. The measurement of tissue levels of dopamine in other studies have failed to verify this hypothesis (Kalivas et al. 1988; Kleven et al., 1988). Likewise, we did not find a reduction in basal levels of extracellular dopamine in the early withdrawal period after repeated cocaine administration. Also, Kalivas and colleagues demonstrated that a challenge injection of methamphetamine after several days of withdrawal from repeated cocaine administration reversed the tolerance observed in dopamine levels after a cocaine challenge (Kalivas and Duffy, 1993a). This finding nullifies the dopamine depletion

hypothesis because methamphetamine releases dopamine by the mechanism of entering the neuron terminal and directly binding to the vesicle and causing release of dopamine. Another possible explanation for the apparent tolerance is that an increase in the dopamine uptake carrier or dopamine transporter would decrease the extracellular dopamine levels observed after withdrawal of repeated administration of cocaine. However, the DAT autoradiography data obtained in this study (Fig. 27), demonstrates that there was no change in labeled DAT sites among naïve, chronically treated, and early withdrawal cocaine groups, thus also weakens the dopamine depletion hypothesis.

These data support a different mechanism of modulating cocaine-evoked extracellular dopamine. We investigated a possible role of cholecystokinin in modulating basal and cocaine-evoked extracellular dopamine. Our hypothesis was that cholecystokinin, acting via CCK-A and/or CCK-B receptors, played a neuromodulatory role in the development of dopamine tolerance to chronic cocaine administration. When perfused with CCK-8S, the low tolerance levels of cocaine-evoked extracellular dopamine were additionally decreased. Interestingly, CCK also had an inhibitory effect on basal dopamine levels before cocaine administration. As a result of the equal-selectivity of CCK-8S for both CCK-A and CCK-B receptors, and the unstable nature of peptide molecules, highly selective non-peptide antagonists (L-364,769, CCK-A; L-369,293, CCK-B) were also perfused into the caudate-putamen in anticipation of obtaining more concrete data. Both L-364,769 and L-369,293 supported the CCK-8S data by reversing the tolerence observed in extracellular dopamine after withdrawal and robustly increasing extracellular dopamine levels up to 425% and 774%, respectively. Similar trends, but with lesser effects were observed in animals that received saline in

place of cocaine on day 11. The same perfusion experiments were conducted in chronic- and acute-cocaine treatment groups. In chronically treated animals, perfused CCK-8S slightly decreased cocaine-evoked extracellular dopamine concentrations, however L-364,769 and L-369,293 increased dopamine levels to as much as 854% and 557%, repectively. When cocaine was replaced with a challenge saline injection, both antagonists significantly elevated extracellular dopamine, but to a lesser degree than cocaine administration. Interestingly, CCK-A antagonism by L-364,769 inhibited while CCK-B antagonism by L-369,293 facilitated dopamine release in naïve animals. Perfused CCK-8S did not alter cocaine-evoked dopamine release, possibly as a result of it mediating opposite effects on extracellular dopamine via CCK-A and CCK-B receptors. Again, these same trends were observed in naïve animals that received saline in place of cocaine. In each study, the slight effects observed in CCK-8S compared to the effects evoked by the non-peptide antagonists is probably due to substantial degradation of CCK by endogenous peptidases. The CCK-B antagonist-induced dopamine elevations are supported by previous data from a study in which CI-988, a CCK-B antagonist perfused into the neostriatum elevated extracellular dopamine, however, this effect was not altered when perfused in a calcium-free aCSF (Corwin et al., 1995).

It is difficult to postulate a mechanism to support the data obtained above because of the marginal amount of information regarding CCK receptor localization and physiology in the caudate-putamen. However, based upon findings from previous studies coupled with the above data, a few possible mechanisms can be constructed. The most conceivable and straightforward model would involve the localization of CCK receptors directly on dopamine terminals. This would allow direct modulation of dopamine release

in the caudate-putamen. This model is plausible due to the findings that CCK reduces the binding affinity of D2 receptors via CCK-B receptors in the striatum (Tanganelli et al., 2001), and that D2 receptors have been demonstrated on dopamine terminals (Silvia et al., 1994). A CCK-evoked subsensitivity of D2 receptors on dopamine terminals would reduce presynaptic inhibition of dopamine and facilitate its release. An increase in striatal extracellular dopamine would cause a positive feed-forward release of more dopamine in the striatum by releasing the thalamus from inhibition via the direct or indirect pathways (Fig. 1). This would allow direct (thalamus) or indirect (cortex) excitatory glutamatergic input into the striatum and more release of dopamine.

CCK-evoked inhibition of D2 receptors on striatal projection neurons would also result in an increase in striatal extracellular dopamine, though indirectly. This hypothesis would also be plausible due to studies demonstrating CCK receptor localization on striatal projection neurons (Beresford et al., 1987; Hill and Woodruff, 1990) and the finding that some striatal neurons co-express D2 and CCK-B receptor mRNAs (Hansson et al., 1998). These striatal neurons would be the projection neurons co-expressing GABA/ENK that initiate the indirect striatonigral pathway because the inhibitory D2 receptors are not found on the direct pathway projection neurons. In this scheme, CCK-induced D2 subsensitization would disinhibit striatal GABA-ergic projection neurons to the globus pallidus, thus inhibiting GP release of GABA into the subthalamic nucleus. This allows the STN to release glutamate and excite dopaminergic dendrites in the sustantia nigra reticulata and release dopamine in the striatum.

Another possible mechanism by which CCK modulates extracellular dopamine would involve an indirect method via striatal interneurons such as the giant aspiny

cholinergic interneuron. Electron microscopy studies revealed that corticostriatal CCK terminals make direct contact with aspiny interneurons, providing a morphological basis for a potential direct interaction of CCK with modulatory interneurons, such as cholinergic interneurons (Grofova et al., 1982). Several studies have shown an interaction between cholinergic and dopaminergic transmissions in the striatum, for example, cholinergic interneurons have been shown to respond to D2 agonists (Gerfen and Engber, 1992; Stoof et al., 1992) and more recent anatomical studies have clearly demonstrated that dopaminergic terminals are in direct apposition to cholinergic terminals in the rat striatum (Sesack and Pickel, 1990). Also, as stated earlier, CCK receptors have been shown to reside on intrinsic striatal neurons, which could include cholinergic interneurons. In addition, it is postulated that cholinergic interneurons operate as information processing and relay centers in the striatum, incorporating input from areas such as the cortex and relaying this information to striatal neurons. In this model, CCK could directly influence acetylcholine release directly by activating CCK receptors on cholinergic soma or dendrites, or, the CCK-evoked D2 subsensitivity discussed above could take place on cholinergic neurons, thus releasing acetylcholine via disinhibition by D2 inactivity. However the case by which CCK influences Ach release, dopamine release could then be regulated by cholinergic input.

Regardless of the mechanism by which CCK mediates the lack of augmented extracellular dopamine during the early withdrawal period or how it modulates dopamine in naïve or chronic animals, the responsiveness of striatal neurons to CCK seems to depend on the active state or activity of the system at a given time. For example, the effects of CCK receptor antagonists are different in naïve animals compared to animals

that have received daily injections of cocaine. In naïve animals, the state of the dopaminergic system is conceivably less active than in animals that have been stimulated daily with cocaine. This would account for the more robust effects of CCK antagonists on extracellular dopamine observed in chronic and early withdrawal groups than seen in naïve animals. Another example is when perfusing CCK-8S into the striatum of chronically treated animals, CCK-8S initially facilitates dopamine release, but when extracellular dopamine levels rise as a result of cocaine administration and the activity level of the striatum increases, CCK-8S appears to inhibit dopamine release when comparing extracellular dopamine levels as a result from cocaine only (Fig. 17). In other words, CCK is a neuromodulator of dopamine, it's role is to keep extracellular dopamine levels at physiological ranges, and when dopamine levels deviate from these ranges (i.e. as in chronic cocaine administration), the release of CCK is increased depending on elevated activity or high frequency stimulation input from the striatum into the cortex, thus modulating brain regions such as the striatum so that neurotransmitter levels, such as dopamine are brought back to normal levels.

This active state hypothesis could also partially explain why endogenous CCK appears to facilitate dopamine release via the CCK-A receptor in naïve animals (Fig. 15) but inhibit dopamine release in chronic (Fig. 17) and earlywithdrawal animals (Fig. 19). It is most likely that CCK-A and CCK-B receptors are located on different neuronal elements. This concept is supported by studies in the nucleus accumbens in which dopamine release is inhibited by CCK-8S via CCK-B receptors in the anterior NAc (Studler et al., 1986; Marshall et al., 1991), and facilited by CCK-8S via CCK-A receptors in the posterior NAc, in naïve animals (Studler et al., 1986; Marshall et al.,

1991). We observed similar findings in the caudate-putamen of naïve animals, in which CCK-A receptor antagonist L-364,769 inhibited basal and cocaine-evoked extracellular dopamine and CCK-B antagonist L-369,293 facilitated extracellular dopamine. However, we observed changes in CCK-A receptor physiology from naïve animals to animals that received several daily injections of cocaine. This could be partially explained by the hypothesis that CCK-A receptors are located on cholinergic interneurons. In the striatum, ACh is thought to also be active-state or activity-dependent when observing its regulatory influence on dopamine release. For example, ACh can influence dopamine release in an excitatory or inhibitory way in the striatum, depending on the activity level of the system (Calabresi et al., 2000). In naïve animals when dopaminergic activity is low in the striatum, CCK could elevate dopamine via CCK-A receptor-evoked release of ACh, which would conceivably facilitate the release of dopamine. However, when dopaminergic activity is elevated as a result of chronic cocaine administration, ACh could possibly be an inhibitory influence on dopamine release. CCK could then inhibit the release of dopamine via CCK-A receptor-evoked stimulation of ACh release, thus inhibiting dopamine.

As a result of a previous study demonstrating the CCK-B receptor antagonist CI-988-evoked extracellular dopamine increase was by a calcium-independent mechanism (Corwin et al., 1995), we investigated if the extracellular dopamine elevations generated by L-364,769 and L-369,293 were via a calcium-dependent mechanism. Given the difficulty and uncertainty of completely removing calcium from the endogenous extracellular fluid, we co-perfused Verapamil, an L-type calcium-channel blocker with either L-364,769 or L-369,293. The L-type channel blocker was chosen as a result of

previous studies demonstrating no difference from the N-type in it's effects on dopamine release (Pierce and Kalivas, 1997). In this study, we demonstrated that both L-364,769- and L-369,293-evoked dopamine release is mediated via a calcium-dependent mechanism (Figs. 20 & 21). This data is important for confirmation that the dopaminergic effects observed with the antagonists were mediated through receptor stimulation and subsequent calcium-mediated release of vescicular dopamine. An example of a possible calcium-independent mechanism for antagonist-induced dopamine release is carrier-mediated release or reverse transport via the dopamine uptake carrier. Carrier-mediated release can be stimulated by passive diffusion of a compound into the nerve terminal and subsequent displacement of vesicular dopamine by binding to the transporter (Corwin et al., 1995). Carrier-mediated release is one mechanism by which amphetamine, methamphetamine, and high glutamate concentrations are thought to cause release of dopamine (Corwin et al., 1995).

An important detail in the above data is that all of the experiments in this study utilized fully conscious animals in obtaining all neurochemical data. The use of awake animals is important, since anesthesia could modify DA release in rats. Striatal neurons are active in freely moving animals (Pierce et al., 1995), whereas in anesthetized animals, are often silent or discharge in a slow, irregular pattern (Wilson, 1993). For example, silent neurons did not respond as often to CCK (13%) as spontaneously active cells did (43%) (Davidowa et al., 1995). This could account for the conflicting reports concerning the effects of CCK on extracellular dopamine, and further strengthens the proposal that CCK is released and modulates dopamine via an activity-dependent mechanism.

The CCK receptor autoradiography data conducted in this study supports the neurochemical groundwork of CCK's major role in the tolerance observed in extracellular dopamine in response to a challenge injection of cocaine after withdrawal. During early withdrawal, we observed a 27% and 30% increase in total CCK receptor binding in the caudate-putamen, compared to CCK receptor binding levels found in naïve and chronic cocaine treatment animals, respectively. The increase in CCK receptor binding on day 11 coincides with the theories discussed previously. If CCK's largest effect on cocaine-evoked dopamine release is in early withdrawal, then it is likely that an up-regulation of CCK receptors or an increase in receptor affinity is involved. This way, the inhibitory effects of CCK on dopamine release observed on day 11 would be augmented by the increase in receptor concentration or binding affinity.

Autoradiographic binding of the CCK-B receptor via selectively blocking the CCK-A receptor with L-364,769, demonstrated no statistically significant change among treatment groups. However, we did observe an increase in CCK-B binding of 9% and 13% when compared to untreated and chronic cocaine treated animals, respectively. Again, this complements the neurochemical data obtained in this study, which demonstrates a greater dopaminergic effect elicited by CCK-B receptor antagonist L-369,293 on day 11 (Fig. 17) than on day 7 (Fig. 19). It also coincides with the observation that total CCK binding is increased to 27% on day 11.

When selectively blocking the CCK-B receptor, CCK-A receptors were not detectible with the autoradiographic methods used. This was not surprising, since other researchers also failed to detect striatal CCK-A receptors by autoradiographical methods (Kirouac and Ganguly, 1993; Suzuki et al., 1993). However, the presence of CCK-A

receptors in the striatum, despite autoradiographical support, is well accepted as a result of physiological evidence (Davidowa et al., 1995; Davidowa et al., 1997). In this study, we also provided physiological evidence for the localization of striatal CCK-A receptors. It is also possible that the increase in total CCK receptor binding observed on day 11, is in part due to increased CCK-A receptors since CCK-B receptor binding was only shown to increase by 9%.

The dopamine system has been the focus of much research during the past 30 years, mainly because alterations in dopamine transmission are involved, directly or indirectly in several brain dysfunctions. As a result of the modulatory actions of CCK via CCK-A and CCK-B receptors on the striatal dopaminergic system found here, and due to the anatomical distribution of CCK within the striatal dopaminergic system, it raises the possibility that CCK-based drugs could provide therapeutic approaches for disorders linked to the dopaminergic system. For example, it has been proposed that drug addiction is the result of neuroadaptive processes within the central nervous system that oppose the acute reinforcing actions of drugs of abuse (Koob and Bloom, 1988; Koob et al., 1998), leading to the emergence of affective changes such as, anxiety, dysphoria, and depression during withdrawal, and potentially leading to drug-seeking behavior and relapse. The data obtained in this study identifies perturbations in brain dopaminergic systems which could possibly be an important element in the neuroadaptive changes induced by chronic cocaine. Many of the pharmacotherapies for cocaine addiction have been based on a replacement strategy, in which it was hypothesized that dopamine is depleted following chronic cocaine use and that treatments to replace dopamine would block craving. However, previous studies have shown that dopamine is not depleted

during early withdrawal from cocaine (Crawley, 1992; Kalivas and Duffy, 1993a; Kalivas et al., 1993) and is shown here that dopamine is decreased by CCK via CCK-A and CCK-B receptors. Thus, by antagonizing neostriatal CCK receptor-mediated inhibition of dopamine transmission, CCK receptor antagonists, which seem to cross the blood-brain barrier and have in vivo haif-lives of several hours (Crawley, 1992), may represent novel compounds for treatment of drug abuse and addiction.

Lastly, the striatum appears to be an important locus for CCK and dopamine interactions, and deficits in these interactions may be reflected in symptoms associated with schizophrenia and Parkinson's disease (Carlsson and Carlsson, 1990). Thus it is of interest to investigate the *in vivo* mechanisms of these neurotransmitters and their interactions with each other. Potential therapeutic implications for CCK receptor compounds could be applied towards psychopathological diseases such as schizophrenia. The hypothesis that the dopaminergic system is overactive is based on the finding that neuroleptics, which are used in the successful management of some symptoms of this disorder, selectively block dopamine receptors (Seeman et al., 1975; Kane and Freeman, 1994; Sigmundson, 1994). The dopamine hypothesis was further strengthened by the fact that psychostimulants, such as cocaine and amphetamine, which increase dopaminergic transmission mainly by increasing extracellular concentrations of dopamine, induce psychotic states resembling those observed in the positive symptoms of schizophrenia (euphoria, auditory hallucinations, and the ability to remain inactive) (Kane and Freeman, 1994). From a pharmacological point of view, dopamine receptor antagonists have been shown to treat some symptoms effectively, but are less effective in managing the negative symptoms of schizophrenia such as loss of motivation and slowed body movement

(Seeman et al., 1975). Also, prolonged treatment with neuroleptics has a major drawback, since 90% of patients under medication suffer from extreme movement disorders known as extrapyramidal side effects (EPS) (Jaber et al., 1996). Moreover, prolonged treatment often leads to irreversible tardive dyskenisia (Jaber et al., 1996). The use of CCK-based drugs for alleviating schizophrenic symptoms is an attractive alternative to the therapeutic tools discussed above. CCK-based drugs could provide more anatomically specific therapeutic approaches, for example, compounds acting at a CCK receptor might have advantages as antipsychotics when given alone or in combination with lower doses of neuroleptics, in that sedation may be less, and the risk of extrapyramidal tardive dyskinesias may be greatly reduced.

CCK receptor-based drugs may play an important role in therapeutic approaches towards alleviating symptoms in dopamine-mediated neuropathological diseases as Parkinson's and Huntington's diseases. The leading model by Graybiel and colleagues for motor disorders such as PD and HD is that the basal ganglia have distinct pathways that compete with each other functionally to release movement (the direct pathway) or to inhibit movement (the indirect pathway) (fig. 4). The competing pathways act like a brake and accelerator in a car. The brake-accelerator model (Graybiel, 1990) suggests that release (disinhibition) of the thalamus by the direct pathway is opposed by the indirect pathway, which inhibits the thalamus via the additional, excitatory subthalamic projection to the substantia nigra (Fig 4). In the simplest view, the poverty of movement in Parkinson's disease results from over activity of the inhibitory indirect pathway, whereas excess movement in disorders such as Huntington's disease represent over-activity of the direct pathway. It has been proposed that antagonism between the direst

and indirect pathways helps select intended movements by suppressing unintended ones and promoting desired movements. In this model, CCK receptor-based compounds could provide therapeutic modulation of motor movement by either inhibiting the indirect pathway, which would help relieve Parkinson's disease symptoms, or could inhibit the direct pathway, which would result in reducing Huntington's disease symptoms. However, at this time, it is necessary to further investigate the physiology of CCK-mediated dopamine release in order to selectively activate or inactivate separate striato-nigral pathways, thus selectively providing therapy towards individual dopaminergic-mediated motor neuropathologies.

In conclusion, these studies indicate that as a result of chronic daily administrations of cocaine, tolerance may develop to cocaine-evoked extracellular dopamine during early withdrawal from drug. We have also demonstrated that this is not a result of an increase in dopamine transporter sites. We hypothesized the possibility of a cholecystokinin-modulation of dopamine. Until recently, a major factor that has hampered progress in understanding the function of CCK in the brain has been the lack of selective antagonists with high affinity and selectivity for CCK receptors. However this situation has changed, and we utilized selective antagonists to selectively block each receptor subtype to investigate each receptors involvement in cocaine-evoked dopamine release, and found that CCK appears to play a major role, via both CCK-A and CCK-B receptors, in the development of tolerance in extracellular dopamine to the effects of daily cocaine administration. We have also demonstrated a modulatory role of basal and cocaine-evoked extracellular dopamine by CCK in naïve and chronically treated animals. CCK's modulatory effects of dopamine seem to depend on the activity or active state of the

dopaminergic system, with the greatest modulation taking place during chronic and early withdrawal cocaine administration. These findings along with others, combined with the increased availability of CCK receptor-based compounds has recently revitalized investigations of the functional role for CCK in the brain and has opened up new possibilities for the treatment of CNS disorders.

Chapter 5

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