THE NEUROKININS AND LOCOMOTOR SENSITIZATION TO COCAINE

by

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ABSTRACT

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This research is an attempt to better understand the role of striatal, neurokinins substance P (SP) and neurokinin A (NKA), in acute cocaine-induced hyperactivity and locomotor sensitization, the escalating locomotor response to chronic, intermittent exposure to an unchanging cocaine dose. We found that preprotachykinin-A mRNA (PPT-A), the precursor for SP and NKA, measured by *in situ* hybridization, is upregulated in the dorsalateral striatum, in response to chronic, intermittent cocaine exposure. These increases in PPT-A mRNA were significantly reduced in rats co-exposed to cocaine and MK-801, an NMDA receptor antagonist known to block the development of locomotor sensitization and associated neurochemical changes. Tissue levels of substance P, measured by radioimmunoassay showed no statistically significant changes in response to a time course of chronic cocaine administration. There was however, a trend toward reduced levels in the substantia nigra in response to acute cocaine suggesting increased utilization. Reductions were no longer observed after chronic exposure or a cocaine challenge, conceivably the result of compensatory increases in substance P expression suggested by the increases in PPT-A mRNA levels.

Two neurokinin receptors, NK-1 and NK-3 which have the highest affinity for SP and NKA respectively, have different distributions and are localized to separate neural populations in the striatum and substantia nigra. We found that the blockade of NK-1

receptors by systemic administration of non-peptide antagonists LY306740 and CP099994 prior to acute cocaine administration attenuated hyperactivity induced by cocaine but the NK-3 antagonist, PD161182, had no effect. NK-1 blockade however had no effect on the development or expression of locomotor sensitization to cocaine. These findings suggest that endogenous neurokinins acting at NK-1 receptors are necessary for the full expression of acute cocaine-induced hyperactivity but do not play a role in sensitization.

NK-3 receptor blockade, by the NK-3 antagonist PD161182, had no effect on cocaine-induced hyperactivity but robustly enhanced the expression of locomotor sensitization. These findings suggest that endogenous neurokinins, acting at the NK-3 receptor, play a homeostatic role by reducing the escalating locomotor activity associated with locomotor sensitization.

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ABBREVIATIONS

5-HT..... 5-hydroxytryptamine (serotonin)
6-OHDA..... 6-hydroxydopamine (compound for lesioning DA neurons)
APO..... apomorphine (synthetic DA agonist)
ACh..... acetylcholine
BG..... basal ganglia (functionally related interconnected nuclei)
CPu..... caudate putamen (dorsal striatum)
D1R..... dopamine 1 receptor
D2R..... dopamine 2 receptor
DA..... dopamine (a catecholamine, precursor is tyrosine)
DARPP-32 ..... dopamine related phosoprotein-32
DAUC ..... dopamine uptake carrier (binding site of cocaine)
EP..... endopeduncular nucleus
EPSP ..... excitatory post synaptic potential
GBR-12909.... synthetic DAUC blocker (binds to different site than cocaine)
GP ..... globus pallidum (a nucleus of the basal ganglia)
GMSPN..... GABAergic medium spiny neurons (striatal projection neurons)
NE ..... norepinephrine (a catecholamine)
NK..... neurokinin
NK-1 ..... neurokinin-1 receptor (highest affinity for SP)
NK-2 ..... neurokinin-2 receptor (not detected in CNS, highest affinity for NKA)
NK-3 ..... neurokinin-3 receptor (highest affinity for NKB, moderate affinity for
NKA..... neurokinin A (a neurokinin sometimes referred to as substance K (SK))
NAc..... nucleus accumbens (ventral striatum)
PFC ..... prefrontal cortex (target of dopaminergic VTA projection)
PPD ..... preprodynorphin (mRNA precursor for dynorphin peptide transmitter)
PPE..... preproenkephalin (mRNA precursor for enkephalin peptide transmitter)
PPT-A ..... preprotachykinin A (mRNA precursor for neurokinins SP and NKA
PPT-B..... preprotachykinin B (mRNA precursor for neurokinin B (NKB)
RIA..... radioimmunoassay
SN ..... substantia nigra
SNc..... substantia nigra compacta (source of DA cell bodies, projects to CPu)
SNr..... substantia nigra reticulata (source of GABAergic output from BG)
SP..... substance P (a neurokinin)
STN..... subthalamic nucleus (projects from GP to SNr)
TH ..... tyrosine hydroxylase (rate limiting enzyme in DA synthesis)
TTX..... tetrodotoxin (blocks voltage gated Na^+ channels
VTA ..... ventral tegmental area (source of DA cell bodies, project to NAc)

1 INTRODUCTION

1.1 Sensitization to Cocaine

The research described here was undertaken to investigate the neurochemical basis of cocaine-induced hyperactivity and locomotor sensitization. Hyperactivity is demonstrated, when rats and other laboratory animals receiving cocaine, exhibit higher activity levels than controls exposed to saline (Kalivas and Duffy, 1993; Roy et al., 1978). Progressive sensitization is defined as the increasing responsiveness over time, to an unchanging dose of cocaine. In addition, after prior exposure to cocaine, a lower dose of drug will elicit greater responsiveness than when administered to drug naïve subjects (Kalivas et al., 1988; Post and Contel, 1983; Post, 1980; Roy et al., 1978; Post and Rose, 1976).

Sensitization, sometimes referred to as reverse tolerance, develops in response to chronic, intermittent drug exposure. In contrast tolerance, the decreasing responsiveness overtime to an unchanging drug dose, is frequently observed in response to continuous or frequent exposure to high doses of cocaine. Thus, the development of sensitization is dose and time dependent (Post,1980). Horizontal locomotion and rearing as well as stereotypies such as head bobbing, sniffing and gnawing have been shown to sensitize in response to repetitive, intermittent cocaine exposure (Post and Contel, 1983; Post, 1980; Roy et al., 1978; Post and Rose, 1976). In addition, the reinforcing properties of cocaine assessed by self-administration and conditioned place preference paradigms also sensitize (Emmett-Oglesby, 1995; Horger et al., 1990).

If, after withdrawal from repetitive, intermittent drug administration, a drug challenge (reintroduction of the drug) results in sustained behavior enhancement, then

enduring sensitization is said to have occurred. A withdrawal period of 3-7 days is referred to as early withdrawal and longer periods are referred to as late withdrawal (Henry and White, 1995; Heidbredder, 1996; Robinson and Becker, 1986; Post and Contel, 1983). Locomotor sensitization to cocaine persists for at least one month following chronic exposure (Henry and White, 1995). These enduring behavioral changes in response to cocaine are indicative of plasticity within the basal ganglia, the brain system which mediates many of the behavioral responses to cocaine.

1.2 Sensitization and Drug Addiction

A person is said to be addicted to drugs when compulsive drug seeking and drug taking, interferes with normal functioning, representing a loss of control. Drugs and stimuli associated with drug taking elicit pleasure, thereby providing positive reinforcement (incentive) for subsequent drug use. Drugs can also provide negative reinforcement by alleviating unpleasant physiological or psychological states associated with drug abstinence or life situations. Sensitization can lead to drug addiction when enhanced pleasure becomes an increasingly powerful incentive for drug taking (Koob et al., 1997; Self and Nestler, 1995; Robinson and Berridge, 1993; Dackis and Gold, 1985).

The incentive-sensitization theory of drug addiction posits that stimuli associated with drug taking become imbued with salience (they engender wanting) and therefore become incentives for drug use. Circuits mediating salience are distinct from circuits mediating pleasure, therefore, wanting can occur independent of pleasure. Incremental enhancements in dopamine circuits (sensitization) that mediate drug wanting produce hypersensitivity to drugs and drug associated stimuli resulting in intense drug craving leading to drug addiction (Robinson and Berridge, 1993).

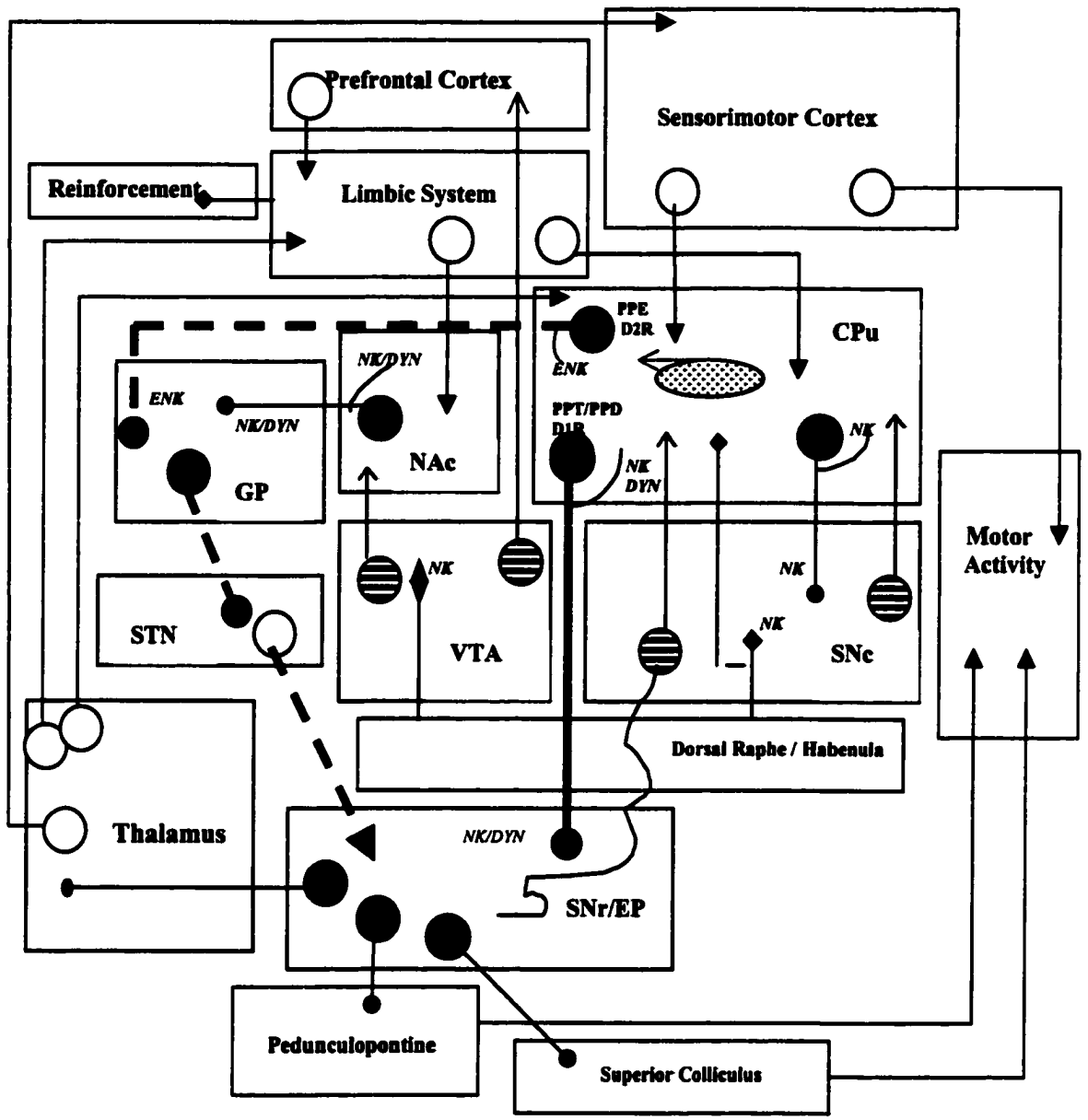
Cocaine is rapidly cleared from the system implying that behavioral changes to chronic cocaine are not due to the cumulative effects of the drug. The study of locomotor sensitization in experimental animals has implications for understanding drug addiction in humans. Cocaine and other psychostimulants are readily absorbed into the brain, are well tolerated and their effects on behavior are easy to observe and measure (Volkow and Fowler, 1995; Post and Contel, 1983). Therefore, cocaine-induced behaviors can be compared with cocaine-induced neurochemical changes revealing relationships between neuroplasticity and behavior.

1.3 Intrinsic Circuits of the Basal Ganglia

1.3.1 Anatomy and Physiology

The motor activating and reinforcing effects of cocaine and other psychostimulants (amphetamines) are mediated by dopamine neurons (Koob and Goeders, 1989; Hubner & Koob, 1990), whose soma are located within the ventral tegmental area (VTA) and substantia nigra compacta (SNc), midbrain nuclei of the basal ganglia (**Fig. 1**). The basal ganglia consist of a group of functionally related and interconnected nuclei located within the forebrain and midbrain. The striatum, which is the primary input region of the basal ganglia, is comprised of the caudate putamen (CPu) and the nucleus accumbens (NAc) that receive intrinsic projections from the dopamine neurons previously mentioned which originate in the SNc and VTA respectively. The striatum also receives widespread extrinsic input from cortical and subcortical regions (Heimer et al., 1995; Fallon and Loughlin, 1995).

FIG. 1 BASAL GANGLIA CIRCUITS



Striatalnigral Pathways and Neuropeptide Transmitters

Direct — *Striatalnigral MSNs co-express Neurokinins(NK) and Dynorphin (DYN)*

Indirect — *Striatalpallidal MSNs co-express Enkephalin (ENK)*

Neurons	Classical Transmitter	Function
	GABA	Inhibitory
	Glutamate	Excitatory
	Dopamine	State Dependent
	Acetylcholine	State Dependent

Basal Ganglia Nuclei

CPu	Caudate Putamen (dorsal striatum)
EP	Endopeduncular Nucleus
NAc	Nucleus Accumbens (ventral striatum)
GP	Globus Pallidum
STN	Subthalamic Nucleus
SNr	Substantia Nigra Reticulata
SNc	Substantia Nigra compacta
VTA	Ventral Tegmental Area

The substantia nigra reticulata (SNr) and endopeduncular (EP) of the midbrain are the primary output nuclei of the BG. GABAergic output neurons of the SNr project to the thalamus. The thalamus in turn sends excitatory projections to the motor cortex whose activation results in motor activity. The nigralthalamic inhibitory projection is tonically active thereby dampening involuntary motor activity (Chevalier and Deniau, 1990). The globus pallidum (GP) and the subthalamic nucleus (STN) form a pathway connecting striatal input and nigral output nuclei (Heimer et al., 1995).

A subset of GABAergic medium spiny projection neurons (GMSPNs), residing within the neurochemically distinct matrix compartment of the CPu, express preprotachykin-A (PPT-A), preprodynorphin (PPD) and dopamine-1 receptor (D1R) mRNA and project directly to the SNr (direct striatalnigral projection, **Fig 1.**) (Surmeier et al., 1996; Le Moine and Bloch, 1995; Gerfen, 1992). PPT-A and PPD mRNA are precursors for the neuropeptide transmitters substance P (SP) and neurokinin A (NKA) (referred to as neurokinins (NK)) and dynorphin (DYN), respectively. Activation of the direct striatalnigral pathway inhibits the nigralthalamic inhibitory projection thereby releasing the thalamus from inhibition. The result is an increase in motor activity (Goldman-Rakic and Selemon, 1990; McGeer and McGeer, Chevalier and Deniau, 1990).

A second population of GMSPNs within the CPu matrix, express preproenkephalin (PPE) and dopamine-2 receptor (D2R) mRNA and project to the pallidum which initiates the indirect striatalnigral pathway (see bolded dashed arrows in **Fig 1.**) (Surmeier et al., 1996; Le Moine and Bloch, 1995; Gerfen, 1992). PPE mRNA is a precursor of the neuropeptide transmitter enkephalin (ENK). This pathway is called indirect because the GABAergic neurons of the globus pallidum (GP) synapse on excitatory glutamatergic

neurons of the subthalamic (STN) nucleus. It is the neurons of the STN which complete the circuit from the striatum to the output neurons of the SNr. Activation of the striatalpallidal projection results in inhibition of the thalamus and therefore inhibition of motor activity (Goldman-Rakic and Selemon, 1990; McGeer and McGeer, 1987; Chevalier and Deniau, 1990).

Striatal dopamine is thought to activate the direct pathway via D1R which activates cAMP and inhibit the indirect pathway via D2R which inhibits cAMP(Gerfen, 1992; Alexander and Crutcher, 1990). Cocaine by increasing striatal DA availability mimics this effect.

GABAergic medium spiny neurons of the NAc project primarily to the ventral globus pallidum and therefore influence the indirect pathway to the SNr (Heimer, 1995). All three neuropeptide mRNA precursors are expressed by one or more NAc projection neurons (Lu et al., 1998; Gerfen, 1992). Dopamine neurons in the VTA project to the NAc and also send a projection to the prefrontal cortex (PFC) a cortical association area with connections to limbic regions (Fallon and Loughlin, 1995).

A subpopulation of PPT-A expressing cells in the CPu project to the SNc. This SNc projecting subpopulation, of GABAergic MSNs, is found in neurochemically distinct regions throughout the CPu, designated as striosomes or patches, which form a labyrinthe within the remaining area called the matrix. The striosomes receive preferential innervation from limbic regions whereas the matrix, receives preferential innervation from sensorimotor regions of the cortex (Heimer et al., 1995, Graybiel, 1990).

Upon dopaminergic activation there is somatodendritic dopamine release in the SNc and VTA as well as axonal DA release in the striatum. A sub-population of

dopaminergic SNc neurons, within the ventral tier, extend dendrites into the SNr (Fallon and Loughlin, 1995). Thus, dopamine effects striatal targets, nigral output and autocrine functions (White et al., 1995; Kalivas, 1993).

The relationship between DA neurons and movement is indirect and complex involving many intermediary neurotransmitters and their receptors. Circuits, containing multiple synapses, intervene between dopamine neurons of the basal ganglia and effector motor neurons.

Our research is an attempt to further elucidate the involvement of striatal neuropeptides in cocaine induced behavioral changes. Our initial approach was to assess neurochemical changes in the striatum of male rats after exposure to a time course of cocaine administration. Using *in situ* hybridization histochemistry we assessed three neuropeptide mRNA precursors: preprotachykinin (PPT), preprodynorphin (PPD) and preproenkephalin (PPE) in coronal sections of the striatum. These peptide precursors are expressed by the striatal projection neurons and their neuropeptide products are in a position to modulate dopaminergic activity, nigralthalamic output and the direct and indirect striatalnigral pathways that mediate locomotor activity. In addition, the striatal projection neurons are a primary postsynaptic target of the dopaminergic neurons that are directly affected by cocaine. We also measured D1 and D2 receptors (D1R and D2R) by autoradiography. We reasoned that the enhanced activity associated with locomotor sensitization could be due to changes in the responsiveness of striatalnigral or striatalpallidal projection neurons to DA, as a consequence of changes in D1 or D2 receptor levels.

1.3.2 Cocaine, Dopamine and Motor Activity

Diminished dopamine due to Parkinson's disease in humans or experimental manipulations (dopamine lesions, genetic engineering) in animals results in severe reductions in movement called hypokinesia (Zhou and Palmiter, 1995; DeLong, 1990; Albin et. al., 1990; Kelly and Iversen, 1976). By increasing dopamine availability in the synapse cocaine acts as an indirect dopamine agonist and increases activity. Although cocaine acts by blocking the reuptake of all the monoamine neurotransmitters: dopamine (DA), serotonin (5-HT) and norepinephrine (NE), its psychostimulant effects are due primarily to blockade of dopamine uptake via binding to the dopamine uptake carrier (DAUC) on axon terminals in the striatum (Ritz et al., 1987). DAUC knockout mice have five times more striatal dopamine than controls and are hyperactive when compared to those controls. Psychostimulant exposure in knockout mice fails to further increase activity or striatal dopamine levels, supporting a crucial role for DAUC blockade in cocaine-induced hyperactivity (Caine, 1998; Rocha et al., 1998).

1.3.3 The Initiation and Expression of Locomotor Sensitization

Microinjections of amphetamine or cocaine within the NAc and CPu but not the VTA or SNc produce enhanced motor activity. NAc infusion produces more activation than CPu infusion at all doses. Repeated intra-VTA but not intra-NAc injections of amphetamine followed by a systemic or intra-NAc drug challenge (amphetamine or cocaine) will result in a sensitized locomotor response. Repeated intra-NAc injections of cocaine sensitize to a subsequent intra-NAc but not a systemic cocaine challenge. These findings suggest that the VTA is necessary for the initiation or induction of sensitization

and the NAc for its expression. (Vezina, P., 1996; Cador et al. 1995; Kalivas and Stewart 1991; Robinson and Becker 1986). According to Kalivas, 1995,

> *"Expression can be defined as the changes in neuronal function that are associated with and causally related to behavioral sensitization. Accordingly, these alterations are relatively permanent. Initiation can be defined as the cellular and molecular alterations in neuronal function that precede the relatively permanent changes mediating the expression of behavioral sensitization. The changes associated with initiation also can be relatively permanent or may be components of transitory neuronal responses."*

Cocaine-induced neurochemical changes have been found to affect both the initiation and expression of locomotor sensitization.

1.4 Basal Ganglia Function

Reciprocal cortical and subcortical innervation of the basal ganglia is extensive involving most brain regions resulting in the basal ganglia having diverse effects on behavior (Graybiel, 1998). While the exact role of each region of the basal ganglia in movement, cognition and drug addiction is not yet completely defined, it is clear that the basal ganglia is of fundamental importance in all of these behaviors (Le Moal and Simon, 1991).

For example, the death of DA neurons within the SNc and VTA of the basal ganglia results in the movement disorders of Parkinson's disease (inability to initiate movement and resting tremor) (Albin et al., 1990; Zigmond et al., 1990; McGeer and McGeer, 1987). Death of striatal neurons results in the uncontrolled movement of Huntington's chorea (Albin et al., 1990; DiFiglia, 1990). DA neurons of the basal ganglia have been implicated in cognitive disorders. For example, DA receptor blockers are a primary treatment for schizophrenia (Feldman et al., 1997).

The reinforcing properties of drugs of abuse have been attributed to increased DA availability in the NAc and prefrontal cortex (PFC) target sites of DA projections from the VTA (Koob et al., 1997; Self and Nestler, 1995). The negative reinforcement associated with withdrawal has been attributed to decreased dopamine function in these areas (Koob et al., 1997; Dackis and Gold, 1984).

Interestingly, approach behavior necessary for obtaining reward requires motor activity and therefore circuits mediating both activity and reinforcement occur within close proximity in the basal ganglia. Psychostimulants activate both motor and reinforcement circuits (Robinson and Berridge, 1993).

The NAc receives its primary innervation from limbic and limbic connected areas and via the thalamus and PFC, communicates back to these regions. Therefore, the NAc and its connections are referred to as the mesolimbic system and have been closely associated with the reinforcing effects of cocaine (Koob, 1992).

The connections of the CPu are called the striatalnigral system and are most closely associated with the selection of motor activities based on sensorimotor inputs (Smith and Bolam, 1992). Both of these circuits have been implicated in locomotor sensitization to cocaine (Robinson and Becker, 1985; Paulson and Robinson, 1995).

It has been proposed that mesolimbic and striatalnigral systems are discrete and segregated parallel circuits (Alexander and Crutcher, 1990) but in fact there is reason to believe that extensive crosstalk between circuits occurs within the basal ganglia (Heimer et al. 1995). For example, the SNr, the primary output region of the basal ganglia, receives innervation from pathways originating in the NAc and pathways originating in the CPu.

1.5 D1 and D2 Receptors and Dopamine Systems of the Basal Ganglia

Dopamine is released by tetrotoxin (TTX) sensitive vesicular exocytosis that is Ca^{2+} and activity dependent. DA, once in the synaptic cleft, is inactivated by efflux away from the synapse and reuptake by the DA uptake carrier (DAUC), the site of cocaine blockade (Feldman et al., 1995). Clearance of DA from the synapse is 100 times slower in DAUC knockout mice (Garris et al., 1994). 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-spannning domains. D1 type receptors (D1 and D5) constitute a subfamily that stimulate adenylyl cyclase activity and the D2 type receptors (D2, D3 and D4) inhibit or have no effect on cAMP (cyclic adenosine-mono-phosphate) formation (Creese et. al., 1983, Kebabian and Calne, 1979). D1 agonists are effective at μM concentrations and D2 agonists at ηM concentrations (Feldman et al., 1995). As previously mentioned, postsynaptic striatal D1 receptors (D1Rs) have been localized to GMSNs of the direct striatalnigral pathway (Gerfen, 1992; Le Moine et al., 1991; Reiner and Anderson, 1990). D2 receptors (D2Rs) are expressed postsynaptically on striatal GMSNs of the indirect pathway (Gerfen, 1992) and as autoreceptors on DA terminals (Silvia et al., 1994). In addition D2Rs are expressed by the large aspiny ACh striatal interneurons and function to inhibit ACh release (Pollack and Wooten, 1992; Stoof et al., 1992; Gerfen et al., 1991).

Lesions of nigral dopamine neurons have been shown to produce small increases in D2R binding levels in the CPu and no changes in the NAc (Joyce, 1991). In keeping with this finding chronic DA receptor blockade by halperidol increased D2R mRNA expression in striatal medium spiny and cholinergic neurons (Bernard et al. 1991).

Chronic, intermittent cocaine exposure consistently resulted in increased D2R binding levels in the NAc (Pecins-Thompson and Peris, 1993; Peris et al. 1990; Goeders and Kuhar, 1987) with inconsistent findings in the CPu (Ziegler et al., 1991; Goeders and Kuhar, 1987). Thus, D2R binding levels in the CPu are responsive to decreases in receptor stimulation but in the NAc they are responsive to increased stimulation. Because cocaine and other psychostimulants increase DA levels one would conclude that D2Rs in the mesolimbic circuit are more responsive to cocaine than those in the striatalnigral circuit.

Lesions of dopamine neurons resulted in a small decrease in D1R binding levels in the dorsal striatum (Joyce, 1991) and a down regulation of D1R mRNA (Gerfen et al., 1990). Chronic, intermittent cocaine exposure followed by 7 days abstinence produced no changes in D1R binding levels in the CPu or NAc (Mayfield et al., 1992). There may be changes in D1R binding levels in response to chronic cocaine exposure that are shortlived and do not persist after 7 days of withdrawal.

Our research is an attempt to add to the current knowledge about changes in dopamine receptor binding levels in response to a time course of chronic, intermittent cocaine administration known to produce progressive locomotor sensitization.

1.6 Opioids and Dopamine Systems of the Basal Ganglia

The preprodynorphin (PPD) gene codes for PPD mRNA that is translated to the propeptide PPD. The PPD propeptide is post-translationally cut and modified within vesicles, to produce the opioid peptides DYN A, DYN B and leu-ENK (Civelli et al., 1985). Similar processing of the preproenkephalin (PPE) gene results in the opioid peptides met-ENK and leu-ENK (Rosen et al., 1984). As previously described PPD

mRNA and D1R are co-expressed in GMSNs of the direct striatalnigral pathway and PPE mRNA and D2R are co-expressed in GMSNs of the indirect striatalpallidal pathway. In the midbrain, enkephalin immunoreactivity is highest in the SNc (Elde et al., 1976) and dynorphin immunoreactivity is highest in the SNr (Van Bockstaele et al. 1994; Vincent et al., 1982).

The endogenous opioids exhibit modest selectivity for receptors. They all have some affinity for the μ receptor, the physiological target for morphine and heroine, but enkephalin shows preferential binding at the delta (δ) receptor and dynorphin at the kappa (κ) receptor (Feldman et al., 1997). In the striatum δ and κ receptor distribution is diffuse whereas μ receptors are restricted to the striosomal compartment (Mansour et al., 1987). δ and μ receptors have been localized to axon terminals within the striatum, globus pallidum and SNr (Trovero et al., 1990; Abou-Khalail et al., 1984; Pollard, 1978). Somatodendritic μ receptors have also been identified in the striatum (Arvidsson et al., 1995). κ receptors have been localized primarily to intrinsic post-synaptic sites in the striatum and substantia nigra (Arvidsson et al., 1995).

Unilateral intranigral injections of μ, δ and κ agonists induce contralateral turning in rodents (Matsumoto et al., 1988; Freidrich et al., 1987; Morelli and DiChiari, 1985, Herrara-Marschitz, 1984) but intrastriatal injections have no effect on turning (Toyoshi et al. 1995) suggesting that opioid induced increases in activity are mediated primarily via the substantia nigra. Evidence from these studies suggests that μ and δ mediated increases in activity are due to their effects on dopaminergic neurons, whereas κ agonists, increase motor activity by directly inhibiting nigralthalmic projection neurons (Morelli

and Di Chiari, 1985). Paradoxically, κ agonists have also been shown to decrease activity by inhibiting dopamine neurons (Reid et al., 1990; Matsumoto et al., 1988).

Naltrindole, a δ-receptor antagonist was shown to inhibit the expression of cocaine-induced sensitization (Heidbreder et al., 1996) and U69593, a κ-receptor agonist was shown to inhibit the development of sensitization (Shippenberg and Rea, 1997; Heidbreder et al., 1993). These results suggest that the endogenous opioid, enkephalin, augments the expression of cocaine sensitization and dynorphin plays a homeostatic role by opposing the development of sensitization. We attempted to confirm these findings using a similar drug treatment paradigm but our results showed that pretreatment with naltrindole and cocaine did not attenuate but in fact augmented the development of locomotor sensitization to cocaine. We found no effect of the dynorphin agonist, U69593, on the expression or development of sensitization (see section 2, **Table 12**). A possible explanation for this discrepancy is that the longer pretreatment paradigm we employed (7 as opposed to 3 days) resulted in the desensitization of opioid receptors.

Hurd and Herkenham (1992), reported increased PPD and PPE mRNA in response to an acute high dose of cocaine (30mg/kg) when rats were sacrificed 2 hours after the last exposure. In a subsequent experiment in which animals were sacrificed 1 hour after the last injection PPD and PPE mRNA levels were unchanged compared to controls. On the basis of these data and prior evidence, the authors conclude that it takes 2-3 hours to detect a change in opioid expression after acute exposure. They also showed that chronic high (30mg/kg) but not lower (10 or 20mg/kg) doses of cocaine increased PPD mRNA levels in the striatum but had no significant effect on PPE mRNA (Daunais and McGinty, 1994). Animals were again sacrificed 1 hour after the final injection.

Our research attempts to extend these findings by assessing opioid prescursor mRNA expression in response to a time course of cocaine at a moderate dose, using multiple time points and a longer period (5 hours) between the last injection and sacrifice.

1.7 The Neurokinins

1.7.1 Expression and Localization

The pre-protachykinin A (PPT-A) and pre-protachykinin B (PPT-B) genes code for the neurokinins (Krause et al., 1987). The PPT-B gene codes for neurokinin B (NKB). PPT-B is expressed at very low levels in the striatum and the NKB peptide is not detectable in the SN (Whitty et al., 1995; Bannon and Whitty, 1995). PPT-A mRNA can be alternatively spliced to produce three different mRNA species, α, β and γ PPT-A mRNA. The three mRNAs are translated to produce three precursor proteins (propeptides) designated with the same name as the mRNA species from which they are derived α, β and γ PPT-A.

Post translational enzymatic processing of the propeptides within terminal vesicles, produces the neurokinins. The β and γ propeptides together constitute 95% of striatal neurokinin precursor species. Substance P (SP) consists of eleven amino acids and is produced from all three PPT-A precursor proteins. Neurokinin A (NKA) consists of 10 amino acids and is derived from β and γ PPT-A. The neurokinins are amidated and share a common sequence at the carboxy terminal (-PHe-X-GLy-LEu-MEt-NH2). The amino terminal confers receptor affinity (Bannon et al., 1990).

The release of peptides is dependent on Ca^{2+} influx and high frequency neural activity. In contrast, the classical neurotransmitters are more likely to be released in response to single action potentials. Neuropeptide inactivation is due to catabolism by

peptidases. Neuropeptides frequently modify the actions of the classical neurotransmitters and therefore are referred to as neural modulators. Substance P, the first tachykinin discovered by von Euler and Gaddum in 1931, in extracts from the intestine and brain, has been the most intensely studied and characterized of the neurokinins (Feldman et al., 1997, Cooper et al., 1996).

Experiments combining local lesions of the striatum or globus pallidus with radioimmunoassay of SP in the SN confirm that nigral SP mainly originates in the striatum (Hong et al., 1977, Brownstein et al. 1977, Kanazawa et al., 1977). Therefore, cocaine-induced changes in striatal PPT-A expression could affect nigral peptide levels leading to changes in nigral output or dopaminergic activity. Triple immunocytochemical studies reveal that GABAergic terminals containing SP make synaptic contact with TH containing dopamine neurons of the SNc (Bolam and Smith, 1990), suggesting that SP may directly modulate dopamine neural activity.

The NAc is a minor source of neurokinins within the VTA (Fallon and Loughlin, 1995). The primary source of SP in the VTA originates in the lateral habenula (LHAB) and interpeduncular (IP) nucleus. Termini of habenula projections have been shown to correspond to DA cell dendrites in the VTA (Herkenham and Nauta, 1979, Emson, P.C. et al., 1977). Equal densities of SP and NKA immunoreactivity are found in the VTA (Kalivas et al., 1985). The lateral habenula, are innervated by the globus pallidum and the SNr and form robust connections with the IP (Herkenham and Nauta, 1979, Emson, P.C. et al., 1977). Therefore, striatal changes can ultimately affect SP levels in the VTA via direct connections from the NAc and indirect connections via the habenula nucleus.

1.7.2 Behavior and Physiology

Microinfusion of the neurokinins SP and NKA, or their analogues, into midbrain regions of theVTA, SNc and SNr, increases motor activity in rats (James and Starr, 1977; Stinus et al., 1978; Kelley and Iversen, 1979; Kelley et al., 1979; Eison et al., 1982; Kelley et al., 1985; Kalivas et al., 1985; Takano et al., 1985; Elliott et al., 1986; Elliott and Iversen, 1986). The neurokinins enhance locomotion via nigralstriatal and mesolimbic circuits (Kelley et al., 1985; Eison et al., 1982) and induce behavioral stereotypies via the nigralstriatal pathway (Stinus et al., 1978; Kelley and Iversen, 1979).

Systemic or striatal blockade of DA receptors or striatal lesions of DA terminals, block the activating effects of neurokinin midbrain infusions (Stinus et al, 1978; Kelley and Iversen, 1979; Kelley et al., 1979; Takano et al., 1985; Eison et al., 1982). This suggests that neuokinin-induced activity is mediated by striatal dopamine transmission.

Intranigral SP and NKA have been shown to activate dopamine neurons of the SNc (Walker et al., 1975; Davies and Dray, 1976) with NKA being far more potent (Innis et al. 1995). Microinfusion of neurokinins in the SNc and VTA results in increased DA and DA metabolites in the CPu (Boix et al., 1992; Baruch et al., 1988; Michelot et al., 1979) and NAc/PFC (Elliott et al., 1986; Kalivas et al., 1985) respectively. Within the VTA, SP increases DA in the PFC, whereas NKA increases DA in the NAc (Elliott et al., 1991; Kalivas et al., 1985). Systemic exposure to haliperidol (HAL), a DA receptor antagonist, blocks neurokinin-induced DA increases.

Both SP and NKA activate non-DA neurons of the SNr and as a result increase dopamine in the striatum (Innis et al., 1985; Pinnock and Dray, 1982). Therefore nigral neurokinins increase striatal DA by direct and indirect activation of DA neurons and

indirectly via activation of the nigro-thalamo-cortico-striatal loop (Reid et al., 1990). Neurokinins have also been shown to locally increase striatal DA (Khan et al., 1994; Tremblay et al., 1992).

Cocaine increases striatal DA levels by blocking its reuptake and the neurokinins mimic this action by the mechanisms enumerated above. Cocaine-induced DA increases the expression of the neurokinin precursor PPT-A and activates the direct striatalnigral pathway, thereby enhancing neurokinin release. This mutual amplification may lead to increases in activity and, over time, produce neural changes necessary for locomotor sensitization.

1.7.3 Cocaine and Dopamine Effects on PPT-A expression

Pharmacological blockade and DA lesions reduce striatal PPT-A mRNA expression providing evidence that DA is necessary for tonic PPT-A mRNA expression (Lindefors, 1992; Gerfen et al., 1991; Bannon et al., 1987; Voorn et al., 1987; Hanson et al. 1981). Following acute and chronic treatment with direct (apomorhpine) and indirect (GBR-12909, amphetamines) dopamine agonists, striatal PPT-A mRNA is upregulated (Zhang et al., 1997; Jolkkonen et al., 1995; Sivam, 1996; Hurd and Herkenham, 1992, Gerfen et al., 1991; Haverstick et al., 1989; Bannon et al., 1987; Li et al., 1987).

Few have looked at the direct effects of cocaine. Chronic, intermittent cocaine exposure at moderate doses is the paradigm that has been shown to elicit locomotor sensitization. Studies that have assessed PPT-A expression in response to cocaine have not used sensitization paradigms. One study used a high acute dose (30mg/kg) and a binge paradigm of 12.5mg/kg 3x/day for 14 days. Both acute and binge type exposure produced increased PPT-A mRNA levels (Mathieu-Kin and Besson, 1998; Hurd and

Herkenham, 1992). Another study measured PPT-A mRNA after 7 days of variable free access to cocaine during self-administration and reported increases in PPT-A mRNA (Hurd et al., 1992). Cocaine can clearly upregulate PPT-A mRNA expression under diverse conditions but it still remains to be shown how PPT-A mRNA expression is influenced by chronic, intermittent exposure. Our study is the first to measure PPT-A mRNA using a paradigm (15mg/kg 2x/day) widely implemented in cocaine sensitization studies (Henry and White, 1995).

Findings from studies using specific D1 and D2 ligands and D1R and D2R knockout mice suggest that, although D1Rs are co-expressed with PPT-A mRNA in GMSNs and their activation can positively influence PPT-A mRNA expression, trans-synaptic events mediated by D2R play a more significant role in DA initiated upregulation of PPT-A mRNA (Drago et al., 1996; Jolkkonen et al., 1995; Pollack and Wooten, 1992; Bannon et al., 1987; Haverstick, et al., 1989). Striatal acetylcholine, serotonin and glutamate have all been shown to increase PPT-A mRNA expression in the striatum (Brenə et al., 1993; Somers and Beckstead, 1992; Pollack and Wooten, 1992).

Our initial results showed that PPT-A mRNA increased over time in response to chronic cocaine in a manner compatible with a role in locomotor sensitization. The opioid mRNA precursors and dopamine receptors showed less compelling patterns of expression. These findings led us to focus on the role of the neurokinins in locomotor sensitization to cocaine for the remainder of our research.

1.7.4 Cocaine and Dopamine Effects on Peptide Levels

Pharmacological blockade or lesions of DA neurons has been shown to reduce substance P levels in the nigra (Bannon et al., 1987; Voorn et al., 1987; Hong et al.,

1978) and NAc (Hanson et al., 1981). Two laboratories found contradictory effects of systemic administration of apomorphine (APO) on neurokinin levels in the striatum. One reported that acute administration decreased striatal SP levels and chronic administration increased peptide levels in the striatum and substantia nigra (Li et al., 1987). The other reported no change in striatal or nigral SP in response to acute or chronic APO exposure but showed upregulation in both regions in response to amphetamine (Bannon et al., 1987). Chronic GBR12909, for 4 days, increased neurokinin peptide in the striatum and nigra (Sivam, 1996) but paradoxically, neither acute nor chronic cocaine had any effect on substance P levels in those areas (Sivam, 1989). Similar to the mRNA results, D2R antagonism decreased neurokinin peptide in the nigra (Bannon et al., 1987).

We used radioimmunoassay to assess tissue levels of substance P after acute, chronic (7 day) and a cocaine challenge to try to clarify these findings.

1.7.5 Receptors

Three neurokinin receptors have been cloned, sequenced and designated neurokinin 1, 2 and 3 (NK-1, NK-2 and NK-3)(Ohkubo, H. and Nakanishi, A 1992) (for reviews see Khawaja and Rogers, 1996, Patacchini and Maggi, 1995, Regoli et al., 1994 and Guard and Watson, 1991). Sequence and structural analysis has revealed homology with G-protein coupled receptors having seven transmembrane domains with extracellular amino termini and cytoplasmic carboxyl termini (Garland et al., 1996). Substance P has the highest affinity for neurokin (NK-1), neurokinin A for (NK-2) and neurokinin B for (NK-3). All neurokinins can interact with each receptor type therefore the presence of any of the receptors in areas of neurokinin availability implies functional relevance. Evidence suggests that the neurokinin receptors are positively coupled to the phospholipase C

signal transduction pathway stimulating the 2nd messengers diacylglycerol and inositol-3-phosphate and to a lesser degree activation of adenylyl cyclases and upregulation of cAMP (Khawaja and Rogers, 1996).

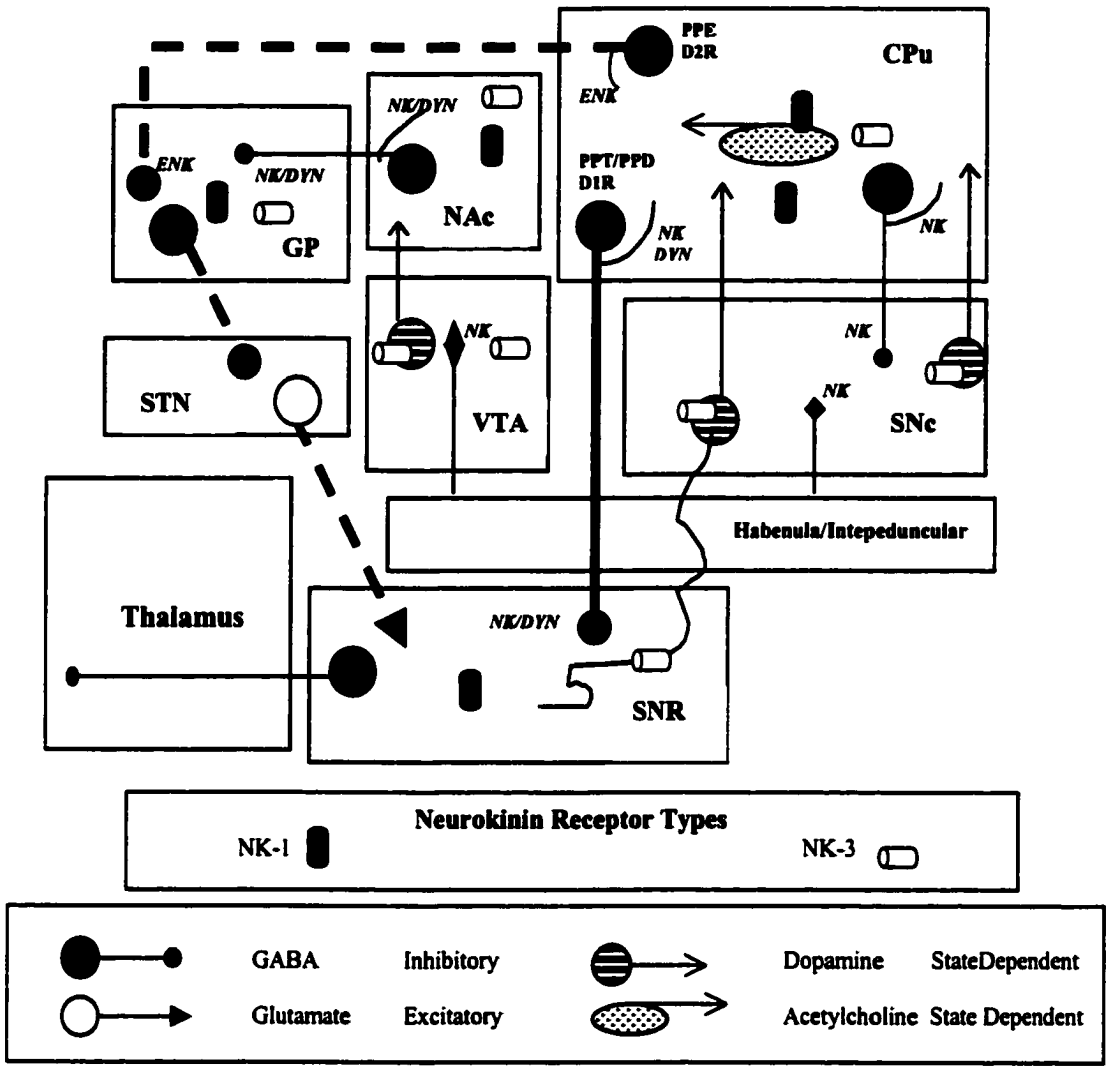
1.7.5.1 Localization

Moderate levels of SP type binding (Saffroy et al.,1988, Quirion, 1985, Mantyh et al., 1984, Quirion et al., 1983), NK-1 message (Whitty et al., 1995) and NK-1 immunoreactivity (Nakaya et al., 1994) and low levels of NKA/NKB binding (Saffroy et al., 1988, Mantyh et al., 1984) and NK-3 message (Whitty et al., 1995) have been detected in the striatum. Striatal NK-1 binding is found primarily in the matrix compartment and NK-3 in the patch (Tremblay et al., 1992). NK-2 receptors have not been detected in the striatum. Neurokinin receptors are expressed on intrinsic neurons of the striatum but some evidence suggests that they may also occur on non-DA afferent terminals (Stoessl, 1994; Arenas et al., 1991). In the striatum, NK-1 receptor mRNA is co-expressed with cholineacetyltransferase (ChAT) mRNA, the rate limiting enzyme in ACh production. SP-containing terminals, derived from collaterals of GABAergic medium spiny projection neurons have been shown to make synaptic contact on ACh interneurons (Aosaki and Kawaguchi, 1996; Gerfen, 1991). These large aspiny ACh interneurons make up only 2% of the striatal neuronal population but have wide reaching effects on extrapyramidal motor function because of their extensive arborizations (Bolam et al., 1984).

NK-3 mRNA has been detected at moderate levels in the GP (Shughrue et al., 1996) but only SP binding has been detected, suggesting NK-3 binding sites are on pallidal

efferent terminals and NK-1 receptors are localized to striatal afferent terminals (Saffroy et al., 1988, Mantyh et al., 1984).

FIG. 2 NEUROKININ RECEPTOR LOCALIZATION



NK-3 receptor message (Shughrue et al., 1996), binding (Stoessl et al., 1994) and immunoreactivity have been observed at moderate to low levels in the SNc. Evidence from double labeling mRNA studies (Whitty et al., 1995) and lesions coupled with autoradioraphy (Stoessl et al., 1994) indicate that NK-3 receptors are expressed on the dendrites and soma of DA neurons in the SNc. In the VTA NK-3 receptors are expressed on DA and non-DA neurons (Chen et al., 1998). NK-1 receptors in the SN have been

undetectable by immunoreactivity (Nakaya et al., 1994) but one autoradiographic (Stoessl et al., 1994) study and a sensitive solution hybridization study (Whitty et al., 1995) detected low levels of nigral NK-1 receptor mRNA. Nigral lesions did not affect NK-1 receptor mRNA levels suggesting that NK-1 receptors are expressed on non-DA cells in the SN. Striatal quinolinic acid lesions caused an increase in both NK-1 and NK-3 receptor mRNA in the SN indicating that the receptors are not located on striatal afferent terminals and that striatal projections tonically inhibit neurokinin mRNA expression (Bannon and Whitty, 1995).

1.7.5.2 Behavior and Physiology

Considerable evidence supports a role for the NK-3 receptor in mediating the locomotor activating effects of NKA in the VTA and SNc. Invivo infusion of specific NK-3 agonists into the SNc (Stoessl et al., 1991) and VTA (Elliott et al., 1991) increase motor activity in rats and increase DA release in the striatum and NAc respectively. In nigral slices, NK-3 agonists increase the firing rate of dopamine cells (Keegan et al., 1995; Seebrook et al., 1993) whose activation is not blocked by specific NK-1 or NK-2 antagonists (Seabrook et al., 1995). These findings support the idea that increased locomotion is a result of NK-3 activation of DA cells resulting in increased striatal dopamine.

The preferred receptor for NKA is NK-2 but it has not been detected in the SN. The NK-3 receptor is the most abundant of the neurokinin receptors in the SN. The NK-3 receptor has twice the affinity for NKA as for SP. Intra-VTA infusion of NKA peptide results in increased locomotor activity in rats (Kalivas et al., 1985). Infusions of NKA in the VTA and SNr increase DA release in the NAc/PFC (Kalivas et al. 1985) and striatum

(for summary see Hokfelt et al., 1991; Reid et al., 1990) respectively. In *in vitro* nigral slice preparations NKA excited DA cells more potently than non-DA cells (Innis et al., 1985). Thus a reasonable interpretation of these data is that NKA is the primary ligand for the NK-3 receptors in the SNc and VTA activating DA neurons and increasing DA release in the striatum resulting in enhanced activity.

Many studies have shown that direct administration of SP into areas of the VTA (Kalivas et al., 1985; Kelley et al., 1985; Pinnock et al., 1983; Eison et al., 1982) and SNr (Kelley et al., 1985; James and Starr, 1977) produces increased locomotor activity in rats. SP has also been reported to increase locomotion when directly administered into the ventral globus pallidum. SP directly applied to the VTA resulted in increased DA release in the NAc (Elliott et al.,1986) and PFC (Elliott et al., 1986; Kalivas et al., 1985) and when applied to the SNr, increases in DA were reported in the striatum (Hokfelt et al., 1991; Reid et al., 1990). Substance P has been reported to be a more potent activator of non-DA cells of the SNr than DA cells of the SNc (Collingridge and Davies, 1982; Pinnock and Dray, 1982). Following administration directly to the SNr or VTA, increases in DA were reported in the striatum and NAc/PFC respectively.

Administered within the SNc, specific NK-1 agonists had no effect on striatal DA release (Elliott et al., 1991) or locomotor activity (Stoessl et al., 1991) but NK-1 agonists administered directly into the VTA increased locomotion and DA release in the NAc and PFC (Elliott et al., 1991). The most reasonable explanation of these findings is that SP, acting at NK-1 receptors in the midbrain, increases DA release in the mesolimbic circuit by direct and indirect effects on DA neurons of the VTA and in the nigralstriatal circuit via the nigral-thalamic-cortical-striatal loop.

Another source of neurokinin mediated increases in striatal DA occurs when neurokinins are released in the striatum via axon collaterals of neurokinin expressing medium spiny neurons. NK-1 and NK-3 agonists elicit ACh from striatal slices in a concentration dependent manner and NK-1 agonists evoke ACh release from 6-OHDA lesioned animals suggesting a non-dopaminergic mechanism (Arenas et al., 1991). Local perfusion of SP in the dorsal striatum of freely moving rats, via reverse microdialysis, showed subsequent elevations of ACh (51%) in a dose dependent manner. Although an NK-1 antagonist by itself had no effect on ACh release, it did prevent SP induced release, suggesting that SP activation of ACh is not tonic (Anderson et al, 1993). Further *in vitro* evidence suggests that neurokinins indirectly upregulate striatal DA via NK-1 mediated ACh release in the patch and matrix and via presynaptic NK-3 activation in the patch (Kahn et al., 1995; Tremblay et al., 1992). The NK-1 receptor has 100-1000 times the affinity for SP as for NKA suggesting that substance P is the primary ligand for striatal NK-1 receptors.

Taken together, this evidence suggests that the locomotor activating effects of NKA are mediated by direct action on midbrain dopamine neurons via NK-3 receptors. The primary effects of SP occur via the nigro-thalamo-striatal loop, activation of large striatal aspiny cholinergic neurons. A common effect of neurokinins acting both in the SN and in the striatum is to increase striatal DA. Psychostimulants and neurokinins increase motor activity via their ability to increase the efficacy of synaptic dopamine in the striatum.

1.8 Hypothesis and Research Aims

1.8.1 Acute Cocaine

Tonically active nigralthalamic output from the basal ganglia inhibits the thalamus thus, inhibiting locomotor activity. Internal basal ganglia circuits facilitate motor activity by inhibiting the nigralthalamic projection thereby releasing the thalamus from inhibition (Chevalier and Deniau, 1993; McGeer and McGeer, 1993). The balanced opposition model, describes a push-pull strategy for regulation of nigralthalamic output and motor activity. Activation of the direct striatalnigral pathway inhibits the nigralthalamic projection thus facilitating motor activity while activation of the indirect striatalnigral pathway, activates the nigralthalamic projection, and inhibits motor activity. Cocaine acting as an indirect dopamine agonist, facilitates motor activity by increasing activation of the direct and inhibiting activity in the indirect pathways (Gerfen, 1992).

Enhanced glucose metabolism and immediate early gene expression have been associated with increased neural activity. Acute systemic cocaine exposure has been shown to increase striatal and nigral glucose metabolism in humans (Volkow and Fowler, 1995) and striatal immediate early genes in rats (Graybiel et al., 1995). In addition, the immediate early gene c-fos is preferentially upregulated in striatal PPT-A expressing neurons in response to acute cocaine (Kosofsky et al., 1995, Johansson, et al. 1994).

We expected that acute cocaine, by increasing striatal dopamine, would activate neurons of the direct striatalnigral pathway thus increasing striatal and nigral neurokinin release. We reasoned that increased neurokinin release should be reflected in decreased tissue levels of peptide in the striatum and substantia nigra due to increased utilization.

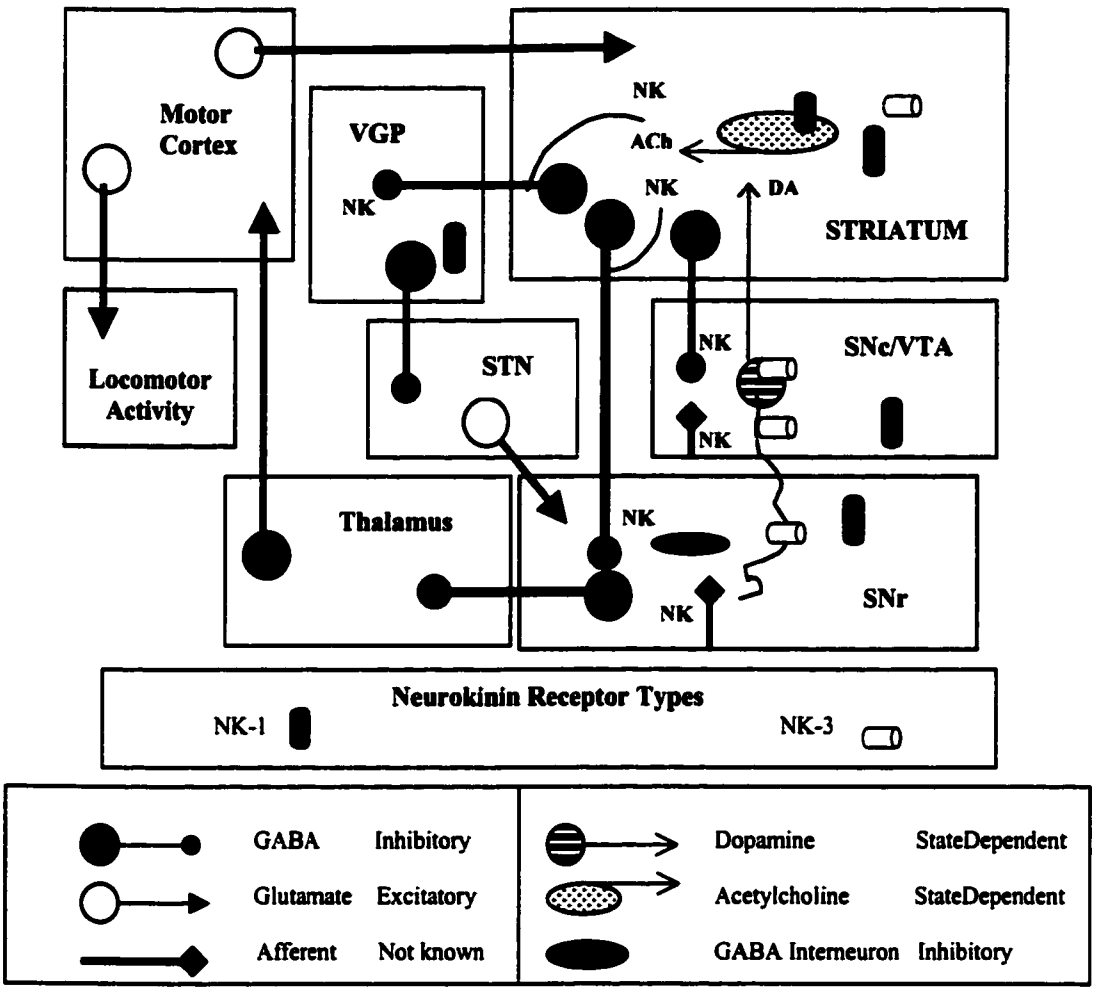
One prior study showed no change in striatal SP measured by RIA in response to acute cocaine (Sivam et al., 1989) but a separate study reported increases in response to acute methamphetamine (Bannon et al., 1987). These differences are most likely due to differences in timing between final drug exposure and animal sacrifice, which would determine whether sufficient time elapsed for compensatory increases in expression to mask reductions due to increased release and utilization.

Increases in PPT-A have been reported in response to acute cocaine (30mg/kg) (Hurd and Herkenham, 1992) and methamphetamine (Zhang et al., 1997). Our aim was to measure tissue levels of SP by radioimmunoassay after acute cocaine exposure and PPT-A mRNA by *in situ* hybridization histochemistry.

The neurokinins acting at NK-1 receptors in the SNr and VTA have been shown to increase motor activity. NK-3 receptors increase activity by actions in the VTA, SNc and SNr. Cocaine, acting as an indirect DA agonist in the striatum, could induce activation of neurokinin expressing cells thereby increasing neurokinin release. Enhanced neurokinin release in the striatum, VGP and midbrain regions of the SNr, VTA and SNc could be responsible for the hyperactivity associated with the response to acute cocaine and other psychostimulants.

To test this hypothesis we conducted experiments in which NK-1 and NK-3 antagonists were administered systemically prior to acute cocaine exposure. We predicted that the neurokinin antagonists would block the expression of acute cocaine induced hyperactivity.

FIG. 3 NEUROKININ CIRCUITS AND MOTOR ACTIVITY



1.8.2 Chronic Cocaine and the Neurokinins

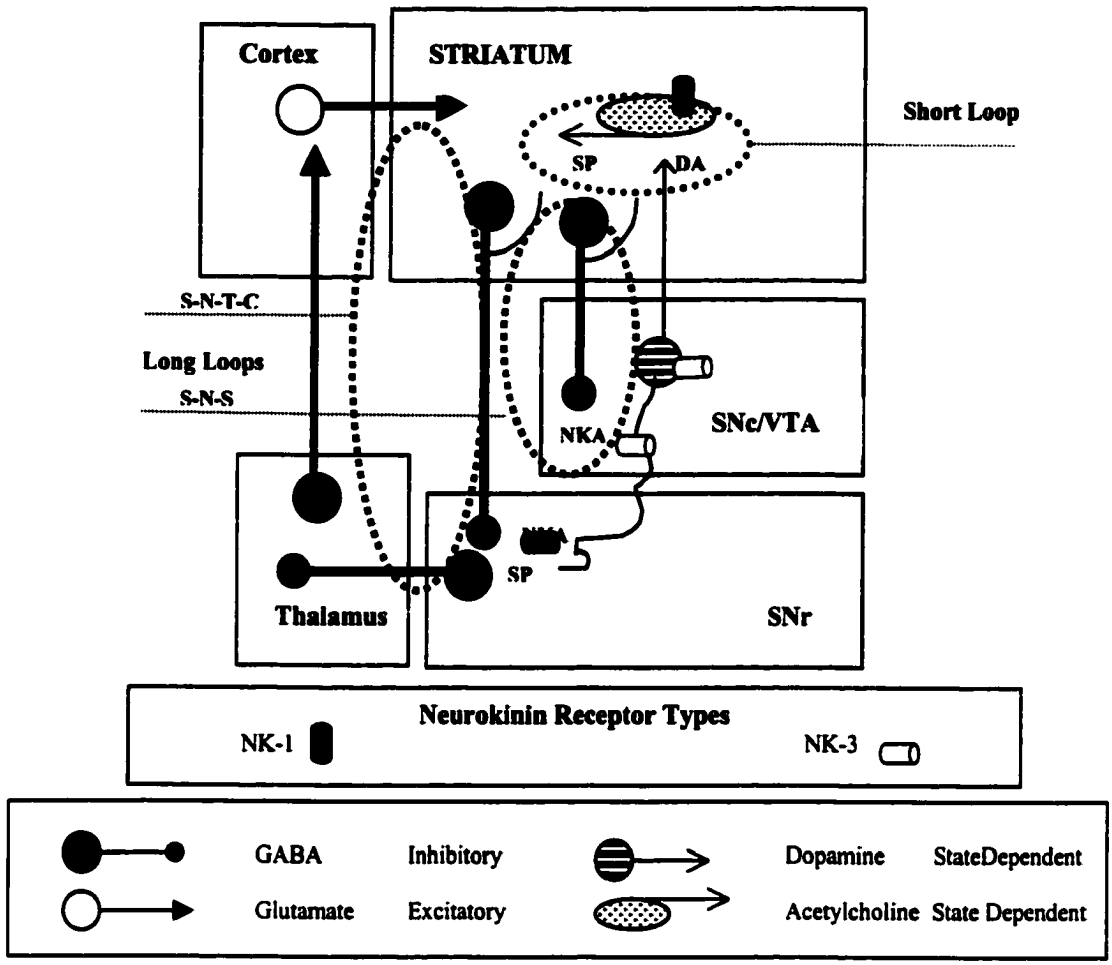
The acute motor stimulatory effects of cocaine, depends on DA transmission in the striatum (Koob and Goeders, 1989) suggesting that enhancements in DA transmission may underlie the escalating motor activity associated with locomotor sensitization. This has been widely supported by findings that pretreatment with cocaine or other psychostimulants results in enhanced extracellular levels of dopamine measured by microdialysis or voltammetry in response to subsequent cocaine exposure (for review see Kalivas, 1995).

The neurokinins, via activation of NK-1 and NK-3 receptors, have been shown to increase striatal DA release (Kahn et al., 1995; Humpel and Saria, 1993; Tremblay et al., 1992; Elliott et al., 1991) and chronic cocaine, acting as an indirect DA agonist, has been shown to upregulate the expression of the striatal neurokinin precursor mRNA PPT-A (Mathieu-Kin, 1998; Hurd and Herkenham, 1992).

Our model suggests that cocaine induced enhancement of neurokinin expression leads to increased nigral and striatal neurokinin release. Elevated levels of neurokinins acting at NK-3 receptors can exert positive feedback on striatal DA release via the striatal-nigral-striatal loop and at NK-1 receptors via a striatal short loop or a striatal-nigral-thalamic-cortical-striatal long loop (**Fig. 4**).

We propose that neurokinin/dopamine circuit amplification is necessary for locomotor sensitization to chronic cocaine and therefore blockade of NK-3 or NK-1 receptors will prevent locomotor sensitization. Because the striatum has been implicated in mediating the expression of sensitization (Vezina, P., 1996; Kelley et al. 1995; Kalivas and Stewart 1991; Robinson and Becker 1986) we predict that blockade of NK-1 receptors, located in the striatum and substantia nigra reticulata, will block the expression of sensitization. Animals pretreated with chronic cocaine will receive either a cocaine or cocaine + antagonist challenge, following three days of withdrawal. We expect that the group receiving the cocaine challenge will show enduring sensitization but the group exposed to a cocaine + antagonist challenge will not.

FIG. 4 NEUROKININ - DOPAMINE AMPLIFICATION



The ventral tegmental area and substantia nigra compacta have been implicated in mediating the development of sensitization (Vezina, P., 1996; Cador et al. 1995; Kalivas and Stewart 1991; Robinson and Becker 1986), therefore we predict that blockade of NK-3 receptors will block the development of sensitization. Animals chronically pretreated with cocaine and challenged with cocaine will exhibit enduring sensitization but animals chronically pretreated with cocaine + antagonist and challenged with cocaine will not.

Based on the incentive-sensitization theory of drug addiction (Robinson and Berridge, 1993) if neurokinin antagonists are able to block the development or expression

of locomotor sensitization they may have the pharmacological potential to block drug craving associated with sensitization of circuits mediating drug craving.

2 MATERIALS AND METHODS

2.1 Animals

Male Sprague-Dawley rats (supplied by Charles River or Harlan) weighing approximately 225-275g at the start of the experiment, were maintained two per cage in a temperature-controlled environment under a 14:10 h light/dark cycle with free access to food and water.

2.2 Drug Administration

Intraperitoneal(i.p.) injections of vehicle or vehicle + drug were administered in a total volume of 0.5ml. The vehicle for cocaine (Sigma, cocaine HCL, C-5776) was phosphate buffered saline (PBS). For experiments which assessed the interaction of cocaine and neuropeptide ligands, vehicle or ligand + vehicle were administered i.p. 15 or 30 minutes prior to cocaine or PBS administration. Ligand/vehicle and cocaine/PBS were injected on opposite sides of the animal. In one experiment neuropeptide ligand was delivered continuously via surgically implanted osmotic mini-pumps (Alzet Inc., 2ML1) located subcutaneously in the mid-scapular region and cocaine was administered systemically as described. The mini-pumps contained a total volume of 2ml (vehicle or vehicle + drug) delivered over the course of 7 days at a flow rate of 10ul/hour. The pumps were implanted between 3:00 and 6:00PM the day before the first cocaine injection at 10:30AM. The pumping rate does not reach steady state for approximately 4-6 hours so prior to the first cocaine exposure rats received approximately 120ul of drug.

2.3 Surgery

For implantation of minipumps the animals were anesthesized with an intramuscular injection of Ketaset/PromAce. The skin on the left mid-scalpular region was shaved with

electrical clippers and swabbed with a sterile pad moistened with 70% isopropanol. A mid-scapular 4.5 cm. incision was made and the subcutaneous tissue spread by the insertion of a hemostat to create a pocket for the 3.5 cm. mini-pump. The filled mini-pump was then inserted with the delivery portal first, closed with wound clips, and treated with topical antibiotic. Animals were closely monitored and kept warm with a heating pad until fully recovered. Seven days after implantation animals were anesthesized with methane gas, the wound reopened, and the mini-pump removed. The wound was reclosed with wound clips and the animals were closely monitored until fully awake and moving freely about at which time they were returned to their home cage.

2.4 Drug Treatment Paradigms

Initial experiments determined the effects of chronic intermittent cocaine on preproenkephalin, preprodynorphin, preprotachykinin and tyrosine hydroxylase mRNA and dopamine (D1R and D2R) and neurokinin-1 (NK-1) receptor levels. Animals were injected i.p.with drug or vehicle 2x/day at 10:00AM and 3:00PM for either 1 (Acute), 3, 6 or 14 days. These experiments consisted of three groups (n=8/group). Group I, the control, received PBS, Group II, cocaine (15 mg/kg) and Group III cocaine (15mg/kg) co-administered with MK-801(1mg/kg). This cocaine paradigm was chosen because it had been reported by others to produce locomotor sensitization in rats (Beitner-Johnson and Nestler, 1991; Henry et al., 1989).

Blockade of NMDA (non-methyl-D-aspartate) receptors by the non-competitive NMDA receptor antagonist MK-801 during cocaine exposure has been shown to block or attenuate locomotor sensitization to cocaine and associated biochemical changes (for review see Wolf, 1998). Our hypothesis was that striatal peptides or receptors that

change in response to a time course of chronic, intermittent cocaine administration may reflect a role for them in locomotor sensitization. This conclusion would be strengthened if the cocaine-induced changes could be blocked or attenuated by concurrent administration of cocaine and the NMDA receptor antagonist MK-801.

On the last day of the experiments animals received only one injection at 10:00 and were sacrificed at 3:00, 5 hours after the last injection. They were decapitated by guillotine, the brains removed and immediately frozen on powdered dry ice and stored at -80° for subsequent processing by *in situ* hybridization histochemistry or receptor autoradiography.

TABLE 1. ACUTE DRUG ADMINISTRATION

	Group I	Group II	Group III	
Day	10:00 AM	10:00 AM	10:00 AM	3:00 PM
1	PBS	Cocaine	Cocaine + MK801	Sacrifice

TABLE 2. CHRONIC INTERMITTENT DRUG ADMINISTRATION

	Group I		Group II		Group III		
Day	10:00 AM	3:00 PM	10:00 AM	3:00 PM	10:00 AM	3:00 PM	3:00 PM
1-2, 1- 5, or 1- 13	PBS	PBS	Cocaine	Cocaine	Cocaine + MK-801	Cocaine + MK801	
3, 6 or 14	PBS		Cocaine		Cocaine + MK-801		Sacrifice

To assess whether changes observed with chronic drug exposure are enduring we measured a subset of the parameters described above after a cocaine challenge. There were two groups, Group I received PBS and Group II received cocaine (15 mg/kg), 2x/day during 14 days of chronic treatment. Animals remained treatment free for 21 days (withdrawl). After withdrawal both groups received a challenge of cocaine (15 mg/kg) at 10:00 AM and were sacrificed that same day at 3:00 PM, five hours after the last drug exposure. They were decapitated by guillotine, the brains removed and immediately

frozen on powdered dry ice and stored at -80° for subsequent processing by *in situ* hybridization histochemistry or receptor autoradiography.

TABLE 3. CHRONIC DRUG ADMINISTRATION, WITHDRAWAL AND DRUG CHALLENGE

	Group I		Group II		Group III		
Day	10:00 AM	3:00 PM	10:00 AM	3:00 PM	10:00 AM	3:00 PM	3:00 PM
1-14	Vehicle	Vehicle	Vehicle	Vehicle	Cocaine	Cocaine	
15-36							
37	Vehicle		Cocaine		Cocaine		Sacrifice

2.5 Behavior

2.5.1 Manual Assessment of Horizontal Locomotion and Stereotypy

The first experiments, undertaken to assess behavior, implemented the 14 day chronic treatment paradigm described in **Table 2.** Two pretreatment days were added to acclimate the animals to the mechanics of the experiment. On the first pretreatment day animals were injected with vehicle in the test cage and behavior was monitored. On the second pretreatment day they were injected with vehicle in their home cage. There were two experimental groups (n=8/group), Group I, the control, received vehicle and Group II received cocaine (15 mg/kg) or methamphetamine (4mg/kg) 2x/day. The behavior of eignt animals was assessed together, four from each experimental group. Behavior was measured manually on days 1, 3, 6, 10 and 14 after the 10:00 AM injections. Plastic cages, identical to the home cage, were taped on the bottom under side with 2 evenly spaced vertical red strips and 2 evenly spaced horizontal red strips, dividing the space into 9 regions. A horizontal locomotor count was registered, on a hand held counter, each time a rat crossed a tape strip with its nose followed by both front paws. Bobbing was measured by a hand held counter, one count was registered for each bob as it

occurred. Rearing and sniffing, were measured by hand held timers. When rearing or sniffing was initiated the counter was started and subsequently stopped when the behavior ceased. Each animal was injected and immediately monitored for 2 minutes. When all animals had been assessed the process was repeated. A total of three 2 min.sessions were conducted approximately 20 mins. apart for each animal. Two investigators each held both a counter and a timer. One investigator monitored and recorded horizontal counts and sniffing and the other investigator monitored and recorded bobbing and rearing. Investigators were not blind to the experimental conditions having administered the injections. This experiment was undertaken to determine if, in our hands, sensitization to psychostimulants could be observed before we made an investment in expensive automated equipment. After successfully measuring behavior sensitization we obtained automated testing equipment with which we conducted the remainder of the experiments.

2.5.2 Determination of a Sensitization Paradigm for Use With Antagonists

A number of drug treatment paradigms (n=10/group) were assessed using automated equipment in order to determine an optimum sensitization paradigm for cocaine and methamphetamine. We assessed locomotor activity every other day for 13 days while administering cocaine (15 mg/kg) 2x/day. The acute response to this drug dose was robust and locomotor activity peaked by day 5. By reducing the dose of cocaine in subsequent experiments we expected to reduce the acute effect thereby producing more reliable differences between acute and chronic activity levels which would be our measurement of sensitization. We next tried 10 mg/kg cocaine and 2 mg/kg methamphetamine 1x/day for 14 days followed by 3 days withdrawal and a subsequent

challenge injection at the same dose (**Table 4**). The cocaine paradigm produced a stepwise increase in locomotor activity over the entire time course and an acute response that was significantly higher than controls but low enough so that later time points resulted in sensitization. The acute response to methamphetamine was too high at 2mg/kg so we repeated the experiment with 1mg/kg and obtained satisfactory results.

2.6 Experimental Design For Behavior

2.6.1 Drug Treatment

Cocaine (10 mg /kg body weight) or methamphetamine (1mg /kg body weight) was administered once per day for seven days followed by three days withdrawal and a subsequent psychostimulant challenge at the same dose (**Table 4**). The drug free withdrawal period provided the time necessary for drug clearance after chronic treatment so that enduring rather than cumulative effects of the psychostimulants could be assessed. The paradigm was used to assess the effects of the neurokinin antagonists on acute cocaine induced hyperactivity and the development and expression of cocaine induced locomotor sensitization. Yong Zhang, another graduate student in the laboratory, implemented the same paradigm to assess the effects of a cocaine or methamphetamine challenge on dopamine and glutamate release after early withdrawal.

TABLE 4. CHRONIC DRUG ADMINISTRATION, 3 DAYS WITHDRAWAL AND CHALLENGE

	Group I	Group II	
Day	10:00 AM or 1:00 PM	10:00 AM or 1:00 PM	3:00 PM or 6:00 PM
1-14, or 1-7	PBS	Cocaine or Methamphetamine	
8-10 or 15-17			
11 or 18	PBS	Cocaine or Methamphetamine	Sacrifice

Ligand or vehicle was administered i.p. 15 or 30 minutes prior to cocaine administration or continuously by osmotic mini-pumps as described. On the first two days of the experiment (pretreatment) all animals received injections of vehicle alone. On the first day vehicle pretreatment was administered in the test cage prior to behavior assessment and on the second day it was administered in the home cage. Pretreatment administration and behavior testing on the first day began between 1:00 and 1:30PM. The behavior measurements recorded on the first day of pretreatment served as a baseline response to the injection and test environment. On the day of the last injection animals were sacrificed between 3:30 and 4:00, 5 hours after the last injection. They were exposed to CO_2 for 2.5min, decapitated by guillotine and the brains quickly removed. The brains were then either immediately frozen on powdered dry ice or dissected and stored at -80° for later processing.

2.6.2 Experimental Paradigm

Five distinct experimental groups were defined in order to assess the interaction of the ligand and cocaine on acute induced hyperactivity and the expression and development of drug sensitization but not all groups were included in all experiments due to the limited supply of antagonists. Group I received ligand vehicle, prior to PBS and Group II received ligand vehicle prior to cocaine at all time points. Group III received vehicle prior to cocaine for 7 days and the ligand prior to cocaine on the challenge day. Group IV was administered ligand prior to cocaine for 7 days and on the challenge day, ligand vehicle prior to cocaine. Group V received ligand alone for 7 days and cocaine alone as a challenge. All groups were injected with ligand vehicle and PBS for two days (pretreatment) prior to starting the regimen described above (**Table 5**).

TABLE 5. DRUG ADMINISTRATION PARADIGM

	Group I Control		Group II Cocaine		Group III Expression		Group IV Development		Group V
Day	i.p. 1 or pump	i.p. 2 or i.p. 1	i.p. 1 or pump	i.p. 2 or i.p. 1	i.p. 1 or pump	i.p. 2 or i.p. 1	i.p. 1 or pump	i.p. 2 or i.p. 1	i.p.
-2	Vehicle	PBS	Vehicle	PBS	Vehicle	PBS	Vehicle	PBS	Vehicle
-1	Vehicle	PBS	Vehicle	PBS	Vehicle	PBS	Vehicle	PBS	Vehicle
1	Vehicle	PBS	Vehicle	Cocaine	Vehicle	Cocaine	Ligand	Cocaine	Ligand
2	Vehicle	PBS	Vehicle	Cocaine	Vehicle	Cocaine	Ligand	Cocaine	Ligand
3	Vehicle	PBS	Vehicle	Cocaine	Vehicle	Cocaine	Ligand	Cocaine	Ligand
4	Vehicle	PBS	Vehicle	Cocaine	Vehicle	Cocaine	Ligand	Cocaine	Ligand
5	Vehicle	PBS	Vehicle	Cocaine	Vehicle	Cocaine	Ligand	Cocaine	Ligand
6	Vehicle	PBS	Vehicle	Cocaine	Vehicle	Cocaine	Ligand	Cocaine	Ligand
7	Vehicle	PBS	Vehicle	Cocaine	Vehicle	Cocaine	Ligand	Cocaine	Ligand
8	----	----	----	----	----	----	----	----	
9	----	----	----	----	----	----	----	----	
10	----	----	----	----	----	----	----	----	
11	Vehicle	PBS	Vehicle	Cocaine	Ligand	Cocaine	Vehicle	Cocaine	Cocaine

To assess acute cocaine effects, activity levels of Group II and Group I were compared on day 1 and the activity levels of Group II after PBS pretreatment and acute cocaine treatment were also compared. Both of these comparisons provided different ways of assessing whether cocaine induced hyperactivity. Activity levels of Group II and Group IV after acute treatment and activity levels of Group IV after PBS pretreatment and acute ligand + cocaine treatment were compared to assess whether the ligand had an effect on the acute cocaine response.

To assess chronic/progressive cocaine effects, activity levels of Group II and Group I were compared on day 7 and activity levels of Group II on day 1 and day 7 were compared. Both of these comparisons provide different ways of assessing whether chronic cocaine induces progressive locomotor sensitization. Activity levels of Group II and Group IV were compared for day 7 and activity levels of Group IV on day 7 and day 1 were compared to assess whether the ligand had an effect on cocaine induced progressive sensitization.

To assess enduring sensitization, after early withdrawal, activity levels of Group II and Group I were compared after drug challenge and activity levels for day 1 and challenge were compared for group II. Results from both comparisons will indicate whether locomotor sensitization to cocaine was sustained after early withdrawal. A comparison of activity levels of Group II and Group IV after challenge and of Group IV on day 1 and challenge will suggest whether the ligand has an effect on the development of sensitization. A comparison between activity levels of Group II and Group III after challenge and of Group III on day 1 and challenge will indicate whether the ligand has an effect on the expression of sensitization. If Group IV is significantly different from Group II on day 7 and Group III and IV are significantly different from Group II after challenge and these changes are in the same direction this would be strong evidence for an effect of the ligand on the expression of sensitization. If, on the other hand, Group IV and Group II are significantly different on day 7 and after challenge but Group III and Group II are not this would be strong evidence that the effect of the ligand is on the induction/development of sensitization.

The NK-1 antagonist LY306740, was provided by Dr. I Iyengar at Eli Lilly. Dr. Iyengar and her colleague Dr. Phebus recommended dose and vehicle based on unreported *in vivo* experiments done in their laboratory. The NK-1 antagonist CP099,994, was provided by Dr. Pagani at Pfizer. An *in vivo* study in rats (Sluka et al. 1997) showed an effect of 30mg/kg CP099,994 on heat hyperalgesia in rats with no ill effects and after discussing this with Dr. Pagani we decided on this dose for our study. A number of other *in vivo* studies employed a wide range of doses for this drug. The NK-1 antagonist SR140333 was provided by Dr. X. Emonds-Alt at Sanofi recherche. In a personal

communication he described two *in vivo* studies in rats employing chronic i.p. administration of 1 and 2 mg/kg SR140333 with no adverse effects. The NK-3 antagonist PD161182 was supplied by Martyn Pritchard of Parke-Davis and he recommended the dose we used.

TABLE 6. SYNTHETIC STRIATAL PEPTIDE RECEPTOR LIGANDS

Compound	Chemical Name		Source	Type	Dose	Vehicle
PD161182	(S)iso-propylbenzyloxycarbonyl(R)α-Me(2,3-difluoro)Phe-$(CH_2)_7NHCONH_2$	Ke 6nM GP Hab IC50 32nM rat CC	Parke Davis	NK3 Antagonist	30mg/kg	PEG-400(45%) EtOH (15%) H20 (40%)
CP-99,994	((2S,3S)-cis-3-(2-methoxybenzylamino)-2-phenyl piperidine) dihyrochloride	PKb 8.0 GPI IC50 CHO 0.53nM IC50 3.0nM Ferret Cortex	Pfizer	NK1 Antagonist	30mg/kg	PBS (45%) H20 (45%) DMSO (10%)
SR140333	((S)-1-{2-[3-(3,4-dichlorphenyl))-1 (3-isopropoxypheylacetyl)piperidin-3-yl] ethyl}-4-phenyl-1 azaniabicyclo[2.2.2] octane	PKb 9.0 GPI IC50 CHO 0.04nM IC50 Ferret 0.5nM	Sanofi Recherche	NK1 Antagonist	2mg/kg	PBS (50%) H20 (50%)
LY306740			Eli Lilly	NK1 Antagonist	3mg/day (pump) 15mg/kg (i.p.)	Dissolved in acidic H2O pH to 6.5 with NaOH
Naltrindole HCL	17-Cyclopropylmethyl-6,7-dehydro-4,5-epoxy-3,14-dihydroxy-6,7,2',3'-indolomorphinian HCL		RBI N-115	δ Antagonist	0.3mg/kg	PBS (48%) H20 (48%) 2% DMSO
U-69593	(5α, 7α, 8β)-(+)-N-Methyl-N[7-(1-pyrrolidinyl)-1-oxaspirol[4,5]dec-8-yl]-benzeneacetamide		RBI	κ Agonist	0.16mg/kg	PBS (80%) Propylene Glycol (20%)

Three different NK-1 antagonists were used. The Eli Lilly compound was only soluble in acidic solvent and could not be chronically injected therefore based on the

manufacturers recommendation it was continuously delivered by osmotic mini-pump in very small volumes to reduce its irritating effects.

2.6.3 Automated Behavior Assessment

Locomotor activity was measured in behavioral testing equipment purchased from San Diego Instruments, San Diego, CA. Plastic activity cages (30 X 50 cm), identical to the animal's home cage, were placed in metal frames equipped with four photoelectric beams that divide the activity cage space into four quadrants (12.5 X 30 cm each). Each activity cage is activated by a separate switch on the controller unit. Photobeam interruptions are registered as digital pulses by a computer and recorded by computer software from the manufacturer. Each time a photobeam is interrupted it is recorded as an activity count. When two photobeams are interrupted sequentially the system registers an ambulation. Ambulations represent horizontal locomotion. The software from the manufacturer generated data files containing total photocell counts and ambulations for each 10 min interval and totals for the each session for each test cage. The testing equipment was contained on a two tiered movable cart which was either placed in the same room in which the animals were housed or in a separate room a short distance away within the animal facility.

Prior to the initiation of behavior testing animals were removed from their home cages and placed in an identical test cage with a fresh covering of beta but no food or water . If the testing apparatus was in a nearby room the animals were placed in their test cages, placed on a rolling cart and transported to the behavior room. Habituation to the test cages was initiated when the test cage was placed within the metal frame on the apparatus and activated via the switch on the controller. Activity levels were recorded in

10 min. intervals for 1 hour. At the end of the hour the activity cages were switched off. The test cages were then transferred, one at a time, to a cart and the animal injected i.p. with psychostimulant drug or vehicle, after which the test cage was immediately returned to the frame and activated. Activity levels were again recorded in 10min intervals for 1.5hours. If the experiment included neuropeptide ligands, the ligand or its vehicle was administered after the 1 hour habituation, in the same manner described, 15 or 30 minutes prior to the administration of psychostimulant drug because the neuropeptide ligands diffuse more slowly than cocaine or methamphetamine. After the testing session animals were immediately returned to their home cages. On the last day of drug exposure animals were taken from their home cages 4.5 hours after drug injection, exposed to CO_2 for 2.5 minutes and sacrificed by decapitation. Brains were removed and processed for *in situ* hybridization or radioimmunoassay as described below.

2.6.4 Experimental and Test Groups

Each experiment had 3 to 5 experimental groups with 10 animals/group. Experiments with the NK-3 antagonist (PD161182) used 6 animals/group because there was a limited amount of ligand provided by Parke Davis. Because there were more animals in an experiment than activity cages available, the animals were randomly placed in two or more test groups (Group A-E, **Table 7**). A test group contained an equal number of animals from each experimental group and these animals were tested in the behavioral apparatus at the same time. The onset of the experiment was staggered by one day for each test group. Locomotor activity was assessed every other day during the initial 14 day trial experiments (7 timepoints) and one of these experiments included 3 days withdrawal followed by a drug challenge (8 timepoints). All subsequent

experiments assessed locomotor activity on the first day of pretreatment, day 1 (acute), day 7 (chronic) and challenge day for all subsequent experiments (4 timepoints).

TABLE 7. TEST GROUPS AND EQUIPMENT SCHEDULING

	Test Groups				
Day	Group A	Group B	Group C	Group D	Group E
-2	pretest	xxxxx	xxxxx	xxxxx	xxxxx
-1	------	pretest	xxxxx	xxxxx	xxxxx
1	Day(1)	------	pretest	xxxxx	xxxxx
2	------	Day(1)	------	pretest	xxxxx
3	------	------	Day(1)	------	pretest
4	------	------	------	Day(1)	------
5	------	------	------	------	Day(1)
6	------	------	------	------	------
7	Day(7)	------	------	------	------
8	www	Day(7)	------	------	------
9	www	www	Day(7)	------	------
10	www	www	www	Day(7)	------
11	Challenge(sac)	www	www	www	Day(7)
12	xxxxx	Challenge (sac)	www	www	www
13	xxxxx	xxxxx	Challenge(sac)	www	www
14	xxxxx	xxxxx	xxxxx	Challenge (sac)	www
15	xxxxx	xxxxx	xxxxx	xxxxx	Challenge (sac)

2.6.5 Quantification and Analysis of Behavior

All data was analyzed by analysis of variance (ANOVA) using software from Statview. Interval data for all timepoints of an experiment, generated by PASF software from San Diego Instruments, was imported into microsoft Excel and arranged by day and interval before importing into Statview. Groups were compared by day and interval using repeated measures ANOVA. Daily interval data was combined and ANOVA comparisons between days for each group were generated. Fisher's protected least significant difference (PLSD) and Student-Newan-Keuls were used for post-hoc comparisons between groups when ANOVA results were significant. Values of $p < 0.05$ were considered significantly different.

2.7 Solid Phase Radioimmunoassay

Radioimmunoassay was performed on dissected tissue homogenate from animals receiving acute, chronic or early withdrawal and challenge drug treatment.

After removal of the brains they were floated in aluminum foil boats on ice water. The brains were placed in a brain matrix (ASI instruments, RBM 4000C) and 1mm coronal sections were cut at the levels of the nucleus accumbens, caudate putamen, globus pallidus and substantia nigra. The sections were placed on moist filter paper resting on a plastic surface (upside down petri dish) over ice and the nucleus accumbens, caudate putamen, ventral striatum and globus pallidus were dissected from either the right or left hemisphere. The caudate putamen was further divided into dorsal and ventral regions. The substantia nigra was taken from both hemispheres and included the SNr, SNc and VTA. The tissue was placed in eppendorf tubes (1.5ml) and frozen at -80°.

All processing of tissue samples, was performed on ice. 10 samples were always processed together, 5 from a control animal (representing all 5 brain regions dissected) and 5 from a cocaine treated animal. 200ul of cold 2N acetic acid was added to each sample and manually homogenized. A grinder was prepared from dental cement that was molded in an eppindorf tube. The grinder was moved up and down 20 times and rotated. The samples were then centrifuged at 4° for 5min at 14,000 RPM. 25ul of supernatant was removed for protein quantification and transferred to a 7ml tube containing 175ul of dH20 (200ul total volume). 100ul of each sample was then removed and placed in a second 7ml tube for duplicate processing. The remaining supernatant (approximately 175ul was transferred to an eppindorf tube for peptide extraction.

2.7.1 Protein Quantification of Dissected Tissue

A serial dilution was prepared from a BSA standard (stored in 2mg/ml aliquots at -80°) at 100, 80, 60, 40, 20 and 10ug/100ul dH2O. 100ul was transfered from each standard dilution to two separate 7ml tubes and 100ul of dH20 was placed in an additional tube ("blank").

All samples and standards were processed in duplicate. 3ml of bradford reagent was added to 100ul of samples, standards and blank, vortexed and allowed to stand for at least 5min but no more than 1hr. A spectrophotometer set to absorb at O.D. of 595 was zero'd with 500ul from the "blank" tube. 500ul of all standards and samples were read and the absorbance was recorded. The standard was plotted using microsoft excel with concentration on the x axis and absorbance on the y axis (**Fig. 5**). Protein concentrations of the samples were derived from extrapolation of absorbance. This amount reflects the amount of protein found in the 12.5 ul, 1/2 of the 25 ul of homogenate taken from the original 200ul total. The protein concentration was then multiplied by 14 in order to reflect the total amount of protein contained in the homogenate to be used in the radioimmunoassay (175ul).

FIG. 5 RIA PROTEIN STANDARD

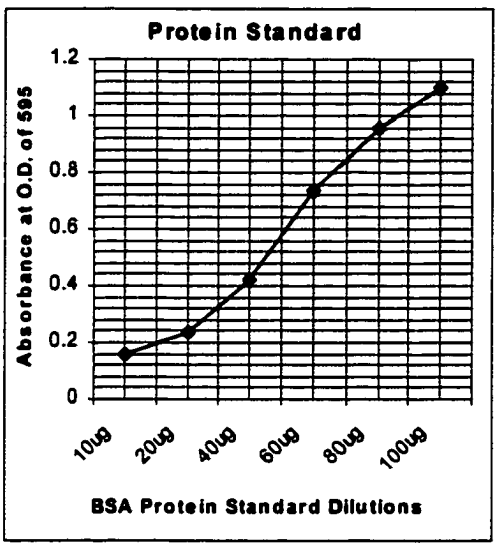


Fig. 5 Example of Protein Standard plotted from diluted samples used to derive protein concentrations of tissue samples dissected from brains of cocaine treated animals

2.7.2 Peptide Extraction

To the 175ul of supernatant extracted from tissue samples 175ul of extraction buffer (50% acetonitile + 0.1% trifluoroacetic acid) was added, samples were vortexed and centrifuged at 4° for 30min at 14,000 RPM. 350ul of chloroform was added to the supernatanat and centrifuged at 4° for 5min. The upper aqueous layer was saved and frozen, dried in a speed vac and stored at -80° until processed by solid phase radioimmunoassay.

2.7.3 Solid Phase Radioimmunoassay

Protein A (P-6031, Sigma) was prepared fresh by dissolving the lyophilized protein in 0.1MNaHC03 (pH 9.0) at 5ug/ml. 100ul was transferred to each of 72 wells of a 96 well Immunolon plate (0.5ug protein A/100ul 0.1M NaHC03) wrapped tightly with plastic wrap and aluminum foil and stored at 4° for no more than 3 weeks. Plates were

prepared at least 24 hours before being used. Wells were emptied by inverting the plates quickly thereby dumping the contents followed by blotting on paper towels. The washing of wells was done with 200ul of wash buffer (0.15M KPO4, 0.2mM ascorbic acid and 0.2% Tween 20)/well) delivered by multipipettor. 10 samples representing 5 brain regions from two animals (control, Group I and cocaine treated, Group II) were always processed together on one plate with its own standard.

Protein A was dumped from the plate and the plate washed 3 times. 200ul assay buffer (wash buffer + 0.1% fish gelatin) was added to each well to block non specific binding to protein A and allowed to stand at room temperature (RT) for 30min, after which the assay buffer was dumped and 50ul assay buffer was again added to 3 wells (reserved for non-specific binding (NSB) of ^{125}I-SP radiolabeled tracer) and 50ul of substance P antibody (RAS 7451, Penninsula Labs) dissolved in assay buffer at a concentration of 1:20,000 - 1:30,000 was added to all remaining wells and allowed to stand at RT for 2hrs. During this time tissue samples were reconstituted in 250ul of dH20 and diluted in assay buffer: NAc,GP, dStriaum 1:100; vStriatum 1:50 and SN 1:500. A Substance P (Y7451, Penninsula Laboratories) standard was prepared by serial dilution from a1.28ug SP/ul dH20 stock at 64.0, 32.0, 16.0, 8.0, 4.0, 2.0, 1.0, 0.5 and 0.1pg/50ul assay buffer concentrations. The contents of the wells were emptied and washed 3 times. Assay buffer alone was added to 6 wells (3 NSB wells and 3 wells reserved for total binding (TB) of tracer (^{125}I-SP peptide). 50ul of each standard and sample were added to wells in triplicate and allowed to stand for 2hrs at RT. During this time the tracer (^{125}I-SP, Y7452, Penninsula Labs) was diluted in assay buffer so that 50ul produced a count of 5000 Counts Per Minute (CPM) on the Wallac Gamma counter.

50ul of tracer was added to each well and incubated at 4° overnight. The next day the contents of the wells were dumped and washed 3 times. The wells were separated and placed in 13mm tubes and counted in a gamma counter.

The antibody concentration used was determined in a separate assay in which multiple concentrations of antibody were assayed with a fixed count of ^{125}I-SP tracer (5000 CPMs in 50ul). The antibody concentration that was approximately 40% of total binding was used in the assay to provide the highest sensitivity.

2.8 Quantification of Peptide in Sample

CPM averages of the three wells were calculated for NSB, TB and each standard and sample. The average for NSB was subtracted from the TB average giving a Total Antibody Binding (TAB) (TB-NSB=TAB). NSB was subtracted from each standard and sample average and divided by TAB to give a percentage of TAB (standard or sample/TAB * 100 = PTAB). PTAB was plotted against known standard concentrations and peptide concentrations were extrapolated from the graph (**Fig. 6**). This number reflects the amount of peptide in 50 ul of diluted reconsituted tissue sample. This peptide amount was multiplied by 5 (1/5 of total sample) and multiplied again by its dilution factor to get the total amount of peptide in the original 175 ul of homogenate reserved for the radioimmunoassay. Amount of peptide in 175ul of original sample was divided by amount of protein in 175 ul of original sample to give picograms of peptide in 1 ug of protein.

FIG. 6 RIA PEPTIDE STANDARD

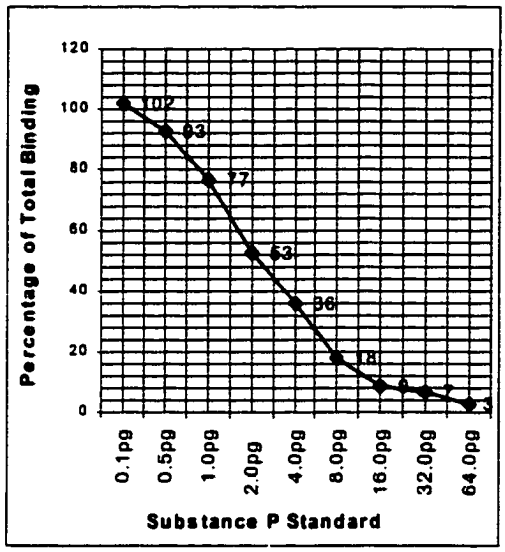


Fig. 6 Example of peptide standard from which Substance P concentrations were extrapolated. % of total binding was calculated based on CPMs of ^{125}I-SP in tissue sample and % of CPMs in total binding sample after CPMs of non-specific binding sample had been subtracted.

2.8.1 Analysis of Peptide Levels

SP levels were compared, by treatment and region using ANOVA factorial. Fisher's protected least significant difference (PLSD) and Student-Newan-Keuls were used for post-hoc multiple comparison when ANOVA results were significant. Values of $p < 0.05$ were considered significantly different. For the experiment in which behavior and radioimmunoassay were combined for the same animals peptide levels were compared to locomotor levels using Statview correlations software.

2.9 Preparation of Frozen Sections

Coronal sections (20 um in thickness) were cut in a cryostat at -15°C and thermally mounted onto Vectabond (Vector Laboratories, Burlingame, CA) treated slides (3-4 sections/slide) and frozen at -80°C.

2.10 *In situ* Hybridization Autoradiography

2.10.1 Hybridization Probes

PPE, PPT and PPD mRNAs were detected with antisense synthetic oligonucleotide probes. Probes were screened with GENBANK and found not to cross hybridize with known nucleic acid sequences. The specificity of the probes was determined as already described (Angulo et al. 1990). The following probes and sequences were used : PPE mRNA, 5'dCAAGTCGTCCTCATCCTGTTTTGCTGCTGCT and 5'dCTCCACGGGGTAAAGCTCATCCATCTTCTT corresponding to amino acids 86-95 and 112-121 of the rat prohormone (Rosen et al. 1984); PPD mRNA, 5'dTTGGCCTTTCTCCAGCTCCTTCAGGAGGGGCTCCAAGAGCTT and 5'dGGCCTGTTTTCTCAAGTCCTCCTCGTTGAAATGGAG corresponding to amino acids 54-67 and 83-94 of the rat prohormone (Civelli et al. 1985); and PPT mRNA, 5'dCATTAATCCAAAGAACTGCTGAGGCTTGGGTCT and 5'dGCCCATTAGTCCAACAAAGGAATCTGTTTTATG corresponding to amino acids 58-68 and 83-93 of the rat prohormone (Krause et al. 1987).

2.10.2 Radiolabeling of the Oligonucleotide Probes

The probes were labeled at the 3' end by terminal deoxynucleotidyl transferase as previously described (Angulo et al. 1990). The reaction mixture was prepared according to instructions for using the terminal transferase kit (Boehringer/ Mannheim) to which was added 70uCi of ^{35}S-dATP (Amersham, specific activity of >1,000 Ci/mmol), and 0.25 ug of probe and allowed to incubate at 37°C for 1 hr. The oligonucleotide probe was separated from unincorporated isotope by chromatography on a Sephadex G25 Quick Spin column (Boehringer/Mannheim) at room temperature.

2.10.3 Localization of mRNA by In situ Hybridization Histochemistry

All procedures were carried out at room temperature unless otherwise specified. The tissue was fixed for 30 min at 4°C in 4% paraformaldehyde/0.1M sodium phosphate buffer (pH 7.2) and washed for 1-2 min in 0.5X SSC buffer (1X SSC = 0.15M sodium chloride/0.015M sodium citrate buffer, pH 7.0). Sections were air-dried, rinsed for 1 min in acetylation buffer (0.1M triethanolamine, pH 8.0) and then acetylated for 10 min with 0.25% acetic anhydride dissolved in acetylation buffer. Slides were given two washes in 1 X SSC for 5 and 2 min and finally air-dried. Sections were hybridized overnight at 37°C in a humidified environment with a solution consisting of 0.2% (w/v) bovine serum albumin, 0.1% (w/v) polyvinylpyrrolidone, 0.2% (w/v) ficoll, 3X SSC buffer, 50% formamide, 10% dextran sulfate, 10mM DTT, 100 μg/ml of sheared and denatured salmon sperm DNA, 400 μg/ml of tRNA, 1mM EDTA, 4 μg/ml of heparin and 6-8 X 10^6 cpm/ml of oligonucleotide probe (12-16 ng/ml). The hybridization solution was applied in a volume of 100 μl and the slide was coverslipped. The next day, coverslips were removed by dipping in 2X SSC buffer. Sections were washed 3 times, 20 min each, in 1X SSC buffer/1mM DTT followed by 2 washes, 20 min each at 45°C in 1X SSC/1mM DTT. Then, one final wash for 20 min at 45°C in 1X SSC. Tissue was dehydrated in successive alcohol solutions (50% and 85%/0.3M ammonium acetate; 100% ethanol) and dried with a fan. Slides were apposed to Hyperfilm MP (Amersham) for 1 week (PPE), 2 weeks (TH), six weeks (PPT) or eight weeks (PPD).

2.11 Receptor Autoradiography

2.11.1 D1R [^{3}H]-SCH 23390

Dopamine 1 receptor (D1R) was detected in coronal sections of brain tissue essentially as described by Mansour et al. (1990). Sections were dried overnight *in vacuo* at 4°C, allowed to warm at RT for 30 min and then preincubated for 10 min at RT in 50mM Tris-Cl, pH 7.4. Slides were incubated for 60 min at RT in a solution containing 1nM of the D1 selective antagonist [^{3}H]-SCH 23390 (71.3 Ci/mmol, New England Nuclear), 0.1μM ketanserin, and T-Salts buffer. The slides were drained, washed 3X 10 min each in ice-cold T-salts buffer and quickly dipped in ice-cold distilled water. Nonspecific binding was assessed with 1μM unlabeled SCH 23390.

2.11.2 D2R [^{3}H]-spiperone

Detection of dopamine 2 receptor (D2R) in coronal sections of brain tissue was carried out essentially as described by Angulo et al. (1991). Sections were air-dried for 10 min at RT and then stored at -70°C until assayed. Brain sections were dried overnight *in vacuo* at 4°C, allowed to warm at RT for 30 min and preincubated for 10 min in 50mM Tris-Cl buffer (pH 7.4). Slides were incubated for 60 min at room temperature in a solution consisting of the selective D2 receptor antagonist 1nM ^{3}H-spiperone (17.5 Ci/mmol, New England Nuclear), 0.1μM ketanserin, 50mM Tris-Cl buffer (pH 7.4), 120mM NaCl, 5mM KCl, 1mM MgCl2, and 1mM CaCl2 (designated as T-Salts buffer). Non-specific binding was assessed with 2μM (+)-butaclamol. The binding reaction was terminated with 3 washes 10 min each in ice-cold (4°C) T-Salts buffer and a rapid wash

in chilled distilled water to remove residual salts. The slides were air-dried at RT and apposed to Hyperfilm-^{3}H (Amersham) for 3 weeks.

2.11.3 NK-1R [^{3}H]-SP

Neurokinin -1 receptor (NK-1) was detected in coronal sections of brain tissue essentially as described by Mantyh et al. (1990). Sections were dried overnight *in vacuo* at 4°C, allowed to warm at RT for 30 min and then preincubated for 10 min at RT in 50mM Tris-Cl, pH 7.4. containing 0.005% polyethylenimine. Slides were incubated for 60 min at RT in a solution containing 2nM of the NK-1 agonist [^{3}H]-SP (??? Ci/mmol, New England Nuclear), 3.0mM MnCl2, 200ug/ml BSA, 40ug/ml bacitracin, 2ug/ml chymostatin, 4ug/ml leupeptin and T-Salts buffer. The slides were drained, washed 2X 2 min each in ice-cold T-salts buffer and quickly dipped 4X in ice-cold distilled water. Nonspecific binding was assessed with 1μM unlabeled SP (Sigma S6883). The slides were quickly dried with a fan, dried over dessicant overnight at RT and apposed to Hyperfilm-^{3}H (Amersham) for 3 weeks.

2.12 Quantification of Autoradiography

Messenger RNA and receptor levels were determined by quantifying grey levels on x-ray autoradiograms of coronal sections at the level of the nucleus accumbens (NAc) and the caudate putamen (CPu) also referred to as the dorsal striatum. Four different regions of the CPu were quantified; the dorsalateral (dl), dorsalmedial (dm), ventralateral (vl) and ventralmedial (vm). In early experiments the nucleus accumbens (NAc) was treated as one region and subsequently was divided into the two regions of the Core and the Shell (**Fig. 7**).

FIG. 7 CORONAL SECTIONS OF THE DORSAL AND VENTRAL STRIATUM

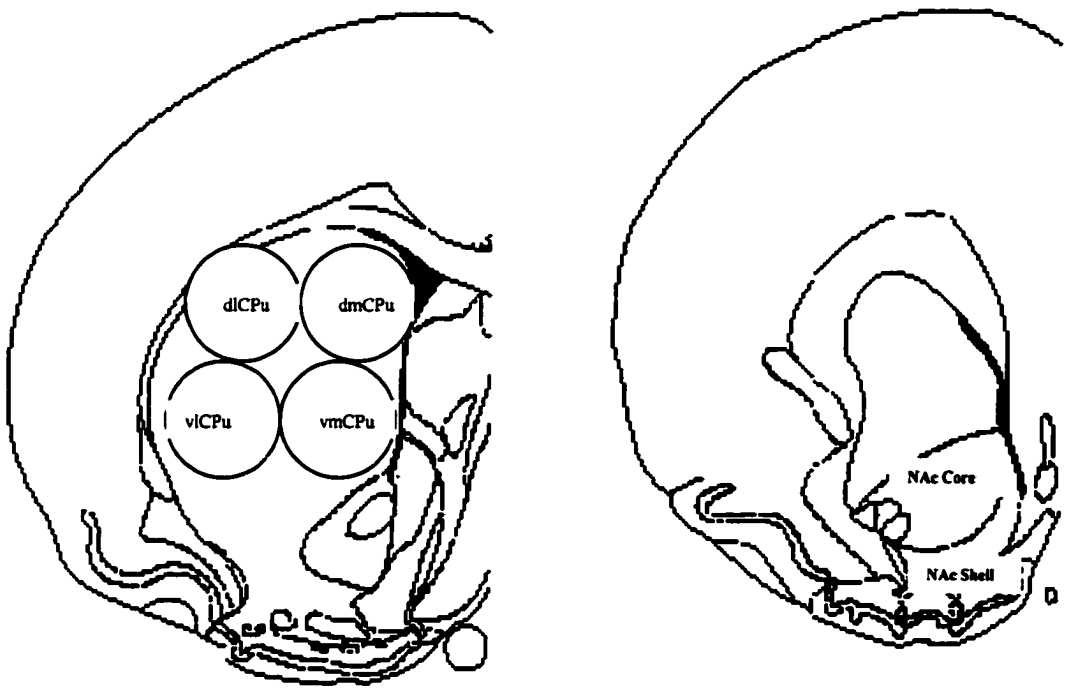


Fig. 7 Messenger RNA and receptor levels were determined by quantifying grey levels on x-ray autoradiograms of coronal sections at the level of the nucleus accumbens (NAc) and the caudate putamen (CPu) also referred to as the dorsal striatum. Four different regions of the CPu were quantified; the dorsalateral (dl), dorsalmedial (dm), ventralateral (vl) and ventralmedial (vm). In early experiments the nucleus accumbens (NAc) was treated as one region and subsequently was divided into the two regions of the Core and the Shell

Grey levels were quantified with an image analysis system utilizing the NIH Image 1.49 software VDM (Rasband 1993). Five sections per animal were quantified and the values averaged to generate an optical density value that corresponds to mRNA or receptor level per animal. Optical density for each treatment group was averaged and the value obtained taken as representative of mRNA or receptor level. ANOVA was used to compare optical density values of treatment groups and brain regions. Controls were normalized to 100% (zero change) and the results were represented in graphic form as

percent change relative to controls (control values represent 0% change and therefore are not shown in the figures).

3 RESULTS

Three striatal neuropeptide precursors preprotachykinin-A (PPT), preprodynorphin (PPD) and preproenkephalin (PPE) were assessed by *in situ* hybridization histochemistry, after acute and chronic (2x/day for 3, 6 and 14 days) exposure to vehicle, cocaine (15mg/kg) or cocaine and the non-competitive NMDA receptor antagonist MK-801 (1mg/kg), in male rats. We assessed mRNA levels in 6 regions of the neostriatum (dorsalateral (dl), ventralateral (vl), dorsalmedial (dm) and ventralmedial (vm)) CPu and the nucleus accumbens (NAc) because the striatum is topographically organized. The dorsalateral and ventralateral CPu receive innervation from the sensorimotor cortex responsible for limb and and head movement respectively. The dorsalmedial and ventralmedial CPu are innervated by visual and auditory cortices respectively.

Our hypothesis was: Striatal peptides or receptors that change in response to a time course of chronic, intermittent cocaine administration may reflect their role in locomotor sensitization. This conclusion would be strengthened if the cocaine-induced changes could be blocked or attenuated by concurrent administration of cocaine and the non-competitive NMDA receptor antagonist MK-801. MK-801 has been shown to block the development of psychostimulant induced locomotor sensitization and the physiological changes associated with it such as subsensitivity of VTA dopamine neurons and supersensitivity of NAc neurons to DA agonists (for review see Wolf, 1998).

3.1 PPT-A mRNA Expression in Response to a Time Course of Cocaine

We found that acute or three days of chronic, intermittent cocaine exposure had no significant effects on PPT-A mRNA levels in the neostriatum (**Fig. 8 A & B**). After six days of chronic cocaine treatment PPT-A mRNA was significantly elevated in the

dl/28%, dm/45% and vm/37% CPu compared with control animals treated with vehicle. The group that received concurrent administration of cocaine and MK-801, had significantly less PPT-A mRNA in these three regions, when compared to the cocaine only group and did not differ significantly from vehicle treated controls (**Fig. 8 C**). Following 14 days of chronic treatment, PPT-A mRNA levels were elevated in all areas of the CPu (dl, 28%, dm, 43%, vl, 18%, vm, 52%) but the group receiving cocaine and MK-801, again had significantly less PPT-A in those areas than the cocaine only group and did not differ significantly from vehicle treated controls (**Fig. 8 D**).

In the dorsalateral CPu not only is there a significant increase in PPT-A mRNA levels in the cocaine treatment group compared to the vehicle treatment groups at 6 and 14 days but there is also a significant increase in PPT-A mRNA levels after 6 (27%) and 14 (28%) days when compared to those in response to acute cocaine treatment. In each case, where there is a significant increase in PPT-A mRNA levels in response to cocaine, the group receiving cocaine and MK-801 had significantly reduced PPT-A mRNA levels for the same region and time point. Based on our initial criteria, change in peptide precursor mRNA expression in response to repeated exposures and reversal by MK-801, these results support a role for the neurokinins in locomotor sensitization.

FIG. 8 PPT-A MRNA ABUNDANCE IN RESPONSE TO A TIME COURSE OF COCAINE OR COCAINE AND MK-801

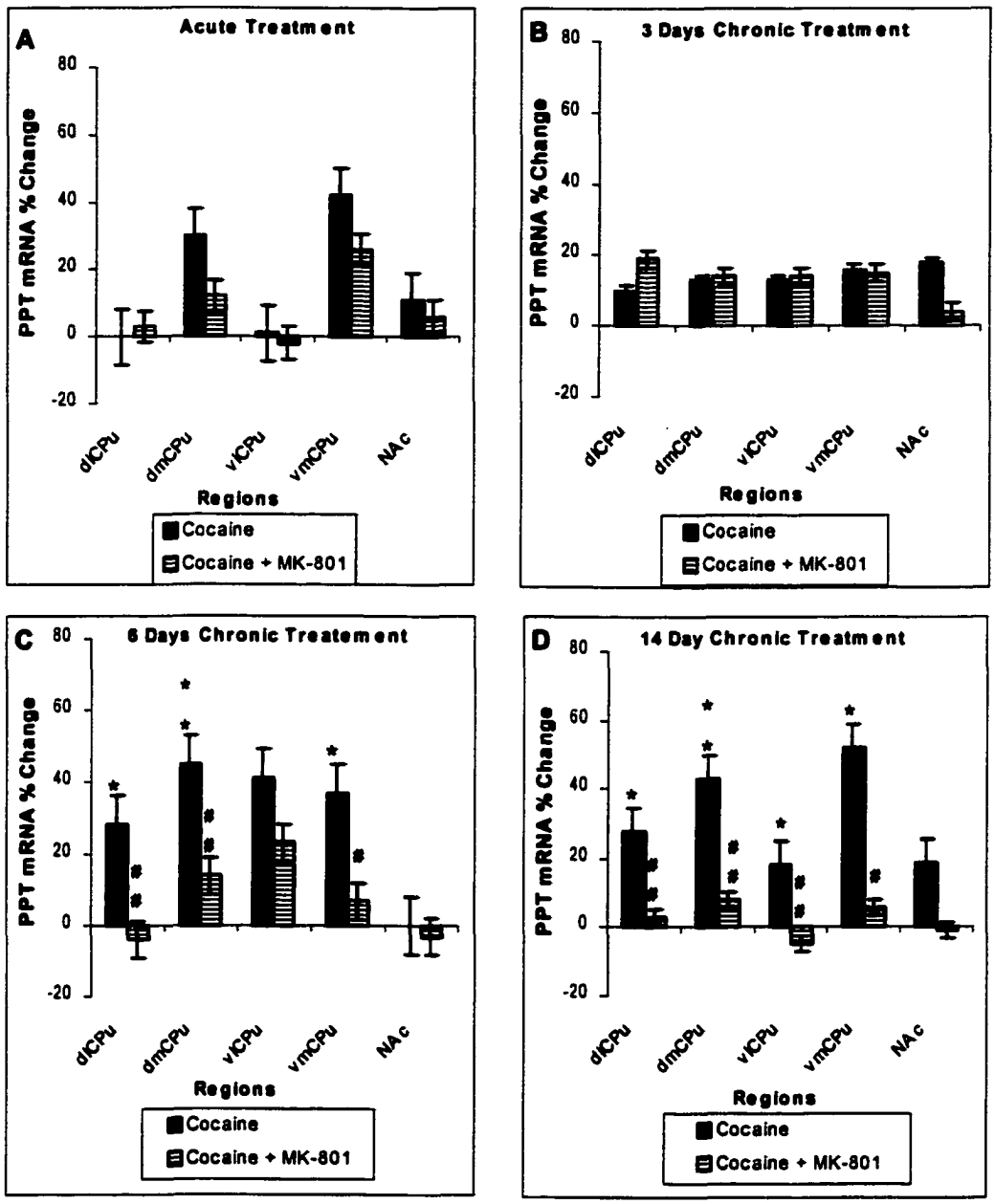


Fig. 8 (A-D) Effects of a time course of acute and chronic cocaine (15mg/kg, 2x/day) or cocaine and MK-801 (1mg/kg) co-administered i.p. one time (acute n=6) or twice a day for 3 (n=6), 6 (n=7) and 14 (n=7) days to rats sacrificed 5 hours after the last drug injection. Coronal sections of the brain were processed by *insitu* hybridization histochemistry and autoradiography. PPT-A mRNA levels are expressed as percent (± SEM) change from mean mRNA levels measured in vehicle treated control animals. *p= <0.05, **p= <0.01represent a significant difference between cocaine treatment and vehicle treated controls. #p= <0.05, ##p= <0.01 represent significant difference between cocaine treated and cocaine + MK-801 treated group. Analyzed by ANOVA followed by Fisher's PLSD.

After early (3 days) withdrawal from 7 days of cocaine pretreatment (10mg/kg, 1x/day) a cocaine challenge (10mg/kg) did not significantly change PPT-A mRNA levels compared to animals pretreated with vehicle and challenged with vehicle (**Fig. 9 A**). After late (21 days) withdrawal from 14 days of cocaine pretreatment (15 mg/kg, 2x/day) a cocaine challenge (15mg/kg) did not significantly change PPT-A mRNA levels compared with animals pretreated with vehicle and challenged with cocaine (**Fig. 9 B**).

FIG. 9 PPT-A MRNA ABUNDANCE IN RESPONSE TO A COCAINE CHALLENGE AFTER WITHDRAWAL FROM CHRONIC COCAINE PRETREATMENT

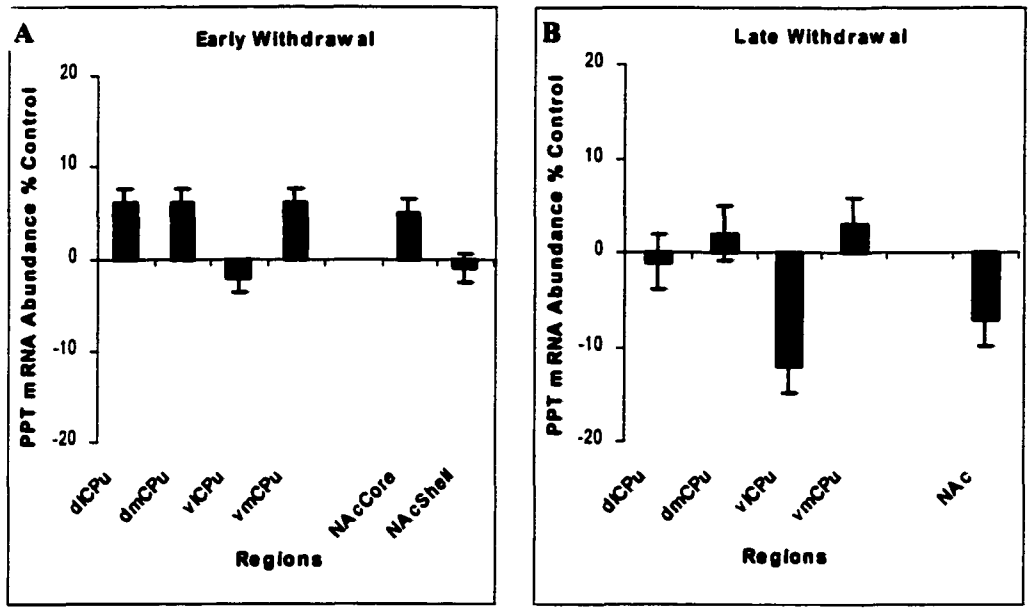


Fig. 9 (A & B) Effects of a cocaine challenge (10mg/kg) after early withdrawal (3 days) following pretreatment with chronic cocaine 10mg/kg, 1x/day for 7 days. PPT-A mRNA levels are expressed as percent (± SEM) change from mean mRNA levels measured in rats pretreated with vehicle and **challenged with vehicle. (B)** Effects of a cocaine challenge (15mg/kg) after late withdrawal (21 days) following pretreatment with chronic cocaine 15mg/kg, 2x/day for14 days. PPT-A mRNA levels are expressed as percent (± SEM) change from mean mRNA levels measured in rats pretreated with vehicle and **challenged with cocaine.** Rats n=8/group were sacrificed 5 hours after the last injection and coronal sections of their brains were processed by *insitu* hybridization histochemistry and autoradiography. Analyzed by Anova followed by Fisher's PLSD

These results (**Table 8**) suggest that increases in PPT-A mRNA in response to chronic cocaine exposure are not sustained after withdrawal and are therefore not essential to the expression of enduring locomotor sensitization but may play a role in its initiation/development.

TABLE 8. SUMMARY OF PREPROTACHYKININ-A MRNA CHANGES IN THE STRIATUM IN RESPONSE TO COCAINE OR COCAINE AND MK-801

		PPT-A mRNA Abundance % Change from Control									
		dlCPu		dmCPu		vlCPu		vmCPu		NAc	
Treatment Paradigm	Figure		+Mk -801		+Mk -801		+Mk -801		+Mk -801		+Mk -801
Acute		0	*3*	30	*12*	1	*-2*	42	*26*	11	*6*
3 Days		10	*19*	13	*14*	13	*14*	16	*15*	18	*4*
6 Days		27*	*-4##*	45**	*14##*	41	*23*	37*	*7#*	0	*-3*
14 days		28**	*3##*	43**	*8##*	18*	*-5##*	52*	*6#*	19	*-1*
Late Withdrawal		-1		2		-12		3		-7	
Early Withdrawal		6		6		-2		6		5	

* $p < 0.05$ one way ANOVA and Fisher's PLSD Cocaine Group compared to Control
** $p < 0.01$ one way ANOVA and Fisher's PLSD Cocaine Group compared to Control
\# $p < 0.05$ one way ANOVA and Fisher's PLSD Cocaine + MK-801 Group compared to Cocaine Group
$p < 0.01$ one way ANOVA and Fisher's PLSD Cocaine + MK-801 Group compared to Cocaine Group

3.2 Opioid mRNA Expression in Response to a Time Course of Cocaine

As shown in **Table 9**, preprodynorphin (PPD) mRNA levels were elevated, after 6 days, in the cocaine treatment group compared to vehicle treated controls in the dorsalateral (27%) and dorsalmedial (54%) CPu and were also significantly increased (44% and 60%) over animals treated with acute cocaine. After 14 days of cocaine treatment PPD mRNA levels were significantly increased over vehicle treated controls in the dorsalmedial (22%) and ventralmedial (31%) CPu and over animals treated with acute cocaine (27% and 34% respectively). However, none of these enhancements in PPD

mRNA levels were attenuated by co-administration of MK-801 (**Table 9**). Dynorphin and dynorphin agonists in the midbrain have been reported to attenuate locomotor activity (Reid et al., 1990; Matsumoto et al. 1988). Increases in PPD mRNA expression may contribute to the maintenance of homeostasis, an attempt by the system to counter the effects of cocaine on escalating motor activity.

TABLE 9. SUMMARY OF PREPRODYNORPHIN MRNA CHANGES IN THE STRIATUM IN RESPONSE TO COCAINE OR COCAINE AND MK-801

		PPD mRNA Abundance % Change									
		dlCPu		dmCPu		vlCPu		vmCPu		NAc	
Chronic Treatment			+Mk-801		+Mk-801		+Mk-801		+Mk-801		+Mk-801
Acute		-8	*22*	1	*25*	2	*33*	-13	*17*	-23	*-3*
3 Days		-9	*3*	-5	*13*	-7	*-1*	-6	*4*	-17	*-2*
6 Days		**27****	*14*	**54****	*32*	24	*20*	32	*14*	18	*18*
14 days		12	*0*	**22***	*17*	2	*-3*	**31****	*19*	11	*5*

* $p < 0.05$ one way ANOVA & Fisher's PLSD Cocaine Group compared to Control
** $p < 0.01$ one way ANOVA & Fisher's PLSD Cocaine Group compared to Control
\# $p < 0.05$ one way ANOVA & Fisher's PLSD Cocaine + MK-801 Group compared to Cocaine Group
\## $p < 0.01$ one way ANOVA & Fisher's PLSD Cocaine + MK-801 Group compared to Cocaine Group

As shown in **Table 10**, preproenkephalin (PPE) mRNA levels were significantly elevated after 6 days, in the cocaine treatment group compared to vehicle treated controls, in the dorsalmedial (26%) and ventralmedial (24%) CPu but were not significantly different from animals treated with acute cocaine. After 14 days PPE mRNA levels in the ventralmedial CPu remained elevated over vehicle treated controls (30%) but again were not significantly different from those of animals treated with acute cocaine. In the ventralmedial CPu, MK-801 significantly attenuated cocaine-induced PPE mRNA increases. After 21 days withdrawal from cocaine pretreatment, a cocaine challenge resulted in PPE mRNA levels in the ventalmedial CPu that were significantly elevated (25%) over controls receiving vehicle pretreatment and a cocaine challenge (similar to

acute cocaine treatment group). PPE mRNA levels in the ventralmedial CPu increase in response to cocaine exposure but upregulation is not significantly affected by repeated exposure (**Table 10**). This suggests that enkephalins may play a role in the expression of sensitization which is supported by the report that naltrindole, a δ receptor antagonist, inhibited the expression but not the development of locomotor sensitization when co-administered with cocaine (Shippenberg and Rea, 1997).

TABLE 10. SUMMARY OF PREPROENKEPHALIN MRNA CHANGES IN THE STRIATUM IN RESPONSE TO COCAINE OR COCAINE AND MK-801

		PPE mRNA Abundance % Change									
		dlCPu		dmCPu		vlCPu		vmCPu		NAc	
Chronic Treatment			+Mk -801		+Mk -801		+Mk -801		+Mk -801		+Mk -801
Acute		14	*4*	16	*-3*	15	*-2*	17	*-3*	15	*8*
3 Days		2	*22*	-3	*13*	5	*17*	8	*22*	-6	*-1*
6 Days		7	*7*	**26****	*19*	17	*17*	**24***	***14#***	15	*6*
14 days		6	*-1*	13	*6*	-2	*-1*	**30****	***10#***	8	*1*
14 Days +21 Withdrawal + Challenge		6		15		5		**25***		-4	

* $p < 0.05$ one way ANOVA and Fisher's PLSD Cocaine Group compared to Control
** $p < 0.01$ one way ANOVA and Fisher's PLSD Cocaine Group compared to Control
\# $p < 0.05$ one way ANOVA and Fisher's PLSD Cocaine + MK-801 Group compared to Cocaine Group
\## $p < 0.01$ one way ANOVA and Fisher's PLSD Cocaine + MK-801 Group compared to Cocaine Group

In summary, PPD mRNA levels are upregulated by repeated cocaine exposure which is unaffected by MK-801 and cocaine induced PPE mRNA upregulation is attenuated by MK-801 but unaffected by repeated cocaine exposure. In contrast, PPT-A mRNA expression is upregulated by repeated cocaine exposure but this PPT-A mRNA response is attenuated by the NMDA receptor antagonist MK-801, suggesting that the cocaine induced increases are dependent on NMDA receptor activation by glutamate.

As described in the introduction, neurokinins increase locomotion (Elliott et al, 1986; Kalivas et al., 1985; Kelley et al., 1985; Takano et al., 1985), dopamine release (Boix et al., 1992; Baruch et al., 1988; Elliott et al., 1986; Kalivas et al., 1985) and dopaminergic activity levels (Innis et al., 1985; Davies and Dray, 1975; Walker et al., 1975). In addition, other laboratories are looking at the role of the opioids in locomotor sensitization but because non-peptide neurokinin receptor antagonists are not readily available there is little work being done with the neurokinins. Taken together, our results, the work of others and the opportunity to look at neurokinins in a way not already being explored led us to focus on the neurokinins and their role in locomotor sensitization.

3.3 Receptor Levels in Response to a Time Course of Cocaine

Binding of the D1 antagonist [3H]-SCH23390 or the D2 antagonist [3H]-spiperone showed little change in response to a cocaine time course (**Table 11**). There was a small but significant decrease in [3H]-SCH23390 binding in the NAc after acute exposure but reductions in D1R binding levels were not sustained over the time course. In fact after 14 days of chronic cocaine exposure [3H]-SCH23390 was upregulated in the dorsalmedial CPu. The only other change detected was a reduction in [3H]-spiperone binding in the NAc after 14 days of chronic cocaine exposure.

Others have reported supersensitivity of striatal projection neurons to dopamine in sensitized rats after chronic cocaine exposure. Increased D1R binding may be partially responsible for increased sensitivity, but, as suggested by other studies, changes in DA receptor responsiveness may occur independent from changes in binding levels. Factors

such as receptor distribution or modifications in signal transduction pathways can affect receptor/ligand interactions.

TABLE 11. SUMMARY OF DOPAMINE RECEPTOR BINDING IN THE STRIATUM IN RESPONSE TO COCAINE OR COCAINE AND MK-801

	D1R Binding Levels % Change									
	dlCPu		dmCPu		vlCPu		vmCPu		NAc	
Cocaine Treatment		+Mk -801		+Mk -801		+Mk -801		+Mk -801		+Mk-801
Acute	6	*9*	-1	*5*	0	*3*	-5	*5*	**-12***	*0*
3 Days	1	*-9*	11	*12*	2	*9*	4	*7*	5	*7*
6 Days	-3	*7*	-4	*3*	-3	*1*	-1	*3*	-1	*0*
14 days	7	*4*	**15***	*8*	9	*4*	8	*3*	-4	*-6*

	D2R Binding Levels % Change									
	dlCPu		dmCPu		vlCPu		vmCPu		NAc	
Cocaine Treatment		+Mk -801		+Mk -801		+Mk -801		+Mk -801		+Mk-801
Acute	5	*6*	1	*4*	-2	*-3*	-1	*-1*	-4	*3*
3 Days	7	*1*	3	*-2*	2	*-4*	4	*4*	4	*0*
6 Days	0	*0*	-4	*0*	0	*-1*	-3	*-5*	-10	*-16*
14 days	-3	*2*	6	*9*	-3	*1*	-1	*2*	**-13***	*-6*

* $p < 0.05$ one way ANOVA followed by Fisher's PLSD Cocaine Group compared to Control

3.4 Progressive Locomotor Sensitization to Chronic, Intermittent Cocaine

Although others have reported locomotor sensitization using the same14 day cocaine treatment paradigm that was used in the *in situ* hybridization studies just described, it was important to show that we could reproduce the behavior results. Animals were treated with cocaine (15mg/kg, 2x/day) for 14 days and locomotion and stereotypies (rearing, bobbing and sniffing) were assessed manually with hand held timers and counters. Horizontal locomotion and rearing sensitized over the time course. After purchasing automated equipment we repeated the experiment but horizontal locomotor activity was

automatically measured and recorded after acute and 3, 5, 7, 9, 11 and 13 days of chronic, intermittent cocaine or vehicle exposure (**Fig. 10**).

Acute cocaine exposure (15mg/kg) significantly enhanced locomotor activity when compared to the response to vehicle on the pretreatment day (74%) or compared with the response to vehicle by the control group on the acute treatment day (87%) indicating cocaine-induced hyperactivity (**Fig. 10**).

Chronic treatment with cocaine 15mg/kg, 2x/day resulted in enhanced locomotion (39% and 37%) after 5 and 13 days respectively, compared with the response to acute cocaine (15 mg/kg) (**Fig. 10**) demonstrating cocaine induced sensitization at these timepoints.

FIG. 10 PROGRESSIVE SENSITIZATION TO COCAINE

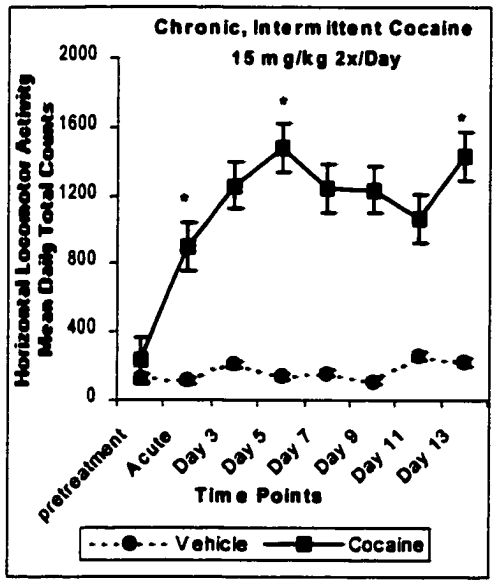


Fig. 10 Effects of a time course of chronic cocaine (15mg/kg, 2x/day) administered i.p. twice a day for 14 days on locomotor activity of rats. Cocaine and PBS vehicle were administered intraperitoneally in a total volume of 0.5ml. Groups (n=10) were pretreated with vehicle alone for 2 days prior to drug administration. Locomotor activity was measured every other day by automated equipment from San Diego Instruments. Animals were habituated to the test cage for 1 hour prior to drug treatment. Locomotor activity is expressed as the daily group mean (± SEM) of total photocell counts, representing locomotor activity. Acute response was compared to pretreatment response for each group. All other timepoints were compared to the acute response for each group. *$p<0.05$, by ANOVA Repeated Measures and Paired t-test.

Fig. 11A below was extrapolated from **Fig. 10** above and **Fig. 11B** from **Fig. 8** (page 59) to illustrate the similarity of the time course of increased locomotor activity and PPT-A mRNA levels in the dorsalateral CPu, in response to the same chronic cocaine treatment (15mg/kg, 2x/day). Locomotor activity levels are shown after acute, 3, 5 and 13

days of cocaine treatment in order to compare it with the PPT-mRNA results for the dlstriatum in which animals were sacrificed after 1, 3, 6 and 14 days of treatment. Locomotor activity at 5 and 13 days is elevated over acute activity levels and PPT-A mRNA levels are elevated at 6 and 14 days over acute levels.

FIG. 11 LOCOMOTOR ACTIVITY AND PPT-A MRNA LEVELS IN RESPONSE TO CHRONIC COCAINE EXPOSURE

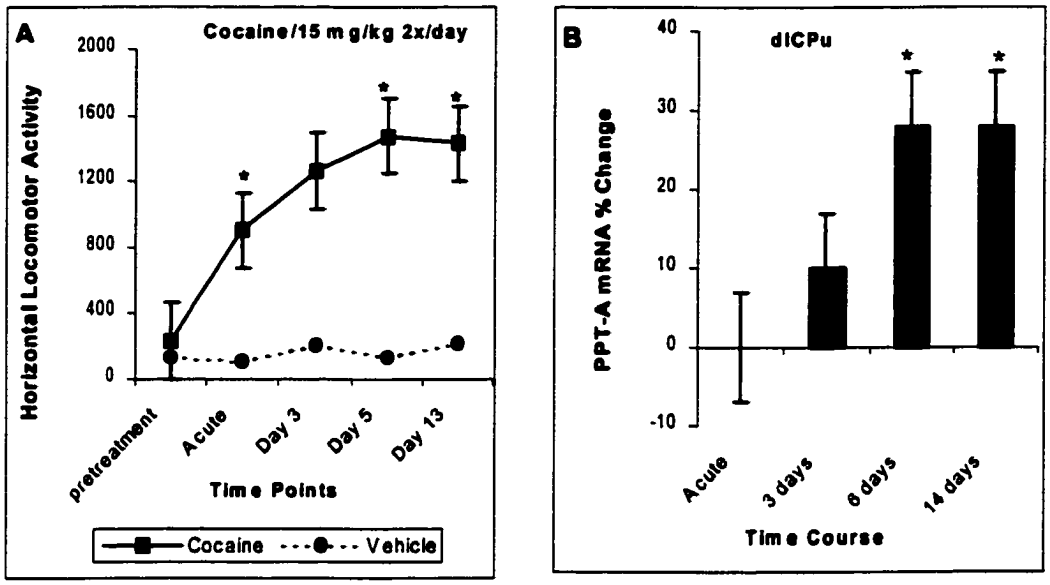


Fig. 11 Effects of a time course of chronic cocaine (15mg/kg, 2x/day) administered i.p. twice a day for 14 days on **(A)** locomotor activity and **(B)** PPT-A mRNA levels. **(A)** Locomotor activity is expressed as the daily group mean (± SEM) of total photocell counts. Activity levels on days 3, 5 and 13 were compared to the acute response. **(B)** PPT-A mRNA levels in coronal sections of the brain processed by *insitu* hybridization histochemistry and autoradiography are expressed as percent (± SEM) change from mean mRNA levels measured in vehicle treated control animals. $^{*}p<0.05$, $^{**}p<0.01$ by ANOVA followed by Fisher's PLSD.

Subsequent experiments were conducted to explore the role of the neurokinins in the development and expression of cocaine-induced locomotor sensitization. Our strategy was to administer cocaine to one group of rats using a drug treatment paradigm and time-course which produces locomotor sensitization. Another group of rats would be treated identically except that they would also be exposed to a neurokinin receptor antagonist during the time course of cocaine treatment. If the group receiving cocaine alone is

sensitized to cocaine and the group receiving cocaine and the antagonist is not, then we can conclude that the neurokinin antagonist blocks locomotor sensitization to cocaine. It was therefore necessary to establish a standardized drug treatment paradigm for these experiments.

The cocaine treatment paradigm (15mg/kg 2x/day) discussed above produced sensitization at 2 out of 6 time-points and locomotor activity reached a plateau on day 5. We reasoned that a reduced dose of cocaine would lead to a reduction in the acute locomotor response, thereby increasing the likelihood of statistically significant enhancements over the acute response at later time-points and a more stepwise increase in activity levels over the time course. We tested a treatment paradigm of 10mg/kg 1x/day over a 14 day time period and included a cocaine challenge at the same dose after 3 days of withdrawal. The withdrawal period is sufficient for cocaine to clear the system (Post and Contel, 1983). Reintroduction of cocaine after withdrawal would allow us to distinguish between the effects of neurokinin antagonists on the development and expression of locomotor sensitization.

FIG. 12 PROGRESSIVE AND ENDURING SENSITIZATION TO COCAINE

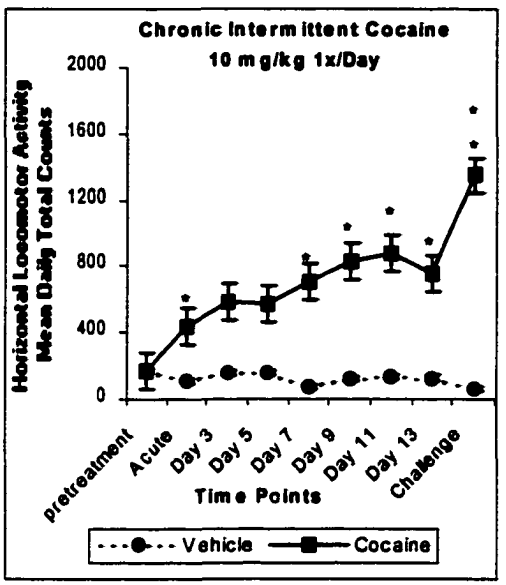


Fig. 12 Effects, of a time course of chronic cocaine (10mg/kg, 1x/day) administered i.p.once a day for 14 days followed by a cocaine challenge after 3 days of withdrawal . Cocaine and PBS vehicle were administered intraperitoneally in a total volume of 0.5ml. Groups (n=10) were pretreated with vehicle alone for 2 days prior to drug administration. Locomotor activity was measured every other day, except during withdrawal, by automated equipment from San Diego Instruments. Animals were habituated to the test cage for 1 hour prior to drug treatment. Locomotor activity is expressed as the daily group mean (± SEM) of total photocell counts, representing locomotor activity. Acute response was compared to pretreatment response for each group. All other timepoints were compared to the acute response for each group. *p<0.05, **p<0.01 by ANOVA repeated measues and paired t-test.

Acute cocaine exposure (10mg/kg) significantly enhanced locomotor activity when compared to the response to vehicle on the pretreatment day (61%) or compared with the response to vehicle by the control group on the acute treatment day (74%) which indicates cocaine-induced hyperactivity (**Fig. 12**).

Progressive treatment with cocaine (10mg/kg 1x/day) increased locomotion in a stepwise manner over the time course. Locomotor activity was significantly elevated over the acute response after days 7 (38%), 9 (48%), 11 (50%) and 13 (42%) and after a cocaine challenge following 3 days of withdrawal (68%) (**Fig. 12**). This chronic cocaine treatment paradigm (10 mg/kg 1x/day) resulted in both progressive and enduring locomotor sensitization and was adopted in subsequent experiments.

The next set of experiments focused on the effect of neurokinin blockade on the development and expression of cocaine-induced locomotor sensitization using the paradigm described above for a period of 7 days followed by a cocaine challenge. The

effect of neurokinin blockade, on the expression of locomotor sensitization, was assessed by comparing the locomotor activity of two groups of rats and by comparing each groups activity after chronic cocaine and cocaine challenge to their own activity after acute exposure. Both groups were pretreated for 7 days with cocaine but one was challenged with cocaine alone (GII) and the other with a neurokinin antagonist prior to the cocaine challenge (GIII). After 7 days of chronic treatment both groups were expected to demonstrate progressive locomotor sensitization. The group treated with the cocaine challenge (GII) was expected to demonstrate enduring locomotor sensitization. The group treated with cocaine + the neurokinin receptor antagonist would not show enduring locomotor sensitization if activity at that receptor is necessary for the expression of locomotor sensitization. For reasons discussed in the introduction, we expected NK-1 receptor antagonists to block the expression of sensitization.

To assess the effect of neurokinin blockade on the development of sensitization, an additional group of rats (GIV) received a neurokinin receptor antagonist prior to cocaine throughout the 7 days of chronic pretreatment. If 7 days of pretreatment with cocaine + a neurokinin receptor antagonist did not result in locomotor sensitization then we would conclude that locomotor sensitization was blocked by the antagonist. Either inhibition of expression or development of sensitization could produce these results. If, after 3 drug free days a cocaine challenge does not produce a sensitized locomotor response we can conclude that the neurokinin antagonist blocked the development of sensitization. On the other hand if a cocaine challenge produces a sensitized locomotor response, then we can conclude that the neurokinin antagonist blocked the expression of sensitization. Based on

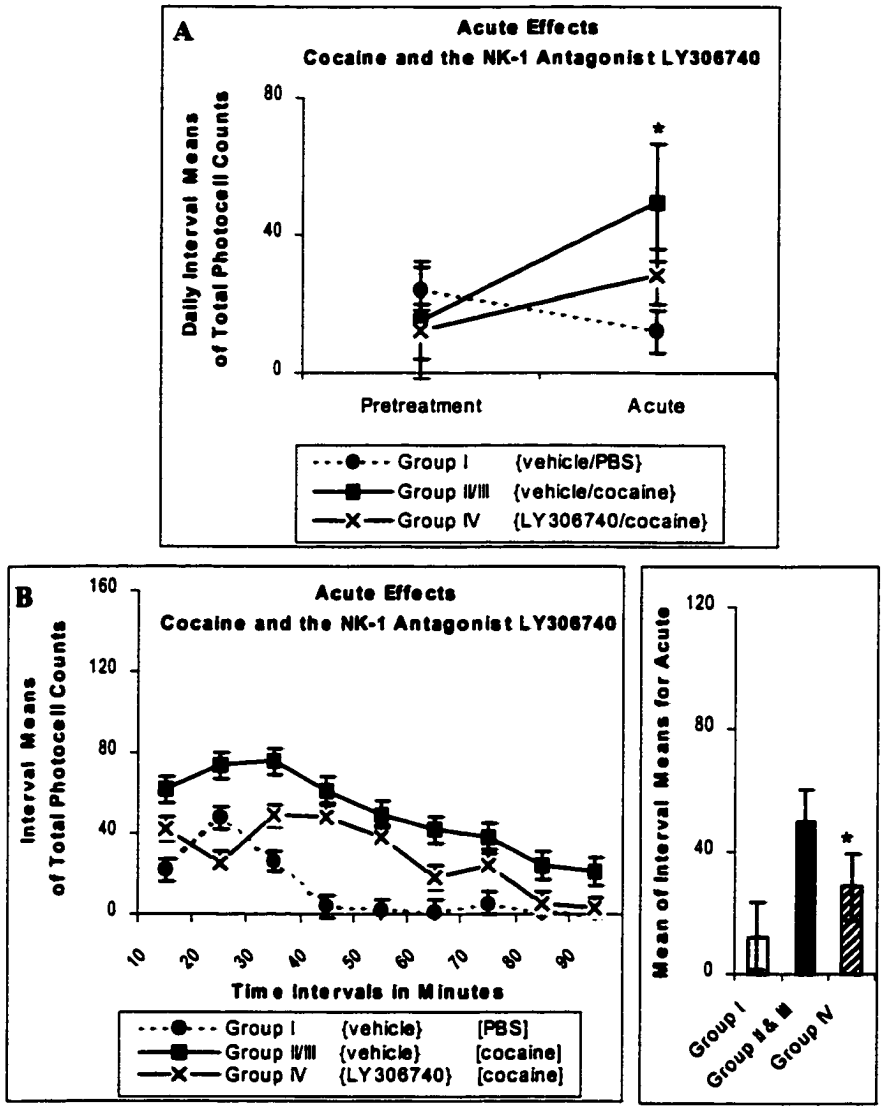
the model outlined in the introduction we expected NK-3 antagonists to block the development of sensitization.

3.5 NK-1 Blockade

The first non-peptide neurokinin antagonist we were able to obtain was the NK-1 antagonist LY306740 from Eli Lilly. This compound is only soluble under acidic conditions, therefore solution volumes necessary to dissolve sufficient compound would cause tissue damage if administered multiple times. The manufacturer recommended continuous delivery of LY306740 via osmotic minipumps because small volumes would be absorbed rapidly, avoiding tissue damage.

Acute exposure to cocaine (GII/III) produced a significant increase in locomotor activity compared to activity levels after pretreatment with saline (68%) and compared to activity levels of animals receiving acute saline treatment (76%) (**Fig. 13A**). NK-1 receptor blockade by LY306740, prior to acute cocaine exposure (GIV), produced activity levels that were not significantly different from the response to saline pretreatment (**Fig. 13A**) but were significantly reduced (-44%) compared to the acute cocaine (GII/III) response (Fig. **13B**).

FIG. 13 NK-1 RECEPTOR BLOCKADE BY LY306740 ATTENUATES ACUTE COCAINE-INDUCED ACTIVITY



Figs. 13 (A & B) All groups received pretreatment with vehicle (i.p., 0.5ml PBS) for 2 days prior to drug administration. All groups were implanted with osmotic minipumps 5 hours after the 2nd pretreatment. The pumps delivered 10ul/hour of vehicle (Groups I, II, III) or LY306740 (3mg/day) (Group IV). Group I received PBS (0.5ml, i.p.) as acute treatment and Groups II/III and IV received cocaine (10mg/kg /0.5ml PBS, i.p). Locomotor activity is expressed as the interval mean (± SEM) of daily total photocell counts, representing horizontal locomotor activity. **(A)** Comparisons of pretreatment vs. acute locomotor activity for each group. *$p < 0.0001$ Group II/III Repeated Measures ANOVA and paired t-test. **(B)** Comparisons of locomotor activity between Group IV and Group II/III after acute treatment. *$p<0.05$. ANOVA followed by Fisher's PLSD. {contents of pump} [i.p. injection]

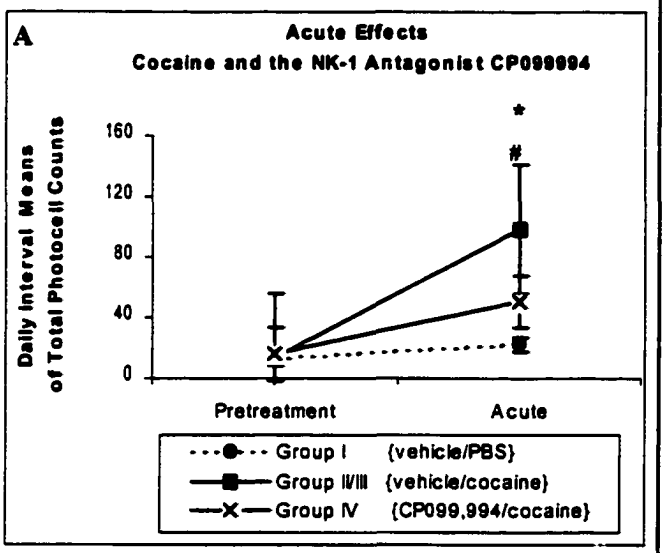
We subsequently obtained the non-peptide NK-1 antagonist SR140333 from Sanofi Recherche. Continuous exposure to a drug may produce different effects than chronic intermittent exposure (Post, 1980). Therefore this compound was used in a similar experiment to the one described above except it was administered intraperitoneally (i.p.) 30 minutes prior to cocaine administration throughout the time course. This compound did not significantly reduce activity levels when administered prior to acute cocaine when compared to the group receiving cocaine alone (-12%) and there were no significant effects on locomotor sensitization (data not shown). The results of a study published shortly after we conducted the experiment presented evidence that this compound does not cross the blood brain barrier (Rupniak et al., 1997). The NK-1 antagonist, CP099994 was then obtained from Pfizer in order to repeat the experiment described above because the same study showed that this compound crossed the blood brain barrier when administered systemically.

In this experiment acute exposure to cocaine (GII/III) produced hyperactivity when compared to the pretreatment response (69%) and when compared to acute exposure to saline (GI) (57%) (Fig.**14A**). Acute exposure to cocaine following NK-1 receptor blockade by CP099994 (GIV) resulted in a significant decrease (-39%) in activity levels compared to the acute response to cocaine alone (GII/III) (Fig. **14B**).

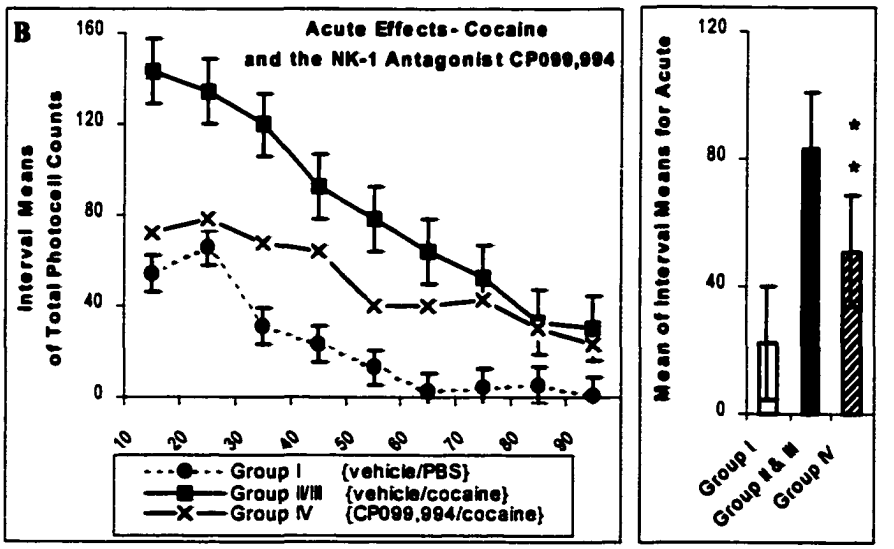
In summary, prior exposure to the NK-1 antagonists, LY306740 and CP099994, reduced acute cocaine-induced hyperactivity, suggesting that the presence of endogenous neuorokinins acting at the NK-1 receptor is necessary for the full expression of this behavior.

However, neither continuous delivery of LY306740 or chronic exposure to CP099994 during the time course of chronic, intermittent cocaine administration significantly affected the development or expression of locomotor sensitization to cocaine (see summary of results **Table 12,** page 81). This suggests that endogenous neurokinins acting at NK-1 receptors are not necessary for the development or expression of locomotor sensitization to cocaine.

FIG. 14 NK-1 RECEPTOR BLOCKADE BY CP099,994 ATTENUATES ACUTE COCAINE-INDUCED ACTIVITY



Figs. 14 (A&B) Drugs were administered i.p. in a total volume of 0.5ml vehicle or vehicle + drug. All groups received pretreatment with vehicle and PBS for 2 days. Group I received vehicle 30 min. prior to a 2nd injection of PBS on day 1. Group II/III received vehicle 30 min. prior to cocaine (10mg/kg) and Group IV received CP099,994 (30mg/kg) 30 min. prior to cocaine (10mg/kg). Locomotor activity is expressed as the daily group mean (± SEM) of total photocell counts, representing horizontal locomotor activity. **(A)** Comparisons of pretreatment vs. acute locomotor activity for each group. **(B)** Comparison of locomotor activity between Group IV and Group II/III after acute treatment, **p<0.0001. ANOVA followed by Fisher's PLSD. {1st i.p. injection/2nd i.p. injection}



3.6 NK-3 Blockade

Although the neurokinins substance P and neurokinin A are expressed together at similar levels in the basal ganglia the NK-1 and NK-3 receptors are expressed on different neurons and have different affinities for the two neurokinin peptides. Therefore the effects of ligand binding at these receptors will have different effects on BG circuits and behavior. The effect of NK-3 blockade on cocaine-induced hyperactivity and locomotor sensitization was investigated using the NK-3 antagonist, PD161182, supplied by Parke Davis. In contrast to the NK-1 antagonists, PD161182 administered i.p. 30 minutes prior to acute cocaine administration had no significant effect on cocaine-induced hyperactivity (**Fig. 15**). Therefore endogenous neurokinins acting at the NK-3 receptor are not necessary for the expression of acute cocaine-induced hyperactivity.

FIG. 15 NK-3 RECEPTOR BLOCKADE HAS NO EFFECT ON ACUTE COCAINE-INDUCED ACTIVITY

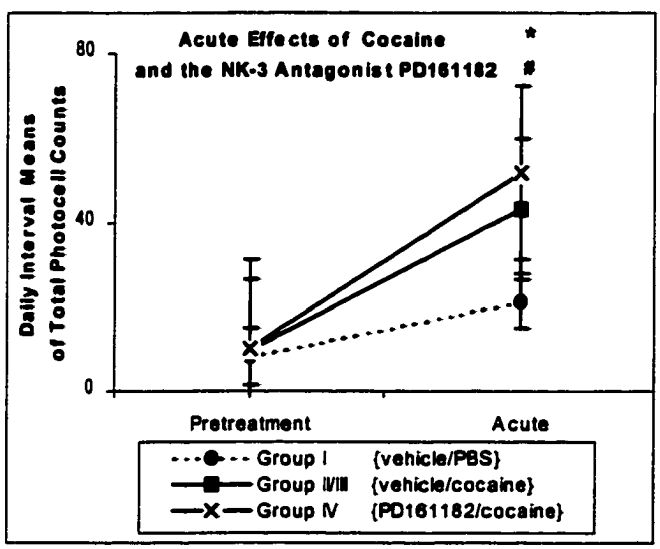


Fig. 15 Drugs were administered i.p. in a total volume of 0.5ml vehicle or vehicle + drug. All groups received pretreatment with vehicle and PBS for 2 days. Group I received vehicle 30 min. prior to a 2nd injection of PBS on day 1. Group II/III received vehicle 30 min. prior to cocaine (10mg/kg) and Group IV received PD161182 (30mg/kg) 30 min. prior to cocaine (10mg/kg). Locomotor activity is expressed as the daily group interval mean (± SEM) of total photocell counts, representing horizontal locomotor activity. Comparisons of pretreatment vs. acute locomotor activity for each group. ANOVA followed by Fisher's PLSD. {1st i.p. injection/2nd i.p. injection}

Seven days of chronic cocaine administration (GII/III) resulted in a significant increase in locomotor activity when compared to the response to acute cocaine (56% increase) (**Figs. 16A & B**) indicating that progressive locomotor sensitization to cocaine

had taken place. The group chronically exposed to PD161182 + cocaine (GIV) exhibited dramatic progressive locomotor sensitization (75% increase over acute response) (**Fig. 16A**), thus, refuting our hypothesis that NK-3 blockade would block the development or expression of progressive sensitization to cocaine.

FIG. 16A NK-3 RECEPTOR BLOCKADE BY PD161182 FACILITATES PROGRESSIVE SENSITIZATION TO COCAINE

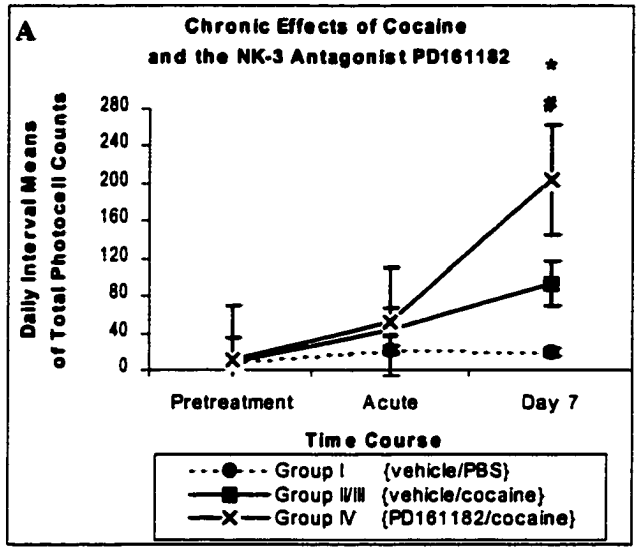


Fig. 16 (A) Drugs were administered i.p. in a total volume of 0.5ml vehicle or vehicle + drug. Group I received vehicle 30 min. prior to a 2nd injection of PBS for seven days. Group II & III received vehicle 30 min. prior to cocaine (10mg/kg) for seven days and Group IV received PD161182 (30mg/kg) 30 min. prior to cocaine (10mg/kg) for seven days. Locomotor activity is expressed as the daily group (n=6/group) mean (± SEM) of total photocell counts, representing horizontal locomotor activity. Comparisons of day 7 vs. acute, locomotor activity by experimental group, $^{*}p< 0.05$ Group II/III, $^{\#}p<0.05$ Group IV. Repeated measures ANOVA and paired t-test.

In fact on day 7 the locomotor activity level of GIV, which received PD161182 + cocaine, was 55% greater than that for GII/III indicating the NK-3 antagonist PD161182 dramatically increases progressive locomotor sensitization.

FIG. 16B LOCOMOTOR ACTIVITY ON DAY SEVEN
OF CHRONIC TREATMENT WITH COCAINE AND PD161182

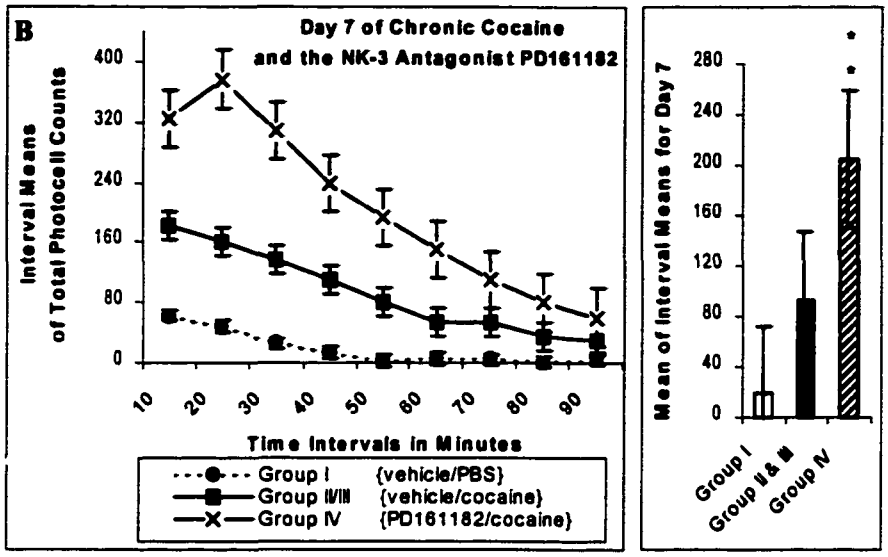


Fig. 16 (B) Group II/III received vehicle 30 min. prior to cocaine (10mg/kg) for seven days and Group IV received PD161182 (30mg/kg) 30 min. prior to cocaine (10mg/kg) for seven days. Locomotor activity is expressed as the daily group (n=6/group) interval mean (± SEM) of total photocell counts, representing horizontal locomotor activity. Comparison of locomotor activity between groups (GroupII/III and Group IV) after 7 days of chronic treatment, **p<0.0001. ANOVA followed by Fisher's PLSD.

In a separate experiment, chronic administration of PD161182 alone for 7 days had no significant effect on locomotor activity (**Fig. 17**). Blockade of the NK-3 receptor does not produce hyperactivity or locomotor sensitization by itself. The results also suggest that endogenous neurokinins do not noticeably impact motor performance in drug free ambulatory rats.

FIG. 17 NK-3 BLOCKADE ALONE HAS NO EFFECT ON LOCOMOTOR ACTIVITY

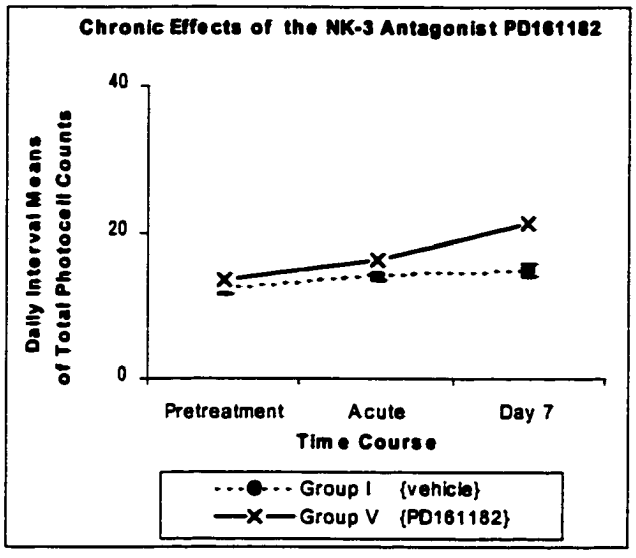


Fig. 17 Drugs were administered i.p. in a total volume of 0.5ml vehicle or vehicle + drug. Group I received vehicle and Group V received the NK-3 antagonist, PD161182 (30mg/kg) for seven days. Locomotor activity is expressed as the daily group (n=6/group) mean (± SEM) of total photocell counts, representing horizontal locomotor activity. Comparisons of day 7 vs. acute, locomotor activity for each group, *p< 0.05 ANOVA followed by Fisher's PLSD.

After 3 days of withdrawal from 7 days pretreatment with cocaine, a cocaine challenge (GII) produced locomotor activity significantly elevated over the acute response (67%) indicating that progressive locomotor sensitization to cocaine observed after 7 days of chronic cocaine exposure (69%) was sustained after early withdrawal (**Fig. 18**). All experimental groups (GII, GIII and GIV) showed significantly enhanced locomotor activity after the challenge compared to activity levels after acute exposure to drug indicating enduring locomotor sensitization in all three groups.

FIG. 18 NK-3 RECEPTOR BLOCKADE FACILITATES ENDURING SENSITIZATION TO COCAINE

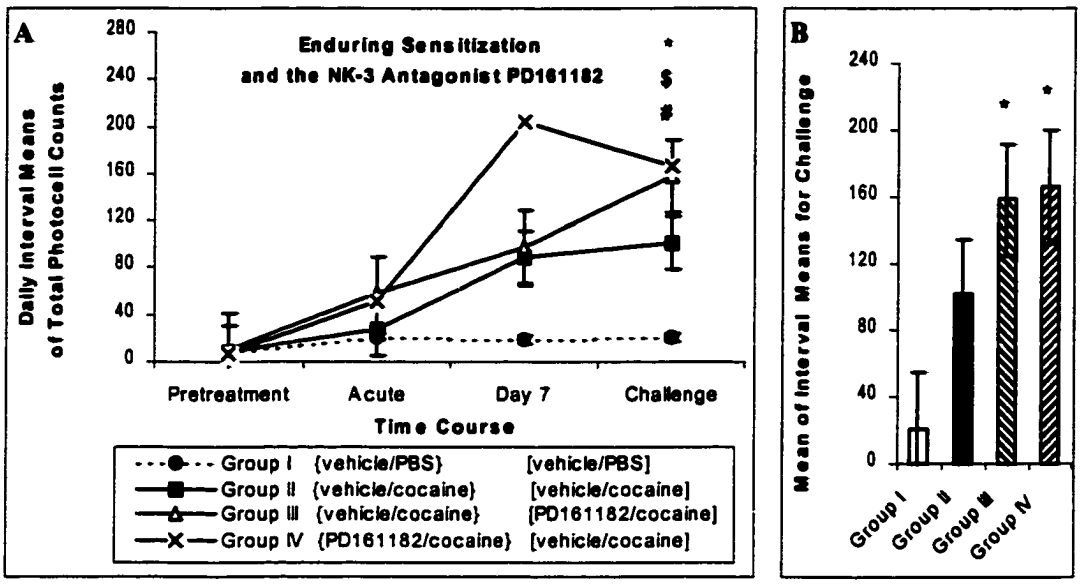


Fig. 18 (A & B) For seven days animals received two i.p. injections each day (0.5ml/injection) 30min apart. The antagonist or its vehicle was always administered first followed by cocaine or PBS. On days 1-7 Group I received, vehicle/PBS, Group II vehicle/cocaine, Group III vehicle/cocaine and Group IV PD161182/cocaine. After 3 drug free days the animals were again given two i.p. injections 30min apart; Group I, vehicle/PBS, Group II, vehicle/cocaine, Group III, PD161182/cocaine and Group IV vehicle/cocaine. Locomotor activity is expressed as the daily group interval mean (± SEM) of total photocell counts, representing horizontal locomotor activity. (n=6/group) **(A)** Comparisons were made between locomotor response on challenge day and the acute response for each group. $^{*}p< 0.05$ Group II, $^{\$}p<0.05$ Group III and $^{\#}p<0.05$ Group IV Repeated Measures ANOVA and paired t-test. **(B)** Comparison of locomotor activity between groups on the challenge day. $^{*}p<0.05$. ANOVA followed by Fisher's PLSD post hoc. {chronic treatment} [Challenge]

As described previously, GII and GIII were pretreated with cocaine alone for 7 days and their locomotor activity on day 7 was similar (**Fig. 18**). Both groups were sensitized. After 3 drug free days group III received the NK-3 antagonist, PD161182, 30 minutes prior to a cocaine challenge and group II received vehicle before the cocaine challenge. The locomotor activity of group III was significantly enhanced (56%) over Group II (**Fig. 18**) suggesting that in the cocaine sensitized state, NK-3 blockade increases the locomotor response to a cocaine challenge thereby enhancing the expression of locomotor sensitization. This suggests that endogenous neurokinins act to block the expression of locomotor sensitization.

Both GII and GIV received a cocaine challenge after either chronic pretreatment with cocaine alone or cocaine + PD161182 respectively. In response to a cocaine challenge after 3 drug free days, the locomotor activity of GIV was significantly higher (64%) than that for GII (**Fig. 18**) suggesting that blockade of the NK-3 receptor during chronic cocaine exposure enhances the development of sensitization. This result suggests that endogenous neurokinins may play a homeostatic role by inhibiting the development and expression of locomotor sensitization to cocaine.

Blockade of the NK-1 receptor just prior to acute cocaine blocks cocaine-induced hyperactivity. Rats sensitized to cocaine show no effect of NK-1 blockade on the expression of locomotor sensitization. Chronic exposure to NK-1 antagonists during chronic cocaine exposure had no effect on the development of sensitization, suggesting that the physiological changes that accompany cocaine sensitization compensate for the effects of an NK-1 blockade. This suggests that endogenous neurokinins augment acute responses (hyperactivity) to cocaine but have no effect on the development or expression of locomotor sensitization.

In contrast to the NK-1 results, blockade of NK-3 receptors just prior to acute cocaine has no effect on cocaine induced hyperactivity but NK-3 antagonists enhance the expression of and augment the development of cocaine induced-locomotor sensitization.

We also looked at the effects of opioids on cocaine induced locomotor sensitization in an attempt to replicate the results of others who had found that systemic administration of delta receptor antagonists and kappa receptor agonists blocked the expression and development of sensitization to cocaine respectively. We found no effect of these ligands on acute cocaine induced hyperactivity or the expression of sensitization (**Table 12**). The

only significant finding was that the delta antagonist, naltrindole enhanced the development of sensitization (neurokinin and opioid results are summarized in **Table 12**).

TABLE 12. SUMMARY OF THE EFFECTS OF LIGANDS ON COCAINE INDUCED HYPERACTIVITY AND LOCOMOTOR SENSTIZATION

			Between Group Comparisons Locomotor Activity By Day			
			Day 1	Day 7	Challenge Day	
			Acute	Progressive	Expression	Development
Compound	Receptor	Dose	(GII/GIII):GIV	(GII/GIII):GIV	GII:GIII	GII:GIV
LY306740	NK1 Ant	3mg/day (pump) 15mg/kg (i.p.)	↓* p=0.0002	No effect	No effect	No effect
CP099,994	NK1 Ant	30mg/kg	↓* p=<0.0001	↑	↑	No effect
SR140333	NK1 Ant	2mg/kg	↓	No effect	No effect	↑
PD161182	NK3 Ant	30mg/kg	No effect	↑* p=<0.0001	↑* p= 0.0260	↑* p= 0.0080
Naltrindole	δ Ant	0.3mg/kg	No effect	↑	No effect	↑* p= 0.0173
U-69593	κ Ag	0.16mg/kg	↓	No effect	No effect	No effect

3.7 Tissue Levels of SP in Response to a Time Course of Cocaine

Tissue levels of substance P were measured by radioimmunoassay after acute, 7 day chronic, and challenge cocaine, in male rats sacrificed 4 hours after the last drug treatment. The following 5 regions of the basal ganglia were assessed: dorsal CPu, ventral CPu, nucleus accumbens, globus pallidus and substantia nigra (includes SNc, VTA and SNr).

There were no significant changes in tissue levels of SP in response to cocaine when compared to vehicle treated controls in the areas and timepoints assessed (**Fig. 19A**). A possible explanation may be that changes in substance P levels in response to a cocaine time course may be masked if increased peptide expression compensates for increases in utilization (increased release and degradation). On the other hand reductions in peptide

levels, due to increased utilization, may be masked if there is sufficient compensation in peptide expression.

FIG. 19 EFFECTS OF A TIME COURSE OF COCAINE ON TISSUE LEVELS OF SUBSTANCE P

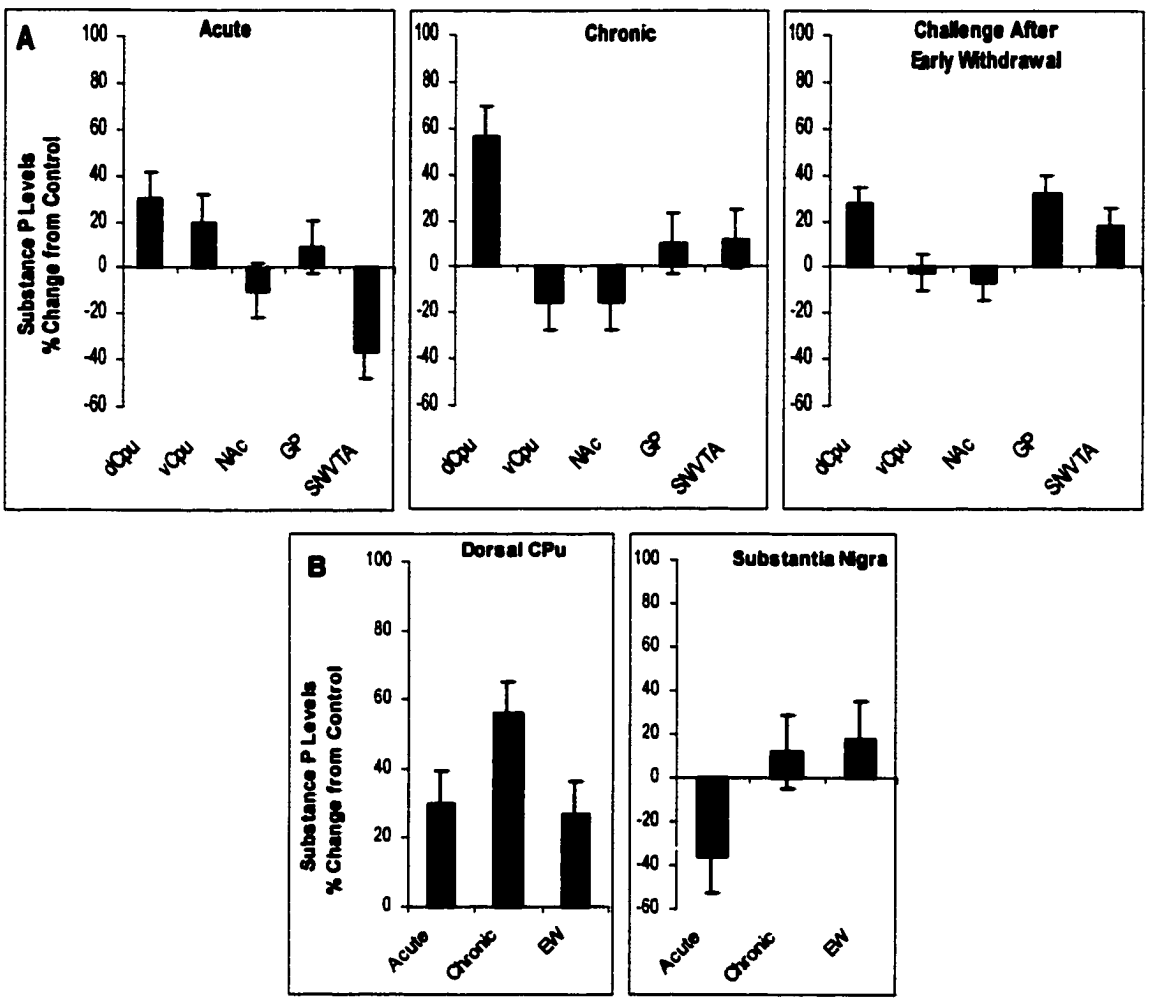


Fig. 19 A & B Effects of acute (10mg/kg), 7 days of chronic cocaine (10mg/kg 1x/day) and a cocaine challenge (10mg/kg) delivered i.p, following 3 days withdrawl from the same chronic cocaine pretreatment, on levels of substance P (SP)immunoreactivity in rat brain tissue assessed by radioimmunoassay. Rats (n=7-8/group) were sacrificed 4 hours after the last drug injection. SP levels are expressed as percent (± SEM) change from mean SP levels measured in vehicle treated control animals. Analyzed by ANOVA followed by Fisher's PLSD. *p= <0.05

In the substantia nigra there was a trend toward reduced SP immunoreactivity in response to acute cocaine (-36%) which could reflect increased utilization due to increased SP release in response to activation of the striatalnigral pathway. In contrast,

enhanced striatal SP levels in response to acute cocaine (+30%), probably reflect compensatory increases in SP production taking place in the soma of SP expressing cells. In keeping with this interpretation, we observed a trend toward increased PPT-A mRNA levels in the dorsalmedial and ventralmedial CPu after acute cocaine exposure (**Fig. 8A** page 59).

After chronic cocaine exposure enhanced SP utilization in the nigra could be masked by an upregulation in SP expression. As noted previously, we observed significant increases in PPT-A expression after chronic cocaine exposure (**Figs. 8 C&D** page 59). Transport of this increased pool of SP to the terminal fields of the nigra could provide sufficient amounts of SP to replenish peptide loss from increased utilization in response to chronic cocaine exposure. Although PPT-A mRNA expression returns to baseline after a cocaine challenge following withdrawal (**Fig. 9** page 60), a sufficient pool of peptide may remain in storage vesicles providing continued compensation for SP release in response to a challenge of cocaine. Thus, tissue levels of SP are similar after chronic cocaine exposure and exposure to a cocaine challenge (**Fig.19**).

4 DISCUSSION

4.1 Neurokinins and Acute Cocaine

4.1.1 Working Hypothesis Underlying Our Experimental Approach

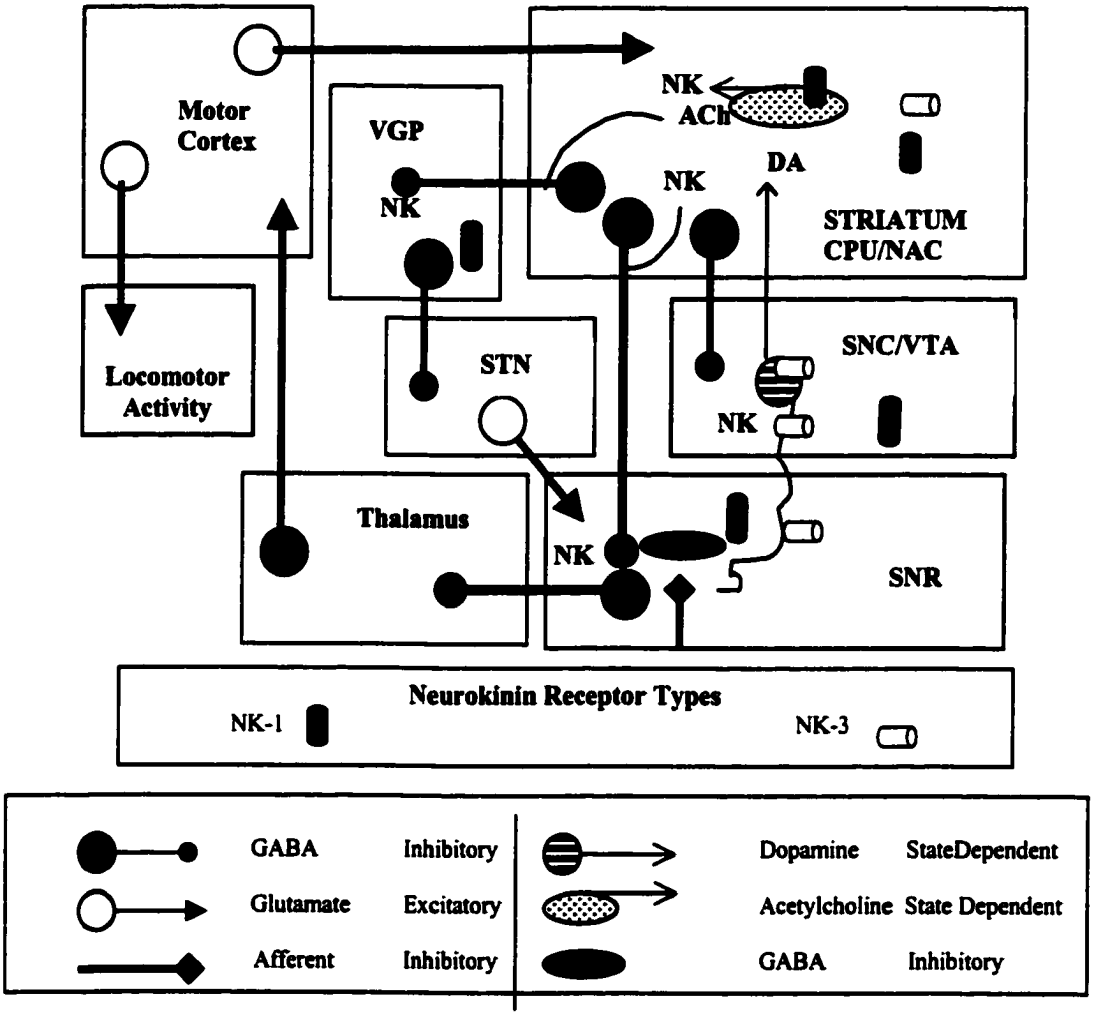
The balanced opposition model of basal ganglia function suggests that cocaine acting as an indirect dopamine agonist, facilitates motor activity by increasing activation of the direct and inhibiting activation of the indirect striatalnigral pathway (Gerfen, 1992). The medium spiny neurons of the direct pathway express the neurokinin precursor, PPT-A mRNA (Surmeier et al., 1996; Le Moine and Bloch, 1995; Gerfen, 1992), and the target regions, the striatum and SNr, are immunoreactive for NKA and SP (Lindefors et al. 1985). A sub-population of PPT-A expressing striatal medium spiny neurons, residing within the striosomal compartment, project to the SNc and may also contribute to tissue levels of SP within the substantia nigra (Heimer et al., 1995, Graybiel, 1990).

We expected that cocaine-induced activity in the striatalnigral pathway would enhance release and utilization of SP in the striatum and substantia nigra. Furthermore, enhanced utilization would be reflected in decreased tissue levels of SP measured by radioimmunoassay and compensatory upregulation in PPT-A mRNA measured by *in situ* hybridization histochemistry. Tissue levels of SP were also measured in the NAc and GP because the NAc projection to the ventral GP (VGP) is made up of neurokinin expressing medium spiny neurons (**Fig. 20**) (Heimer, 1995; Napier et al. 1995).

Specific NK-3 and NK-1 agonists, administered directly into the SNc/VTA (Stoessl et al., 1995; Elliott et al., 1991) and VTA respectively (Elliott et al, 1991) have been shown to increase locomotor activity in rats. NK-1 agonists applied to the SNr and

striatum and NK-3 agonists directly administered in the VTA, SNc, SNr and striatum (Humpel and Saria, 1993; Tremblay et al., 1992; Elliott et al., 1991) increase striatal DA.

FIG. 20 SITES OF POTENTIAL NEUROKININ-INDUCED LOCOMOTOR ACTIVITY



The locomotor activating effects of cocaine and other psychostimulants is primarily due to their ability to increase striatal DA by blocking reuptake (Ritz et al., 1987). The neurokinins, released in response to cocaine, may also contribute to enhanced striatal dopamine. We hypothesized that acute cocaine-induced neurokinin release is necessary for the locomotor activating effects of cocaine. Therefore systemic blockade of NK-1 and NK-3 receptors with antagonists would attenuate acute cocaine-induced hyperactivity.

The SP preferring NK-1 and NKA preferring NK-3 receptors were blocked by systemic administration of non-peptide antagonists prior to acute cocaine administration, to assess the affects of functional loss of neurokinin receptor activation on locomotor activity.

4.1.2 Effects of Acute Cocaine on Neurokinin Expression

We observed a trend toward upregulation of PPT-A mRNA in the dorsalmedial and ventralmedial CPu in response to acute cocaine (15mg/kg) (**Fig. 8A** page 59)although these changes were not statistically significant. Support that these trends may reflect real changes comes from other studies that have shown PPT-A upregulation at higher doses of cocaine (30mg/kg) and in response to other indirect DA agonists such as methamphetamine and GBR12909 (Zhang et al., 1997; Hurd and Herkenham, 1992).

We had predicted that tissue levels of SP measured by radioimmunoassay in the substantia nigra and striatum, would decrease in response to acute cocaine, due to increased release and utilization. In keeping with our prediction, we observed a trend toward reduced levels of SP in the SN (-36%) four hours after acute cocaine exposure (10mg/kg) although the decreases did not reach statistical significance. In contrast to the nigral reductions, SP immunoreactivity in the striatum showed a trend toward increase (+30%). In a similar study, increases in striatal tissue levels of SP were reported in response to acute methamphetamine (Bannon et al., 1987) although, another study found no changes in response to acute cocaine (20 or 30mg/kg) (Sivam, 1989). Enhanced striatal levels of SP may be the result of compensatory increases in peptide expression occurring rapidly in striatal regions containing the soma of SP expressing cells. Evidence that striatal SP release increases in response to psychostimulants was reported in a study which used an antibody-coated microprobe inserted into the striatum of anesthesized rats,

to directly measure SP release in response to acute d-amphetamine (Furmidge et al., 1992). Further clarification of the effects of acute cocaine on SP release could be obtained by performing RIA or some other sensitive SP detection method on dialysate collected *in vivo*, from cocaine treated rats. Alternatively, an RIA done on tissue taken from rats immediately following the final drug treatment should more accurately reflect dynamic changes in SP before compensatory increases in expression could mask increased utilization.

4.1.3 Effects of Neurokinin Receptor Blockade on Acute Cocaine-Induced Hyperactivity

In two separate experiments, systemic blockade of NK-1 receptors by NK-1 non-peptide antagonists (LY306740 (**Fig. 13** page 72) and CP099994 (**Fig. 14** page 74)), prior to acute cocaine exposure, attenuated (56% and 61% respectively) but did not completely block cocaine-induced hyperactivity. This suggests, that cocaine-induced neurokinin release, acting at NK-1 receptors, is necessary for the full expression of cocaine induced hyperactivity. In future experiments it would be important to determine whether acute systemic administration of NK-1 antagonists alone affects locomotor activity.

Trends described above, showing plasticity in SP tissue levels after acute cocaine, suggest that the substantia nigra and the dorsal striatum are the most likely sites for the NK-1 antagonist's attenuation of cocaine-induced hyperactivity. NK-1 receptors have been localized to the VTA and SNr, subregions of the substantia nigra, and to the large aspiny cholinergic interneurons of the striatum (Aosaki and Kawaguchi, 1996; Gerfen, 1991). To determine whether one or more of these sites are responsible for the effects of NK-1 blockade, NK-1 antagonists could be administered directly into these regions by reverse microdialysis, prior to acute, systemic cocaine administration. If direct

administration of NK-1 antagonists into either one of these regions, attenuates activity levels when compared to a group receiving sham injections of antagonist then one could conclude that such sites are responsible for these effects.

Striatal cholinergic interneurons arborize extensively in the matrix compartment (Graybiel et al., 1986; Bolam et al., 1984) and they are in a position to modulate the direct and indirect striatalnigral output neurons, thereby affecting locomotor behavior. Substance P containing nerve terminals of the striatum make synaptic contact with cholinergic neurons (Bolam and Izzo, 1988) and neurokinins evoke ACh release by activation of NK-1 receptors (Steinberg et al., 1995; Anderson et al, 1993; Arenas et al., 1991). When medium spiny neurons, which are usually silent, are phasically activated they release SP which in turn evokes long lasting excitatory post-synaptic potentials (EPSPs) in large aspiny ACh interneurons increasing their firing probability and increasing ACh release (Aosaki and Kawaguchi, 1996). Dopamine and acetylcholine modify the activity of medium spiny neurons by changing their responsiveness to other transmitters (Chiara et al., 1994). Striatal neurokinins may effect locomotor activity by changing the balance between these two modulatory transmitters. If NK-1 antagonists applied directly to the striatum attenuate cocaine-induced hyperactivity then it would be interesting to look at the effects of systemic NK-1 blockade on striatal DA and ACh release levels measured by high performance liquid chromatography (HPLC) on striatal dialysate.

The current pharmaceutical treatment for Parkinson's disease consists of the oral administration of the dopamine precursor levodopa in combination with the decarboxylase-inhibitor carbidopa to slow the conversion of levodopa to dopamine in

peripheral tissue (Springhouse, 1995). Our results raise the possibility that a non-peptide NK-1 agonist may be able to augment the motor affects of this treatment.

Acute systemic administration of NK-3 antagonists had no effect on locomotor activity when administered alone or when administered prior to cocaine, suggesting that, endogenous neurokinins acting at NK-3 receptors do not play a role in acute cocaine induced hyperactivity. This conclusion is supported by a study in which an NKA antibody coated microprobe inserted into the SNc did not detect basal levels of NKA or d-amphetamine stimulated NKA release (Furmidge et al., 1993). Since NKA is the preferred ligand for NK-3 receptors the absence of basal and acute psychostimulant-induced NKA in the SNc suggests that the perferred NK-3 ligand is unavailable under these conditions. These data are consistent with our finding that NK-3 blockade has no effect on basal locomotor activity or acute cocaine-induced hyperactivity. A follow-up study detecting NKA peptide in dialysate from the nigra would be important in confirming this. Although the neurokinins, SP and NKA are found at similar levels in the brains of drug naïve rats it is possible that psychostimulants affect peptide processing and release differently.

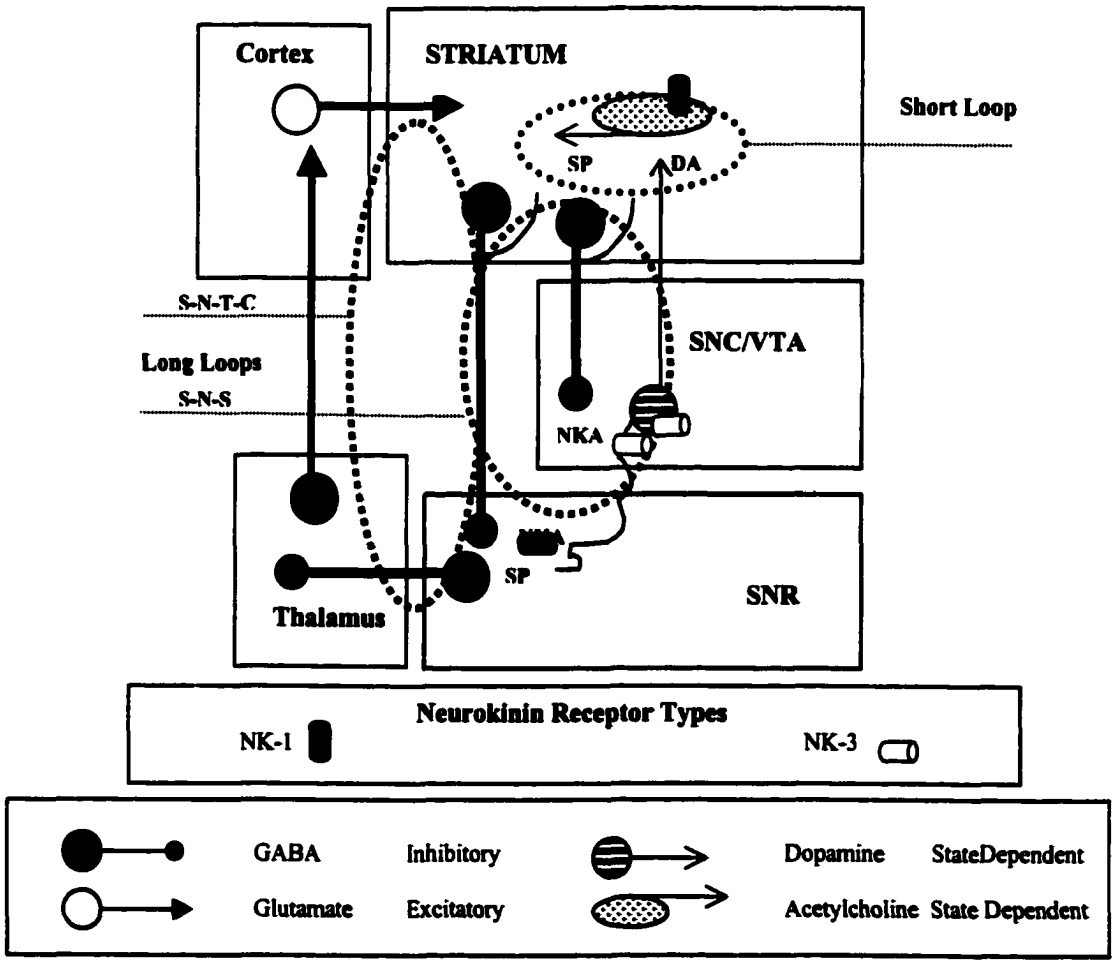
It should be noted that NK-3 agonists microinjected into the SNc and VTA have been shown to increase locomotor activity (Stoessl et al., 1995; Elliott et al., 1991) and striatal DA release (Humpel and Saria, 1993). NK-3 agonists have also been shown to increase DA cell activity in nigral slices (Seabrook et al., 1995; Keegan et al., 1992). This system can obviously be stimulated by exogoneous application of NK-3 agonists but our results suggest that under physiological conditions this does not occur.

4.2 Neurokinins and Locomotor Sensitization Cocaine

4.2.1 Working Hypothesis Underlying Our Experimental Approach

We predicted that, chronic, intermittent cocaine exposure would upregulate the expression of PPT-A mRNA over acute levels resulting in enhanced neurokinin production and release, in response to repeated cocaine exposure. The enhanced release of neurokinins, in turn, would increase dopaminergic activity and dopamine release resulting in further increases in neurokinin expression and release, creating a self-enhancing amplification loop (**Fig. 21**).

FIG. 21 NEUROKININ – DOPAMINE AMPLIFICATION LOOPS



Our hypothesis was that amplificaton within these circuits is responsible for the escalating locomotor response associated with cocaine sensitization. Three different amplification loops were postulated to mediate the effects of chronic cocaine (**Fig. 21**).

To test this hypothesis we measured substance P precursor mRNA expression by *in situ* hybridization histochemistry and tissue levels of SP by radioimmunassay in response to a time course of chronic, intermittent cocaine administration and a cocaine challenge after three and twenty one days of drug abstinence. We predicted that PPT-A expression would be upregulated in response to increasing cocaine exposure but would be attenuated by co-administration of MK-801 and tissue levels of substance P would be decreased due to increased release and utilization.

NK-1 receptors are associated with the short intra-striatal and the long striatal-nigral-thalamic-cortical-striatal (S-N-T-C-S) loops and NK-3 receptors are associated with the long striatal-nigral-striatal (S-N-S) loop. To test this hypothesis the SP preferring NK-1 receptor and the NKA preferring NK-3 receptor were blocked by systemic administration of non-peptide receptor antagonists prior to cocaine administration to assess the effects of loss of neurokinin receptor function on the development and expression of locomotor sensitization. If NK-3 receptor blockade attenuated the development or expression of sensitization, we would conclude that sensitization was dependent on the amplification of the striatal-nigral-striatal loop. Alternatively, if blockade of NK-1 receptors attenuated the development or expression of sensitization, we would conclude that sensitization was dependent on the intrastriatal or striatal-nigral-thalamic-cortical-striatal loop. Certainly, there could be dependence upon all three pathways. In that case we would find that both NK-1 and NK-3 antagonists blocks locomotor sensitization to cocaine.

4.2.2 Chronic Cocaine and Neurokinin Expression

As expected, we found that chronic cocaine increased PPT-A mRNA levels, after six and fourteen days of exposure but not after one or three days and that these increases were attenuated by concurrent administration of cocaine and the NMDA receptor antagonist MK-801 (**Fig. 8** page 59). This confirmed earlier work by others, showing that NMDA activation is necessary for the development of neurochemcial changes associated with chronic cocaine exposure and locomotor sensitization (for review see Wolf, 1998). Although others had reported upregulation of PPT-A mRNA in response to chronic, intermittent administration of a number of direct and indirect DA agonists (Zhang et al., 1997; Sivam, 1996; Hurd & Herkenham, 1992) as far as I know, this study is the first to show a similar result for cocaine. Because locomotor sensitization to cocaine is sustained in response to a cocaine challenge following withdrawal (**Fig. 12** page 69) we expected that PPT-A mRNA levels would also remain enhanced. Contrary to what we expected however, PPT-A mRNA levels returned to baseline when measured after a cocaine challenge following either three or twenty one days of withdrawal from drug (**Fig. 9** page 60). This suggests that cocaine exposure does not have an enduring affect on PPT-A mRNA upregulation and therefore elevated levels of PPT-A are not necessary for the expression of sensitization but may be necessary for its initiation or development, possibly by increasing vesicular pools of neurokinins.

We found that tissue levels of striatal and nigral substance P were unchanged after chronic cocaine or cocaine challenge when compared to saline treated controls. These results are consistent with the findings of Sivam, 1989, who also showed no change in SP levels after chronic cocaine administration. In contrast, a similar study reported enhanced

SP immunoreactivity in response to chronic amphetamine (Bannon et al., 1987). This same study found no change in SP levels in response to apomorphine, a direct DA agonist whereas a similar study reported increases (Li et al., 19887). Reported increases are in keeping with an enhanced vesicular pool of neurokinin. None of these studies reported decreases in striatal SP levels in response to chronic cocaine exposure suggesting that SP release is not enhanced in response to chronic cocaine as suggested by our hypothesis. Alternatively, increased utilization may be masked by increased peptide pools, and over-compensation may even explain the reported increases in tissue levels of SP. Evidence from our experiments with NK-3 blockade (see below) shows that locomotor behavior is responsive to an NK-3 ligand after chronic but not acute cocaine exposure which raises the possibility that NKA rather than SP release, may be modified by chronic, intermittent cocaine. Changes in NKA release in response to a cocaine time course could be determined more accurately by collecting dialysate *in vivo* from cocaine treated rats followed by peptide detection by radioimmunoassay or some other sensitive detection method.

4.2.3 NK-3 & NK-1 Blockade and Locomotor Sensitization to Cocaine

NK-3 blockade by systemic administration of PD161182 during 7 days of chronic cocaine administration resulted in a robust enhancement in progressive locomotor sensitization (119%) when compared to animals receiving cocaine alone (**Fig. 16** pages 76 & 77). A challenge of PD161182 + cocaine after 3 days withdrawal from chronic cocaine, significantly enhanced locomotor activity (56%) over animals receiving a challenge of cocaine alone (**Fig.18** page 79). In addition, this group had significantly higher activity after the challenge than after 7 days of chronic exposure to cocaine (63%)

whereas the group challenged with cocaine alone although still sensitized their response to the challenge was not significantly increased over their response to chronic cocaine (13%). This result suggests that NK-3 blockade enhances the expression of locomotor sensitization.

Rats pretreated with cocaine + PD161182 for 7 days followed by a challenge of cocaine alone also showed higher locomotor activity over animals pretreated with cocaine and challenged with cocaine (64%), however activity levels after the challenge were significantly decreased compared to the response to chronic pretreatment with PD161182 + cocaine (-19%). Chronic pretreatment with PD161182 alone for 7 days prior to acute cocaine administration resulted in activity levels similar to those observed in response to acute cocaine. Therefore, chronic NK-3 blockade by itself does not change the locomotor response to subsequent cocaine exposure. Taken together, these findings suggest that the primary effect of NK-3 blockade is on the expression and not the development of locomotor sensitization. In cocaine-sensitized rats, endogenous neurokinins acting via NK-3 receptors, inhibit locomotor activity thereby providing homeostatic regulation that attenuates but does not completely block the escalating locomotor behavior associated with sensitization.

NK-3 receptors have been localized to dopamine cell bodies and dendrites in the SNc and VTA. In rats naïve to cocaine, exogenously applied neurokinins within these areas activate DA neurons, DA release and motor activity but endogenous neurokinins do not appear to play this role. Existing evidence suggests that NKA is not tonically released in the VTA and SNc and moreover acute exposure to psychostimulants does not induce NKA release in these areas (Furmidge et al., 1993). To my knowledge there are no

studies that have measured NKA release in the nigra in response to chronic psychostimulant exposure. An experiment, that measured NKA by radioimmunoassay in SNc/VTA dialysate or tissue collected immediately following chronic drug exposure from the VTA and SNc, would provide this information. Evidence from this study suggests that NKA release in the VTA and SNc should increase in response to chronic cocaine and would act via NK-3 receptors to inhibit DA neurons and reduce striatal DA release. This would suggest that NK-3 blockade prior to cocaine administration in sensitized animals would result in enhanced DA release in the striatum compared to animals receiving cocaine alone. This prediction could be tested by measuring DA levels in dialysate collected from the striatum of rats after exposure to chronic cocaine or cocaine + PD161182. It is interesting to note that striatal DA levels measured by microdialysis were found to be lower after a cocaine challenge than after acute cocaine administration (Zhang, 1997, unpublished results). This supports the assertion that there is homeostatic regulation of dopamine neurons in the sensitized state and our results suggest that the neurokinins may be the mediator of this effect.

Sustained activation of DA neurons has been shown to result in depolarization block, a mechanism that under less intense stimulation results in a shift of neural activity into the bursting mode. Depolarization block decreases responsiveness to glutamate, providing a homeostatic balance to over-stimulation (Kalivas, 1993). In a non-sensitized state, dopaminergic activity levels are kept in check by somatodendritic D2 receptors which when activated by DA, inhibit DA neurons (Henry and White, 1992). Subsensitivity of D2 receptors in response to chronic cocaine results in increased dopamine activity that may unmask the excitatory actions of neurokinins at NK-3

receptors. The NK-3 antagonist PD161182 may relieve the block by eliminating depolarizing effects at the NK-3 receptor increasing activity in this pathway and facilitating locomotor activity. In a recent article it was reported that blockade of NK-3 receptors antagonizes depolarization block of midbrain dopamine neurons in guinea pigs (Guedet et al., 1999)

MK-801 administered to rats has been shown to increase locomotor activity and to sensitize to chronic administration but when administered with cocaine it has the opposite effect attenuating the development of locomotor sensitization (Wolf, 1998). MK-801 acting alone on DA neurons may reduce depolarization block thereby contributing to increased activity levels and increased striatal DA. In the cocaine-sensitized state however, endogenous neurokinins are performing this function so that adding MK-801 further reduces activity in these neurons.

Our research suggests that activation of NK-3 receptors in cocaine sensitized animals plays a homeostatic role in reducing sensitized locomotor activity. The incentive-sensitization theory of drug addiction proposes that sensitization of dopamine circuits involved in drug wanting contribute to the craving experienced by drug addicts (Robinson and Berridge, 1993). The motor activating and reinforcing affects of cocaine are mediated by dopaminergic circuits of the basal ganglia (Koob and Goeders, 1989). It has been suggested that the biological mechanisms underlying locomotor sensitization and drug craving may be similar (Henry and White, 1992). If NK-3 receptor blockade during cocaine exposure, potentiates the reinforcing effects of cocaine as it potentiates the locomotor activating effects, then NK-3 agonists may be able to reduce craving and provide a potential treatment for drug addiction.

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