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Synaptic Depression by Dopamine in Nucleus Accumbens

by

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1. Introduction

This work demonstrates a novel mechanism for the modulation of synaptic transmission by dopamine in the rat nucleus accumbens. Dopamine is known to increase intracellular levels of cyclic adenosine 3'-5'-monophosphate (cAMP) via postsynaptic D_1 receptors which are positively coupled to adenylate cyclase (AC). The results in this thesis suggest that D_1 receptor activation then modulates synaptic transmission via the following steps. Intracellular cAMP is pumped by nucleotide transport molecules into the extracellular space where it is metabolized to adenosine monophosphate and subsequently adenosine. The extracellular adenosine then acts on presynaptic adenosine A_1 receptors causing a decrease in glutamate release which is seen electrophysiologically as a depression of EPSP amplitude. Therefore, the postulate is that DA inhibits synaptic transmission in rat nucleus accumbens neurons via an adenosine A_1 mediated pathway. This mechanism/pathway is shown diagramatically in Figure 1.

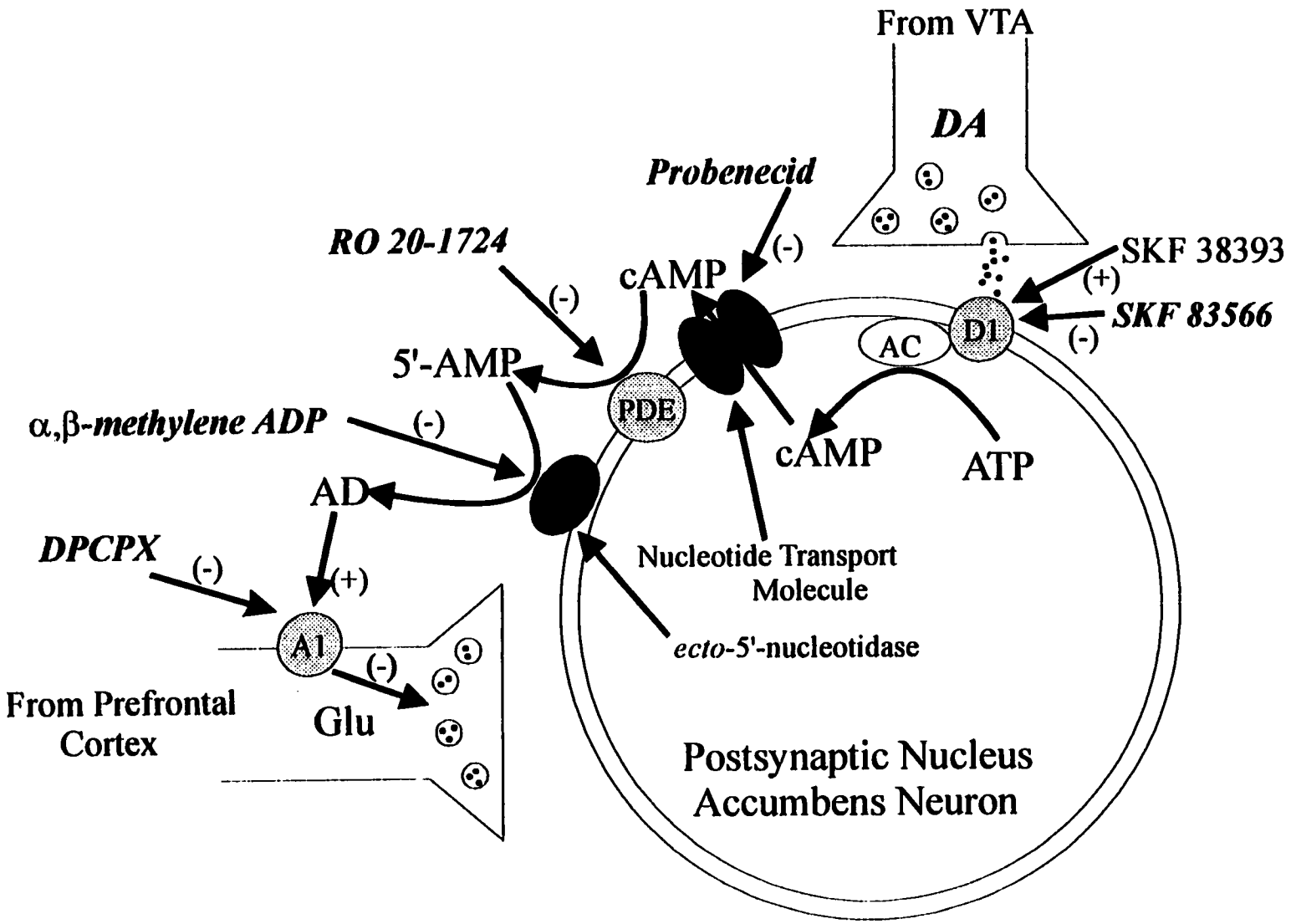
1.1 Abbreviations

6-OHDA = 6-hydroxy-dopamine

8-CPT = 8-cyclopentyltheophylline

8-pSPT = 8-(p-sulfophenyl)-theophylline

Figure 1: A diagrammatic representation of the postulated pathway for dopamine-mediated inhibition of fEPSPs in NAcb slices. Dopamine (DA) is released from presynaptic cortico-striatal neurons and acts on postsynaptic D1 receptors. D1 receptors are positively coupled to adenylate cyclase (AC), and initiate an increase in intracellular cAMP. This cAMP is pumped out of the cell by the nucleotide transport molecule, and subsequently metabolized by *ecto*-enzymes to adenosine (AD). Adenosine then acts on presynaptic A_1 receptors, causing a decrease in glutamate release from presynaptic terminals. Shown in ***bold italics*** on the diagram are the various drugs applied to support the hypothesized mechanism.


From VTA
DA
Probenecid
(-)
RO 20-1724
(-)
cAMP
SKF 38393
(+)
SKF 83566
(-)
D1
AC
5'-AMP
PDE
α,β-methylene ADP
(-)
cAMP
ATP
AD
DPCPX
(-)
(+)
Nucleotide Transport
Molecule
ecto-5'-nucleotidase
A1
(-)
From Prefrontal
Cortex
Glu
Postsynaptic Nucleus
Accumbens Neuron

aCSF = Artificial cerebrospinal fluid

AC = Adenylate cyclase

AD = Adenosine

ADP = Adenosine 5'-diphosphate

AMP = Adenosine 5'-monophosphate

ATP = Adenosine 5'-triphosphate

cAMP = Cyclic adenosine 3',5'-monophosphate

CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione

DA = Dopamine

DMSO = Dimethyl sulfoxide

DPCPX = 8-Cyclopentyl-3,7-dihydro-1,3-dipropyl-1H-purine-2,6-dione

EPSPs = Excitatory postsynaptic potentials

fEPSPs = Field excitatory postsynaptic potentials

GABA = γ-Aminobutyric acid

IBMX = 3-Isobutyl-1-methylxanthine

IPSPs = Inhibitory postsynaptic potentials

mEPSCs = Miniature excitatory postsynaptic currents

mGluRs = Metabotropic glutamate receptors

NAcb = Nucleus accumbens

NECA = 5'-N-ethylcarboxamideadenosine

PIA = N^6-phenylisopropyladenosine

RO 20-1724 = 4-[(3-Butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone

VTA = Ventral tegmental area

1.2 The Nucleus Accumbens

1.2.1 Behaviour and Physiology

The NAcb (also referred to as the ventral striatum) is an area of the brain which has been implicated in many diverse behaviour patterns. Studies in rats and other rodents over the past twenty years or more have demonstrated that behavioural activity is modified by injection of various neurochemicals into NAcb. For example, 6-OHDA lesions have been shown to affect displacement behaviour (Robbins and Koob, 1980) and more specific behaviours such as chewing (Lund and Dellow, 1971) and regulation of forelimb muscle tone (Ellenbroek *et al.*, 1988). The most intensively studied behaviours related to NAcb function have been locomotor activity and learning/memory functions. The NAcb has been linked to processes such as selective attention, secondary reinforcement, reward, social memory, and attention to cue directed behaviours (e.g. Bos *et al.*, 1991; Ploeger *et al.*, 1991).

Additionally, the NAcb is thought to play a role in addictive and behavioural sensitization behaviours, such as amphetamine self-administration (Piazza *et al.*, 1991) and place preference (Carr and White, 1983). Several researchers have shown that drugs of abuse such as ethanol, amphetamine, cocaine, morphine, and nicotine stimulate the release

of DA from mesolimbic structures (Di Chiara and Imperato, 1988; Imperato and Di Chiara, 1985; Imperato *et al.*, 1986). Further electrophysiological studies have shown that cocaine acts in the NAcb to potentiate the actions of dopamine (Uchimura and North, 1990). Intracellular recordings were made in this study from NAcb neurons, and it was demonstrated that low concentrations of cocaine (1-10 μM) increased the effects of applied DA by blocking DA uptake (Uchimura and North, 1990). Results of dialysis experiments in the NAcb showed that administration of nicotine caused the release of DA from limbic afferents to the NAcb (Imperato *et al.*, 1986). Similar dialysis results have been obtained in the NAcb using other drugs of abuse, such as opiates, ethanol, amphetamine, and cocaine (Di Chiara *et al.*, 1988). This is important evidence linking the rewarding actions of such drugs to the mesolimbic DA system, which has its major terminus in the NAcb. It is also an important finding that drugs of such varied pharmacological classes (e.g. central depressants, central stimulants, narcotic analgesics, to name a few), which likely act through different primary mechanisms, seem to have a common final pathway mediating their rewarding properties (Di Chiara and Imperato, 1988).

Among the most clinically important and interesting aspects of the NAcb are its postulated roles in disease states such as Parkinson's disease and schizophrenia. It has been known since the early 1960s that in parkinsonian patients the striatum demonstrated a loss of dopamine (Barbeau, 1960). As a result of these findings, there has been much interest in the use of dopaminergic agents (e.g., L-Dopa) in the treatment of Parkinson's disease. In schizophrenia, the "dopamine hypothesis" has been favoured as an explanation

(Bachus and Kleinman, 1996). In this hypothesis, the symptoms of schizophrenia are brought about via a degeneration of the limbic DA system (Joyce, 1993). However, evidence supporting this hypothesized dopaminergic framework for schizophrenia has been elusive, and recently a neural systems approach has gained support (Bachus and Kleinman, 1996). This theory is based on evidence which links the limbic cortex to the neuropathology of schizophrenia, and since the limbic cortex modulates dopaminergic function in the NAcb, the focus has shifted to a possible glutamatergic dysfunction underlying schizophrenia (Bachus and Kleinman, 1996).

1.2.2 Anatomy and Connectivity

The NAcb is a rostral region of the brain, which is similar in its subcortical connections to the rest of the striatum. However, where the NAcb differs is in the inputs it receives from such areas as the hippocampus,. ventral tegmental area, amygdala, sublenticular substantia innominata, and lateral hypothalamus (Merideth *et al.*, 1992). In general, it can be stated that the NAcb is a target for afferents from limbic regions (including the prefrontal cortex), whereas the remainder of the striatum receives inputs from sensory- and motor-related cortical areas (O'Donnell and Grace, 1993). At the upper level of anatomical differentiation, the NAcb can be divided into two regions: the core (central region, surrounding the rostral limb of the anterior commissure), and the shell (peripheral zone) (Meredith *et al.*, 1993). The core region has been suggested to

function as an extension of the striatum, where the shell seems to represent a rostral extension of the amygdala (Heimer *et al.*, 1991). Core and shell regions of the NAcb can be differentiated based on several criteria. For example, differences exist in terms of immunohistochemistry, binding of various receptor ligands, efferent and afferent connections, density of cholinergic neurons, levels of peptides, and in the membrane properties of neurons (Meredith *et al.*, 1992, 1993). In core neurons, for instance, the resting potential is more negative, and the input resistance lower than in shell neurons (Pennartz *et al.*, 1992), suggesting that shell neurons will be more excitable than core neurons (Meredith *et al.*, 1993). Neither core nor shell neurons have been seen to be spontaneously active at resting membrane potentials, though depolarization by current injection in either type can generate both single spikes and trains of action potentials (O'Donnell and Grace, 1993).

The principal neuron of the striatum and NAcb is the medium spiny neuron which receives inputs from many brain regions and relays them to midbrain structures such as the globus pallidus and substantia nigra (Chang and Kitai, 1985; Pennartz and Kitai, 1991). The medium spiny neurons have been described in detail by Meredith *et al.* (1992) in a study in which NAcb neurons were filled with the dye Lucifer Yellow, and were reconstructed by computer. Their results show small to medium neurons (9-15 μm diameter) with extensive dendritic arborizations. The dendrites are spiny, with neurons in the core showing an average of 20% more spines than neurons in the shell (Meredith *et al.*, 1992). Subsequently, glutamate decarboxylase immunoreactivity studies have shown that nearly 90% of the neurons in the NAcb are GABAergic in nature as determined by

glutamate decarboxylase (GAD)-immunoreactivity (Kita and Kitai, 1988). In addition, staining studies have revealed that approximately 3% of neurons in the NAcb are different from the typical medium spiny neuron in that they are generally larger and more sparsely spined on both dendrites and somata (O'Donnell and Grace, 1993). Approximately equal proportions of these large neurons stain positive for GAD or choline acetyltransferase (ChAT) (Kita and Kitai, 1988).

Chang and Kitai (1985) used antidromic stimulation to show that axons of the NAcb neurons project to the VTA and substantia nigra, and followed intracellularly labeled NAcb axons to the ventral pallidum. Similarly, they demonstrated that orthodromic stimulation of the amygdala was sufficient to produce both IPSPs and EPSPs in the NAcb. Other studies have shown afferent inputs to the NAcb from a wide range of brain areas, including amygdala, neocortex, hippocampus, thalamic nuclei, and VTA (Christie *et al.*, 1987). Of these, only hippocampal and cortical inputs have been shown to use the neurotransmitter glutamate as their signalling molecule (Walaas, 1981), whereas afferents from VTA are known to be dopaminergic (Beckstead *et al.*, 1979). Prefrontal cortical fibres have been shown to form asymmetrical synapses in NAcb, and nearly 100% terminate on spines in the core region (Sesack and Pickel, 1992). In a similar study, it has been shown that dopaminergic afferents from VTA also synapse primarily on the shafts and spines of dendrites from neurons in the NAcb (Zahm, 1992). Significantly, corticostriatal afferents seem to converge on the same elements as dopaminergic inputs in both the shell and core regions (Toterdell and Smith, 1989), suggesting a potential

morphological substrate for the presynaptic interactions seen in these areas (Meredith *et al.*, 1993).

In a study integrating several of these ideas, Yang and Mogenson (1984) showed a functional relationship between excitatory inputs to the NAcb from the hippocampus and mesolimbic dopaminergic inputs from the VTA. In this study, the subiculum of the hippocampus was stimulated, and recordings were made from cells of the NAcb. It was shown that this stimulation resulted in excitation of the NAcb cells. However, when trains of conditioning pulses were delivered to the VTA 100 ms prior to stimulation of the hippocampus, the excitation of NAcb neurons was attenuated (Yang and Mogenson, 1984). In order to demonstrate that the attenuation seen was a result of a dopaminergic action, it was shown both that iontophoretically applied DA mimicked, and that pretreatment of the VTA with 6-OHDA reduced the attenuation (Yang and Mogenson, 1984).

These studies fit with pharmacological, electrophysiological and behavioural studies which have suggested that NAcb neurons stimulate locomotion via a disinhibition, rather than a direct stimulation, of their target neurons. Excitatory input via glutamatergic cortical afferents (Christie *et al.*, 1987) stimulates striatal neurons, which are GABAergic (Kita and Kitai, 1988), and these neurons in turn innervate GABAergic neurons in midbrain nuclei. In this way, stimulation of striatal neurons causes an inhibition of inhibitory neurons in the output nuclei (such as globus pallidus). Many researchers have now provided evidence that glutamatergic excitation and GABAergic inhibition are major components of the excitation of spiny projection neurons (Chang and Kitai, 1986;

Uchimura *et al.*, 1989; and Pennartz *et al.*, 1990). For example, the $GABA_A$ antagonists picrotoxin and bicuculline enhance and prolong EPSPs in spiny projection neurons (Pennartz and Kitai, 1991) and glutamate receptor antagonists strongly depress IPSPs (Horne *et al.*, 1990; Pennartz and Kitai, 1991), both in slices.

1.3 Adenosine

In the early 1970s it was first noted that there were extracellular AD receptors which modified physiological function and possibly cAMP concentration in many different cell types. The basic properties of these receptors were first described by Sattin and Rall (1970), and Van Calker *et al.* (1979) first recognized that there were probably two classes of receptor, which either stimulated or inhibited adenylate cyclase activity. The two receptor classes were first distinguished on the basis of pharmacological properties. A comparison of the actions of AD and two of its analogs, NECA and PIA, on the adenylate cyclase pathways in three different tissues revealed two different potency sequences (Londos *et al.*, 1980). In rat adipocyte cells, PIA > adenosine > NECA, whereas in liver and Leydig cells, NECA > adenosine ≥ PIA. Van Calker *et al.* (1979) referred to these two types as the A_1 and A_2 receptors, respectively, with A_1 receptors having a ten-fold higher affinity for AD.

Iontophoretic applications *in vivo* (e.g. Phillis *et al.*, 1979; Kostopoulos and Phillis, 1977) or in brain slice preparations (Dunwiddie and Hoffer, 1980) show that AD

has inhibitory electrophysiological effects. Scholfield (1978) used extracellular and intracellular recording techniques to demonstrate that AD, at various concentrations up to 1 mM, could depress postsynaptic potentials in guinea-pig olfactory cortex. Similar results have been achieved in the CA1 region of the rat hippocampus (Dunwiddie and Hoffer, 1980) and in the neostriatum (Malenka and Kocsis, 1988).

In order to distinguish whether the electrophysiological actions of AD were mediated through A_1 or A_2 receptors, selective antagonists were needed. The synthesis of DPCPX, the 1,3-dipropyl-8-cyclopentyl substitution of xanthine, as a selective A_1 antagonist (Lohse *et al.*, 1987) fulfilled this need. In *in vitro* rat hippocampus preparations, Thompson *et al.* (1992) showed that bath applications of 200 nM DPCPX blocked the actions of 50 μM AD, and concluded that since this block was total, any involvement of A_2 receptors could be excluded. A similar study involving frog neuromuscular junctions showed that DPCPX blocked AD-mediated neuromuscular depression by acting on A_1 receptors (Redman and Silinsky, 1993).

Scholfield (1978) showed that action potential amplitude, membrane potential, and resting input resistance were not affected by AD. These factors led to the hypothesis that AD acted to reduce the efficacy of synaptic transmission, possibly via reduced transmitter release (Phillis and Wu, 1981). Dunwiddie and Haas (1985) provided further evidence of the presynaptic action of AD when they showed that AD increased paired-pulse facilitation of Schaffer collateral responses in CA1 hippocampal neurons. Their major evidence was the fact that AD had a greater depressant effect on the first response in a conditioning-testing pair, thereby enhancing the observed facilitation. This result was

consistent with previous findings that agents which reduce transmitter release (i.e. reduced calcium) increase synaptic facilitation (Harris and Cotman, 1983).

There are at least three mechanisms to be considered, which are not necessarily exclusive of one another, by which AD might inhibit synaptic transmission (Greene and Haas, 1991). The first is the direct suppression of presynaptic calcium influx by AD, the second an indirect suppression due to a shunt by an increased potassium conductance, and the third an intracellular effect on calcium dependent release in the presynaptic cell (Greene and Haas, 1991).

In the nucleus accumbens, glutamate is a major excitatory transmitter for input from several different brain regions (Nauta *et al.*, 1978; Kelley and Domesick, 1982; Christie *et al.*, 1987). Uchimura and North (1991) showed that application of AD to *in vitro* slices of rat NAcb reduced glutamate-mediated postsynaptic potentials. They concluded that this was representative of a presynaptic action by AD to inhibit the release of glutamate from cortical fibers (Uchimura and North, 1991). This result was consistent with autoradiographic studies showing [^{3}H]cyclohexyladenosine binding sites (A_1 receptors) in the nucleus accumbens (Goodman and Snyder, 1982) and on the terminals of corticostriatal fibers (Alexander and Reddington, 1989). Additional support for the presynaptic action of AD has come from *in vitro* studies. Proctor and Dunwiddie (1987) showed that the decrease in EPSP amplitude mediated by AD is not accompanied by a decrease in sensitivity to glutamate, indicating that the effect of AD is on the presynaptic release of the excitatory transmitter. Also, Lupica *et al.* (1992) used whole cell recording and variance analysis to investigate the site of action of AD in rat hippocampus. They

showed that both AD and the AD receptor antagonist cylcohexyladenosine reduced the number of neurotransmitter quanta released, but not the size of quanta or postsynaptic sensitivity.

Goodman and Snyder (1982) also comment on the relationship between the localization of A_1 receptors and the AD-generating enzyme 5'-nucleotidase in striatum (Scott, 1967). The NAcb is shown to contain a moderately high level of A_1 receptors (Goodman and Snyder, 1982), and a very high level of 5'-nucleotidase activity (Scott, 1967). These results further suggest that AD is formed in the NAcb through the actions of the enzyme 5'-nucleotidase, and is then used as a transmitter, acting on A_1 receptors, which are likely located presynaptically.

1.4 cAMP Efflux

Since it is known that AD has inhibitory effects in the NAcb and many other brain regions (Phillis and Wu, 1981), the origin of endogenous AD must be considered. One possibility is cAMP which is generated intracellularly and pumped into the extracellular space. This hypothesis is supported by early evidence from Woodruff *et al.* (1976) showing that the depressant actions of dopamine and its analogue ADTN are mimicked in most cells by application of the cAMP analogue dibutyryl cAMP (Woodruff *et al.*, 1976).

Unidirectional anion pumps have been described in many tissue types (Steinberg *et al.*, 1987; Tisa and Rosen, 1990). C6 rat glioma cells have been shown to be capable of extruding intracellular cAMP by a unidirectional nucleotide efflux pump, which can be

blocked by probenecid, alkaloids, and prostaglandin A_1 (Penit *et al.*, 1974; Doore *et al.*, 1975; Rindler *et al.*, 1978; Henderson and Strauss, 1991). Probenecid has also been used to block cholate (Henderson and Strauss, 1991) and methotrexate (Henderson and Tsuji, 1987, 1988) efflux in these same cells. In rat cerebral cortex cultures, it has also been shown that probenecid blocks the accumulation of extracellular cAMP stimulated by isoproterenol (Rosenberg *et al.*, 1994).

Due to the large differences in structure and charge among the possible substrates for the transporter, it has been suggested that various other structurally diverse molecules might also be substrates of this transport molecule. If this is the case, this anion transporter may play a role in the removal of potentially toxic anions from within the cell (Henderson and Tsuji, 1990). This hypothesis is strengthened by descriptions of such an anion transport molecule in multidrug-resistant cells, which show resistance to anti-tumor drugs such as anthracyclines, actinomycin D, and epipodophylotoxins (Beck, 1987; Gottesman and Pastan, 1988; Moscow and Cowan, 1988).

cAMP has been shown to be an anion transport substrate in many tissues (Penit *et al.*, 1974; Zumstein *et al.*, 1974; Doore *et al.*, 1975; Plagemann and Erbe, 1977; Brunton and Mayer, 1979; Heasley and Brunton, 1985). Related studies show that cAMP accumulates in rat striatal tissue in response to DA applications (Stoof and Kebabian, 1982; Headley and O'Shaughnessy, 1986; O'Shaughnessy *et al.*, 1987). An interesting possibility is that adenylate cyclase itself might be the anion transport molecule (Rosenberg, 1992). This was suggested when Krupinski *et al.* (1989) published an amino

acid sequence and structure for adenylate cyclase which was strikingly similar to the suggested structure of the mutlidrug-resistance transporter (Gottesman and Pastan, 1988).

1.5 Dopamine D_1 Effect

The fact that DA acts in the nucleus accumbens has long been known. In the early 1970s, Pijnenburg and Van Rossum (1973) discovered that injections of DA into the rat NAcb enhanced locomotor activity. Other researchers were able to duplicate the effect with DA agonists such as apomorphine, and with amphetamine, which stimulates dopamine release (Kelly *et al.*, 1975; Swerdlow and Koob, 1987). Electrophysiologically, DA has been shown to attenuate EPSPs in the NAcb studied *in vivo* (Yim and Mogenson, 1988). With this knowledge came the question of the physiological actions of DA in the NAcb. Woodruff *et al.* (1976) addressed this question in the context of DA as it relates to the actions of locomotor stimulant drugs. In their 1976 report, Woodruff and colleagues made extracellular recordings from single neurons in an *in vivo* preparation. Cells were induced to repetitive firing by applications of DL-homocysteic acid (2-4 nM) and the effect on firing rate of various iontophoretically applied drugs was investigated. It was shown that dopamine and the dopamine analogue ADTN (2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene) exerted strong inhibitory effects on the firing of neurons of the NAcb (Woodruff *et al.*, 1976).

Kebabian and Calne (1979) first classified two receptors for DA, termed D_1 and D_2. It is now known that there are at least five distinct subtypes of the DA receptor,

which are broadly grouped into two families: D_1-like , including D_1 and D_5 receptors, and D_2-like, including D_2, D_3, and D_4 receptors (Waddington and Deveney, 1996). The two families of receptors are distinguished based on pharmacology, function, and localization. The D_1-like receptors are coupled positively to adenylate cyclase, while the D_2-like receptors inhibit this enzyme or act through different effector systems such as phospholipase C and A_2, or Ca^{2+} and K^+ channels (Griffon *et al.*, 1996). The effects of all DA receptors are mediated through G-proteins (Griffon *et al.*, 1996; Jensen *et al.*, 1996).

Uchimura *et al.* (1986) determined that the hyperpolarizing action of DA on NAcb neurons was mediated by D_1 receptors, since it was unaffected by the selective D_2 antagonist sulpiride. It was shown subsequently that dibutyryl cAMP selectively enhanced the hyperpolarization, but had no effect on the depolarization seen in some cells and shown to be mediated by D_2 receptors (Uchimura *et al.*, 1986). In their 1996 paper, Nicola *et al.* further addressed the question of D_1 and D_2 receptors. They showed that the D_1 antagonist SCH-23390, but not the D_2 antagonist sulpiride, attenuated the effects of DA applications. Similarly, the D_1 agonist SKF-38393 (100 μM), but not the D_2 agonist quinpirole, mimicked the DA effect. Harvey and Lacey (1996) showed the same thing with a different D_1 agonist, SKF-81297A. In further support of these biochemical investigations, autoradiographic studies using [^{3}H]SCH-39166 and [^{3}H]SCH-23390 showed that the NAcb and caudate nucleus contain high densities of D_1 receptors (Wamsley *et al.*, 1992). As an extension of the evidence supporting a role for a D_1-like receptor in the NAcb, Kebabian *et al.* (1972) showed early on that DA acted to increase levels of cAMP in the caudate nucleus.

In addition to the discrimination between D_1 and D_2 actions of DA, Nicola *et al.* (1996) and Harvey and Lacey (1996) both presented evidence that the D_1 effect is presynaptic. Nicola *et al.* used whole-cell voltage clamp to study mEPSCs in NAcb cells. They were able to show that amphetamine application (a drug which induces DA release and subsequent synaptic depression similar to that seen with direct DA application) caused a decrease in frequency and not amplitude of mEPSCs. This is consistent with the idea that DA acts by causing a decrease in presynaptic transmitter release. Similar whole-cell methods were used by Harvey and Lacey (1996) to show a presynaptic site of action for DA. None of the drugs applied, including DA agonists, cocaine, and amphetamine, caused a change in membrane conductance or holding current in cells that were inhibited. This indicates that the inhibition is due solely to actions on the presynaptic cell. All of this evidence fits well with the hypothesis that DA acts via AD, which has already been shown to act at presynaptic receptor sites.

There has been speculation about possible synergistic actions of D_1 and D_2 receptors in the brain (Beaulieu, 1987). It has been demonstrated that D_2 receptor activation can counteract the effects of D_1 activation in some brain regions (Stoof and Kebabian, 1981) but this is not the case in nucleus accumbens (Stoof and Verheijden, 1986). However, there is evidence that D_2 receptors play a role in DA reuptake in the NAcb. Cass and Gerhardt (1994) showed in an electrochemical study that applications of raclopride, an antagonist of D_2 receptors, increased both the amplitude and duration of DA effects. In contrast, local applications of SCH-23390 had no effect. This evidence

suggests that D_2 and not D_1 receptors modulate the activity of the DA transporter such that D_2 receptors cause a reduction in DA reuptake.

1.6 Adenosine from Extracellular cAMP

Very early in the study of DA in the striatum, it was shown through biochemical studies that DA acted in the caudate, or dorsal striatum, through activation of adenylate cyclase and subsequently increased levels of cAMP (Kebabian *et al.*, 1972). Subsequently, also in the caudate, Siggins *et al.* (1974) showed that iontophoretic applications of cAMP depressed spontaneous firing of the neurons. This evidence, along with the discovery that cAMP is secreted from many cells, leads to the discussion of possible extracellular roles for cAMP in NAcb.

Barber and Butcher (1983) proposed several possible functions for cAMP secretion, one of which is that cAMP may act as an extracellular messenger, either directly, through cAMP receptors, or indirectly, as a major source of extracellular AD. In fact, Dunwiddie and Hoffer (1980) had shown that cAMP applications depressed field recordings in rat hippocampus, a response which was blocked by theophylline, suggesting that cAMP acted at the extracellular adenosine receptor and supporting the idea that cAMP acts via metabolism to AD (Dunwiddie, 1985). As mentioned in a previous section, Rosenberg *et al.* (1994) showed that probenecid blocked the isoproterenol stimulated accumulation of cAMP in rat cerebral cortex cultures. An additional finding of this report was that probenecid had significantly less effect on the accumulation of AD.

The conclusion reached was that the endogenous AD derived from an extracellular source, possibly cAMP, and was not directly transported out of cells (Rosenberg *et al.*, 1994).

If extracellular cAMP in NAcb is converted to AD, there must be an extracellular mechanism in place. Hydrolysis of cAMP to AD requires at least two steps. First, the extracellular cAMP has to be hydrolyzed to AMP, and second, the AMP must be hydrolyzed to AD. The extracellular hydrolysis of AMP to AD requires an extracellular 5'-nucleotidase, or *ecto*-5'-nucleotidase, which is known to be present on glial cells (Kreutzberg *et al.*, 1978) and has been shown to be important in NMDA-evoked AD release in rat cortical slices (Craig and White, 1993). The hydrolysis of cAMP to AMP would require an extracellular cyclic nucleotide phosphodiesterase, for which there is evidence (Rosenberg and Dichter, 1989; Vasquez and Rosenberg, 1989). Rosenberg and Dichter (1989) used thin-layer chromatography to show that accumulated extracellular cAMP was degraded into AMP and AD, and that this degradation was independent of serum or serum components. This evidence suggested that cAMP was degraded by an extracellular phosphodiesterase and acted in cerebral cortex via AD receptors.

There is supporting evidence for the extrusion and subsequent degradation of cAMP from the spinal cord (Sweeney *et al.*, 1990). Whereas the data of Rosenberg and Dichter (1989) support the hypothesis that in cortex cAMP is derived from astrocytes, Sweeney *et al.* (1988) suggest that in spinal cord the source of cAMP is primary afferent neurons.

A series of studies by Gereau and Conn (1994) provides an example of a system in the rat hippocampus which is similar to that proposed here for the NAcb. They proposed

that in hippocampal slices the depression of EPSCs by cAMP depends on the metabolism of cAMP and the subsequent release of either adenosine or 5'-AMP which acts on presynaptic adenosine receptors. In previous work on mGluRs, Winder and Conn (1992, 1993) had shown that activation of a certain subtype of mGluR resulted in an increase in cAMP accumulation in hippocampal slices. Gereau and Conn (1994) used the protein kinase inhibitor staurosporine to show that the cAMP which accumulated was not acting via a kinase pathway. Likewise, they were able to block the depression completely by applying the membrane impermeable adenosine receptor antagonist 8-pSPT, which suggested that the cAMP was having an extracellular action on adenosine receptors. Furthermore, 8-sSPT and the phosphodiesterase inhibitor RO 20-1724 were used together to suggest that cAMP is not metabolized extracellularly.

In another similar system, Bonci and Williams (1996) have recently shown that in the guinea pig VTA, D_1 receptor activation causes an inhibition of IPSPs. This inhibition can be blocked by adenosine receptor antagonists 8-CPT and DPCPX. By examining the effects of D_1 antagonists and RO 20-1724, they also demonstrated that the metabolism of cAMP was an integral part of the mechanism. In association with this, probenecid was used, and the results suggested that cAMP is transported to the extracellular space before being metabolized.

2. Hypothesis

Adenosine acts as an intercellular second messenger to transduce presynaptic dopamine D_1 receptor mediated depression of excitatory postsynaptic potentials in rat nucleus accumbens neurons.

3. Questions

There are four main questions to be answered by this research:

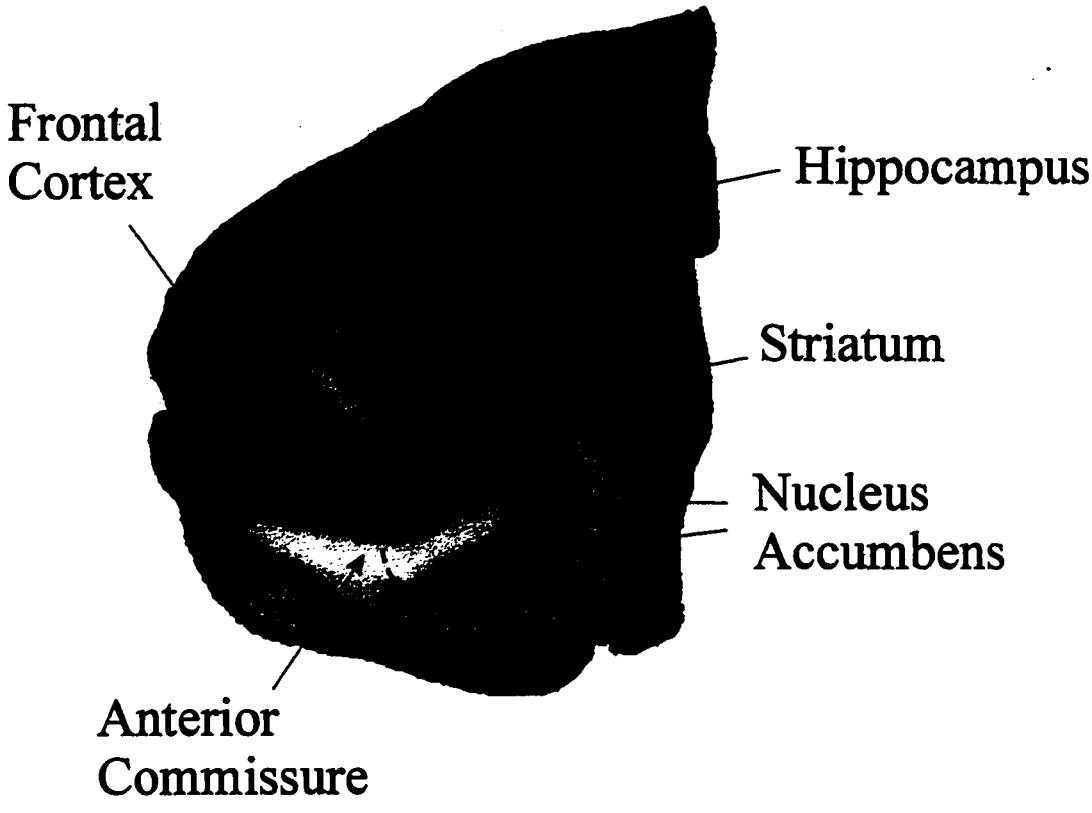
1) Is the dopamine-mediated depression of fEPSPs in rat NAcb mediated by dopamine D_1 receptors?
2) Is the depression of fEPSPs mediated by adenosine A_1 receptors?
3) Is the action of dopamine dependent on the release of cAMP from cells in the NAcb?
4) Are the A_1 receptors in this pathway activated by AD which accumulates via conversion from extracellular cAMP?

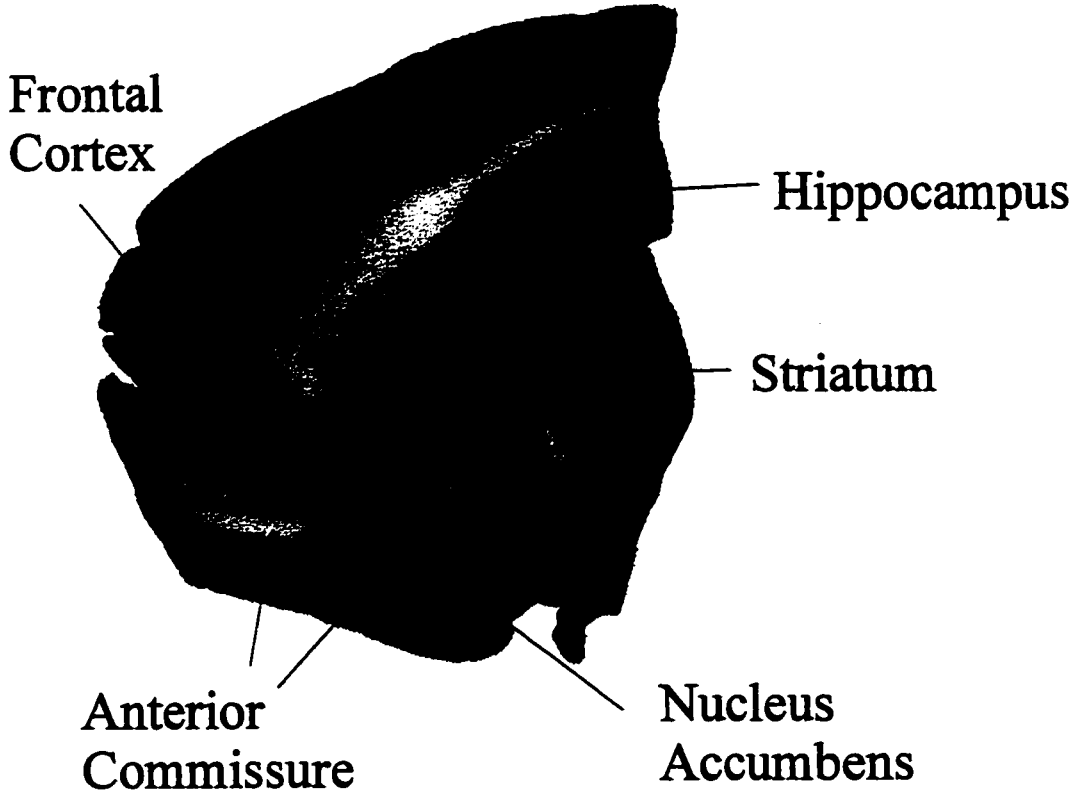
4. Materials and Methods

4.1 ***Nucleus accumbens slice preparation.*** Sprague-Dawley rats (3-4 weeks postnatal) were anesthetized with diethyl ether, and 400-450 μm parasagittal slices of nucleus accumbens were prepared from both hemispheres using a vibratome (DSK Microslicer Model DTK-1000, Lancer Vibratome Series 1000, or Leica VT1000S) (see Figure 2). Slices were prepared in ice-cold artificial cerebro-spinal fluid (aCSF), which was bubbled continuously with 95% O_2/5% CO_2. The composition of the aCSF was (in mM): 126 NaCl, 2.5 KCl, 2.0 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2.0 $CaCl_2$, and 10 D-glucose. In order to increase the excitability of cells in the slices, the concentration of KCl was subsequently increased to 5.0 mM. Following at least sixty minutes of incubation at room temperature, slices were transferred to a recording chamber where they were submerged in continuously flowing aCSF (all solutions were bubbled with 95% O_2/5% CO_2 continuously) at about 3 mL/min (Gilson Medical, Minipulse-3) at a temperature of 26-30°C, monitored by a digital thermometer (Omega HH-52). Although the temperature varied between experiments, for any individual experiment temperature was controlled to within approximately 1°C. Slices were held in place in the recording chamber using two platinum wires.

4.2 ***Electrophysiological recording.*** Prelimbic cortical afferent fibers were stimulated at 0.1 Hz (Digitimer Ltd. Isolated Stimulator, Model DS2) by a bipolar stimulating electrode

Figure 2: Photographs of the two typical parasagittal rat brain slices used in all experiments. In this case, the slices are oriented so that "up" is dorsal, and "left" is rostral. The upper photograph shows a slice in which the nucleus accumbens (outlined by the dotted line) is easily defined as the region immediately surrounding the anterior commissure. It is well differentiated from the striatum. The bottom photograph is a more medial slice, in which the anterior commissure can still be used as a landmark. Again, the nucleus accumbens is relatively well differentiated from the striatum. In all cases, stimulating electrodes were placed at the border between the nucleus accumbens and the frontal cortex, typically dorsal to the anterior commissure. Recording electrodes were then placed varying distances into the nucleus accumbens.


Frontal Cortex
Hippocampus
Striatum
Nucleus Accumbens
Anterior Commissure


Frontal Cortex
Hippocampus
Striatum
Anterior Commissure
Nucleus Accumbens

placed in the white matter at the cortex/NAcb border. Extracellular recordings of field EPSPs (fEPSPs) were made with glass micropipettes filled with 3 M NaCl. Glass pipettes were pulled from borosilicate glass (World Precision Instruments, Inc.) using the Sutter Instruments Co. Model P-87 electrode puller. Electrodes were pulled with wide tips (~10 MΩ), similar to patch electrodes. The stimulating electrode was manoeuvered into position in the NAcb with a mechanical manipulator, and fEPSP amplitude was monitored using PCLAMP software (Axon Instruments). Stimulating pulse amplitude and duration, as well as electrode position in the slice, were adjusted to achieve a sub-maximal fEPSP amplitude. All recordings were made using an AC coupled amplifier (A-M Systems Differential AC Amplifier, Model 1700). Due to the possible influence of GABAergic inhibition on fEPSPs in the NAcb, the $GABA_A$ inhibitor picrotoxin (25 μM) was included in all solutions. The antioxidant sodium metabisulfite (50 μM) was also included in all solutions to reduce dopamine oxidation.

4.3 ***Data acquisition and analysis.*** The Peak Detection capability of PCLAMP6 was used to record amplitudes of fEPSPs relative to a baseline period recorded just before stimulation. These values were saved to a second computer every half hour, at the completion of 60 runs, to allow for online analysis. Peak amplitude data was manipulated in Microsoft Excel, and plotted against time with GraphPad Prism. Several criteria were monitored in this fashion to determine which recordings would be pursued, and which would be discarded. The first criterion was a stable baseline period of at least 20 minutes,

during which the amplitude of the fEPSP peaks did not change appreciably. A second criterion was a minimal stimulation artifact. In some cases, stimulation artifacts were large enough to obscure part or all of the fEPSPs, and in these cases, the slice and data were discarded. The third criterion was an initial response to DA after the stable baseline period. Slices which did not show an appreciable decrease in fEPSP peak amplitude in response to an initial application of DA were also discarded. Once an acceptable data set had been recorded, further analyses were possible. Percent inhibitions were measured by averaging nine peak values immediately prior to drug application, and comparing this value to an average of nine peak values at the end of the drug application period. Statistical significance was determined by Friedman two-way ANOVA analysis, using the Dunn's (Bonferroni) *post hoc* comparison.

4.4 ***Drugs.*** Probenecid (p-[dipropylsulfamoyl]benzoic acid), α,β-methylene-ADP, dopamine (3,4-dihydroxyphenylethylamine), picrotoxin, and sodium metabisulfite were obtained from SIGMA. RO 20-1724 (4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone), CNQX-HBC complex (6-cyano-7-nitroquinoxaline-2,3-dione complexed with 2-hydroxypropyl-β-cyclodextrin for water solubility), SKF-83566, and DPCPX (8-cyclopentyl-3,7-dihydro-1,3-dipropyl-1H-purine-2,6-dione) were obtained from RBI. Probenecid was prepared as a 100 mM stock solution in 110 mM NaOH, diluted 300 μL in 100 mL for a concentration of 300 μM in final solutions. DPCPX and RO 20-1724 were prepared as stocks in DMSO (dimethyl sulfoxide, SIGMA; final

solutions with DMSO concentrations less than 0.05%), and aliquoted and stored in the freezer. In all experiments involving these two compounds, all solutions included equal concentrations of DMSO. α,β-methylene-ADP and SKF 83566 were prepared as stocks in water, and aliquoted and stored in the freezer.

4.5 ***Experimental Protocol.*** After establishing a minimum stable baseline period of at least 20 minutes, dopamine (75 μM) was bath applied to slices for 20-25 minutes. This was followed by a wash period of no less than 30 minutes. After a successful DA application (one showing a significant depression of fEPSP amplitude during the application, followed by a rebound to baseline values during the washout), the period of drug application began. Either DPCPX (100 nM), Probenecid (300 μM), SKF 83566 (100 nM), RO 20-1724 (50 μM), or α,β-m-ADP (25 μM) was applied for at least 15 minutes to provide a stable period of recording. After this time, DA was applied in the presence of these drugs for a further 20-25 minutes. Following the DA/drug application, DA was removed and the slice was bathed for a further 5-10 minutes with the drug. This was followed again by a wash period of no less than 30 minutes. After this wash period, a second 20-25 minute DA application was attempted, followed by a further 30 minute wash period. To terminate the experiments, CNQX (~1-5 μM) was applied to selectively block the synaptic component of the recorded potentials. The length of application varied from slice to slice, as CNQX was applied for as long as it took to completely block the fEPSP (typically no more than 5 minutes). Finally, in a similar fashion to CNQX, TTX (1 μM)

was applied to block the remaining presynaptic population spike (for example, see Figure 3 and Figure 4).

In a small number of experiments, an alternate protocol was used. In these cases, DA was again applied after a minimum of 20 minutes of stable recording. However, instead of removing DA and washing after 20-25 minutes of DA application, the drugs were then co-applied with DA for a further 20-25 minutes. After a period of washout, CNQX and TTX were applied as in the regular protocol.

Figure 3: Three sample traces from a typical experiment. The single trace labeled as Control shows all the basic components of a stimulated response. fEPSP peak amplitudes were measured as the difference between a baseline period recorded immediately prior to stimulation and a maximum peak value for the fEPSP. The two overlapping traces at the bottom show the use of CNQX, an AMPA/kainate glutamate receptor antagonist, to selectively block the field potential, while leaving the presynaptic volley unaffected. TTX was used finally to block the presynaptic component as well.

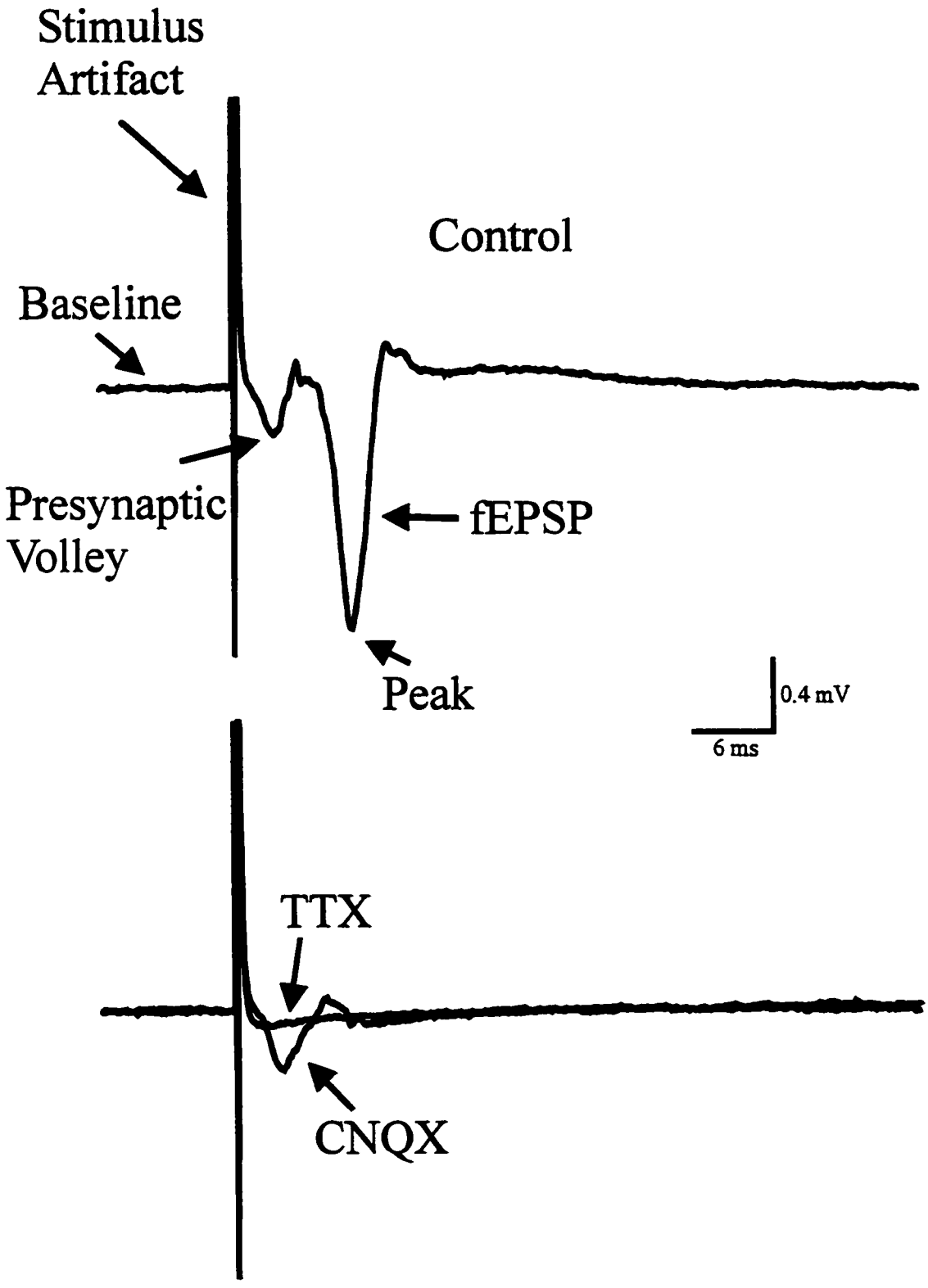
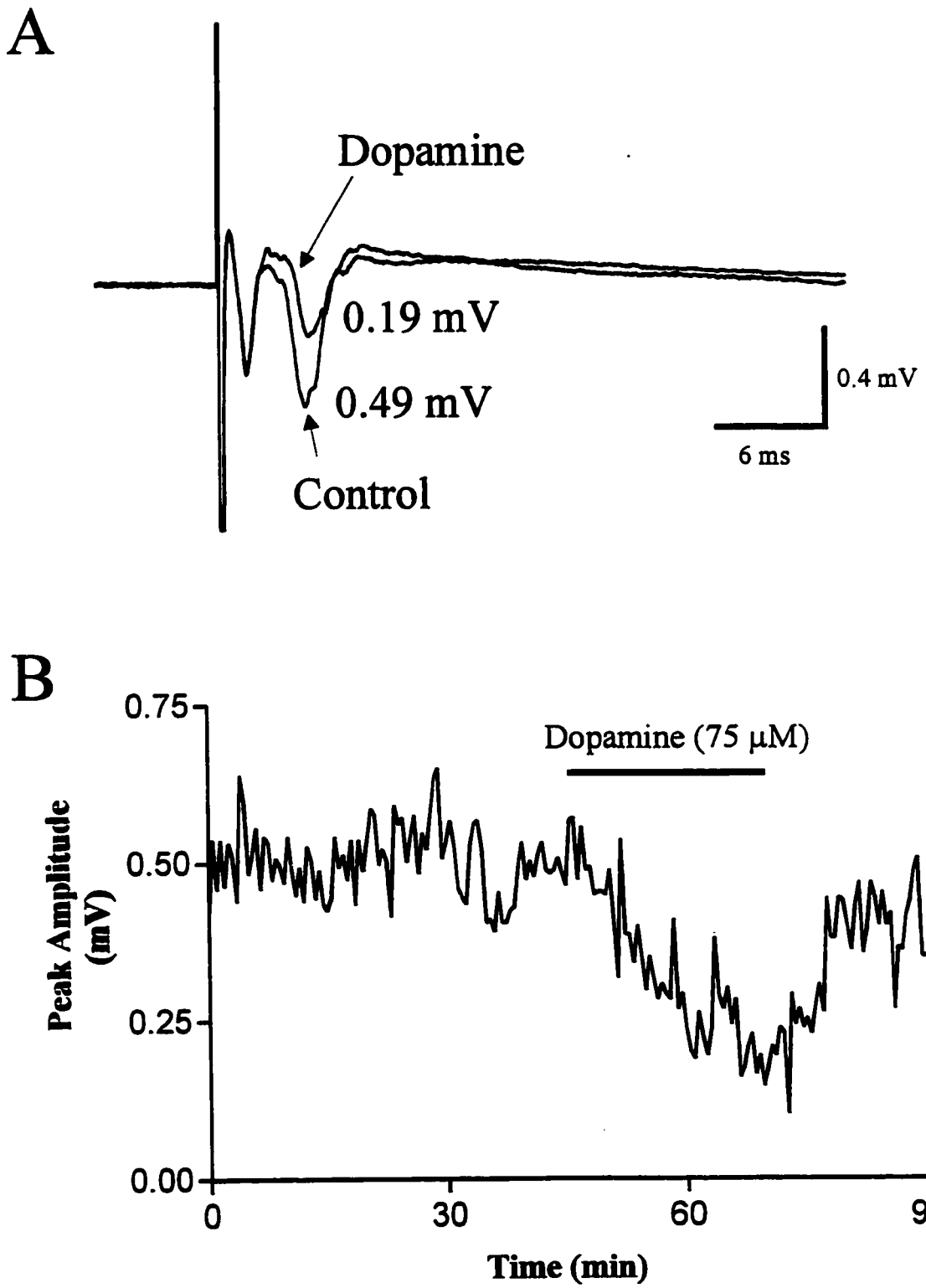

Stimulus
Artifact
Control
Baseline
Presynaptic
Volley
fEPSP
Peak
0.4 mV
6 ms
TTX
CNQX

Figure 4: DA caused a reduction of fEPSP amplitude. **A:** sample traces. In this case, DA had the effect of reducing the peak amplitude from 0.49 mV to 0.19 mV, a decrease of 61%, while at the same time having no effect on the amplitude of the presynaptic volley. **B:** Graphical representation of data from the same experiment showing the effect of DA on the synaptic component of the field recording. Peak amplitude in mV is plotted against time in minutes.


A
Dopamine
0.19 mV
0.49 mV
Control
0.4 mV
6 ms
B
Dopamine (75 μM)
Peak Amplitude (mV)
0.75
0.50
0.25
0.00
0
30
60
90
Time (min)

5. Experiments and Results

5.1 Dopamine Acts Through D_1 Receptors

The first goal of this research was to verify that the DA effect we see in rat NAcb is indeed mediated through dopamine D_1 receptors. Several previous studies have shown that the actions of DA in the NAcb are mediated via D_1 receptors (Uchimura *et al.*, 1986; Harvey and Lacey, 1996; Nicola *et al.*, 1996). This series of experiments was conducted simply to show that we could demonstrate the same response to DA in our slice preparation, using a selective antagonist of the D_1 receptor to block the effects of bath applied dopamine.

Figure 5 shows an experiment in which the selective D_1 antagonist SKF 83566 was used to block the DA-mediated depression of fEPSP amplitude in the rat NAcb. The accumulated data from three such experiments are shown in Figure 6. In the presence of SKF 83566 and DA, fEPSP amplitude was changed to 104 +/- 13.8 % of control. This was significantly different than the effect of DA on its own, which depressed fEPSP amplitude to 64.9 +/- 3.21 % of baseline ($n=3$; $p<0.01$). As was the case with most experiments, little or no recovery of the depressant action of DA on fEPSP amplitude was seen.

As demonstrated in panels E to H of Figure 5, applications of DA made after washout of SKF 83566 were unable to depress fEPSP amplitude. This failure to recover

Figure 5: The dopamine D_1 receptor antagonist SKF 83566 prevented dopamine-mediated fEPSP depression. The graphs (A, C, E, G) show peak amplitude of fEPSPs in mV against time in minutes. On the right (B, D, F, H) are representative traces from the same experiment. **A/B:** Dopamine caused a 27% depression of fEPSP amplitude. **C/D:** This effect was completely blocked by SKF 83566. **E/F:** The effect did not recover after washout of SKF 83566. **G/H:** CNQX and TTX application shows that stimulus artifact did not significantly contribute to peak values.

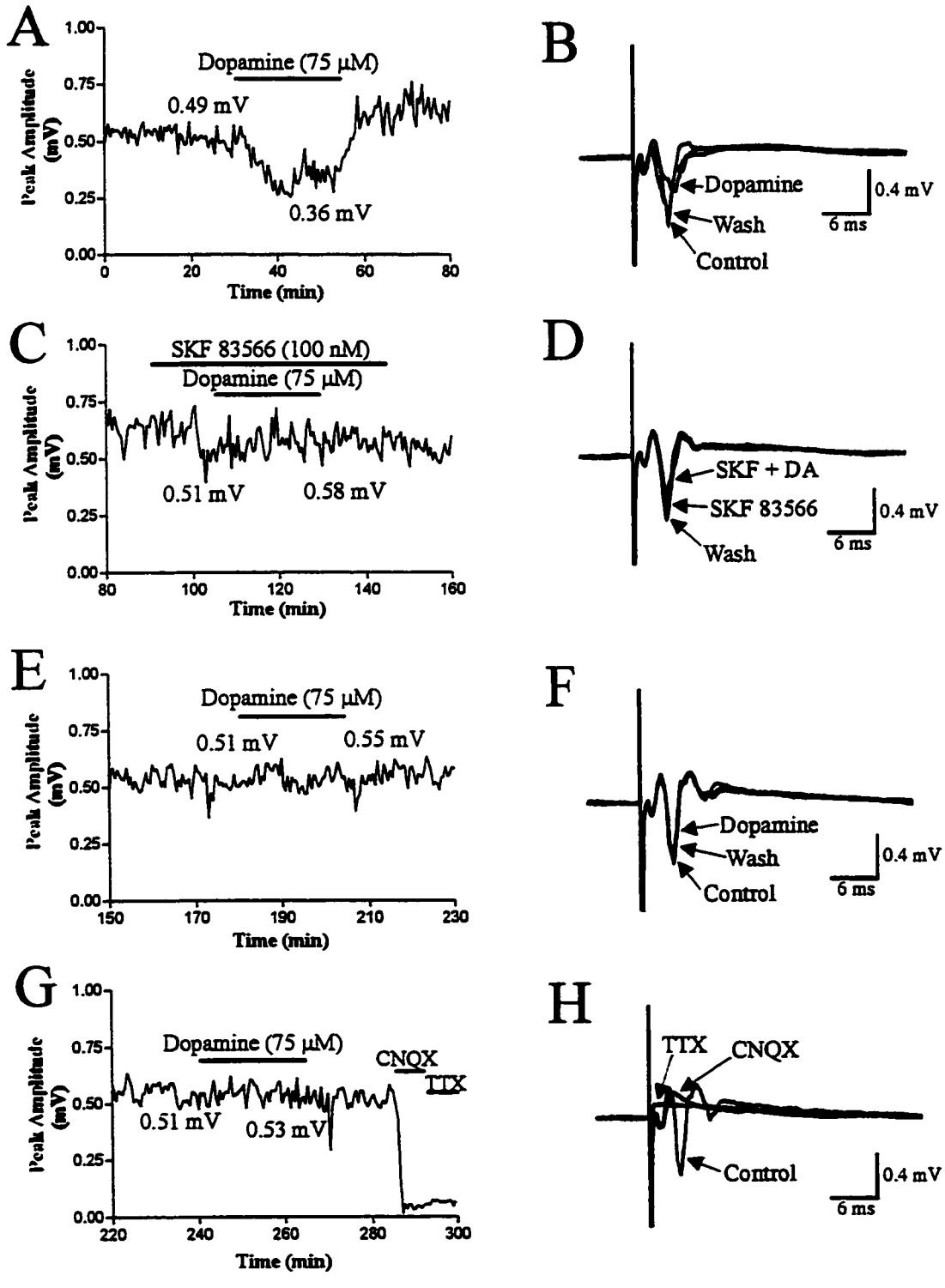
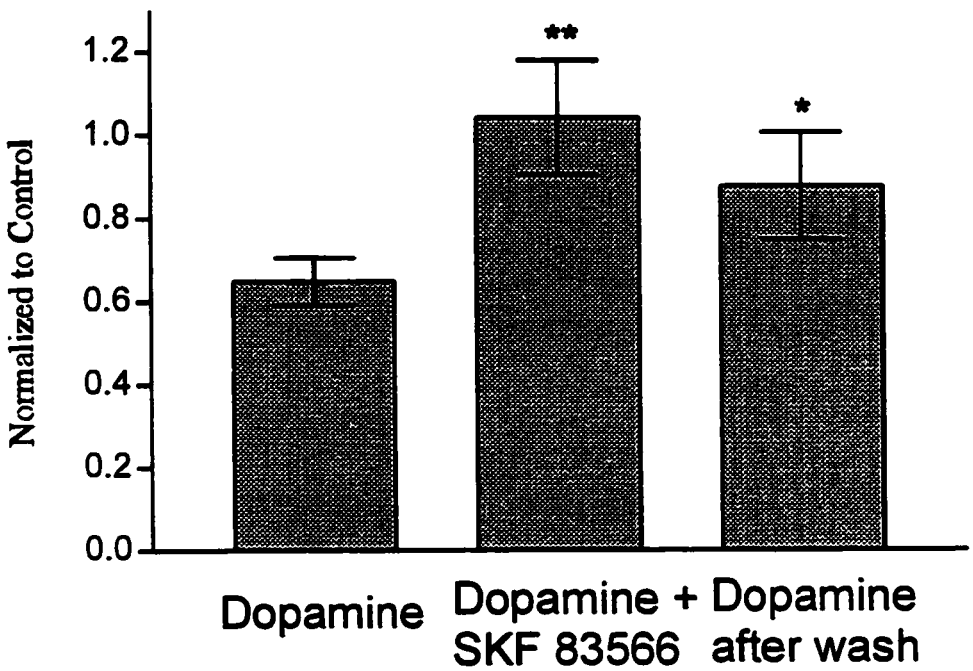

A
Dopamine (75 µM)
0.49 mV
0.36 mV
Peak Amplitude (mV)
Time (min)
B
Dopamine
Wash
Control
0.4 mV
6 ms
C
SKF 83566 (100 nM)
Dopamine (75 µM)
0.51 mV
0.58 mV
Peak Amplitude (mV)
Time (min)
D
SKF + DA
SKF 83566
Wash
0.4 mV
6 ms
E
Dopamine (75 µM)
0.51 mV
0.55 mV
Peak Amplitude (mV)
Time (min)
F
Dopamine
Wash
Control
0.4 mV
6 ms
G
Dopamine (75 µM)
CNQX
TTX
0.51 mV
0.53 mV
Peak Amplitude (mV)
Time (min)
H
TTX
CNQX
Control
0.4 mV
6 ms

Figure 6: Bar chart showing accumulated results of experiments using the D_1 receptor antagonist SKF 83566 to block DA-mediated fEPSP depression. Results indicate that SKF 83566 caused a significant block of fEPSP depression. The inhibitory effect of DA did not recover after a period of washout of SKF 83566 ($n=3$; * $p<0.05$; ** $p<0.01$).

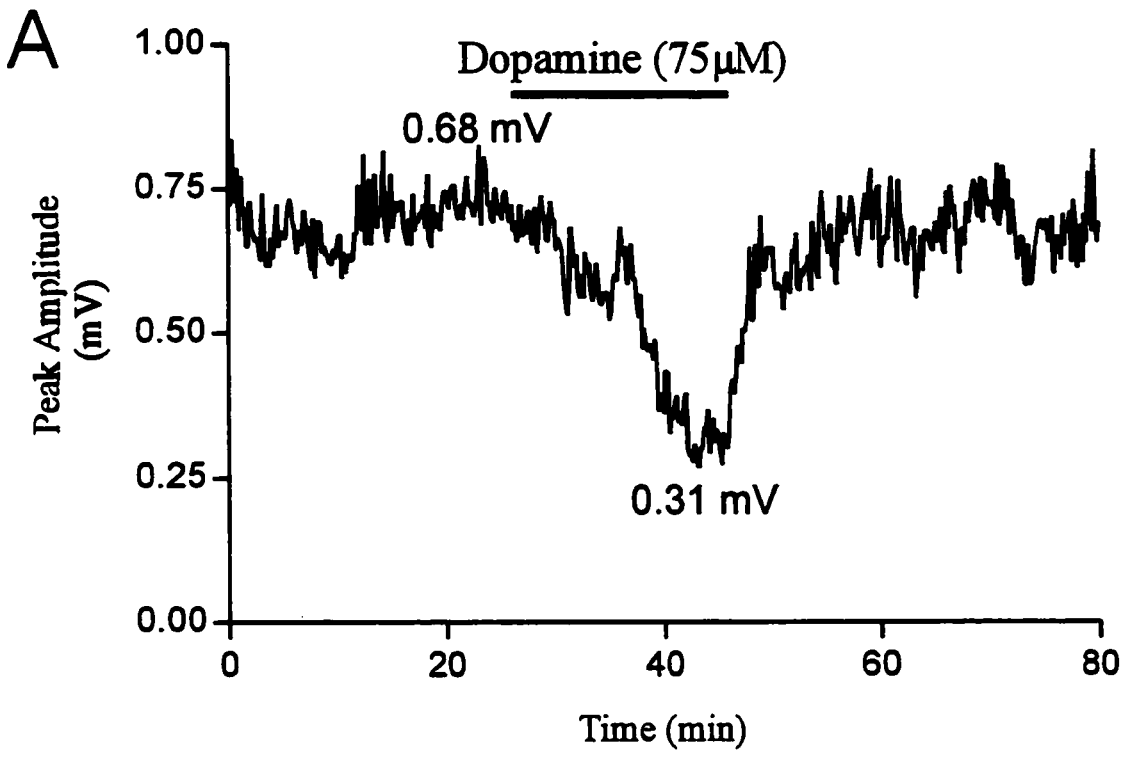

Normalized to Control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Dopamine
Dopamine + SKF 83566
Dopamine after wash

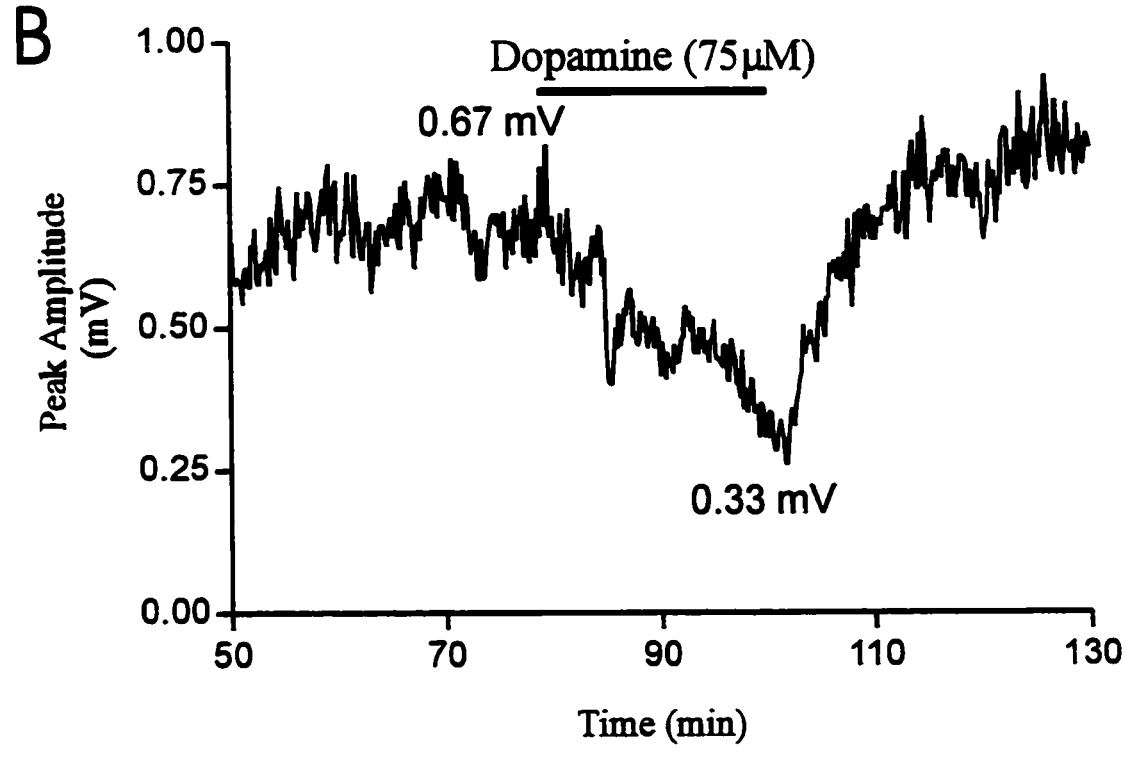
the DA effect prompted a subset of experiments to show that in the absence of a drug application, multiple applications of DA could in fact depress fEPSP amplitude. Figure 7 shows the graphed results from an experiment in which two subsequent applications of DA resulted in similar inhibitions of fEPSPs. Similar results were seen in three experiments, where a second DA application resulted in fEPSP inhibitions of 101 +/- 3.90 % of the first application. This result leads us to assume that the failure to recover a DA effect in this preparation is due to the fact that drug washout is incomplete, rather than to a loss of sensitivity to DA. The fact that fEPSP amplitude itself does not deteriorate over the long time course of these experiments also reassures that there is no loss of viability in the slices.

5.2 Depression is Mediated by A_1 Receptors

The second objective of this research was to determine whether or not depression of fEPSPs caused by dopamine application to *in vitro* slice preparations of rat NAcb is mediated by adenosine A_1 receptors. Studies in the rat NAcb and hippocampus have shown that AD presynaptically inhibits transmitter release by activating DPCPX sensitive A_1 receptors (Uchimura and North, 1991; Thompson *et al.*, 1992). In the rat NAcb, DA has been shown to mimic the inhibitory effects of AD, and further evidence suggests that the inhibition is due to a decrease in presynaptic transmitter release (Uchimura and North,

Figure 7: Two consecutive applications of DA to the same slice both resulted in a depression of fEPSP amplitude. In **A**, DA results in a 55% decrease in fEPSP amplitude. Values returned to control levels after washout of DA. **B** shows the second application of DA 30 minutes after the first application was stopped, resulting in a 52% decrease in fEPSP amplitude.


A
Dopamine (75μM)
0.68 mV
0.31 mV
Peak Amplitude (mV)
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
Time (min)


B
Dopamine (75μM)
0.67 mV
0.33 mV
Peak Amplitude (mV)
1.00
0.75
0.50
0.25
0.00
50
70
90
110
130
Time (min)

1991). The overlapping effects of AD and DA suggest the possibility that DA acts via a pathway which includes presynaptic AD receptors.

Several experiments demonstrated the ability of 75 μM DA to depress fEPSPs in rat NAcb (e.g. Figure 4). A number of experiments were conducted to show that two applications of DA to the same slice could both elicit a depressant effect (Figure 7). Subsequently, several experiments have shown that DPCPX blocks this effect (e.g. Figure 8). Data from four experiments showed that DA caused fEPSP depressions of 32.6 ± 13.5 % of control values. DA in the presence of DPCPX in these same slices produced changes of 3.93 ± 3.31 % from control in the fEPSPs. After long washout periods, further DA applications resulted in depressions of 11.7 ± 6.88 % (Figure 9). These data strongly suggest that the depressant effect of DA in these slices occurs via an A_1 adenosine-mediated pathway.

5.3 Depression is Dependent on cAMP Extrusion

The third aim was to show that the AD-mediated effect of DA on fEPSPs in rat NAcb slices is dependent on the release of cAMP from cells in the NAcb. In various brain regions, probenecid-sensitive nucleotide transport molecules have been shown to be involved in the extrusion of large anions from cells (Steinberg *et al.*, 1987; Tisa and Rosen, 1990; Henderson and Strauss, 1991; Rosenberg *et al.*, 1994). In a number of

Figure 8: The adenosine A1 receptor antagonist DPCPX prevented dopamine-mediated fEPSP depression. The graphs (A, C, E) show peak amplitude of fEPSPs in mV against time in minutes. On the right (B, D, F) are representative traces from the same experiment. **A/B:** Dopamine caused a 52% depression of fEPSP amplitude. **C/D:** This effect was completely blocked by DPCPX. **E/F:** The effect recovered partially after washout of DPCPX.

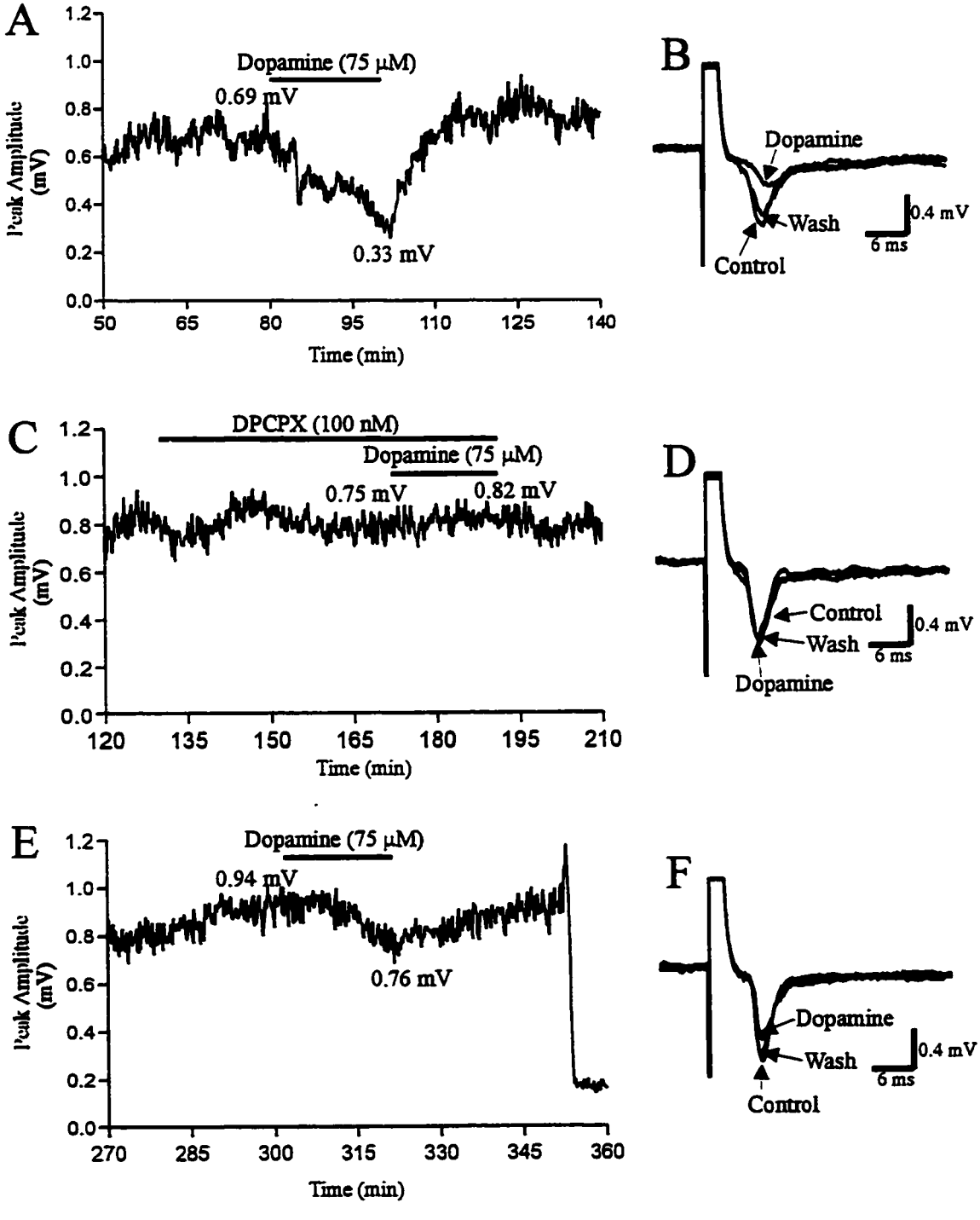
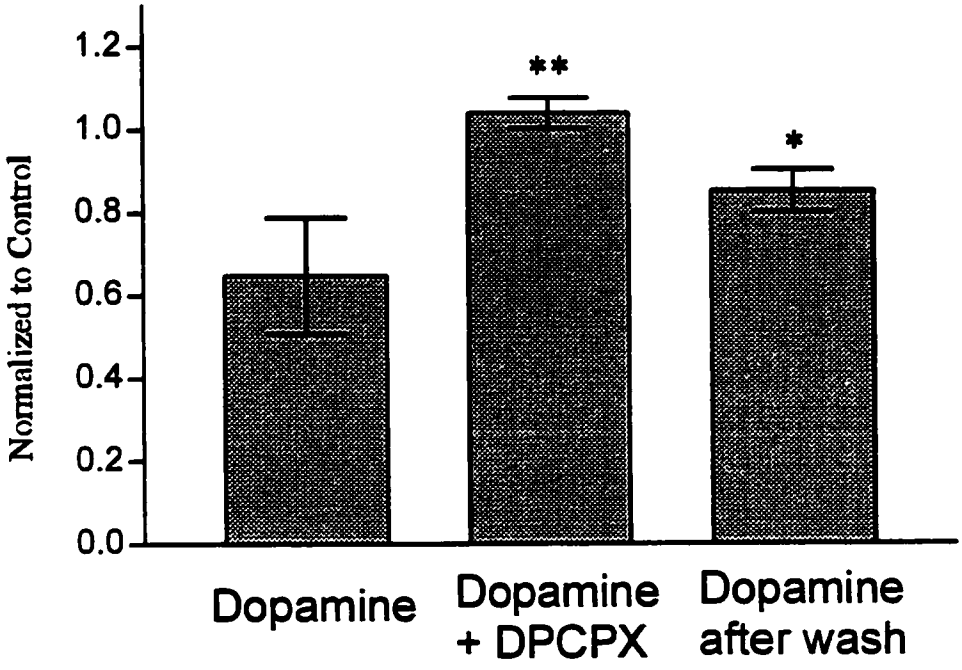

A
Dopamine (75 μM)
0.69 mV
0.33 mV
Peak Amplitude (mV)
Time (min)
B
Dopamine
Wash
Control
0.4 mV
6 ms
C
DPCPX (100 nM)
Dopamine (75 μM)
0.75 mV
0.82 mV
Peak Amplitude (mV)
Time (min)
D
Control
Wash
Dopamine
0.4 mV
6 ms
E
Dopamine (75 μM)
0.94 mV
0.76 mV
Peak Amplitude (mV)
Time (min)
F
Dopamine
Wash
Control
0.4 mV
6 ms

Figure 9: Bar chart showing accumulated results of experiments using the A1 antagonist DPCPX to block DA-mediated fEPSP depression. Results indicate that DPCPX caused a significant block of fEPSP depression. The blocking effect of DA was only partially recovered after a period of washout of DPCPX (n=4; * $p<0.05$; ** $p<0.01$).


Normalized to Control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Dopamine
Dopamine + DPCPX
Dopamine after wash

these cell types, cAMP is a substrate for the transporter (Penit *et al.*, 1974; Zumstein *et al.*, 1974; Doore *et al.*, 1975; Plagemann and Erbe, 1977; Brunton and Mayer, 1979; Heasley and Brunton, 1985), and in rat striatum, cAMP has been shown to accumulate extracellularly in response to DA application (Stoof and Kebabian, 1982; Headley and O'Shaughnessy, 1986; O'Shaughnessy *et al.*, 1987). Also, cAMP in some tissues mimics the inhibitory effects of AD and DA application (Woodruff *et al.*, 1976). Along with the conclusion that DA acts via a pathway involving adenosine A_1 receptors, these data lead to the hypothesis that extracellular AD is derived from cAMP which is pumped out of cells by the nucleotide transport molecule.

Figure 10 shows the results of an experiment in which 100 μM probenecid was used to block the DA-dependent depression of fEPSP amplitude. In this experiment, an application of DA caused a 45.7% decrease in fEPSP amplitude from a control level of 0.81 mV. fEPSP amplitude subsequently recovered to 85.0% of control. In 100 μM probenecid, an application of DA produced a 19.1% decrease in amplitude. Applications of DA subsequent to washing out probenecid produced no further depressions of fEPSP amplitude. The accumulated data in Figure 11 show that in the presence of probenecid, DA causes a depression of fEPSP amplitude to 88.0 +/- 11.6 % of control, and that this differs significantly from the effect of DA alone ($n=5$; $p<0.01$). No significant recovery of the DA effect was ever seen following washout.

Figure 10: The nucleotide transport inhibitor probenecid blocked the dopamine-mediated fEPSP depression. The graphs (A, C, E, G) show peak amplitude of fEPSPs in mV against time in minutes. On the right (B, D, F, H) are representative traces from the same experiment. **A/B:** Dopamine caused a 46% depression of fEPSP amplitude. **C/D:** This effect was significantly blocked by Probenecid. **E/F:** The effect did not significantly recover after washout of Probenecid. **G/H:** CNQX and TTX application shows that approximately 0.2 mV of the peak amplitude values represents stimulus artifact.

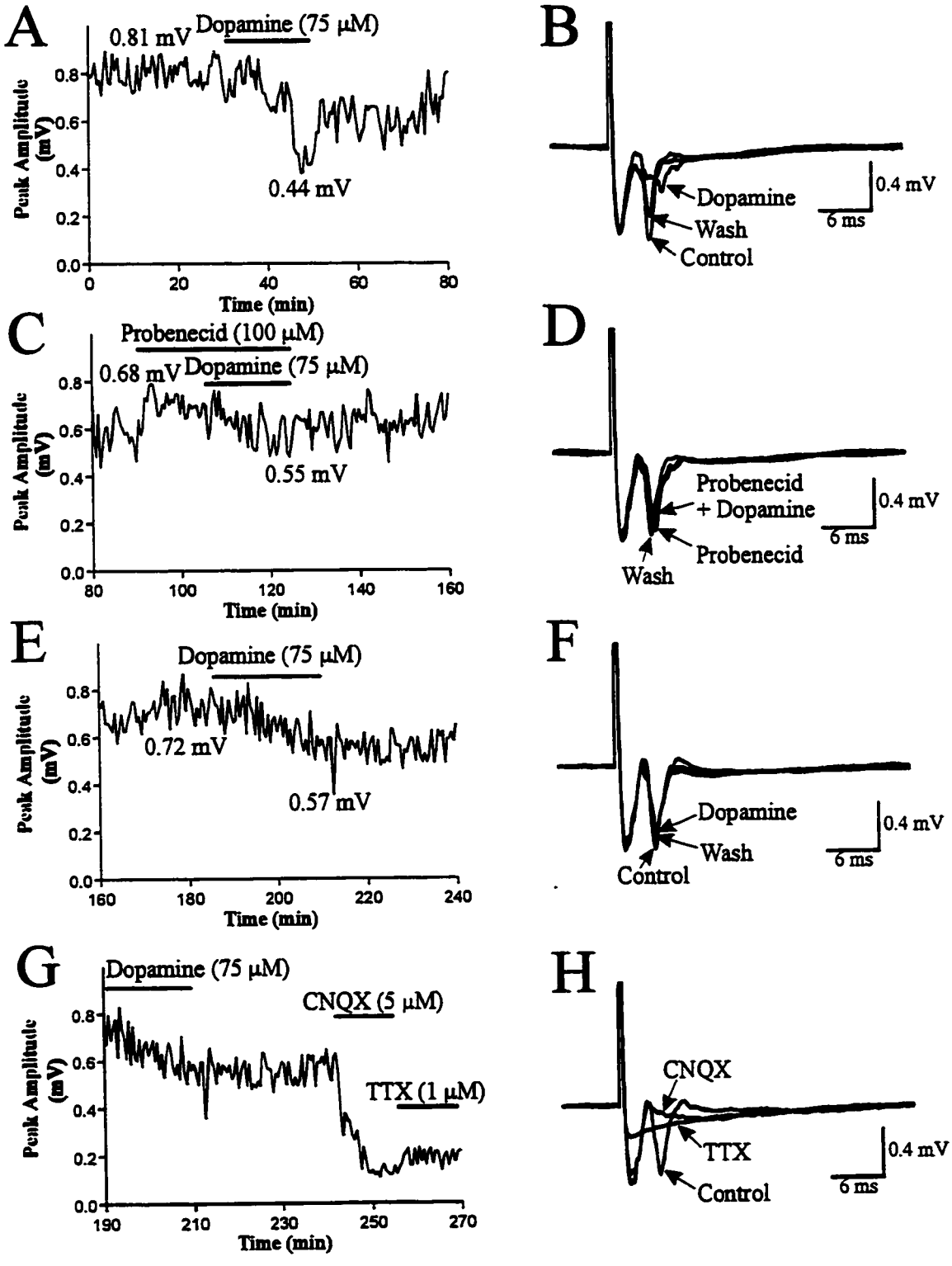
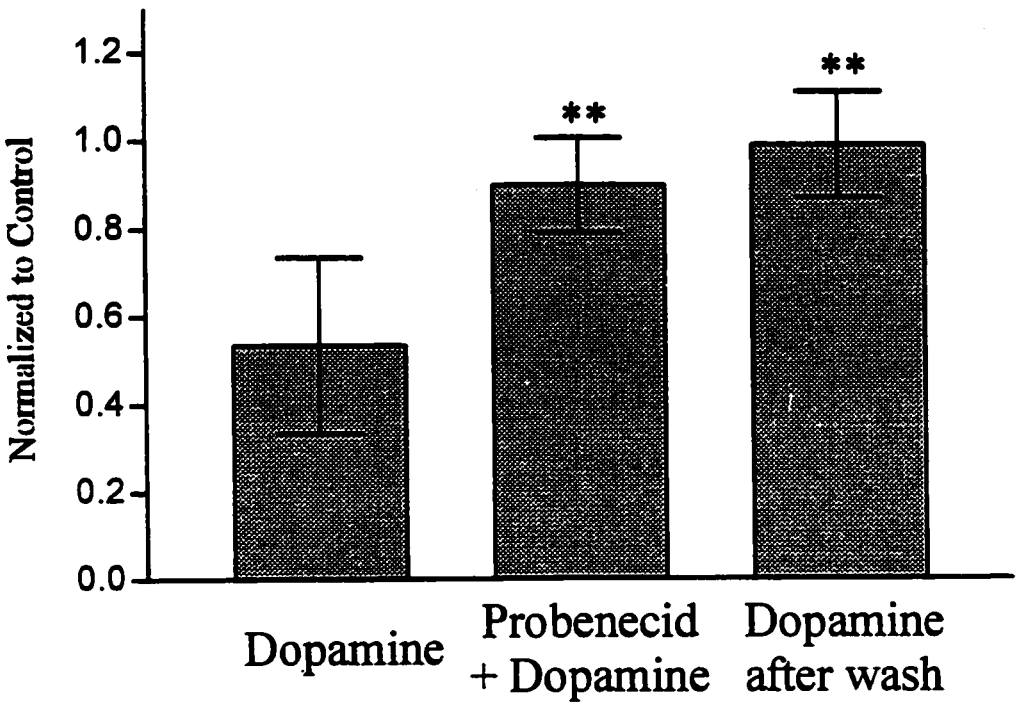

A
0.81 mV
Dopamine (75 μM)
0.44 mV
Peak Amplitude (mV)
Time (min)
B
Dopamine
Wash
Control
0.4 mV
6 ms
C
Probenecid (100 μM)
0.68 mV
Dopamine (75 μM)
0.55 mV
Peak Amplitude (mV)
Time (min)
D
Probenecid
+ Dopamine
Probenecid
Wash
0.4 mV
6 ms
E
Dopamine (75 μM)
0.72 mV
0.57 mV
Peak Amplitude (mV)
Time (min)
F
Dopamine
Wash
Control
0.4 mV
6 ms
G
Dopamine (75 μM)
CNQX (5 μM)
TTX (1 μM)
Peak Amplitude (mV)
Time (min)
H
CNQX
TTX
Control
0.4 mV
6 ms

Figure 11: Bar chart showing accumulated results of experiments using the nucleotide transport inhibitor probenecid to block DA-mediated fEPSP depression. Results indicate that probenecid caused a significant block of fEPSP depression. The blocking effect of DA did not recover after a period of washout of probenecid (n=5; ** $p<0.01$).

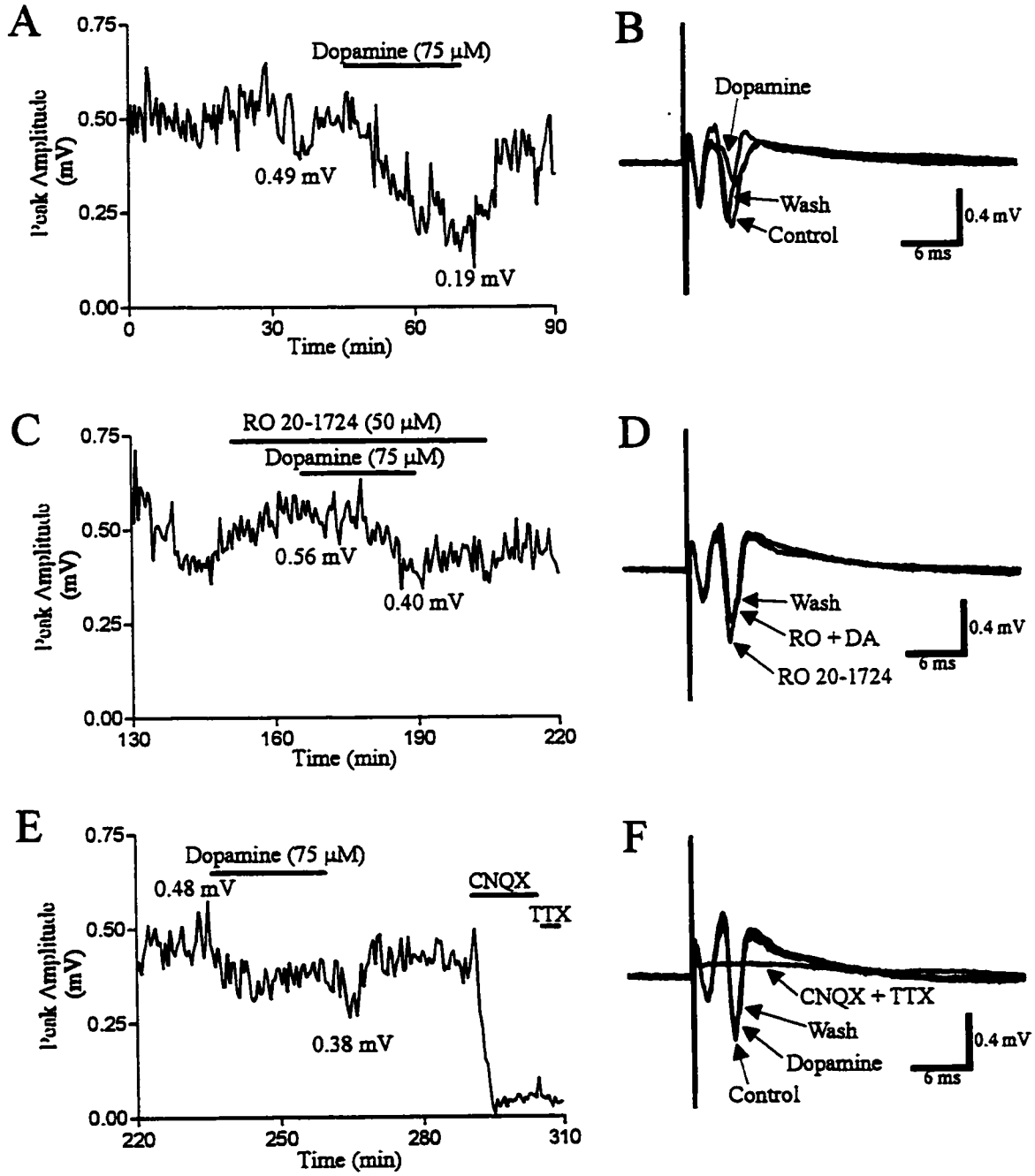

Normalized to Control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
Dopamine
Probenecid + Dopamine
Dopamine after wash

5.4 cAMP is Converted Extracellularly to Adenosine

The final objective of this research was to show that the AD which is responsible for the DA effect in NAcb is derived from extracellular cAMP. The discovery that in many tissues cAMP is secreted from cells leads to the investigation of extracellular roles for cAMP. cAMP has been found to mimic the effect of AD in rat hippocampal cells (Dunwiddie and Hoffer, 1980), and chromatographic evidence supports the theory that extracellular cAMP is metabolized to AD (Rosenberg and Dichter, 1989). Studies using probenecid have shown that this transport blocker reduces the accumulation of extracellular cAMP but not AD in cortical cultures (Rosenberg *et al.*, 1994). This suggests that it is not AD itself which is transported out of the cells in this case, but rather that cAMP is transported out and converted to AD. All of this data suggests to us that a similar process may be at work in the NAcb, whereby cAMP is transported out of cells and subsequently converted into AD which then acts on A_1 receptors.

Six experiments have shown that the cAMP-specific phosphodiesterase inhibitor RO 20-1724 can significantly reduce the depression of fEPSP amplitude caused by DA (e.g. Figure 12). Dopamine alone was seen to reduce fEPSP amplitude to 44.5 +/- 13.3 % of control values. In the presence of RO 20-1724 however, this depression was significantly blocked ($n=6$; $p<0.05$), and DA only changed fEPSP amplitude to 93.2 +/- 16.3 % of control. After washing out the RO 20-1724, little or no recovery of the

Figure 12: The cAMP-dependent phosphodiesterase inhibitor RO 20-1724 prevented dopamine-mediated fEPSP depression. The graphs (A, C, E) show peak amplitude of fEPSPs in mV against time in minutes. On the right (B, D, F) are representative traces from the same experiment. **A/B:** Dopamine caused a 61% depression of fEPSP amplitude. **C/D:** This effect was significantly blocked by RO 20-1724. **E/F:** The effect did not significantly recover after washout of RO 20-1724.


A
Dopamine (75 µM)
0.49 mV
0.19 mV
Peak Amplitude (mV)
0.75
0.50
0.25
0.00
0
30
60
90
Time (min)
B
Dopamine
Wash
Control
0.4 mV
6 ms
C
RO 20-1724 (50 µM)
Dopamine (75 µM)
0.56 mV
0.40 mV
Peak Amplitude (mV)
0.75
0.50
0.25
0.00
130
160
190
220
Time (min)
D
Wash
RO + DA
RO 20-1724
0.4 mV
6 ms
E
Dopamine (75 µM)
0.48 mV
CNQX
TTX
0.38 mV
Peak Amplitude (mV)
0.75
0.50
0.25
0.00
220
250
280
310
Time (min)
F
CNQX + TTX
Wash
Dopamine
Control
0.4 mV
6 ms

depression by DA was seen, and fEPSP amplitude was only reduced to 82.7 +/- 11.7 % of control (see Figure 13). However, even this small reduction in amplitude was not significantly different than the original dopamine effect.

Similarly, four experiments using α,β-m-ADP have shown that this inhibitor of *ecto*-nucleotidase activity can block the DA-dependent depression of fEPSP amplitude (Figures 14 and 15). Figure 14 shows data gathered with the same protocol used in the previous sections. In Figure 15, data was accumulated with a slightly different protocol. In this case, DA was applied to depress fEPSP amplitude and this effect was reversed by the application of α,β-m-ADP. To control for this longer single period of DA application, Figure 16 shows an experiment in which DA was applied for 50 minutes. The inhibition caused by DA was sustained throughout this time, and quickly reversed when DA was removed from the bath.

On its own, DA depressed fEPSP amplitudes to 57.4 +/- 3.21 % of baseline. α,β-m-ADP significantly blocked this action, reducing depressions to 89.5 +/- 8.76 % of baseline ($n=4$; $p<0.01$). Following washout, fEPSP amplitudes of 93.2 +/- 7.62 % indicate that there was no recovery of DA-mediated depression (see Figure 17).

Figure 13: Bar chart showing accumulated results of experiments using the cAMP-dependent phosphodiesterase inhibitor RO 20-1724 to block DA-mediated fEPSP depression. Results indicate that RO 20-1724 caused a significant block of fEPSP depression. The blocking effect of DA partially recovered after a period of washout of RO 20-1724 ($n=6$; * $p<0.05$).

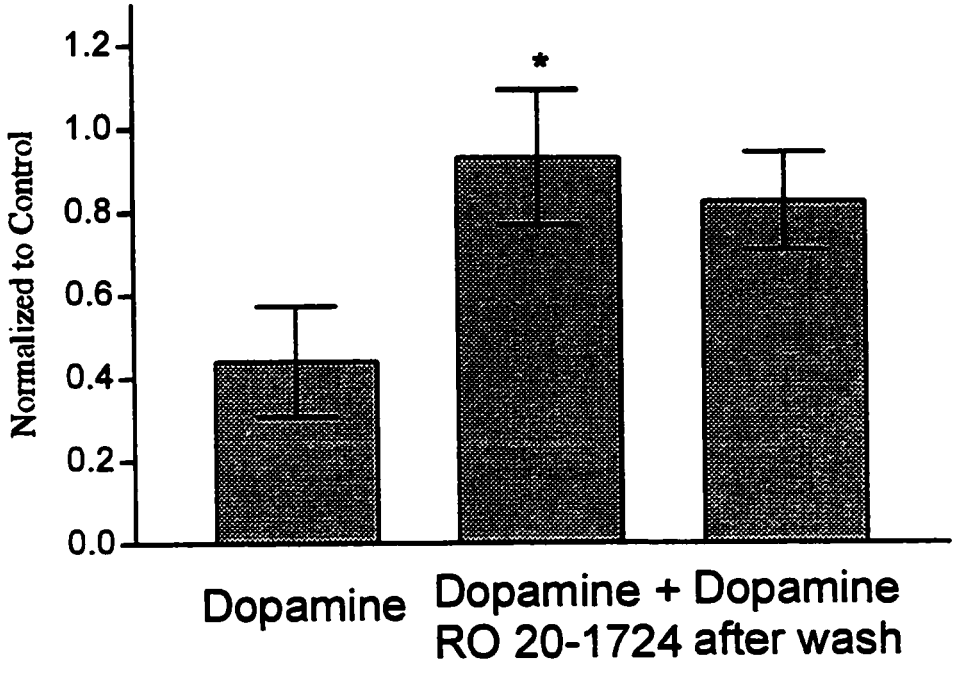

Normalized to Control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Dopamine
Dopamine + Dopamine RO 20-1724 after wash

Figure 14: The *ecto*-nucleotidase inhibitor α,β-m-ADP prevented the dopamine-mediated fEPSP depression. The graphs (A, C, E) show peak amplitude of fEPSPs in mV against time in minutes. On the right (B, D, F) are representative traces from the same experiment. **A/B:** Dopamine caused a 36% depression of fEPSP amplitude. **C/D:** This effect was completely blocked by α,β-m-ADP. **E/F:** The effect did not significantly recover after washout of α,β-m-ADP.

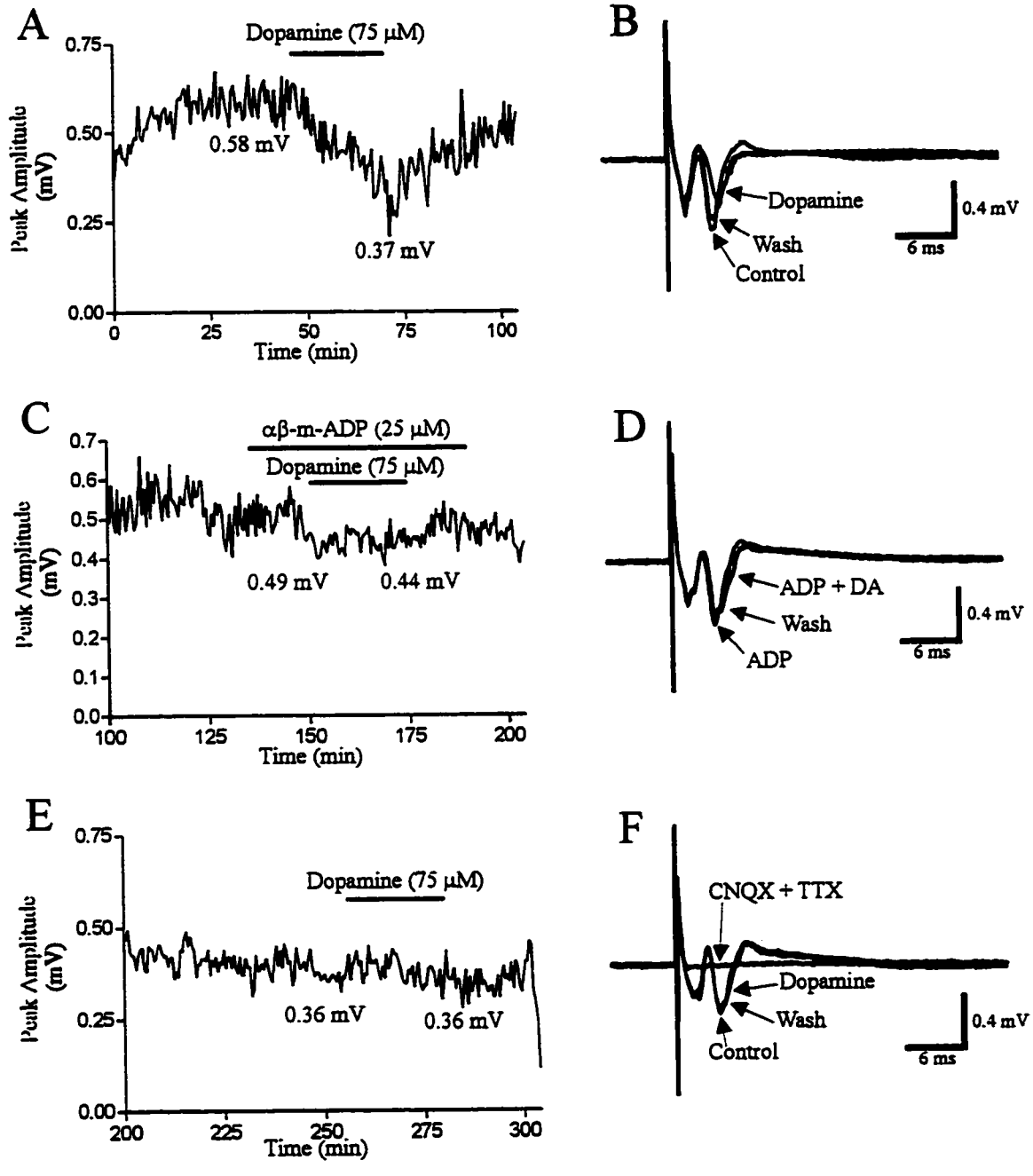
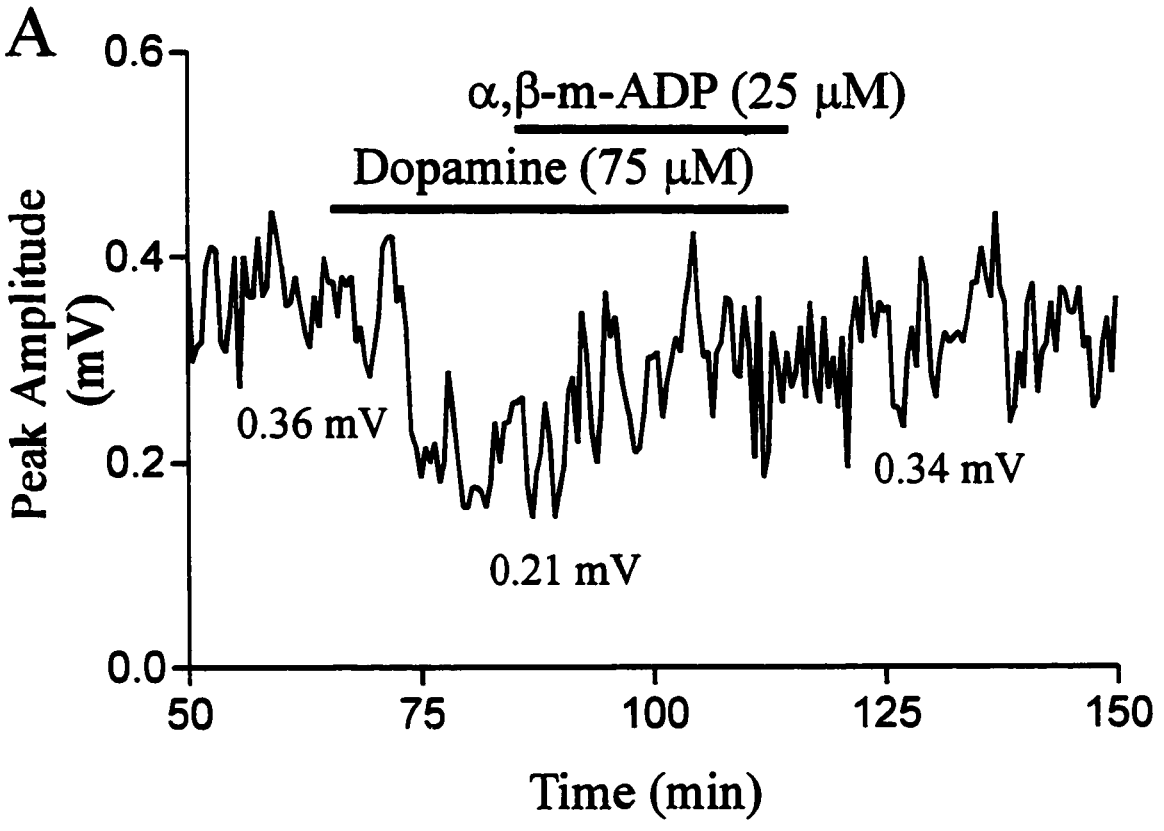

A
Dopamine (75 μM)
0.58 mV
0.37 mV
Peak Amplitude (mV)
Time (min)
B
Dopamine
Wash
Control
0.4 mV
6 ms
C
αβ-m-ADP (25 μM)
Dopamine (75 μM)
0.49 mV
0.44 mV
Peak Amplitude (mV)
Time (min)
D
ADP + DA
Wash
ADP
0.4 mV
6 ms
E
Dopamine (75 μM)
0.36 mV
0.36 mV
Peak Amplitude (mV)
Time (min)
F
CNQX + TTX
Dopamine
Wash
Control
0.4 mV
6 ms

Figure 15: The *ecto*-nucleotidase inhibitor α,β-m-ADP on dopamine-mediated fEPSP depression using a slightly different protocol. The graph in **A** shows peak amplitude in mV against time in minutes. The traces in **B** are sample traces from the same experiment **A/B:** Dopamine caused a 42% depression of fEPSP amplitude, and this depression was reversed to 94% of control with the application of α,β-m-ADP.


A
α,β-m-ADP (25 μM)
Dopamine (75 μM)
Peak Amplitude (mV)
0.6
0.4
0.2
0.0
0.36 mV
0.21 mV
0.34 mV
50
75
100
125
150
Time (min)

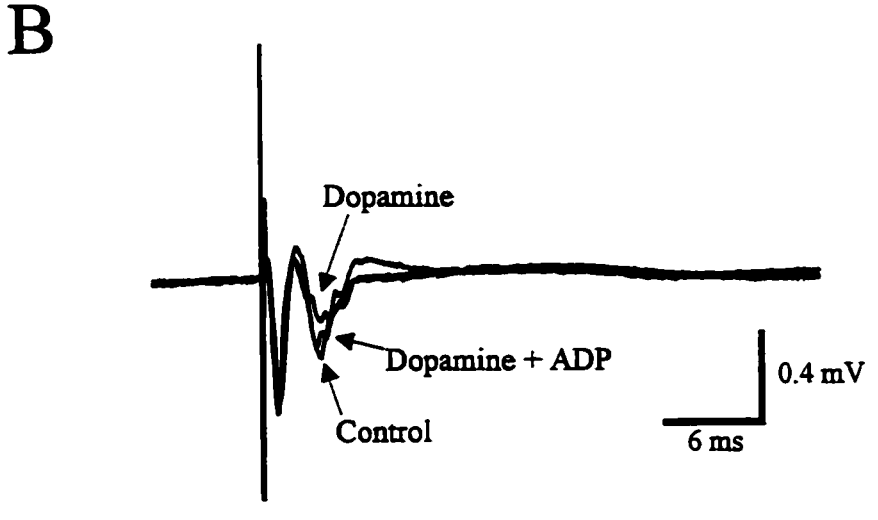

B
Dopamine
Dopamine + ADP
Control
0.4 mV
6 ms

Figure 16: An experiment showing a 50 minute application of DA to control for experiments like that seen in **Figure 15** which require an extended period of dopamine application. DA caused a 63% decrease in fEPSP amplitude, and this depression was maintained through the duration of application. The response recovered to 80% of baseline after DA washout.

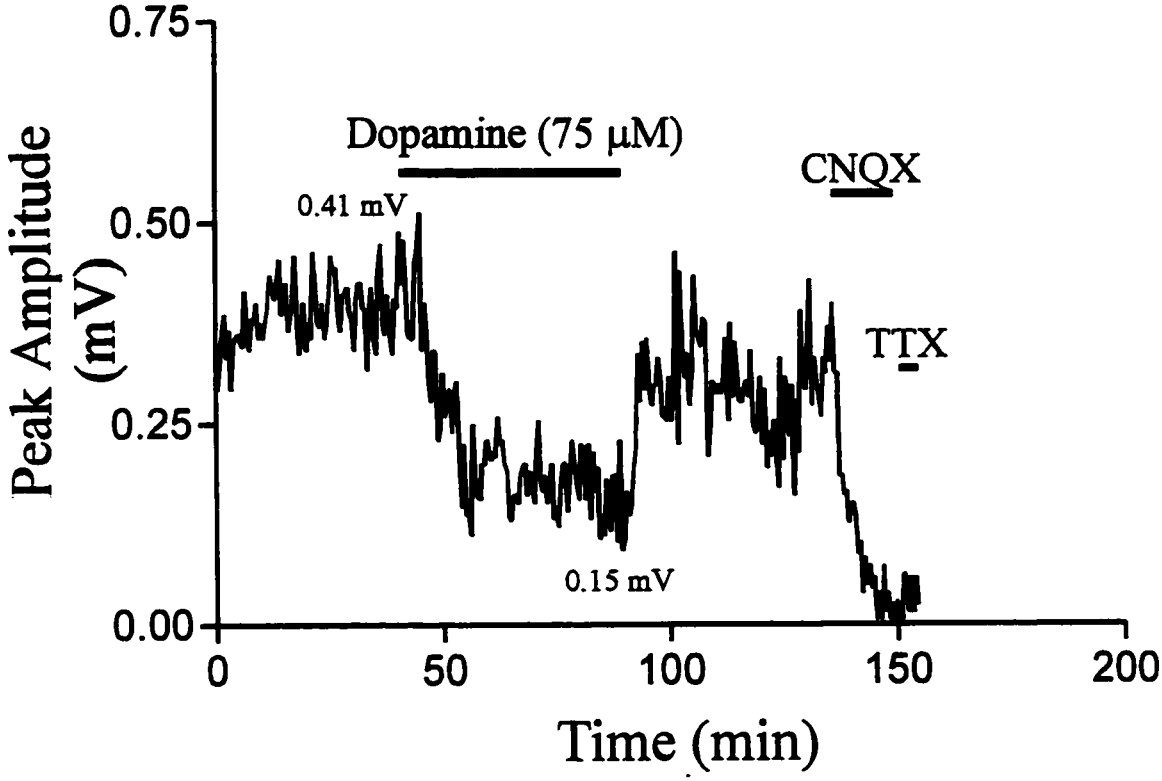
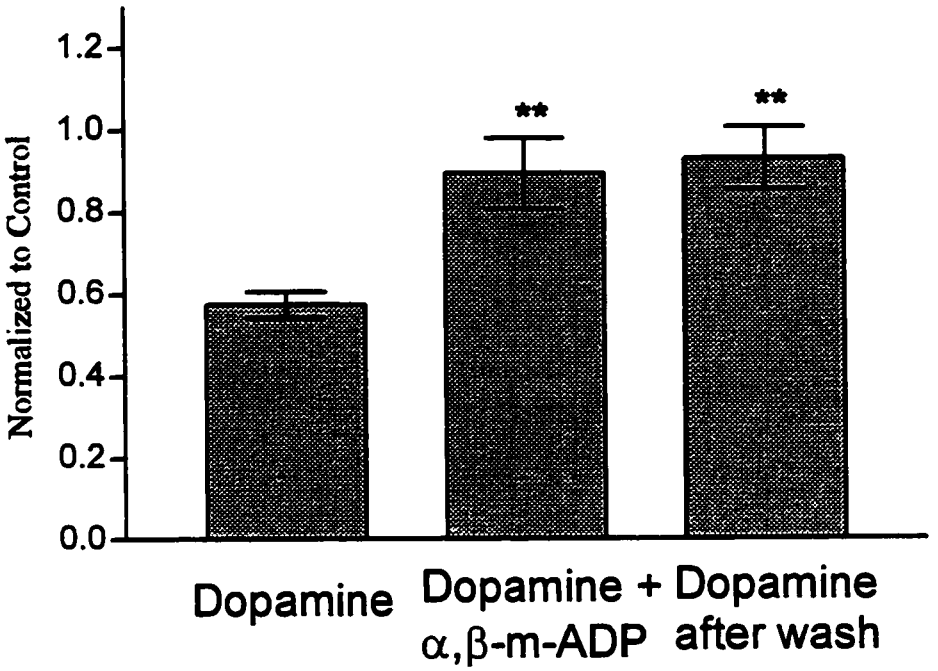

0.75
0.50
0.25
0.00
Peak Amplitude (mV)
Dopamine (75 μM)
CNQX
TTX
0.41 mV
0.15 mV
0
50
100
150
200
Time (min)

Figure 17: Bar chart showing accumulated results of experiments using the *ecto*-nucleotidase inhibitor α,β-m-ADP to block DA-mediated fEPSP depression. Results indicate that α,β-m-ADP caused a significant block of fEPSP depression. The blocking effect of DA did not recover after a period of washout of α,β-m-ADP (n=5; ** $p<0.01$).


Normalized to Control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
Dopamine
Dopamine + α,β-m-ADP
Dopamine after wash

6. Discussion

In the course of this thesis, several questions were considered in attempting to demonstrate a novel mechanism for the modulation of synaptic transmission by dopamine in the rat nucleus accumbens (Figure 1). First, I addressed the question of whether DA was acting to depress field EPSPs in the NAcb via D_1 receptors. In repeatable experiments, DA was shown to depress fEPSPs in parasagittal NAcb slices. This depression was blocked by the selective D_1 receptor antagonist SKF 83566. In the second series of experiments, I considered the possibility that the inhibitory action of DA involved adenosine A_1 receptors. The selective A_1 antagonist DPCPX was shown to block the inhibitory effect of applied DA, demonstrating the involvement of these receptors in the mechanism. The third set of experiments was designed to show that export of cAMP from cells of the NAcb is integral to the mechanism of inhibition by DA. The nucleotide transport inhibitor probenecid was shown to block the depression of fEPSPs by DA in this preparation. The final two series of experiments were designed to investigate the possible role of extracellular conversion of cAMP to AD in the inhibitory action of DA. First, the cAMP dependent phosphodiesterase inhibitor RO 20-1724 was shown to block the actions of applied DA, implicating the conversion of cAMP to AMP in this mechanism. Second, the *ecto*-5'-nucleotidase inhibitor α,β-m-ADP was also shown to block the action of DA in NAcb slices, showing that the hydrolysis of AMP to AD is equally important in the mechanism of action of DA in this system. The accumulated evidence strongly supports

the hypothesized mechanism of action of DA in decreasing the amplitude of fEPSPs in slices of rat NAcb.

6.1 Dopamine Inhibits fEPSPs via D_1 Receptors

With my first series of experiments, the goal was twofold. First, to show that extracellularly recorded postsynaptic potentials in rat nucleus accumbens could be inhibited with bath-applied dopamine, and second, to show that this inhibition could be blocked by the D_1 receptor antagonist SKF 83566. SKF 83566 has been demonstrated to posses very high affinity (approximately 1 nM) for the D_1 receptor (Balmforth *et al.*, 1988; Boyson *et al*, 1986). The results from this section confirm previously published results showing the effects of DA in the NAcb.

As the results show, I was able to depress fEPSPs in an *in vitro* preparation of rat NAcb by bath application of DA (75 μM). There has been a significant amount of previous work in the rat NAcb to demonstrate this effect of applied dopamine (Woodruff *et al.*, 1976; Harvey and Lacey, 1996; Nicola *et al.*, 1996). The earliest studies involving the NAcb and DA showed that psychostimulant drugs like the alkaloid ergometrine (Pijnenburg, *et al.*, 1973) or the DA analogue 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) (Elkhawad and Woodruff, 1975), when injected into the NAcb, caused long-lasting stimulation of locomotor activity in rats. This evidence of a stimulatory action for DA seemed contradictory to *in vitro* studies with the same

compounds showing that their actions on cells in the NAcb were inhibitory (Woodruff *et al.*, 1976). However, some researchers have also shown that the effect of applied DA in the NAcb mimics the effect of DA endogenously released by the stimulation of the ventral tegmental area (VTA; Yim and Mogenson, 1988).

The second goal for this series of experiments was to show that the inhibitory action of DA was occurring via the activation of D_1 receptors. I have shown that the inhibitory effect of DA on fEPSPs in the rat NAcb is blocked by the selective D_1 antagonist SKF 83566. These results are in agreement with the current literature suggesting that the action of DA in the NAcb of the rat involves the D_1 receptor. Harvey and Lacey (1996) and Nicola *et al.* (1996) showed that the D_1 agonists SKF 38393 and SKF 81297A both mimicked the effect of applied DA, and that the D_1 antagonist SCH 23390 completely blocked the depression of EPSPs caused by DA.

Radioligand binding studies have provided important evidence for the localization of the D_1 receptor to the NAcb. Several different compounds have been used, but among the most selective is the D_1 antagonist SCH 39166. The radioactive analogue of this antagonist, [^{3}H]SCH 39166, has demonstrated a high density of D_1 binding in the NAcb (Wamsley *et al.*, 1992). Other studies on the location of the DA-sensitive adenylate cyclase have provided evidence that these D_1 receptors are located postsynaptically on neurons in the NAcb, rather than on the dopaminergic terminals themselves. In 6-OHDA lesion studies, the activity of the DA-sensitive adenylate cyclase in the accumbens was not altered by lesion of the dopaminergic input fibres (Krueger *et al.*, 1976).

There is also evidence for the localization of other types of DA receptors in the rat NAcb. Specifically, both D_2 and D_3 receptors have been shown to be present in this region (Bouthenet *et al.*, 1991; Lévesque *et al.*, 1992). However, the actions of these receptors in the NAcb seem to be quite different from those of the D_1 receptor. The D_2 receptor has been implicated in the regulation of DA reuptake (Cass and Gerhardt, 1994), and both D_2 and D_3 receptors have been shown to differentially regulate neurotensin mRNA expression (Diaz *et al.*, 1994).

In order to rule out the action of DA at D_2 receptors, researchers have shown that the results obtained with DA and D_1 agonists and antagonists could not be attained using the D_2 agonist quinpirole or the D_2 antagonist (-)-sulpiride (Harvey and Lacey, 1996; Nicola *et al.*, 1996). In the same paper, Harvey and Lacey also demonstrate that DA and the SKF compounds had no effect on the membrane conductances or holding currents of cells in the NAcb. This data suggests a presynaptic site of action for DA. Similarly, Nicola *et al.* (1996) showed that DA increased paired-pulse facilitation, and reduced the frequency but not the amplitude of miniature EPSCs, all evidence that the action of DA was presynaptic. Unlike in the proposed mechanism for this thesis, both Harvey and Lacey (1996) and Nicola *et al.* (1996) suggested that it was the D_1 receptors themselves which were located presynaptically. However, in a more recent paper, Harvey and Lacey (1997) have proposed a mechanism which does involve D_1 receptors located postsynaptically on the medium spiny neurons of the NAcb.

6.2 fEPSP Inhibition by Dopamine Depends on A_1 Receptor Activation

The second series of experiments was designed to demonstrate that the inhibitory action of DA in the rat NAcb depends on a mechanism which involves the activation of adenosine A_1 receptors. I have shown that the selective A_1 receptor antagonist DPCPX (100 nM) blocked the inhibitory action of DA on NAcb slices. There is again substantial evidence in the literature for the depressant actions of AD in the brain from as far back as the 1970s (Kostopoulos and Phillis, 1977; Scholfield, 1978). Adenosine and the A_1 agonist R(-)N^6-(2-phenylisopropyl)adenosine (R-PIA) have been shown to induce synaptic depression in the rat striatum (Lovinger and Choi, 1995), and Uchimura and North (1991) showed that glutamate-mediated postsynaptic potentials could be reduced by the application of adenosine to rat NAcb slices. This result fits well with the data of Malenka and Koscis (1988), who showed that adenosine depressed field potentials in the striatum from cortical stimulation. Recent data has shown a possible role for A_1 receptors in the process of short-term depression in the NAcb as well (Lovinger and Choi, 1995). Perhaps most relevant to the current work is the recent paper by Harvey and Lacey (1997). Their research again demonstrates that glutamate release from corticostriatal fibers is decreased by the actions of AD through presynaptic A_1 receptors.

Adenosine A_1 receptors have been shown to be localized in the nucleus accumbens by autoradiographic labeling studies (Goodman and Snyder, 1982; Alexander and Reddington, 1989). Alexander and Reddington (1989) also showed several other interesting results. First, they were able to demonstrate that lesions of the corticostriatal

input fibers resulted in a decrease in A_1 binding in the NAcb. This result helps to further localize A_1 receptors to the presynaptic terminal of the corticostriatal inputs. Second, there was no effect on A_1 binding in this region following lesions of the dopaminergic inputs, indicating the A_1 receptors are postsynaptic to these terminals. In addition to A_1 receptors, several other subtypes are known to exist, including A_{2a}, A_{2b}, and A_3. Radioligand binding studies show that A_{2a} receptors are also localized to the NAcb (Alexander and Reddington, 1989; Olah and Stiles, 1995). A_{2b} receptors do show a limited amount of expression in the brain, though the majority seems to be in the cecum, large intestine, and urinary bladder (Olah and Stiles, 1995). The A_3 receptor is mainly expressed in the testis, though lower levels are detected in the lung, kidney, and heart, and lower levels still in some brain regions (Olah and Stiles, 1995). Although the preponderance of the evidence shows that the main receptor involved in the inhibitory actions of AD is the A_1 receptor (agonists include CPA; antagonists include CPT, DPCPX), there is also evidence implicating the A_2 receptor (agonists include CGS 21680; antagonists include DMPX) (Popoli *et al.*, 1994).

Similar to the NAcb, significant evidence exists showing the actions of AD in the hippocampus. Dunwiddie and Hoffer (1980) showed that AD and various derivatives thereof were able to depress the amplitude of extracellular potentials in the rat hippocampus. A study by Dunwiddie and Fredholm (1984) demonstrated that these electrophysiological effects of adenosine in the hippocampus were mediated by the A_1 type of receptor, rather than the A_2 type. This determination was made on the basis of potency studies, where it was shown that the receptor involved displayed the order of potency L-

PIA ≥ CHA > NECA > 2CA, which is typical for the A_1 receptor. A large body of evidence also exists for a presynaptic site of action for adenosine in the hippocampus. Studies have shown that applications of AD reduce the release of the stimulatory neurotransmitter glutamate in the hippocampus (Corradetti *et al.*, 1984), which directly indicates a presynaptic action. Dunwiddie and Haas (1985) provided evidence to verify the presynaptic action of AD by showing that AD application to hippocampal slices resulted in paired pulse facilitation. Lupica *et al.* (1992) analyzed several parameters at the Shaffer collateral-commisural synapse in the hippocampus. Their results show that adenosine and the A_1 agonist cyclohexyladenosine act to decrease average EPSP amplitude, and that they do so by decreasing the number of quanta of transmitter released, and not by reducing the size of individual quanta, or the postsynaptic sensitivity to transmitter. Similarly, Thompson *et al.* (1992) demonstrated that adenosine has actions at presynaptic receptors, located on excitatory fibres as determined by the effect of adenosine on EPSPs elicited in the presence of GABA.

Although the evidence is convincing that AD acts presynaptically to inhibit the release of transmitter, not much is known about the possible mechanisms of this inhibition. Scanziani *et al.* (1992) showed that in the CA3 region of the hippocampus, the inhibition of glutamate release by adenosine does not depend on an inhibition of the presynaptic Ca^{2+} current. However, evidence from the goldfish brain suggests that the action of AD through A_1 receptors to decrease glutamate release does indeed involve an inhibition of Ca^{2+} influx (Poli *et al.*, 1993), so the problem is not yet solved.

The xanthine compound DPCPX has long been used as a selective antagonist for A_1 receptors in the brain (e.g. Thompson *et al.*, 1992) and other regions (e.g. Redman and Silinsky, 1993), and is used as such in this thesis, so an important consideration is the selectivity and cross-reactivity of the drug. Radioligand binding studies provide the best evidence for the selectivity of DPCPX. Lohse *et al.* (1987) compared the actions of [^{3}H]DPCPX with other A_1 antagonists such as 1,3-diethyl-8-phenylxanthine (DPX), 8-{4-[({(2-aminoethyl)amino}carbonyl)methyl]oxy}phenyl-1,3-dipropyl-xanthine (XAC, for "xanthine amine congener") and theophylline. They showed that DPCPX had a K_i value of 0.45 nM at the A_1 receptor, and 330 nM at the A_2 receptor, indicating an A_1 selectivity of over 700-fold. Similarly, DPCPX showed an affinity for A_1 receptors ten times greater than that of the next most potent compound, XAC. An important consideration when dealing with xanthine compounds is the potential for inhibition of cyclic nucleotide phosphodiesterases. Lohse *et al.* (1987) also address this problem. DPCPX showed an IC_{50} value for the camp-phosphodiesterase of greater than 100 μ M, and less than 10 % inhibition at this level. These values indicate significantly less effect on phosphodiesterase activity than the other xanthine compounds tested. There is potential evidence that DPCPX has actions that occur through systems other than the classical A_1 receptor. Guay-Broder *et al.* (1995) have shown that in cystic fibrosis models, DPCPX activates chloride efflux, but may do so through direct actions on the cystic fibrosis transmembrane regulator. However, with the known binding and specificity values, the 100 nM concentrations used in the present work should provide selective action at A_1 receptors, and should prevent cross-reactivity.

6.3 The Mechanism of Inhibition by Dopamine Involves cAMP Efflux

A critical part of the proposed mechanism of action of DA in this thesis is the extrusion of cAMP from cells in the NAcb. I have hypothesized that DA acts on the postsynaptic NAcb cells to activate adenylate cyclase and increase the intracellular level of cAMP, and the data support this hypothesis by demonstrating the involvement of D_1 receptors. Additionally, there is support from the literature for a DA-sensitive adenylate cyclase in the NAcb (Kebabian *et al.*, 1972; Kebabian and Calne, 1979). One potential complication of the proposed mechanism is the possible down-regulation of D_1 activity by activation of D_2 receptors (Stoof and Kebabian, 1982). However, it has been demonstrated that this inhibition does not occur in the rat NAcb (Stoof and Verheijden, 1986).

I also propose that this DA effect is mediated by AD, and this is supported by results showing the involvement of adenosine A_1 receptors, thought to be located on presynaptic corticostriatal terminals.

In this section, I have addressed the possible link between these two events. I have hypothesized that the cAMP which is produced by activation of D_1 receptors is pumped out of the cells and into the extracellular space where it is subsequently hydrolyzed to adenosine. There is a substantial body of literature showing the extrusion of cAMP from various tissue types, in response to various stimuli (e.g. Brunton and Mayer, 1979). As far back as thirty years ago, cAMP accumulation was demonstrated in guinea pig brain (Sattin and Rall, 1970), and not long after this, a direct correlation between applied DA

and cAMP levels was demonstrated in the rat caudate nucleus (Kebabian *et al.*, 1972). Among the first work to demonstrate an actual release of cAMP from intact tissue was a study by Zumstein *et al.* (1974) showing that cAMP could be produced and released by rat adipose tissue *in vitro*. Also in more than one cell type, the transport inhibitor probenecid has been shown to block the extracellular accumulation of cAMP, demonstrating that cAMP efflux from cells is not a simple diffusional process (Penit *et al.*, 1974; Doore *et al.*, 1975; Rosenberg *et al.*, 1994). This suggestion is further strengthened by work showing that the efflux of cAMP is an energy-dependent process, requiring ATP to function (Rindler *et al.*, 1978). However, probenecid has been demonstrated to effect the transport of more than one compound. For example, Henderson and Tsuji (1987) showed that in L1210 mouse leukemia cells, probenecid blocks the efflux of methotrexate. Later, they further demonstrated that cholate shared the same probenecid-sensitive transport mechanism (Henderson and Tsuji, 1990; Henderson and Strauss, 1991). There is also evidence that in some tissue types the probenecid-sensitive transport of cAMP contributes to organic ion influx as well as efflux (García and Burnside, 1994).

There is some evidence against this action of DA in the NAcb. In particular, a 1996 study by Harvey and Lacey raises some important questions about the role of cAMP in the DA-mediated depression of EPSCs in the rat NAcb. They demonstrate that agents such as the adenylate cyclase stimulator forskolin, the non-specific phosphodiesterase inhibitor IBMX, the cAMP-specific phosphodiesterase inhibitor rolipram, and the non-hydrolizable cAMP analogue dibutyryl cAMP, which should act to elevate intracellular levels of cAMP do not appear to mimic or enhance the DA effect. Similarly, in their 1997

paper, Harvey and Lacey again postulate a mechanism which does not involve the actions of adenylate cyclase or cAMP.

However, the idea that cAMP extrusion is a part of this mechanism is supported by evidence in the literature. Dunwiddie and Hoffer (1980) showed that the inhibition of field potentials by adenosine in the rat hippocampus could be mimicked by application of cAMP. They demonstrated that both effects were blocked by adenosine receptor antagonists, indicating that applied cAMP as well as adenosine itself acts through adenosine receptors in this tissue. More recently, Brundege *et al.* (1997) have further demonstrated this in the rat hippocampus. They show that bath application of cAMP or forskolin leads to the formation of extracellular AD and the activation of AD receptors in a manner mimicking that seen with application of AD itself. In the NAcb, Stoof and Verheijden (1986) showed that application of DA or the D_1 agonist SKF 38393 stimulated an increase in cAMP efflux as measured by radioimmunoassay.

Therefore, my third series of experiments was designed to determine whether cAMP efflux is indeed required in the DA-mediated inhibition of fEPSPs in this preparation. In order to show this, I used probenecid (100 μM) to block the actions of DA. The data clearly demonstrate that this transport antagonist can inhibit the DA-mediated depression of fEPSPs in the rat NAcb slice preparation. This result is a clear indication that the transport of a nucleotide, possibly cAMP, out of cells in the NAcb is required for the inhibitory actions of DA.

6.4 cAMP is Converted to Adenosine Extracellularly

The final segment of this thesis had one goal: to demonstrate that the extracellular conversion of cAMP to AD is a necessary step in the mechanism by which DA inhibits fEPSP amplitude in the rat NAcb. There has been substantial work showing the presence of plasma membrane enzymes whose active sites occur on the extracellular surface, also called *ecto*-enzymes, in a variety of cell types (for review, see Manery and Dryden, 1979). More recent work demonstrates this further, in other areas, including the rat brain (Clemow and Brunjes, 1996). Also, the recent study by Brundege *et al.* (1997) shows that cAMP in the bath mimics the actions of AD.

The first step in the conversion of cAMP to AD is the hydrolysis of cAMP to AMP, a process involving the cAMP-specific phosphodiesterase (potentially PDE IV). Immunoreactivity and Western blot analysis have demonstrated the presence of the cAMP-specific PDE IV in striatum (e.g. Lobban *et al.*, 1994). There is a fair amount of data from the literature to support the hypothesis that conversion of cAMP to AD is involved in the actions of DA and AD in the brain. Zumstein *et al.* (1974) were among the first to demonstrate this. In their studies on the release of cAMP from rat adipose tissue, they discovered that application of theophylline, a phosphodiesterase inhibitor, increased the levels of cAMP detected in the extracellular medium. This showed that at least part of the accumulated cAMP was being hydrolyzed after being released. Similar results have been obtained in other tissues, including pigeon erythrocytes (Brunton and Mayer, 1979) and rat hippocampus (Gereau and Conn, 1994). Rosenberg and Dichter

(1989) showed in cultures of rat cerebral cortex, that epinephrine could stimulate the accumulation of both intracellular and extracellular cAMP, and that the extracellular cAMP was degraded into AMP and AD. Their evidence showed that this hydrolysis occurred independently of the serum, suggesting the presence of extracellular nucleotidase and phosphodiesterase. In a later study, also in rat cortical culture, Rosenberg *et al.* (1994) have shown that several inhibitors of cAMP phosphorylation, including RO 20-1724, could increase the levels of secreted cAMP. Gereau and Conn (1994) also demonstrated that the application of the cyclic nucleotide phosphodiesterase inhibitor RO 20-1724 could completely abolish the inhibitory effects normally seen following the stimulation of increased cAMP levels. To this extent, their results also support my data, suggesting that cAMP hydrolysis, presumably to AD, is required to mediate the inhibitory effects on fEPSPs (Gereau and Conn, 1994). However, they further showed in the hippocampus that the inhibitory effect of bath applied cAMP was not blocked by RO 20-1724. This result indicated that cAMP was not converted to AD outside the cell, and lead them to conclude that cAMP was first intracellularly hydrolyzed, and that either AD or 5'-AMP was released to the extracellular space.

RO 20-1724 has been used in several preparations as an inhibitor of cyclic nucleotide phosphodiesterase. However, there is the possibility of cross-reactivity here as well. At concentrations above 0.1 mM, RO 20-1724 is known inhibit cAMP efflux from cells (Brunton and Mayer, 1979). I have avoided this problem by using a concentration of the compound which is significantly below this value. Also, RO 20-1724 is known to have some effects on protein kinase activity and platelet aggregation (Kinner and Wilson,

1977). Phillis and Wu (1981) tested RO 20-1724 along with several other phosphodiesterase inhibitors for possible competitive binding to adenosine receptors, since some compounds such as theophylline are known to show this effect. At concentrations up to 100 μM, RO 20-1724 only very weakly inhibited L-PIA binding, demonstrating that it has a very low affinity for adenosine receptors. In general, this compound is accepted as a potent and selective inhibitor of cAMP phosphodiesterase.

Therefore, my results showing that RO 20-1724 blocks the effect of applied DA strongly support the hypothesis that the cAMP hydrolysis is integral to the mechanism.

The final step in the conversion of cAMP to AD involves the hydrolysis of AMP to AD. There is support in the literature for the hypothesis that this process occurs extracellularly. It has long been known that the enzyme 5'-nucleotidase is localized to the plasma membranes of cells in the brain (Kreutzberg *et al.*, 1978), and α,β-m-ADP has been shown to be a potent inhibitor of this *ecto*-enzyme (Orford and Saggerson, 1996). James and Richardson (1993) have studied the purified striatal cholinergic synapse, and have demonstrated the presence of *ecto*-5'-nucleotidase in this preparation. Additionally, they blocked the action of this enzyme with α,β-m-ADP, and showed that the production of adenosine in the striatum is at least partially an extracellular process. In contrast, some work in the rat hippocampus has shown that adenosine in this region can be produced intracellularly, and released as adenosine *per se* (Lloyd *et al.*, 1993; Mitchell *et al.*, 1993). Lloyd *et al.* (1993) showed that the release and extracellular accumulation of AD was not inhibited by the *ecto*-5'-nucleotidase inhibitor α,β-m-ADP. In further support of the

intracellular formation of AD, Lloyd *et al.* (1993) used L-homocysteine thiolactone to trap intracellular AD and found that this did decrease extracellular AD accumulation. This data, taken with that of Gereau and Conn (1994), shows the possibility for more than one method of accumulation of AD in the extracellular space in the hippocampus. It is possible that AD levels outside the cell can be generated by the direct release of AD, or by the release and subsequent hydrolysis of cAMP. Some evidence suggests that NMDA receptors may be involved in this process. Manzoni *et al.* (1994) the depression of excitatory synaptic transmission in the hippocampus caused by NMDA could be blocked by the A_1 antagonist CPT. There is very similar evidence for more than one method of AD release in the rat cortex. Craig and White (1993) showed that NMDA and non-NMDA receptors evoked AD accumulation by separate mechanisms. Their data show that α,β-m-ADP decreased AD release stimulated by NMDA or glutamate, but had no effect on the release triggered by kainate or AMPA. This leads to the conclusion that non-NMDA receptors stimulate the release of AD *per se*, while NMDA receptors stimulate the release of a precursor nucleotide.

Thus the possibility exists that there is more than one mechanism for AD release in the NAcb as well. Recent work by Harvey and Lacey (1997) shows that DA-induced EPSCs in the rat NAcb could not be blocked by the cAMP-specific phosphodiesterase inhibitor rolipram. Likewise, they showed that the adenylate cyclase stimulator forskolin could not mimic the effect of DA in this preparation. Interestingly, this data is in direct conflict with the data I have gathered. In my hands, the cAMP phosphodiesterase did significantly reduce the inhibition of fEPSPs by DA, and I was able to show that the

extracellular conversion of cAMP to AD was critical to the effect. This leads me to the conclusion that as in the hippocampus and cortex, there may be more than one mechanism for AD accumulation in the extracellular space of the rat NAcb.

6.5 Conclusions

The data accumulated in the course of this thesis support the hypothesis illustrated in Figure 1. In the NAcb slice preparation, DA causes the depression of field potentials, and this depression is mediated by D_1 receptors as demonstrated by the selective D_1 antagonist SKF 83566. The mechanism by which DA inhibits fEPSPs involves the efflux of a nucleotide, perhaps cAMP, through the probenecid-sensitive nucleotide transport molecule. cAMP is converted to adenosine in the extracellular space, as demonstrated with the cAMP phosphodiesterase inhibitor RO 20-1724, and the of *ecto*-5'-nucleotidase inhibitor α,β-m-ADP. Finally, I have shown that this extracellular adenosine acts to decrease fEPSP amplitude via A_1 receptors using the selective A_1 antagonist DPCPX.

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